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For further information contact:

Mr. Ernest Mann, Joint Managing Director
International Food Information Service
Lane End House, Shinfield
Reading RG2 9BB, United Kingdom
Telephone: 0044-734-88 32 67, Telex: 0051-84 72 04
or
Dr. Udo Schutzsack, Joint Managing Director
International Food Information Service
Arabella Center, Lyoner-Strasse 44-48
6000 Frankfurt 71, West Germany
Telephone: (06 11) 66 87-338, Telex: 4 14 351 GIDFM

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Quality and Nutritional Changes in Frozen Breaded Shrimp Stored in Wholesale and Retail Freezers

KEITH W. GATES, JACKIE G. EUDALY, AMANDA H. PARKER, and LAURA A. PITTMAN

ABSTRACT

Chemical, microbiological, and sensory changes were monitored in breaded fantail shrimp held in upright and coffin style freezers at two retail markets and in a large warehouse freezer. Quality and nutritional changes in a test lot of shrimp were followed for 13 months. Significant sensory deterioration can be detected as early as 3 - 4 months after production when shrimp are stored in a retail freezer. Minimum retail freezer temperatures reached ≤ −20°C between 1% and 36% of the monitored days and had daily temperature variations of 12 - 18°C. Storage at the wholesale level below −20°C with maximum daily temperature variations of 2 - 3°C proved to be an effective method to maintain the quality of frozen shrimp for at least 13 months of storage.

INTRODUCTION

THE QUALITY and nutrition of frozen breaded shrimp available to the consuming public have been a recent concern of breaded shrimp manufacturing companies, state and federal regulatory agencies, retail merchants, and consumers. Breaded shrimp manufacturers were convinced that they were producing a safe, nutritious, and appetizing product for public consumption. Periodic consumer complaints and regulatory actions led the processors to believe that the consuming public was not necessarily purchasing the same quality product that left their manufacturing facilities. Two previous papers, Rao et al. (1975) and Williams et al. (1981) addressed the problems encountered by moisture migration from shrimp to surrounding breading. Decreased weight of shrimp coupled with an increased breading weight caused the product to fall below the United States Food and Drug Administration Standard of identity for frozen breaded shrimp which specifies a minimum shrimp content of 50% (FDA, 1976). Additional questions have been raised regarding the nutritional and hedonic quality of breaded shrimp in the market place. Again, breaded shrimp manufacturers believed that they were producing fine products, but handling abuses at the retail level sometimes deprived consumers of expected quality. The following study was designed to determine chemical, microbiological, and sensory changes in frozen breaded shrimp as it moved through the wholesale-retail distribution system.

MATERIALS & METHODS

A LARGE Georgia seafood processor provided a single lot of 8-ounce frozen breaded fantail brown shrimp (*Penaeus aztecus*) produced and blast frozen in Texas on July 6, 1982. The lot was trucked to a wholesale freezer in Glynn County, GA on July 11, 1982. Initial and replacement product was supplied from the freezer to two retail merchants in Brunswick, Georgia. The first market stored and displayed product for retail sale in a vertical or upright closed door freezer case, Warren/ Sherer model HRL-5U. The second cooperator displayed and stored the frozen shrimp in a horizontal or coffin freezer open to the air, Hill model EZ 6KF. Product was loaded into the upright freezer on July 29, 1982 and into the coffin freezer on August 10, 1982. Additional product was supplied to each retail store when it was requested by the manager. In all cases, product was delivered following completion of each month's sampling program. Table 1 presents the dates that frozen breaded shrimp were delivered from the warehouse freezer to retail upright and coffin freezers.

Authors Gates, EuDaly, Parker, and Pittman are affiliated with the Univ. of Georgia, Marine Extension Service, P.O. Box Z, Brunswick, GA 31523.

Sampling

Ryan battery powered thermographs continuously recorded temperatures at each storage location. A model K-45 thermograph was installed in the warehouse freezer while a model K-10 was installed in each retail display case.

Five 8-oz packages were collected monthly from each storage area for chemical, microbiological, and sensory analyses. Samples from each location were composited for all determinations. A subsample of 20 whole shrimp was collected to determine breading content. Thirty shrimp were held for the taste panel and the remaining whole breaded shrimp were ground for chemical, vitamin, and microbiological analyses. Frozen breaded shrimp collected from the warehouse freezer were first analyzed in July 1982. Initial retail samples from each display case were taken in August 1982. Monthly samples from each storage location were collected through July 1983. The retail store providing the coffin freezer discontinued sales of seafood in August 1983. Final breaded shrimp samples were collected from the upright and the wholesale freezers in August 1983.

The following chemical and bioassay vitamin analyses were completed monthly in duplicate, except for percent breading, on breaded shrimp collected from each location: (1) moisture (Horwitz, 1980), (2) Kjeldahl protein (Horwitz, 1980), (3) ash (Horwitz, 1980), (4) breading (Horwitz, 1980), (5) ammonia, specific ion electrode [$NH_{3(aq)}$ and NH_4^+ are converted to $NH_{3(aq)}$] (Ward et al., 1978), (6) riboflavin (Difco Laboratories, 1977; Horwitz, 1980), and (7) thiamin (Difco Laboratories, 1977; Horwitz, 1980).

Microbiological levels were determined for all samples: (1) aerobic plate count (Food and Drug Administration, 1978); (2) MPN total coliforms and *E. coli* (FDA, 1978); and (3) MPN coagulase positive staphylococci (FDA, 1978). Aerobic plate counts were completed in duplicate.

A taste panel consisting of seven trained members evaluated samples fried for 3 min at 177°C in peanut oil for textural and flavor characteristics. A modified flavor profile was used to characterize each shrimp sample presented in duplicate to panel members. A continuous sensory scale of 0 - 5 was used to describe each flavor and textural characteristic. A score of zero indicated lack of detection by a panel member for a given trait while a socre of five indicated the strongest impression of that trait (Civille and Szczesniak, 1973; Civille and Liska, 1975). The textural characteristics defined were: hardness, chewiness, fibrousness, and moistness. Flavor characteristics (Cardello, 1981) were defined as: overall shrimp intensity, sweet, nutty buttery, old seafood, freezer burn, and rancidity. Panel members were presented with duplicate control samples of breaded shrimp in addition to duplicates of three experimental

Table 1—Schedule of product delivery dates to the retail freezers

Coffin Freezer	Upright Freezer
	29 July 1982 (Month0)
10 August 1982 (Month 1)	25 August 1982 (Month 1)
19 October 1982 (Month 3)	
	12 November 1982 (Month 4)
14 December 1982 (Month 5)	
2 March 1983 (Month 8)	23 March 1982 (Month 8)
27 April 1983 (Month 9)	27 April 1983 (Month 9)
6 July 1983 (Month 12)	6 July 1983 (Month 12)

samples. The control sample was the latest code date breaded fantail shrimp produced by a local seafood processor.

All chemical, microbiological, and sensory data sets containing two or more values for each dependent variable were analyzed by the Statistical Analysis System (SAS). Methods included General Linear Regression Model (GLM) utilizing an analysis of variance procedure to compare monthly data for each dependent variable with each storage location. Means were compared for significant differences at the 0.05 level using Tukey's studentized range test (HSD) (Ray, 1982). In the remainder of the paper, a significant difference among means refers to Tukey's studentized range test with $p < 0.05$.

RESULTS & DISCUSSION

Storage conditions

The warehouse freezer exhibited maximum daily temperature variations of 2 - 3°C, the smallest deviation of any freezer studied. Temperatures reached ≤−20°C on 70% of the monitored days. The maximum recorded temperature of −10°C occurred in the first month following a freezer malfunction. The minimum temperature of −24°C occurred during the fifth and sixth months of storage. No consistent defrost cycle was noted.

The maximum daily temperature variation of 34°C occurred for the coffin freezer in the seventh month. Normal daily temperature variations were approximately 12°C with a 6 - 8 hr defrost cycle. Daily minimum temperatures reached ≤−20°C on 1% of the monitored days and ≤−7°C on 99% of the days. The coffin freezer exceeded 0°C on 5% of the days and reached a maximum temperature of 17°C following malfunctions in the fourth and sixth months.

The upright freezer had a maximum daily temperature variation of 25°C in the third month. A general 6 - 12 hr defrost cycle with a 14 - 18°C temperature variation was observed for the freezer. Product temperature reached ≤−20°C on 36% of the monitored days and ≤−7°C on all days. The freezer exceeded 0°C on 18% of the days, reached a maximum temperature of 3°C in the third month, and a minimum temperature of −24°C in both the third and fourth months.

Chemical and physical characteristics

Tukey's studentized range test applied to the chemical data indicated that net weights (Fig. 1), percent moisture (Fig. 2), and ammonia levels (Fig. 3) were most useful in differentiating the three treatment conditions. The mean net weight of product stored in the upright freezer increased significantly in the third and fourth months, concurrent with observed ice crystal formation, but registered a net decrease by the end of the study (Fig. 1, Table 2). Weight loss in the coffin freezer was greatest. The mean net weights of the coffin freezer samples were significantly less than those of the upright and warehouse freezer samples from the third through the twelfth month of storage, except in the eighth and tenth months.

Moisture values reflected the net weight data (Fig. 2, Table 2). Significant percent moisture increases in shrimp from the upright freezer in the third and fourth months paralleled net weight gains during the same period. Product dessication was indicated by significantly lower moisture levels determined for upright samples during six of the last nine months of storage, including the final sample. Moisture levels of coffin freezer shrimp were significantly less than the other storage area samples from one month until the end of the project, except for the ninth month.

Final ammonia levels exceeded initial values in all cases (Fig. 3, Table 2). Ammonia levels from coffin stored shrimp were significantly greater than the other sample values from the fifth month of storage through the eleventh month. The ninth and tenth months were exceptions. The freezer had been restocked one month prior to sample collection.

The percent shrimp determined for all wholesale and retail samples failed to meet the Standard of Identity for breaded shrimp that specifies a minimum content of 50% shrimp for frozen raw breaded shrimp (Food and Drug

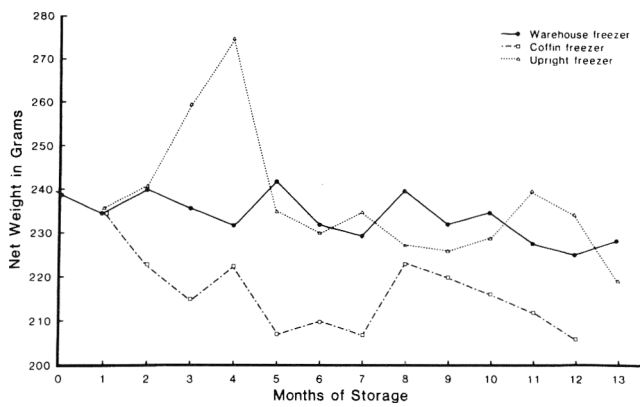


Fig. 1—Mean monthly net weight in grams of shrimp stored in warehouse, coffin, and upright freezers.

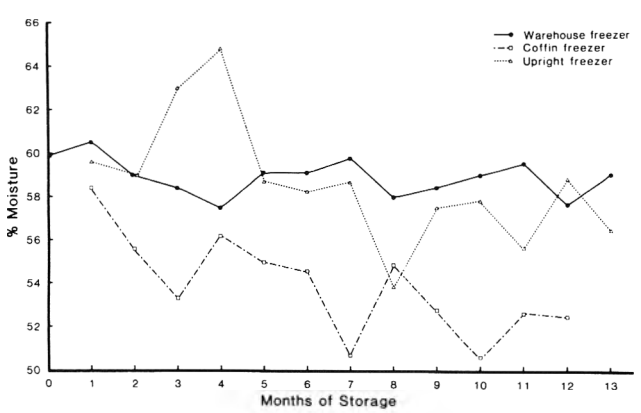


Fig. 2—Mean monthly moisture content of breaded shrimp stored in the warehouse, coffin, and upright freezers.

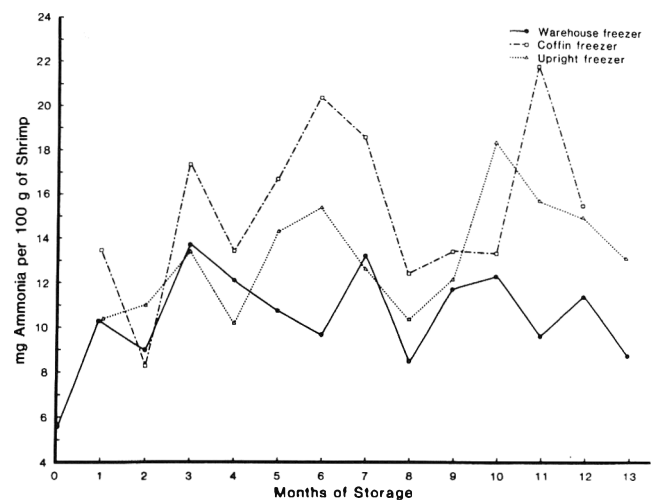


Fig. 3—Mean monthly ammonia levels (mg/100g) of shrimp stored in the warehouse, coffin, and upright freezers.

Administration, 1976). The initial wholesale freezer sample, six days after the production run, contained 5.26% less shrimp (46.24%) than was determined at the Texas facility (51.50%) on the production line. The rapid decrease in percent shrimp, net weight decreases, and percent moisture changes attributed to moisture migration from the shrimp into the surrounding breading and out of the package are consistent with studies by Rao et al. (1975) and Williams et al. (1981). Williams' samples lost 6.8% shrimp following blast freezing which reduced mean percent shrimp levels from 51.1% to 44.3%. The relative percent loss of shrimp was reduced to 4.3% by the second week. The current study determined a 5.26% shrimp loss at the end of 2 wk storage in the warehouse freezer. Rao reported 14.24% and 13.62% losses following 1 and 2 wk of storage. Overall decrease in percent shrimp was greater for the retail freezers than the warehouse freezers in Williams' and the present study. In the current study, percent shrimp loss decreased for both wholesale and retail freezers, after initial freezing, reached a minimum at 6 months and increased through the next 6 months of storage.

Williams et al. (1981) found that shrimp net weights stored in the retail freezer decreased more rapidly than shrimp stored in the warehouse freezer. Mean net weight of shrimp frozen for Williams' study decreased 0.6% following freezing by a blast freezer. After freezing, percent decreases for the wholesale freezer at 1, 6 and 9 months were less than the present study (Table 3). Our vertical freezer samples lost less weight than Williams' retail freezer while the coffin freezer results were mixed.

Moisture transfer to and from packaged breaded shrimp and the movement of moisture within the product itself is a continuing quality and regulatory problem. Storage of breaded shrimp at the retail level compounds the problem and hastens quality deterioration. Constant defrosting and higher storage temperatures of the coffin and upright freezers reduced moisture contents, net weights, and percent shrimp of products stored at the retail level more rapidly than in the controlled conditions encountered in the wholesale freezer. Higher mean temperatures observed in the coffin freezer produced significant reductions in the mean net weight of samples by the second month of storage and significant reductions in moisture content by the first month. The upright freezer samples experienced a significant net weight gain in the third and fourth months. Large ice crystals collected in the packages following 2 months of freezer defrost temperatures that exceeded 0°C. Significant desiccation was also observed in the upright freezer by the end of the study.

The nutritional quality of shrimp stored in the wholesale and retail freezers was maintained throughout the study. No significant differences in protein, thiamin, or riboflavin levels were determined among the three experimental conditions. Moisture free/ash free protein levels ranged from 23.57 - 33.05% with a mean value of 28.72% (Table 2). Thiamin levels fell rapidly during the first 2 months of storage (Table 2), stabilized and randomly ranged from 0.014 - 0 043 mg/100g shrimp with a mean of 0.025 mg/100g for the remainder of the study. The value was within 0.005 mg/100g of the 0.03 mg/100g figure presented by the Department of Agriculture (Watt and Merrill, 1975). Initial riboflavin levels decreased (Table 2). Following the fourth month, the levels ranged between 0.016 and 0.057 mg/100g bracketing the Department of Agriculture value of 0.03 mg/100g (Watt and Merrill, 1975). The mean of 0.037 mg/100g exceeded the published value.

Microbiological analyses

All samples examined microbiologically were within Georgia Department of Agriculture Guidelines for raw breaded shrimp. Mean monthly plate counts ranged from 2.40×10^4 to 5.25×10^5 organisms per gram with an average value for all treatments of 1.81×10^5 organisms per gram (Table 2). MPN total coliform organisms ranged from 7 - 110 organisms per gram. MPN coagulase positive staphylococci ranged from <3 to 23 organisms per gram. No consistent microbiological differences were noted among the three experimental conditions.

Sensory characteristics

Textural properties proved to be effective criteria to detect quality changes in breaded shrimp. Textural changes in the wholesale shrimp were less than those observed in the retail samples. The greatest changes occurred in the coffin freezer samples and these changes were detected much earlier in the storage study than quality changes associated with the upright freezer. Upright freezer sample rating differences with storage time were greater than those perceived for the wholesale freezer.

Panel members detected lower perceived moistness in samples from the coffin freezer as early as the first month of storage (significantly different by the third month). Coffin and upright freezer moistness ratings fell while warehouse samples increased by the end of the study (Table 2). Significantly greater moistness ratings were given to the upright freezer samples in the third and fourth months. The ratings correlated with significantly higher percent

Table 2—Mean initial and final physical, chemical, and sensory levels determined for shrimp stored in warehouse, upright, and coffin freezers

Parameter	Warehouse		Upright		Coffin	
	Initial	Final	Initial	Final	Initial	Final
Net Weight (g)	238.76	228.47	235.65	219.26	234.58	206.68
Moisture (%)	58.96	58.93	59.63	56.44	58.43	52.47
Ammonia (mg/100g)	5.60	8.75	10.40	13.20	13.50	15.60
Protein (% mf/af)	28.25	30.02	28.27	29.36	29.36	30.89
Thiamine (mg/100g)	0.150	0.020	0.194	0.019	0.069	0.020
Riboflavin (mg/100g)	0.071	0.031	0.063	0.045	0.068	0.037
Aerobic Plate Count (org/g x 10^4)	12.4	6.6	14.1	2.4	45.0	3.2
Moistness	2.79	2.93	2.86	2.75	2.93	1.14
Hardness	3.07	2.00	2.14	2.75	2.29	4.07
Chewiness	3.14	2.07	2.29	2.75	2.36	4.21
Fibrousness	2.43	2.21	2.36	2.68	2.57	3.82
Overall Shrimp	2.71	2.39	1.79	1.71	1.64	0.71
Nutty Buttery	2.57	2.43	2.64	1.89	2.71	0.96
Sweetness	1.93	2.39	1.93	1.68	1.86	0.61
Old Seafood	1.36	0.29	2.07	1.68	1.64	3.39
Freezer Burn	0.93	0.75	1.86	2.54	1.14	4.14
Rancidity	0.79	0.00	1.43	0.36	1.07	2.04

moisture values determined for the same samples. Significant desiccation in upright freezer samples was detected in the eleventh and twelfth months of storage when compared to the warehouse samples. The thirteenth month, 1 month after product was delivered to the upright freezer from the warehouse freezer, showed no significant difference.

Coffin freezer samples registered significantly greater hardness ratings (Fig. 4) by the fourth month of storage. The pattern continued through the twelfth month, except for the eighth month's sample when no significant difference between upright and coffin freezer samples was observed. The upright freezer was last restocked 4 months before the samples were collected. Hardness ratings for the coffin and upright freezers increased. Warehouse ratings decreased by the end of the study (Table 2).

Warehouse freezer chewiness sample ratings decreased over 13 months (Fig. 5). Coffin freezer sample ratings increased from 2.36 to 4.21 and were significantly chewier than the warehouse and upright freezers by the third month of storage. Upright ratings were significantly chewier than the warehouse freezer in the twelfth and thirteenth months (Table 2). Coffin freezer fibrousness increased from 2.57 to 3.82 and was significantly greater than the other sample ratings for 5 through 12 months of storage (Table 2).

Modified flavor profiles effectively differentiated samples collected from the three storage conditions. Flavor profiles detected sample deterioration within 3 - 5 months of storage for the coffin freezer samples, and within 11 months of storage for the upright freezer samples. Samples stored in the wholesale freezer changed least during storage.

The detected level of overall shrimp flavor decreased from the initial to final sample for all freezers (Table 2). Overall shrimp flavor detected from the coffin freezer shrimp was significantly less than the other samples by the fifth month. The flavor levels of the upright freezer shrimp remained significantly less than those of the warehouse shrimp following the eleventh month of storage. Nutty buttery ratings exhibited the same pattern as the overall shrimp values except for the tenth month when no significant differences were determined among the samples (Table 2). New product had been transferred from the warehouse freezer to both retail freezers 1 month earlier. Sweetness levels were greater at the end of the study for warehouse shrimp, but decreased for upright and coffin samples (Table 2). Sweetness showed significantly lower levels for the coffin freezer samples during the eleventh and twelfth months of storage.

Detected old seafood flavors (Fig. 6) increased most rapidly in the coffin stored shrimp and significantly exceeded other sample levels by the third month (Table 2). Levels remained highest except for the fourth and ninth month samples which were collected 1 month after the coffin freezer was restocked. Perceived old seafood flavor decreased for the warehouse and upright freezers with time, but the warehouse level fell more rapidly and remained significantly below the upright freezer ratings by the eleventh month (Table 2). Additionally, the upright freezer old seafood flavor ratings for the third and fourth months were significantly greater than the warehouse shrimp. The data reflected elevated temperatures detected in the upright freezer, when maximum defrost temperatures exceeded 0°C. The seventh month's sample, collected three months after the upright freezer was restocked, had a significantly higher old seafood rating than the warehouse freezer.

Detectable freezer burn (Fig. 7) increased with time for both retail freezer samples (Table 2). Warehouse freezer burn ratings decreased. Freezer burn results closely paralleled the patterns developed by overall shrimp flavor, nutty buttery flavor, and old seafood flavor. The coffin freezer had significantly greater ratings, indicating poorer quality, from the third through the twelfth month. Elevated temperatures in the upright freezer registered as significantly greater freezer burn than warehouse samples in the third and fourth months. Samples collected in the seventh and eighth months rated significantly higher freezer burn levels than the warehouse freezer, 3 and 4 months, respectively, after the upright freezer had been restocked. The final 3 months of the storage study provided significantly greater freezer burn levels for upright freezer samples than warehouse samples.

Table 3—Percent net weight losses in shrimp stored in wholesale and retail freezers from the present study and from that of Williams et al. (1981).

	1 Month	6 Months	9 Months
Williams' Wholesale Freezer	−1.30%	−0.20%	−1.80%
Wholesale Freezer	−1.79%	−2.79%	−2.68%
Williams' Retail Freezer	−1.40%	−6.20%	−9.30%
Upright Freezer	−1.30%	−2.34%	−5.21%
Coffin Freezer	−1.75%	−12.18%	−7.84%

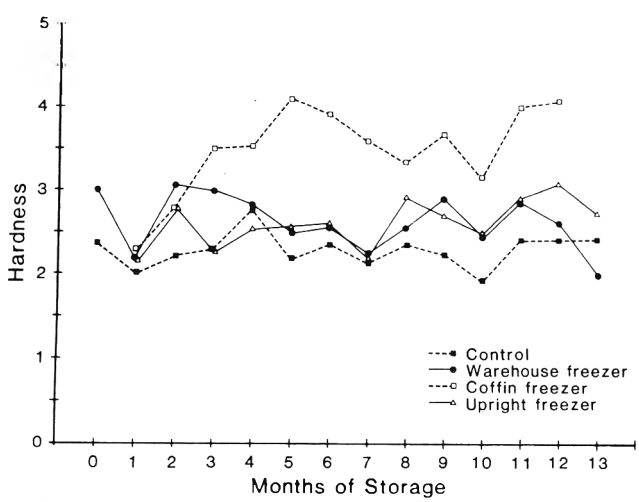


Fig. 4—Mean monthly hedonic hardness ratings for control, warehouse, coffin, and upright freezer samples.

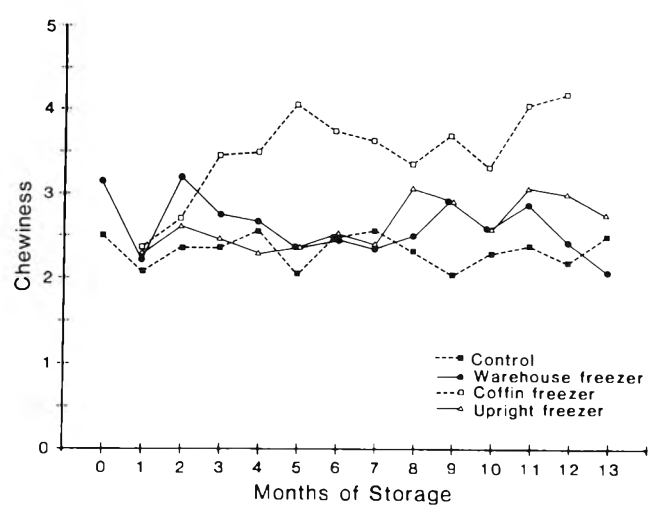


Fig. 5—Mean monthly hedonic chewiness ratings for control, warehouse, coffin, and upright freezer samples.

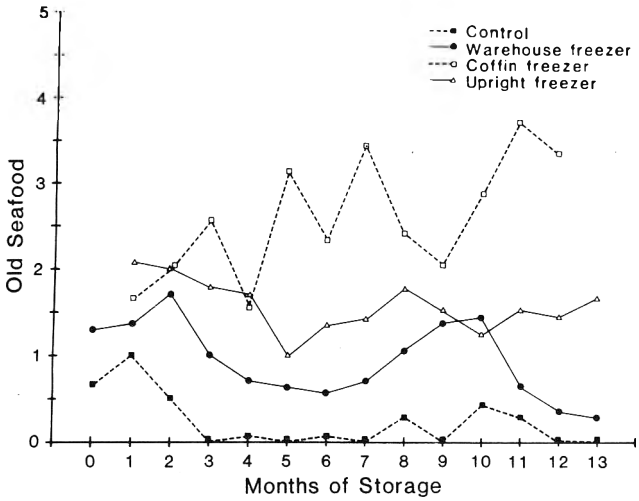


Fig. 6—Mean monthly hedonic old seafood flavor for control, warehouse, coffin, and upright freezer samples.

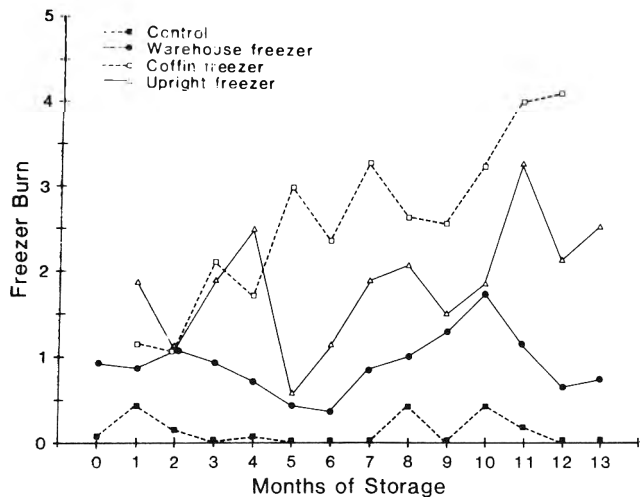


Fig. 7—Mean monthly hedonic freezer burn flavor for control, warehouse, coffin, and upright freezer samples.

Rancidity ratings increased with storage time for the coffin freezer samples while the other samples decreased with time (Table 2). The coffin freezer rancidity levels were significantly greater than warehouse values from the third through twelfth months. Again, the warehouse freezer samples fell below the upright freezer levels.

The nutritional quality of frozen breaded shrimp showed little deterioration following thirteen months of storage at the wholesale and retail level. Protein, thiamin, and riboflavin levels revealed no differences among the freezers. Vitamin levels decreased rapidly following the first 2 months of storage; however, levels remained fairly constant for the next 11 months and within 0.007 mg/100g of published U.S. Department of Agriculture nutritional data (Watt and Merrill, 1975).

Monthly monitoring of percent moisture, net weights, ammonia levels, and sensory qualities proved effective methods to evaluate product quality and differentiate frozen breaded shrimp stored in a wholesale warehouse freezer, a retail upright freezer, and retail coffin freezer. Product stored in the wholesale freezer exhibited the least deterioration with time. Sensory evaluation of warehouse samples against control products indicated no consistent significant differences following 13 months of storage.

The quality of the retail product decreased more rapidly in the coffin freezer than the upright freezer. Approximately 4 months into the study, the degraded quality of shrimp stored in the retail coffin freezer was evident when compared to upright and warehouse samples. Product quality in the coffin freezer was judged to be below the minimum consumer standards by the end of the study. Product stored in the coffin freezer was exposed to consistently higher minimum temperatures than were encountered in the upright or warehouse freezers. Daily minimum temperatures were 5 – 10°C greater than the minimum upright freezer temperatures, with few differences in daily maximum temperatures.

Elevated temperatures in the third and fourth months were readily detected by chemical and sensory monitoring of product stored in the upright freezer. Eleven months into the study, noticeable and consistent quality deterioration was detected in the shrimp samples. Product samples from the upright freezer in the seventh and eighth months (3 and 4 months after breaded shrimp had been restocked) showed significantly different sensory qualities than those observed for the warehouse freezer.

SUMMARY

RESULTS of the study indicate that significant sensory deterioration can be detected as early as 3 – 4 months after production when shrimp are stored in a retail freezer. Net weight and percent moisture changes support the contention. As the age of the product increases, shorter storage times at the retail level will precipitate measurable sensory and chemical deterioration within the product. Storage at the wholesale level below −20°C proved to be an effective method to maintain the quality of frozen breaded shrimp for at least 13 months of storage.

All tested product failed to meet the U.S. Standard of Identity for frozen breaded shrimp of a minimum 50% shrimp content, despite the fact that the breaded product contained 51.5% shrimp before freezing. Product held in the warehouse freezer had the greatest percent shrimp levels after 13 months of storage. Williams et al. (1981) and Rao et al. (1975) suggest rapid freezing and the use of finer grained breading to reduce the relative loss of percent shrimp through moisture migration. Introduction of improved moisture barrier packaging would also help alleviate quality and regulatory problems associated with moisture migration.

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Heat Transfer During Brining of Cuartirolo Argentino Cheese

JULIO A. LUNA and JUAN A. BRESSAN

ABSTRACT

The heat transfer which occurs during the brining of Cuartirolo Argentino cheese is mathematically modelled as a conduction process. The thermal diffusivity of the cheese was determined experimentally and shown to agree with the value calculated from thermal conductivity, density and heat capacity measurements. The theoretical and experimental temperature profiles in the time and spatial domains showed good agreement, thus demonstrating the applicability of the proposed model.

INTRODUCTION

THE PURPOSES of the brining of Cuartirolo Argentino cheese are to achieve cooling, uptake of salt, and moisture loss. Both heat and mass transfer processes are important in determining microbial and enzyme activity which, in turn, influence the quality of the final product (Robinson, 1981). The uptake of salt and moisture loss have been studied for several other cheese varieties (Gilles, 1977; Geurts et al., 1974, 1980; Sutherland, 1977), but these authors did not take into account the cooling of the cheese. However, the heat transfer problem is as important as the mass transfer problem for the proper design and control of the brining process. Thus the objective of this work was to design a simplified model to describe the cooling of Cuartirolo Argentino cheese. Since the heat and mass transfer phenomena occurring during brining of the cheese can be treated separately, based upon the characteristics of the process, the cooling of the cheese was mathematically modelled as a conduction process, and experimental work was carried out in order to validate the proposed model.

The physical system considered was that of batch brining in which the cheese is immersed in well-stirred brine kept at a low constant temperature. The key physicochemical parameter, thermal diffusivity, was determined experimentally and is shown to agree with the value calculated from thermal conductivity, density and heat capacity measurements.

Theory

The system under consideration consists of a well-stirred brine and the cheese is assumed to be a finite rigid medium. During the brining process the brine is kept at a low constant temperature allowing the cooling of the cheese. Simultaneously, salt diffuses into the cheese and water is lost. It is assumed that there is no chemical reaction in the system, and that there is no convective mass transfer into the pores but only diffusion.

The governing equations are (Luikov, 1964):

$$\frac{\partial C_w}{\partial t} = \text{div}\,[D_w\,(\underline{\nabla} C_w + \delta\,\underline{\nabla} T)] \tag{1}$$

$$\rho_o\,C_p\,\frac{\partial T}{\partial t} = \text{div}\,(k_{eff}\,\underline{\nabla} T) + L\,\xi\,\rho_o\,\frac{\partial C_w}{\partial t} \tag{2}$$

$$\frac{\partial C_i}{\partial t} = \text{div}\,(D_i\,\underline{\nabla}\,C_i) \tag{3}$$

Eq. (1) and (2) describe a coupled heat and mass transfer system based on the assumption that the water content in the liquid phase of the porous medium is the coupling variable. Eq. (3) describes the diffusion of other species. In Eq. (1) and (2):

$$C_w = \sum_{k=1}^{2} C_{w_k} \qquad \begin{matrix} k = 1\text{: water vapor} \\ k = 2\text{: liquid water} \end{matrix} \tag{4}$$

and

$$C_{w_k} = \frac{m_{w_k}}{m_o} \tag{5}$$

During the brining step the cheese behaves as a "porous moist body" ($C_w > C_{w_c}$), where the relative water concentration is equal to the liquid water concentration, C_{w_2} (Luikov, 1964):

$$C_w = C_{w_2} = \frac{m_{w_2}}{m_o} \tag{6}$$

and is called moisture content of the body. For the case under consideration the thermogradient coefficient:

$$\delta = \frac{D^T_{w_2}}{D_{w_2}} \tag{7}$$

is $\delta = 0$ (Luikov, 1964; Whitaker and Chou, 1983). In addition, the phase conversion coefficient, defines as (Luikov and Mikhailov, 1965):

$$\xi = \frac{D_{w_1}}{D_w} \tag{8}$$

is zero for $C_w > C_{w_c}$ because $D_{w_1} = 0$.

The former consideration leads to the following set of governing equations:

$$\frac{\partial C_i}{\partial t} = \text{div}\,(D_{eff}\,\underline{\nabla}\,C_i) \tag{9}$$

$$C_p\,\rho_o\,\frac{\partial T}{\partial t} = \text{div}\,(k_{eff}\,\underline{\nabla} T) \tag{10}$$

where "i" stands for all the diffusive species including water. The D_{eff_i} and k_{eff} are effective coefficients in an isotropic homogeneous medium (Whitaker, 1967). The coefficients k_{eff} and C_p can be assumed constant (Luikov,

Work was performed at INTEC (U.N.L.-CONICET) Guemes 3450, 3000 Santa Fe (Argentina) where author Bressan is a faculty member and author Luna is a Graduate Student. Inquiries should be directed to Dr. Bressan.

1964) in the intervals $\Delta C_w \cong 5\%$ and $\Delta T \cong 25^\circ C$, which occur during the brining step. Based on these assumptions, the heat transfer problem can be solved separately.

If we consider the cheese as a finite slab, Eq. 10 becomes:

$$\frac{\partial T(x, y, z, t)}{\partial t} = \alpha_{eff} \nabla^2 T(x, y, z, t) \qquad (11)$$

where α_{eff}, the effective thermal diffusivity of the cheese, is:

$$\alpha_{eff} = \frac{k_{eff}}{\rho_o C_p} \qquad (12)$$

and

$$\begin{aligned} -R_1 < x < +R_1 \\ -R_2 < y < +R_2 \\ -R_3 < z < +R_3 \end{aligned} \qquad (13)$$

For the case under consideration, experimental evidence shows that the initial temperature profile is flat and that a first class boundary condition ($\frac{\alpha}{hR} \to 0$) can be applied.

Accordingly, the initial and boundary conditions are:

IC:

at t = 0, at any position

$$T(x, y, z, o) = T_o = \text{constant} \qquad (14)$$

BC:

$$\begin{aligned} T(\pm R_1, y, z, t) = T_c \\ T(x, \pm R_2, z, t) = T_c \\ T(x, y, \pm R_3, t) = T_c \end{aligned} \qquad (15)$$

The solution to the problem can be expressed, as the product of three one-dimensional solutions as (Crank, 1975; Luikov and Mikhailov, 1965):

$$\frac{T - T_c}{T_o - T_c} = \sum_{\ell=1}^{\infty} \sum_{m=1}^{\infty} \sum_{n=1}^{\infty} A_\ell B_m C_n \cdot \cos(\mu_\ell \frac{x}{R_1}) \cdot \cos(\lambda_m \frac{y}{R_2}) \cdot \cos(\gamma_n \frac{Z}{R_3}) \cdot \exp[-(\mu_\ell^2 K_1^2 + \lambda_m^2 K_2^2 + \gamma_n^2 K_3^2) F_o] \qquad (16)$$

where

$$A_\ell = (-1)^{\ell+1} \cdot \frac{2}{\mu_\ell}; \quad B_m = (-1)^{m+1} \cdot \frac{2}{\lambda_m}; \quad C_n = (-1)^{n+1} \cdot \frac{2}{\gamma_n};$$

$$\mu_\ell = (2\ell - 1)\pi/2; \quad \lambda_m = (2m-1)\pi/2; \quad \gamma_n = (2n-1)\pi/2.$$

$$\frac{1}{R^2} = \frac{1}{R_1^2} + \frac{1}{R_2^2} + \frac{1}{R_3^2}; \quad K_j = \frac{R}{R_j} (j = 1, 2, 3); \quad F_o = \frac{\alpha_{eff} \cdot t}{R^2}$$

MATERIAL & METHODS

Cuartirolo Argentino cheese

The Cuartirolo Argentino cheese used in this study is a soft-type cheese made from whole milk. Coagulation is achieved in 25 min at 35°C by adding bovine rennet, calcium chloride and lactic starters. Curd is cut in 1 cm cubes, agitated in vat for 40 min, drained and poured into stainless steel molds 24 cm x 24 cm x 7 cm in size. Cheeses in molds are allowed to attain desired acid development in a 30°C room while stacked in piles three molds high. Cheeses are usually brined by immersion, for 5-6 hr, in a 22° Be brine at 7-8°C. Ripening is accomplished in 15 days in a room kept at 2-8°C and 98% humidity. The resulting product contains a maximum moisture of 55%, 18% fat, and weighs approximately 3.8 kg.

Sampling and cooling experiments

For a 10-wk period, four molded cheeses a week were taken from a commercial factory and transported to the pilot plant. After the cheeses reached the desired acidity one was used to measure the initial temperature profile (t = 0) and then sampled to determine thermal diffusivity, heat capacity, density and thermal conductivity as indicated in Fig. 1. The remaining three cheeses were brined for 6 hr in a 22° Be well stirred brine kept at 7-8°C. Two of the cheeses were sampled for physicochemical determinations, as indicated in Fig. 1, at t = 2 hr and t = 4 hr. The evolution of the temperature profile of the remaining cheese was followed by a set of eight hypodermic probes (ϕ = 1 mm). They were mounted on a special device to place them at the desired positions into the cheese. Continuous data acquisition and processing were accomplished by means of a MINC-11 microprocessor. At t = 6 hr, the end of the brining process, this cheese was also sampled for physicochemical measurements.

Average heat capacity values were determined by mixing equal weight samples from the three sampling points for t = 0, 2, 4 and 6 hr. Density, thermal conductivity and thermal diffusivity values were determined for each sampling point (Fig. 1) at t = 0, 2, 4 and 6 hr. All samples studies were duplicated.

Physicochemical measurements

The thermal diffusivity of the cheese samples was determined, applying the method proposed by Dickerson (1965). The method is based on the measurement of the transient heat transfer through a thin-walled cylindrical probe containing the cheese sample by recording the time-temperature profiles at the wall and in the center of the cylinder. The cylinder was constructed of copper with teflon

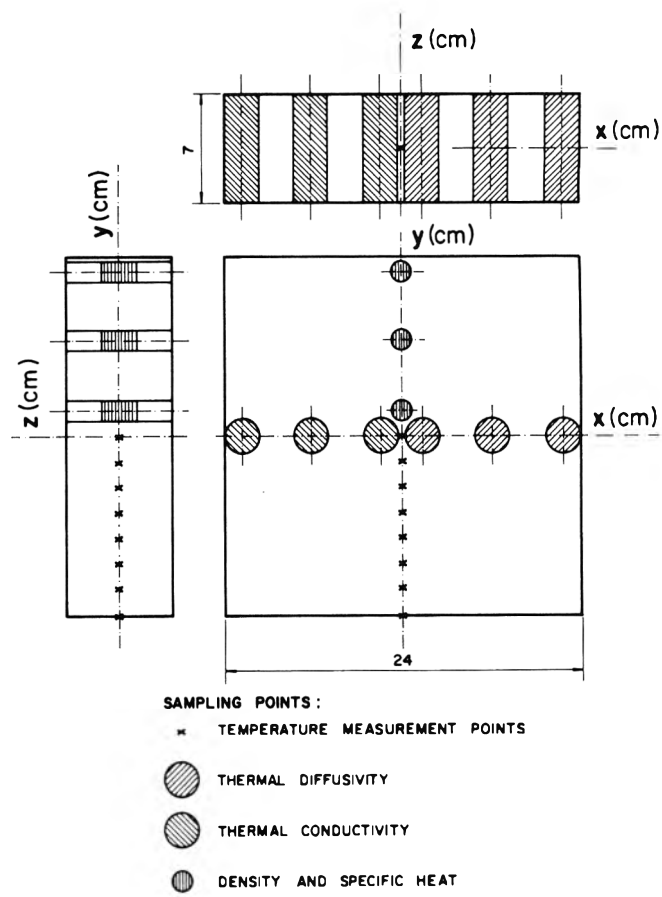


Fig. 1—Sampling and temperature measurement points.

end caps. According to Dickerson's design equation, the cylinder diameter was 2.5 cm. The cylindrical probe containing the cheese sample was submerged into a well-stirred 2°C water bath. After the probe reached the bath temperature, a constant rate heating step was initiated and the time-temperature profiles were recorded by a MINC-11 microprocessor until the temperature of both thermocouples (center and wall) was constant.

Average cheese density was determined by dividing a mass of cheese by its volume as determined by water displacement.

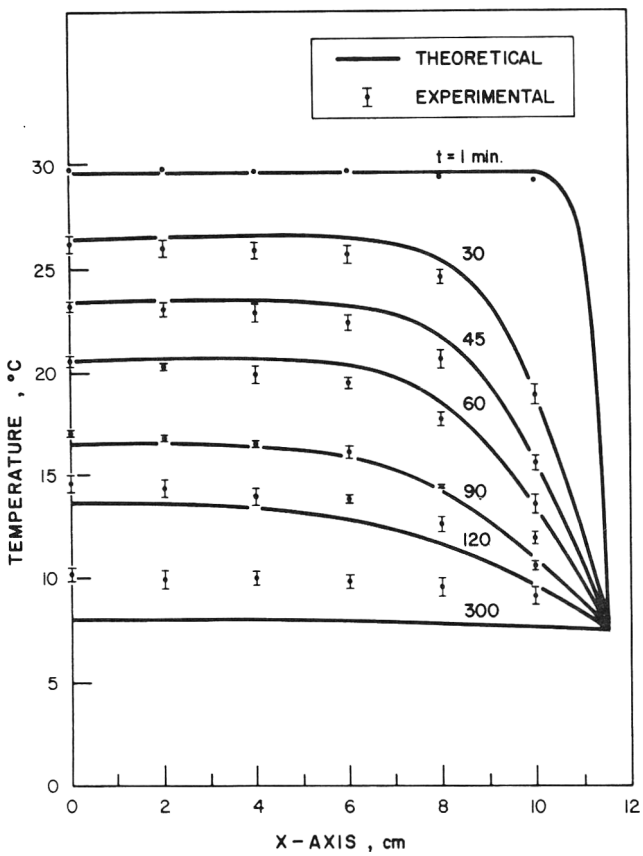


Fig. 2—Theoretical and experimental spatial (x-axis) temperature profiles during brining of Cuartirolo Argentino cheese. Points represent average experimental temperature values of 10 runs. Bars represent standard deviations.

Heat capacity was measured by Differential Scanning Calorimetry in a Metler TA 3000 system using the following scan parameters: Rate: 5°C/min; Initial temperature: 0°C; Final temperature: 40°C; Sample weight: ∼ 50 mg. From the specific heat-temperature profile a mean C_p value was calculated over the temperature interval.

The thermal conductivity of the cheese samples was determined by the Line Heat Source Method (Moshenin, 1980; Sweat and Parmelee, 1978). Logarithmic-time-temperature data were recorded on a MINC-11 microprocessor. A correlation coefficient was calculated to describe the linearity of the curve. The run was rejected if the correlation coefficient was less than 0.99.

RESULTS & DISCUSSION

THE THERMAL DIFFUSIVITY of the cheese is a critical parameter in determining the theoretical temperature profiles. Table 1 presents the experimental values of density, specific heat, thermal conductivity and thermal diffusivity.

The $\bar{\rho}_i$, $\bar{C}_{pi}$, $\bar{k}_{eff_i}$ and $\bar{\alpha}_{Di}$ are mean values over 24 samples (six samples taken at four different times). S_i values are sample average standard deviations. The $\bar{\rho}$, $\bar{C}_p$, $\bar{k}_{eff}$ and $\bar{\alpha}_D$ are mean values for ten runs. S values are pooled sample standard deviations (Himmelblau, 1970). A Bartlett test was used to check the homogeneity of the variances.

From $\bar{\rho}$, $\bar{C}_p$ and $\bar{k}_{eff}$ an $\bar{\alpha}^{calc} = 1.017 \times 10^{-3}$ cm^2/sec was calculated. The S value obtained by propagation of errors was 2.44×10^{-5}. This value shows close agreement with the $\alpha_D^{exp} = 1.015 \times 10^{-3}$ cm^2/sec ($S = 7.02 \times 10^{-5}$) determined by the Dickerson's method.

By replacing this value $\alpha_D^{exp} = 1.015 \times 10^{-3}$ cm^2/sec in Eq. (16), the evolution of the theoretical temperature profiles can be obtained (i.e. the theoretical cooling of the cheese during the brining process). The experimentally determined spatial average temperature profiles are shown in Fig. 2, along with the theoretical ones on the x-axis ($y = 0$, $z = 0$). Theoretical and experimental data at different brining times compared by means of percent deviation values (Heldman, 1974) show to agree closely as indicated in Table 2. A further check of the validity of the proposed model is the comparison of the theoretical and experimental evolution of the cheese center. Fig. 3 shows that the theoretical and experimental temperatures of the cheese center agree closely. The preceding results show that the simplified theoretical model describes very well the cooling of the cheese immersed in a well stirred brine.

The proposed model can be applied to any cheese variety of similar geometry provided the characteristic thermal transport parameter, i.e., the thermal diffusivity, is known.

Table 1—Experimental values of the four physicochemical parameters used in this study

	Parameter							
	ρ (g/cm^3)		C_p (cal/g·°C)		K_{eff} (10^4 cal/cm·sec·°C)		α_D^{exp} (10^3·cm/sec)	
Run No.	$\bar{\rho}_i$	$S_i \cdot 10^3$	$\bar{C}_{pi}$	$S_i \cdot 10^2$	$\bar{K}_{eff_i}$	$S_i \cdot 10^5$	$\bar{\alpha}_{Di}$	$S_i \cdot 10^5$
1	1.0487	10.60	0.8280	0.89	8.9138	0.96	1.107	9.10
2	1.0735	7.49	0.8433	0.95	8.7222	1.00	0.932	4.36
3	1.0508	4.49	0.8413	1.73	8.6805	1.71	0.928	7.87
4	1.0675	5.79	0.8290	1.65	9.1111	1.46	1.036	9.04
5	1.0554	7.63	0.8075	1.70	8.8725	1.11	1.032	4.70
6	1.0592	6.80	0.8215	1.68	8.9121	1.23	0.933	8.57
7	1.0519	8.66	0.7955	1.54	9.0034	0.99	1.115	4.12
8	1.0642	5.80	0.8504	1.02	8.9457	1.02	0.910	6.28
9	1.0605	4.01	0.8301	1.79	8.8312	1.25	1.023	7.90
10	1.0600	9.17	0.8027	1.81	8.8978	1.33	1.121	5.77
	$\bar{\rho}$	$S \cdot 10^3$	$\bar{C}_p$	$S \cdot 10^2$	$\bar{k}_{eff}$	$S \cdot 10^5$	$\bar{\alpha}_D^{exp}$	$S \cdot 10^5$
	1.0601	7.32	0.8245	1.52	8.8890	1.22	1.015	7.02

Fig. 4 shows the evolution of the cheese center temperature with time for several values of thermal diffusivity. Higher water contents (softer cheeses) will mean higher thermal diffusivity values and in turn faster cooling rates.

CONCLUSION

A RIGOROUS STATEMENT of the transport phenomena occurring during brining of Cuartirolo Argentino cheese followed by simplifying assumptions, based on the real system, led to a theoretical model of the cooling of the cheese. Experimental data support the validity of the proposed model. The critical thermal diffusivity value was measured by two different experimental procedures showing close agreement. The theoretical model can be used for any type of cheese provided α is known.

NOMENCLATURE

C_i : specific mass content of component "i."
C_p : reduced specific heat of the body, cal/g °C.
C_w : specific mass content = specific moisture content.
C_{w_k} : specific moisutre content of the bound water in its various states.
C_{w_c} : specific moisutre content in the moist-state range.
D_{eff_i} : effective diffusivity, cm^2/sec.
D_i : diffusivity of component "i", cm^2/sec.
D_w : moisture diffusivity, cm^2/sec.
D_{w_k} : diffusivity of the bound water in its various states, cm^2/sec.
$D^T_{w_k}$: thermal moisture diffusivity of the bound water in its various states, cm^2/sec °C.
F_o : Fourier number.
h : heat transfer coefficient, cal/m^2·sec °C.
k_{eff} : effective conductivity, cal/cm·sec °C.
L : specific vaporization heat, cal/g.
m_o : mass of dry body (the mass of the body skeleton), g.
m_{w_k} : mass of the bound water in its various states, g.
R : characteristic length, cm.
S : pooled sample standard deviation.
S_i : sample average standard deviation.
t : time, sec.
T : temperature, °C.
x, y, z : spatial coordinates, cm.
α : thermal diffusivity, cm^2/sec.
α_{eff} : effective thermal diffusivity, cm^2/sec.
δ : thermal gradient coefficient, 1/°C.
ξ : phase conversion coefficient.
ρ_o : density of the dry body, g/cm^3.

Subscripts

0 : initial state or state of dry body.
C : surrounding medium.
D : Dickerson.

Table 2—Percent deviation between experimental data and theoretical temperature profiles (Eq. 16) at corresponding times

	Time (min)						
	1	30	45	60	90	120	300
% Dev.	1.43	3.32	4.51	5.02	3.09	4.92	17.58

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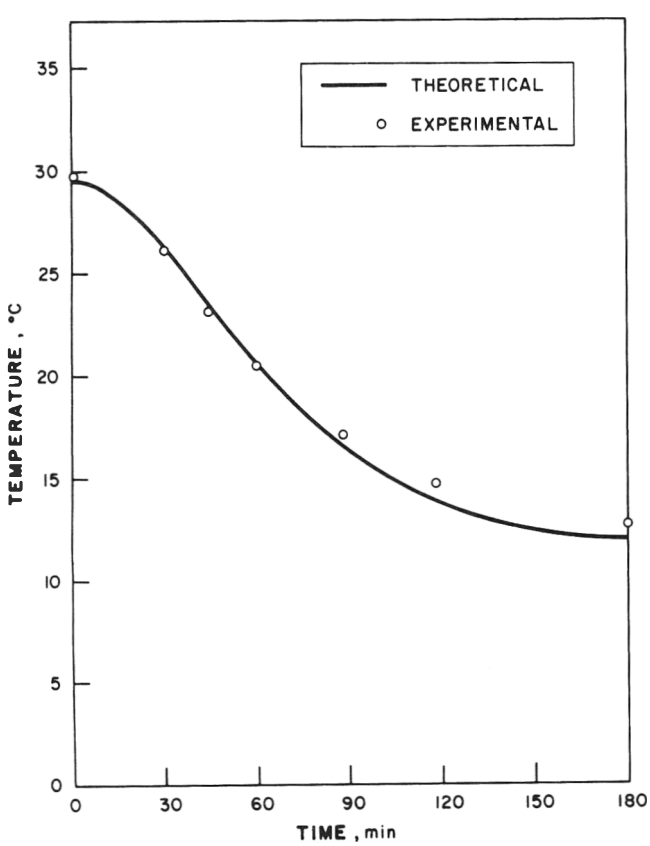


Fig. 3—Cheese center temperature evolution (x = 0, y = 0, z = 0).

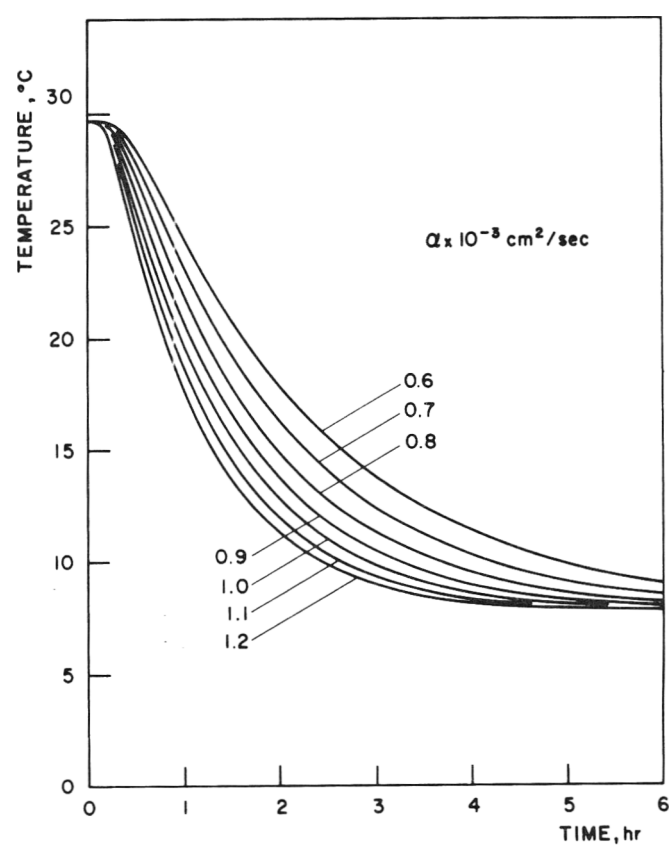


Fig. 4—Temperature profiles for several thermal diffusivity values.

Air Drying Bacon Slices to Reduce a_w: An Anti-Clostridial Alternative to Sodium Nitrite

R. P. KONSTANCE and C. C. PANZER

ABSTRACT

Low temperature air drying studies were conducted on bacon slices to determine the practicality and effectiveness of this method for reducing water activity (a_w) as an anti-clostridial alternative to sodium nitrite. Isotherms were developed to determine the extent of moisture removal required to achieve a_w = 0.92 or lower; these levels have been found to inhibit *C. botulinum*. Drying to these levels of inhibition can be accomplished in approximately 1.75 hr. Drying rates are significantly affected by air temperature, air flow rate, and slice overlap. Experimental data indicate drying can be accomplished with no substantial change in product quality.

INTRODUCTION

THE PROPOSED REDUCTION or elimination of sodium nitrite as a curing component in processed meats has been the subject of considerable research over the last decade. During this time, attention has been focused on the study of bacon, where residual nitrite has shown a high degree of correlation with the formation of carcinogenic N-nitroso compounds (Pensabene et al., 1979, 1980). Nitrite has several well-known beneficial effects in meat curing, the most important of which is the role that sodium nitrite plays in retarding *Clostridium botulinum* growth and toxin production. In the United States and Canada, studies have shown that the risk of toxin production is minimal for both normal (120 - 150 ppm) and low (50 - 60 ppm) nitrite levels even when the bacon has undergone temperature abuse for 1 wk at 27°C (Hauschild and Helsheimer, 1980). Unfortunately, trace amounts of nitrosopyrrolidine were found even in low nitrite bacon (Sen et al., 1974). This fact, coupled with the potential for more severe temperature abuse, especially in underdeveloped countries, indicates the need for continued research for an alternative to sodium nitrite as an anti-botulinal agent. Sofos and Busta (1980) list a number of physical and chemical controls as potential alternatives to sodium nitrite. One such alternative, dehydration, is the subject of this paper.

The dehydration of meat has been practiced for centuries as a method of preservation. Removing water by drying concentrates the water-soluble nutrients and reduces the water activity (a_w) thereby inhibiting or preventing the outgrowth of spoilage and toxinogenic microorganisms. Processed bacon exhibits a water activity ranging from 0.97 to 0.99 and is thus liable to spoilage through the growth of a wide range of organisms. By reducing the water activity below 0.93, many of the organisms, and most importantly *C. botulinum*, can be inhibited (Ohye and Christian, 1967; Riemann, 1973; Tompkin and Christiansen, 1976).

Various attempts have been made at lowering the water activity of bacon by drying. Hennergardt (1975) has reported on a method of freeze-drying bacon slices. These freeze-dried slices were stored in vacuum packages for 6 months at 38°C with no reported adverse effects. For most applications, however, the freeze-drying process is economically prohibitive. A number of researchers have employed a precook or prefrying step to accomplish the same purpose. Mattson (1978) demonstrated that precooking could be used to lower the bacon a_w to 0.85. It has also been established that a combination of microwave and hot air or steam reduced the a_w of bacon to acceptable levels for *C. botulinum* inhibition (Anon., 1977). A large amount of fat rendering is, however, inherent to the precooking operation, leaving a product that although acceptable for hotel/restaurant applications, is not generally salable to individual consumers.

In order to minimize the amount of fat rendering as well as investigate a more economical method of drying bacon, low temperature, low humidity air drying techniques were studied. This paper presents the results of that study.

MATERIALS & METHODS

THE BACON used in this study was purchased locally as fully processed slabs. Specific formulations for the curing step were unknown, but composition included sodium chloride, sucrose, sodium ascorbate, sodium nitrite (≤100 ppm), sodium tripolyphosphate, and hydrolyzed plant protein. The bacon was cooked in the smokehouse for 9 hr yielding a product that was approximately 99.5% of green weight. The bacon was sliced to the desired thickness (±0.01 cm) at this laboratory using a Berkel Model 180 GS high speed slicer. Slice order was carefully maintained allowing for the selection of specific slices for certain analytical evaluations (i.e., fat analysis, water activity measurement, and moisture analysis). By using alternate slices for drying, the fat content of each dried slice was estimated by a fat analysis of the two surrounding slices. Because of the high losses of fat encountered when preparation by grinding was used, the fat analysis was done on bacon cut into 3-cm squares. These pieces were soaked in petroleum ether for 18 hr and then extracted for 5 hr using Soxhlet extraction.

A number of bacon slabs were used for the drying experiments. Replicate experiments were conducted on adjacent or near adjacent slices from the same belly. Drying of all individual bacon slices, including pieces for the separate fat and lean study, was accomplished in a cylindrical, Plexiglas laboratory scale dryer, with a diameter of 2 inches. Type T thermocouples were placed inside the dryer to measure surface and dry bulb temperature. A rotameter was used to measure the air flow rate. Air velocities over the slices in this system ranged from 15 - 50 cm/sec. The air humidity was controlled by bubbling the drying air through specific concentrations of sulfuric acid to achieve a desired humidity of 10 - 50%. The individual bacon slices were hung vertically to minimize surface contact and heating due to conduction. Moisture loss (measured as weight loss) was determined every 30 min. Drying curves were constructed based on these measurements. Duplicate data are shown for all drying curves. These duplicate experimental data were compared by analysis of their representative regression equations using the method described by Neter and Wasserman (1974). Experimental data were then pooled and a composite regression curve is presented.

Water activities were measured using a Kaymont "Rotronic Hygroskop (Model DT)." The system was calibrated using saturated salt solutions of known relative humidity (Greenspan, 1977). The accuracy of the system was determined to be ±0.015 a_w units. A water jacket on the sample holder fed by a constant temperature bath maintained the desired temperatures (±0.1°C) for all water activity measurements.

Multiple, shingled slices were dried in a forced circulation air dryer (Sinnamon et al., 1968). In this dryer, dehumidification was

Authors Konstance and Panzer are with the USDA-ARS, Eastern Regional Research Center, 600 E. Mermaid Lane, Philadelphia, PA 19118.

accomplished using a desiccant column packed with 4 mesh $CaSO_4$. An American Meter "Diacon" dew-point apparatus was used to monitor and control downstream humidity by proportioning the amount of air passed through the desiccant column and the air which bypassed desiccation. The air rate was measured with a Meriam Instrument Model 50 MCZ-4P laminar flow element connected to an inclined manometer. Air velocities ranging from 50 - 180 cm/sec were used for these experiments. Drying curves were constructed in the same manner used for the individual slices. All slices were analyzed for moisture content by the standard vacuum oven method (AOAC, 1970).

A sensory evaluation, using 16 trained taste panelists, was conducted to establish differences in the bacon slices due to drying. Slices from a given bacon slab were separated sequentially into groups of eight. Within each group, four slices were identified for analysis in the undried state (fat, peroxide, and two taste samples). The remaining four slices were air dried and prepared for analysis (fat, moisture, peroxide, and taste). Samples were maintained in refrigerated storage until time of analysis. The bacon slices were fried at 175°C in a preheated electric pan. Frying time for the undried bacon was 6 min (3 min each side) and for the dried bacon slices was 4 min (2 min each side). After frying the bacon was drained to remove excess grease. The extent of rancid off-flavor development was assessed with a scoring system based on a six-point scale (1 = very strong; 6 = none). Acceptability of the bacon samples was determined with a nine-point hedonic scale (1 = dislike extremely; 9 = like extremely) of Peryam and Pilgrim (1957). Taste scores were evaluated using an analysis of variance and Duncan's multiple range test (Duncan, 1955). Samples were panelled at 1-wk intervals over a 3-wk span. Peroxide determinations were made using AOAC Method 28.023 (AOAC, 1980).

RESULTS & DISCUSSION

Moisture sorption isotherms

Konstance et al. (1983) showed that the type of drying had an impact on the desorption isotherms of bacon slices at 25°C and constant fat level. In Fig. 1, the isotherms for air and freeze-dried bacon slices are shown. Freeze-dried slices exhibited higher water activities at a given moisture content. This may be due to changes in the water-holding capacity created by the freezing process (Lawrie, 1966).

These differences indicate that sorption data for freeze-dried bacon slices will not adequately describe the requirements for an air drying system. In developing the moisture desorption data for air-dried bacon, two parameters, bacon composition and storage temperature, were considered.

Variations in bacon composition, especially in terms of fat:lean ratio, are large. The effects of this variability on the desorption isotherm and in turn the amount of drying required to assure a "safe" water activity level of 0.92 was of primary concern. In Fig. 1, for example, the desorption isotherms (25°C) are shown for air-dried bacon slices at 10%, 50%, and 70% fat. At constant water activity, the quantity of water sorbed increased with decreasing fat content.

The desorption isotherms for air-dried bacon slices, stored at temperatures ranging from 5 - 40°C were, for all practical purposes, identical at $a_w > 0.85$. These findings agree with the data of Konstance et al. (1983) for freeze-dried slices. In Fig. 2 moisture reduction is plotted against water activity. The data represent 25 separate experiments from three bacon bellies with essentially the same cure composition. In each experiment a bacon slice of known fat content was dried for a specific period of time to a desired level of water activity. The fat contents investigated were 40%, 50% and 60%. Moisture reduction was calculated from the initial weight, final weight, and moisture content of each dried bacon slice. The accuracy of the water activity measurements was ±0.015 a_w units. Although a fat-independent relationship was observed, a knowledge of the initial moisture content of the bacon was necessary for a_w prediction. A moisture reduction of approximately 50% was required to achieve $a_w = 0.92$.

Single-sliced drying

Drying rate curves of the major components of bacon, fat and lean, were developed. Drying was accomplished in air under mild conditions. Bacon fat (Fig. 3), which has a low moisture content, exhibited a drying rate that is characteristic of internal diffusion control. Bacon lean (Fig. 4), on the other hand, underwent an initial falling rate period that appeared to be linear with moisture content (section AB, Fig. 4). This falling rate period has been described by Treybal (1968) as unsaturated surface drying. Constant rate drying was not observed in bacon fat or bacon lean.

Commercially produced bacon slices are typically 0.24 - 0.27-cm thick, with a fat content ranging from 45 - 65% and a moisture content ranging from 25 - 40% (wet basis). Fig. 5 shows a drying rate curve for a typical slice of bacon. As with bacon lean, a period of unsaturated surface drying occurred initially. This linear falling rate period continued until the moisture content was reduced to approximately 0.17g H_2O/g solids. The moisture content required to achieved inhibition levels of a_w (0.90 - 0.92), was approximately 0.25g H_2O/g solids. Dehydration of a typical bacon

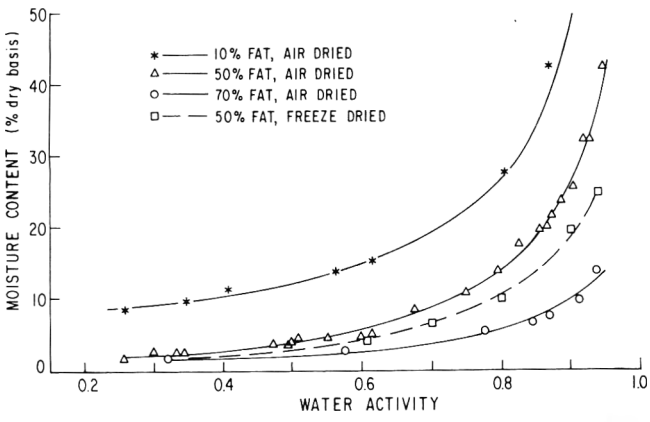


Fig. 1—Moisture desorption isotherms of bacon slices at 25°C. Effects of composition.

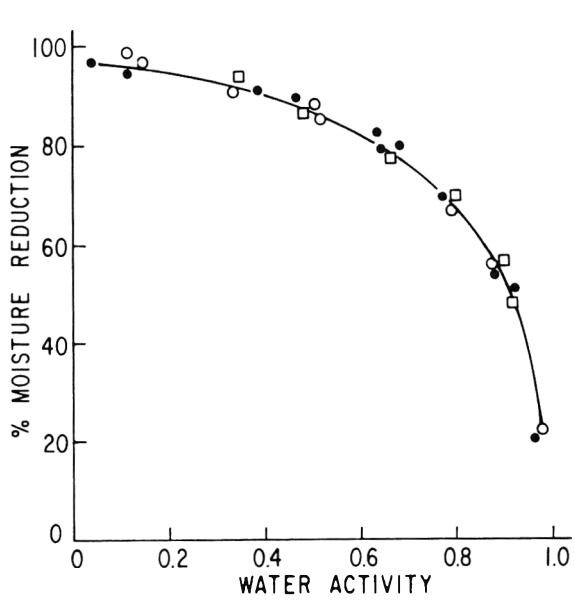


Fig. 2—Relationship of moisture reduction and a_w at various fat levels (○ - 40%, ● - 50%, and □ - 60%).

slice to achieve this a_w reduction would occur entirely in the region of unsaturated surface drying. The derivation of the equations describing the falling rate period were presented by Treybal (1968).

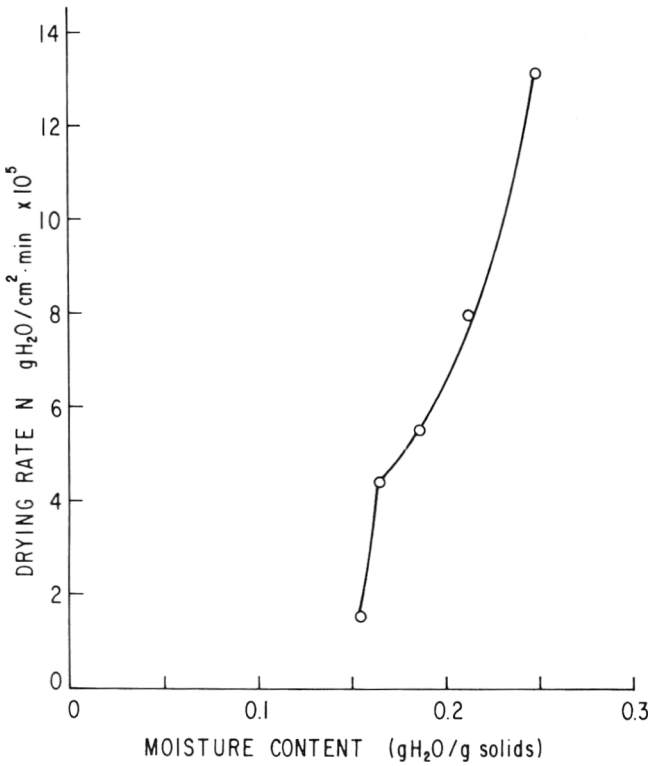


Fig. 3—Rate of drying bacon fat (25°C, 10% RH, 36 cm/sec).

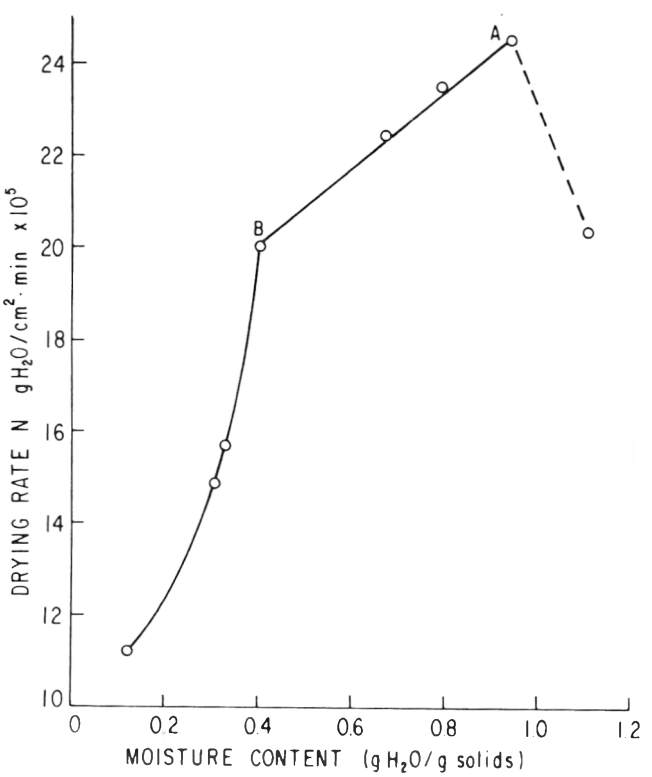


Fig. 4—Rate of drying bacon lean (25°C, 10% RH, 36 cm/sec). Section A-B is the linear falling rate period.

Based on the assumption of unsaturated surface drying, the rate of drying during the falling rate period can be defined generally by Eq. (1):

$$N = \frac{-L_s dx}{A\, d\theta} \tag{1}$$

For any given set of drying conditions, the drying rate of a given bacon is only a function of moisture content and time. Rearranging to yield the drying time gives:

$$\int_0^{\theta} d\theta = \frac{L_s}{A} \int_X^{X_1} \frac{dX}{N} \tag{2}$$

where: θ = drying time (min); A = drying surface area (cm^2); X_1 = initial moisture content (g H_2O/g solids); X = moisture content at time θ (g H_2O/g solids); N = drying rate (g H_2O/(min · cm^2); L_s = weight of dry solids (g).

As derived by Treybal (1968), in the special case where N is linear in x, as occurs during unsaturated surface drying, and when N_m is the logarithmic average of the rate N at moisture X, and N_1 at moisture X_1, then Eq. (2) can be written as:

$$\theta = \frac{L_s (X_1 - X)}{A N_m} \tag{3}$$

Noting that $L_s = \rho_s Z_s A$, where ρ_s = apparent bacon density (g solids/cm^3) and Z_s = slice thickness (cm), and expressing Eq. (3) in terms of a moisture ratio yields

$$\frac{X}{X_1} = 1 - \frac{N_m \theta}{\rho_s Z_s X_1} \tag{4}$$

The mechanism of evaporation during the falling rate period is the same in a constant rate period. The effects of such variables as slice thickness, air velocity, temperature, and relative humidity are thought to be the same as for constant rate drying (Treybal, 1968). In order to establish the validity of the assumption of a linear falling rate period for the data presented in this study, the effect of these variables on drying rate was investigated.

Effect of slice thickness

Drying data were obtained for bacon slices at four thick-

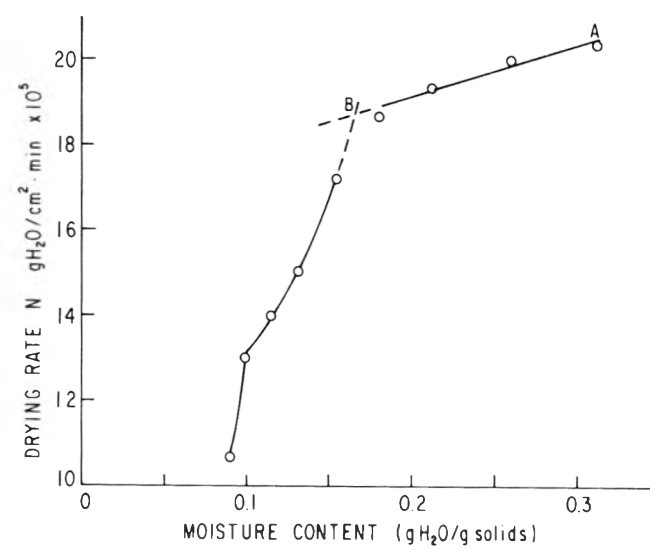


Fig. 5—Rate of drying whole bacon slice (25°C, 10% RH, 36 cm/sec, 0.24 cm thickness). Section A-B is the linear falling rate period.

nesses (0.16, 0.24, 0.32, and 0.48 cm). As before, these slices were air dried at a temperature of 25°C, a relative humidity of 10%, and an air velocity of 36 cm/sec. The drying curves (Fig. 6) appear to be linear in the first 120 - 150 min of drying. The slopes of these linear portions, calculated using regression analysis, were 4.91×10^{-3}, 3.28×10^{-3}, 2.19×10^{-3}, and 1.68×10^{-3} at thickness of 0.16 cm, 0.24 cm, 0.32 cm, and 0.48 cm, respectively. In unsaturated surface drying, the slopes of these lines should be proportional to $1/Z_s$. Comparison of the slopes shows good agreement to this expected proportionality.

The drying curves were predicted from Eq. (4), using the log mean average of the drying rates determined from Fig. 5 ($N_m = 1.98 \times 10^{-4}$ g $H_2O/(cm^2 \cdot min)$) and an apparent density of $0.6 g/cm^3$ as determined by volume weight measurements. The predicted curves showed excellent agreement with the experimental data through 120 min of drying. These results support the hypothesis that the mechanism of bacon drying to anticlostridial water activity levels is unsaturated surface drying. Deviation from predictions after 120 min may be attributable to the end of unsaturated surface drying.

Effects of air velocity

Fig. 7 illustrates the course of drying of bacon slices, 0.16-cm thick, that were air dried at 25°C and three different air velocities (18, 36, and 46 cm/sec). The calculated values of N_m increased from 0.84×10^{-4} to 2.43×10^{-4} g/(cm^2 – min) as the air velocity was increased from 18 to 46 cm/sec at 10% RH. For most foods dried with the air flow parallel to the drying surface, the drying rate during constant rate drying, N_c, was proportional to the mass velocity, G, g/(cm^2 · sec) (Van Arsdel and Copley, 1963) as shown in Eq. (5):

$$N_c = d\, G^{0.8} \quad (5)$$

where d is a constant. This empirical relationship was essentially confirmed in this study by correlation of initial drying rates (calculated from initial slopes of the drying rate curves in Fig. 7) to G via a power law expression with an exponent of 0.72.

The drying data shown in Fig. 7 support the assumption of unsaturated surface drying through the first 120 min of drying for the hither air velocities (36 and 46 cm/sec). At an air velocity of 18 cm/sec, this model of drying was not followed for the entire period required to reach an a_w of 0.92.

Effect of temperature and humidity

The effects of drying temperature (20°C, 25°C, 29°C, 35°C) for bacon slices, 0.32-cm thick, dried at a relative humidity of 10%, an air velocity of 36 cm/sec were examined. For unsaturated surface drying, the drying rate (N), was proportional to the difference between the air temperature (T_q) and the surface temperature of the bacon (T_s). These temperature differences varied by less than 10% for slices dried at temperatures less than 35°C, and the rate of drying in this small temperature range was essentially identical. During drying at these temperatures surface temperature remained below 30°C and no melting of fat was apparent. When the dry bulb temperature was increased to 35°C, the slices exhibited a coating of melted fat over most of the bacon surface area. Surface temperature increased to 32°C and a reduction in the drying rate was noted. Drying temperature should, therefore, be controlled at 29 - 30°C to prevent melting of fat while obtaining the best drying rate.

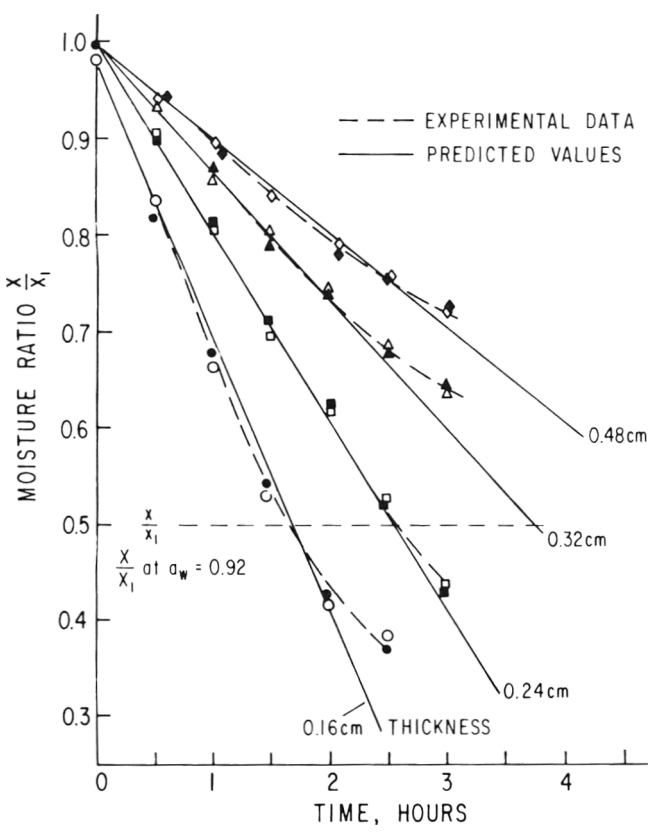


Fig. 6—Effect of slice thickness on drying of whole bacon slices at 25°C, 10% RH, and 36 cm/sec. (○,●; □,■; △,▲ represent duplicate data.)

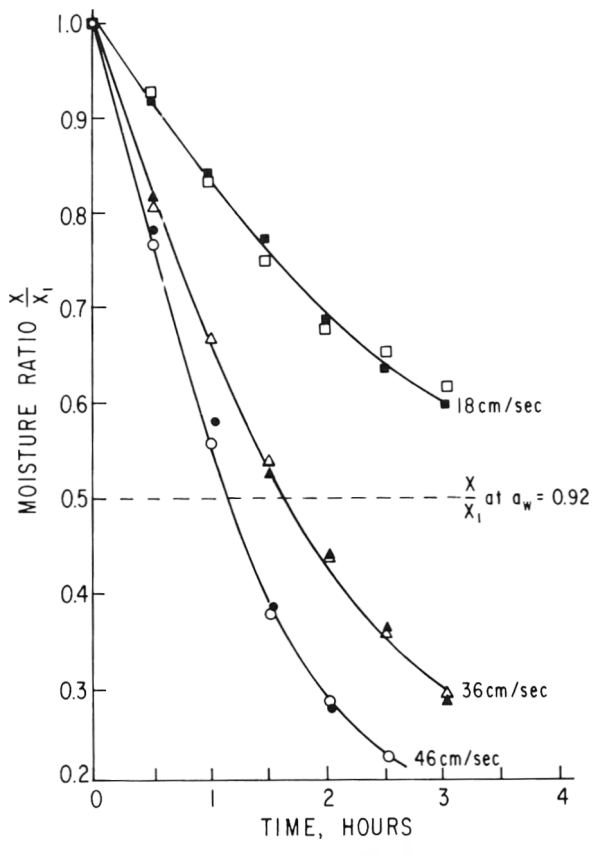


Fig. 7—Effect of air velocity on drying of whole bacon slices at 25°C, 10% RH, 0.16 cm thickness. (○,●; □,■; △,▲ represent duplicate data at air velocities of 18, 36, and 46 cm/sec, respectively.)

Humidity effects on the drying curves for bacon slices 0.24-cm thick dried at 25°C and 36 cm/sec are shown in Fig. 8. Reduction in drying rate accompanied the increase in humidity. Drying rates from Fig. 8 in the unsaturated surface drying period were 10% RH – 1.98×10^{-4} g/cm^2 · min; 50% RH – 1.02×10^{-4} g/cm^2 · min; 85% RH – 0.55×10^{-4} g/cm^2 · min. At higher relative humidities total drying time would extend beyond the unsaturated surface drying region and significantly longer drying time would be required.

As can be seen from the data, individual bacon slices from the bacons studied generally followed an unsaturated surface drying regime when typical commercial bacon slices were used. Deviation from linearity was experienced with thicker slices and at the lowest air velocity. In these cases, the unsaturated surface drying did not extend to the required level of water activity.

Table 1—Analysis of drying data using the Yates algorithm[e]

Source of variation	Observed variable	Estimated effects
a	0.00180	0.0135
b	0.00289	0.0121
ab	0.00446	0.0059
c	0.00189	0.0053
ac	0.00321	0.0010
bc	0.00262	0.0028
d	0.00222	0.0058
ad	0.00340	0.0008
bd	0.00229	0.0030
cd	0.00250	0.0015
⩾ third order	–	<0.0012

[a] Shingling factor
[b] Air velocity
[c] Air temperature
[d] Air humidity
[e] Box et al. (1978)

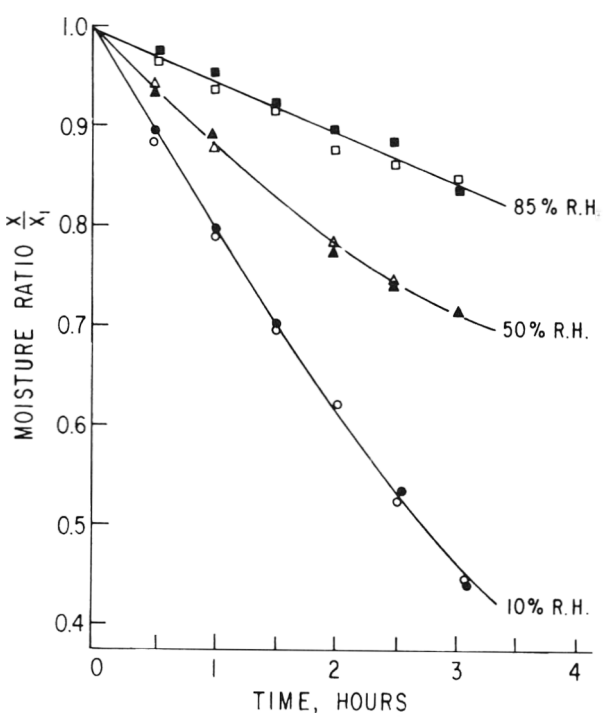


Fig. 8—Effect of humidity on drying of whole bacon slices at 25°C, 36 cm/sec, 0.24 cm thickness. (○,●; □,■; △,▲ represent duplicate data.)

Shrinkage

Shrinkage was the most evident change in product characteristics that occurred during air drying of bacon slices. During drying at 25°C, 36 cm/sec, and 10% RH, for typical bacon slices where no fat losses occurred, weight reduction was due solely to moisture removal. Shrinkage was caused primarily by the self-contraction of the material as moisture was removed. The volume of the material is often a linear function of the mean moisture content during shrinkage (Keey, 1972), as expressed in Eq. (6):

$$V_f = V_o (1 + Cx) \quad (6)$$

where: V_f = product volume at final moisture (cm^3) V_o = moisture free volume (cm^3); C = bulk shrinkage coefficient; x = moisture content, dry basis, g H_2O/g dry bacon. Bacon shrinkage was evaluated in terms of surface area because (a) no measurable change in slice thickness occurred during drying, and (b) slice thickness was small, relative to surface area. Shrinkage was evaluated using a surface area analog of Eq. (6).

$$A_f = A_o (1 + gx) \quad (7)$$

where A_f = product area at final moisture (cm^2); A_o = surface area at zero moisture (cm^2); g = area shrinkage coefficient; and x = moisture content, dry basis, g H_2O/g dry bacon.

In Fig. 9 A_f/A is plotted against moisture. Here, A is the surface area of a raw bacon slice (40 cm^2). The bacon was dried at 25°C with the drying air at 10% RH and an air velocity of 36 cm/sec.

Regression analysis results inthe linear relationship:

$$A_f/A = a + bx \quad (8)$$

(a = 0.668, b = 0.667, and r, the correlation coefficient = 0.965). The intercept a, in this case, is equal to A_o/A giving the following relationship:

$$A_f = A_o \left(1 + \frac{b}{a} x\right) \quad (9)$$

and

$$A_f = 26.53 (1 + 0.998 x) \quad (10)$$

Slices from the bacon studies, dried to an a_w = 0.92 (corresponding to moisture content of 29%), would shrink approximately 13% during drying.

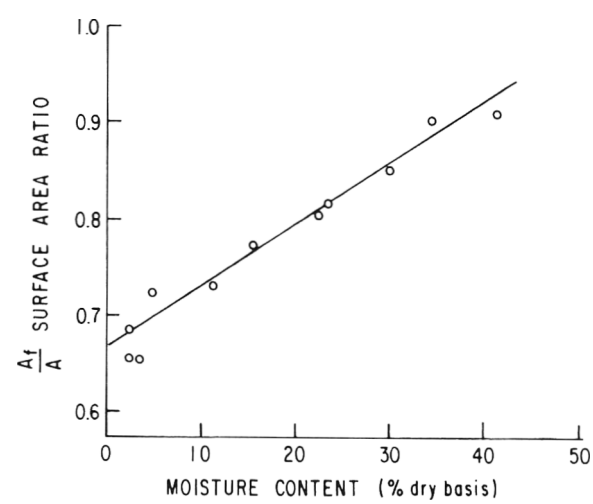


Fig. 9—Shrinkage of whole bacon slice. (Air dried at 25°C, 10% RH, 36 cm/sec, 0.24 cm.)

Packaged bacon drying

Since continuous drying of individual bacon slices presents serious packaging and handling problems from a processing standpoint, studies were conducted on air drying bacon with the slices overlapped (shingled). The data obtained from the single-slice studies were used as the basis for determining the range of the parameters investigated in the shingled experiments. A 2^4 experimental design, incorporating the following parameters, was used to determine drying rate response: S_f = shingling factor (%) – 50, 75; V = air velocity (cm/sec) – 180, 50; T = air temperature (°C) – 25, 18; and RH = air humidity (%) – 10, 50. Shingling factor represents the amount of product overlap: i.e., at a shingling factor S_f = 75, 25% of the product surface was exposed. Using the Yates algorithm (Box et al., 1978) the effects of these parameters on the slope of the drying cuve $N/\rho_s Z_s X_1$ were analyzed (Table 1). The variance was estimated by assuming that the three and four-factor interaction effects reflect contribution to experimental error alone. Calculation of the pooled estimate of the variance (s_R^2) was made utilizing the sum of squares (ss) from these higher order interactions. Locating the mean of the reference distribution at zero and comparing the estimated effects showed that: shingling facator (a), air velocity (b), the two-factor interaction of shingling and air velocity (a, b), and humidity (d), significantly affected the slope of the drying curves. The effects of these parameters are shown in Fig. 10 (shingling factor and air velocity) and Fig. 11 (temperature and humidity). Utilizing a 50% shingling factor 24 slices of bacon (average thickness 0.26 cm) can be dried to a water activity of 0.92 in approximately 100 min using 10% RH, 25°C, and an air velocity of 180 cm/sec.

Table 2—Sensory evaluation for rancidity development[a]

Time	0 wk	1 wk	2 wk	3 wk
Sample				
Undried	4.93	5.07	5.31	5.09
Dried	4.64	5.47	5.31	5.27

[a] 1 = very strong; 6 = none.

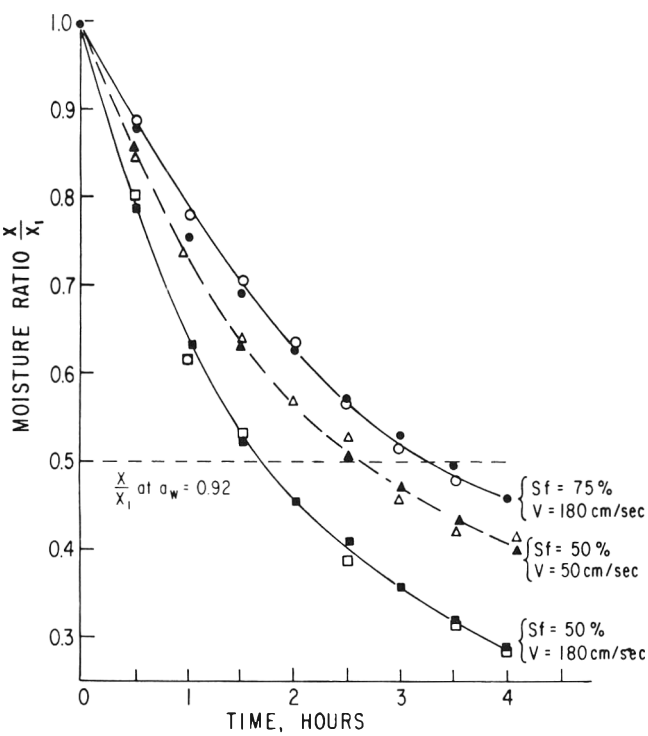


Fig. 10—Effect of air velocity (v) and shingling factor (S_f) on drying of shingled bacon, 24 slices at 25°C, 10% RH, 0.26 cm thickness. (○, ●; □,■; △,▲ represent duplicate data.)

Product quality

The uniformity of drying of the bacon slices when shingled at 75% was poor. A large variation in the moisture content, when individual slices in a package were compared, approached 30%. When a 50% shingle was used, uniformity was excellent. With the exception of end effects, where slices were 5 – 10% drier, variation of the water activity across a package of 24 slices was insignificant ($\sigma^2 = 7 \times 10^{-5}$ at a mean a_w = 0.904). The water activity of each end slice was about 6% lower (a_w = 0.850). The decrease in uniformity due to the end effects would be minimized in a continuous operation.

The taste panel was unable to detect any significant differences (P = 0.95) in rancidity between the dried and undried samples (Table 2). All samples were characterized as "very faint rancidity" (score = 5). Tasters rated all of the samples in the "like moderately" range in the hedonic test (Table 3). Differences in the scores of the hedonic test were not statistically significant (P = 0.95). Although sodium nitrite is not a strong antioxidant (relative to most phenolic antiox.dants) the low concentrations of this compound in the bacon samples may in part have contributed to the delay of oxidation.

Table 3—Overall sensory evaluation (Hedonic evaluation)[a]

Time	0 wk	1 wk	2 wk	3 wk
Sample				
Undried	7.07	7.27	7.38	7.00
Dried	7.00	7.33	7.23	6.91

[a] Hedonic scores: 1 = dislike extremely; 9 = like extremely.

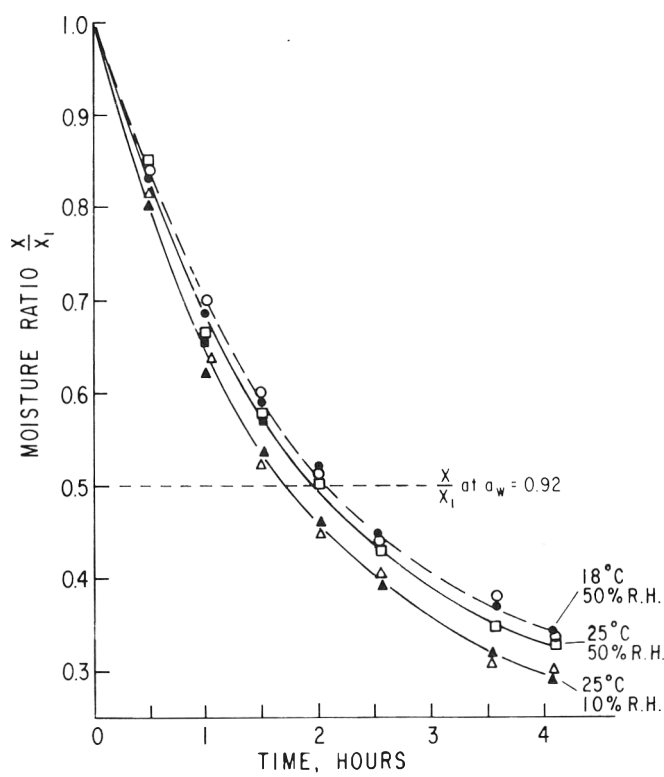


Fig. 11—Effect of temperature and humidity on drying of shingled bacon, 24 slices at 50% shringling, 180 cm/sec, 0.26 cm thickness. (○,●; □,■; △,▲ represent duplicate data.)

Peroxide values were determined during storage as a means of monitoring fat oxidation. In all cases the peroxide values were less than 1.0 meq/1000g fat. A duplicate experiment to measure peroxide development in the dried bacon samples showed the same low peroxide levels during the 3-wk storage period. These low peroxide levels indicate that autoxidation occurs in the early phases of the induction period and supported the findings of the taste panel.

CONCLUSIONS

THE MOISTURE SORPTION relationships for dried bacon slices were dependent upon the drying method. Within an air-dried system the most significant change in the sorption relationship occurred with differences in the fat/lean ratio. Using a fat-independent relationship where percent moisture reduction was compared to water activity, it was found that removing 50% of the water in bacon lowered water activity levels to 0.92, which is adequate in inhibiting growth and toxin production by *C. botulinum*.

Drying of individual bacon slices within the limits of the drying schemes studied generally followed an unsaturated surface drying regime. The drying rate of typical bacon slices was a linear function of moisture content throughout the period of interest ($a_w \geqslant 0.92$). During this period, the drying rate of these bacon slices exhibited a typical response to parameters such as air velocity, humidity, and slice thickness. Deviation from this linearity was experienced as slice thickness increased and at very low air velocities. In the narrow range of temperatures studied, little differences in the drying rate was noted as temperature was increased. When the drying temperature reached 35°C however, the drying rate decreased as fat began to melt. When shingled slices were dried, the drying rate decreased. Drying at a 50% shingling factor, 25°C, and 180 cm/sec air velocity reduced the water activity of bacon slices (0.26-cm thick) to 0.92 in approximately 1.75 hr with no melting of fat and no discernable adverse effect on product quality. The slices shrank linearly in this first 2 hr of drying to approximately 85% of the original size. Taste tests for rancidity and overall flavor, as well as peroxide determinations, indicate that bacon slices dried in air were not different from their undried counterparts. Air drying of bacon can be considered a possible anticlostridial alternative.

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Chemical and Sensory Properties of Thirteen Major Beef Muscles

F. K. MC KEITH, D. L. DE VOL, R. S. MILES, P. J. BECHTEL, and T. R. CARR

ABSTRACT

Chemical and sensory properties of the following thirteen muscles from ten Angus steers were evaluated: semimembranosus, semitendinosus, adductor, biceps femoris, rectus femoris, gluteus medius, psoas major, longissimus – loin portion (LD-L), longissimus – rib portion (LD-R), triceps, infraspinatus, pectoral, and supraspinatus. Steaks (3.8 cm) were cooked to 70°C internally on open hearth grills for sensory evaluation. Muscles from the round and chuck, excluding the infraspinatus, were generally scored lower by the sensory panel than muscles from the loin and rib. The psoas major, LD-L, LD-R, and infraspinatus were more tender ($P < 0.05$) than seven other muscles; WBS values followed the same trend. Large ranges were observed among muscles for percent fat, sarcomere length, and total collagen.

INTRODUCTION

FEW STUDIES have been conducted evaluating differences in tenderness and other sensory properties among beef muscles. Ramsbottom et al. (1945) compared 25 major muscles from three "U.S. Good" heifer carcasses and found significant differences in tenderness and Warner-Bratzler shear force (WBS) between many muscles. The study also concluded that muscles with smaller amounts of connective tissue had lower WBS values, but found no relationship between intramuscular fat content and WBS values. Ritchey and Hostetler (1964) compared sensory properties of the biceps femoris, semimembranosus, semitendinosus and longissimus and reported lower sensory panel detectable connective tissue values and lower total collagen contents in the longissimus than other muscles. Prost et al. (1975), Zinn et al. (1970) and Hiner and Hankins (1950) all reported differences in tenderness (as measured by shear force and/or sensory panel) among muscles.

Marsh (1977) gives two major structural reasons for variation in tenderness, collagen (connective tissue) and the contractile status which refers to the decrease in sarcomere length caused by cold shortening. Intramuscular fat content has traditionally been thought to affect palatability (Tatum, 1981); however, that theory has been difficult to prove. Cover et al. (1956), McBee and Wiles (1967), Campion and Crouse (1975), Jennings et al. (1978), and Tatum et al. (1980) have reported positive relationships between marbling (or percent fat) and tenderness; however, in most instances, the correlations were low. Tatum et al. (1980) reported that large differences in marbling affected tenderness but there was not necessarily a consistent linear relationship between the two parameters. In addition, Cross et al. (1973) found virtually no relationship between percent fat and palatability traits of beef varying in chronological age.

Current industrial practices make single muscles and muscle groups available to consumers and processors, thus knowledge of the palatability characteristics of these muscles or muscle groups combined with current technology could improve their palatability traits. This study was conducted to investigate the differences in palatability of 13 major beef muscles and the physical and chemical properties which may contribute to differences in sensory properties.

Authors McKeith, DeVol, Miles, Bechtel, and Carr are affiliated with the Dept. of Animal Science, 205 Meat Science Lab., Univ. of Illinois, 1503 S. Maryland Drive, Urbana, IL 61801.

MATERIALS & METHODS

TEN ANGUS STEERS of similar genetic background were fed an 83% concentrate diet, then slaughtered (426 days old) at the Univ. of Illinois Meat Science Laboratory. Carcass pH was measured in the longissimus muscle at the 13th rib using an EXTECH Model 420 portable pH meter and probe (inserted approximately 5 cm deep) at 1, 6, 12, 18 and 24 hr postmortem. Carcass temperature was measured in the longissimus muscle at the 12th rib after 1, 6, 12, 18 and 24 hr postmortem by inserting a thermocouple 5 cm deep into the longissimus from the split surface of the vertebral column. After a 24 hr chill period, grade data (USDA, 1975) was collected and color scores were evaluated on the longissimus (1 = purple; 8 = youthful pink). Carcasses were fabricated into wholesale cuts (NAMP, 1981) from which the thirteen muscles were removed (Table 1). Three steaks (3.8 cm) were cut consecutively from the center or thickest portion of each muscle perpendicular to the muscle fibers and were frozen (–30°C) for subsequent analysis. Two of these steaks were used for sensory panel and WBS determination and the third steak was used for proximate analysis, collagen content and sarcomere length determinations.

Samples for proximate analysis were trimmed of all fat and epimysium, ground twice, and duplicate 5-g samples were analyzed. Percent fat was determined by extraction according to Koniecko (1979) with the exception of warm chloroform-methanol (2:1)

Table 1—Thirteen muscles and their respective wholesale and retail cuts[a]

Muscle	Wholesale Cut	Retail Cut
Semimembranosus	Round	Beef Round Top Round
Adductor	Round	Beef Round Top Round
Semitendinosus	Round	Beef Round Bottom Round, Beef Round Eye Round
Biceps femoris	Round	Beef Round Bottom Round
Rectus femoris	Round	Beef Round Tip
Gluteus Medius	Loin	Beef Loin Sirloin Steak (Wedge, Round, Flat and Pin Bone)
Psoas major	Loin	Beef Loin Tenderloin
Longissimus-Loin	Loin	Beef Loin Porterhouse Steak, Beef Loin T-Bone Steak, Beef Loin Top Loin Steak
Longissimus-Rib	Rib	Beef Rib Eye Steak, Beef Rib Roast (small and large end)
Triceps	Chuck	Beef Chuck Blade Roast, Beef Chuck 7-Bone Pot Roast, Arm Pot Roast
Infraspinatus	Chuck	Beef Chuck 7-Bone Pot Roast, Beef Chuck Blade Roast
Pectoral	Chuck	Beef Chuck Arm Pot Roast, Brisket
Supraspinatus	Chuck	Beef Chuck 7-Bone Pot Roast, Beef Chuck Blade Roast

[a] Retail cuts are named as recommended in the Uniform Retail Meat Identity Standards published by the National Live Stock and Meat Board (1973).

being used as the solvent instead of petroleum ether. Samples for collagen analysis were ground twice, powdered in liquid nitrogen, and 1.0-g samples were weighed into screw-cap culture tubes and hydrolyzed in 20 mL of 6M HCl for 24 hours at 118°C. Hydroxyproline content was determined using the procedure of Blumenkrantz and Asboe-Hansen (1975); a conversion factor of 8 was used to convert hydroxyproline to collagen (expressed as mg collagen per gram wet tissue). Sarcomere length was determined by homogenizing 3g of muscle in 25 mL of 0.25M sucrose for 20 – 40 sec in a Virtis "23" homogenizer. Average sarcomere lengths were determined from measurements of 10 different fragments using an eyepiece micrometer calibrated to a stage micrometer of a Zeiss phase contrast microscope.

Steaks for a six member, experienced sensory panel (composed of University faculty and graduate students) were broiled to an internal temperature of 70°C on Farberware Open Hearth electric broilers. Internal temperature was monitored using copper constantan wire with thermocouples placed in the approximate center of each steak and attached to a Campbell Scientific CR5 recorder. Steaks were removed from the broiler and cut into cubes (American Meat Science Association and National Live Stock and Meat Board, 1978). Samples were served warm (six steaks per sitting) to panel members in individual booths. Panelists were provided water to cleanse the palate between samples. Panel scores were based on an 8-point defined scale (juiciness, 1 = extremely dry, 8 = extremely juicy; muscle fiber and overall tenderness, 1 = extremely tough, 8 = extremely tender; detectable connective tissue, 1 = abundant, 8 = none; flavor desirability, 1 = extremely undesirable, 8 = extremely desirable).

Steaks for WBS determination were cooked in the same manner as sensory panel steaks. After steaks had cooled to room temperature, six 1.27 cm diameter cores were removed parallel to the muscle fibers and WBS values were obtained.

A complete block design (using individual animals as blocks) was used to remove variation due to differences among steers from the error sum of squares (Neter and Wasserman, 1974). Results were analyzed using the General Linear Models (GLM) and Correlation (CORR) procedures of the Statistical Analysis System (SAS, 1982). Differences between muscles were analyzed by Duncan's Multiple Range test (Duncan, 1955; SAS, 1982). Correlations are included in the discussion of data; however, there was limited variation in the population (N = 10) studied.

Table 2—Means and ranges of carcass characteristics

Trait	N	Mean	Minimum	Maximum	S.E.[a]
Color[b]	10	6.70	4.0	8.0	.37
Maturity[c]	10	A^{28}	A^{20}	A^{40}	2.49
Marbling[c]	10	$Small^{31}$	$Slight^{40}$	$Moderate^{0}$	26.3
Carcass wt. (kg)	10	258.0	227.7	282.7	5.38
Ribeye area (cm^2)	10	68.0	60.0	73.5	1.35
Fat thickness (cm)	10	0.94	0.76	1.27	0.05
% Kidney, heart & pelvic fat	10	2.4	1.8	3.2	0.17
Yield grade[c]	10	2.7	2.1	3.2	0.1
Carcass temp (°C)					
1 hr	8	38.7	37.6	41.1	0.40
6 hr	10	18.5	16.0	21.2	0.66
12 hr	10	13.2	12.3	15.7	0.32
18 hr	10	9.2	8.2	10.8	0.21
24 hr	10	7.8	6.6	8.6	0.20
Carcass pH					
1 hr	10	6.14	5.54	6.44	0.09
6 hr	10	5.48	5.35	5.72	0.04
12 hr	10	5.50	5.39	5.76	0.03
18 hr	10	5.49	5.28	5.74	0.05
24 hr	10	5.53	5.34	5.76	0.04

[a] Standard error of the mean.
[b] Subjective color score: 1 = purple, 8 = youthful pink.
[c] Based on descriptions included in USDA (1975) beef grade standards.

RESULTS & DISCUSSION

THE MEANS and ranges of carcass characteristics for the ten Angus cattle used in the study are given in Table 2. The cattle had average yield and quality grades of 2.7 and low Choice, respectively.

Sensory panel evaluations

Sensory evaluation scores and WBS evaluation are presented in Table 3. Muscles of the loin and rib were more

Table 3—Rank of thirteen muscles by sensory evaluation scores and Warner-Bratzler shear force[h]

Muscle rank	Muscle fiber tenderness	Connective tissue amount	Overall tenderness	Warner-Bratzler shear force (kg)	Muscle juiciness	Flavor desirability
1	Psoas major[a] (7.30)	Psoas major[a] (7.37)	Psoas major[a] (7.10)	Psoas major[a] (2.64)	Infraspinatus[a] (6.43)	Psoas major[a] (6.55)
2	Infraspinatus[b] (6.74)	LD-Rib[b] (6.74)	Infraspinatus[b] (6.50)	Infraspinatus[ab] (3.28)	Biceps femoris[ab] (6.18)	Infraspinatus[a] (6.48)
3	LD-Rib[bc] (6.46)	LD-Loin[bc] (6.43)	LD-Rib[b] (6.42)	LD-Loin[abc] (3.46)	Supraspinatus[ab] (6.12)	LD-Loin[a] (6.37)
4	LD-Loin[cd] (6.26)	Infraspinatus[c] (6.23)	LD-Loin[bc] (6.17)	Gluteus medius[abc] (3.48)	LD-Loin[ab] (6.07)	LD-Rib[ab] (6.35)
5	Rectus femoris[de] (5.88)	Gluteus medius[d] (5.75)	Rectus femoris[cd] (5.74)	Semitendinosus[abc] (3.57)	Triceps[abc] (5.91)	Rectus femoris[abc] (6.19)
6	Biceps femoris[e] (5.83)	Rectus femoris[d] (5.75)	Gluteus medius[de] (5.63)	Rectus femoris[bc] (3.68)	Rectus femoris[abc] (5.89)	Biceps femoris[bcd] (6.00)
7	Gluteus medius[e] (5.63)	Semitendinosus[de] (5.60)	Triceps[de] (5.57)	LD-Rib[bc] (3.78)	Psoas major[bc] (5.82)	Gluteus medius[cde] (5.87)
8	Semitendinosus[e] (5.48)	Triceps[de] (5.55)	Semitendinosus[de] (5.53)	Triceps[bc] (3.89)	LD-Rib[bc] (5.82)	Triceps[cde] (5.84)
9	Triceps[e] (5.48)	Adductor[def] (5.34)	Semimembranosus[de] (5.52)	Semimembranosus[bc] (3.94)	Semimembranosus[cd] (5.47)	Supraspinatus[de] (5.72)
10	Adductor[e] (5.45)	Semimembranosus[ef] (5.14)	Biceps femoris[de] (5.44)	Adductor[bc] (4.16)	Adductor[cd] (5.40)	Semitendinosus[de] (5.66)
11	Semimembranosus[e] (5.40)	Supraspinatus[ef] (5.08)	Adductor[de] (5.42)	Supraspinatus[c] (4.45)	Pectoral[cd] (5.27)	Semimembranosus[de] (5.64)
12	Supraspinatus[e] (5.40)	Biceps femoris[f] (4.91)	Supraspinatus[e] (5.19)	Biceps femoris[d] (5.49)	Gluteus medius[de] (5.02)	Adductor[e] (5.58)
13	Pectoral[f] (4.77)	Pectoral[g] (4.02)	Pectoral[f] (4.57)	Pectoral[d] (6.34)	Semitendinosus[e] (4.79)	Pectoral[e] (5.52)

[a-g] Means in the same column with a common superscript are not significantly different ($P > 0.05$).
[h] Mean sensory panel values are shown in parenthesis with the scale ranging from 1 to 8 (a higher number being more desirable).

tender and had less detectable connective tissue than muscles from the round and chuck. The psoas major was the most tender muscle and had less panel detectable connective tissue than all other muscles ($P < 0.05$) but the infraspinatus, LD-loin and LD-rib were also ranked high for this trait. The pectoral was the least tender muscle and had the most panel detectable connective tissue ($P < 0.05$); however, few tenderness differences were observed between muscles of the round and chuck. The differences may have been larger if the carcasses utilized had lower USDA quality grades. Results of WBS evaluations followed the same general trend as overall tenderness, with shear forces ranging from 2.64 kg (psoas major) to 6.34 kg (pectoral). These results are consistent with Prost et al. (1975), Hiner and Hankins (1950), and Ramsbottom et al. (1945), who reported the psoas major, infraspinatus, gluteus medius and longissimus to be more tender than the pectoral and adductor. However, data reported by Zinn et al. (1970) differ with the results of this study for the triceps brachii, longissimus and semimembranosus.

Juiciness scores are not consistent within wholesale cuts (Table 3). The infraspinatus had the highest numerical juiciness score, although not significantly different from five other muscles, and the semitendinosus was less juicy ($P < 0.05$) than 11 other muscles. Flavor desirability scores of the infraspinatus and muscles of the loin and rib were generally higher than other muscles (Table 3) and are similar to the tenderness evaluation results.

Percent fat analysis

The thirteen muscles were ranked by percent moisture and percent fat (Table 4). A large range in percent fat (determined on lean tissue trimmed of all external fat and epimysium) was observed; the infraspinatus and LD-rib had the highest fat contents (7.7% and 6.8%, respectively) and adductor, the lowest (1.5%). The four muscles that were rated most tender and flavorful (Table 3) had the highest fat contents, suggesting a relationship between fat content and palatability. However, low correlations were obtained between percent fat and overall tenderness ($r = 0.28$; $P < 0.01$), percent fat and juiciness ($r = 0.19$; $P < 0.05$), and percent fat and flavor desirability ($r = 0.44$; $P < 0.01$). These low correlations agree with Tatum et al. (1980), Jennings et al. (1978), Campion and Crouse (1975) and Cover et al. (1956) who reported positive but low correlations between marbling (or percent fat) and tenderness, but disagree with the results of McBee and Wiles (1967), who found a linear relationship between marbling and tenderness, juiciness, and flavor.

Sarcomere length

A wide range of sarcomere lengths were observed among the thirteen muscles (Table 4). The psoas major, pectoral, triceps, infraspinatus, and rectus femoris had relatively long sarcomere lengths and the semimembranosus, gluteus medius, LD-loin, and biceps femoris had relatively short sarcomere lengths. These results are in agreement with the results of Herring et al. (1965) and Hostetler et al. (1972) for carcasses suspended in the traditional manner. There was a weak relationship between sarcomere length and tenderness ($r = 0.25$; $P < 0.01$); however, a strong correlation between sarcomere length and tenderness would not have been expected in this study since all muscles had relatively long mean sarcomere lengths. Cold shortening was absent and the temperature and pH data (Table 2) between 6 and 12 hr postmortem indicate the carcasses reached a low pH (approximately 5.5) while the temperature (15°C) was still high enough to prevent cold shortening.

Collagen content

Total collagen content of the muscles was determined (Table 4). A wide range of values was observed among muscles; the infraspinatus and rectus femoris had high collagen contents and the LD-loin, LD-rib, and psoas major had the lowest collagen contents in this study.

Total collagen content was not a good predictor of tenderness. The correlation between collagen content and overall tenderness for all thirteen muscles was −0.10 ($P > 0.05$); however, it improved ($r = -0.36$; $P < 0.01$) when the infraspinatus muscle was excluded from the corre-

Table 4—Rank of thirteen muscles by percent moisture, percent fat, sarcomere length, and collagen content

Muscle rank	Percent moisture	Percent fat	Sarcomere length (μm)	Total collagen (mg/g wet tissue)
1	Supraspinatus[a] (76.4)	Infraspinatus[a] (7.7)	Psoas major[a] (3.46)	Infraspinatus[a] (17.81)
2	Triceps[b] (74.4)	LD-Rib[ab] (6.8)	Pectoral[b] (2.81)	Rectus femoris[b] (13.96)
3	Adductor[bc] (74.1)	LD-Loin[bc] (6.1)	Triceps[c] (2.55)	Triceps[c] (10.45)
4	Semimembranosus[cd] (73.4)	Psoas major[bc] (5.9)	Infraspinatus[cd] (2.45)	Biceps femoris[cd] (9.60)
5	Semitendinosus[cd] (73.4)	Rectus femoris[c] (5.7)	Rectus femoris[d] (2.37)	Pectoral[cd] (8.79)
6	Pectoral[cd] (73.3)	Biceps femoris[c] (5.6)	Sem tendinosus[e] (2.21)	Superspinatus[cd] (8.72)
7	Biceps femoris[de] (72.6) 8	Pectoral[c] (5.6)	Adductor[e] (2.17)	Semitendinosus[cd] (8.32)
8	Infraspinatus[de] (72.6)	Gluteus medius[cd] (5.1)	Supraspenatus[e] (2.11)	Gluteus medius[cd] (8.13)
9	Rectus femoris[de] (72.6)	Triceps[d] (4.3)	LD-Rib[e] (2.10)	Semimembranosus[de] (7.30)
10	Psoas major[ef] (72.3)	Semimembranosus[de] (4.0)	Semimembranosus[f] (1.96)	Adductor[ef] (5.52)
11	Gluteus medius[ef] (71.8)	Semitendinosus[de] (4.0)	Gluteus medius[fg] (1.90)	LD-Loin[ef] (5.04)
12	LD-Loin[f] (71.5)	Supraspinatus[e] (3.0)	LD-Loin[g] (1.84)	LD-Rib[f] (4.66)
13	LD-Rib[f] (71.5)	Adductor[f] (1.5)	Biceps femoris[g] (1.81)	Psoas major[f] (3.23)

[a-g]Means within a column with a common superscript are not significantly different ($P > 0.05$).

lation. The correlation of twelve muscles excluding the infraspinatus muscle was also evaluated because the large seam of connective tissue in the infraspinatus muscle was included in the sample used for hydroxyproline determination but was not included in samples served for sensory evaluation. The low correlation between total collagen and tenderness found in this study is in agreement with the results of Cross et al. (1973), Field et al. (1970), Kruggel et al. (1970), and Herring et al. (1967). Ritchey and Hostetler (1964) found lower total collagen contents in the longissimus muscle than in the biceps femoris, semitendinosus, and semimembranosus, which is in agreement with this study. Percent soluble collagen may be an important factor relating to meat tenderness (Hill, 1966; Field et al., 1970; Kruggel et al., (1970) or sensory panel detectable connective tissue (Cross et al., 1973). Although percent soluble collagen was not evaluated in this study, this fraction may be important to beef tenderness.

CONCLUSIONS

RESULTS of sensory panel evaluation indicate that several muscles were consistently superior in tenderness and flavor desirability. The muscles of the loin (LD-loin and psoas major), rib (LD-rib) and the infraspinatus were ranked high while the pectoral was consistently ranked low. None of the chemical properties evaluated had strong correlations to the palatability traits, although a trend was observed for muscles with higher percent fat to be more tender and flavorful. These data indicate that some muscles would have a potentially greater economic value if they were separated and used independently.

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Destruction of Pathogenic Bacteria in Turkeys Roasted in Microwave Ovens

J. A. G. ALEIXO, B. SWAMINATHAN, K. S. JAMESEN, and D. E. PRATT

ABSTRACT

Whole turkeys inoculated with *Salmonella typhimurium*, *Staphylococcus aureus*, or *Clostridium perfringens* were cooked in a microwave oven (2450 MHz) to an end-point temperature of 76.6°C. The cooking procedure did not completely eliminate any of the three pathogenic bcteria from the turkeys. The extent of survival of *C. perfringens* on the cooked unstuffed turkeys was proportional to the number of spores in the initial inoculum. Similar results were observed when turkeys stuffed with inoculated stuffing were cooked in microwave ovens. Cooking in brown-in-bags increased the destruction of *C. perfringens* and *S. typhimurium* in unstuffed turkeys even though the end-point temperature was only 68.3°C.

INTRODUCTION

TURKEYS and other meats are often incriminated in outbreaks of foodborne diseases (CDC, 1983). Raw turkeys may be contaminated with *Salmonella* spp., *Staphylococcus aureus* and *Clostridium perfringens* as they are purchased and brought into the kitchen (Dawson et al., 1979). Inadequate cooking may allow such pathogenic bacteria to survive. Also, since turkeys are almost always prepared a day before consumption, pathogenic bacteria that have survived the cooking process or that have been introduced in the product after cooking, through poor handling practices, may multiply and cause infection or intoxication if the turkeys have not been rapidly cooled to below 7°C and stored properly between preparation and consumption. Bryan and McKinley (1974) reported that turkeys cooked in conventional ovens set at 163°C to an end-point temperature of at least 74°C were free from *Salmonella* and *S. aureus*. However, spores of *C. perfringens* survived the cooking process. Bryan and McKinley (1974) made several recommendations for preparing, cooking, cooling, and reheating of turkeys in institutional food service units.

The authors have been conducting studies on the development of optimal time-temperature-microwave intensity combinations for the roasting of whole turkeys in microwave ovens. Sensory evaluation data indicated that turkeys cooked in microwave ovens at high setting (100% power) initially for 30 to 45 min, followed by cooking at roast setting (80% power) initially for 30 to 45 min, followed by cooking at roast setting (80% power) until temperature reached 76.6°C, had desirable sensory attributes (Jamesen, 1984). The sensory evaluation studies also indicated that turkeys placed in Brown-in-Bags and cooked in microwave ovens to an end-point temperature of 68.3°C were preferred by the panelists. Turkeys have a previous history of involvement in foodborne disease outbreaks and microwave cooking has been reported to be less efficient than conventional cooking for the destruction of microorganisms (Bengtsson and Green, 1970; Ockerman et al., 1976; Leon Crespo et al., 1977; Leon Crespo and Ockerman, 1977; Fung and Cunningham, 1980). Therefore, the objective of this investigation was to evaluate the extent of destruction of three common foodborne pathogenic bacteria in turkeys cooked in microwave ovens according to the protocols developed by Jamesen (1984).

Authors Aleixo, Swaminathan, Jamesen, and Pratt are affiliated with the Dept. of Foods & Nutrition, Purdue Univ., W. Lafayette, IN 47907.

MATERIALS & METHODS

Microorganisms used in the study

Staphylococcus aureus ATCC 27660 and *Clostridium perfringens* ATCC 14810 were obtained from the American Type Culture Collection, Rockville, MD; *Salmonella typhimurium* was from the culture collection maintained at the Dept. of Foods & Nutrition, Purdue Univ., IN.

Turkeys and stuffing

Turkeys of two different weight classes (2.7 - 3.7 kg and 6.3 - 7.3 kg) were purchased frozen from local supermarkets or from a distributor. Unstuffed and stuffed birds were utilized in the study. The stuffing consisted of bread crumbs (40%), 0.85% sodium chloride solution (saline, 40%), and margarine (20%). Six hundred fifty-five grams of stuffing were used for the larger birds and 327g were used for the smaller birds.

Preparation of inocula

S. typhimurium and *S. aureus* were grown overnight at 37°C in 100 mL of tryptic soy broth (TSB, Difco); cells were harvested by centrifugation at 1000 x *g* for 20 min, washed once with sterile 0.85% saline, and resuspended in 8L of saline. For *C. perfringens* the same procedure was followed substituting fluid thioglycollate medium (Difco) for TSB. Suspensions of *S. typhimurium* and *S. aureus* contained approximately 10^8 cells/mL (determined by plating appropriate dilutions on tryptic soy agar) and that of *C. perfringens* contained approximately 10^4 spores/mL (determined by plate counts after heating the bacterial suspension at 80°C for 10 min). Unstuffed thawed turkeys were inoculated by dipping them for 5 min in the bacterial suspension in saline and then draining by allowing to drip for 2 min. In the case of stuffed turkeys, the bacterial suspension in saline were used instead of water to rehydrate the bread crumbs; melted margarine was then added and well mixed with the bread crumbs. Each experimental trial used five turkeys, four to observe the destruction of bacteria after cooking and one as an uncooked control to verify the uptake of inoculum by the bird.

Cooking protocol

Turkeys were cooked in five 600W Litton (Model 520) microwave ovens. The ovens were operating at 2450 MHz and were equipped with Vari-Cook controls, which varied the time over which the magnetron was in operation from 100% on high setting to 10% at warm setting. The turkeys were initially placed breast down on microwave proof pans and cooked on high for 30 - 45 min. The setting was then changed to roast and the birds placed breast up for the remainder of the cooking time, i.e., until the temperature reached the level necessary for development of desirable sensory attributes (76.6°C or 68.3°C). The temperature was monitored by two microwave thermometers inserted on each side of the breast almost touching the breast bone. The position of the turkeys was changed every 15 min after the initial cooking to allow even cooking of the product.

Bacterial enumeration

A rinse method (Barnes et al., 1973) was used for sampling unstuffed turkeys for enumeration of bacteria. The method consisted of rinsing each turkey with 300 mL of 0.1% peptone solution

for 1 min in a sterile polyethylene bag and recovering the rinse liquid in a sterile flask. For uncooked control turkeys, decimal dilutions prepared from the rinse liquid were used for surface inoculation of appropriate selective differential plating media (BAM, 1978). The number of bacteria in the cooked turkeys was estimated by a 3-tube MPN technique which used a 10 mL portion of the rinse liquid in the highest dilution. Pre-enrichment (18 hr, 37°C) in buffered peptone water and TSB were employed to resuscitate sublethally injured cells of *S. typhimurium* and *S. aureus*, respectively. Fluid thioglycollate medium was used for the enrichment of *C. perfringens*. Tubes showing growth after 24 hr incubation at 37°C were tested by standard procedures (BAM, 1978) to confirm the presence of each bacterium. The number of bacteria in the stuffing from stuffed uncooked control turkeys was determined from 50g samples by plating in selective agar; the number of bacteria in stuffed turkeys surviving microwave cooking was estimated by a 3-tube MPN technique as above.

RESULTS & DISCUSSION

THE SURVIVAL DATA for *C. perfringens* are shown in Table 1. Unstuffed hens (2.7 - 3.7 kg weight range) which were inoculated with approximately 7.0×10^5 cells and 1.2×10^4 spores, showed 1 to 2 log reduction in the number of cells of *C. perfringens* after cooking and *C. perfringens* was isolated from all four cooked turkeys. Unstuffed toms (weight range 6.3 - 7.3 kg) contained less than nine cells of *C. perfringens* after cooking. However, the bacterial suspension which was used to inoculate the unstuffed toms, contained a very low number of spores of *C. perfringens*. Unstuffed toms, which had been cooked in brown-in-bags showed increased destruction of *C. perfringens* spores, although the turkeys were cooked to an internal temperature of 68.3°C and the cooking time was almost half of that required for unstuffed turkeys cooked without brown-in-bags. Stuffed turkeys, in which the stuffing had been inoculated with *C. perfringens* showed a destruction pattern similar to that observed in the case of unstuffed turkeys cooked without brown-in-bags. It appears that the ultimate number of survivors in the cooked turkeys was dependent upon the number of spores originally present in inoculum.

The results reported in Table 1 on unstuffed turkeys are in agreement with those reported by Blanco and Dawson (1974) on the survival of *C. perfringens* on chicken parts cooked with microwave energy. The above investigators observed a two log cycle reduction in the resident and inoculated *C. perfringens* cells on chicken parts cooked to 98°C. Also, Blanco and Dawson (1974) reported that the reduction in the number of spores of *C. perfringens* by microwave energy was minimal and that the cooking environment provided an efficient activation and germination step for the spores of *C. perfringens*.

Table 1—Survival of Clostridium perfringens in turkeys[a] roasted in microwave ovens

Weight range (kg)	Cooking conditions	Final temp (°C)	Range of cooking time (min)	Initial counts per turkey or g of stuffing: Vegetable cells	Initial counts per turkey or g of stuffing: Spores	Range of MPN per turkey or g of stuffing after cooking	No. of cooked turkeys positive for C. perfringens per no. tested
			A. Unstuffed turkeys				
2.7 - 3.7	30 min high 31.3 min roast	76.6	57 - 65	7.2×10^5	1.2×10^4	7.5×10^3 to 4.0×10^4	4/4
6.3 - 7.3	45 min high 48.3 min roast	76.6	91 - 109	2.3×10^6	$<1 \times 10^2$	< 9	0/4
6.3 - 7.3	Brown-in-bag 45 min high 12 min roast	68.3	54 - 63	9.9×10^6	1.8×10^4	<9 to 69	3/4
			B. Stuffed turkeys				
6.3 - 7.3	45 min high 111.7 min roast	76.6 76.6	142 - 167	1.8×10^5	1.7×10^4	1.0×10^2 to 4.8×10^2	4/4

[a] Mean of four turkeys

Table 2—Survival of Salmonella typhimurium in turkeys[a] roasted in microwave ovens

Weight range (kg)	Cooking condition	Final temp (°C)	Range of cooking time (min)	Initial count of cells per turkey or g of stuffing	Range of MPN per turkey of g of stuffing after cooking	No. of cooked turkeys positive for S. aureus per no. tested
			A. Unstuffed turkeys			
2.7 - 3.7	45 min high 24.5 min roast	76.6	55 - 87	6.0×10^8	< 9 to 45	3/4
6.3 - 7.3	45 min high 62 min roast	76.6	97 - 117	9.2×10^7	45 to 129	4/4
6.3 - 7.3	Brown-in-bag 45 min high 12.3 min roast	68.3	47 - 62	3.1×10^8	< 9 to 129	2/4
			B. Stuffed turkeys			
6.3 - 7.3	45 min high 64 min roast	76.6	99 - 128	7.1×10^5	<0.3 to 1.1	2.4

[a] Mean of four turkeys

The extent of survival of *S. typhimurium* in turkeys roasted in microwave ovens is shown in Table 2. *S. typhimurium* was isolated from 11 of 16 cooked turkeys. Unstuffed turkeys of larger weight range yielded maximum numbers of *S. typhimurium* after cooking. Cooking the larger weight turkeys in brown-in-bags increased *S. typhimurium* destruction. Two of four stuffed turkeys were positive for *S. typhimurium* after cooking to an end-point temperature of 76.6°C. Although the destruction rate of *S. typhimurium* in turkeys after microwave cooking was significant (4 to 7 log cycles), the presence of *S. typhimurium* in cooked turkeys at low levels is disturbing. Other investigators were able to recover *S. typhimurium* from inoculated chocolate foam pie (Baldwin et al., 1968) and fish muscle (Baldwin et al., 1971) after microwave cooking.

The results on the survival of *S. aureus* in turkeys roasted in microwave ovens are shown in Table 3. *S. aureus* was isolated from 13 of 16 cooked turkeys. All four unstuffed turkeys (2.7 - 3.7 kg) yielded *S. aureus* after cooking to an end-point temperature of 76.6°C. Unstuffed large turkeys cooked in brown-in-bags yielded greater numbers of *S. aureus* (84 - 3300 cells/turkey) than unstuffed turkeys which were not cooked in brown-in-bags. Stuffed turkeys, in which the stuffing was inoculated with *S. aureus*, yielded very low numbers of *S. aureus* after cooking. Only two of four stuffed turkeys were positive for *S. aureus*. However, part of the destruction of *S. aureus* in the stuffing could be attributed to the use of large amounts of melted margarine in the stuffing. As shown in Table 4, a 3 to 4 log-reduction in the numbers of *S. aureus* was observed after the addition of melted margarine to the stuffing-water mix.

Limited trials on the effect of holding turkeys in microwave oven in warm setting (10% power) for 30 min after cooking to 76.6°C to allow the temperature to equilibrate and let the coldest point reach the end-point temperature showed an increase of 2 - 3°C in temperature after the holding. There was a reduction in the numbers of *S. typhimurium* and *S. aureus* but not complete destruction (Table 5). In one trial the turkeys were cooked to an end-point temperature of 85°C to determine if complete destruction of *S. aureus* and *S. typhimurium* was possible, but results were unsuccessful, as evident from the data presented in Table 6.

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Table 3—Survival of Staphylococcus aureus in turkeys[a] roasted in microwaves ovens

Weight range (kg)	Cooking condition	Final temp (°C)	Range of cooking time (min)	Initial count of cells per turkey or g of stuffing	Range of MPN per turkey of g of stuffing after cooking	No. of cooked turkeys positive for S. aureus per no. tested
			A. Unstuffed turkeys			
2.7 - 3.7	45 min high 10.3 min roast	76.6	53 - 57.5	1.3×10^8	279 to 720	4/4
6.3 - 7.3	45 min high 63.3 min roast	76.6	102 - 113	3.8×10^8	< 9 to 12	3/4
6.3 - 7.3	Brown-in-bag 45 min high 11.6 min roast	68.3	50 - 66.5	3.7×10^8	84 to 3300	4/4
			B. Stuffed turkeys			
6.3 - 7.3	45 min high 40.8 min roast	76.6	70 - 106	5.9×10^7	<0.3 to 4.3	2/4

[a] Mean of four turkeys

Table 4—Destruction of Staphylococcus aureus by melted margarine added to stuffing

Stuffing	Counts of *S. aureus* (CFU/g)					
	Water + stuffing	Time (min) of incubation at room temperature after addition of margarine				
		0	15	30	60	75
Melted margarine added	2.5×10^7	1.3×10^2	3.5×10^3	2.2×10^3	1.2×10^3	1.2×10^3
Without margarine	3.0×10^7	2.3×10^7	2.0×10^7	2.2×10^7	5.5×10^6	7.0×10^5

Table 5—Effect of holding turkeys in warm setting for 30 min on the survival of S. typhimurium and S. aureus[a]

Organism	Initial count per turkey	MPN of organisms/turkey after cooking	MPN of organisms per turkey after cooking and holding for 30 min in warm setting
S. typhimurium	3.1×10^8	> 3300	279 - 720
S. aureus	2.7×10^8	> 3300	279

[a] Weight range of turkeys: 2.9 - 3.3 kg; Cooking conditions: 45 min high, 13 - 20 min roast.

Table 6—Effect of cooking turkeys to a final temperature of 85°C on the survival of S. typhimurium and S. aureus[a]

Organism	Initial count per turkey	Range of MPN of organism/cooked turkey	No. of cooked turkeys positive for organisms/no. tested
S. typhimurium	3.2×10^8	63 - 720	4/4
S. aureus	1.6×10^8	69 - 3300	4/4

[a] Weight range of turkeys: 2.6 - 3.3 kg; Cooking condition: 45 min high, 0 - 30 min roast. Range of cook ng time: 45 - 75 min.

Effect of Poly- and Pyrophosphates on the Natural Bacterial Flora and Inoculated *Clostridium sporogenes* PA 3679 in Cooked Vacuum packaged Bratwurst

R. A. MOLINS, A. A. KRAFT, H. W. WALKER, and D. G. OLSON

ABSTRACT

Survival and growth of inoculated *Clostridium sporogenes* PA 3679 and of natural aerobic and anaerobic bacterial flora were studied in cooked, vacuum packed bratwurst containing 0.5% phosphates during refrigerated (5°C) and subsequent temperature abuse storage (24°C). Sodium acid pyrophosphate (SAPP), sodium tripolyphosphate (STPP), tetrasodium pyrophosphate (TSPP) and sodium polyphosphate glassy (SPG) were tested. No significant bacterial inhibition by any phosphate was observed during refrigerated storage, nor was there appreciable growth in the control bratwurst. However, SAPP significantly inhibited aerobic and anaerobic bacteria (including *C. sporogenes*) upon temperature abuse, followed in effect by TSPP and STPP. Cooking to 65.5°C helped retain antimicrobial properties of phosphates to some extent. Enzymatic hydrolysis of phosphates is postulated as a major factor in loss of antimicrobial properties of phosphates in processed meats.

INTRODUCTION

RENEWED INTEREST in the possible use of phosphates as antimicrobial agents in meat products has derived from the search for compounds that may enable the meat industry to substitute or reduce amounts of long-accepted additives such as nitrites and sodium chloride. Protection must still be provided against the growth of pathogenic bacteria and possible production of microbial toxins. The approval given to the use of certain poly- and pyrophosphates in processed meats by the USDA in 1982 and the information available on the chemical and physical advantages derived from their use have resulted in the inclusion of phosphates in a large variety of meat products (Knipe, 1982).

In the microbiological field, however, the effects of phosphate addition to meat products is still a subject of some controversy. In studies with vacuum packed, sliced berliner sausage, Mol et al. (1971) found that the presence of 0.45% polyphosphates did not affect the growth of unclassified *Streptobacterium* LL3 during storage of 12°C. Recent work by Nielsen and Zeuthen (1983) with vacuum packed, sliced bologna to which a low pH mixture of sodium tripolyphosphate (STPP), tetrasodium pyrophosphate (TSPP) and sodium polyphosphate glassy (SPG) (0.3%) or STPP alone had been added in solution, showed that the blend had no influence on the growth of an atypical *Streptobacterium* inoculated on the surface, but had a pronounced inhibitory effect on similarly inoculated *Brochothix thermosphacta* and *Serratia liquefaciens*, while STPP alone was ineffective. Wagner and Busta (1983) observed that sodium acid pyrophosphate (SAPP) delayed toxin production by *Clostridium botulinum* for 12–18 days when used in combination with 40 ppm of sodium nitrite and 0.26% potassium sorbate in beef/pork frankfurter emulsions at 27°C, but that mesophilic bacterial counts were no different from those in the absence of phosphates. Earlier experiments by Ivey and Robach (1978) involving canned comminuted pork showed no significant interactions between SAPP or SPG concentrations and sodium nitrite in preventing *C. botulinum* outgrowth and toxin production, but a synergistic relationship was found between those phosphates and potassium sorbate. The authors suggested that botulism-safe meat products could be produced without $NaNO_2$. Other researchers have reached similar conclusions in studies with pork slurries prepared from high pH meat (6.3–6.8) but not with slurries from meat in the pH range 5.5–6.3, in which 0.3% of a polyphosphate blend enhanced toxin production by *C. botulinum* (Roberts et al. 1981a, b).

Our work with uncooked refrigerated bratwursts packed in air tight bags and kept for 7 days at 5°C led us to conclude that 0.5% SAPP, STPP, TSPP or SPG are ineffective as inhibitors of the natural aerobic mesophilic or psychrotrophic spoilage flora of uncooked bratwurst or against inoculated *Staphylococcus aureus* (Molins et al., 1985). The contrast between these negative findings and the strong inhibitory properties exhibited by phosphates on the growth of bacteria in less complex laboratory media as we reported earlier (Molins et al., 1984) and as stated by other authors (Post et al., 1963; Gray and Wilkinson, 1965; Seward et al., 1982; Firstenberg-Eden et al., 1981) might be due to multiple interactions between these compounds and meat components. Reports by Awad (1968) and Sutton (1973) have demonstrated that phosphates added to meats and fish are hydrolyzed to orthophosphates by naturally occurring phosphatases. Mild heat treatment of meats to deactivate such enzymes may substantially decrease, though not eliminate, hydrolysis of the phosphates over time.

Consequently, the objective of this study was to determine the effect of commonly used, food-grade phosphates at the maximum level allowed in processed meats (0.5%) on the survival and growth of the natural bacterial flora and inoculated *Clostridium sporogenes* PA 3679 spores in bratwurst cooked and vacuum packed immediately following preparation.

MATERIALS & METHODS

Bratwurst preparation

A 300-lb batch of ground pork butts ground through a 1/8 in diameter plate was obtained from the Iowa State Meat Science Laboratory, packed in 12-lb portions in polyethylene freezer bags, twist-tied, frozen in an air blast freezer to −28°C and kept frozen until needed. Prior to preparation, 12-lb portions were allowed to thaw for 48 hr in a walk-in cooler at 2-4°C. Bratwurst spice mix (Saratoga Specialities, Elmhurst, IL) was added to the meat and blended for 5 min at medium speed by means of a Hobart C-10 mixer. The spice mix used was 2% (w/w), equivalent to 1.7% NaCl in the product. Portions (1000g) of meat were weighed and each received 53 mL of a 10% (w/v) aqueous solution of a single phosphate previously sterilized by filtration though a 22μ Millipore membrane filter (Millipore Corp., Bedford, MA), to obtain the desired concentration of 0.5%, the maximum limit allowed by the USDA, in the product. The phosphates used were sodium acid pyrophosphate (SAPP), sodium polyphosphate glassy (SPG) formerly known as sodium hexametaphosphate (Stauffer Chemical Co., Westport, CT), tetrasodium pyrophosphate (TSPP) and sodium tripolyphosphate (STPP) (Monsanto, St. Louis, MO). Sterile distilled water was added

Authors Molins, Kraft, Walker, and Olson are with the Dept. of Food Technology, Iowa State Univ., Ames, IA 50011. Author Olson is also affiliated with the Dept. of Animal Science, Iowa State Univ.

to one of the 1000-g portions to be used for preparation of control sausages. After being blended for one minute at each low and high speeds in a Kitchen-Aid Model 4 mixer, the 1000-g batches were divided in half; one was inoculated and the second received an amount of sterile distilled water equal to the volume of the inoculum added to the first portion. The inoculum consisted of 10 ml *Clostridium sporogenes* PA 3679 spore suspension in sterile, deionized water prepared according to the procedure outlined by Swank (1971) and targeted to give approximately 1000 spores/g sausage. The inoculum was introduced into the meat by blending, as described above. The culture was obtained from the ISU Food Technology Department collection and selected for rhizoid colony formation to facilitate differentiation from other anaerobic bacteria during plate counting. Uninoculated controls were balanced for added water as specified. The mixes were stuffed into 1 in. diam edible collagen casings (Devro, Inc. Somerville, NJ) by means of an Oster Food and Meat grinder (Oster, Milwaukee, WI). Immediately after stuffing and tying, each batch of sausages was cooked to an internal temperature of 65.5°C in a separate 3000 mL beaker containing distilled water and kept at 80°C in a Magni Whirl Constant Temperature Bath (Blue M Electric Co., Blue Island, IL) equipped with bottom paddle agitation. The cooking end-point was determined by inserting a copper-constantan thermocouple longitudinally through one bratwurst of each batch and monitoring the internal temperature with a Hewlett Packard 3476A Multimeter Potentiometer. Average cooking time was 11 min. Cooking losses that could have affected the level of phosphates were previously determined and found to be negligible (0.64 to 1.1%). After cooking, the bratwurst was wrapped in aluminum foil, rapidly cooled to an internal temperature of 4–5°C, separated and placed in small, individual, polyethylene bags to avoid cross-contamination. One sausage from each treatment, for a total of five sausages, was placed in Curlon 892 (Curwood Inc., New London, WI) vacuum packaging bags (O_2 permeability < 1 mL/645 cm^2/24 hr at 22.8°C and 0% RH) and vacuum sealed in a Multivac MG-2 packaging machine. Inoculated and uninoculated bratwurst were packed separately. The bags were stored for 7 days in a display case at about 5°C, followed by 48 hr of temperature abuse (24–25°C). The order of batch preparation was randomly assigned, and all experiments were replicated three times.

Sampling

For each of the three replications, one package each of inoculated and uninoculated sausages was taken from storage on days 0, 1, 3, 5, and 7 and on days 8 and 9 (corresponding to 24 and 48 hr of temperature abuse at 24°C) for microbiological examination. The sausage was weighed in 30g amounts and blended in a jar with 270 ml 0.1% sterile peptone water for 30 sec at each of low and high speeds in an Osterizer blender. Serial dilutions were then made according to standard methods. Trypticase soy agar (TSA, BBL) was used to plate aerobic mesophilic (48 hr, 30°C) and psychrotrophic (10 days, 5°C) bacterial populations. TSA was also used for total anaerobic counts, while TSA containing 0.1% soluble starch (Fisher Scientific Co., Fair Lawn, NJ) was the medium used to determine anaerobic viable spore counts after heat shocking the appropriate dilutions at 80°C for 20 min (Swank, 1971; Neal, 1976). BBL Gas Pak Anaerobic Systems were used to obtain anaerobic incubation conditions (48 hr, 37°C). *C. sporogenes* PA 3679 vegetative cell and viable spore counts were determined from the same plates used for total anaerobic and total viable spore counts, respectively, by counting typical rhizoid colonies characteristic of the culture used as inoculum, and which was determined to be a stable property. In order to confirm the identity of the typical rhizoid colonies, 10 isolated colonies were randomly picked from counted plates at each sampling time, examined under the microscope and subcultured in cooked meat medium (BBL, 48 hr at 37°C). Observations were made for gas production, proteolysis, and single terminal spores. No typical rhizoid colonies were observed from uninoculated bratwurst. The pH of the suasages was determined throughout the sampling period using a Radiometer 28 meter equipped with an Orion 9163 probe. Sampling order was randomized among treatments.

Plate count data were transformed to logarithms and analyzed by means of an SAS program with GLM option. Comparison of means was based on Duncan's multiple range test.

RESULTS

Fig. 1 and 2 show mesophilic and psychrotrophic bacterial counts in phosphate-containing samples. Bacterial numbers were not significantly different ($P > 0.05$) from those of controls (no phosphate) throughout the 7-day refrigerated storage period. However, upon temperature abuse (24–25°C), SAPP significantly ($P < 0.05$) inhibited the growth of both mesophiles and psychrotrophs, limiting their numbers to less than 10^5 CFU/g of sausage even after 48 hr at 24°C. SAPP was equally effective in inhibiting naturally occurring ("wild") anaerobes (Fig. 3), which were similarly inhibited by TSPP during the first 24 h of temperature abuse. SPG proved to be ineffective as an inhibitor of aerobic mesophiles, psychrotrophs or "wild" anaerobic bacteria, while TSPP provided no stable pattern of inhibition in any case.

Anaerobic growth curves obtained from samples inoculated with *C. sporogenes* PA 3679 are shown in Fig. 4. The addition of 0.5% SAPP resulted in significantly ($P < 0.05$) lower numbers of total anaerobes as well as populations of *C. sporogenes* PA 3679 than those found in control sausages

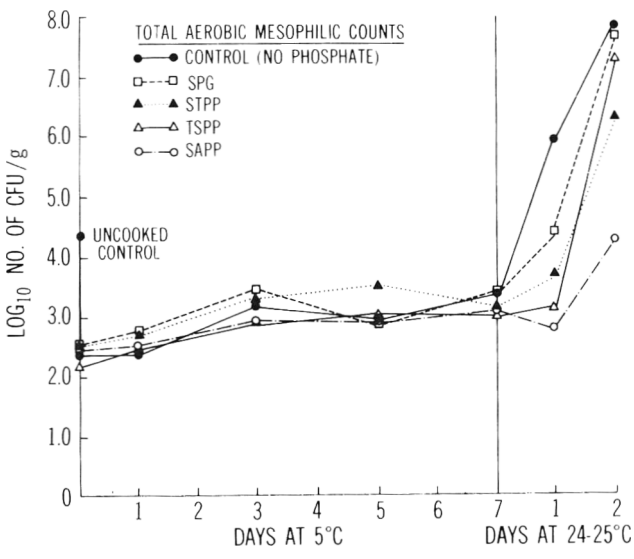


Fig. 1—Effect of phosphates on total mesophilic counts of cooked bratwurts.

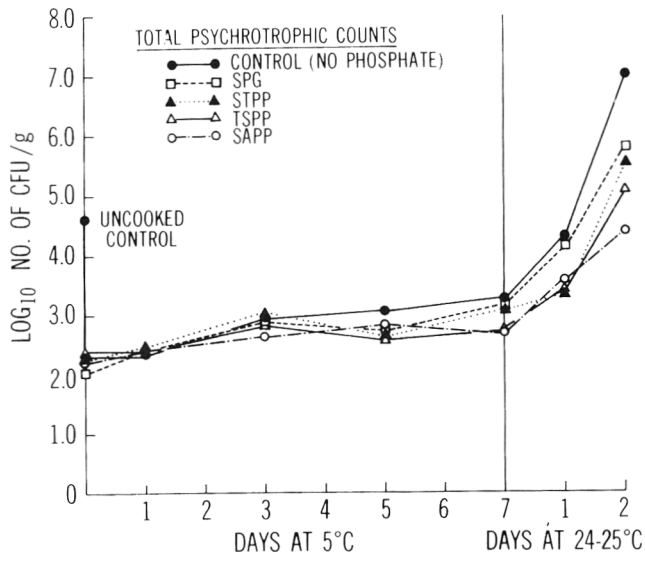


Fig. 2—Effect of phosphates on psychrotrophic counts of cooked bratwurst.

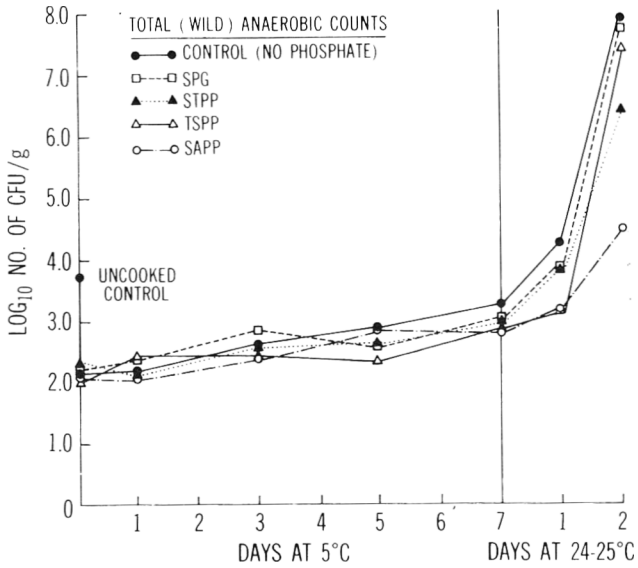


Fig. 3—Effect of phosphates on naturally occurring anaerobic bacteria in cooked bratwurst.

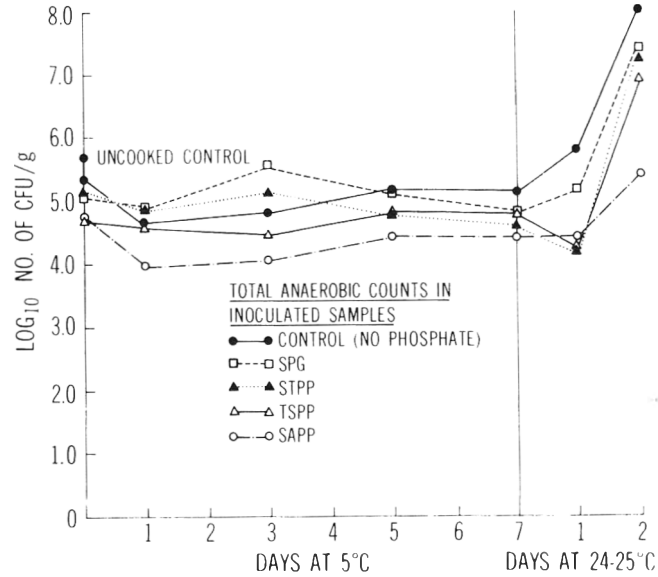


Fig. 4—Effect of phosphates on total anaerobic counts of bratwurst inoculated with C. sporogenes *PA 3679.*

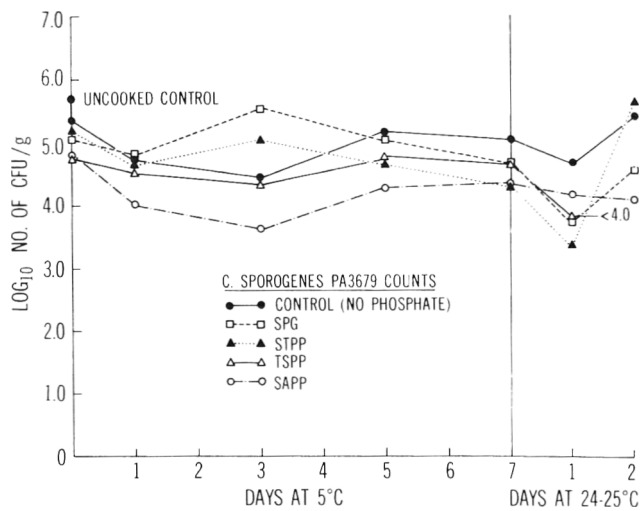


Fig. 5—Effect of phosphates on counts of C. sporogenes *PA 3679 in cooked bratwurst.*

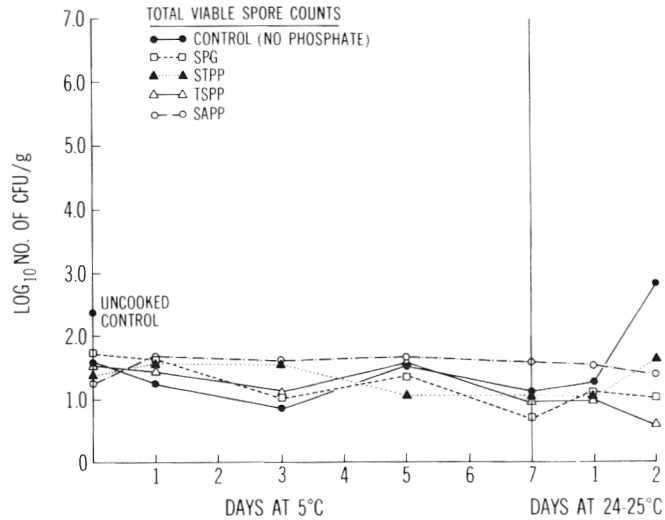


Fig. 6—Effect of phosphates on viable spore counts of cooked bratwurst.

(Fig. 4 and 5, respectively) during the entire refrigerated storage period at 5°C. However, differences among phosphate treatments were not significant ($P > 0.05$). After 24 hr of elevated temperature (24–25°C), all sausages formulated with STPP and TSPP contained significantly ($P < 0.05$) lower numbers of "wild" anaerobes and PA 3679 vegetative cells than those with SPG or no phosphates, while SAPP allowed no anaerobic growth during that time. Prolonged holding at 24–25°C produced more difference, after 48 hr, only SAPP remained strongly and significantly ($P < 0.05$) inhibitory. Fig. 5 shows 10^5 organisms per gram initially, which includes vegetative cells as well as spores.

Viable spore counts in uninoculated bratwurst did not differ greatly during storage at 5°C (Fig. 6), and no significant ($P > 0.05$) effects on "wild" anaerobic spore viability could be attributed to any phosphate tested during refrigerated storage at 5°C. After 24 hr of temperature abuse (24–25°C), however, SAPP-containing samples had higher total anaerobic spore counts than controls but the difference was not significant ($P > 0.05$). At that time, use of SPG and SAPP also resulted in slightly higher numbers of PA 3679 viable spores than for controls (Fig. 7). At 48 hr of abuse temperature, control sausages had significantly ($P < 0.05$) higher total viable, but not PA 3679 spore counts, than sausages treated with any of the phosphates, as shown in Fig. 6 compared with Fig. 7.

A comparison was made of bacterial and spore numbers prior to and after cooking the bratwurst to an internal temperature of 65.5°C. No significant ($P > 0.05$) differences were observed among treatments, indicating that phosphates did not affect the heat resistance of vegetative cells or that of spores.

Determinations of pH demonstrated a strong buffering capacity by all phosphates (Table 1), since the pH values of bratwurst containing phosphates decreased by only 0.02–0.15 unit even after 48 hr at 24°C. At that time most bacterial counts were about 10^7–10^8 CFU/g of sausage (except for sausage containing SAPP) and the pH of control samples had decreased by 0.25–0.55 unit.

DICUSSION

THE EFFECTS attributable to the various phosphates tested are related to heating, storage temperatures and

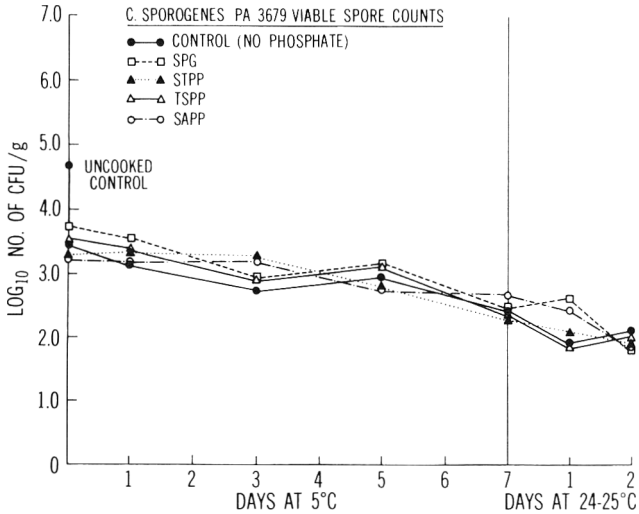


Fig. 7—Effect of phosphates on counts of viable spores of C. sporogenes *PA 3679 in cooked bratwurst.*

Table 1—Mean pH values of cooked, vacuum packed bratwurst throughout refrigerated and abuse temperature storage

Time (days)	Storage Temp (°C)	Phosphate treatment None (control)	SAPP	STPP	TSPP	SPG
0[a]	5°C	6.45	6.10	6.65	6.80	6.38
1	5°C	6.44	6.08	6.60	6.78	6.33
3	5°C	6.40	6.10	6.60	6.83	6.33
5	5°C	6.43	6.08	6.60	6.80	6.35
7	5°C	6.38	6.08	6.60	6.78	6.35
8	24-25°C	6.39	6.08	6.58	6.78	6.33
9	24-25°C	6.08	6.08	6.50	6.75	6.28

[a] Uncooked control mix had a pH of 6.30.

times. On day 0, cooking the bratwurst to an internal temperature of 65.5°C significantly ($P < 0.05$) decreased vegetative cells and spore counts in all samples, but no additional effect by any phosphate on the heat resistance of vegetative cells or spores was observed.

SAPP caused a significant ($P < 0.05$) reduction in numbers of anaerobic bacteria present in inoculated bratwurst during the first day of refrigerated storage at 5°C (Fig. 4). Anaerobic organisms that decreased were *C. sporogenes* PA 3679, shown in Fig. 5, and not "wild" anaerobes since populations of the latter were not affected in inoculated sausages (Fig. 3). In general, little bacterial growth occurred during refrigerated storage and no significant ($P > 0.05$) antimicrobial effect of phosphates was observed during that period.

After 24 hr of temperature abuse at 24°C, SAPP, STPP and TSPP significantly decreased anaerobic counts in inoculated bratwurst (by approximately two log cycles) as well as mesophilic and psychrotrophic bacterial numbers. However, only SAPP and TSPP were inhibitory to "wild" anaerobes. Viable spore counts were unaffected by STPP or TSPP, but significantly higher numbers of viable *C. sporogenes* spores were recovered from samples treated with SPG or SAPP. The food safety implication of these findings is that if 24 h of temperature abuse occurred, TSPP and STPP might contribute to inhibition of clostridia at the spore outgrowth and/or vegetative cell growth stages, while SAPP and SPG possibly inhibit spore germination.

After "temperature abusing" the bratwurst for 48 hr, a very strong inhibitory effect on all bacterial growth by SAPP resulted in a reduction of total aerobic and anaerobic counts 3 to 5 log cycles below those of control samples.

The possible contribution of pH to our results cannot be overlooked, since we did not attempt to control it. In studies by Nelson et al. (1983) with 0.4% SAPP, STPP, or SPG, SAPP effectively inhibited growth and toxin production by *Clostridium botulinum* in temperature-abused (27°C) chicken frankfurter emulsions over a pH range of 5.87–6.19. Ingredients also included 40 ppm sodium nitrite and/or 0.26% potassium sorbate. Similar results were reported by Wagner and Busta (1983) for temperature-abused beef-pork frankfurter emulsions at various pH levels. In terms of enzymatic activity, Awad (1968) found that a pH range of 5.7–7.0 was optimum for tripolyphosphatase, while pyrophosphatase exhibited maximum activity at pH 7.0. He also demonstrated that 100 mg of STPP/100g meat would be hydrolyzed in fresh or cooked (80°C) beef homogenates by 47% and 33%, respectively, in less than 1 hr and by 88% and 33% after 4 hr at pH 5.8 (fresh beef) or 6.0 (cooked beef) and 4–5°C. Equivalent values for TSPP hydrolysis in less than 1 hr were 10% and 23% (pH values 6.0 and 6.2, 4–5°C), 27% and 30% at 4 hr. For SPG, hydrolysis was 34% and 7% in less than 1 hr (pH values 5.7 and 6.0, 4–5°C), and 41% and 13% at 4 hr. These factors may be of importance in understanding the differences in bacterial inhibition observed in SAPP-containing formulations (pH 6.10) when contrasted with the apparent ineffectiveness of STPP (pH 6.65), TSPP (pH 6.80) and SPG (pH 6.38). Except for sausages containing SAPP, all other sausages had pH values that would optimize the activity of the hydrolytic enzymes corresponding to each phosphate. In our study, cooking the bratwurst to an internal temperature of 65.5°C could have decreased but not totally eliminated enzymatic hydrolysis of the poly- and pyrophosphates (Awad, 1968). Hydrolysis may still have occurred so that the concentration of SAPP, STPP, TSPP and SPG in the final product may have been considerably lower that the initial 0.5%. These results and those of previous studies in this laboratory (Molins et al., 1985) also suggest that any delay in cooking after addition of phosphates to bratwurst formulations would render these compounds useless or less effective as antimicrobial agents. However, the effects of hydrolysis of the condensed phosphate species to orthophosphates on microorganisms is still not clear. Many other factors are concerned with bacterial growth inhibition. Residual levels of added phosphates, pH, ionic strength, other inhibitors, temperature and presence of metal ions, are all involved in considering effectiveness of phosphates as bacterial inhibitors in processed meats.

CONCLUSIONS

ADDITION OF SAPP, STPP, TSPP or SPG at a level of 0.5% to vacuum packaged, cooked bratwurst has no significant ($P > 0.05$) effect on bacterial survival or growth during refrigerated storage for 7 days. However, the addition of 0.5% SAPP inhibited aerobic and anaerobic bacterial growth and may possibly contribute a safety margin against *Clostridium* spp in vacuum packed, cooked bratwurst in the absence of nitrite even when "temperature abused" at 24°C for 48 hr. TSPP and STPP were also effective during 24 hr of elevated temperatures, but their inhibitory action on mesophilic bacteria does not persist.

Cooking the product as soon as feasible after addition of phosphates to the formulation is suggested to help retain the antimicrobial properties of phosphates in bratwurst.

Consideration should be given to hydrolysis of phosphates, and hence residual poly- and pyrophosphates in relation to bacterial growth inhibition in future microbiological research involving these compounds and meat products.

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This study confirms previous observations that microwave cooking is not as effective as conventional cooking for destruction of microorganisms. There could be several reasons for the inefficient destruction of bacteria in the microwave cooked turkeys. Heating patterns of substances in a microwave oven vary depending upon the position in the oven where the substance is placed (Leon Crespo et al., 1977). Also, food constituents absorb microwave energy with different intensities (Curnutte. 1980): thus one should expect variability in the heating patterns of different areas of the food being cooked by microwaves. This phenomenon would be more pronounced when cooking foods of large mass, as in the case of whole turkeys.

It is possible that the proper time-temperature relationship which governs thermal destruction of microorganisms was not achieved in our cooking protocol. When turkeys were held for 30 min in warm setting (10% power) after cooking to the end-point temperature of 76.6°C there was a reduction in the numbers of *S. typhimurium* and *S. aureus* but not completel destruction. This indicates that if the turkeys were held longer in warm setting complete inactivation of bacteria would eventually be achieved.

It should be pointed out that the abnormally high numbers of bacteria used in this study make comparisons with the normal situation, where turkeys are minimally contaminated, rather difficult. There is obviously a need for further research in this area. In the meantime, any recommendations made to consumers on microwave roasting of turkeys should take into account the possibility of survival of pathogenic microorganisms in the product after cooking.

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Effects of Beef Carcass Electrical Stimulation and Hot Boning on Display Color of Unfrozen Vacuum Packaged Steaks

J. R. CLAUS, D. H. KROPF, M. C. HUNT, C. L. KASTNER, and M. E. DIKEMAN

ABSTRACT

Ninety-six beef sides from 48 carcasses were used to determine effects of control (C, chilled 48 hr at 5°C), electrical stimulation (ES, 45 min postmortem, 400 volts for 2 min, pulsed), and hot boning (HB, 2 hr postmortem), and combination (ESHB) treatments on muscle color of longissimus (LD) and semimembranosus (SM), vacuum packaged steaks. HB muscles frequently were visually brighter purplish-red than other treatments. Compared to ESHB, ES LD was not different, but ES SM was duller purplish-red in color. Reflectance indicators of reduced myoglobin and metmyoglobin were essentially the same across treatments in both muscles. Vacuum packaged fresh beef steaks from all treatments were acceptable in color at 0, 3, 7, and 14 days of display. Vacuum packaging appears suitable for steaks from any of these carcass treatments but is especially useful for steaks from hot boned cuts.

INTRODUCTION

PACKAGING retail cuts in oxygen permeable film like polyvinylchloride is well established and used throughout the industry. Use of oxygen permeable film promotes formation of a desirable, bright cherry red muscle color that consumers associate with freshness. However, in oxygen permeable film, beef color deteriorates rather rapidly and the growth of spoilage organisms is permitted which limits shelflife (Landrock and Wallace, 1955).

Vacuum packaging in oxygen impermeable film is relatively new and is primarily used for packaging primal and sub-primal cuts. This sytem severely limits available oxygen and suppresses growth of spoilage organisms (Kraft and Ayres, 1952; Jaye et al., 1962). Vacuum packaged pork loins had less surface discoloration and lower bacterial counts than polyvinylchloride wrapped loins (Smith et al., 1974). Vacuum packaged loins were still acceptable at 21 days and produced highly acceptable retail cuts in appearance, odor, and bacterial counts evaluated at 7 days after cutting (2°C storage). Vacuum packaged wholesale beef cuts maintained an acceptable product for at least 15 days (Hodges et al., 1974). Pierson et al. (1970) found that anaerobically packaged beef top round steaks stored for 10 days were comparable to fresh beef in color, odor, and flavor, but aerobically packaged beef was unacceptable after 4 days of storage. Griffin et al. (1982) indicate that vacuum packaged steaks can be displayed for 10 days if steaks are from relatively fresh sub-primals (or carcasses), are packaged in high oxygen-barrier film and are displayed at not over 2°C.

When electrical stimulation (ES) affects longissimus dorsi color of ribbed beef carcasses or beef steaks wrapped in oxygen permeable film, it is in a favorable direction (Smith et al., 1977; Savell et al., 1978; Hall et al., 1980). However, hot boning (HB) results in darker colored muscle when excised too early postmortem (Kastner et al., 1973).

Authors Kropf, Hunt, Kastner, and Dikeman are affiliated with the Dept. of Animal Science & Industry, Kansas State Univ., Manhattan, KS 66506. Author Claus is with Sugardale Foods Inc., Canton, OH 44711.

Our objectives were to determine the effects of ES, HB, and their combination on muscle color display life of fresh, vacuum packaged beef steaks.

MATERIALS & METHODS

FORTY-EIGHT crossbred steers sired by 7/8 Simmental x 1/8 Hereford or Angus bulls out of crossbred dams were obtained from the R.L. Hruska U.S. Meat Animal Research Center in Clay Center, Nebraska. The cattle were about 8 months old and averaged 263 kg when placed on a feeding trial at Kansas State University.

Cattle were fed ad libitum under one of two feeding regimens. First, three accelerated groups (ACC) were stepped-up to a finishing diet over a 28-day period. The final diet (dry matter basis) consisted of 9.6% forage sorghum silage, 84.4% corn, and 6.0% protein and mineral supplement. ACC cattle were slaughtered in three groups after reaching either 441 kg (139 days, ACC 1), 494 kg (178 days, ACC 2) or 560 kg (242 days, ACC 3). Secondly, the conventionally fed cattle (CONV) were fed a high roughage diet for 110 days, followed by a 21-day pre-finishing adjustment phase and then finished on the same diet as the ACC groups. These cattle were slaughtered after 284 days at a mean live weight of 596 kg.

Cattle were slaughtered at the Kansas State Univ. meat laboratory. Carcasses ranged from a group mean yield grade of 2.2 - 3.1, a quality grade of Good 21% to Good 95%, and had average carcass weights of 262 kg (ACC 1), 309 kg (ACC 2), 351 kg (ACC 3), and 358 kg (CONV).

Animals were stunned and bled. Bleeding time was used as time zero for all treatments. Each of 96 sides was assigned to one of four treatments in a completely randomized block design: control (C), electrical stimulation (ES), hot boning (HB), or electrical stimulation plus hot boning (ESHB).

The C sides were chilled at 5°C for 48 hr postmortem. ES was applied through 6 mm diameter stainless steel probes, one inserted in the inside round about 8 cm below the attachment of the achilles tendon and the other laterally along the humerus. Sides were stimulated at 45 min postmortem with 400 - 600v of alternating current (60 Hz and 0.6 amp delivered through the carcass) for 2 min with a sequence of 1.6 sec on and 0.8 sec off and chilled until 24 hr postmortem at 5°C. The longissimus (LD) and semimembranosus (SM) muscles from the HB and ESHB sides were excised 2 hr postmortem and stored until 24 hr postmortem at 2°C in an oxygen impermeable bag.

After chilling, an LD steak (2.5 cm) was cut from the posterior portion (over 2nd to 3rd lumbar vertebrae) of the LD and an SM steak (2.5 cm) was cut from the distal section of the SM. Steaks were vacuum packaged with a Bivac 1 packager using high oxygen-barrier film, 3.3 mil base, and a 5.2 mil top web (oxygen transmission rate = 10 cc/m^2/24 hr at 23°C). Steaks were stored in the dark 6 days at 5°C prior to display to allow for myoglobin reduction and more closely simulate a commercial situation with distribution and storage time.

Fresh, vacuum packaged steaks were displayed at 2 - 4°C under General Electric Natural® fluorescent (40 watt) lighting providing 1076 lux continuously (24 hr/day) at the meat surface. Visual appraisal, individually by a four member panel, and reflectance spectrophotometry were used to evaluate muscle color on days 0, 3, 7, and 14 of display. Visual scores were estimated to the nearest 0.5 on a five-point scale: 1 = bright purple red, 2 = dull purple red, 3 = slightly brownish purple red, 4 = brownish purple red, and 5 = brown. Reflectance at 474, 525, 580, and 630 nm was measured using a Bausch and Lomb 600 reflectance spectrophotometer adjusted to 100% reflectance with a $MgCO_3$ block. One reading per steak was taken each time at a location considered "average" for the steak. Reflectance values were used to estimate reduced myoglobin (%R474 nm/%R525 nm), metmyoglobin (%R630 nm/%R525

nm), and oxymyoglobin (%R630 nm-%R580 nm). Data were analyzed by analysis of variance. Least significant different was calculated to determine differences between means when appropriate.

RESULTS & DISCUSSION

VISUAL COLOR SCORES (Table 1) for HB LD and SM steaks were frequently lower (brighter purple-red) than for other treatments. ES LD steaks were visually the same ($P > 0.05$) as ESHB LD steaks, whereas ES SM steaks were duller and less bright in appearance ($P < 0.05$) than ESHB SM steaks at all display times. ES steaks were not different ($P > 0.05$) from C steaks for the LD or SM, except on day 14 in which ES SM steaks were visually less bright ($P < 0.05$) than C SM steaks.

Measurements to estimate reduced myoglobin (%R474 nm/%R525 nm) and metmyoglobin (%R630 nm/%R525 nm) indicated these chemical states were uniform across treatments in both muscles. ES LD steaks tended to have more oxymyoglobin (higher %R630 nm-%R580 nm) than C LD steaks, whereas ES SM steaks were not different ($P > 0.05$) from C SM steaks. HB steaks tended to have lower indications of oxymyoglobin (lower %R630 nm-%R580 nm) compared to ES for both muscles and compared to C steaks from SM muscle.

Since vacuum packaging removes oxygen and reduces its partial pressure in the package, the primary chemical state of myoglobin with a good vacuum likely is purple-red reduced myoglobin. Visually, HB muscles were mostly brighter purplish-red than muscles from conventionally handled carcasses. Working on the same carcasses and muscles, Axe et al. (1983) found that HB muscles had slightly faster temperature declines than those cold boned at 48 hr. The faster temperature decline could conserve reducing pathways in the muscle. In Claus et al. (1984), we proposed that HB muscles may have a greater reducing capacity in a polyvinylchloride (oxygen permeable) packaging system. However, in vacuum packaged muscle, evidence of this conservation was not supported by reflectance estimates of reduced myoglobin. Other measurements of metmyoglobin reducing activity were not made and these should be more reliable than reflectance measurements.

ES muscle appeared more faded and lighter colored than HB muscles. These differences may be due to a more rapid drop in pH and subsequent changes in the water holding capacity, muscle structure, and light reflecting properties. Also the greater oxygen incorporation into ES muscle could result in increased oxymyoglobin, but with oxygen utilization by muscle, partial oxygen pressure may decrease to a level favoring metmyoglobin formation and a duller browner meat color. Sleper et al. (1983) reported LD muscle from ES to have greater Hunter CIE "L" values, less reduced myoglobin, and more oxymyoglobin for LD muscle bloomed 0, 1, 3, 4, 5, and 5 min when done 6 days postmortem. This applies particularly well to the SM, as the ES SM deep within the round muscles of the hindquarter would have a higher temperature at a given pH than C or HB muscles. HB SM muscles were more rapidly chilled (Axe et al., 1983) thereby possibly preserving metmyoglobin reducing ability, muscle cell membrane integrity and hydrophilic nature of proteins and other constituents. In cold-boned muscle, the inner portion of the SM that was located deep within the muscles of the round was lighter in color than the outer portion. However, in HB muscle this difference was not as severe, an observation supported by Taylor et al. (1980).

Savell et al. (1979), Dutson et al. (1980), and Judge et al. (1980) proposed that ES improves the tenderness of muscles by a direct physical disruption or by some chemical effect on the muscle or connective tissue. Consequently, the tenderness improvement and the more rapid pH decline (Axe et al., 1983) of ES may be associated with a more open muscle structure, which in turn may result in greater

Table 1—Effects of electrical stimulation and hot boning on the visual color score[d] and reflectance measurements of unfrozen vacuum packaged beef steaks

Day	Carcass treatments[e]							
	D	ES	HB	ESHB	C	ES	HB	ESHB
	Longissimus				Semimembranosus			
	Visual color score							
0	1.2[b]	1.2[b]	1.0[b]	1.1[ab]	1.5[b]	1.5[b]	1.2[a]	1.3[a]
3	1.4[a]	1.4[a]	1.2[a]	1.4[a]	1.7[bc]	1.9[c]	1.5[a]	1.6[ab]
7	1.6[b]	1.7[b]	1.4[a]	1.5[b]	2.1[c]	2.1[c]	1.7[a]	1.9[b]
14	2.1	2.0	1.9	2.1	2.6[b]	2.8[c]	2.3[a]	2.5[b]
	%R474 nm/%R525 nm							
0	1.59	1.60	1.60	1.60	1.60	1.59	1.61	1.60
3	1.60	1.61	1.59	1.62	1.62[b]	1.61[a]	1.58[a]	1.59[ab]
7	1.59	1.61	1.57	1.55	1.62	1.59	1.59	1.60
14	1.59	1.58	1.59	1.59	1.62[b]	1.56[a]	1.60[ab]	1.58[ab]
	%R630 nm/%R525 nm							
0	2.24	2.29	2.27	2.26	2.37	2.37	2.33	2.37
3	2.27	2.25	2.26	2.23	2.38[b]	2.38[b]	2.33[ab]	2.32[a]
7	2.21	2.21	2.20	2.16	2.32	2.31	2.26	2.30
14	2.24	2.24	2.28	2.26	2.45[b]	2.36[ab]	2.39[ab]	2.33[a]
	%R630 nm-%R580 nm							
0	15.8[a]	17.3[b]	16.0[a]	16.5[ab]	16.3[b]	16.6[b]	14.6[a]	15.1[a]
3	16.4[ab]	17.2[b]	16.2[a]	16.8[ab]	16.3[bc]	16.4[c]	15.2[a]	15.6[ab]
7	16.5[a]	17.4[b]	16.5[a]	17.0[ab]	16.4[b]	16.7[b]	15.5[a]	16.0[ab]
14	15.6	16.3	15.6	15.9	15.6	15.5	15.2	15.1

[abc] Means for the same muscle and row with the same or no superscript letter are not different ($P > 0.05$).
[d] Visual color score: 1 = bright purple red, 2 = dull purple red, and 3 = slightly brownish purple red.
[e] C-control, ES-Electrical stimulation, HB-not boning, and ESHB-electrical stimulation plus hot boning.

reflective properties of the muscle causing it to appear lighter. These factors would also be related to greater oxygen incorporation by the muscle. Sleper et al. (1983) had reported the greater brightness of LD muscle from ES to be due largely to greater Hunter CIE "L" values rather than a greater percentage oxymyoglobin.

Few differences existed between treatments in reflectance measures of reduced myoglobin and metmyoglobin. Pierson et al. (1970) studied the initial pigment change at 3.3°C of anaerobically packaged beef. They determined that the oxymyoglobin content of freshly vacuum packaged SM steaks rapidly decreased and was negligible after 4 hr. During the oxymyoglobin decline, both metmyoglobin and reduced myoglobin increased and at about 4 hr metmyoglobin peaked and then declined until no longer measurable after 8 hr. Reduced myoglobin continued to increase until 100% of the pigment was in this state (8 hr). Reduced myoglobin content of anaerobically packaged sliced beef remained very constant (100%) throughout a 15-day storage period.

Muscle color of vacuum packaged unfrozen beef steaks appeared to be much more uniform across treatment groups than in polyvinylchloride packaged steaks (Claus et al., 1984). Also, vacuum packaging provided a more stable color over longer display times as Pierson et al. (1970) found in anaerobically packaged beef steaks. Vacuum packaged retail cuts also have the advantage of being more microbiologically stable and consequently have a longer shelf-life compared to cuts packaged in oxygen permeable film (Kraft and Ayres, 1952; Smith et al., 1974). If consumers were introduced properly to the purplish-red color of vacuum packaged beef and would accept it, then this packaging system could be used to extend the shelf-life of unfrozen beef. In addition to lack of consumer acceptance and the cost of vacuum packaging, a major disadvantage is the lack of a visual indicator of freshness to assess microbial and/or sensory soundness. Also, with extended display time, some off odors have been observed upon opening the package (Sutherland et al., 1976; Griffin et al., 1982).

Although subtle visual differences in muscle color between C, ES, HB and ESHB were observed, few differences were found in the reflectance indicators of reduced myoglobin, metmyoglobin, and oxymyoglobin. Additionally, regardless of treatment, muscle color was acceptable at all display times up to 14 days.

In summary, vacuum packaging of steaks seems appropriate for all of the postmortem carcass treatments of the study, especially for steaks from hot boned cuts.

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Effect of Electrical Stimulation and Boning Temperature on Distribution and Migration of Curing Ingredients (Nitrite, Salt, Glucose) in Pork Tissue

H. W. OCKERMAN and K. KWIATEK

ABSTRACT

Hot and cold-boned pork samples, both electrically stimulated and nonstimulated were placed below a brine solution in tubes, held at 3 - 5°C and sampled at 24, 48 and 72 hr at four segments depths of 1.25 cm each. Results indicated that electrical stimulation caused improvement ($P < 0.01$) in absorption and distribution of curing ingredients both in hot and cold-boned pork tissue. The migration of nitrite was higher ($P < 0.01$) for hot-boned pork tissue electrically stimulated and nonstimulated. The salt level was higher ($P < 0.05$) in cold-boned tissue than in hot-boned tissue and the level of glucose, in general, was higher ($P < 0.01$) in cold-boned than in hot-boned tissue for each time at each depth.

INTRODUCTION

IN PREVIOUS RESEARCH, Ockerman and Kwiatek reported that electrical stimulation caused a significant ($P < 0.01$) improvement in absorption and migration of curing ingredients (nitrite, salt, glucose) into all depths of pork samples which were prepared from cold-boned and hot-boned *triceps brachii* pork muscles (Ockerman and Kwiatek, 1985a, b). The authors speculated that this overall increase in migration and concentration of curing ingredients in both cases was probably influenced by the disruption of muscle tissue caused by electrical stimulation (Savell et al., 1978; Sonaiya et al., 1982; Cross, 1979). Electrical stimulation has also been shown to alter the chemical, physical, sensory and cooking characteristics of meat (Chrystall and Hagyard, 1975, 1976; Grusby et al., 1978; Gilbert and Davey, 1976; Gilbert et al., 1976; Davey et al., 1976; Smith et al., 1977, 1980; Savell et al., 1977, 1979 and Sorinmade et al., 1978).

Meat processed from prerigor hot-boned carcasses has been shown to be superior to cold-boned postrigor tissue in palatability (Cross et al., 1979; Seideman et al., 1979; Wells et al., 1980) and hot processing of primal cuts of beef and pork has become increasingly popular in the meat industry (Butcher, 1980; Cross et al., 1979; Taylor et al., 1980). It is likely that in the future a good percentage of the carcasses providing those primals will be electrically stimulated. Prerigor hot-boned muscle with or without electrical stimulation may respond differently to various meat processing methods than chilled muscle tissue. Therefore it would be interesting from a technological and a research point of view to evaluate time of boning (hot-boned vs. cold-boned) and its interactions with electrical stimulation on the influence of migration and distribution of curing components in muscle tissue.

This study was designed to analyze differences and interactions in migration and distribution of curing ingredients in pork tissue that was hot-boned and cold-boned with and without electrical stimulation.

Author Ockerman is affiliated with the Dept. of Animal Science, The Ohio State Univ., Columbus, OH 43210 and The Ohio Agricultural Research & Development Center, Wooster, OH 44691. Author K. Kwiatek is affiliated with Department of Hygiene of Animal Products, Veterinary Research Institute, Pulawy, Poland.

MATERIALS & METHODS

PIGS were conventionally slaughtered and the left side of each carcass was electrically stimulated after splitting within 30 min post-slaughter. The carcasses were stimulated using a High Voltage JASEC Electrical Meat Stimulator. Each treated side received 15 electrical impulses of 500V alternating current of 2.5 sec each in duration followed by 2.5 sec of no current. The other side (right) was used as a control (nonstimulated). The triceps brachii muscles were removed for hot-boned carcasses directly after electrical stimulation or within 30 min postmortem and 24 hr postmortem after chilling (3 ± 2°C) for cold-boned samples from both sides of the carcasses. This muscle was used because it was easy to obtain early postmortem with minimum destruction to the carcass. Cylindrical samples of 1.5 cm in diameter and 5 cm in length (parallel to muscle fibers) were prepared from the muscle using an electrical cork borer. These cylindrical samples were tightly placed into plastic tubes (1.5 cm internal diameter x 9 cm in length) in an effort to prevent migration of curing solution between the sample and side of the tube. Two and one-half mL curing solution composed of 20% NaCl, 6% glucose and 0.16% sodium nitrite was added to the tubes above the tissue samples. This volume of cure was proven in preliminary research to be sufficient to have cure remaining after the storage period. Both stimulated and nonstimulated samples were held at 3° - 5°C and sampled at 24, 48, and 72 hrs. At each sampling time, the excess cure above the sample was discarded and the cylindrical sample was removed from the tube and divided into 4 cylindrical segments of 1.25 cm in depth from the top (adjacent to curing solution) to the bottom of the sample. These segmented samples were each individually analyzed for nitrite level using the procedure described by Ockerman (1981) with two necessary changes: (1) samples were blended in a Stomacher Lab-Blender with 80 mL hot 70°C distilled water for 2 min, then quantitatively transferred into 500 mL volumetric flasks; (2) no mercuric chloride was added to the flasks after 2 hrs extraction in a water bath to avoid the addition of any extra chloride ions. Ockerman and Dowiercial (1980) have reported that elimination of mercuric chloride did not affect the analysis for nitrite in cured tissue. Thus, it was possible to use the same extract to determine the content of nitrite, sodium chloride and glucose. Salt was analyzed by the Dicromat® (Diamond Crystal Salt Co., St. Clair, MI) procedure (Anonymous, 1977) using the salt analyzer. Glucose was determined by the procedure described by Koniecko (1979) for meat products. Ten *triceps brachii* cylindrical samples were prepared for each treatment (stimulated vs. nonstimulated), each boning temperature (hot vs. cold), each time period (24, 48, 72 hr) for the individual tissue segments (0 - 1.25, 1.26 - 2.50, 2.51 - 3.75, and 3.76 - 5.0 cm). All samples were analyzed for NaCl, $NaNO_2$ and glucose. This resulted in a total of 40 determinations for nitrite, salt and glucose for each treatment and for each period of time.

Analysis of variance (Harvey, 1968) was used to determine the significance of stimulation, boning temperature, depth of sample, storage time and their interactions. Means were separated by Duncan Multiple Range Test (Ockerman, 1983).

RESULTS & DISCUSSION

TABLE 1 shows the statistically significant ($P < 0.01$) four-way interactions (boning temperature x stimulation x depth x time) for nitrite, indicating the interdependence of all of these treatments on the nitrite concentrations. As would be anticipated, the average level of nitrite decreased at each segment depth during the curing process. This concentration was influenced by electrical stimulation which resulted in a statistically significant ($P < 0.01$) im-

Table 1—Comparison of least square means for the level of nitrite (ppm) for hot and cold-boned meat samples electrically stimulated and non-stimulated at various depths for various period of time

Depth in cm		0–1.25		1.26–2.50		2.51–3.75		3.76–5.0		Interaction for time X stimul. X boning temp	
Time in Hours	Treatment	Hot boned	Cold boned	Hot boned	Cold boned	Hot boned	Cold boned	Hot boned	Cold boned	Hot boned	Cold boned
24	Stimulated	399^{ax}	313^{bx}	172^{ax}	114^{bx}	83^{ax}	74^{ax}	62^{ax}	47^{ax}	179^{ax}	137^{bx}
	Nonstimulated	397^{ax}	316^{bx}	137^{ay}	82^{by}	59^{ay}	30^{by}	39^{ay}	33^{ax}	158^{ay}	115^{by}
48	Stimulated	479^{ax}	408^{bx}	177^{ax}	140^{bx}	89^{ax}	91^{ax}	64^{ay}	64^{ax}	202^{ax}	176^{bx}
	Nonstimulated	435^{ay}	373^{by}	170^{ax}	92^{by}	77^{ax}	42^{by}	46^{ax}	36^{ay}	182^{ay}	136^{by}
72	Stimulated	478^{ax}	447^{bx}	213^{ax}	142^{bx}	126^{ax}	98^{bx}	97^{ax}	76^{ax}	229^{ax}	191^{bx}
	Nonstimulated	433^{ay}	408^{by}	146^{ay}	125^{bx}	83^{ay}	47^{by}	46^{ay}	44^{ay}	177^{ay}	156^{by}
Interactions for depth X stimulation X boning temp.	Stimulated	452^{ax}	390^{bx}	187^{ax}	132^{bx}	99^{ax}	88^{ax}	74^{ax}	62^{ax}		
	Nonstimulated	421^{ay}	366^{by}	151^{ay}	100^{by}	73^{ay}	40^{by}	42^{ay}	32^{ay}		

^{a,b} Means for the same item within the same row at the same depth bearing different superscripts are different at $P<0.01$.
^{x,y} Means for the same item within the same column at the same time period bearing different superscripts are different at $P<0.01$.

provement in migration and absorption of nitrite both in hot and cold-boned meat tissue. For nitrite concentration the 3-way interactions depth x stimulation x boning temperature and time x depth x boning temperature were also statistically significant ($P < 0.01$). These interactions and the least square means suggest that, in addition to stimulation, time and depth, hot-boning in conjunction with these also increased the rate of migration and penetration of nitrite. Higher levels of nitrite at each depth and each time period were observed in both unstimulated or electrically stimulated hot-boned muscle tissues when compared to cold-boned unstimulated or electrically stimulated tissue. These differences were large enough to be significant except after 24 hr at the 2.51 – 3.75 cm (stimulated) and 3.76 – 5.0 cm (stimulated and nonstimulated) depths, after 48 hr at the 2.51 – 3.75 cm (stimulated) and 3.76 – 5.0 cm (stimulated and nonstimulated) depths and after 72 hr at the 3.76 – 5.0 cm (stimulated and nonstimulated) depth. In these cases the levels of nitrite in hot-boned samples were not sufficiently higher to be statistically significant. As also shown by the 3-way interaction (time x stimulation x boning temperature) in Table 1, the least square means for nitrite in hot-boned tissue at each treatment time were significantly higher ($P < 0.01$) for stimulated than for nonstimulated tissue as well as being higher for hot-boned, in comparison to cold-boned tissue. This suggests that both electrical stimulation and hot-boning improved the migration and distribution of nitrite. The same tendency for hot boning to increase the nitrite level was observed in the other 3-way interaction (depth x stimulation x boning temperature) in Table 1 for nitrite values for each sample depth except for the 2.51 – 3.75 cm (stimulated) and 3.76 – 5.0 cm (stimulated and nonstimulated) depths, when stimulated hot- and cold-boned tissues were compared, and in these cases the increase in nitrite was not large enough to be statistically significant. Mullins et al. (1958) also reported that cure penetration was somewhat better in hot-injected hams than in chilled hams.

The significant 2-way interactions for the migration and distribution of salt in pork tissues are shown in Fig. 1 (time x depth) and Fig. 2 (stimulation x depth). The comparison of salt concentrations for each time period at each level (Fig. 1) indicated a general increase with time and in all cases the 72 hr level was significantly higher ($P < 0.01$) than the 24 hr time period concentrations. The 48 hr time period salt level, although always intermediate, was not significantly different from the 72 hr salt level at 0 – 1.25 cm and 2.51 – 3.76 cm depths and not significantly different from the 24 hr salt level at 1.26 – 2.50 cm and 3.75 –

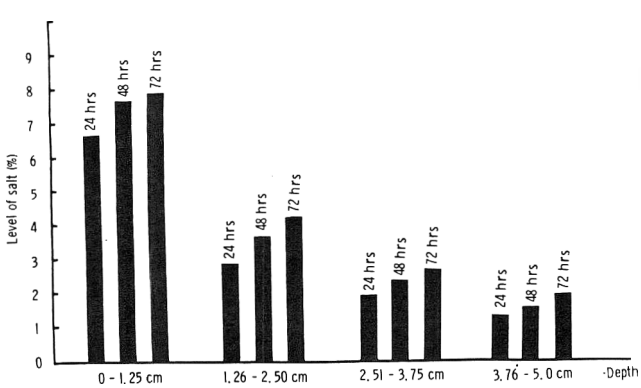


Fig. 1—Effect of time on the concentrations of salt at several depths in pork tissue (n = 20).

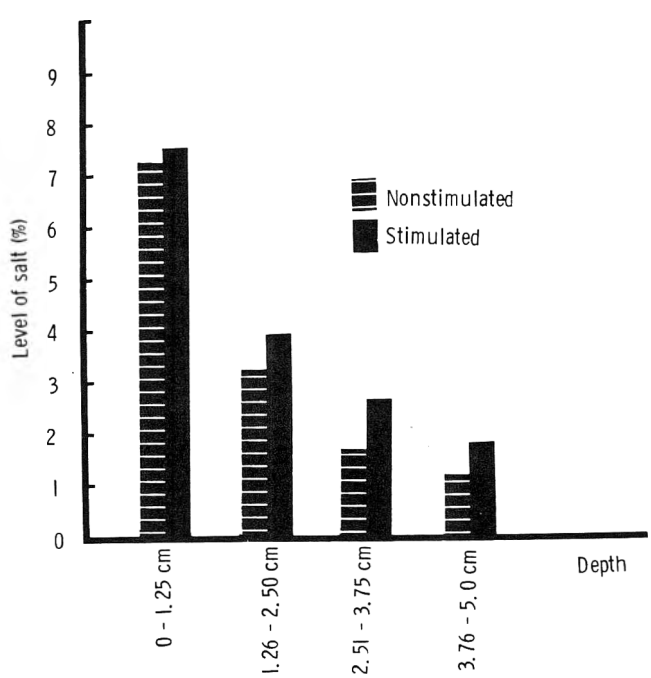


Fig. 2—Effect of electrical stimulation on the concentrations of salt at several depths in pork tissue (n = 20).

5.0 cm depths. The significant ($P < 0.01$) stimulation x depth interaction for salt concentrations (Fig. 3) suggests that electrical stimulation increased salt concentration at all depths in pork tissue during the curing process.

The other 2-way interactions (stimulation x boning temperature, stimulation x time, time x boning temperature, depth x boning temperature) and the 3-way interactions (stimulation x time x depth, stimulation x depth x boning temperature, stimulation x time x boning temperature, time x depth x boning temperature) and the 4-way interaction (stimulation x time x depth x boning temperature) for salt levels were not statistically significant ($P < 0.05$). However, the influence of boning temperature was significant ($P < 0.05$) with cold-boned tissue having slightly higher level of salt (3.8%) than hot-boned tissue (3.6%). The slower penetration of salt into hot-boned compared to cold-boned pork tissue was probably caused by the higher pH of hot-boned muscle tissue at the beginning of the experiment. This agrees with the report of Lawrie (1979) that the higher the pH, the slower the penetration of salt into pork tissue, and also agrees, in general, with the research reported by Arganosa and Henrickson (1969).

The glucose concentration three-way interactions for time x depth x boning temperature, stimulation x depth x boning temperature and stimulation x time x depth were statistically significant ($P < 0.01$). As illustrated in Fig. 3, and shown in the mean separation calculation, the level of glucose for each time at each depth was significantly ($P < 0.01$) higher in cold-boned than in hot-boned tissues except for the 0 - 1.25 cm depth at 24 hr, where the glucose concentration was significantly higher for hot-boned tissue, for the 0 - 1.25 cm depth at 48 and 72 hr there were not significant differences and at the 1.25 - 2.50 cm depth at 48 hr where the increased level for cold-boned tissue was again not large enough to be significant.

Fig. 4 illustrates the stimulation x depth x boning temperature interaction for glucose. Mean separation calculations indicated that electrical stimulation caused a significant increase in the level of glucose in muscle tissue, when compared to nonstimulated tissue, in both hot and cold-boned treatments, except for hot-boned tissue at the 2.51 - 3.75 cm depth. Electrical stimulation resulted in an increase in glucose level at each time and depth (Fig. 5), and these increases were all large enough to be significant except for the 24 hr glucose values at the 0 - 1.25 cm depth and the 48 and 72 hr glucose levels at the 2.51 - 3.75 cm depth.

The overall results for the influence of time, depth and electrical stimulation on the curing components levels are as would be expected and agree in general with the literature (Ockerman and Kwiatek, 1985a, b; Ockerman and Dowiercial, 1980). The results of this research on hot vs cold-boning were not as consistent; the cure ingredients (salt, glucose) in higher concentration generally migrated faster in cold-boned tissue, and the nitrite level migrated faster in hot-boned tissue. The differences in tissue pH, water-holding capacity, state of fiber swelling, ionic charge and concentrations of curing ingredients all offer possible explanations but this research did not distinguish between these and other possible explanations for this difference in migration speed of the individual cure components in hot and cold-boned tissue.

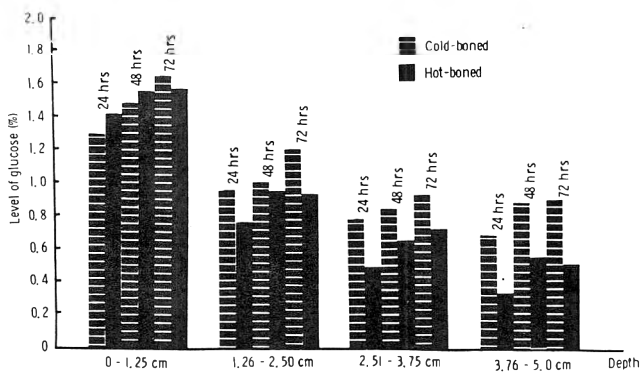


Fig. 3—Effect of boning temperature (hot and cold) and time on the concentrations of glucose at several depths in pork tissue (n = 20).

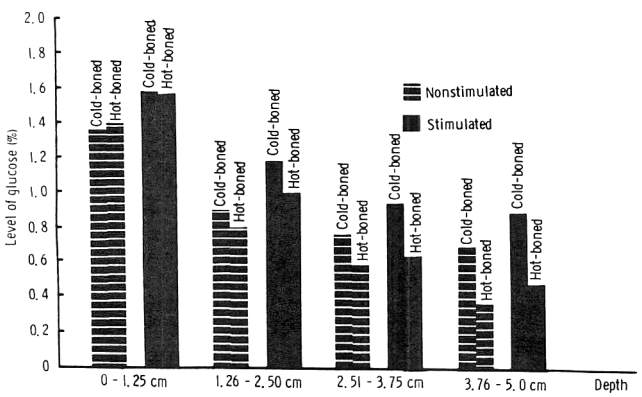


Fig. 4—Effect of electrical stimulation and boning temperature (hot and cold) on the concentrations of glucose at several depths in pork tissue (n = 20).

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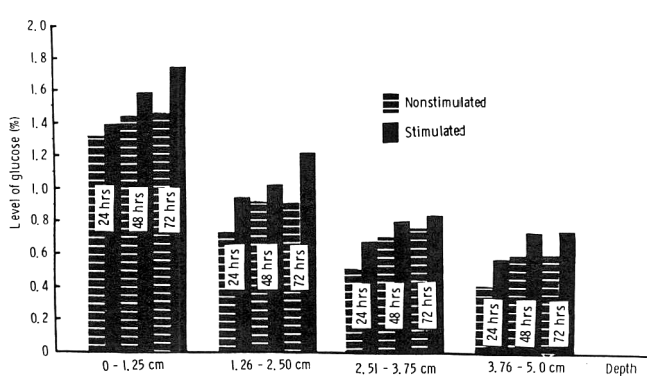


Fig. 5—Effect of electrical stimulation and time on the concentrations of glucose at several depths in pork tissue (n = 20).

Effect of Coconut Cream Addition to Soymilk on the Composition, Texture, and Sensory Properties of Tofu

ELIAS E. ESCUETA, MALCOLM C. BOURNE, and LAMARTINE F. HOOD

ABSTRACT

Coconut cream was added to soymilk at 0, 4.7, 9.0, 16.6, 33.3, and 50% levels prior to coagulation with calcium sulfate. The protein and moisture content of the tofu decreased while fat increased with increasing levels of added coconut cream. The percent yield of tofu did not increase up to 4.7% added coconut cream but increased significantly when 9.0% or more coconut cream was added to soymilk. Hardness, springiness, cohesiveness and gumminess were increased by low levels of added coconut cream and decreased with high levels of coconut cream. This simple technology of coconut cream enrichment increased the caloric density of the tofu without affecting its acceptability.

INTRODUCTION

IN AN EXTENSIVE nationwide survey, the Food and Nutrition Research Council of the Philippines (1969) reported that the available food energy per capita in the Philippines is 1804 calories per day which is only 88.6% of the recommended allowance despite the large intake of cereal products, sugar and syrups. This is attributed to the low intake of fats, oils, nuts, and seeds. Bautista (1979) reported that the average Filipino consumes only 27% of the recommended fats and oils intake. It is also apparent that protein which is reported to be adequate (Intengan and Marfori, 1971) is not being utilized efficiently as a body building nutrient since part of this protein intake is utilized for energy needs.

Coconut cream is rich in fat and there is an abundant supply of coconuts in the Philippines. Malabrigo (1977) reported that the Philippines produced 26% of the world's coconut output and accounted for 66% of the world's total combined copra and coconut oil exports. In 1982, the country exported 191,788 metric tons of copra and 948,942 metric tons of oil valued at $52.8 and $410 million, respectively. Moreover, coconut fat is generally accepted to be more digestible than most other fats and oils because it is liquid at body temperature (Banzon, 1977).

The goal of this study was to explore the possibility of increasing the caloric density of tofu (soybean curd), a product that is already part of the Filipino diet. The study was specifically designed to determine the effect of coconut cream enrichment of soymilk on the yield, composition, textural properties and sensory qualities of tofu.

MATERIALS & METHODS

Preparation of soymilk

Philippine grown soybeans of L-114 variety were used. A modification of the boiling-water grind technique described by Bourne et al. (1976) was used in the preparation of soymilk. One kg of soybeans was soaked in three times its weight of tap water at ambient temperature for approximately 8 hr then drained, washed in fresh water and drained again. A 1-gal. capacity Waring Blendor was used to grind the soaked beans with boiling tap water. The weight ratio of dry beans to water was at 1:10, taking into account the water absorbed during soaking. The slurry was filtered through 2 layers of cheesecloth to remove the insolubles. The soymilk contained 4.5% total solids, 1.2% fat and 3.4% protein.

Preparation of coconut cream

Mature coconuts used in this study were purchased from the local "sari-sari" (variety) store. Spoiled nuts (usually detected by a softening in the eye of the kernel) were culled. The husked mature coconuts were halved by striking the shell sharply with a bolo knife. The water was drained off and the white meat was separated from the shell by grating in a motorized rotary grater then ground in a Waring Blendor at high speed with an equal amount of water (w/w). The slurry was pressed through two layers of cheesecloth to obtain the cream. The coconut cream had the following composition: moisture, 77.2%; protein, 2.2%; fat, 17.3%; ash, 0.8%; and other constituents by difference, 2.5%.

Preparation of tofu

The following combinations of soymilk and coconut cream (w/w) were made:

Soymilk only (1:10);
20 parts soymilk + 1 part coconut cream (20:1);
10 parts soymilk + 1 part coconut cream (10:1);
5 parts soymilk + 1 part coconut cream (5:1);
2 parts soymilk + 1 part coconut cream (2:1); and
1 part soymilk + 1 part coconut cream (1:1).

The mixtures were homogenized at ambient temperature under 4,000 psi in a single stage Manton Gaulin homogenizer to prevent separation of the fat layer. A stainless steel bucket containing each mixture was placed in a steam jacketed kettle in boiling water for 10 min which brought the milk to about 98°C. Then it was immersed in cold tap water to bring the temperature to about 80°C. Calcium sulfate was added at the rate of 0.25% of the weight of the milk which was stirred vigorously for a few seconds. The mixture was allowed to coagulate for 1 hr. The curd was poured into a wooden box (12 cm x 25 cm x 15 cm high) lined with cheesecloth and the supernatant allowed to drain through the 1.2 cm diameter holes in the sides and bottom of the box for 1 hr. The curd was allowed to set overnight under a weight of approximately 6.65 g/cm^2 before analyses were carried out.

Texture analysis

Texture profile analysis using an Instron Testing Machine following the method of Bourne (1968) was used to determine the textural qualities of tofu. The Instron crosshead was cycled with a vertical reciprocating movement at a constant speed of 50 mm per min with a stroke length of 7.5 mm. The maximum clearance between the moving horizontal plate and the stationary horizontal bedplate of the machine was 10 mm and the minimum clearance was 2.5 mm high giving a 75% compression. Chart speed was 500 mm/min. Eight to ten 2 cm diameter by 1 cm high cylinders of tofu per treatment were prepared using a cork borer. Each piece of tofu was compressed twice to give a first and a second bite at room temperature. The following textural parameters were derived from the force-time curve based on the method of Bourne (1968); hardness, springiness, cohesiveness, gumminess and chewiness.

Chemical analysis

Analysis of all samples was made in duplicate. Total nitrogen was determined by the micro-Kjeldahl method (AOAC, 1975, method no. 47.021) on solid samples and protein calculated using a

Authors Bourne and Hood are affiliated with the Institute of Food Science, Cornell Univ., Ithaca, NY 14850. Author Escueta's present address is Institute of Food Science & Technology, Univ. of the Philippines at Los Banos, College, Laguna 3720, Philippines.

factor of 6.25 x N. Crude protein of liquid samples was determined by the method of Lowry et al. (1951). Fat was determined by petroleum ether extraction in the Goldfisch fat extractor (AOAC, 1975; method no. 14.080) and moisture and total solids in a forced draft hot air oven at 100 - 102°C for 24 hr

Percentage recoveries

The yields of tofu components protein, fat and total solids were calculated by dividing the amounts of these components in tofu by their respective amounts in the soymilk-coconut cream blend and multiplied by 100. The recovery of tofu was calculated by dividing the weight of tofu by the weight of soymilk-coconut cream blend and then multiplied by 100.

Sensory evaluation

A trained panel comprised of nine young adult Filipinos who were experienced in sensory evaluation evaluated flavor, mouthfeel, moistness and overall acceptability using a 7-point scale. Acceptability ranged from 1-dislike very much to 7-like very much; moistness from 1-very dry to 7-very moist; mouthfeel from 1-very coarse to 7-very smooth; and flavor from 1-very strong off-flavor to 7-very pleasant flavor. The tofu was fried and cooled to room temperature (28 - 30°C) before presenting a 40 - 50g sample to the panelists for evaluation. All samples were coded with randomly assigned numbers and presented to the panelists in a randomized arrangement to equalize the sample sequence effect on food preference (Eindhoven et al., 1964).

Statistical design

The experiment were performed using the randomized complete block design (RCB). Each experiment was done independently with three replications, where each replication (block) consisted of a separate batch. Analysis and measurements were made in duplicate, giving a total of six values per observation. Analysis of variance was performed using the RCB design, followed by a multiple range test during the least significant difference (LSD) when the F value was significant (Cochran and Cox, 1957).

Table 1—Composition of soymilk, tofu and whey as affected by coconut cream enrichment of soymilk (percent as is basis)

	Coconut cream added (%)					
Component	0	4.7	9.0	16.6	33.3	50
Soymilk-coconut cream blend						
Total solids	4.5[a]	5.5[b]	6.5[c]	7.7[d]	10.3[e]	12.7[f]
Fat	1.2[a]	2.0[b]	2.7[c]	4.2[d]	6.6[e]	9.2[f]
Protein	3.4[a]	3.4[a]	3.3[a]	3.3[a]	3.1[b]	2.8[c]
Tofu						
Moisture	77.2[a]	73.5[b]	70.3[c]	68.3[d]	66.9[e]	63.0[f]
Fat	6.3[a]	8.3[b]	11.9[c]	17.3[d]	21.1[e]	27.6[f]
Protein	12.3[a]	12.0[a]	11.4[b]	10.6[c]	8.5[d]	6.5[e]
Whey[g]						
Total solids	2.4[a]	2.1[a]	2.3[a]	2.5[a]	2.2[a]	2.4[a]
Fat	0.2[a]	0.3[a]	0.3[a]	0.3[a]	0.3[a]	0.3[a]
Protein	0.6[a]	0.6[a]	0.6[a]	0.6[a]	0.6[a]	0.6[a]

[a-f]Row means with the same letter are not significantly different (P = 0.05). LSD values of each parameter from top to bottom are: 0.59, 0.39, 0.27, 1.06, 0.79, 0.74.
[g] LSD comparison not done since ANOVA not significant

Table 2—Recovery of tofu, protein, fat and total solids from soymilk with various levels of added coconut cream (percent of original weight in milk)

	Coconut cream added (%)					
Component	0	4.7	9.0	16.6	33.3	50
Tofu	22.6[a]	22.7[a]	24.1[b]	26.9[c]	27.7[d]	31.3[e]
Protein	89.6[a]	88.2[b]	88.1[b]	88.3[b]	86.8[c]	86.6[c]
Fat	82.5[a]	82.7[a]	84.4[b]	87.8[c]	93.4[d]	96.6[e]
Solids	84.4[a]	85.5[b]	87.1[d]	87.7[d]	90.1[e]	91.7[f]

[a-f]Row means with the same letter are not significantly different (P = 0.01). LSD values: Tofu, 0.16; protein, 0.21; fat, 0.51; solids, 0.24.

RESULTS & DISCUSSION

Chemical composition

The composition of soymilk-coconut cream blend, tofu and whey as affected by levels of coconut cream enrichment of soymilk is shown in Table 1. Total solids and fat of the soymilk-coconut cream blend increased significantly with each level of added coconut cream, while protein was almost unchanged up to 16.6% levels of added coconut cream and then decreased significantly after 33.3% of added coconut cream. Moisture and protein of the tofu decreased significantly while fat increased significantly with added coconut cream. The composition of whey was practically unchanged for all levels of coconut cream addition.

These changes in composition of the soymilk-coconut cream blends and the tofu reflect the fact that soymilk contains about 3% protein but is low in fat (about 1%) while coconut cream contains about 2% protein but is high in fat (about 17%).

Recovery of tofu components

The recoveries of tofu, protein, fat and solids from different soymilk-coconut cream blends are shown in Table 2. The yield of tofu was not significantly affected from 0 - 4.7% of added coconut cream but was significantly affected when 9.0% or more of the coconut cream was added. The recoveries of fats and solids increased significantly as levels of added coconut cream were increased. The protein yield, decreased from 89.6% at 0% coconut cream to 86.6% at 50% added coconut cream to soymilk. Increasing fat of the blend may have interfered with the protein coagulation with calcium sulfate. These data, together with that in Table 1, indicate that the increasing yield of tofu from the coconut cream enriched soymilk is primarily due to the higher amount and higher recovery of fat in the tofu.

Texture profile analysis

The texture profile analysis parameters are shown in Table 3. The general trends of the changes in textural

Table 3—Texture profile analysis of tofu affected by coconut cream enrichment of soymilk

TEXTURE PARAMETER	0	4.7	9.0	16.6	33.3	50.0
Hardness (Newtons Force)	3.9[a]	3.8[a]	4.0[a]	4.0[a]	3.4[b]	3.3[b]
Springiness (mm)	2.5[a]	3.2[c]	2.9[abc]	3.0[bc]	2.9[abc]	2.6[ab]
Cohesiveness (A_2/A_1)	0.35[a]	0.38[a]	0.42[ab]	0.49[b]	0.40[ab]	0.39[a]
Chewiness (Newtons Force)	3.4[a]	4.6[bc]	5.0[cd]	5.8[d]	3.8[ab]	3.4[a]
Gumminess (Newtons Force)	1.3[a]	1.4[a]	1.7[b]	1.9[c]	1.3[a]	1.3[a]

[a-d]Row means with the same letter are not significantly different (P = 0.05). LSD values: Hardness, 0.30; Springiness, 0.49; Cohesiveness, 0.09; Chewiness, 1.14; Gumminess, 0.15.

parameters was a slight increase after 4.7% or 9.0% added coconut cream and then a slight decrease after 33.3 or 50% of added coconut cream. This suggests that a tofu of greater springiness, cohesiveness, chewiness and gumminess could be produced by adding coconut cream up to 16.6% of the soymilk. Further addition of coconut cream to soymilk would decrease the values of these textural parameters including that of hardness.

The shape of the force distance curves changed as the amount of coconut cream increased. Tofu made from pure soymilk spread out under progressively increasing compression and the force increased steadily up to the point of maximum compression. Tofu made from soymilk-coconut cream blends fractured and then spread out as compression was continued. The point of fracture is shown by the humps on the LHS of the curves in Fig. 1.

The percent of samples showing the fracture break and mean peak force values of the fracture breaks are shown in Table 4. The number of fractures increased with increasing coconut cream, reaching the highest number of 81% at 33.3% added coconut cream. The force value of the fracture peaks increased from 1.37N at 0% to 1.60N at 16.6% coconut cream, and then decreased to 1.33N at 50% added coconut cream. This behavior is similar to hardness (Table 3) which increased though not significantly at 9% and 16.6% added coconut cream and then decreased significantly as the level of added coconut cream was increased to 3.4% and 50%. At high levels of coconut cream the fat dominated the curd structure making it softer resulting in less fractures during compression. The fatty acid composition of coconut fat may also have an effect on the texture of tofu.

Yeo (1976) reported that the addition of lard to soymilk decreased the hardness of the resulting tofu. At 0% lard the reported hardness value was 7.4 (units not given), at 2% lard hardness was 4.8, and at 10% lard hardness was down to 3. The reason Yeo (1976) did not observe any increase in hardness, as is reported in this paper, could be because the 2% lard addition to soymilk (Yeo's lowest level) gave a tofu with 15% fat which is almost equivalent to the tofu produced with 16.6% added coconut cream in this experiment. The different physical and chemical properties of lard and coconut oil may also have influenced the textural properties of tofu.

Table 4—Number and force value of the fracture peaks in the texture profile analysis of tofu

Coconut cream added (%)	No. of samples measured	% Fracture[a]	Avg force (Newtons)
0	27	33	1.37
4.7	27	26	1.56
9.0	26	65	1.58
16.6	27	74	1.60
33.3	27	81	1.58
50	28	75	1.33

[a] $\% \text{ Fracture} = \frac{\text{No. of samples showing fracture}}{\text{Total no. of samples measured}} \times 100$

Table 5—Sensory scores of tofu prepared from soymilk enriched with coconut cream

	% Added coconut cream					
Sensory parameters	0	4.7	9.0	16.6	33.3	50.50
Mouthfeel	4.1^{a}	4.8^{b}	4.7^{b}	5.0^{b}	5.5^{c}	4.9^{b}
Flavor	3.9^{a}	4.5^{b}	4.7^{b}	4.4^{ab}	4.2^{ab}	4.2^{ab}
Moistness	4.9^{ab}	4.7^{a}	4.7^{a}	5.2^{bc}	5.4^{c}	5.1^{bc}
Acceptability[d]	4.6^{a}	4.8^{a}	4.9^{a}	4.8^{a}	4.8^{a}	4.6^{a}

[a–c] Row means with the same letter are not significantly different (P = 0.05). LSD Values: Mouthfeel, 0.44; Flavor, 0.52; Moisture, 0.37.
[d] ANOVA not significant.

Sensory qualities

The overall acceptability of tofu was the only sensory parameter that was not affected by levels of added coconut cream to soymilk (Table 5). Flavor, moistness and mouthfeel were significantly affected by the different levels of added coconut cream to soymilk. Mouthfeel increased at 4.7% coconut cream added and remained fairly constant up to 16.6%, then increased significantly at 33.3% of added coconut cream. This had an effect on most of the sensory parameters, however, it did not affect the acceptability of tofu. Yeo (1975) increased the acceptability of tofu even with low levels of animal fat (lard, pork fat) and beef trimmings which is understandable because of the inherent flavor in these animal fats. A disadvantage cited by Yeo (1976) was the increase in the total saturated fatty acids as the amount of animal fat added to tofu was increased.

Energy value of tofu

The gross energy value and the available energy value of tofu (cal/100g) are shown in Table 6. The energy value of

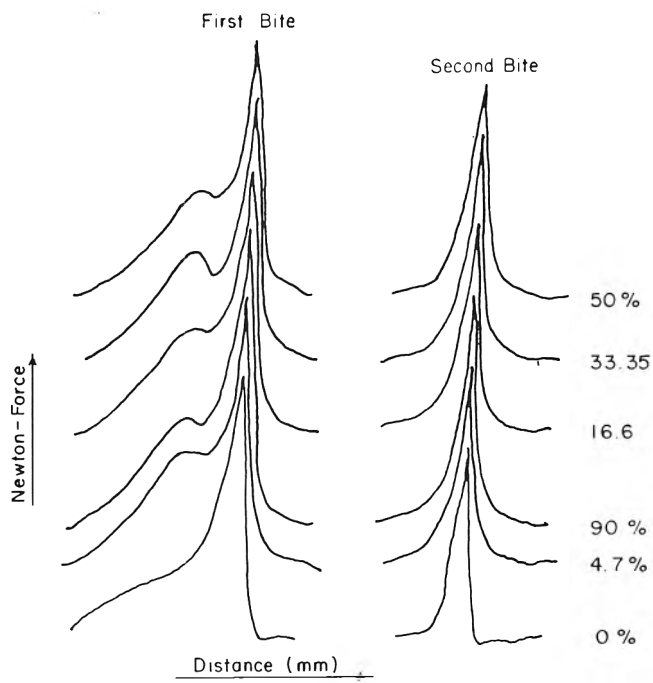


Fig. 1—Texture profile analysis of tofu made from soymilk containing 0, 4.7, 9.0, 16.6, 33.3 and 50% coconut cream.

Table 6—Energy value of tofu produced from coconut cream enriched soymilk[a]

Coconut cream added	Gross energy value[b] Cal/100g	Available energy value[c] Cal/100g
0	146	122
0.47	172	148
9.00	203	178
16.66	238	213
33.33	260	238
50.50	308	286

[a] Calculations of energy value based on Pike and Brown. (1975).
[b] Caloric value (cal/kg of carbohydrate, 4.15; fat, 9.4; protein, 5.65.
[c] Caloric value (cal/kg) of carbohydrate, 4.0; fat, 9.0; protein, 4.0.

Table 7—Ingredient cost of tofu as affected by coconut milk addition[a]

Ingredients			Ingredient cost (US cents[b])				
Soybean milk (g)	Coconut cream (g)	Tofu yield (g)	Soybean milk	Coconut cream	$CaSO_4$	Total cost	Tofu cost cents/100g
1000	0	226	4.4	0	2.5	6.9	3.1
953	47	227	4.2	1.5	2.5	8.2	3.6
910	90	241	4.0	3.0	2.5	9.5	3.9
834	166	269	3.6	5.5	2.5	11.6	4.4
667	333	277	2.5	11.1	2.5	16.1	5.8
500	500	313	2.2	16.6	2.5	21.3	6.8

[a] Based on prices prevailing in the Philippines in 1984 and exchange rate of P18.00/US dollar
[b] Soybeans, US $0.42/kg; coconut, US $0.16/nut; $CaSO_4$, US $4.72/lb.

tofu increased when higher amounts of coconut cream were added to soymilk. Since the acceptability of tofu was not affected by added coconut cream to soymilk (Table 5), this experiment suggests that one could tailor the energy content of tofu according to specific calorie needs without affecting the total consumption of the product. This is particularly so in the Philippines where calorie intake is about 88% of RDA and the protein intake is 95% of RDA.

Ingredient cost of coconut cream enriched tofu

Table 7 summarizes the effect of added coconut cream on ingredient cost of tofu at prices prevailing in 1984. The total cost of the ingredients increases as the amount of coconut cream increases since the cost of coconut cream per unit weight is higher than the cost of soymilk per unit weight. The unit cost of tofu increased only slightly up to 16.6% added coconut cream because the higher yield of tofu offset most of the increased ingredient cost. At higher levels of coconut cream (33.3% and 50%), the unit cost of tofu increased substantially because the increase in yield failed to balance the increased cost of coconut cream addition.

CONCLUSION

THE ADDITION of coconut cream to soymilk decreased protein and moisture and increased fat in tofu made from the coconut cream addition. The textural properties of tofu were significantly affected by added coconut cream. Hardness increased but not significantly up to 16.6% added coconut cream and then decreased significantly from 16.6% to 50% added coconut cream. Springiness, cohesiveness, and gumminess exhibited similar trends, increasing at first and then decreasing with further addition of coconut cream to soymilk.

The acceptability of tofu was not changed by adding coconut cream to soymilk. Hence, the caloric density of tofu can be increased by adding coconut cream without affecting the acceptability. The addition of 16.6% coconut cream to the soymilk increases the caloric density of the resulting tofu by 75% while increasing the ingredient cost by only 15%.

This simple technology of tofu manufacture with coconut cream enrichment warrants consideration by those involved in the planning and delivery of health and nutrition services in developing countries like the Philippines.

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Time, Temperature, Microbial and Sensory Quality Assessment of Chicken and Noodles in a Hospital Foodservice System

MARION L. CREMER, TONG-KYUNG KWAK YUM, and GEORGE J. BANWART

ABSTRACT

Time and temperature, sensory and microbial quality of chicken and noodles were assessed in a hospital where food in plastic bags was stored refrigerated for 1 month. Respective end-point temperatures and times were: cooking, 88.3 ± 6.4°C, 15.0 ± 8.3 min; cooling, 8.7 ± 2.9°C, 2.0 ± 0.6 hr; storage, 0.2 ± 0.6°C, 3 to 31 days; heating, 63.2 ± 13.4°C, 1.8 ± 0.1 hr; holding (convection oven), 83.4 ± 10.8°C, 1.7 ± 0.1 hr; and holding (steam table), 79.8 ± 9.2°C, 27.2 ± 15.2 min. Respective mesophilic, psychrotrophic, coliform, staphylococcal and anaerobic spore counts (colony forming units or most probable number per g) were: 387, 197, <3, <10 and 6. Scores for general acceptability (60-point scale) were 29.7 for the fresh product and 25.4 after 4 wk storage; this difference was significant.

INTRODUCTION

A READY-PREPARED type foodservice system (Unklesbay et al., 1977) in which food is cooked in a steam jacketed kettle, pumped into plastic bags which are mechanically clipped shut, tumbled in a cold water bath and stored at refrigerator temperature for 1 month is being marketed and implemented in large-scale operations in the United States. This system is designed to reduce labor time and cost by preparation of one large vs. several small batches of products.

Similar systems were developed and generally discontinued in hospitals in Sweden and the United States (Bjorkman and Delphin, 1966; McGuckian, 1969; Wadsworth, 1974). Jakobsson and Bengtsson (1972) compared the quality of frozen and refrigerated cooked, sliced beef under conditions representative of commercial practice in Sweden and found no bacterial growth in any sample pasteurized and stored at refrigerator temperatures for up to 3 wk. Zallen et al. (1975) examined the effects of refrigerated storage on sensory and microbiological quality of beef loaves that were cooked, cooked and pasteurized, or cooked, frozen and thawed before refrigerated storage. Refrigerated loaves and those pasteurized prior to refrigerator storage were not significantly different, but a significant decrease in sensory quality occurred after 9 days.

Numerous studies (Bunch et al., 1976, 1977; Tuomi et al., 1974a, b; Cremer and Chipley, 1980a, b; Rollin and Matthews, 1977; Dahl et al., 1978; Bobeng and David, 1978a, b) have been conducted on ready-prepared type foodservice systems, but no published data are available on the current system in which food is packaged in plastic bags and stored for as long as 1 month at refrigerator temperatures. Since this system is used extensively in large-volume operations, these data may be critical for assurance of food quality and safety. The purpose of this study was to determine time and temperature conditions and microbial and sensory quality of food during various phases of product flow and after product storage times which occurred in actual foodservice operation.

Author Cremer is affiliated with the Dept. of Human Nutrition & Food Management and Ohio Agricultural Research & Development Center, The Ohio State Univ., 1787 Neil Ave., Columbus, OH 43210. Author Yum is with the Dept. of Food & Nutrition, College of Home Economics, Yonsei Univ., 134 Shinchon-Dong, Sudaemun-Ku, Seoul 120, Korea. Author Banwart is with the Dept. of Microbiology, The Ohio State Univ. 484 W. 12th Ave., Columbus, OH 43210.

MATERIALS & METHODS

General

This study was conducted in a 1,029-bed general, metropolitan hospital with all conditions including holding, storage, heating times and temperatures the same as occurred within the operation. Chicken and noodles was the menu item selected for use in the study of the system based on service frequency, refrigerator storage time and food composition. Chicken and noodles was generally prepared once per month, served once a week and stored for a month before all portions were used. Phases in product flow and the sampling plan for time, temperature, sensory, and microbial evaluations are shown in Table 1. Time, temperature, sensory, and microbial evaluations were made during phases of initial preparation which occurred one time per month; microbial, time, and temperature evaluations at the end of storage and for service of the portion of product used after 1, 2, 3, and 4 wk storage; and sensory evaluation for the freshly prepared product and product heated after 2 and 4 wk storage. The entire study was repeated three times.

Product

The formula for preparation of chicken and noodles was: liquid margarine, 5.5 kg; all purpose flour, 2.1 kg; fresh milk with 2% fat, 113.3L; chicken base, 2.1 kg; onion powder, 44.4 mL; canned mushroom pieces and stems, no. 10 cans, 3; cooked frozen chicken meat, 34.1 kg; frozen raw egg noodles, 24.6 kg; modified starch, 3.4 kg; water, 5.7L; salt, 38.4g; diced frozen carrots, 5.5 kg; and chopped dried parsley, 177.4 mL.

For preparation, a 424L steam-jacketed kettle, equipped with a mechanical stirrer (Groen Model INA/2-100), was sprayed with a silicon based anti-sticking agent; margarine and flour were placed in the kettle and cooked 10 min to make a roux; milk, chicken base, onion powder, and mushrooms were added to make a sauce; then chicken was added and the mixture simmered. Noodles were cooked in boiling salted water, rinsed with water and set aside; carrots and parsley were added to the sauce; starch was dissolved in cold water, and then added to the sauce mixture; the cooked noodles were added, and the completed mixture was heated.

After cooking, the product was pumped hot from the kettle into 254 X 762 mm plastic casings which were closed mechanically with a metal clip by means of Cryovac Model No. 8125 Filling Station and W/X 4135 clipper. Casings were a cross-linked, coextruded, multi-layer transparent material with water vapor transmission rate of 0.5g per 250 sq cm per 24 hr at 27.8°C, 100% relative humidity; and oxygen transmission rate of 20 - 100 cc per sq cm per 24 hr at 22.8°C at atmospheric pressure (according to the manufacturer's specification). Each casing held approximately 6.3L and each batch filled 35 casings. (The casings were placed in a casing cooler (Chester-Jensen, Model P-1 Cooler S/N 1213) which was essentially an air-agitated water bath at 3.4 ± 1.1°C with a Model HGC parallel flow heat exchanger (Chester-Jenson, Chester, PA). After cooling, the product was stored in a walk-in refrigerator at 1.3 ± 0.9°C. On the day of service, three to five bags of chicken and noodles were removed from storage and heated in a water bath. After heating, the bags were cut and food was poured into 30.5 x 50.8 x 7.6 cm pans which were covered with aluminum wrap, placed in a forced gas convection oven for holding and then placed on a steam-table. Time

Table 1—Phases in product flow and scheme for microbial sampling and time and temperature recording

Phases	Time	Food temperature	Environmental temperature
Cooking*	1. Beginning to all ingredients added 2. Completed mixture	At end of cooking by inserting the thermomemter into center of food in steam jacketed kettle	
Casing	Beginning to end	For first and last bags filled by folding filled bags in center and placing thermomemter probe in fold	Room temperature at beginning
Cooling	1. Beginning to end, for first bag 2. Beginning to end, for last bag	At end of cooking from 7 of 35 bags selected at random by folding bag 1/3 from top and bottom and placing thermometer probe in folds	Water temperature in cooler at 30 min intervals
Storage*[ab]	Beginning to end	From 1 bag selected at random from 3 - 5 removed from storage at one time, by opening the bag and inserting the thermometer from the top into two random points in the food mass	Refrigerator temperature recorded morning and evening through the study period
Heating* (water bath)	Beginning to end	At end of heating for 2 of 3 - 5 bags removed from storage by puncturing bags and inserting the thermometer in the top, middle, and bottom of the food mass	Water temperature recorded at 30 min intervals
Holding* (convection oven)	Beginning to end	At end of holding at 2 points in each of 2 pans	Air temperature of convection oven recorded at 30 min intervals
Holding and* assembly (steam table)	1. Beginning to first portioning 2. Beginning to last portioning	In the center of the mas at time of first and last portioning of first pan	Water temperature in steam table at beginning of holding

[a] Sample drawn from same bag as used to record food temperature
[b] Bags 1 and 2 corresponded to pans 1 and 2, respectively, through phases of heating in a water bath to holding on a steam table; microbial samples were drawn from Bag or Pan 1 throughout
* Indicates phases at the end of which samples were drawn for microbial evaluation

and temperatures in various phases in product flow are indicated in Tables 2 and 3.

Time and internal temperature measurement

Time, food temperature and environmental temperature readings were made as indicated in Table 1. Food surface and interior and water temperature readings were made with a Model 392 Heatprober digital thermometer (Wahl Instruments, Inc., Culver City, CA), room temperature readings from a Taylor utility thermometer, Model 5135; refrigerator temperatures from a thermometer built into the equipment; and in convection ovens, from a Taylor oven thermometer. The thermometers used were checked for accuracy and calibrated when necessary in either ice water or boiling water. Temperatures in food were read 45 sec after the thermometer was inserted. For food temperature readings in the phases of casing and cooling, surface temperature readings were made because the bag would be punctured through direct insertion of a thermometer. The plastic bag was folded over and the probe of the thermometer was placed between the folds of the bag.

Sensory evaluation

Sensory evaluations were made by quantitative descriptive analysis with score sheet and scoring procedures similar to those previously described (Anon, 1981: Stone et al., 1974, Zook and Wessman, 1977). The score sheet was developed in relation to the sensory qualities considered important in the product and was tested prior to use. Evaluations were made by placing a vertical mark across an unstructured line 15 cm long and anchored 1.25 cm from either end by pairs of terms. Evaluations were translated into scores from 0 to 60 by placing a ruler with 0.25 cm gradients against the 15 cm lines.

Sensory chracteristics evaluated are indicated in Table 5. Evaluations were made by a 13-member experienced panel composed of hospital dietitians who were selected through use of the triangle test (Amerine et al., 1965). The bases for selection were ability to discriminate between (a) water and each of the following solutions: 0.1% sodium chloride, 0.5% sucrose, 0.005% tartaric acid, 0.3% caffeine, 0.2% sodium chloride, 1% sucrose, 0.01% tartaric acid, and 0.06% caffeine; (b) freshly cooked chicken compared to chicken chilled 24 hr and reheated in a microwave oven; (c) noodles cooked 10 min in boiling water compared to noodles cooked 15 min; and (d) sauce made with conventional starch compared to sauce made with modified starch. The concentration of the four basic test solutions was based on threshold value data (Amerine et al., 1965). The criterion for panelists' selection was 60% or higher of correct responses as recommended by ASTM (1968). After the panelists were selected, the objectives of the study, the meaning of terms, and use of the score sheet were explained. Panelists were given two practice sessions to evaluate the product with the score sheet.

Evaluations were made in a conference room adjacent to the hospital kitchen with considerable daylight in combination with fluorescent lighting. One sample was evaluated at each session of 30 min. The food was taken from the patient tray assembly line in the middle of the serving period and presented to panelists in a compartmentalized insulated tray with white plastic dish inserts in the same portion size as served to patients.

Microbiological evaluation

Sampling for microbiological tests was conducted at the points indicated in Table 1. Approximately 100g of each sample were aseptically collected into a sterile plastic bag (Whirl Pak, NASCO, Fort Atkinson, WI), packed immediately in ice in a plastic foam ice chest and held in the walk-in refrigerator (during the time period for collecting samples) until transported to the laboratory for analysis. The total time of transport for any of the samples was less then 0.5 hr.

After transportation, a 20g portion of each sample was blended with 180 mL of sterile Trypticase Soy Broth in a sterile laboratory blender for 60 sec and incubated for 1 hr at 35°C. This is basically the procedure reported by Speck (1976) for the repair and enumeration of injured coliforms and staphylococci. Serial dilutions were prepared with 90 mL of sterile 0.1% peptone water, and immediately inoculated onto plates or tubes depending on the method used for each test.

Microbiological tests used to evaluate product quality included (a) total mesophilic aerobic plate counts (APC-m) by using Plate Count Agar and incubating at 35°C for 48 hr; (b) total psychrotrophic aerobic plate counts (APC-p) with Plate Count Agar and incu-

Table 2—Measurements for time and internal temperature[a] for chicken and noodles in phases in product flow

Phase in product flow	Unit	Time Mean SD[b]	Time Range	Temperature °C Mean SD	Temperature °C Range	Observations[c]
Cooking				88.3 ± 6.4	81.6 – 94.4	3
Beginning to all ingredients added	min	70.7 ± 7.1	63 – 77			
Completed mixture	min	15.0 ± 8.5	6 – 23			3
Overall	min	85.7 ± 2.5	83 – 88			3
Casing						
Beginning to end	min	21.7 ± 2.5	19 – 24			3
First bag				79.4 ± 2.8	76.1 – 81.2	3
Last bag				69.2 ± 3.7	65.4 – 72.8	3
Cooling				8.7 ± 2.9	4.5 – 18.1	42
Beginning to end, first bag	hr	2.3 ± 0.6	1.8 – 3.0			3
Beginning to end, last bag	hr	1.9 ± 0.6	1.5 – 2.6			3
Storage (refrigerator)						
Week 1	day	3.0 ± 0.0	3	0.8 ± 1.2	−0.1 – 3.3	3,6
Week 2	day	10.0 ± 0.0	10	0.2 ± 0.2	−0.1 – 0.6	3,6
Week 3	day	17.0 ± 0.0	17	0.1 ± 0.4	−0.4 – 0.7	3,6
Week 4	day	24.0 ± 0.0	24	0.1 ± 0.2	−0.2 – 0.4	3,6
Week 5	day	31	31	−0.2 ± 0.1	−0.2 – 0.1	1,2
Overall	day	14.8 ± 9.2	3–31	0.2 ± 0.6	−0.4 – 3.3	13,26
Heating (water bath)				63.2 ± 13.4	38.7 – 87.5	66
Beginning to end	hr	1.8 ± 0.1	1.6 – 2.0			13
Holding (convection oven)				83.7 ± 10.8	55.9 – 99.0	44
Beginning to end	hr	1.6 ± 0.1	1.5 – 1.7			13
Holding and assembly (steam table)						
Beginning to first portioning	min	14.1 ± 5.3	5 – 21			13
Beginning to last portioning	min	40.3 ± 9.0	28 – 53			13
First portioning				81.3 ± 9.8	62.3 – 93.7	13
Last portioning				78.2 ± 8.7	61.7 – 91.0	13
Overall	min	27.2 ± 15.2	5 – 53	79.8 ± 9.2	61.7 – 93.7	26

[a] Values are for end-point temperatures except for first portioning at the phase of holding and assembly; values for casing and cooling are for surface temperature whereas all other measurements were directly in food.
[b] SD refers to standard deviation.
[c] Where two value are indicated, the first refers to time, the second to temperature recordings; where only one value is given, the number of observations is the same for both time and temperature.

Table 3—Environmental temperature means, standard deviations, and ranges for various phases in product flow of chicken and noodles

Phase in product flow	Mean SD[a] (°C)	Range (°C)	Observations
Casing (room temp.), beginning	22.8 ± 2.2	20.2 – 24.1	3
Cooling (water temp.), 30 min intervals	3.4 ± 1.1	2.0 – 5.3	19
Storage (refrigerator temp.)	1.3 ± 0.9	0.0 – 3.3	141
Heating (water temp.), 30 min intervals	92.4 ± 9.4	58.1 – 99.5	64
Holding (air temp. in convection oven), 30 min intervals	124.3 ± 24.3	82.2 – 182.2	45
Holding (water temp. in steam table), beginning	71.8 ± 3.9	59.8 – 74.7	13

[a] Refers to standard deviation

bating at 7°C for 10 days; (c) coliform counts by using the most probable number (MPN) method with Lauryl Sulfate Tryptose Broth for the presumptive test and incubating at 35°C for 48 hr, and 2% Brilliant Green Lactose Bile Broth for the confirmed test with incubation at 35°C for 48 hr; (d) staphylococcal counts using Baird-Parker Medium with the spread plate method and incubating at 35°C for 48 hr, with colonies typical of coagulase-positive staphylococci picked and tested for the coagulase reaction with coagulase plasma (Difco) using the tube method; and, (e) anaerobic spore counts using an MPN method with Beef Heart Infusion Broth, heating these cultures at 80°C for 20 min and, after heating, stratifying the infusion tubes with sterile 2% agar and incubating at 35°C for 72 hr.

The procedures for microbiological evaluation were the same as those stated in the FDA *Bacteriological Analytical Manual* (FDA, 1978) and the APHA *Compendium of Methods for the Microbiological Examination of Foods* (Speck, 1976). Colonies were counted with the aid of a Darkfield Quebec Colony Counter (Model 3330, American Optical Company Instrument Division, Buffalo, NY).

Statistical analysis

Variability of time and temperature was determined by obtaining means and standard deviations in each phase of product flow. Differences in the individual sensory characteristics scored were determined by a three-way analysis of variance (Snedecor and Cochran, 1974) according to judge, batch and storage time. When significant differences were found ($P<0.05$ or $P<0.01$) among mean scores for storage time, differences among samples were determined by Duncan's New Multiple Range Test (Duncan, 1955).

RESULTS & DISCUSSIONS

Time and temperature

Time and temperature for product in various phases of product flow and environmental temperatures during processing and storage are indicated in Tables 2 and 3. The ranges in time indicate variability in all phases of product flow. Refrigerator storage time of 3–31 days was relatively

long compared to recommendations of 1 day (Longree, 1980), 5 days (Rowley et al., 1972), or 20 hr (Bobeng and David, 1978a, b) but was according to the operational plan. This points to a potential microbial hazard and a need for the establishment of strict temperature standards for systems of this type. The average total time of 3.9 hr for heating and holding (includes time for heating in a water bath, holding in a convection oven and holding on a steam table) prior to assembly was also long and might be reduced to avoid the potential for deterioration in sensory and microbial quality of food. Cooling times were acceptable compared to recommendations of <4 hr (HEW, 1978; Longree, 1980; Bobeng and David, 1978a, b) but marginal compared to recommended time of 2 hr (Rowley et al., 1972). Furthermore, the standard cooling time was established in relation to a specific end-point temperature, and the temperature standard was not met.

A preliminary study was done to determine the potential difference between surface and interior temperature measurements in the phases of casing and cooling. Temperatures were read in the same bag by both direct and indirect methods after storage and after heating. Based on 21 bags, two surface readings per bag and three interior readings per bag, values after heating were 63.0°C, 39.3-87.5°C, and 12.6°C for the mean, range and standard deviation, respectively, when the thermometer was put directly into food and 73.0°C, 43.0–86.0°C and 8.9°C for surface measurements. For temperature after storage for 11 bags and two readings per bag by both methods, values were 0.3°C, −0.4 to 3.3°C, and 0.9°C for the mean, range, and standard deviation, respecitvely, when interior measurements were made and 1.5°C, 0.5–2.6°C, and 0.6°C when measured on the surface. These data indicate variable temperatures within the same bag and that the interior is lower than the surface temperature. After food had been refrigerated at least 3 days, the temperature in the interior was lower than at the surface but the difference was not great.

Data for end-point cooling temperatures (Table 2) reveal unacceptable temperatures related to a standard of 4.4–7.2°C (Longree, 1980) and indicate the ineffectiveness of the air agitated water bath used for cooling. A casing cooler with rotating drum which is sold as a system component but had not been purchased for use with this operation might provide effective cooling. Problems with cooling have been identified in studies of other sytsems (Bobeng and David, 1978b, Cremer and Chipley, 1980a; Rollin and Matthews, 1977). Bryan (1978) reported that ineffective cooling was a principal cause of food related illness.

End-point temperatures after heating in a water bath (Table 2) were unacceptable as related to recommendations of 74°C (HEW, 1978; Longree, 1980; Rowley et al., 1972) and point to the ineffectiveness of a water bath in heating food. Ineffective heating has been cited as a major cause of foodborne illness (Bryan, 1978) and has been found in relation to other equipment and foodservice systems (Cremer and Chipley, 1977; Cremer, 1978; Cremer and Chipley, 1980a, b). While being held in a convection oven to maintain the temperature, the mean temperature of the chicken and noodles was raised by 20 degrees but the temperatures were quite variable, and values below 60°C that is recommended (Longree, 1980; HEW, 1978) for holding heated food were observed. Wide ranges in water temperature at heating, air temperature during holding in the convection oven and in water temperature in the steam table (Table 3) may account for the variation in food temperatures during these phases.

End-point cooking temperatures were variable but higher than the HEW (1978) recommended temperature of 74°C and as high as 82.2°C recommended by Longree (1978) for cooking poultry. Food temperatures at the end of

Table 4—Microbiological evaluation of chicken and noodles at various phases in product flow.

Storage, refrigerator (weeks)	Phase in product flow[a]	Total plate count (Mesophilic)[b]	Total plate count (Psychrotrophic)[b]	Coliforms[c]	Staphylococci[b]	Anaerobic spores[c]
	Before initial cooking					
	Chicken, cooked frozen	1,800	610	46	90	3
	Noodles, raw, frozen	11,000	8,000	36	310	18
0 (freshly prepared)	A	162	63	<3	10	<3
1	D	203	100	<3	10	<3
	E	123	52	<3	<10	<3
	F	102	40	<3	15	<3
	G	103	42	<3	10	<3
2	D	242	150	<3	10	<3
	E	122	82	<3	20	<3
	F	90	60	<3	15	<3
	G	97	63	<3	15	<3
3	D	342	183	<3	10	<3
	E	163	87	<3	<10	<3
	F	115	57	<3	10	<3
	G	132	60	<3	10	<3
4	D	387	197	<3	<10	6
	E	170	93	<3	10	<3
	F	120	80	<3	20	<3
	G	127	85	<3	<10	<3

[a] Letters indicate phase in product flow (end of phase) where samples were taken for evaluation according to the following: A, cooking mixture; D, storage; E, heating in water bath; F, holding in convection oven; and G, portioning from steam table.
[b] Expressed as colony forming unit per gram (CFU/g) of sample; mean of three trials.
[c] Expressed as most probable number per gram (MPN/g) of sample; mean of three replications.

refrigerator storage also appeared acceptable compared to −1.1 to 4.4°C (Longree, 1980); and 4.4°C or less (Rowley et al., 1972), but temperatures were based on recommended storage time of less than 1 or 5 days and may not be acceptable related to the system of operation in which longer storage is planned. The refrigerator temperature was quite constant and may be an important factor in results of uniform temperatures of the refrigerated food.

Microbiological results

Results for the microbiological evaluation of chicken and noodles are presented in Table 4. Data indicated good microbial quality in food before and after cooking in all phases of product flow and after all periods of storage. The chicken basic material (frozen cooked) was somewhat better than the noodles (raw frozen) and during cooking the combined product, a substantial reduction in the microbial population occurred. The mean total plate counts increased in numbers as the refrigerated storage time increased from 0 to 4 wk (24 days). However, considering a generation time of 19 days (for APC-m) or 15 days (for APC-p), these numbers were probably insignificant.

During heating in a water bath (after storage), some reduction in the microbial popultion occurred, and a further reduction occurred during holding in the convection oven. Microbial counts did not increase appreciably during holding on a steam table.

Sensory quality

Mean scores for sensory characteristics of chicken and noodles and any significant ($P < 0.05$ or < 0.01) differences according to storage time are presented in Table 5. For the combined product, scores for general acceptability, flavor and appearance (conformation and moistness) at or close to the midpoint of the evaluation scale indicated average quality in the product. General acceptability and conformation (mushiness) in heated chicken and noodles were significantly different from the fresh after 4 wk storage, but no difference occurred after 2 wk. After 2 wk storage and heating, the appearance of clumping and firmness of noodles, color and consistency of sauce and moistness in the combined product were significantly different from the fresh, but no differences in these characteristics occurred between 2 and 4 wk. No component of flavor in the product or tenderness of chicken was affected.

Greater clumping of noodles after 2 or 4 wk of refrigerator storage and heating than for the freshly prepared product may be attributed to the effects of starch gelatinization in noodles which were cooked and chilled. Noodles which were significantly ($P < 0.01$) less firm after 2 or 4 wk of storage were probably produed by a combination of long heating, long holding after heating, and storage. The consistency of sauce for the freshly prepared product was at about the midpoint of the evaluation scale but after 2 or 4 wk of storage and heating, the sauce appeared significantly ($P < 0.01$) stiffer. This occurred despite the use of modified starch and may be involved with the finding of increased clumping of noodles. Also, long heating and holding times may have increased the consistency of the sauce as compared to the fresh product. Significant ($P < 0.01$) differences in moistness in the combined product may be related to differences in consistency of sauce. The color of freshly prepared sauce appeared fairly close to white but was

Table 5—Mean scores[a] for sensory evaluations of chicken and noodles and significant differences[b] related to refrigerator storage time

Characteristic	Storage time		
	0 week[c]	2 weeks	4 weeks
Noodles			
Appearance (strands clump together to individual strands apparent)	**39.6x	29.0y	25.9y
Firmness (extremely soft to extremely firm)	**29.7x	21.3y	21.3y
Intensity of off-flavor (no off-flavor to strong off-flavor)	14.7	16.4	12.9
Chicken			
Intensity of chicken flavor (weak to strong)	27.2	26.7	25.7
Intensity of off-flavor (no off-flavor to strong off-flavor)	14.7	14.0	14.9
Tenderness (extremely tough to extremely tender)	25.2	25.0	24.5
Sauce			
Color (white to bright yellow)	*16.2y	19.0x	19.2x
Blendedness of flavor (unblended to well-blended)	37.7	36.9	37.3
Intensity of desirable flavor (low flavor strength to high flavor strength)	30.8	31.3	31.2
Intensity of off-flavor (no off-flavor to strong off-flavor)	13.2	12.8	14.2
Consistency (stiff, no flow to considerable flow)	**33.0x	19.4y	16.4y
Chicken and noodle combination			
Appearance: moistness (looks dry to looks moist, juicy)	**42.1x	33.2y	30.7y
Appearance: conformation (looks mushy to each component apparent)	**28.8x	28.4x	22.6y
Flavor (low quality to high quality)	32.3	30.9	29.8
General acceptability (low quality to high quality)	*29.7x	29.0x	25.4y

[a] Means based on evaluation of 13 judges, 3 replications of study, and scale of 0 to 60.
[b] Means on same line followed by different letters are significantly different according to Duncan's New Multiple Range Test at $P < 0.05$.
[c] Freshly prepared sample.
** Indicates significant difference at $P < 0.01$ and * significant difference at $P < 0.05$.

significantly ($P < 0.01$) more yellow after 2 or 4 wk of storage and heating. The color change could have been produced by a Browning reaction in heating.

Scores generally indicated moderately good quality of the product. The changes with storage were primarily textural and could be related to heating and holding as well as to effects of storage. No significant difference in general acceptability until after 4 wk of storage and no flavor change may indicate the effectiveness of the plastic storage bags in preserving food quality. However, the data showed a definite quality advantage for the freshly prepared product and for product held for 2 rather than 4 wk.

CONCLUSION

FOOD may be cooked, placed in plastic bags with very low oxygen permeability and stored at refrigerator temperatures for up to 4 wk with good preservation of microbial and sensory quality. However, significant differences in sensory quality occurred, particularly after 1 month of storage. Further, temperatures of food after heating and cooling, which were unacceptable for food safety, point to potential hazards in the system, particularly since temperature recommendations are generally associated with relatively short storage time. Since storage of food at refrigerator temperatures in plastic bags for fairly long periods of time may be potentially hazardous, temperatures at initial cooking, cooling, and heating for service should be rigidly monitored and controlled.

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Individual Packaging of Apples for Shelf Life Extension

C. R. ANZUETO and S. S. H. RIZVI

ABSTRACT

Red Delicious apples after 2 wk and 4 months of cold storage and after 4 months of CA storage were individually packaged in two heat-shrinkable copolymers. Packaged and control apples were stored at 3°C and 90% RH, 21°C and 30°C and 50% RH, and at 30°C and 50% and 70% RH and the effect of packaging on modification of the microatmosphere inside the fruit and on various chemical and physiological changes were monitored with time. Temperature was the dominant storage factor. At 3°C, packaged and control apples had good eating quality and did not show significant differences ($P > 0.05$) after 3 months of storage. At 21°C, packaged apples reached a minimum acceptable condition at week 6 of storage which represented a shelf life extension of 3-4 wk over nonpackaged controls. At 30°C, internal quality of packaged apples deteriorated faster than controls. The respiration rate of packaged apples was lower than that of controls.

INTRODUCTION

IN ADDITION to temperature, control of equilibrium concentrations of respiratory gases in produce packaged in a polymeric film may have an additive effect in extending the postharvest life of perishable commodities. The possibility of obtaining modified atmosphere (MA) conditions has been put forward as one of the advantages to be gained by the use of plastic films (Tomkins, 1962; Henig, 1975; Deily and Rizvi, 1982). However, gas concentration during fruit storage in plastic films can not be controlled accurately. The range of this change can be regulated only partially by the selection of films with suitable properties, varieties of fruit, and storage temperature (Moiseyeva, 1966). In such a system, a dynamic equilibrium is established between two simultaneous processes of respiration and permeation. The exogenous components can be partially manipulated to control the rate of diffusion of endogenous components. In this way, an internal atmosphere within the commodity is established which, if maintained within certain limits, should be conducive for shelf-life extension. This study was undertaken to investigate the influence of individual packaging of apples in heat-shrinkable polymeric films on the quality of the fruits.

MATERIALS & METHODS

Origin of the apples, packaging methods, and storage treatments

Three batches of apples were studied in this investigation as follows:

Experiment I: Harvest season apples. Red Delicious apples harvested in early October 1983 from the Cornell orchards and kept for approximately 2 wk in cold storage (0°C, 95% RH) were sorted for size and physical damage and individually sealed in Cryovac D-925, 1 mil (0.001 in) thick film using a Multivac machine. Fruit packages were then shrunk with hot air by means of a Milwaukee Heat-Blo gun Model 500x at approximately 82°C (180°F) for 5-10 sec. Subsequently, apples were submerged in a 0°C water bath to avoid heat injury. Properties of the film used are listed in Table 1. Packaged and unpackaged (control) fruits were then randomly stored under the following conditions: Treatment I: 21°C, 50% RH; Treatment II: 30°C, 50% RH; Treatment III: 30°C, 70% RH; and Treatment IV: 3°C, 90% RH, (within ± 1°C and ± 5% RH). Storage facility for treatment IV was a semi-commercial size cold room. Three Percival environmental chambers were used for treatments I-III.

Experiment II: Apples after 4 months of cold storage. Red Delicious apples harvested in the 1983 season from the Cornell orchards and stored for about 4 months in a cold storage room (0°C; 95% RH) were sorted for size and physical damage. Apples were then individually packaged on a Weldotron Bar Heat Sealer, Model 6401L in Cryovac D-925, 1 mil and D-950, 0.6 mil films. Fruit packages were shrunk in a Weldotron Hot Air Shrink Tunnel, Model 7142, operating at 190.5°C (375°F) and conveyor speed of 50 for the D-925 film and at 163°C (325°F) and conveyor speed of 60 for the D-950 film. Two small holes were pierced on each package to permit air evacuation during heat-shrinking. Apples packaged in both D-925 and D-950 films and unpackaged apples were then randomly stored under the following conditions: Treatment I: 21°C, 50% RH; Treatment II: 3°C, 90% RH.

Experiment III: Apples after 4 months of CA storage. Red Delicious apples harvested during the 1983 season from the Cornell orchards and stored for four months in CA rooms (3% CO_2 – 3% O_2 and 0°C) were individually packaged under the same conditions and using the same equipment as mentioned in Experiment II above. The D-925, 1 mil film packages were not pierced this time. Apples packaged in both D-925 and D-950 films and nonpackaged fruit were then randomly stored under the following conditions: Treatment I: 21°C, 50% RH; Treatment II: 3°C, 90% RH.

Respiration rate and quality measurements

Respiration rate measurements. Respiration rates at 20°C of individually packaged and control apples were measured to determine the effect of type of film, film thickness, and packaging method. Weighed triplicates of 5-7 units were placed in 5L containers. The containers were tightly closed and purged with a continuous flow of air (200 mL/min). Carbon dioxide concentrations were determined with a Beckman non-dispersive infrared analyzer Model 865 for a period of approximately two weeks. Results were reported as cc of CO_2 produced per hour per kg of fruit.

Fruit quality measurements. In addition to weight loss, the following quality indices (in triplicate) were measured for both packaged fruits and controls, at day zero and then once a week.

Flesh firmness. Firmness was measured on three spaced faces of each apple, after removing a skin disk from the cheek. An Effegi fruit firmness tester with an 11 mm (7/16 in.) diameter plunger was used and firmness reported in kg-f.

Soluble solids. The juice resulting from each of the three apples during firmness testing was used for soluble solids content determination (at ambient temperature) with a hand refractometer Atago Model N1. Results were reported as total sugars (Brix %).

Titratable Acidity and pH. A representative juice sample was prepared from each of the three apples after the firmness and soluble solids determinations. Acidity was measured by titrating 50 mL juice, after dilution with 50 mL distilled water, against

TABLE 1—Gas transmission rates of films tested[a]

	at 21°C (1000 cc/m²-24 hr-atm)			at 37°C; 100% RH (g/100 in²-24 hr)
	O_2	CO_2	C_2H_4	$H_2O_{(v)}$
D-925 (1 mil thick)	4-5	15-16	7.2	0.7
D-950 (0.6 mil thick)	7-8	25-26.5	12	1.17

[a] Cryovac Division, W.R. Grace & Co. Duncan, SC 29334

Author Angueto is Graduate Student and Author Rizvi, Associate Professor, Institute of Food Science, Cornell Univ., Ithaca, NY 14853.

0.1N NaOH to pH 7. The results were expressed as mg malic acid/100 mL juice.

Gas analysis. Internal gas samples drawn from the core cavity of the apples with a hypodermic syringe were analyzed in a Fisher Gas Partitioner, Standard Model 1200. The apparatus was installed with two aluminum columns for O_2 and CO_2 determination, one packed with 80/100 mesh Columpak PQ and the other with 60/80 mesh Molecular sieve 13 X, and with a thermal conductivity detector. Gas samples were drawn from the package headspace (stem and calyx ends) and analyzed for O_2 and CO_2 concentrations as well. In addition, ethylene determinations were done on apples in experiments II and III from internal and from package headspace gas samples. A Varian Gas Chromatrograph, Model 3700 installed with alumina columns and a flame ionization detector was used for this determination.

Fruit appearance and sensory evaluation. Packaged and control apples were assessed weekly by the authors for appearance (i.e. color and degree of shrivelling on a qualitative basis). A sensory panel of ten non-trained panelists ranked the apples for overall eating quality every other week. Attributes evaluated include flavor, odor, and texture. The apples were ranked on a 9-point hedonic scale, where 9 represented "like extremely" and 1 "dislike extremely." Each panelist was provided with one half of an apple from each treatment. Samples were served at ambient temperature and under normal illumination.

Statistical analysis

Data were subjected to analysis of variance using Duncan's Multiple range test and Least Significant Difference (LSD) procedure to determine differences, by time, between variables studied in packaged and non-packaged apples stored under different conditions (SAS, 1982).

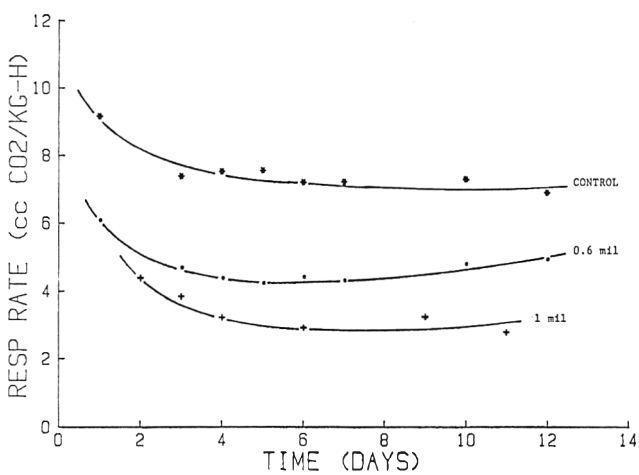


*Fig. 1—Effect of individual film packaging on respiration rate of apples at 20°C. CA apples, vacuum packaged. * = Control; o = 0.6 mil film + = 1 mil film.*

TABLE 2—Effects of film thickness and storage conditions in experiment II on acceptability of apples

Storage conditions[a]	Storage time (weeks)				
	0	2	4	6	8
I P (0.6 mil)	6.8	6.7a[z]	6.7a	5.5a	4.3a
I P(1.0 mil)	6.8	6.0a	5.1a	5.0a	4.9a
I C	6.8	6.3a	4.7a	[b]	[b]
II P(0.6 mil)	6.8	7.3a	6.9a	7.8b	7.1b
II P(1.0 mil)	6.8	7.7a	7.0a	7.9b	7.1b
II C	6.8	6.7a	7.3a	7.0b	7.5b

Hedonic Scale: 9 = Like extremely; 1 = Dislike extremely. Scores less than 5 are considered below consumer acceptability.
[a] Storage conditions I (21°C, 50% RH); II (3°C, 90% RH); P = packaged; C = control.
[b] Test discontinued due to severe shrivelling in the apples.
[z] Scores within columns followed by the same letter are not significantly different at 5% level using Duncan's Multiple Range Test.

RESULTS & DISCUSSION

Effect of film packaging on respiration rate of apples

A general decrease in respiratory activity of individually packaged apples, as compared to unpackaged controls, was observed. Greater reductions in CO_2 production were observed in apples packaged using the vacuum method (Fig. 1). In this respect vacuum packages were followed by nonvacuum packages without pierced holes (1 mil film packages in Experiment III), and then by nonvacuum packages where holes were pierced in bags prior to packaging (1 and 0.6 mil film packages in Experiment II and 0.6 mil film packages in Experiment III) (data not shown). The 1-mil film tended to cause greater reduction in respiration rates than the 0.6 mil film, probably due to accumulation of higher CO_2 and lower O_2 levels in apples packaged in the thicker film. With the vacuum method, differences observed in the respiration rates between apples packaged in the two films and between those observed for packaged apples and controls were statistically significant ($P < 0.05$). Film in nonvacuum packages became loose and thus the controlled permeation-respiration system was partially destroyed. The greater reduction in respiratory activity in vacuum packaged apples may be explained by the effects of higher CO_2 and lower O_2 levels established in the internal atmosphere in these apples. Better film integrity and tightness around the fruit, as well as the effects of the vacuum operation on pulling out some of the O_2 initially present in the fruit's intercellular spaces, may have contributed too.

Fidler and North (1966) have reported that the effect of accumulation of CO_2 was primarily to lower the respiration quotient. They also stated that storage life is governed by other considerations, namely, by the effect of the storage conditions on physiological disorders, on rotting, and on color and hardness of the fruit. Moiseyeva (1966) and Fidler and North (1966) stated that although a reduction of respiratory intensity had some effect on retarding the processes associated with respiration, i.e. oxidation of sugars and organic acids, development of coloring, transformation of pectic substances, and retardation of fruit tissue density, it was not possible to forecast storage life from respiration data alone. In this study, modified atmosphere conditions increased the storage time at which apples can be kept at ambient temperature (Table 2) and reduced rates of respiration, but there was no precise relationship between these phenomena.

Weight loss

Although weight loss of both packaged and nonpackaged fruits was observed to increase with time it was significantly reduced ($P < 0.05$) by individual film packaging (Fig. 2). Water loss in packaged apples, even at 30°C, was negligible compared to that in control fruits. Weight loss of packaged apples after 8 wk at 21° and 30°C was only around 2% and 4%, respectively, as opposed to 15% and 22% for their nonpackaged counterparts. Packaged and control apples in cold storage showed the least weight loss among all treatments tested (<1.5% after 8 weeks) and differences between packaged and control fruits were not significant ($P > 0.05$). The thinner film (0.6 mil) appeared to allow more moisture loss than the 1 mil thick film under both ambient and cold storage conditions. However, differences due to film thickness were not significant ($P > 0.05$) for apples kept in cold storage.

Loss of water from apples was not only manifested in weight loss but also in the impairment of appearance and eating quality. Control fruits at 21° and 30°C showed visible shrivelling when 4-5% of their original weight was lost after 1-2 weeks of storage as compared to their film packaged counterparts. By this time controls were very

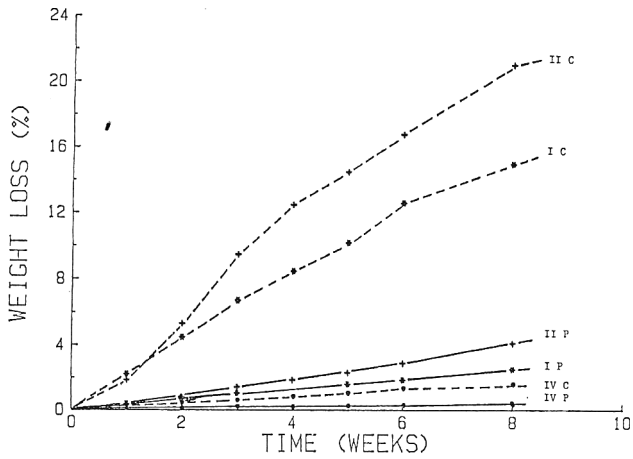


*Fig. 2—Weight loss in apples individually packaged in 1 mil film (P) and nonpackaged apples (C) under storage conditions I, (21°C; 50% RH) *; II, (30°C; 50% RH) +; and IV, (3°C; 90% RH) o in experiment I.*

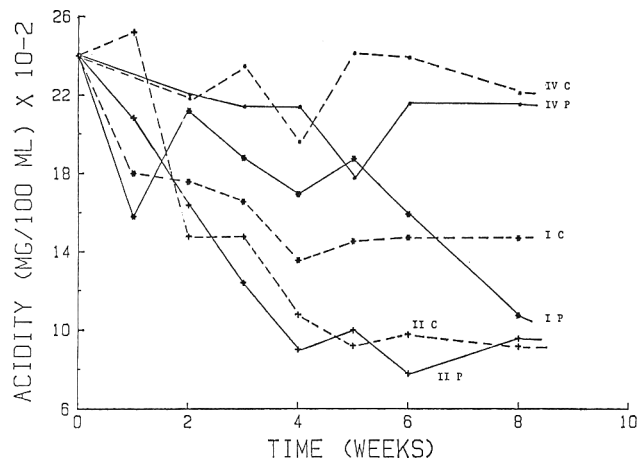


*Fig. 3—Changes of acidity in apples individually packaged in 1 mil film (P) and nonpackaged apples (C) under storage conditions I, (21°C; 50% RH) *; II, (30°C; 50% RH) +; and IV, (3°C; 90% RH) in experiment I.*

unattractive because of the wrinkled appearance of the skin. Considering weight loss only, the potential economic life of apples was as much as quadrupled by packaging. The degree of shrivelling observed in nonpackaged apples held at 21° and 30°C was not reflected by the analytical methods used to evaluate the quality attributes of the fruit. From the results obtained, temperature and humidity appeared to be the main storage factors affecting moisture loss. The storage conditions tested and the packaging material showed significant interaction ($P < 0.05$) affecting moisture loss. Moisture condensation was never observed either inside or outside of the tight film. In terms of moisture loss, apples after 4 months of CA storage behaved practically the same way as those after 4 months of cold storage (data not shown).

Soluble solids

Soluble solids (total sugars) content of the samples fluctuated over a relatively wide range during the storage period studied (data not shown). A general decline in both packaged and control apples in the three experiments was observed. In Experiment I an increase in total sugars occurred during the first 2-3 wk of storage (data not shown). This was associated with starch hydrolysis (Burton, 1978). The rate of sugar consumption was reduced during the storage of the fruit at 3°C and 90% RH, which may be due to reduction in respiration rate and metabolic processes. At 21° and 30°C, nonpackaged apples generally had higher sugar levels than packaged fruit. This behavior can be attributed to a concentration effect (i.e. dehydration) due to higher water loss in nonpackaged apples.

Titratable acidity

A trend of decline in acidity was observed for individually packaged and nonpackaged apples under all storage conditions studied. Fig. 3 shows such a trend for Experiment I. Packaged and nonpackaged apples stored at 3°C and 90% RH maintained much higher levels of acidity than any other storage treatment. Packaged apples held at 21°C tended to show slower rates of acid loss than their nonpackaged counterparts. This may be partially explained by the effects of internally modified atmospheres created in packaged apples on better retention of acids. The same effect has been found for apples in controlled atmosphere storage as opposed to apples stored in air (Schommer and Stainsbury, 1957; Olsen and Schommer, 1964; Fidler and North,

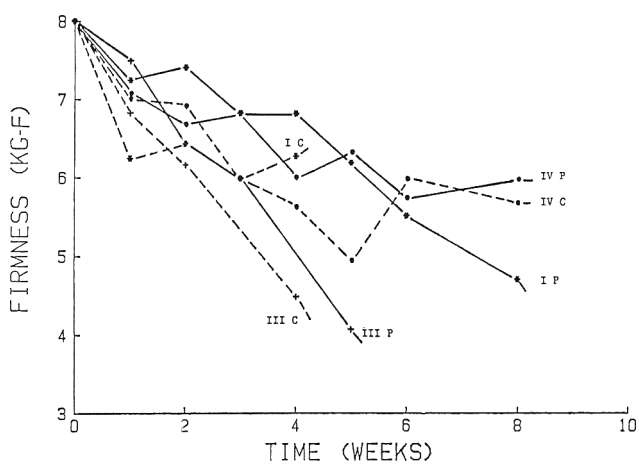


*Fig. 4—Changes of firmness in apples individually packaged in 1 mil film (P) and nonpackaged apples (C) under storage conditions I, (21°C; 50% RH) *; III, (30°C; 70% RH) +; and IV, (3°C; 90% RH) o in experiment I.*

1966). Control apples kept at 30°C showed higher acid levels than their packaged counterparts, possibly because of the high dehydration observed in the former increasing acid content. The same behavior was observed at the end of the storaged period in apples kept at 21°C. Temperature appeared to be the factor more critically affecting acid loss of the fruit. Higher acidity levels were generally maintained by apples packaged in 1 mil than in 0.6 mil film. (Data for 0.6 film packages not shown).

Flesh firmness

In all storage treatments (Experiment I) individually packaged apples were firmer than controls during the 8-wk storage period (Fig. 4). However, in experiments II and III, packaged apples kept at 3°C tended to be slightly softer than controls. Such differences were not significant ($P > 0.05$). In nonpackaged apples stored at 21° and 30°C, an increase in firmness occurred after a few weeks of storage (represented in Fig. 4 by the interruption of the corresponding curves around week 4 of storage). Such behavior was believed to be due to excessive water loss from the fruit. This caused the apple flesh to become spongy and dry and thus more resistant to penetration by the pressure

tester. This behavior was noticeable after about 2-3 wk of storage. Subsequently, flesh characteristics changed to a somewhat mealy and soft condition. Main effects, that is, storage conditions and packaging material, independently of each other (i.e. without interaction) significantly affected ($P < 0.05$) flesh firmness. No consistent pattern was observed regarding the effect of film thickness on softening rates and differences in firmness between 0.6 and 1 mil film packages were not significant ($P < 0.05$).

Concentration of CO_2 and O_2 in headspace and cavity

Changes in internal CO_2 and O_2 concentrations of the apples followed the same pattern in all storage conditions studied. However, the gaseous levels attained in packaged apples and the time such levels were maintained in the fruits differed from treatment to treatment especially in Experiment I. Fig. 5 shows the results obtained for apples at 21°C in Experiment I.

Higher than normal concentrations of CO_2 (and lower than normal concentrations of O_2) in the internal atmosphere of individually packaged apples were associated with lower respiratory activity and vice versa. In general, at 21°C average concentration of internal O_2 was reduced from about 19% to about 14-15% as a result of packaging; and that of internal CO_2 increased from about 2% to about 4-6%. The concentration of these gases in the packages' headspace was similar to that of ambient air. Refrigerated apples had lower internal CO_2 and higher O_2 levels than their counterparts at higher temperatures. The 1 mil-film packages retained higher levels of CO_2 (and lower levels of O_2) than the 0.6 mil-film packages in both ambient and cold storage conditions. However, these differences were not always significant throughout the storage period owing to fluctuations observed in internal gas levels.

Ethylene concentration

The quality of both packaged and control apples under ambient or cold storage conditions did not appear to be affected by the ethylene levels accumulated within them. These levels fluctuated within a range of 30 to 500 ppm. No general pattern was followed regarding ethylene levels in packaged and control apples at either 3° or 21°C. In the case of the apples under refrigerated conditions, fruit ripening was not noticeably affected by ethylene since this gas does not stimulate ripening under refrigerated storage. For the apples kept at ambient conditions, ethylene has been reported not to affect postclimacteric fruits (Smock and Neubert, 1950; Knee et al., 1975). (Apples used in this study were postclimacteric).

Blanpied et al. (1972), reported that there was no consistent effect of naturally evolved ethylene in stimulating ripening of apples and pears under CA conditions. Liu (1977) stated that the effects of ethylene removal in CA storage still remain unclear.

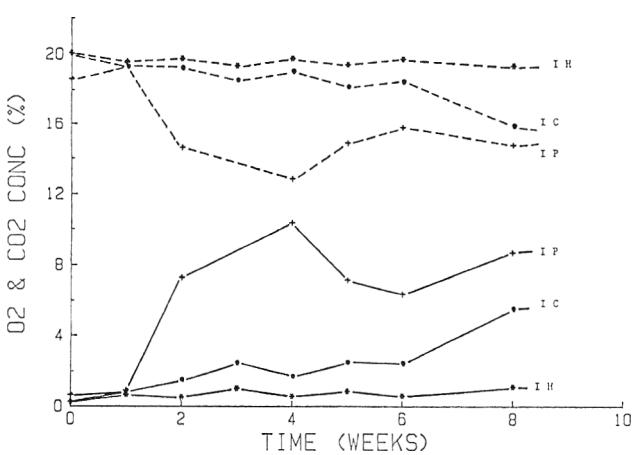


*Fig. 5—Changes in concentration of respiratory gases (O_2 ——— and CO_2) in headspace (H, *) of 1 mil film packages and in internal atmosphere of individually packaged (P, +) and non-packaged (C, o) apples under storage conditions I (21°C; 50% RH) in experiment I.*

Taste panel and overall quality evaluation

According to taste panel scores (Table 2), individually packaged apples in both 0.6 and 1 mil films held under ambient conditions (21°C, 30-50% RH) reached minimum acceptable quality after 6 weeks of storage in the three experiments (i.e. scores below 5 points). Regardless of the origin of the apples, i.e. freshly harvested, or after 4 months of cold or CA storage, shelf life extension of packaged apples was practically the same in all cases (data shown only for apples after 4 months of cold storage). This suggests that after a certain period of storage under less than optimum conditions, fruit deteriorated reaching approximately the same quality level regardless of other factors. This result can be explained by Fidler and North's (1966) finding that the effects of given concentrations of CO_2 and O_2 were additive in respiration effects but not necessarily on storage life.

Packaged apples held at 30°C and 50-70% RH were unacceptable at week 6 of storage and of marginal quality at week 4. Sensory evaluation of the fruit was generally in good agreement with objective measurements, especially for packaged fruit. Control apples under ambient conditions were unacceptable after 2-3 wk of storage, mainly due to external deterioration. Packaged apples held at ambient conditions lost some of their redness during storage.

CONCLUSIONS

THE PHYSIOLOGICAL DETERIORATION of Red Delicious apples at 21°C and 30-50% RH, was delayed by individual packaging in heat-shrinkable copolymeric films. Shelf life of packaged apples was extended 3-4 wk over nonpackaged fruits, which, after 2-3 wk under such storage conditions, were noticeably shrivelled. Packaged fruit held at 30°C tended to deteriorate faster than nonpackaged counterparts. Shelf life of individually packaged apples held at ambient temperature was practically the same (around 6 wk) regardless of the postharvest storage practice adopted before packaging, film thickness or the packaging method used. Effectiveness of individual film packaging was primarily manifested by substantial reduction of water loss which, in turn, maintained external quality of the packaged apples. The respiratory rate of individually packaged apples was consistently lower than that of their nonpackaged counterparts due to the effect of higher CO_2 and lower O_2 levels on inhibition of CO_2 production. However, storage life of packaged fruit was not strictly dependent on either respiratory levels or internal CO_2 and O_2 concentrations.

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Stabilization of Anthocyanins of Blood Orange Fruit Juice

EMANUELE MACCARONE, ANGELO MACCARRONE, and PAOLO RAPISARDA

ABSTRACT

The stability of the red color of pigmented orange fruit juice was somewhat improved by pasteurization with microwaves and addition of tartaric acid and gluthathione as mildly acidic and antioxidant agents, respectively. Highest stability was obtained through the formation of complexes between anthocyanins and phenolic compounds, such as rutin and caffeic acid.

INTRODUCTION

STRUCTURE AND STABILITY of the anthocyanins have been extensively studied during the last decade, owing to the growing interest in the use of natural colorants in foods (Brouillard, 1982; Hrazdina, 1982; Markakis, 1982). Anthocyanins are very sensitive to discoloration by various agents, because of the electron deficiency of the flavylium nucleus. In particular, previous investigations of the color degradation of orange juice have evaluated the effect of pH and temperature (Galoppini et al., 1968), addition of SO_2 (Galoppini and Russo, 1969) and 5-hydroxymethylfurfural (Porretta et al., 1966), and treatment with enzymatic systems (Casoli et al., 1969). The stabilization of the anthocyanins of orange juice has today become an important problem because that part of the pigmented orange production which is not used as fresh fruit cannot be easily transformed into industrial juices.

The previous studies on separation and identification of the anthocyanins of blood oranges (Maccarone et al., 1983, 1985) stimulated an investigation of improving the color stability of the fruit juice by the following treatments: (a) clarification by pectolytic enzyme and by bentonite; (b) pasteurization by microwaves; (c) addition of acidic and antioxidant agents; (d) formation of anthocyanin-copigment complexes.

The clarifications by pectinase and bentonite were used to determine if the absence of pectins and their degradation products affected the color stability of the juice. Pasteurization by microwaves was adopted because it has been successfully applied in other food processing (Mudgett and Schwartzberg, 1982). Tartaric acid was utilized as an acidulant in order to shift the anthocyanin equilibria towards the more highly colored flavylium forms. Glutathione was used as a mild antioxidant to reduce chemical and enzymatic oxidation. The juice of the *Moro* orange fruit was used because it contains more anthocyanins than the other pigmented varieties (Russo and Galoppini, 1969).

MATERIALS & METHODS

SAMPLES OF MORO oranges, produced in the Plain of Catania, were cut in half and squeezed by a commercial presser. The juice was centrifuged to remove much of the yellowish fine pulp and clarified by adding 25 mg/L of "Ultrazym 100" (Ciba-Geigy, Basle, Switzerland). The solution was held at 25°C for 14 hr to allow enzymatic reactions and then centrifuged. The treatment by bentonite was performed by adding 1 g/L of "Pluxbenton" (Agenzia Enologica Italiana, Milan, Italy) to the juice. After mixing, the suspension was held at 20°C without agitation for 14 hr and then centrifuged to remove additional fine pulp.

Samples (100 mL) were placed in colorless glass bottles (headspace: 10 mL). In one series, 40 mg of sodium benzoate was added to inhibit fermentation. Another series of samples was pasteurized by heating with microwaves (2450 MHz) for 3 min in a Sharp oven (model 5600, Sharp Co., Osaka, Japan). The maximum temperature of the juice was 95°C. The samples were then removed from the oven and quickly cooled. The bottles were capped and stored at 16–18°C.

The juices were analyzed by standard methods (Tateo, 1969). The intensity of the color was measured at 535 nm with a Coleman-Hitachi spectrophotometer (Tokyo, Japan) after dilution of the juice with absolute ethanol (15→50 mL) and filtration through filter paper to remove precipitates.

Anthocyanins were isolated from the juice by ionic exchange chromatography (Fuleki and Francis, 1968; Maccarone et al., 1985). The juice (500 mL) was introduced into a 40 cm column (ϕ_i = 4 cm) packed with CG 50(H) resin (Carlo Erba, Milan, Italy). The resin was first treated with a buffered solution of sodium citrate/HCl at pH 4.5 and then washed with deionized water to pH 7. The anthocyanins were selectively absorbed on the resin, whereas the other components of the juice (flavonoids, sugars, organic acids) were removed from the column by washing with deionized water and methanol. The pigments were then eluted using MeOH-1% formic acid; the solution was evaporated under vacuum at 25°C. The crude anthocyanins were dissolved in a few mL of MeOH-1% HCl and precipitated with dry ether.

RESULTS & DISCUSSION

TABLE 1 shows the results of the analysis of the juice after the clarification processes. The treatment with enzyme did not modify the chemical characteristics compared with those of the nonclarified juice, except for a negligible reduction of ascorbic acid, total nitrogen and anthocyanins; but the clarification by bentonite caused a 17% decrease in the color intensity and 30% decrease in the total nitrogen.

Table 2 (Series no. 1,2 and 3) shows the absorbance at 535 nm of the above juices at various times of storage at 16–18°C. The bright red color became gradually brown as

Table 1—Analysis of the Moro orange juice

	Control	Clarified by enzyme	Clarified by bentonite
pH value	3.23	3.10	3.10
Ash (g/100 mL)	10.5	10.5	10.5
Total acidity (g/100 mL) as citric acid	1.43	1.42	1.41
Reducing sugar (g/100 mL)	3.70	3.72	3.70
Total sugar (g/100 mL)	7.36	7.40	7.32
Sucrose (g/100 mL)	3.47	3.50	3.44
Formol number	1.85	1.85	1.75
Ascorbic acid (mg/100 mL)	43	41	40
Total nitrogen (g/100 mL)	0.171	0.166	0.111
Absorbance at 535 nm (dil. 15→50 mL)	0.93	0.89	0.78

Authors Maccarone and Rapisarda are affiliated with the Dept. of Chemical Sciences, Univ. of Catania, 95125 Catania, Italy. Author Maccarrone is affiliated with the Inst. of Agr. Industries, Univ. of Catania, 95123 Catania, Italy.

Table 2—Stability of the color of the Moro orange juice

Series no.	Pre-treatment	Additive[b]	pH	Absorbance at 535 nm[a] Days of storage 0	12	28	45	56	60	75	84	90	112
1	None	B	3.2	0.93		0.48		0.32			0.29		0.25
2	Clarification by enzyme	B	3.1	0.89		0.46		0.32			0.28		0.27
3	Clarification by bentonite	B	3.1	0.78		0.33		0.23			0.17		0.16
4	Anthocyanins		3.2	0.81		0.77	0.73			0.67		0.60	
5	None	B,T(300 mg)	3.0	1.06		0.72		0.48			0.40		0.34
6	None	B,G	3.2	0.93		0.55		0.39			0.35		0.28
7	None	B,A	3.2	0.93		0.46		0.31			0.24		0.20
8	Pasteurization		3.2	0.93	0.69	0.54	0.47		0.39	0.35		0.31	
9	Pasteurization	G,T(150 mg)	3.1	0.97	0.85	0.62	0.50		0.44			0.40	
10	Pasteurization	G,T(250 mg)	3.0	1.03	1.03	0.93	0.82		0.71			0.45	
11	Pasteurization	G,T(500 mg)	2.9	1.23	0.99	0.80	0.68		0.55			0.40	
12	Pasteurization	R	3.2	0.85	0.80	0.69	0.56		0.48	0.43		0.40	
13	Pasteurization	C	3.2	0.88	0.88	0.82	0.71		0.64	0.55		0.45	

[a] After dilution of the juice with absolute ethanol (15→50 mL). The absorbances are average values from duplicate runs.
[b] B, Sodium benzoate (40 mg/100 mL); T, Tartaric acid. G, Gluthathione (10 mg/100 mL); A, Ascorbic acid (100 mg/100 mL); R, Rutin (50 mg/100 mL); C, Caffeic acid (50 mg/100 mL).

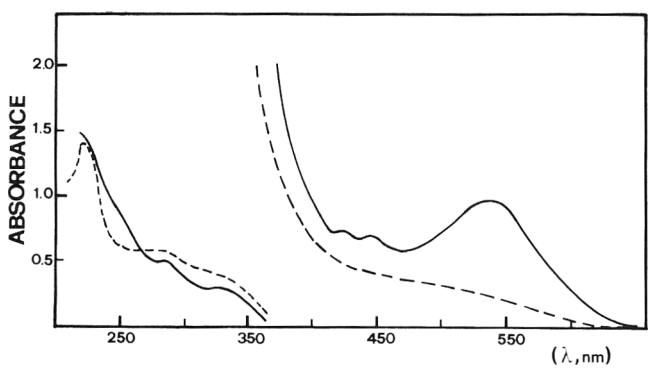


Fig. 1—UV-Visible absorption spectra of the Moro orange juice: ——, Fresh juice; ------, Juice after 4 months of storage. UV range, dilution 1→50 mL; Visible range, dilution 15→50 mL.

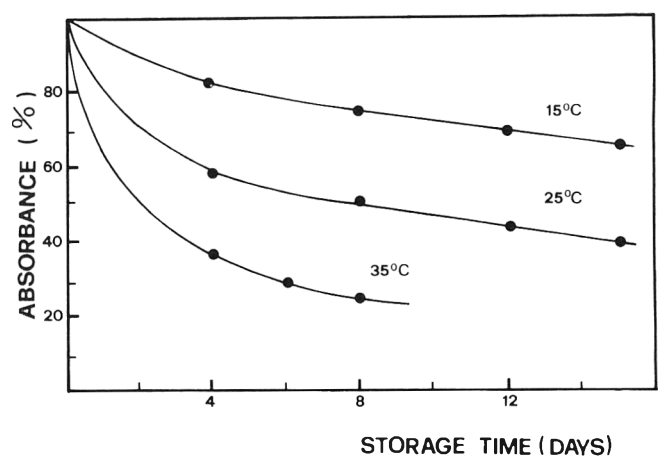


Fig. 3—Effect of the storage temperature on the color degradation of the orange juice.

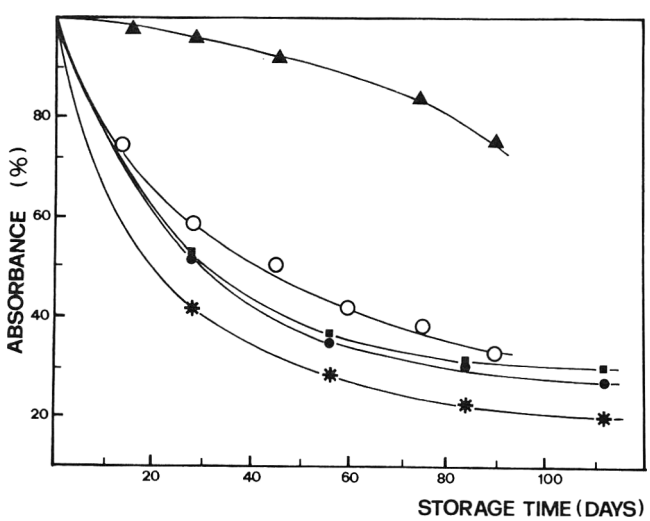


*Fig. 2—Effect of pre-treatments on the color stability of the orange juice: ●, Nonclarified; ■, Clarified by enzyme; *, Clarified by bentonite; ○, Pasteurized by microwaves; ▲, Standard anthocyanins in aqueous solution at pH 3.2.*

indicated by the changes in 535 nm absorbance; the anthocyanins were completely destroyed after 4 months (Fig. 1).

The enzyme-pretreated juice showed a degradation profile similar to that of the nonclarified juice (Fig. 2). In both cases the half life time of anthocyanins ($t_{1/2}$) was ca. 30 days. The bentonite treatment appeared to reduce the color stability ($t_{1/2}$ = 20 days). Since the clarification processes did not improve color stability, further experimentation was carried out using the nonclarified juice.

As expected, an increase in the storage temperature reduced color stability. Fig. 3 shows the disappearance of the anthocyanins in the juice samples stored at 15°, 25°, and 35°C: the rate of degradation doubled for increases of 10 degrees, confirming previous results using model systems (Calvi and Francis, 1978; Kearsley and Rodriguez, 1981).

Decoloration of the anthocyanins appeared to be caused by various chemical and enzymatic systems which were present in the juice. In fact, an aqueous solution of the purified pigments, isolated from the juice and buffered at pH 3.2, gave only a 25% color loss after 3 months of storage at 16–18°C (Table 2, Series no. 4), in comparison with 65% degradation of color when pigments were in the juice (Fig. 2). Therefore, stability studies utilizing pasteurized juice were performed to evaluate the effect of enzymatic activity. Since pasteurization by a conventional heat

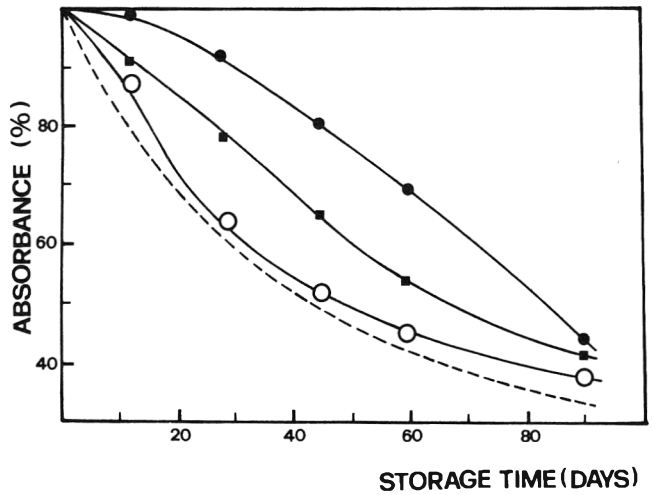


Fig. 4—Effect of tartaric acid (T) at different concentration, together with gluthathione, on the color stability of the orange juice: ○, T = 150 mg/100 mL; ●, T = 250 mg/100 mL; ■, T = 500 mg/100 mL; ------, Reference juice.

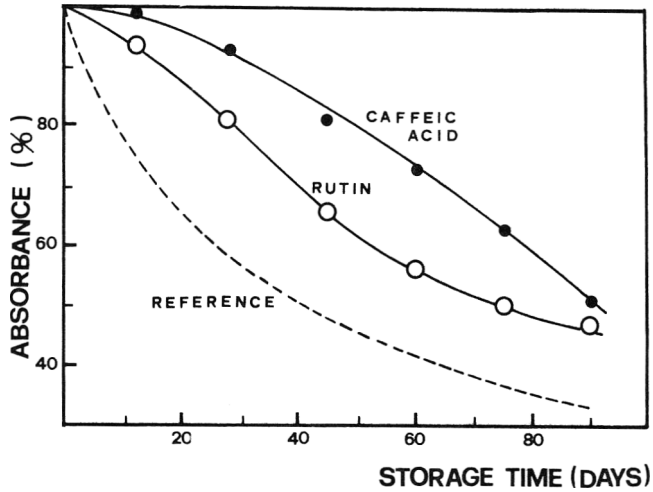


Fig. 5—Effect of phenolic additives on the color stability of the orange juice.

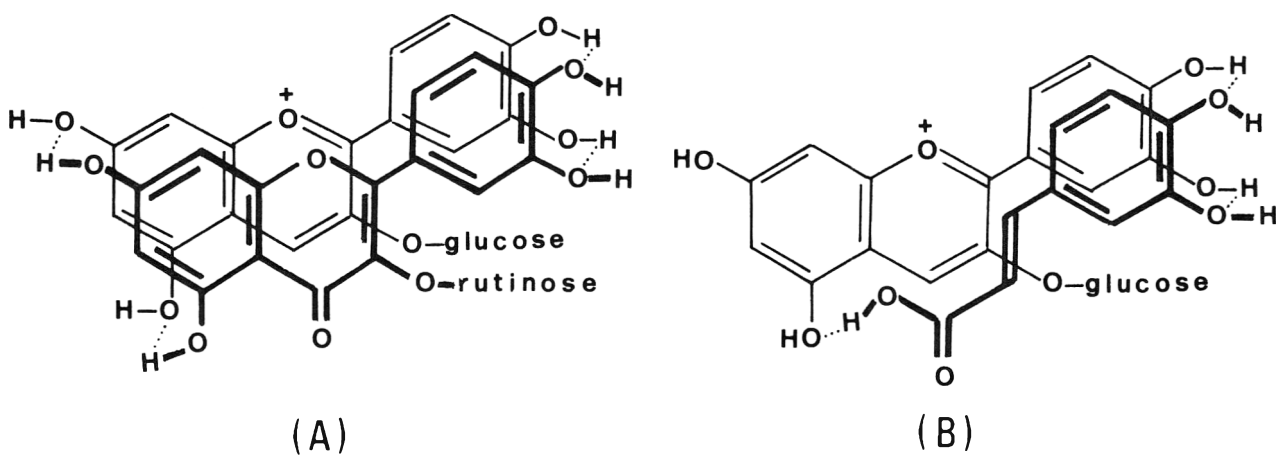


Fig. 6—(A) Anthocyanin-Rutin complex; (B) Anthocyanin-Caffeic acid complex.

exchanger caused damage to the color, heating by microwaves was adopted. The advantages of the latter technique for food processing have been clearly reviewed (Mudgett and Schwartzberg, 1982).

Pasteurization by microwaves did not change the initial color intensity of the orange juice and somewhat improved the stability (Table 2, Series no. 8). Although the stability was not as good as that for the isolated pigments, the time of half disappearance of anthocyanins was about 45 days (Fig. 2), compared to the 30 days for the juice to which sodium benzoate was added. In this case, the absorption curves were qualitatively similar to those reported in Fig. 1, confirming that the apparent color stability was due to anthocyanin stabilization.

It is well known that increasing the acidity of an aqueous solution of anthocyanins causes an increase of both λ_{max} and band intensity, due to a shift of the equilibria of the anthocyanins towards the flavylium salts. Moreover, addition of an antioxidant to the juice reduces chemical and enzymatic oxidation of anthocyanins. For these experiments tartaric acid and gluthathione (De Rosa and Maglitto, 1972) were used as mildly acidic and antioxidant additives, respectively.

Preliminary trials using 300 mg of tartaric acid, 100 mg of gluthathione and 100 mg of ascorbic acid per 100 mL of juice (Table 2, Series no. 5, 6 and 7) showed that tartaric acid and gluthathione partially stabilized color, whereas the changes observed with ascorbic acid were small enough to be negligible. Decrease of stability of the anthocyanins in the presence of ascorbic acid has been observed in other fruit juices (Trifirò and Landi, 1965) and in model systems (Starr and Francis, 1968; Calvi and Francis, 1978).

Further esperiments were then carried out with orange juice pasteurized by microwaves using tartaric acid at different concentrations together with gluthathione (Table 2, Series no. 9, 10 and 11). The stabilizing effect of both additives on the color was negligible with 150 mg of added tartaric acid. The best result was obtained using 250 mg ($t_{1/2}$ = 82 days). A further increase of tartaric acid (500 mg) yielded less satisfactory results (Fig. 4). The latter amount of tartaric acid did not exceed the solubility; therefore, above a critical concentration of the acid, an acceleration of the degradation rate of anthocyanins was observed. This trend is probably due to a slow acid hydrolysis of glucosides with formation of the more degradable aglycones (Jurd, 1972).

The effects of tartaric acid could be partly due to the initial color increase: i.e., the pH color shift (Table 2), but

an actual additional stabilization was indicated by the absorbance value (Fig. 4).

In the last few years copigmentation of the anthocyanins with various phenolic compounds (flavonols, catechins) has been studied (Scheffeldt and Hrazdina, 1978; Williams and Hrazdina, 1979; Chen and Hrazdina, 1981; Osawa, 1982). The presence of such substances also has a protective effect on the degradation of the ascorbic acid (Shrikande and Francis, 1974). Some phenolic compounds for stabilizing the anthocyanins of orange juice were tested. Most of the pigments of *Moro* orange juice are based on the structure of cyanidin (Chandler, 1958; Maccarone et al., 1983) which bears two vicinal OH-groups in the benzene ring; therefore, two phenolic compounds with a similar structure were used: rutin (quercetin-3-rutinoside) and *trans*-caffeic acid (Table 2, Series no. 12 and 13). In the presence of rutin (50 mg per 100 mL) or caffeic acid (50 mg per 100 mL) the $t_{1/2}$ values were 75 days and 90 days, respectively (Fig. 5). The improved stabilization of the color of these juices is ascribed to the reduced availability of the anthocyanins to undergo nucleophilic reactions with water or other agents, because of the probable formation of more stable intermolecular complexes (Sweeny et al., 1981), as in Fig. 6.

CONCLUSIONS

RESULTS on pasteurized juice with the addition of tartaric acid and gluthathione or by the formation of anthocyanin complexes afforded some insight into methods to improve the commercial stability of the red color of *Moro* orange juice. Complexation showed the most merit, but pH, oxygen and antioxidants should also be considered.

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Evaluation of Vanillin Assay for Tannin Analysis of Dry Beans

S. S. DESHPANDE and M. CHERYAN

ABSTRACT

Several parameters that might influence the vanillin assay for tannin analysis of dry bean (*Phaseolus vulgaris L.*) varieties were investigated. The assayable tannin content decreased with decreasing particle size of the bean flours. Time elapsed after grinding as well as storage under high humidity conditions markedly influenced tannin assays. The solubility of tannins was dependent on the type of solvent used, with methanolic extraction requiring shorter periods as compared to extraction with acidified methanol. Color differences within the bean samples appeared to be the prime cause of variation in tannin analysis of beans. Extractions on seed coats alone increased the assayable tannin by 1.1–2.5 times compared to extractions of bean flours.

INTRODUCTION

IN RECENT YEARS, increasing concern over nutritionally harmful effects of certain biological compounds such as phytate and phenolics is evident (Reddy et al., 1982; Deshpande et al., 1984a). These compounds occur naturally in the seeds of cereals and legumes, and if present in appreciable quantities, can lower the nutritional value and biological availability of dietary proteins and minerals.

Literature data suggest that phenolic substances occur primarily in the seeds of certain pigmented cultivars of sorghum, millets, and some legumes (Deshpande et al., 1984a; Salunkhe et al., 1982). One of the major difficulties encountered in polyphenol research is the lack of a standard quantitative method for the analysis of phenolics that would be suitable for a wide range of seeds, forage crops and food products under varying experimental conditions. Also, not all phenolics are of nutritional concern, and thus the "total phenol" content of foods as routinely expressed may not be a true index of the nutritional quality of foods. Some of the methods commonly employed in tannin analyses suffer from the lack of specificity. They do not seem to distinguish polyphenols of nutritional concern from other low molecular weight phenols that also occur naturally in these products. The use of tannic acid (a hydrolyzable gallotannin) as a "reference standard" is also questionable since its biological properties differ from those of tannins of flavonoid origin. Polyphenols of cereals and legumes are predominantly of the latter type. Also, commercially available tannic acid has been shown to be a mixture of four phenolic compounds, the relative proportions of which vary between the samples (King and Pruden, 1970). Thus the choice of a suitable standard for tannin analysis is also important.

Literature data on tannin methodology have been diverse and at times conflicting. Currently available methods for tannin analysis range from simple colorimetric, UV spectrophotometric, chromatographic, and enzymic to more sophisticated and expensive nuclear magnetic resonance techniques. The commonly used methods to determine tannins include the vanillin test (Burns, 1963; Bate-Smith and Lerner, 1964), the Prussian Blue test (Price and Butler, 1977), and the Folin-Denis test (Burns, 1963). Although all these methods are based on the chemical characteristics of tannins, the latter two redox methods are not very specific and detect all phenols with varying sensitivity. Hence, when screening for condensed tannins in plants, the vanillin test is preferred because of its sensitivity, specificity and simplicity Although this reagent detects monomeric as well as polymeric flavonoids, it is quite specific to a narrow range of flavanols and dihydrochalcones which have a single bond at 2,3 position and free meta-oriented hydroxy groups on the B-ring (Sarkar and Howarth, 1976).

Price et al. (1978) have critically reevaluated the vanillin reaction for the detection of tannins in sorghum. In spite of the modifications suggested by these researchers, the method still did not appear to give reproducible results with several dry bean varieties. Such differences may be caused by the inherent variation between sorghum and bean tannins. Wide differences were also observed within the same genotype (*Phaseolus vulgaris* L.) of dry beans. The present investigation was therefore undertaken to study the influence of several parameters such as extraction time, time required for maximum color development, relative humidity, storage time, particle size, and day-to-day variation in tannin analyses of four varieties of dry beans of widely differing tannin content and seed coat color. In addition, the effects of tannin on color variation within a given sample of dry beans were also evaluated.

MATERIALS & METHODS

Source of beans

Dry beans (*Phaseolus vulgaris* L.) of Pinto (mottled light brown), Small Red (dark red), Viva Pink (light pink), and Black Beauty (dark black) varieties, grown and harvested in 1980, were obtained from Roger Brothers Seed Co. (Twin Falls, ID). The beans were stored in a cold room at about 10°C prior to the initiation of these experiments. The beans were ground to obtain flours of desired particle size using the U.S. Standard sieve system. The mesh size given is that through which 100% of the sample passed. Typical particle size distribution within each mesh size for the flours of the four bean varieties selected is shown in Fig. 1. Unless mentioned otherwise, all chemicals used were of reagent grade.

Particle size variation

Bean flour samples of desired particle size were extracted (within 6 hr of milling) with absolute methanol for 1 hr with mechanical shaking and centrifuged at 1500 × *g* for 10 min. Aliquots of the supernatant were assayed for tannin. Based on these data, flours of 820 micron particle size (20-mesh) gave the highest values for tannin. Hence, all subsequent experiments were carried out with flours of this particle size.

Effect of storage time

Bean flour samples were stored in air-tight plastic bags at room temperature (25°C), and analyzed daily for tannin over a period of 7 days. Tannin determined immediately after grinding represented the control value.

Authors Deshpande and Cheryan are affiliated with the Dept. of Food Science, 382-D, Ag. Eng. Sci. Bldg., University of Illinois, Urbana, IL 61801.

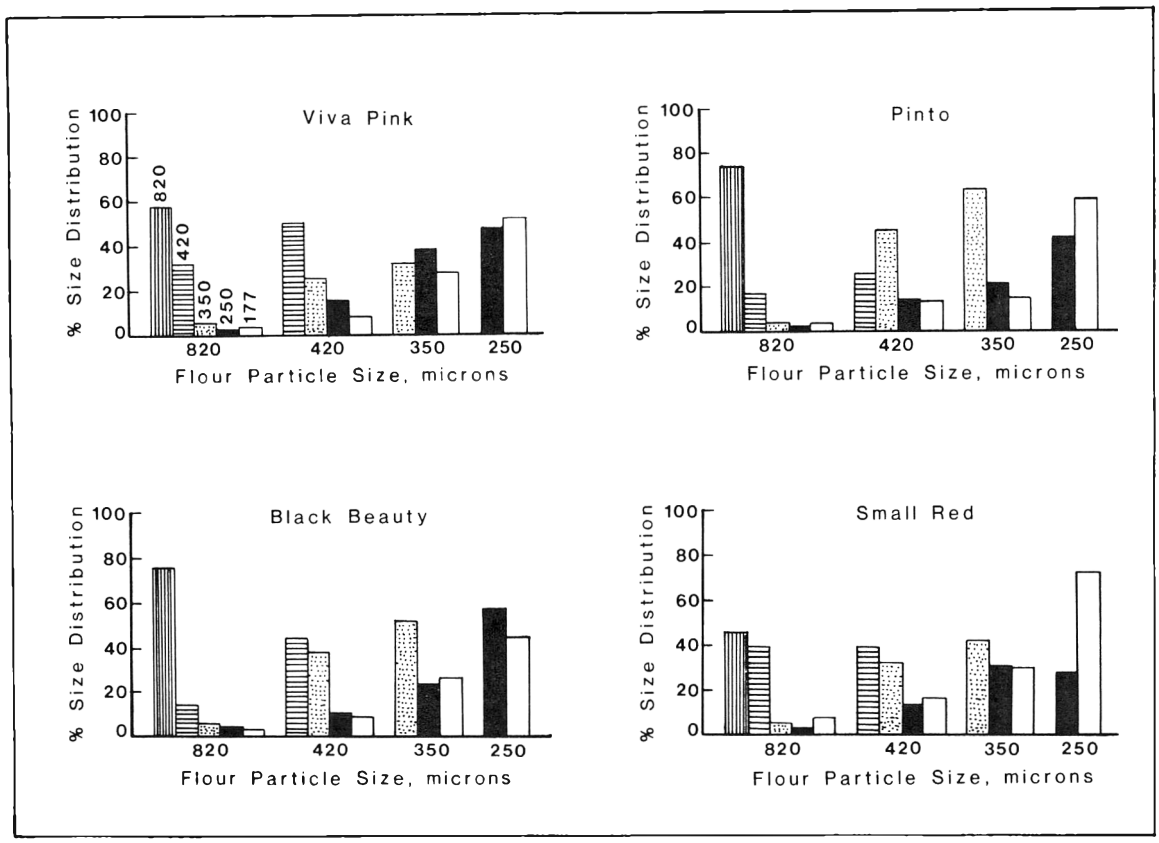


Fig. 1—Average particle size distribution of the dry bean flours. The numbers over each bar represent microns.

Effect of relative humidity

Equilibrium relative humidities of 33, 45, and 57% were maintained in desiccators containing magnesium chloride, potassium carbonate, and sodium bromide solutions, respectively, as described by Rockland (1960). Freshly ground samples of Viva Pink bean flours were placed in each desiccator, and tannin was assayed over a period of 6 days.

Day-to-day variation

Beans were ground on each of three separate days. Samples were assayed for tannins within 6 hr of milling.

Time for maximum color development

The vanillin reagent (0.5%) was added to the aliquots of freshly ground samples extracted with either absolute methanol or 1% HCl in methanol. The optical density was recorded for a period of 30 min at 1 min intervals.

Extraction time

Samples were extracted with absolute methanol or 1% HCl in methanol for 10, 20, 30, 40, 50, and 60 min with mechanical stirring, filtered through sintered glass funnels under vacuum, and analyzed for tannins.

Effect of color variation in bean samples

The light and dark colored seeds from a representative sample of Small Red beans were separated and analyzed for tannin.

Tannin assay

A 2-3g bean flour sample (accurately weighed) was extracted with 10 mL absolute methanol (or 1% HCl in methanol) for desired time with mechanical stirring. Except where the effects of extraction time were under study, the samples were centrifuged at 1500 x *g* for 10 min and aliquots were immediately analyzed for tannin using the 0.5% vanillin assay (Price et al., 1978) or the 2% vanillin assay (Burns, 1963). Optical density measurements at 500 nm were made using a Beckman Model 34 spectrophotometer attached to a printer. Appropriate standard curves using catechin (+) (3.5 moles of H_2O per mole of catechin, Sigma Chemical Co., St. Louis, MO) were prepared and tannin was expressed as mg catechin equivalents on a dry weight basis.

All the extractions were carried out at least in duplicate and the individual assays run in triplicate using appropriate sample and reagent blanks. All the assays were conducted at room temperature (25 ± 1°C, 28–34% RH).

Statistical analysis

Wherever applicable, the data were analyzed by the analysis of variance procedures of the Statistical Analysis System (SAS, 1979). Least significant differences ($p = 0.05$) were used for multiple mean comparison tests.

RESULTS & DISCUSSION

Effect of particle size

The particle size greatly influenced the tannin content of beans (Table 1). Assayable tannin decreased by about 25–49% with 2% vanillin assay and by 36–50% with the 0.5% assay, as the minimum particle size was reduced from 820 to 250 microns. In most cases, the differences between particle sizes were statistically significant, although as expected, a greater variation was observed among the four varieties investigated. The 2% vanillin assay, which does not include correction for background color of the sample, consistently gave higher values than the 0.5% vanillin assay. In fact, with the former method, Black Beauty beans yielded higher values for tannins with the decreasing particle size. However, when corrections were applied for background color, the same relationship as described above was observed between assayable tannin and particle size.

It was interesting to note that for two of the four varieties selected, viz., Pinto and Viva Pink beans, statistically significant differences in tannin were not observed when either of the two assays were used with 820 micron particle size. Both these varieties had light colored seed

Table 1—Effects of particle size on tannin analysis of dry beans[a]

Minimum particle size, microns		2% Vanillin assay				0.5% Vanillin assay			
		Pinto	Small Red	Viva Pink	Black Beauty	Pinto	Small Red	Viva Pink	Black Beauty
820	(20)[b]	337	304	169	154	328	248	162	60
420	(40)	269	262	111	172	251	210	118	45
350	(45)	245	250	105	213	226	191	99	36
250	(60)	223	227	87	258	186	159	95	30
LSD[c] (p = 0.05)		9.8				5.6			

[a] Values represent mg catechin equivalent/100g beans on dry weight basis. Samples were extracted in absolute methanol for 1 hr.
[b] Numbers in the parentheses are U.S. Standard mesh sizes.
[c] Least significant difference. Differences of two means within/between the varieties exceeding this value are significant.

coats. On the other hand, the remaining two dark colored varieties, specially the Black Beauty beans, gave much sharper differences between the two assays. This may presumably be due to the presence of monomeric anthocyanin-like compounds which turn red under the acidic conditions employed for the assay. Also, the differences between the two assays were more pronounced with decreased particle size, suggesting that such monomeric compounds may be attached to some seed component and are freed to yield a positive reaction only when separated from them. This may be the reason the Black Beauty beans gave higher values with the 2% assay.

The loss of assayable condensed tannins appeared to be directly related to the time and extent of milling the seeds. Although no studies with either sorghum or legumes have been reported regarding the influence of varying particle sizes on the determination of tannin, the degree of chopping or macerating the fresh plant material of *Lespedeza cuneata* was found to reduce its tannin content (Burns, 1966; Lyford et al., 1967). On the other hand, the tendency of many phenols to oxidize during sample preparation and extraction is well known. Possibly, the greater loss of some seed component rich in tannin with increased milling time could also cause such variation. Also, external cooling was not provided during grinding of bean samples. The heat and moisture generated during grinding may create a microenvironment in which tannins are easily oxidized or can complex with other seed components such as proteins. The vanillin assay also fails to detect such complexed tannins. All these factors would lead to a decrease in the assayable tannin of the seeds with increased time and extent of milling.

The effect of particle size on assayable tannin may also be a function of exposed surface area of flour particles which contain protein that can bind tannin. These relationships are summarized in Table 2. The surface area and volume ratios for the bean flours were calculated using the percent particle size distribution within each mesh size (Fig. 1). Assayable tannin was directly related to the volume to surface area ratios of varying particle size flours (r = 0.96-0.99). Davis and Hoseney (1979) observed that the biological activity values calculated as percent alpha-amylase activity for sorghum tannins were much higher when expressed on a per kernel basis as compared to those expressed on flour weight basis. They also found that the amount of condensed tannins per kernel of grain sorghum decreased with maturity. Since beans vary in size, both within variety and between varieties (Deshpande et al., 1984b), this may also influence the assayable tannin.

Table 2—Relationship between surface area and volume of bean flours and assayable tannin (0.5% vanillin assay)

Flour particle size, microns	Surface area $\mu^2 \times 10^6$	Volume $\mu^3 \times 10^8$	Volume/Surface area, μ	Assayable tannin[a]
Pinto				
820	1.69	2.23	131.8	328
420	0.36	0.22	60.5	251
350	0.30	0.17	54.8	226
250	0.14	0.05	36.7	186
r = 0.97				
Small Red				
820	1.22	1.50	122.7	248
420	0.38	0.24	62.7	210
350	0.25	0.13	51.0	191
250	0.13	0.04	34.7	159
r = 0.96				
Viva Pink				
820	1.42	1.81	127.2	162
420	0.42	0.27	64.3	118
350	0.23	0.11	49.2	99
250	0.15	0.05	37.5	95
r = 0.99				
Black Beauty				
820	1.69	2.22	131.3	60
420	0.42	0.27	63.8	45
350	0.27	0.14	52.8	36
250	0.16	0.06	38.3	30
r = 0.97				

[a] Values represent mg catechin equivalents/100g beans on a dry weight basis.

Effect of storage time

Assayable tannin of the four varieties investigated decreased steadily with time elasped after milling (Fig. 2). A much steeper decline was observed with Pinto beans during the first four days than with beans of the other three varieties. At the end of 7 days storage, the values decreased by about 16-56% and 16-60% using the 2% and the 0.5% vanillin assay, respectively. The greatest decrease was observed with Black Beauty beans suggesting that the monomeric anthocyanin-like pigments were more prone to oxidation than the polymeric condensed tannins. Price et al. (1978) observed only about 15% decrease in tannin of BR-54 sorghum variety after 18 days of storage at room temperature. This again indicates tremendous inherent variability in the nature and the type of tannins present in different species of food crops.

Effect of relative humidity

The relative humidity of storage also influenced assayable tannin depending on time before analysis (Table 3). Significant differences, however, were observed only under relatively high humidity conditions. The time elapsed after grinding also affected assayable tannin. The relative humidity of the environment may be a factor in influencing the assay only in laboratories where such conditions are not controlled, and in high humid weather conditions particularly during the summer months.

The decrease in assayable tannin when seeds are stored under high humidity conditions seems to have practical applications in tannin detoxification to improve the nutritive value of grain sorghum and legumes. Lichtenwalner et

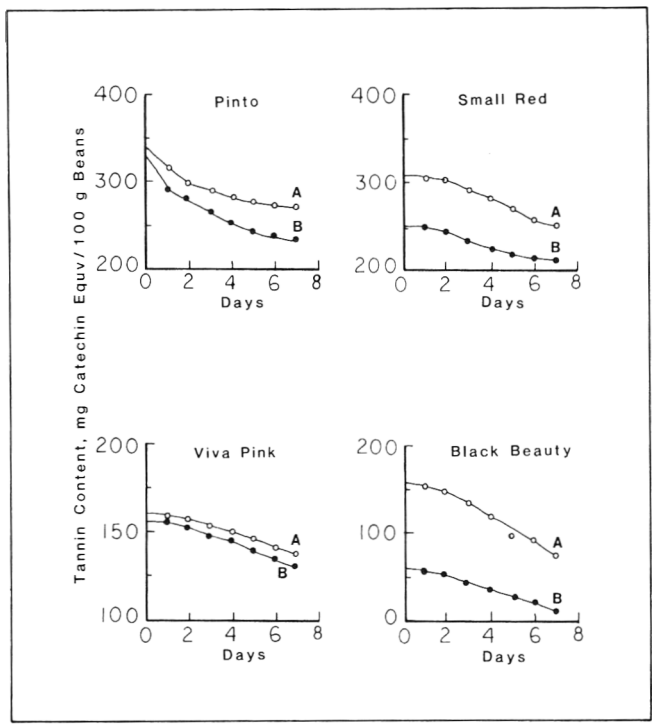


Fig. 2—Effect of storage time on the assayable tannin of dry beans. Curve A: 2% vanillin assay; Curve B: 0.5% vanillin assay

Table 3—Effects of relative humidity of storage conditions on tannin analysis of Viva Pink beans using 0.5% vanillin assay[a]

Time of analyses days	Relative humidity		
	33%	45%	57%
Control		(161)	
2	154	151	144
4	147	142	118
6	129	132	102
LSD[b] (p = 0.05)		4.8	

[a] Values represent mg catechin equivalents/100g beans (820 micron particle size) on a dry weight basis. Samples were extracted in absolute methanol for 1 hr.
[b] Lease significant difference. Differences of two means within/between the varieties exceeding this value are significant.

al. (1979) coined the term "reconstitution" for the process of adding sufficient moisture back to grain and sealing the moist grain from the environment so that fermentation can occur. Recently, Mitaru et al. (1983) reported that such high moisture storage or reconstitution of high tannin sorghums decreased extractable tannin to a level similar to that of low tannin sorghum. The greatest reduction in assayable tannin occurred within the first 10 days of storage and then gradually levelled off. Although the mechanism of tannin deactivation during reconstitution is not known, Mitaru et al. (1983) suggested that the process may probably be similar to the polymerization of tannins during the ripening of the seed. The process ceases when the moisture drops. Davis and Hoseney (1979) also observed that the amount of condensed tannin per kernel of grain sorghum gradually declined as the grain approached harvest. Several researchers have reported an improvement in nutritional quality of high tannin sorghum using reconstitution processes (Reichert et al., 1980; Price and Butler, 1980; Price et al., 1979).

Day-to-day variation

Data on the tannin of bean samples ground on each of three separate days are summarized in Table 4. Similar to the observations of Maxson and Rooney (1972) and Price et al. (1978) with sorghum grain, values between batches of seeds ground independently showed considerably more variation than did replicate assays within one batch of ground seeds. In most cases, at least one value was significantly different from the others. Since most parameters were carefully controlled, the loss of some tannin-rich fraction during milling and probably the seed-to-seed variation in tannin appeared to be the primary reasons for such variation.

Time for maximum color development

Since the vanillin reaction with bean tannins consistently showed a variable response, the adequacy of the 20 min reaction time prior to absorbance measurements was also evaluated. The absorbance patterns for the four bean varieties after the addition of the 0.5% vanillin reagent to both the methanolic (curve B) and acidified-methanolic (curve A) extracts are shown in Fig. 3. Some of the relevant data are summarized in Table 5. A rapid reaction between the tannins and the vanillin reagent was observed within the first few minutes of the assay. Absorbance increased much more rapidly when acidified-methanol was used as the extracting solvent, and the absorption peak was reached significantly earlier for three of the four varieties studied. Consequently, the 'optimum absorbance range', arbitrarily defined as 0.005 OD units on either side of the peak, occurred prior to the suggested 20-min measurement time. However, with the exception of Small Red beans, the total time span for the desired absorbance range was similar when either of the solvents was used for tannin extraction. Based on our data, the 20-min assay appeared to be valid for only Pinto and Viva Pink beans using methanol as the extracting solvent.

Extraction time

The extraction pattern was markedly affected by the solvent used. With absolute methanol, maximum absorbance was reached within 10–20 min of extraction (Fig. 4), and the values slowly decreased with longer extraction time, presumably due to tannin oxidation. On the other hand, at least 50–60 min extraction was required using acidified methanol. Among the varieties, the extraction pattern was quite different for Black Beauty beans. The absorbance was almost twice as high in acidified-methanol as in methanolic extracts. Once again, this clearly differentiated the types of phenolic compounds in this variety.

Effect of color variation in bean samples

Since batches of seeds ground separately consistently cause greater variation in tannin, the effects of color differences within a given sample of seeds were also evaluated. Small Red beans were selected primarily because of the visible differences in their seed coat color. The light and dark colored seeds (approximately 20:80 distribution) were subjectively separated and ground in different batches prior to extraction. Tannin as determined by the 0.5% vanillin test ranged from 228 ± 9 mg and 254 ± 14 mg catechin equivalents/100g beans for the light and dark colored seeds, respectively. The inherent color variation within a given sample of seeds, therefore, seemed to be one of the major factors influencing the tannin assay.

The results of this study on bean tannins indicated that several factors other than those already reported by Price et al. (1978) may also influence the vanillin assay. To avoid these sources of variability as much as possible, analyses were conducted using all the optimum parameters described earlier. For each variety, three representative samples of 20g each were ground to obtain 820 micron particle flours. Extractions were made in absolute methanol and acidified-

Table 4—Day-to-day variation in tannin analysis of dry beans[a]

Variety	2% Vanillin assay			0.5% Vanillin assay		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Pinto	337	309	340	328	296	317
Small Red	304	315	298	248	269	251
Viva Pink	169	181	173	162	165	154
Black Beauty	154	143	163	60	56	67
LSD[b] (p = 0.05)		6.4			4.7	

[a] Values represent mg catechin equivalents/100g beans (820 micron particle size) on dry weight basis. Samples were extracted in absolute methanol for 1 hr.
[b] Least significant difference. Differences of two means within/between the varieties exceeding this value are significant.

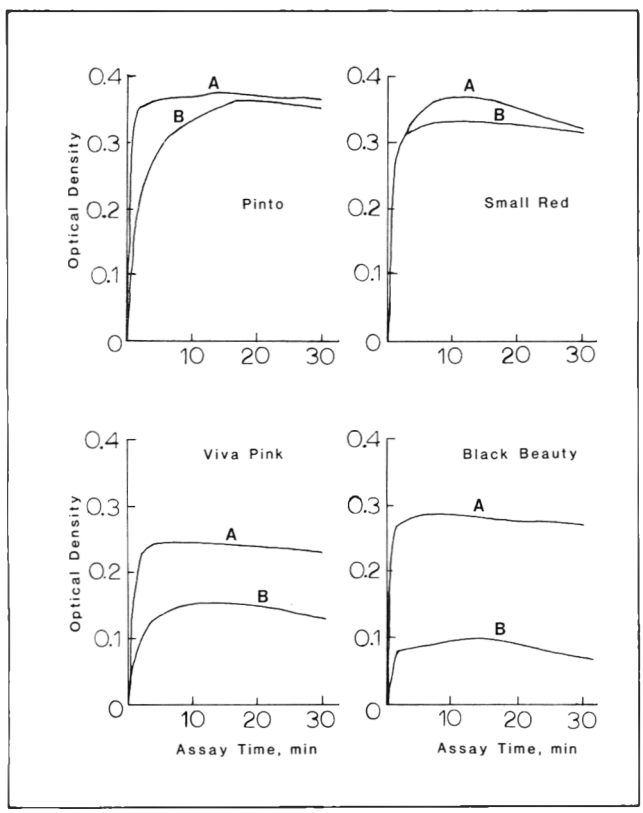


Fig. 3—Absorption pattern of tannin extracts with assay time. Curve A: Acidified-methanolic extract; Curve B: Methanolic extract.

methanol for the desired period of time (Fig. 4) for each variety within 6 hr of milling. The 0.5% vanillin assay was conducted for the usual 20-min period as well as when the peak absorbance was observed (Table 5). The data on these experiments are summarized in Table 6. Significant differences in tannin were observed as determined by the 'optimum' and the 20-min assay. No definite pattern was observed with regard to the solvent used. Pinto and Viva Pink beans gave significantly higher values for tannins with acidified-methanol. The dependance of tannin solubility on the type of extraction solvent suggested that the tannins of Pinto and Viva Pink beans may have different chemical characteristics than those of the other two varieties studied. Price et al. (1978) also reported similar observations for tannins of group II sorghum varieties. They suggested that the group II tannins may be nutritionally inert because they are bound to some grain component and unless released, do not dissolve and, therefore, do not precipitate proteins. The extraction of such tannins is dependent on an acid-catalyzed reaction to release the tannins. Price et al. (1978) further suggested the possibility of group II 'tannins' being actually monomeric flavonoids which gave a vanillin-positive test when extracted with methanolic HCl.

Table 5—Absorption characteristics of tannin extracts of dry beans using 0.5% vanillin assay[a]

Variety and solvent	Amax	Amax at min	Optimum absorption range[b], min
Pinto			
MeOH	0.360	20	15–25
1% HCl in MeOH	0.377	12	8–18
Small Red			
MeOH	0.369	10	8–14
1% HCl in MeOH	0.331	11	6–20
Viva Pink			
MeOH	0.154	13	9–18
1% HCl in MeOH	0.243	8	4–14
Black Beauty			
MeOH	0.097	11	7–16
1% HCl in MeOH	0.284	5	2–12

[a] Absorbances are not corrected for background color.
[b] See text for definition.

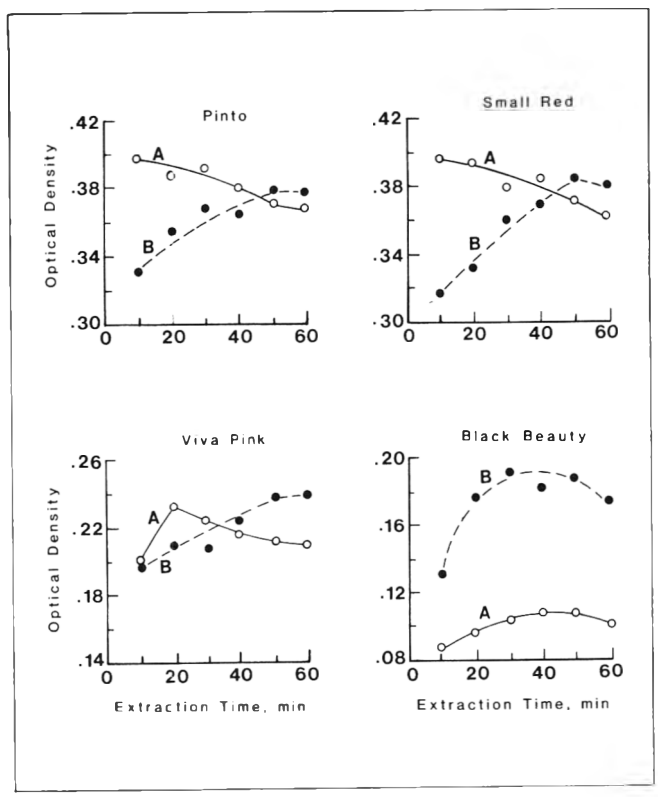


Fig. 4—Effects of extraction time on the optical density of tannin extracts. Curve A: Methanolic extract; Curve B: Acidified-methanolic extract.

The assayable tannin in the seeds was dependent on the volume to surface area ratios of flours (Table 2) and therefore to the amount of protein exposed on the surface of the flour particles. To eliminate tannin binding by seed storage proteins, extraction was done on intact beans. However, only 10–20% of the total tannins were detected in absolute methanol with prolonged extraction periods (> 48 hr, data not shown). Apparently, some sort of grinding was essential to release tannins from intact seed coat cells. Therefore, extractions were carried out on seed coats alone. The hulls were scraped off the seed surface with a sharp razor blade and were coarsely ground in a mortar. Data on assayable tannins extracted from seed coats alone are presented in Table 7. Seed coats contained approximately 0.5–7.0% tannins. When expressed on total seed weight basis, these values were 1.1–2.5 times higher compared to extractions of bean flours. This again proved that seed proteins were the major interfering components with tannin assay. Laurena et al. (1984) also detected 2-3 times greater tannins in 8 varieties of cowpeas when tannins were extracted from seed coats alone.

The results of this investigation clearly suggested a need for a standardized vanillin assay for bean tannins and a careful selection of conditions and parameters to ensure reproducibility within and between laboratories. The 0.5% vanillin assay as suggested by Price et al. (1978) appears to be much more sensitive than the 2% assay. Appropriate sample blanks also need to be used to eliminate variation due to the background color caused by the anthocyanin-like pigments. The inherent color variation within a given sample could be minimized by the careful selection of at least three representative samples and grinding them separately prior to the analysis. Extractions with seed coats should be preferred to eliminate variation caused by tannin-protein binding when bean flours are used. Since scraping off the seed coats is tedious where a large number of samples are routinely analyzed for tannins on a daily basis, perhaps a relationship could be established between protein content and assayable tannins. In such cases, the beans should be ground as coarsely as possible (> 820 micron particle size). The use of a laboratory rice polisher or a sorghum pearling instrument such as those used in cereal laboratories to scrape off the surface layers to a desired degree would considerably speed up tannin analyses. Such an approach would mimimize variation caused by tannin-protein binding and give an accurate indication of the amount of tannin present in cereals and legumes. The present study was done with only four bean varieties, and yet it showed wide differences between tannin properties that could influence their determination. Also, unlike sorghum tannins, dry bean tannins have not yet been isolated and chemically characterized. A large number of dry bean varieties and other legumes, preferably grown under different environmental conditions, thus need to be screened for the presence and the type of condensed tannins prior to the development of a standardized assay which would permit comparison of results among laboratories. Such standardization will also permit meaningful determination of the influence of tannins on the nutritional quality of legumes.

Table 6—Tannin of dry beans using the 20-min and the 'optimum' 0.5% vanillin assays[a]

	Absolute Methanol		1% HCl in Methanol	
Variety	20-min assay	Optimum assay	20-min assay	Optimum assay
Pinto	306	301	317	329
Small Red	257	283	239	245
Viva Pink	160	167	181	209
Black Beauty	49	56	26	38
LSD[b] (p = 0.05)	6.2		9.7	

[a] Values represent mg catechin equivalents/100g beans (820 micron particle size) on dry weight basis.
[b] Lease significant difference. Differences of two means within/between the varieties exceeding this value are significant.

Table 7—Tannin of dry bean seed coats extracted with absolute methanol

		Assayable tannin	
Variety	Seed Coat[a] %	g catechin equv./ 100g seed coat	mg catechin equv./ 100g seed
Pinto	8.2	6.85	562
Small Red	8.1	5.95	482
Viva Pink	8.0	6.48	518
Black Beauty	8.1	0.51	44

[a] Data taken from Deshpande et al. (1982).

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Objective Measurements of the Process of Curd Formation during Rennet Treatment of Milks by the Hot Wire Method

TOMOSHIGE HORI

ABSTRACT

A new thermal method for nondestructive, continuous and objective measurements of the progress of curd development was proposed to obtain much more uniform industrial cheese production. The temperature measurements of the electrically heated (direct electric current, 0.7A) platinum wire (0.1 mm$^{\Phi}$ x 106 mm) immersed axially in the renneted whole milk (volume, 660L) adequately monitored the whole process of milk curd coagulation in the actual cheesemaking. In addition, the temperature of the hot wire referred to the initial temperature resulted in the kinematic viscosity of the coagulating milks.

INTRODUCTION

COAGULATION of milk is the fundamental process in cheesemaking. This is usually accomplished by adding rennet extracted from the stomachs of calves. The curd formation is believed to occur in two phases, a primary (proteolytic) and a secondary (clotting) phase. The extensive but not complete conversion of casein to paracasein by the enzyme rennin (EC 3.4.23.4) in rennet resulted in milk coagulation in the presence of calcium ions (Green et al., 1978; Dalgleish, 1979; Dalgleish et al., 1981). The milk coagulum becomes firmer as linkages between casein micelles increase, until the curd structure consists of fat droplets and some whey trapped in a continuous matrix of casein (Zviedrans, 1983).

Objective measurements of the curd-firming of renneted milk would be of great value in industry. The determination of the optimum time for curd cutting will allow the whey to be drained properly and keep losses of curd fines and fat in whey to a minimum; curdmaking conditions affect the yield and quality of cheese and must be strictly controlled (Thomasow and Voss, 1977; Marshall et al., 1982). An optimum cutting time is established by the cheesemaker who "feels" the curd and uses his own subjective judgment. This technique is accurate and acceptable if the evaluation is made properly. No subjective methods, however, may be useful for commercial operations with closed vats.

Few objective methods are available for direct measurement of the clotting phase during the treatment in the actual cheesemaking vat. Various methods based mainly on rheological, spectroscopic or ultrasonic principle have been developed but each has some drawback (Thomasow and Voss, 1976). The instruments for the viscometric methods (e.g., Scott Blair and Oosthizen, 1961; Kopelman and Cogan, 1976; Garnot and Olson, 1981; Bynum and Olson, 1982) which cut curd or exposed it to strong mechanical stress had the disadvantage that whey was released due to a syneresis which gave varying results. Moreover, not more than one measurement can normally be made with the viscometric methods. The torsion viscometers (e.g., Olson and Bottazzi, 1977 for "Thrombelastograph"; McMahon and Brown, 1982 for "Formagraph") and the pressure transducer (e.g., Hatfield, 1981; Marshall et al., 1982) still exposed the curd to stress and deformation. The application of spectroscopic (e.g., Dalgleish et al., 1981; Hardy and Fanni, 1981; Surkov et al., 1982) and ultrasonic (e.g., Everson and Winder, 1968) methods were limited to scientific usage.

A thermal, or line heat source, method would be a new procedure for monitoring the process of curd formation, because heat conduction does not destroy the coagulum. Fundamental studies of milk coagulation require nondestructive, continuous and objective measurements. These measurements will determine the physical properties which significantly characterize and describe the structural changes of the curd during rennet treatment.

The objective of this research is to establish a new objective method for direct measurements of the process of curd formation during rennet treatment in cheesemaking.

MATERIALS & METHODS

Sample

Skim milk and whole milk were used in the study. Spray-dried skim milk (protein, 36.1% db; fat, 0.8% db) was reconstituted with distilled water to obtain the skim milk sample (solids, 10.0% wb; $\nu = 1.368 \times 10^{-6}$ m^2/s at 30.0°C). Fresh whole milk (solids, 12.0% wb; fat, 3.5% wb; protein, 3.1% wb; ash, 0.7% wb) was pasteurized at 75°C for 15 sec immediately before rennet treatment . The ν value of the pasteurized whole milk sample was 1.377×10^{-6} m^2/s at 30.0°C.

Distilled water (4.4-51.9°C) was used as the standard liquid.

Rennet treatment of milk samples

Milk samples were treated with rennin from calf stomach. The skim milk sample (volume, 900 mL) in a cylindrical flask (90 mm$^{\Phi}$ x 180 mm) was treated at pH 6.59 and 30.0 ± 0.1°C with crystallized and lyophilized enzyme rennin (40,000 and 200,000 units/kg-milk, from Sigma Chemical Co., St. Louis, MO). Skim milk coagulation was also done at pH = 5.53-6.59, θ = 30.0-40.0°C and C_T = 5,400-595,000 units/kg-milk; Glucono-δ-Lactone (C_g = 0-0.20% wb) was added to acidify milk samples. One unit enzyme coagulated 10 mL milk per min at 30°C. The whole milk samples (volume, 130L or 660L) in the cheesemaking vat (545 mm x 850 mm x 305 mm or 900 mm x 1,600 mm x 480 mm) were coagulated at pH 6.55 and 30.0 ± 0.1°C by calf rennet (0.0035% wb, approximately 5,000 units/kg-milk, from Chr. Hansen, Copenhagen).

Annato food color (0.005% wb), KNO_3 (0.02% wb) and starter bacteria culture (0.5% wb, pH 4.53 at 5°C) were added to the whole milk sample.

Cheesemaking

St. Paulin cheese was manufactured from the whole milk curds cut under three conditions. Whole milk samples (130L) in the smaller vats were treated with calf rennet for 35, 50, and 80 min prior to cutting curd with a wire knife (opening, 10 mm). The cubic curd granules cooked for 45 min at 30°C and another 45 min at 34°C were subjected to static pressure [980 Pa for the initial 30 min in the vat, and 9.8 kPa for another 30 min in the cheese mould (87 mm$^{\Phi}$ x 65 mm)] to obtain the green cheese samples. The green cheese was salted (2.3% wb) and coated with cheese wax (thickness, 2 mm) prior to ripening (12 wk at 11°C) for sensory evaluation of cheese quality.

Author Hori is affiliated with Snow Brand Milk Products, Co., Ltd., Technical Research Institute, 1-2, Minamidai 1-chome, Kawagoe, Saitama, 350 Japan.

Sensory evaluation for curd firmness and cheese quality

Subjective measurements were made on the acceptability (firmness) of the curd to determine the empirical optimum cutting time. An index finger was inserted at an acute angle in the curd and was brought up gently to form a cleavage. This cleavage, if clean and smooth, indicated the curd was ready; if it was ragged, it indicated the curd was not ready. Sensory judgments for curd firmness were carried out for 18 vats (3 vats/wk) with 660L whole milk.

Flavor, hardness, springness, mouthfeel, and acceptability of the ripened cheese samples were evaluated with the Scheffé's method for paired comparisons (Scheffé, 1952). The sensory tests were made under the following conditions: Experienced (about once every 2 wk for more than 2 yr) panelists affiliated with our technical research institute evaluated the paired cheese samples (about 20g, 10°C; each on separate white plate). All judgments were conducted in individual, partitioned booths maintained at 20°C, under 100 W white bulbs in each booth. Paired comparisons by the Scheffé's method (rating, −3, −2, −1, 0, +1, +2, +3) were made by 40 panelists. Each panelist judged three different pairs for 15 min. All judgments were finished in 2 days.

Hot wire measurements

The development of temperature was observed in an electrically heated wire for monitoring the process of curd formation. A platinum wire (0.1 mm$^{\Phi}$ x 106 mm, Fig. 1) immersed axially in the samples (900 mL for skim milk or water in the flask; 130L or 660L for whole milk in the cheesemaking vat) were heated with constant direct electric current (0.7A for milks, 0.5-1.0A for water). The temperature measurements were made automatically at regular intervals (5 sec) during rennet treatment with a data acquisition and control unit (model 3497A from Hewlett Packard Co., California) directly connected to a desk top computer (model 86 from H.P. Co.). The electric current was supplied with 0.014% accuracy to attain constant heat generation in the platinum hot wire with a power supply (model 2853/2862 from Yokogawa Electric Works, Ltd., Tokyo), while the temperature of the hot wire was calculated to 0.044% accuracy by:

$$\theta_w = (R/R_o - 1)/\alpha_w \quad (1)$$

The exact values of R_o and α_w of the platinum wire used in this study were 1.364892Ω and 3.8166 x 10^{-3} 1/K.

The temperature measurements for the water samples (4.4-51.9°C) were repeated 12 times at regular intervals (5 sec) in each measurement. The samples were initially in thermal equilibrium.

For the whole milk sample, the hot wire sensor (Fig. 1) was lowered in the cheesemaking vat and placed in a fixed position prior to the steam sterilization of the vat.

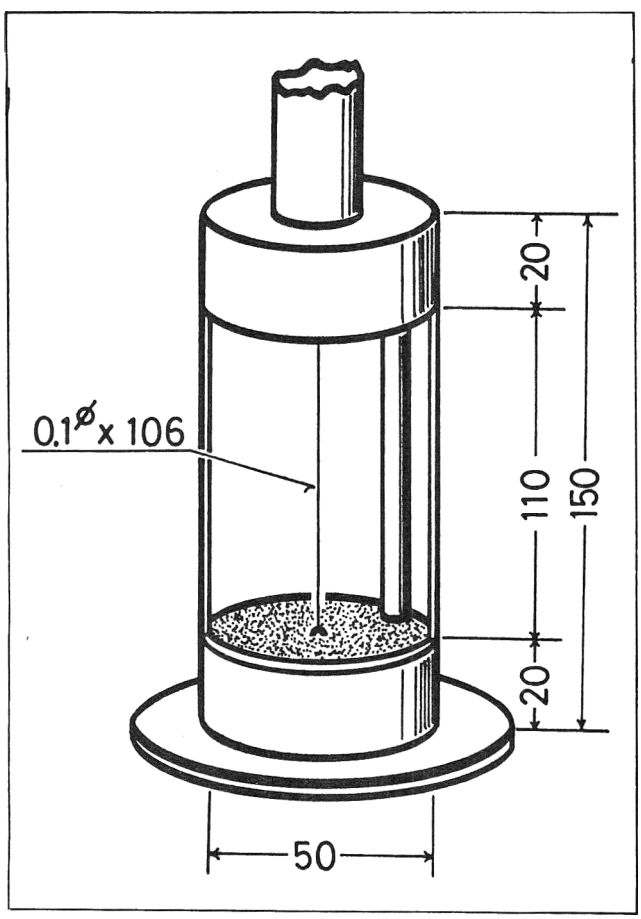


Fig. 1—Hot wire sensor for the nondestructive, continuous and objective measurements of milk coagulation.

RESULTS & DISCUSSION

Monitoring the process of curd formation in cheesemaking vat by the hot wire method

The temperature of the electricaly heated platinum wire was sensitive to the process of curd formation of the milk samples. The θ_w value which was independent of time for several minutes after rennet addition increased drastically when visual clotting was observed; the ($d\theta_w/dt$) value decreased with time until the θ_w value reached the equilibrium value (Fig. 2). The initial linear portion of the θ_w vs t_r curve suggested the enzymatic reaction or splitting of κ-casein by rennin (the primary phase of coagulation), while the convex portion showed the nonenzymatic changes or coagulation of casein micelles (the secondary phase).

The hot wire detected the clotting time and adequately monitored the whole process of milk coagulation. The θ_w vs time curve characterized the curdling process, when the whole milk sample (volume, 660L) was rennet-treated in the actual cheesemaking vat as follows (Fig. 3): After the pasteurized milk was stirred gently to cool it to the required coagulation temperature, or 30°C, the starter culture was added (Fig. 3, #1); calf rennet (0.0035% wb or approximately 5,000 units/kg-milk) was added to the milk (pH 6.55) acidified for 15 min (Fig. 3, #2); visual clotting when the θ_w value increased abruptly (Fig. 3, #3) was followed by the progress of curd development, until the

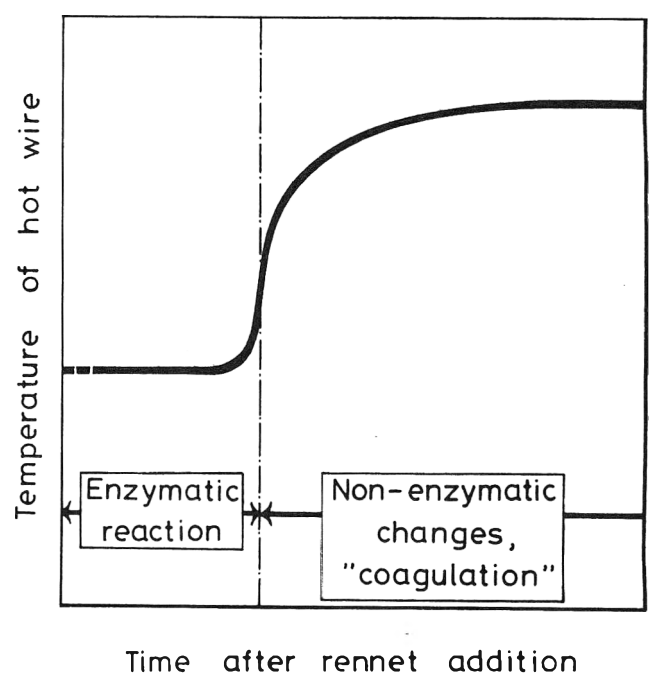


Fig. 2—Illustrative relationship between temperature of the hot wire and time after rennet addition during rennet treatment of milks.

coagulum reached the empirical optimum firmness for cutting at t_r = 60 min (Fig. 3, #4).

Effect of cutting time for curd on the yield and quality of cheese

The yield of solids content in cheese decreased with decreasing cutting time. The curdmaking in the smaller vats (130L) for three different cutting times (Δt = 15.7, 31.0, and 60.0 min for t_c = 19.34, 19.00, and 20.00 min, respectively; Fig. 4) led to different yields of solids content in cheese (the yield is the % content of whole milk). Although the yield values of fat and protein content in curds 45 min after cutting were independent of Δt, these values in cheese for Δt = 15.7 min decreased by 6–7% as compared to those for Δt = 31.0 and 60.0 min. That is, higher yield for solids content in cheese was obtained with longer cutting time. In addition, the yield of cheese, taking account of changes in moisture of cheese, increased with increasing Δt (Fig. 5).

The quality of cheese, on the contrary, decreased with

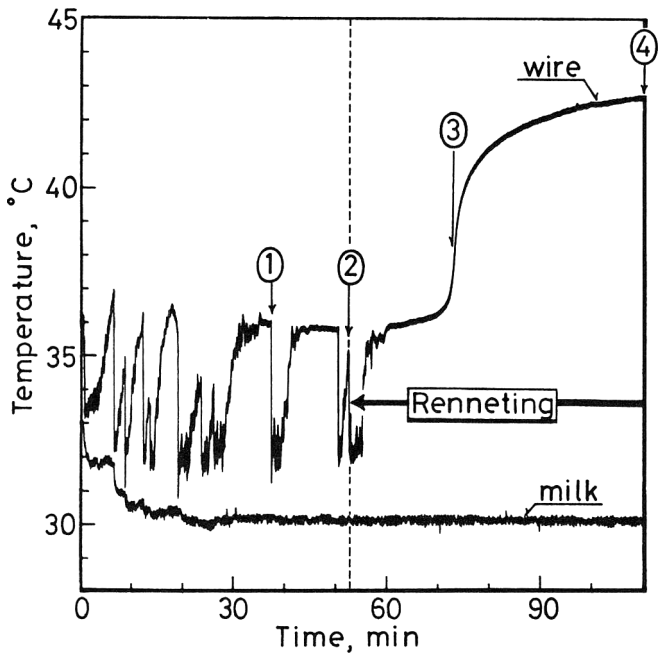


Fig. 3—Temperature measurements of the hot wire during the process of curd formation of whole milk (volume, 660L; C_r = 5,000 units/kg-milk) at 30°C in the actual cheesemaking: #1, starter culture addition; #2, rennet addition; #3, clotting time; #4, empirical required firmness for cutting curds.

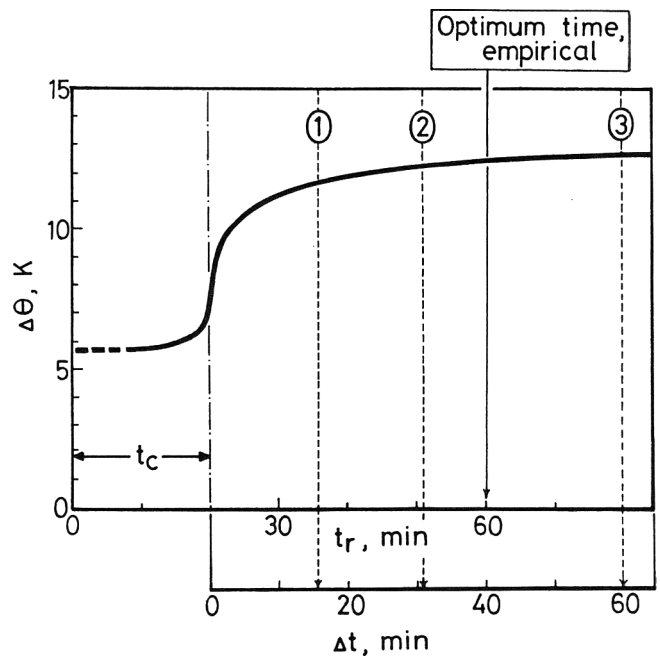


Fig. 4—Relationship between the increasing temperature of the hot wire referred to initial temperature during renneting of whole milk (volume, 660L; C_r = 5,000 units/kg-milk) at 30°C and the times for cutting curds (#1, 2, 3) to evaluate the effect of Δt on the yields and quality of cheese.

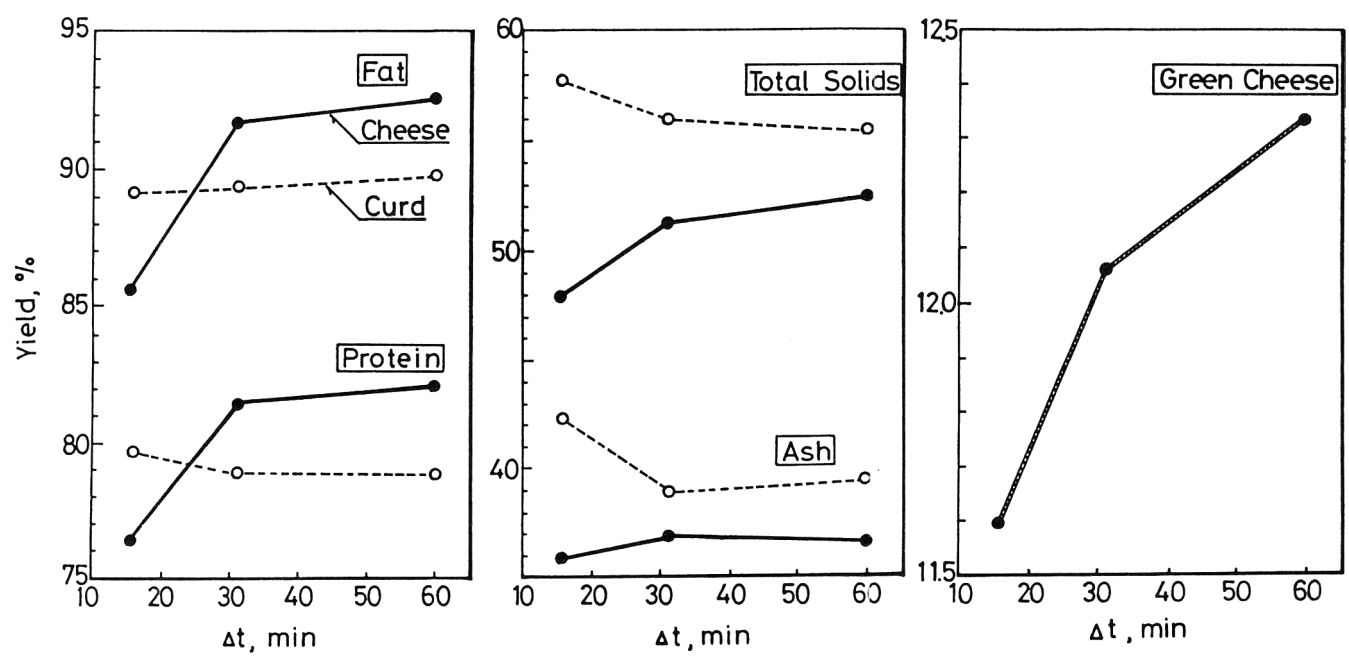


Fig. 5—Effect of cutting time for curds on the yields of green cheese and solids content in curds during cooking (at 45 min after cutting) and in green cheese.

the increasing cutting time. The rating for flavor, springness, mouthfeel, and acceptability significantly decreased with the increasing Δt, while hardness increased with the increasing Δt (Fig. 6).

The objective measurements by the present method determine the proper time at which the milk curd should be cut. The curd would be ready if the time referred to the hot wire coagulation time reached about 30 min, because the acceptability of ripened cheese samples significantly decreased for $\Delta t > 30$ min (Fig. 6) despite the small increase of the yields of fat and protein content in green cheese (Fig. 5).

No knowledge of the general composition of the cheese milk was required first so that the hot wire could be adjusted before starting. The higher the viscosity of the milk increased, the higher the initial temperature in the linear portion of the curve (Fig. 2). Initial readings of θ_w were increased by up to one degree by build-up of milk stone or calcium deposits on the wire used in this study. The increase was measurable but its effect was minimal, because the relative, not absolute, temperature behavior of the wire determiend the optimum time for cutting curd.

Effect of rennet concentration, pH and temperature on milk clotting time

The effect of rennet concentration on milk clotting time decreased with increasing acidity. The hot wire measurement showed that t_c of skim milk sample decreased exponentially with increasing C_r, C_g and θ (Fig. 7). In addition, the interaction between C_r and C_g was significant (Table 1); the effect of C_r on t_c was maximum at lowest acidity (C_g = 0% wb) of milk sample and minimum at highest acidity (C_g = 0.20% wb).

Skim milk coagulation monitored by the present method led to the quantitative relationship between t_c, pH, C_r and θ. This coagulation for t_c ranging from 2.8–209.0 min was measured under 60 conditions (C_r = 5,400–595,000 units/kg-milk, pH = 5.53–6.59, θ = 30.0–40.0°C). The following regression formula suggested that pH of milk would be the fundamental factor which must be strictly controlled in automation of curdmaking (Fig. 8):

$$\log t_c = 0.0776 (\log C_r)^2 - 0.446 \log C_r + 1.29 (pH)^2 - 14.3\ pH - 0.00410\ \theta^2 + 0.414\ \theta - 0.0272\ \theta \times \log C_r + 35.3 \quad (2)$$

where multiple correlation coefficient was 0.962 for n = 60.

Theoretical considerations

The θ_w measurements by the present method characterized the heat transfer behavior at the surface of the electrically heated wire. In the steady-state, Fourier's equation for heat conduction in the radial direction in an infinite cylinder immersed axially in the liquid,

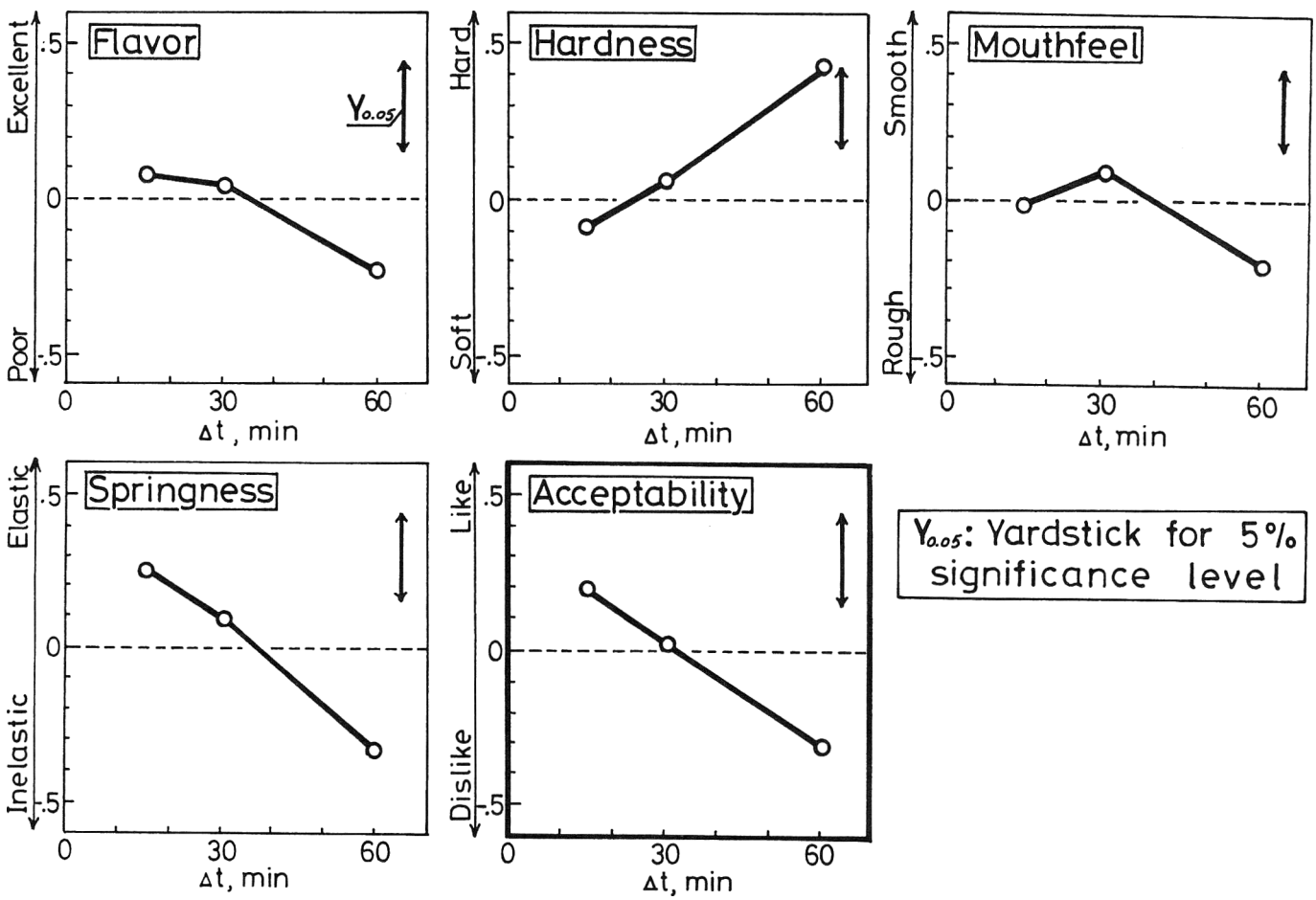


Fig. 6—Effect of cutting time for curds on the quality of St. Paulin cheese ripened for 12 wk at 11°C. The Y axis for each sensory profile indicates the difference between pairs. If the absolute value of the difference between the population means is larger than the yardstick or $Y_{0.05}$, the difference is significant.

$$\frac{\partial T}{\partial t} = a\left(\nabla^2 T + \frac{1}{\lambda} W\right) \quad (3)$$

$$\nabla^2 = \frac{\partial^2}{\partial r^2} + \frac{1}{r}\frac{\partial}{\partial r} \quad (4)$$

resulted in the following formula (Grigull, 1961):

$$\alpha = \frac{W \cdot d}{4\Delta\theta} \quad (5)$$

Thus, the decreasing α value at the surface of the hot wire led to the increasing θ_w during the progress of curd formation (Fig. 3); i.e., the heat transfer with the free convection current around the wire varied with the coagulation. The rapid decrease of θ_w with time in the θ_w vs time curve resulted from the stirring of milk for cooling and mixing; the rapid increase of α value with forced convection current resulted in the sudden decrease of θ_w.

In particular, the accurate determination of α value by the present method described the heat transfer around the hot wire. The precise $\Delta\theta$ measurements for the distilled water (4.4-51.9°C for Pr = 3.11-10.47) showed that the heat transfer around the wire in the steady-state was characterized by the significant relationship between Nusselt number and Grashof number as follows (Fig. 9, coefficient of correlation between log Nu and log Gr = 0.998 for n = 18):

$$Nu = 121\ Gr^{0.221} \quad (6)$$

where

$$Nu = \alpha d/\lambda \quad (7)$$

$$Gr = d^3 g\beta\Delta\theta/\nu^2 \quad (8)$$

To determine the values of Nu and Gr for the water sample, the g and β values of the water were postulated to be constant in the temperature range, 4.4-51.9°C, while the ν and λ values for the water were calculated by the following regression formulae:

$$\nu = (1.7907 - 6.0037 \times 10^{-2}\,\theta + 1.3427 \times 10^{-3}\,\theta^2 - 1.7548 \times 10^{-5}\,\theta^3 + 9.5963 \times 10^{-8}\,\theta^4) \times 10^{-6},\ m^2/s \quad (9)$$

for $0°C < \theta < 60°C$, which was obtained by the present author on the basis of the recommended ν values (Tokyo Astronomical Observatory, 1980), and

$$\lambda = 0.5617 + 2.005 \times 10^{-3}\,\theta - 8.49 \times 10^{-6}\,\theta^2,\ W/(m \cdot K) \quad (10)$$

Table 1—Analysis of variance for milk clotting time of skim milk (solids, 10% wb)

Factor	d.f.	V	F	
C_r	2	19510.	440.818	***
C_g	3	14580.	329.500	***
θ	2	980.7	22.158	***
$C_r \times C_g$	6	4352.	98.325	***
$C_r \times \theta$	4	43.02	0.972	
$C_g \times \theta$	6	18.52	0.418	
Error	12	44.26		
Total	35			

*** significant (0.1%)

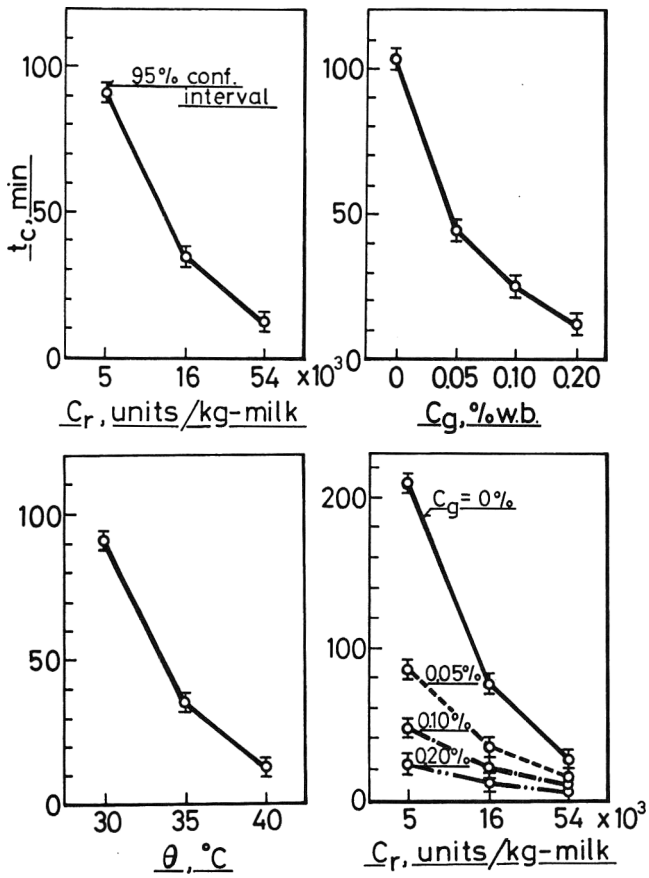


Fig. 7—Effect of rennet concentration, Glucono-δ-Lactone concentration and temperature on milk clotting time (skim milk; solids, 10% wb) measured by the hot wire method.

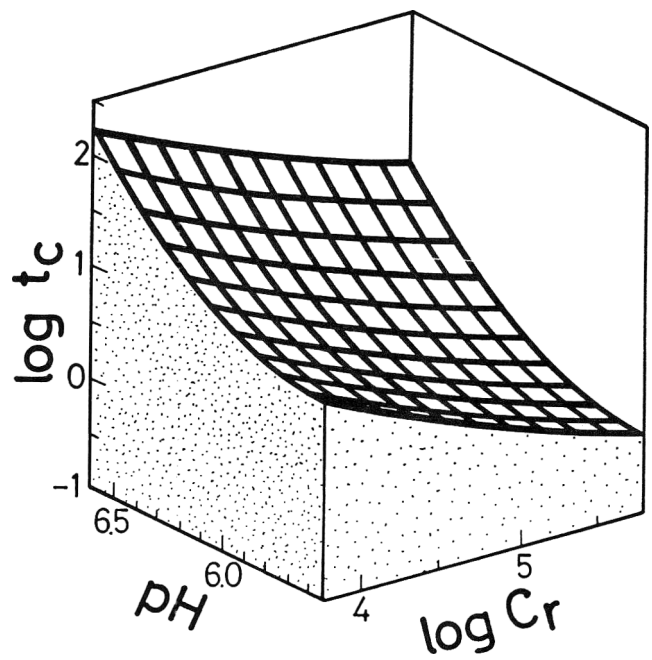


Fig. 8—Relationship between rennet concentration, pH and milk clotting time (skim milk; solids, 10% wb; 30°C) measured by the hot wire method.

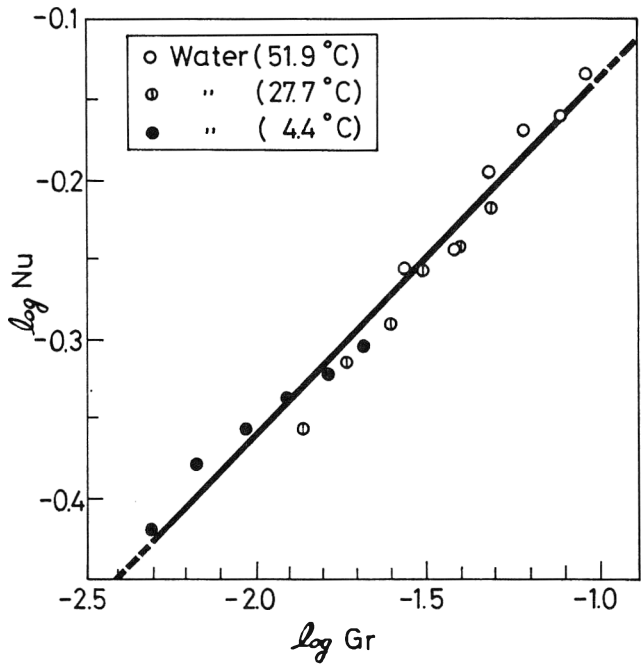


Fig. 9—Relationship between Nusselt number and Grashof number, describing the heat transfer at the surface of the hot wire (platinum, 0.1 mmΦ x 106 mm) immersed axially in the distilled water (4.4-51.9°C).

for $0°C < \theta < 100°C$ (Nagasaka and Nagashima, 1980). Eq. (6) is applicable to the milk coagulation system, because the Pr values for milk samples are postulated to be equal those for the distilled water at a given temperature.

The theory of heat transfer for free convecton current showed the reliability and acceptability of Eq. (6) for scientific use. Nusselt (1915) proved that the heat transfer in the free convection current system was described by the formula:

$$Nu = f(Gr, Pr) \quad (11)$$

At sufficiently small Reynolds number of the system, Eq. (11) is rewritten as follows (Lorentz, 1881):

$$Nu = f(Gr \cdot Pr) \quad (12)$$

Eq. (6) indicated that Eq. (12) can be rewritten by:

$$Nu = c_1 f(Gr) \quad (13)$$

for Pr = 3.11–10.47.

Thus, the $\Delta\theta$ measurements directly resulted in the kinematic viscosity of the milk samples during rennet treatment. From Eq. (5) and (6), the ν value was determined by:

$$\nu_f = 35.4 (g\beta)^{0.5} d^{-3.03} (\lambda_f/W)^{2.26} \Delta\theta^{2.76} \quad (14)$$

where the subscript, f, means that the values are taken at $\theta = \theta_w - \Delta\theta/2$. In addition to the normally constant g and β values, the λ value of milk samples in Eq. (14) was postulated to be constant during rennet treatment (Hori, 1983). Therefore, when constant heat generation was made in the wire, Eq. (14) can be rewritten by:

$$\nu_f = c_2 \Delta\theta^{2.76} \quad (15)$$

where c_2 is related to the sample and to the sensoring wire used.

Moreover, Eq. (15) indicated that the $\Delta\theta$ measurements characterized the process of curd formation by the following formula:

$$\nu^* = (\Delta\theta/\Delta\theta_o)^{2.76} \quad (16)$$

The $\Delta\theta$ measurements by the present method characterized the kinematic viscosity behavior of the coagulating milks: e.g., the dimensionless ν^* value increased by approximately nine times as compared to the initial ν^* value, when the curd of whole milk sample reached the empirical required firmness. The ν^* value for the skim milk samples, on the other hand, increased by approximately five times (C_r = 40,000 units/kg-milk) or by approximately 6.5 times (C_r = 200,000 units/kg-milk) (Fig. 10); neither curds of skim milk sample reached the required firmness for cutting.

In general, the ν^* vs time curve characterized the coagulation "rate" behavior of the casein micelles in the renneted milks. Overbeek (1949) proved that the collision frequency of the micelles ($k_d = 4kTN/3\nu\rho$) equals a von Smoluchowski rate constant for coagulation (k_s) (von Smoluchowski, 1918) if the reaction is a fast coagulation, having no energy barrier to the coagulation of colliding particles. Dalgleish (1983) showed that the ratio of the experimental k_s values to the calculated k_d values was postulated to be constant at a given temperature, although the direct measurement of these k_s values from the turbidity measurements proved that the coagulation reaction had a significant activation energy. Thus, the θ_w measurements which lead to the ν value can characterize the rate of coagulating casein micelles, providing an excellent contribution to the existing scientific literature on milk coagulation.

CONCLUSIONS

THE NONDESTRUCTIVE, continuous and objective measurements by the hot wire method adequately monitored the whole process of milk curd formation in cheesemaking; this indicated the practical potential of the present method and use to the cheese industry.

NOMENCLATURE

θ	Temperature, °C
T	Temperature, K
θ_w	Temperature of the wire, °C
$\Delta\theta$	Temperature of the wire referred to the initial temperature, K or °C
$\Delta\theta_o$	Initial value of $\Delta\theta$, K or °C
R	Electric resistance of the wire, Ω
R_o	R at 0°C, Ω
α_w	Coefficient for R varying with temperature, 1/K
t	Time, sec
t_r	Time after rennet addition, min
t_c	Milk clotting time or t_r at which milk begins to clot, min
Δt	Time for cutting curds referred to t_c, min
a	Thermal diffusivity, m^2/s
λ	Thermal conductivity, W/(m·K)
λ_f	λ at $\theta = \theta_w - \Delta\theta/2$, W/(m·K)
W	Heat generated in the wire, J/m^3
α	Heat-transfer coefficient, $W/(m^2 \cdot K)$
r	Distance from the center of the wire in the radial direction, m
d	Diameter of the wire, m
g	Gravitational acceleration, m/s^2
β	Coefficient of volumetric expansion, 1/K
ν	Kinematic viscosity, m^2/s
ν_f	ν at $\theta = \theta_w - \Delta\theta/2$, m^2/s
ν^*	Relative kinematic viscosity, –
Nu	Nusselt number, –

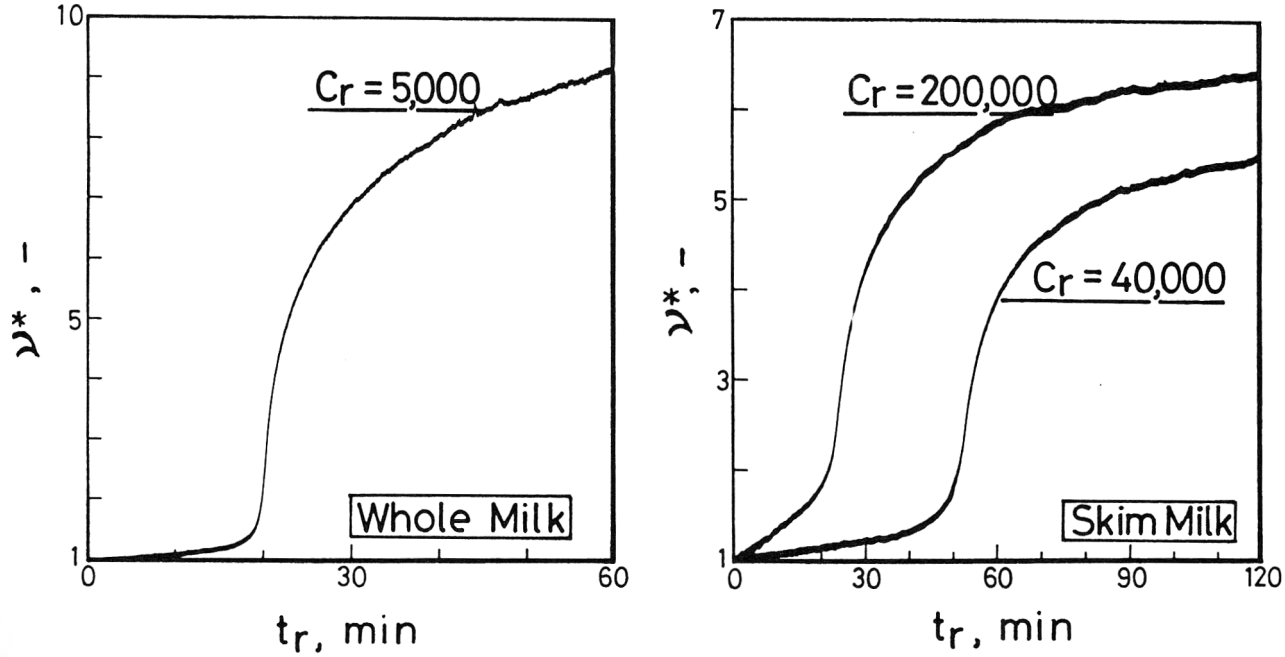


Fig. 10—Development of relative kinematic viscosity during the rennet treatment of milks with Cr = 5,000, 40,000 and 200,000 units/kg-milk.

Gr	Grashof number, –
Pr	Prandlt number, –
C_r	Rennet concentration, units/kg-milk
C_g	Glucono-δ-Lactone concentration, % wb
k_d	Collision frequency of the micelles, $m^3/(s \cdot mol)$
k_s	von Smoluchowski rate constant for coagulation, $m^3/(s \cdot mol)$
k	Boltzmann constant, J/K
N	Avogadro's number, 1/mol
ρ	Density, kg/m^3
c_1	Numerical constant in Eq. (13), –
c_2	Numerical constant in Eq. (15), $m^2/(s \cdot K \cdot A)$
n	Number of measurements, –
db	Dry basis, –
wb	Wet basis, –

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Characterization and Bioconversion of Great Northern Bean Blancher Effluent

B. N. MUGGIO, M. A. UEBERSAX, G. L. HOSFIELD, and S. S. THOMPSON

ABSTRACT

Chemical characterization of blanching effluent and bioconversion by *Hansenula anamola* was performed. The effluent contained 27,000 mg/L BOD and 31,000 mg/L total solids including 49.5% starch and 23.6% protein (dry basis). A high biochemical oxygen demand (BOD): chemical oxygen demand (COD) ratio (0.95) indicated a waste easily and completely degradable, with nitrogen and phosphorus levels above those necessary for unimpeded microbial growth. Batch fermentations of raw effluent produced 12g dry cells/L, containing 53% protein, with a corresponding 84% reduction in BOD. Great Northern bean blanching effluent was demonstrated to be a suitable growth medium for *H. anomala*.

INTRODUCTION

MOST CANNERY WASTES contain more than ten times the biochemical oxygen demand (BOD) found in domestic sewage (Weckel, 1967). Fruit and vegetable processing, especially blanching and cooling operations, results in a large amount of wastewater (Bomben, 1977). Analysis of individual unit operations in canning indicated that blanching is a major source of effluents, resulting in an average of 40% of cannery wastes (Lund, 1974; Ralls and Mercer, 1973). Studies by Olson et al. (1974) have shown that dry bean processing results in the largest amount of wastewater and the highest BOD per ton of raw product among twenty-six fruit and vegetable processes investigated. Detroy and Hesseltine (1978) also observed thirteen fruit and vegetable processing operations and found legume waste loads among the highest for BOD and total solids per ton. Legume cooking water has been found to contain measurable amounts of minerals, with relatively high amounts of magnesium, phosphorus and potassium (Meiners et al., 1976a). Hang and Woodams (1979) reported high BOD in baked bean processing wastewater and noted that municipal activated sludge treatment produced foul odors when this waste was treated.

Food processing wastewater can be characterized as being readily biodegradable, nontoxic and of relatively high organic concentration (Eckenfelder, 1976). Traditional substrates for growth of microbial biomass or single cell protein (SCP) have been carbohydrates, either sugars or starchy materials readily degraded to sugars (Reed, 1981). During the past decade substrates and organisms have been investigated, such as: cheese whey (*Aeromonas hydrophilia*), tapioca starch (*Candida utilis*), corn and pea wastes (*Trichoderma viride*), brewery wastes (*Calvatia gigantia*) and sauerkraut brine (*Candida utilis*) (Butany and Ingledew, 1973; Thanh and Wu, 1975; Church et al., 1973; Shannon, 1973; Kyung, 1978). Many of these studies, aimed at reduction of BOD in effluents rather than production of biomass, resulted in reduced BOD in the range 50 - 90% and production of biomass, 35 - 83% protein.

Objectives of this study included calculation of a solid mass balance for a commercial bean blanching operation, chemical analysis of the blancher effluent, use of wastewater composition information to assess the biological treatability of the waste and selection of a suitable microorganism for reduction of BOD and production of microbial protein.

Author Muggio is with Gordon Food Service, Grand Rapids, MI 49501. Author Uebersax is with the Dept. of Food Science & Human Nutrition, and Author Thompson is with the Dept. of Microbiology and Public Health, Michigan State Univ., East Lansing, MI 48824. Author Hosfield is affiliated with the USDA/Dept. of Crop & Soil Sciences, Michigan State Univ., East Lansing, MI 48824.

MATERIALS & METHODS

Wastewater samples and material balance

Wastewater samples for all analyses and fermentations were obtained from a commercial dry bean processor and stored at −20°C until needed. Effluent from three consecutive blanchings of Great Northern beans (*Phaseolus vulgaris* L.) was collected along with dry and blanched bean samples. Dry beans were prepared with an initial 23 min hot water and steam blanch/cook cycle, followed by direct water cooling. These components were analyzed for moisture (AOAC, 1970) to provide data for calculation of a material or mass balance.

Wastewater analysis

All wastewater analyses except mineral determinations were performed in triplicate on each of the runs. Total solids and ash were determined using methods 224A and 224B of *The Standard Methods for the Examination of Water and Wastewater* (APHA, 1971). Measurement of organic nitrogen as an indicator of crude protein was done using AACC method 46-13 (AACC, 1962). BOD determinations were made using Standard Methods (APHA, 1971) and a Beckman Model 0206 Oxygen Analyzer (Beckman Instruments, Inc., Irvine, CA). Chemical oxygen demand (COD) determinations were done according to Section 220 of the Standard Methods (APHA, 1971). Measurement of pH was with an Orion Model 901 Microprocessor Ionalyzer (Orion Reserach, Cambridge, MA).

Glucose, sucrose, m-inositol, raffinose and stachyose were analyzed using high-pressure liquid chromatography (HPLC) (Agbo, 1982). Starch analysis (Agbo, 1982) utilized the precipitate remaining after the third ethanol extraction for sugars. This method required enzymatic hydrolysis and glucose quantitation by the YSI Model 27 Industrial Analyzer (Yellow Springs Instrument Co., Inc., Yellow Springs, OH).

Mineral analysis

Eleven minerals were analyzed by direct-coupled plasma emission spectroscopy: potassium, phosphorus, magnesium, calcium, zinc, iron, aluminum, copper, manganese, molybdenum and boron. Sample preparation was based on methodology by Jones and Warner (1969) and Jones and Steyn (1973), utilizing a Spectraspan IIIA Emission Spectrophotometer (Spectrometrics, Inc., Andover, MA).

Fermentations

All fermentations were carried out in a 7-L capacity bench-top Microferm Fermentor (New Brunswick Scientific Co., Inc., Edison, NJ), which included automatic temperature, aeration and agitation control. Fermentations were done as batch operations and employed the following parameters: 30°C, 1L air/L wastewater/min, and 800 rpm agitation. Analyses of BOD, COD and pH were performed on each 3L batch prior to sterilization by autoclaving in the fermentation vessel at 121°C for 15 min. A 10% inoculum of a 30-hr culture of *H. anomala* (Dept. Food Science, Michigan, State U.) in acidified malt extract broth (Dehydrated Bacto-Malt Extract,

Difco B186) was added after the medium was cooled to 30°C. Samples of the fermentation medium were asceptically removed every 3 hr during the 36 hr fermentation period for COD and pH determination. *H. anomala* was enumerated by plating in duplicate on Acidified Potato Dextrose Agar (Difco B13). Colonies on representative plates were enumerated after 5 day incubation at 25°C.

Yeast cells were harvested from the fermentation broth by centrifugation at 1300 x *g* for 10 min. Cells were washed with distilled deionized water and re-centrifuged twice following the initial centrifugation. Yeast dry weight (total solids) was determined on washed cells according to AOAC method 10.173 (AOAC, 1970). Protein analysis on the dried yeast was performed using AACC method 43-16 (AACC, 1962).

RESULTS & DISCUSSION

Material balance

Material balances are useful in comparing blancher performance with theoretical values for the mapping of nutrient loss and waste generation (Bomben et al., 1975; Bomben, 1977). Water and solids balance (Fig. 1) for the blanching operation illustrates water added during the 23 min steam blanching which hydrated and softened the beans. Generally, this process required 2700 kg water to prepare 800 kg of dry beans. This operation resulted in 2400 kg of waste effluent per batch. No wastewater was generated during the hydration period but high temperatures and rapid swelling of beans caused splitting of the seed coats. Following blanching, cooling water entered into the bottom of the blancher tank and flowed up through the beans and out of the blancher as a hot, translucent yellow waste stream.

Wastewater analysis

Composite results of chemical analyses of three effluents are presented in Table 1. Triplicate measurements on three blanch runs were used to characterize the typical waste and nutrient profile produced from this operation. Values in Table 1 for BOD, N, total solids, and ash were within ranges published for baked bean processing wastewater by Hang and Woodams (1979). Protein (N x 6.25) was similar to that found in legume cooking water by Meiners et al. (1976b). Carbohydrates accounted for greater than half of all total solids in the effluent. Although ash was similar to that of Hang and Woodams (1979), phosphorus in the current study was greater. However, even higher phosphorus and potassium were reported by Meiners et al. (1975a). Over half (62%) of the ash was potassium and the four "macrominerals", potassium, phosphorus, magnesium and calcium together accounted for 95.8% of the total.

Fermentation

Results of wastewater characterization indicated a favorable potential as a fermentation substrate. Several investigators have identified nutritional requirements for biolog-

Table 1—Average composition of three effluents obtained from the Great Northern bean blanching and cooling operation, reported as mg/L (except pH)

Composite characteristics[a]	Mean ± 1 s.d.	Range	Coefficient variability
	mg/L		
BOD	27413 ± 489	1500	1.8
COD	28976 ± 233	804	0.8
Total solids	31000 ± 899	2286	2.9
Nitrogen	1169 ± 25	75	2.2
Carbohydrates:			
Starch	15337 ± 261	806	1.7
Glucose	704 ± 145	453	20.6
Sucrose	106 ± 13	44	11.9
m-Inositol	470 ± 11	34	2.3
Raffinose	46 ± 3.8	14	8.3
Stachyose	541 ± 48	160	8.8
Ash	5799 ± 165	554	2.8
Minerals:			
Potassium	3700 ± 112	335	3.0
Phosphorus	1155 ± 58	116	5.0
Magnesium	472 ± 24	70	5.1
Calcium	240 ± 17	54	7.3
Zinc	42.3 ± 3.4	11.8	8.1
Iron	25.5 ± 1.6	5.1	6.3
Aluminum	11.0 ± 1.0	3.1	8.8
Copper	5.2 ± 0.3	1.0	6.1
Manganese	4.6 ± 0.4	1.2	7.6
Molybdenum	0.2 ± 0.01	0.05	4.4
Boron	0.1 ± 0.02	0.05	16.7
pH	6.98 ± 0.03	0.11	0.4

[a] Determinations made on effluent from three consecutive blanch cycles. Triplicate analyses were made on all characteristics except minerals, duplicate.

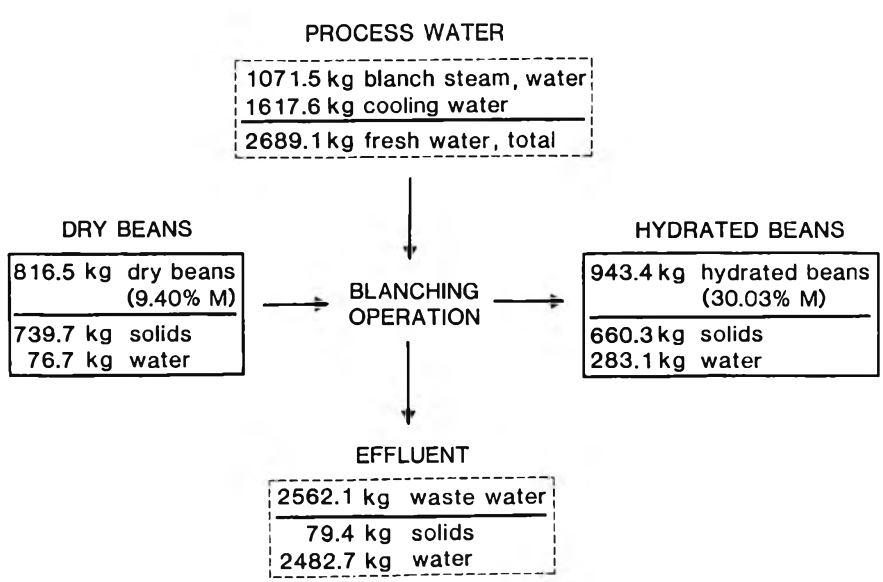


Fig. 1—Typical moisture-solids balance for Great Northern beans, prepared with an initial 23 min hot water and steam blanch/cook cycle, followed by direct water cooling. Dry and hydrated beans, process water and effluent are expressed as weight per blanch cycle (bean moisture, %M).

ical treatment of wastewaters (Helmers et al., 1952; Symons et al., 1960; Sawyer, 1956). Great Northern bean blancher effluent dramatically exceeded these general requirements for growth (Table 2).

H. anomala was chosen for this study based on a comparison of this organism's biochemical abilities with the nutrient profile of the wastewater substrate. *H. anomala* assimilates glucose, sucrose, raffinose, and starch aerobically (Lodder, 1970) and is capable of vigorous growth on vitamin-free media, synthesizing all necessary vitamins (Phaff et al., 1978).

Fig. 2 shows changes in COD, cell number and pH vs time for the same batches. A typical microbial growth curve consisting of lag, logarithmic growth and death phases is observed in Fig. 2. Removal of COD from the medium paralleled the growth of the cells. A rapid drop in pH to 6.3 was noted after 5 hr. Stable pH was maintained until 18 hr after which pH steadily increased, reaching 7.36 in 36 hr. Fermentation and harvest of cells yielded a reduction of 84.3% of the BOD (79.7% of COD) in the wastewater. The resulting effluent had a BOD of 4.25 g/L and a pH near neutrality thus indicating that untreated Great Northern bean blancher effluent was a good medium for growth of *H. anomala* and met criteria for industrial fermentation stock as expressed by Weinshank and Garver (1967); a good medium for industrial fermentations consists of an inexpensive carbon and energy source, an inexpensive nitrogen source and sufficient biological adjuncts to meet specific nutritional requirements of the organism in question.

Data showing changes in substrate BOD, COD and pH, as well as cell yield and protein content of *H. anomala* biomass harvested during three 36-hr batch fermentations of whole, untreated wastewater are presented in Table 3. Cell yields were 0.42 g cells/g substrate, falling within the range reported in the literature: 1.07 g cells/g substrate for *Pseudomonas* sp. grown on n-paraffins (Wodzinski and Johnson, 1968) to 0.37 g cells/g substrate for *Hansenula polymorpha* grown on methanol (Levine and Cooney, 1973). Protein (Kjeldahl N x 6.25) was 53.4%, similar to literature yeast values (Cooney et al., 1980; Kihlberg, 1972). A considerable fraction of the nitrogen in microbial biomass, however, is non-protein nitrogen, a fact generally recognized by experts in the field but often overlooked in practice (Reed, 1981). Yeast cells contain 6% to 12% nucleic acids (Kihlberg, 1972), as well as lesser amounts of choline, glucosamine and other nitrogen-containing non-protein constituents. According to Abdul-Hafez et al. (1977), *H. anomala* contains 1.78% total nonprotein nitrogen, thus application of the 6.25 factor may lead to over-estimates of protein by 10% to 20%.

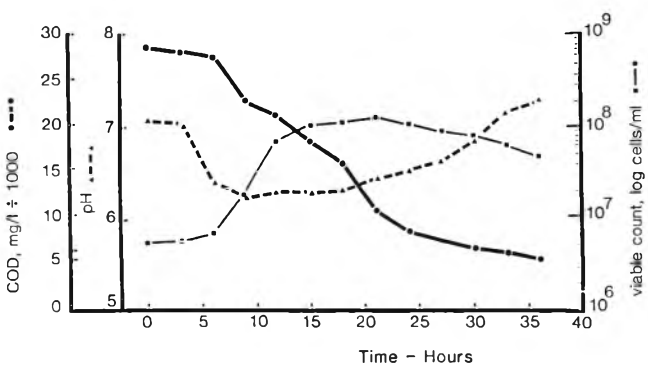


Fig. 2—Changes in cell count, COD and pH during 36 hr growth period of H. anomala in effluent obtained from a Great Northern bean blanching and cooling operation.

Table 2—Comparison of literature values for nutritional requirements for biological treatability of wastes with values provided by Great Northern bean blanching and cooling effluent

Nutritional Requirements	Required values from literature	Provided Values in Effluent
BOD:N:P (Sawyer, 1956)	100:3.1:0.67	100:4.3:4.2
BOD:N (Helmers et al. 1952)	25 – 33:1	23.4:1
BOD:P	143 – 200:1	23.7:1
BOD:COD (Symons et al. 1960)	0.6:1	0.95:1
COD:N	20 :1	24.8:1
COD:P	100 :1	25.1:1

Growth in pretreated wastes

Three additional series of fermentations were performed to evaluate filtration, nitrogen supplementation and pH control of the effluent (data not shown). Temperature, aeration and agitation were maintained the same as for whole wastewater for these triplicate experiments. Filtration through Whatman filter paper No. 1 prior to fermentation removed 23.2% of the BOD (25.8% of COD), and growth of *H. anomala* in this medium resulted in an overall reduction in BOD of 93.2% (COD, 92.5%). Nitrogen supplementation was accomplished by addition of 0.1% ammonium sulfate (w/v) to the wastewater, increasing the N by 18.1%. Reductions of BOD and COD in this waste were 85.3% and 90.7%, respectively, while cells harvested from these runs contained 8.93% Kjeldahl N, greatest of all trials. This is similar to reports by Thanh and Wu (1975) that addition of N, while not appreciably increasing cell yield, resulted in cells containing 39.4% more protein. In water receiving pH control, fermentations were run for 48 hr. Fermentation of wastewater acidified to pH 6.5 and buffered with 0.1M phosphate buffer resulted in BOD and COD reductions of 97.0% and 92.8%, respectively (93.0% and 90.7% at 36 hr). Cell yield (14.84 g/L) was greatest for this treatment.

SUMMARY

GREAT NORTHERN BEAN blanching effluent was a good substrate for bioconversion by *H. anomala.* This waste contained adequate nitrogen and phosphorus for microbial growth, reducing waste BOD and producing a high-protein biomass. Chemical analysis of whole wastewater in this study indicated that unsupplemented waste could serve as a suitable substrate.

Table 3—Results of 36 hr batch fermentation of Great Northern bean blanching and cooling effluent by H. anomala

Measurement		
Effluent:		
BOD:	initial, mg/L	27055
	final, mg/L	4245
	reduction, %	84.3
COD:	initial, mg/L	28596
	final, mg/L	5816
	reduction, %	79.7
pH:	initial	7.08
	final	7.36
Harvested cells:		
	yield, g dry cells/L	12.64
	N, % (Kjeldahl)	8.54
	protein, % (%N x 6.25)	53.4

n = 9.

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Physicochemical Differences Between Milled Whole Kernel Rice and Milled Broken Rice

A. PROCTOR and D. E. GOODMAN

ABSTRACT

The purpose of this study was to demonstrate the existence of distinct differences between selected physicochemical properties of head and broken milled rice. Polyuronic acids were found to affect hardness of the rice kernels. The broken kernels were measurably softer than intact kernels for all cultivars. Values for alkali spreading and amylographic pasting properties indicated that broken kernels were more porous, and exhibited lower peak and setback viscosities than intact kernels. This suggests differences in the relative amounts of amylose and amylopectin in head rice and brokens. A protein-phytin complex may be more prominent in broken kernels than head rice.

INTRODUCTION

RICE is unique among cereals in that it is primarily consumed as the whole grain following the removal of the hull and, usually, of the bran and germ. The processing steps employed in dehulling and bran removal are abrasive, and grain breakage often occurs. The breakage of whole kernel rice lowers its quality, its market value (Spadaro et al., 1980), and the amount of rice available for direct human consumption. The importance of increasing rice milling yields is a worldwide problem, particularly in developing countries where agronomic yields are much lower than those in developed countries (Adair et al., 1966). The many factors which influence the breakage of rice during milling are summarized by Spadaro et al. (1980).

Much work has attempted to correlate various physicochemical properties of the rice kernel with whole kernel milled rice yields (head rice yields). Goodman and Rao (1983) reported head yields to be weakly but significantly correlated to hardness ($r = +0.22^{*}$), alkali spreading ($r = +0.30^{**}$), amylose ($r = -0.48^{**}$), and length-width ratio ($r = -0.38^{**}$). They also noted correlations between hardness and area-volume ratio of the kernel ($r = +0.38^{**}$), moisture of the rough rice (i.e., prior to milling) and hardness ($r = -0.31^{**}$), and between alkali spreading and amylose ($r = -0.64^{**}$). Chang and Somrith (1979) state that hardness and absence of chalky kernels are associated with high rice milling recoveries. Kernel hardness or apparent hardness has been routinely measured by several workers in conjunction with rice quality evaluation (Goodman and Rao, 1983; Vidal and Juliano, 1967). Zoerb and Hall (1960) reported that moisture content had the greatest influence on the strength properties of grains. Juliano (1970) found that kernel hardness of rice was significantly correlated with protein content. Kongseree and Juliano (1972) found a significant correlation between alkali spreading value and gelatinization temperature ($r = -0.96^{**}$). From this they postulated that the alkali spreading value reflected in a positive fashion the relative porosity of the rice endosperm to various agents such as potassium hydroxide. That is, as the alkali spreading value increases, so does the kernel porosity. Lastly, polyuronic acid has been reported in the cell wall material of the rice kernel (Pascual and Juliano, 1983), but, no direct correlation with endosperm hardness in rice has been reported.

To better understand the cause of breakage of rice kernels, we studied the physicochemical properties of the milled head rice fraction and of the broken milled rice fraction. Intuitively, differences in properties must exist between these two rice types or fractions that cause breakage occurring during processing. Differences found in yields have been reported for different grain-types, i.e. short, medium, long, (Webb, 1980). Also, yields for specific lots of rice held at similar conditions are reproducible. Thus, breakage is probably neither a random nor a stochastic event. A major objective of this study was to demonstrate physicochemical differences between head rice and broken kernels. This work also attempted to establish a suite of parameters indicative of rice kernel hardness.

MATERIALS & METHODS

Rice varieties

The rice samples used for this study were taken from the 1982 crop at the University of Arkansas Rice Experiment Station. Of the five cultivars, three were medium grain varieties (Mars, Chinese Hybrid #4, and Chinese Hybrid #5), and two were long grain varieties (Lebonnet and Starbonnet). Mars, Lebonnet, and Starbonnet are varieties of established commercial importance in both Arkansas and the United States; the hybrids were included because of their high agronomic yields.

Shelling and milling

Duplicate samples of each cultivar were dehulled and milled in accordance with standard USDA procedures (USDA, 1976). A McGill sheller and a McGill No. 2 mill were used. All samples were well milled, removing between 10 and 12% of the weight of the brown rice, leaving no visible evidence of bran left on the kernels. The moisture of the rough rice was determined with a Motomco model 919 moisture tester.

Head rice yields were determined by separating the broken fractions with a rotating Satake rice size grader, Model TRG. The head rice yield was calculated as a percent of the weight of the rough rice.

Sample preparation

For analyses requiring a flour, rice kernels were ground with a Udy mill with a 1.0 mm screen. The resulting flour was approximately 75 mesh.

Physical tests

The hardness of 100 head rice kernels from each variety was measured using the Instron Universal Testing Instrument model 1132. The instrument was equipped with a 100 kg compression cell. Hardness was defined as the peak force during the first compression cycle (Bourne, 1978). In determining hardness values for the broken fractions, only sound second heads were selected.

Amylographic pasting viscosities were determined according to the method of Webb (1981), with a Brabender Visco/amylo/graph model VA-1B. The samples were ground to a flour as described above. In each case, a 50g aliquot was blended with 300g distilled, deionized water in a Waring Blendor for 45 sec, and the resulting slurry was washed into the amylograph cup with an additional 150g

Author Proctor is a graduate assistant in the Dept. of Food Science, Univ. of Arkansas, Rt. 11, Fayetteville, AR 72701. Author Goodman is presently employed with The Kellogg Co., 966 N.F. St., P.O. Box 3866, Omaha, NE 68103.

Table 1—Physical properties of the rice varieties[a]

Rice type	Variety	Rough[a] rice moisture (%)	Head[b] rice yield (%)	Instron[c] hardness (kg)	Alkali[b] spreading value	Amylographic Pasting Properties (B.U.)[d,e] peak viscosity	setback viscosity
Head	Starbonnet	11.22	52.20a	7.59ab	2.10d	655d	1120f
Head	Lebonnet	9.98	52.56a	8.35a	2.05d	565g	1195d
Head	Mars	9.73	56.60a	6.71ab	3.35c	810b	1050h
Head	Hybrid#4	9.31	52.80a	7.83a	3.85bc	895a	1355b
Head	Hybrid #7	9.31	50.30a	7.49ab	4.00b	895a	1375a
Head	C.V.%	10.29	5.65	33.86	29.20	19.41	11.73
Broken	Starbonnet	11.22	N/A	3.89c	2.00d	540h	990i
Broken	Lebonnet	9.98	N/A	5.92b	2.00d	575f	1070g
Broken	Mars	9.73	N/A	3.50c	3.90bc	575f	855j
Broken	Hybrid#4	9.31	N/A	3.99c	5.75a	680c	1250c
Broken	Hybrid #7	9.31	N/A	3.23c	5.70a	610e	1155e
Broken	C.V. %	10.29	N/A	53.05	43.58	8.91	14.26

[a] Readings within columns with the same letter are not significantly different using Duncan's multiple range test.
[b] Average of duplicate determinations.
[c] Readings are the mean of 100 determinations.
[d] Viscosity was expressed in Brabender units.
[e] Single determinations.

water. The initial temperature was 25.0°C and the slurry was heated to a final temperature of 92.5°C at the constant rate of 1.5°C per min. The temperature was maintained at 92.5°C for 15 min and then lowered at 1.5°C per min back to the initial starting temperature. The hot paste and setback viscosities were determined from the amylogram.

Chemical tests

Alkali spreading values were determined according to the method of Little et al. (1958).

Polyuronic acids were determined using the alcohol-insoluble materials following the method of Kinter and Van Buren (1982). The flour for each fraction was first extracted with absolute ethanol, and the dried alcohol insoluble material was subjected to analysis. Spectrophotometric measurements were done on a Varian 634S spectrophotometer. This procedure is for extracting pectic materials, but since no qualitative tests were run on the extracts, the extracted substance will be referred to as polyuronic material.

Total nitrogen was determined according to AACC Method No. 46-12 (AACC, 1976). The protein content was determined by multiplying the nitrogen level by 5.95.

Atomic absorption, using an IL 3500, was used to determine magnesium according to the method of Gaines and Mitchell (1979a). Phosphorous was determined using the method of Murphy and Riley (1962) with modification by Gaines and Mitchell (1979a). Boron was determined by the method of Gaines and Mitchell (1979b).

Statistical analysis

The SAS statistical package, release 79.6, was used for all of the statistical analyses.

RESULTS & DISCUSSION

DIFFERENCES between head rice and broken kernels were seen in several properties. The physical property data for both rice types are given in Table 1. The broken kernels were measurably softer than the intact kernels for all cultivars. The three commercial cultivars were classified into two categories based on the hardness of their respective head rice fractions.

To demonstrate that differences found between hardness for the two rice types existed prior to processing, a sample of a milled cultivar with a known hardness was broken by shattering intact kernels in a Wig-L-Bug laboratory amalgamator for 1 sec, and then determining the hardness of the resulting brokens. For consistency within this study, only second heads were tested. Table 2, shows that no statistical difference in hardness was found for the two rice types (intact vs broken). This indicates that the observed lower

Table 2—Comparison of hardness values for intact and broken fractions from the same milled rice sample[a]

Fraction	Mean[b]	N	Grouping[c]
Head	4.22	20	A
Broken	3.71	20	A

[a] Broken samples resulted from breaking intact milled kernels.
[b] LSD = 0.57.
[c] Means with the same letter are not different according to Duncan's Multiple Range Test.

hardness values exhibited by the fractions broken on milling were inherently lower in hardness than those which did not break. This further suggests that the brokens were not less hard because of prestressing from the processing. Accordingly, some differences in hardness among the rice kernels within a cultivar truly existed prior to milling, and, during processing, those less hard kernels broke, giving the broken fraction or rice type.

Results of the alkali spreading tests indicated no differences in birefringence end point temperature or kernel porosity between rice types for the commercial cultivars, but clear differences were found between the intact and broken fractions for the two Chinese hybrids (Table 1). Alkali spreading values indicated that Mars was more porous than either of the two other medium grain cultivars.

Values for amylographic pasting properties tended to indicate that broken kernels exhibited lower pasting viscosities than intact kernels. This indicates that the starch granules of the broken kernels do not swell as easily or to as great an extent as those of head rice. The lower setback viscosity of the brokens, relative to head rice, would suggest that the retrogradation of the brokens is less than for head rice. This would occur if there were differences in the types of amylose between the two rice types, as is supported by the differences in hardness and alkali spreading values.

The significant correlation between peak viscosity and alkali spreading value in head rice reflects the fact that both of these parameters are influenced by the degree of swelling of the starch granule.

The presence of polyuronic acids in the rice endosperm (Table 3) has not previously been reported. These materials were found to be significant in generating a descriptive model for hardness (see below), and were marginally correlated with hardness (Table 4). The inclusion of polyuronic

acid levels into the descriptive model provides evidence for a role of polyuronic acids in effecting kernel hardness.

The amounts of polyuronic acids found indicated limited rice type differences, with only Starbonnet and Mars showing distinct difference in these levels (Table 3).

Minerals and protein levels did not differ substantially between rice-types (Table 3). Boron was the only mineral measured that exhibited any detectible influence on rice kernel hardness.

Duncan's Multiple Range test for each of the physicochemical properties measured, using pooled data, indicated the same type of results, with differences found between the two rice type fractions for hardness, alkali spreading value, levels of polyuronic acids, and magnesium levels.

A protein-phytin complex may be more prominent in broken kernels, which would explain the significant correlation between protein and phosphorus in the brokens (Table 3). The highly significant correlation between phosphorus and magnesium in broken kernels (Table 3) strongly suggests an ionic association. This may be in the form of phytate compounds.

Table 3—Chemical properties of the rice varieties

Rice type	Variety	Protein[a,b] (%)	Polyuronic acid[a,c] materials (%)	P[a,b] (%)	Mg[a,b] (%)	B[a,b] (P.P.M.)
Head	Starbonnet	8.34a	0.55ab	0.23a	0.05bc	0.45a
Head	Lebonnet	8.33a	0.57a	0.15ab	0.05bc	0.50a
Head	Mars	8.04a	0.42d	0.15ab	0.05bc	0.35a
Head	Hybrid #4	8.63a	0.48cd	0.16ab	0.05bc	0.57a
Head	Hybrid #7	7.89a	0.47cd	0.10b	0.03d	0.48a
Head	C.V. %	5.91	12.26	35.00	26.97	36.95
Broken	Starbonnet	8.63a	0.46cd	0.23a	0.07a	0.50a
Broken	Lebonnet	8.33a	0.58a	0.18ab	0.055abc	0.55a
Broken	Mars	8.04a	0.34e	0.18ab	0.06ab	0.70a
Broken	Hybrid #4	8.04a	0.44d	0.17ab	0.06ab	0.65a
Broken	Hybrid #7	7.74a	0.49c	0.12b	0.04cd	0.60a
Broken	C.V. %	4.91	19.20	22.30	20.34	19.63

[a] Readings within columns with the same letter are not significantly different using Duncan's multiple range test.
[b] Readings are the mean of duplicate analyses and reported on a fresh weight basis.
[c] Readings are the mean of four analyses and reported on a fresh weight basis.

Table 4—Correlation analysis of the physicochemical properties of head rice

	Hardness	Rough rice moisture	Head rice yield	Alkali spreading value	Peak viscosity	Setback viscosity	Protein	Polyuronic acids	P	Mg	B
Hardness	–	–	–	–	–	–	–	–	–	–	–
Rough rice moisture	0.07	–	–	–	–	–	–	–	–	–	–
Head rice yield	−0.59	0.01	–	–	–	–	–	–	–	–	–
Alkali spreading value	−0.42	−0.82[a]	−0.06	–	–	–	–	–	–	–	–
Peak viscosity	−0.49	−0.69	−0.04	0.98**	–	–	–	–	–	–	–
Setback viscosity	0.43	0.61	−0.74	0.60*	0.54	–	–	–	–	–	–
Protein	0.55	0.16	0.01	−0.24	−0.19	0.12	–	–	–	–	–
Polyuronic acids	0.83[a]	0.58	−0.49	−0.80[a]	−0.81[a]	−0.01	0.39	–	–	–	–
P	0.11	0.87*	0.14	−0.67	−0.51	−0.51	0.57	0.47	–	–	–
Mg	0.29	0.29	0.53	−0.51	−0.48	−0.43	0.79[a]	0.29	0.60	–	–
B	0.80[a]	−0.25	−0.64	0.11	0.08	0.78[1]	0.66	0.43	−0.01	0.15	–

**($p < 0.01$)
*($p < 0.05$)
[a] Significant at the 10% level.

Table 5—Correlation analysis of the physicochemical properties of broken kernels

	Hardness	Rough rice moisture	Head rice yield	Alkali spreading value	Peak viscosity	Setback viscosity	Protein	Polyuronic acids	P	Mg	B
Hardness	–	–	–	–	–	–	–	–	–	–	–
Rough rice moisture	0.16	–	–	–	–	–	–	–	–	–	–
Head rice yield	N/A	N/A	–	–	–	–	–	–	–	–	–
Alkali spreading value	−0.64	−0.79[a]	N/A	–	–	–	–	–	–	–	–
Peak viscosity	−0.16	−0.77	N/A	0.81[a]	–	–	–	–	–	–	–
Setback viscosity	0.08	−0.47	N/A	0.56	0.79	–	–	–	–	–	–
Protein	0.46	0.91*	N/A	−0.86*	−0.61	−0.34	–	–	–	–	–
Polyuronic acids	0.70	0.08	N/A	−0.31	−0.01	0.50	0.20	–	–	–	–
P	0.24	0.87*	N/A	0.71	−0.51	−0.43	0.94*	−0.13	–	–	–
Mg	0.13	0.72	N/A	−0.55	−0.32	−0.39	0.82[a]	−0.32	0.98**	–	–
B	−0.53	−0.57	N/A	0.57	0.43	−0.21	−0.57	−0.84[a]	−0.27	−0.035	

**($p < 0.01$)
*($p < 0.05$)
[a] Significant at the 10% level.

Tables 4 and 5 present the correlation analysis of the selected physicochemical properties for the head fractions and the broken fractions, respectively. Peak viscosity was significantly related to alkali spreading value ($r = +0.98^{**}$) and rough rice moisture content was correlated with phosphorus content ($r = +0.87^{*}$). Hardness for the intact kernels was marginally correlated with polyuronic acid levels ($r = +0.83$). For the broken fractions, rough rice moisture and phosphorus contents were also correlated ($r = +0.87^{*}$). In addition, three other correlations were significant for the brokens: rough rice moisture and protein content ($r = +0.92^{*}$), phosphorus and protein content ($r = +0.94$), and magnesium and phosphorus content ($r = +0.96^{**}$).

Multiple regression analysis was performed on the hardness data, on the basis of rice type. For the head rice fractions, a single model was developed:

$$\text{hardness} = 6.39 + 1.19^{*}\ \text{polyuronic acid level} - 0.48^{*}\ \text{rough rice moisture content}$$

The R-square value was 0.96, and the model was found to be significant at the 5% level.

For the broken fractions, no significant model was found.

Differences between head rice and broken kernels were found in hardness, alkali spreading value and amylographic pasting properties. Broken kernels are softer and exhibit a lower peak viscosity and setback viscosity than head rice. Thus the differences that cause some rice kernels to break may be attributed to differences in the relative amounts of amylose and amylopectin.

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Chemical Treatment and Process Modification for Producing Improved Quick-Cooking Rice

D. A. SMITH, R. M. RAO, J. A. LIUZZO, and E. CHAMPAGNE

ABSTRACT

Quick-cooking rice was produced by soaking raw, white, long grain rice in a 1% aqueous sodium citrate and calcium chloride solution (50:50) at 50°C for 15 min. The soaked sample was cooked in an autoclave at 121°C for 3 min and freeze-dried to 20% moisture followed by convective air-drying to a final moisture of 12%. Nutrient analysis of the rice chemically treated and dried by a combination of both methods contained maximum amounts of thiamin (2.0 μg/g), niacin (27.4 μg/g), and iron (25.1 μg/g). The rehydrated sample, prepared by boiling the rice in water at 100°C for 5 min, received high sensory scores.

INTRODUCTION

STANDARD milled white rice, depending on variety and grain size (long, medium or short), requires from 20 – 35 min to cook to a satisfactory culinary acceptability when boiled according to usual recipe directions. In some instances the rice is soaked and boiled, then washed and steamed, which requires a total attention time of about an hour. The relatively long preparation time in the home and an occasional pot of sticky, pasty cooked rice has contributed to restricted rice consumption in the United States for many years. Thus, considerable efforts have been made in recent years to develop methods which will shorten preparation time and develop new improved products (Keneaster, 1974; Robbins, 1976; Roberts et al., 1980). Furthermore, numerous attempts have been made to relate the chemical and physical properties of rice kernels and rice starch with the hydration and cooking characteristics of commercially milled rice (Carlson et al., 1979; Juliano, 1970; Luh and Liu, 1980; Wadsworth et al., 1979; Goodman and Rao, 1983; Hogan and Plank, 1958; Bhattacharya and Sowbhagya, 1971). Although the general procedure for producing most instant rice products involves the basic steps of soaking in water, cooking and drying, variations in these procedures have been shown to have a significant effect on the cooking time and the final sensory qualities of the finished product (Roberts, 1972; Lewis, 1965; Tanakam and Yukami, 1969; Cox and Cox, 1975).

Chemicals believed to act principally to modify the protein structure by attenuation, disruption and/or disintegration are citrates, including magnesium citrate, sodium citrate, and calcium citrate. Such citrates are not sufficiently effective alone to produce a desirable quick-cooking rice product in conjunction with heat treatment (Gregory, 1976).

Auxiliary chemicals reported as having beneficial effects are fatty acid glycerides, which deter adhesion, and primary or secondary calcium phosphates which facilitate absorption of water by the starch and enhance the whiteness of the rice product. However, use of these chemicals has been shown to result in higher percent of broken rice and to adversely affect the storage life (Rutledge et al., 1972; Gregory, 1976).

Milling, soaking and steaming methods that are presently used for the production of quick-cooking rice remove much of the vitamins and minerals occurring naturally in the rice grain. However, when rice grains are enriched with vitamins such as riboflavin, yellow-orange color is imparted to the rice particles which distinguishes the riboflavin enriched material from the conventional product of commerce, making it unacceptable to consumers. Coatings which are conventionally applied to fortify rice fail to mask the color.

Of the numerous quick-cooking and enrichment processes and their respective combinations which have been reported over the past several years, only a very few have been proven satisfactory. There is still active interest among instant rice manufacturers in developing new and improved quick-cooking rice products with the view toward increasing yields, retaining higher percent nutrients, shortening process time, and improving appearance and other sensory qualities all of which should ultimately increase the total consumption of rice in the United States and elsewhere.

This investigation was designed to develop improved methods for the production of a quick-cooking rice with pre-selected properties. The end product should have the normal appearance of presently available commercial rice, and the grains should be appreciably different in size from unmodified rice. Such rice should have substantially the same characteristics of swelling as natural rice, and when finally cooked will have flavor, texture, taste and appearance very similar to ideally cooked conventional milled rice. The processing methods used for the preparation of such quick-cooking rice must result in whole grains which are strong and not fractured or mutilated. Avoidance of mutilation of the rice grains minimizes losses of starch and nutrients in the process of preparing the quick-cooking rice.

MATERIALS & METHODS

Materials

Raw, white, long-grain Bluebonnet variety rice of known history, obtained from the Louisiana Rice Experiment Station in Crowley, LA, was used in this investigation. Both nonenriched and enriched rice were studied. The enriched rice was prepared by thoroughly mixing 99.5% of nonenriched rice with 0.5% premix. The premix was enriched with 400 mg of thiamin, 3200 mg of niacin, and 2600 mg of iron per 454g of rinse-proof rice. The premix was furnished by Mr. S.L. Wright of Crowley, LA, who processes and distributes this material to commercial rice brokers. Adding 0.5% premix to 99.5% nonenriched rice brings the nutritional level of the rice-premix mixture to the level set forth in the Standard of Identity for Enriched Rice which requires that enriched rice have 2 – 4 mg of thiamin, 16 – 32 mg of niacin, and 13 – 26 mg of iron per 454g of rice (Houston, 1972).

Methods

The laboratory operations for the preparation of quick-cooking rice includes five major steps: (1) soaking, (2) cooking, (3) drying, (4) rehydration, and (5) sensory evaluation (Luh and Liu, 1980).

Soaking. The effects of four aqueous soaking media – distilled water, and water containing 1%, 3.5%, and 7% of a 50-50 mixture of sodium citrate and calcium chloride – together with two levels

Authors Rao, Liuzzo, and Champagne are affiliated with the Dept. of Food Science, Louisiana Agricultural Experiment Station, Louisiana State Univ. Agricultural Center, Baton Rouge, LA 70803. Author Smith's present address is Dept. of Food Science, Auburn Univ., Auburn, AL 36830.

of soaking temperature (21° and 50°C) and two periods of soaking times (1 and 15 min) were chosen for this study. The soaking process consisted of placing 100g sample in a 2-L beaker to which was added 800 ml of the appropriate aqueous soaking medium. The mixture was stirred manually while soaking at the selected temperature for the selected duration of time. At the end of the soaking period, the solution was decanted and the excess moisture was removed by blotting the samples over filter paper. The samples were then placed in a tared 300 ml evaporating dish and weighed.

The four levels of soaking media and two levels each of soaking temperature and periods yielded a total of 32 groups of soaked rice (16 samples for nonenriched and 16 samples for enriched rice). Preliminary data from soaking indicated that, without exception, the 15 min soaking period yielded fewer discolored products (other samples had undesirable discoloration) than the 1 min soaking. Therefore, the 16 discolored samples were not investigated further.

Cooking. Aliquots of the 16 groups of rice that had been soaked for a period of 15 min were subjected to two methods of cooking: (1) heating in an autoclave at 121°C, 15 psig, for 2 and 3 min; and (2) heating in excess water at 85° and 100°C for 5, 10, and 15 min. After each cooking process, the cooking solution was decanted and the cooked rice was rinsed and chilled with cold water (0 - 2°C). The excess moisture was removed by spreading the cooked rice over filter paper. The sample was then weighed in tared evaporating dishes.

The cooking procedure yielded a total of 128 groups of cooked rice (32 from cooking in an autoclave and 96 from hot water cooking). Inspection of the data from cooking indicated that those groups of samples which were soaked in 3.5% and 7% solutions of sodium citrate and calcium chloride were of inferior quality in terms of clumpiness, color and texture. Therefore, these 64 samples were not investigated further.

From the remaining 64 groups of cooked rice, a total of 16 groups were selected for drying on the basis of optimal percent moisture uptake, volume increase, and gelatinization: these 16 groups also represented matched groups with respect to: (1) enriched and non-enriched rice; (2) soaking media – distilled water and 1% solution of sodium citrate and calcium chloride; (3) soaking conditions – 21° and 50°C for 15 min; and (4) cooking methods – heating the soaked rice in an autoclave at 121°C at 15 psig for either 2 or 3 min and in hot water at 100°C for 10 min.

Drying. The moisture of the selected 16 groups of cooked rice was reduced to 12% by using two different drying methods: (1) convective air drying (two rates of air flow, 88 and 120 cfm, and two air temperatures, 82° and 93°C); and (2) freeze-drying the cooked rice to an initial moisture of 20% (pressure 60μ and shelf temperature 4°C) followed by convective air drying (air velocity 120 cfm at 93°C) the product to final moisture of 12%.

The convective air drying equipment used in this study was especially designed and built by the Louisiana State Univ. Agricultural Engineering Dept. It consisted of a 28 cm fan which forced air through an insulated square (22.9 cm x 22.9 cm) sheet metal duct fitted with electric heating elements, baffles, and a test section to introduce a wire mesh tray holding the rice samples. Heat was supplied to the drier by four banks of electric finstrip-type heaters controlled by rheostats. The four baffles provided prior to the test section of the duct maintained uniform air flow through out the duct. The manifold fitted to the fan regulated the air flow rates.

The equipment used for freeze-drying was a USM-15 model Virtis freeze-dryer.

Two rates of air flow, 88 and 120 cfm, and two levels of air temperatures, 82° and 93°C, yielded 64 groups of dried samples, while the procedure involving partial freeze-drying followed by convective air drying at 120 cfm and 93°C yielded 16 additional groups of cooked and dried samples. From these 80 groups, based on the optimum expanded volume ratio (1.5 or above) and the absence of clumpiness and breakage of the rice grains, a total of 28 groups were selected for rehydration; of these, 20 were from the 64 groups and 8 were from the 16 groups. These 28 groups also represented matched groups with respect to (1) enriched and nonenriched rice and (2) soaking media distilled water and 1% solution of sodium citrate and calcium chloride.

Rehydration ratio. Duplicate 10g samples of the quick-cooking rice were weighed on a torsion balance, placed in individual 600-ml pyrex beakers, to each of which was added excess water. The beakers were covered with watch glasses, placed on electric heaters and allowed to boil for 5 min at 100°C. The contents of the beakers were then transferred into corresponding 75-mL Buchner funnels

Table 1–Effect of soaking on percent moisture uptake and percent volume increase of raw rice[a]

	Control				1% Chemical treatment				3.5% Chemical treatment				7% Chemical treatment			
Soaking temp. (°C)	21		50		21		50		21		50		21		50	
Soaking time (min)	1	15	1	15	1	15	1	15	1	15	1	15	1	15	1	15
Nonenriched samples, Total 16																
% Moisture uptake	9.2	14.0	13.4	28.0	12.0	16.5	14.0	34.0	11.9	15.6	14.0	33.0	11.0	13.0	11.5	33.0
% Volume increase	3.8	4.8	3.8	5.0	3.7	4.7	3.7	4.9	3.5	4.6	3.7	4.8	3.7	4.5	3.6	4.7
Enriched samples, Total 16																
% Moisture uptake	9.1	14.0	13.4	28.0	11.9	16.4	14.1	34.0	11.9	15.6	13.9	33.0	11.0	13.0	11.5	33.0
% Volume increase	3.8	4.8	3.8	5.0	3.9	4.6	3.7	4.9	3.5	4.6	3.7	4.7	3.7	4.5	3.6	4.6

[a] Selection: High percent moisture uptake preferred; High percent volume increase preferred.

Table 2–Effect of cooking on percent moisture uptake, volume increase ratio and percent gelatinization of control group[a]

	Soaking temperature, 21°C; time, 15 min								Soaking temperature, 50°C; time, 15 min							
Temperature	85°C			100°C			121°C		85°C			100°C			121°C	
Time (min)	5	10	15	5	10	15	2	3	5	10	15	5	10	15	2	3
Nonenriched rice																
% Moisture uptake	44.7	53.3	64.0	59.3	63.4	75.0	58.3	61.8	48.9	57.2	67.1	62.0	67.0	77.0	63.0	65.0
Volume increase ratio	1.8	2.0	2.4	2.2	2.4	2.7	2.1	2.3	1.9	2.1	2.5	2.3	2.5	2.8	2.3	2.6
% Gelatinization	65.0	71.0	79.0	81.0	79.0	88.0	80.0	84.0	70.0	74.0	83.0	77.0	83.0	90.0	86.0	100.0
Enriched rice																
% Moisture uptake	44.5	53.0	63.9	59.2	63.9	75.1	58.2	62.0	48.7	57.2	67.4	61.8	67.1	77.2	62.9	65.0
Volume increase ratio	1.8	1.9	2.4	2.2	2.4	2.7	2.1	2.3	2.0	2.1	2.5	2.2	2.5	2.8	2.3	2.6
% Gelatinization	65.0	70.0	79.0	80.0	80.0	88.0	80.0	84.0	71.0	74.0	83.0	75.0	83.0	92.0	86.0	100.0

[a] Criteria for selection: (1) high % gelatinization, (2) moderate moisture uptake, (3) moderate to high volume increase.

fitted with coarse, porous filter paper and gentle suction was carefully applied for 0.5 - 1 min or until the drip from the funnel had almost ceased. The solids from the funnel were removed and weighed. The rehydration ratio was calculated by dividing the weight of the rehydrated rice by the weight of the initial sample of dry quick-cooking rice.

Percent gelatinization. The percent gelatinization was measured microscopically by slicing thin sections of the cooked rice with a razor blade and staining these sections with a 0.5% solution of Congo Red dye which differentiates gelatinized (red) granules from ungelatinized (uncolored) granules (Bhattacharya and Sowbhagya, 1971).

Percent volume increase. The volumes of the rice samples were determined before and after rehydration by placing an aliquot in a graduated cylinder. Percent volume increase was determined by dividing the increase volume of the rehydrated rice by the original volume of raw rice and multiplying by 100.

Clumpiness and breakage. The degree of clumpiness and breakage was determined by visual examination since it is still one of the accepted procedures in international trade (Houston, 1972).

Sensory evaluation. The rehydrated rice samples were evaluated on a hedonic scale by a panel of five judges for color, cohesiveness, off-flavor, and doneness. The four samples which received the highest sensory scores were then analyzed for thiamin, niacin and iron.

Nutrient analysis. Procedures outlined in AOAC (1970) were used for the determination of thiamin, niacin and iron. Moisture was determined by using a Motomco moisture meter. The accuracy of the instrument was periodically tested by the official air-oven method described in the USDA Announcement (USDA, 1976).

RESULTS & DISCUSSION

Soaking

Table 1 shows the results of soaking on percent moisture uptake and percent volume increase of uncooked rice. The data indicated a maximum percent moisture uptake of 28% for both the nonenriched and enriched control groups at a soaking temperature of 50°C and 15 min soaking period.

Table 3—Effect of cooking on percent moisture uptake, volume increase ratio and percent gelatinization of 1% chemically treated rice[a]

	Soaking temperature, 21°C; time, 15 min								Soaking temperature, 50°C; time, 15 min							
Temperature	85°C			100°C			121°C		85°C			100°C			121°C	
Time (min)	5	10	15	5	10	15	2	3	5	10	15	5	10	15	2	3
Nonenriched rice																
% Moisture uptake	47.1	55.4	66.0	61.5	65.5	79.2	60.5	64.0	51.0	59.6	69.4	65.0	69.2	70.4	65.2	67.1
Volume increase ratio	1.8	2.1	2.4	2.3	2.4	2.6	2.1	2.4	2.0	2.2	2.3	2.3	2.5	2.9	2.4	2.7
% Gelatinization	68.0	74.0	83.0	79.0	83.0	92.0	85.0	88.0	73.0	78.0	87.0	80.0	87.0	95.0	91.0	100.0
Enriched rice																
% Moisture uptake	47.2	55.4	59.9	61.4	65.6	79.3	60.4	64.0	51.3	59.4	69.2	65.0	69.3	79.5	65.1	67.2
Volume increase ratio	1.8	1.9	2.5	2.3	2.4	2.7	2.1	2.4	2.0	2.1	2.5	2.3	2.5	2.9	2.4	2.7
% Gelatinization	68.0	75.0	83.0	80.0	83.0	92.0	85.0	88.0	74.0	77.0	78.0	80.0	87.0	96.0	91.0	100.0

[a] Criteria for selection: (1) high % gelatinization, (2) moderate moisture uptake, (3) moderate to high volume increase.

Table 4—Effect of cooking on percent moisture uptake, volume increase ratio and percent gelatinization of 3.5% chemically treated rice[a]

	Soaking temperature, 21°C; time, 15 min								Soaking temperature, 50°C; time, 15 min							
Temperature	85°C			100°C			121°C		85°C			100°C			121°C	
Time (min)	5	10	15	5	10	15	2	3	5	10	15	5	10	15	2	3
Nonenriched rice																
% Moisture uptake	43.1	51.1	52.1	53.1	63.0	71.3	55.9	60.8	44.2	52.1	52.5	55.0	63.2	72.4	56.4	63.0
Volume increase ratio	1.6	1.9	2.0	2.0	2.3	2.7	1.9	2.1	1.7	2.0	2.0	2.2	2.4	2.8	2.2	2.4
% Gelatinization	63.0	69.0	70.0	71.0	77.0	88.0	73.0	83.0	65.0	69.0	70.0	72.0	77.0	89.0	74.0	90.0
Enriched rice																
% Moisture uptake	43.1	50.9	52.1	53.0	63.1	71.1	55.7	61.0	44.2	52.0	52.6	55.0	63.3	72.3	56.4	63.1
Volume increase ratio	1.6	1.9	2.0	2.0	2.3	2.6	1.9	2.0	1.7	2.0	2.1	2.2	2.5	2.8	2.2	2.4
% Gelatinization	63.0	69.0	70.0	71.0	77.0	87.0	72.0	84.0	65.0	69.0	70.0	72.0	78.0	89.0	74.0	91.0

[a] Criteria for selection: (1) high % gelatinization, (2) moderate moisture uptake, (3) moderate to high volume increase.

Table 5—Effect of cooking on percent moisture uptake, volume increase ratio and percent gelatinization of 7% chemically treated rice[a]

	Soaking temperature, 21°C; time, 15 min								Soaking temperature, 50°C; time, 15 min							
Temperature	85°C			100°C			121°C		85°C			100°C			121°C	
Time (min)	5	10	15	5	10	15	2	3	5	10	15	5	10	15	2	3
Nonenriched rice																
% Moisture uptake	41.6	43.8	44.1	52.1	62.0	63.5	50.9	59.0	42.3	44.0	45.1	54.0	62.8	63.8	51.2	59.7
Volume increase ratio	1.4	1.5	1.6	1.7	2.1	2.1	1.6	1.9	1.4	1.6	1.6	1.8	2.2	2.3	1.7	1.9
% Gelatinization	60.0	63.0	64.0	70.0	75.0	77.0	68.0	80.0	61.0	63.0	65.0	71.0	75.0	78.0	69.0	82.0
Enriched rice																
% Moisture uptake	41.6	43.7	44.0	52.3	62.1	63.4	50.8	59.2	42.5	43.9	54.0	54.1	62.8	63.8	51.1	59.7
Volume increase ratio	1.4	1.5	1.6	1.8	2.1	2.1	1.5	1.9	1.5	1.5	1.7	1.8	2.2	2.3	1.7	1.9
% Gelatinization	60.0	63.0	63.0	70.0	75.0	76.0	68.0	80.0	61.0	63.0	65.0	71.0	75.0	78.0	68.0	82.0

[a] Criteria for selection: (1) high % gelatinization, (2) moderate moisture uptake, (3) moderate to high volume increase.

Under the same soaking conditions, the maximum volume increase for these two groups was 5.0%. The chemically treated rice (1.0% chemical treatment, 50°C soaking temperature and 15 min soaking time) resulted in maximum percent moisture uptake of 34% and 4.9% volume increase. Examination of the data showed that enrichment of the samples did not affect the degree of moisture uptake or volume increase; however, samples which were chemically treated had almost 50% more moisture uptake than the controls soaked under identical conditions in distilled water. Increasing the concentration of chemicals in the soak water from 1 to 3.5% to 7% did not improve either moisture uptake or volume increase; the 1% solution was as effective as the others.

Soaking increased the depth and uniformity of the migration of the cooking water into the rice grain. The rate of migration of soak water into the rice grain depends upon the soaking period and temperature of the soak water. Such penetration reduces the tendency of the rice grain to disintegrate or be mutilated from the action of internal osmotic pressure acting to burst the rice grains during subsequent boiling which results in starch being lost to the cooking water. Addition of calcium chloride to the soak water deters adhesion between the grains which would cause mutilation if the grains were forced apart. Sodium citrate in the soak water acts principally to modify the protein structure of the rice by disruption and disintegration (Yaghoubian, 1974).

Cooking

The cooking of rice results essentially in the gelatinization and swelling of the starch granules within the rice endosperm. It is believed that the internal structure of a rice grain may include an integumental web formed by the protein components which sequester the starch to deter imbibition of water by the starch for gelatinization. This protein shielding of the starch is believed to be responsible for the long time normally required to cook rice in boiling water (Cox and Cox, 1975). Cooking can be affected by the rice being processed in hot or boiling water at atmospheric pressure or by the rice being steamed in an autoclave. Steaming the rice under pressure in an autoclave increases the rate of gelatinization and thus reduces the heating time.

The differences in percent moisture uptake, volume increase and gelatinization in nonenriched and enriched controls and nonenriched and enriched chemically treated groups due to each cooking variable, viz., cooking time, cooking temperature and cooking methods, are shown in Tables 2 through 5. The data indicated that those groups which had been soaked in the 3.5 and 7% solutions of sodium citrate-calcium chloride were of inferior quality; consequently, these groups of cooked rice were not investigated further and will not be included in the discussion.

A comparison of percent gelatinization of both the enriched and non-enriched groups in the control (Table 2) when soaked and cooked under different conditions indicated that cooking the soaked rice in an autoclave at 121°C and 15 psig for 3 min resulted in maximum gelatinization. Similar observations were made with respect to chemically (1%) treated groups. However, the percent moisture uptake and percent volume increase (67.1 and 100%, respectively) of these chemically treated groups were found to be slightly higher than the untreated control groups which were soaked and cooked under identical conditions. Apparently, the groups which were soaked in aqueous solutions of sodium citrate and calcium chloride had a modified protein and/or starch component as a result of the chemical treatment which facilitated penetration of the water into the interior of the rice grains and expedited the imbibition of such water by the starch during gelatinization. For optimal drying and superior quick-cooking rice, it is desirable to have a high percent gelatinization, moderate moisture uptake and moderate to high percent volume increase in the cooked product.

Drying

The variations in volume ratio, clumpiness and breakage in the nonenriched and enriched control groups and the chemically treated enriched and nonenriched groups due to each variable (convective air drying-air flow 88 and 120 cfm, air temperature 82° and 93°C; and freeze-drying followed by convective air drying) are presented in Tables 6 and 7. Highest volume ratio (1.5 – 1.6), minimum clumpiness and breakage (<5%) were observed in the nonenriched and enriched control groups when they were dried by convective air drying at 120 cfm air flow and 85° and 93°C. However, under the same drying methods and conditions, the chemically treated groups showed higher volume ratios than the respective controls. From these data it appears that enriching the rice did not affect the volume

Table 6—Effect of convective air drying variables on volume ratio, clumpiness and breakage in cooked rice

	Sample no.	1		2		3		4		5		6		7		8	
	Air flow	Air temperature (°C)															
	(cfm)	82	93	82	93	82	93	82	93	82	93	82	93	82	93	82	93
Water soaked control (1 - 4 nonenriched, 5 - 8 enriched)																	
	88	1.4	1.5	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.5	1.4	1.4	1.4	1.4	1.4	1.4
Volume ratio	120	1.5	1.6	1.4	1.5	1.4	1.5	1.5	1.6	1.6	1.6	1.4	1.5	1.4	1.5	1.5	1.6
% Clumpiness	88	+	+	++	+	+++	++	+++	++	+	++	++	++	++	++	+++	++
	120	++	+	++	++	++	++	++	++	+	++	++	++	++	++	++	++
	88	+	+	+	<5	+	<5	+	+	++	+	+	+	+	<5	+	+
% Breakage	120	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	+	
Samples: nonenriched (1 - 4), enriched (5 - 8), 1% chemical treatment in soaking																	
	88	1.6	1.6	1.5	1.5	1.5	1.5	1.5	1.4	1.6	1.6	1.5	1.5	1.5	1.5	1.5	1.4
Volume ratio	120	1.7	2.0	1.7	1.8	1.6	1.7	1.5	1.7	1.7	2.0	1.6	1.8	1.6	1.7	1.5	1.7
	88	+	+	+	+	+	+	+	++	+	+	+	+	+	+	+	++
% Clumpiness	120	+	<5	+	+	+	+	+	+	+	<5	+	+	+	+	+	+
% Breakage	88	+	+	+	<5	+	+	+	+	+	+	+	<5	+	+	+	+

[a] Criteria for selection for further processing: high volume ratio, low clumpiness and breakage. Number of samples selected for further processing: 10 + 10 = 20 (underlined samples). Clumpiness and breakage rating: <5%; 5 – 25% = +; 25 – 75% = ++; 75 – 100% = +++.

Table 7—Effect of freeze-drying and convective air drying on volume ratio, clumpiness and breakage in cooked rice[a]

	Sample number[b]							
	1	2	3	4	5	6	7	8
Water soaked control								
Volume ratio	2.0	1.8	1.6	1.7	2.0	1.8	1.6	1.7
Clumpiness	<5	<5	<5	<5	<5	<5	<5	<5
Breakage	<5	<5	<5	<5	<5	<5	<5	<5
Chemically soaked (1%)								
Volume ratio	2.2	2.0	2.0	1.9	2.2	2.0	2.0	1.9
Clumpiness	<5	<5	<5	<5	<5	<5	<5	<5
Breakage	<5	<5	<5	<5	<5	<5	<5	<5

[a] Freeze-dry conditions: pressure, 60 microns; temperature, 4°C. Cooked samples were freeze-dried to 20% moisture followed by convective air drying at 93°C and an air flow velocity of 120 cfm until the moisture reached 12%.
[b] Underlined samples were selected for further processing.

ratios. Chemical treatment (1%) of the rice did result in an increased volume ratio.

The highest volume ratios (2.0) for the non-enriched and enriched control groups and (2.2) for chemically treated enriched and non-enriched groups were observed when the cooked samples were freeze-dried followed by convective air drying. Clumpiness and breakage were maintained at the same level (<5%). These data indicated that a combination of freeze-drying followed by convective air drying of the cooked rice resulted in an increased volume ratio (2.2 to 2.0), which is a desirable attribute in quick-cooking rice. The cooked rice which was frozen prior to freeze-drying was somewhat less sticky and had better dry flow characteristics than the conventionally dried rice product. The freezing process appeared to toughen the gel structures to a considerable extent, thus reducing the extreme stickiness of some of the starch-glutelin gel complexes which resulted from the pre-cooking process; in addition, there was coagulation and shrinking of the starch gel and the glutelins. These glutelins become hydrated to a considerable degree under the freezing conditions. The coagulation releases free water into the voids which are opened by coagulation and shrinkage, and the liberated water migrates and increases the size of any ice crystal nuclei which are present. The freezing process thus liberates water from these gels while partially denaturing the protein lattice. This sufficiently reduces the tendency of the individual grains to stick together, and to mash and break at the slightest pressure. During freeze-drying all the liberated water formed into ice crystals is removed in the vapor state without passing through the liquid state. This avoids rehydration of the starch during thawing and the resultant collapsing of the granules, starch granule breakage and the production of a more sticky and pasty final product.

Rehydration

The rehydration ratios of nonenriched and enriched control groups and chemically treated nonenriched groups dried by convective air drying are presented in Table 8. Chemically treated freeze-dried groups showed a maximum rehydration ratio of 2.2; whereas, chemically treated convective air-dried groups reached a maximum rehydration ratio of 2.0. A rehydration ratio range of 1.2 - 1.9 was obtained in controlled nonenriched and enriched groups which were not chemically treated. From these data it appeared that enrichment did not significantly affect the rehydration ratios. However, chemical treatment and partial freeze-drying improved the rehydration ratios of the samples.

Sensory evaluation

The data on sensory evaluation are presented in Table 9. An inspection of these data showed that there was little or no difference in terms of color. All samples were white to the same extent and received the highest score. However, in terms of cohesiveness, off-flavor and doneness, the taste panelists rated groups 1 and 2 as superior to groups 3, 4, 5. These scores indicated that groups 1 and 2 were well separated with no off-flavor and were cooked to perfect doneness. The sensory results indicated that chemically treated raw rice, soaked at 50°C for 15 min, cooked in an autoclave at 121°C for 3 min and followed by a combination of freeze-drying and convective air drying produced a superior final product.

Nutrient analyses

The nutrient analyses for thiamin, niacin and iron for six groups of quick-cooking rice are presented in Table 10. These six groups consisted of 4 quick-cooking rices which received the highest overall sensory scores, and one each of enriched and nonenriched rice samples. The enriched and nonenriched groups were included in the test to determine the variations, if any, in the values of thiamin, niacin and iron from the theoretical data obtained by mixing 0.5% premix with 99.5% raw rice. The other four groups were analyzed to determine the effect of soaking, cooking and drying conditions on the percent nutrient retention.

An examination of the data obtained by nutrient analysis indicated that Group 1 (chemically treated raw rice, soaked at 50°C for 15 min and cooked in an autoclave at 121°C for 3 min followed by a combination of freeze drying and convective air drying) contained the maximum amounts of thiamin (2.0 μg/g), niacin (27.4 μg/g), and

Table 8—Effect of processing variables on rehydration ratios of instant rice

	Samples									
	Nonenriched					Enriched				
	1	2	3	4	5	6	7	8	9	10
Convective air dried samples										
Water soaked	1.9	1.8	1.7	1.8	1.7	1.9	1.8	1.7	1.8	1.7
1% Chemically soaked	2.0	2.0	1.9	1.9	1.9	2.0	2.0	1.9	1.9	1.8
Freeze-dried + convective air drying										
1% Chemical treatment (1,2,5,6)		1	2	3	4		5	6	7	8
Water soaked (3,4,7,8)		2.2	2.1	2.0	2.0		2.2	2.1	2.0	2.0

Table 9—Sensory score sheet for cooked instant rice[a]

	Sample[b]				
	1	2	3	4	5
Color	9	9	9	9	9
9. White					
7. Cream					
5. Grey-yellow					
3. Tan					
1. Brown					
Cohesiveness	9	9	7.5	7.5	8
9. Well-separated grains					
7. Partially separated					
5. Sticky					
3. Very sticky					
1. Pasty					
Off flavor	9	9	8	8	8
9. None					
7. Perceptible					
5. Slightly strong					
3. Moderately strong					
1. Very strong					
Doneness	5	5	6.5	6.5	4.5
9. Underdone					
7. Slightly underdone					
5. Done					
3. Slightly overdone					
1. Overdone					

[a] Scores are averages of ratings by five judges.
[b] Sample #1 — Chemically (1%) treated raw rice, soaked at 50°C for 15 min and cooked by autoclave at 121°C for 3 min before freeze-drying and convective air drying.
Sample #2 — Same as Sample #1, except no chemical treatment.
Sample #3 — Same as Sample #1, except convective air dried only.
Sample #4 — Same as Sample #3, except no chemical treatment.
Sample #5 — Commercially available enriched instant rice.

iron (25.1 μg/). However, groups 3 and 4 which were dried by convective air drying alone showed considerably lower values of thiamin (1.25 - 1.40 μg/g), niacin (14.8 - 16.5 μg/g), and iron (24.9 μg/g). These results indicated that partial freeze drying of the cooked rice reduced the nutrient losses during the preparation of quick-cooking rice.

CONCLUSIONS

THE RESULTS of this investigation have shown that improved quick-cooking rice with a high retention of nutrients and with characteristics very similar to cooked tabletop rice can be produced by chemical treatment of raw rice followed by some modifications in conventional drying procedures. The chemical treatment recommended includes soaking the raw rice in an aqueous solution of 1% sodium citrate and calcium chloride (50:50) at 50°C for 15 min and drying the product by freeze and convective air drying.

Table 10—Effect of processing on the nutritive value of instant rice[a]

	Non-enriched	Enriched	Group			
			1	2	3	4
Thiamin, μg/g	0.72	2.1	2.00	1.90	1.40	1.25
Niacin, μg/g	14.0	31.0	27.0	23.1	16.5	14.8
Iron, μg/g	1.6	27.0	25.1	25.0	24.9	24.9

[a] Group 1 — Chemically (1%) treated raw rice, soaked at 50°C for 15 min and cooked by autoclave at 121°C for 3 min before freeze-drying and convection air drying.
Group 2 — Same as Group 1, except no chemical treatment.
Group 3 — Same as Group 1, except convective air dried only.
Group 4 — Same as Group 3, except no chemical treatment.

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Storage Stability of a Processed Ginger Paste

J. D. BARANOWSKI

ABSTRACT

A processed paste was prepared from fresh ginger so that a convenient, shelf-stable product with the odor characteristics of fresh ginger could be manufactured from ginger produced in excess of fresh market demand. The paste was sampled periodically for color by Hunter YI index, odor by trained panel and (6)-gingerol (the major pungent component) by HPLC. This product was found to have a fair shelf-life under refrigeration, but degradation of color, odor and (6)-gingerol made the paste unacceptable after 8 wk storage at 25°C or 37°C.

INTRODUCTION

GINGER, the rhizome of *Zingiber officinale,* has long been used both in medicine and as a spice. In Hawaii, ginger farming has undergone recent expansion, with 160 acres under cultivation in 1983 producing 2300 metric tons worth 2.8 million dollars and is thus becoming an important crop in this locality (Anon., 1984). At present this crop is destined for the fresh market. However, considering the ginger farming expansion rate of the past 5 yr, presumably some form of processing will be necessary to accomodate the excess future production.

Fresh ginger root is characterized as having a subtle, distinct aromatic odor, which is seldom carried over into processed ginger. Chemically the essential odor of ginger is composed of a complex mixture of mono- and sesquiterpene hydrocarbons, with lesser amounts of oxygenated terpenoids (Govindarajan, 1982). Bednarczyk and Kramer (1971) found that the contribution of four peaks from a GLC fingerprint could predict ginger odor of the dried rhizome, correlating with a trained panel at $R^2 = 0.85$. Some of these peaks were later found to be composed of several components which were identified as β-sesquiphellandrene, cis-β-sesquiphellandrol, ar-curcumene, α-terpinol, neral, geranial, and nerolidol (Bednarczyk and Kramer, 1975).

Ginger pungency is attributed largely to phenolic ketones (Connell and Sutherland, 1969). In fresh ginger, the major pungent components are the gingerols. Connell and Sutherland (1969) reported that the gingerols were present in Queensland ginger in a ratio of 53:17:30 for the (6)-, (8)- and (10)- gingerols, respectively. Dehydration products of the gingerols (shogaols) are not found in any quantity in the fresh root but are found in the oleoresin and are assumed to be due to chemical changes occurring with the extraction process and during storage. Based on taste panel analysis of both crude preparations and pure compounds, the shogaols seem to be more pungent than their parent gingerols (Narasimhan and Govindarajan, 1978; Deniff et al., 1981).

Processing by dehydration of the rhizome is carried out for preservation since the fresh root is susceptible to wilting, sprouting and mold attack, especially after preparation for market. Regretfully though, few would agree that the delicate odor of fresh ginger is comparable to that of the dried form. In this study, the objective was to prepare a processed ginger product, a paste, which would be in a form acceptable to consumers, would be shelf stable, and would retain the delicate, sweet-spice odor of fresh ginger root. This paste was then examined for odor and color stability during storage.

Author Baranowski is affiliated with the Dept. of Food Science & Human Nutrition, Univ. of Hawaii at Manoa, 1920 Edmondson Road, Honolulu, HI 96822.

METHODS & MATERIALS

FRESH, mature ginger rhizomes were obtained locally, washed and stored at 25°C until processing. Preliminary heat processing studies indicated that the fresh ginger odor was extremely unstable over time at elevated temperatures, so citric acid was added to reduce the pH (to 4.15) so that simple pasteurization would suffice as a heat process. Roughly 12 kg paste was prepared by blending 8 batches of 1.5 kg each. Each batch consisted of 750g each of freshly sliced ginger root and water, 15g xanthan gum stabilizer ("Keltrol", Kelco, San Diego, CA) and 7.5 mL 30% w/v citric acid solution, which were comminuted for 2 min in a food processor. The paste was heated in a steam kettle to 80°C, packed into 202x314 cans, seamed, inverted for 3 min, cooled in running water for 15 min and air dried. The process time from application of heat to finish seaming was roughly 12 min.

In the first study, designed to evaluate odor changes, cans were stored at either -10° or 25°C. The pastes were samples at 0, 4, 8, 16 and 24 wk of storage. The 0 wk stored samples were evaluated immediately after their preparation. Odor was evaluated by a 10 member trained panel. Roughly 1 hr prior to presentation, samples were prepared by placing 2.0 ± 0.1g paste into a 1-oz paper cup which was capped. Four panels were conducted at each sampling period on separate days, so that each panelist rated each sample, on a scale of 7 = intense fresh ginger odor, to 1 = no ginger odor, four times. Panels were conducted in partitioned booths with red lights to mask color differences. Samples were coded with three digit random numbers and presented in random order. One can of paste served for 2 days' panels, with refrigerated storage in the interim. Statistically significant differences between the -10°C and 25°C stored samples at each sampling were determined using ANOVA run on a Statistical Analysis System (SAS, 1982) program.

In the second study, to evaluate changes in color and flavor components, samples were prepared as above, but with storage at -10,° 10,° 25,° and 37°C. Color was monitored by Hunter color difference meter values. Samples were taken at the same time intervals as in the first study.

(6)-Gingerol content of these pastes was determined by high performance liquid chromatography (HPLC), using 40g paste for sample preparation (Baranowski, 1985). The methanol extracts prepared from the samples were stored under nitrogen at -10°C until the end of the study so that all HPLC analyses could be carried out at the same time. Each sample was analyzed in duplicate. Quantification was made by preparing a standard curve from the isolated compound using a molar extinction coefficient of 3341 at 282 nm (Baranowski, 1985).

RESULTS & DISCUSSION

THE ODOR of ginger is a complex chemical mixture. A steam-distilled volatile oil is normally prepared from ginger for evaluation of the spice potential. Depending upon storage condition, the loss of volatile oil can be fairly rapid from dried ginger and it has been reported that ground, dried ginger can lose up to 50% of its volatile oil during 6 months of storage depending upon the container (Richardson, 1967). Canned ginger paste (Table 1) lost a considerable amount of its fresh ginger odor during storage, with the 25°C stored samples having ca 70% of the odor of a frozen

Table 1—Panel evaluation of ginger pastes for fresh ginger odor during storage

Wk of Storage	Odor rating[a]	
	−10° C[b]	25° C[b]
0	5.72	5.25 (91.8)[c]
4	5.90	5.17 (87.6)[d]
8	5.85	4.02 (68.7)[d]
16	4.55	3.30 (72.5)[d]
24	5.35	3.77 (70.5)[d]

[a] Odor rating scale — 7 = fresh ginger odor to 1 = no ginger odor
[b] Storage temperature
[c] (% odor of −10° C stored paste)
[d] Significantly different (P=0.01) from the rating of the −10°C stored sample at that sampling.

control after 24 wk storage. With hermetically sealed containers it is not expected that this loss is due to simple volatilization of the odor components. During storage of ginger essential oils and oleoresins in other studies, it was noted that the zingiberine and β-sesquiphellandrene decreased markedly during storage, with a concurrent increase in ar-curcumene (Salzar, 1975; Connell and Jordan, 1971). Bednarczyk and Kramer (1971) reported that β-sesquiphellandrene and ar-curcumene were the major determinants of ginger odor, so that a change in the ratio of these compounds and the breakdown of others may be the cause of the loss of fresh ginger odor in the pastes.

In all, six Hunter color scores were determined during the study: L, a, b, YI, YII, and WI. The latter three are mathematical manipulations of the first three designed to describe yellowness or whiteness of the sample (Anon., 1979). Fig. 1 is a plot of the YI index, which described browning in the product. The other two complex color scores gave data similar in trend to that of YI, with a general increase in YII and a decrease in WI with storage. Changes in this index occurred even under frozen storage and were dependent upon storage temperature, although color development was not directly proportional to temperature. These changes occurred most dramatically during the first 4 wk of storage at all temperatures. The trends for L, a, and b values were similar, with a general decrease in L values, and increases in both a and b values. While these measurable color changes did occur, the −10°C and 10°C stored samples were still visually acceptable at the end of the study; the 25°C stored samples were borderline; and the 37°C samples were probably unacceptable from a consumer standpoint.

The stability of (6)-gingerol, the major pungent component in the paste, was also dependent on a time-temperature relationship with gradual decreases at all temperatures over time, and progressive losses with increasing temperature for any given sampling period (Table 2). Connell (1969) noted a half-life of 6.3 hr at pH 4.2 for gingerols in oleoresin held at 120°C. Assuming a Q_{10} = 2, the half-life would be ca. 12.5 wk at 37°C, while the data herein would indicate a half-life of ca. 19 wk at this temperature. Data were also presented indicating that acid conditions hastened the breakdown, so that the use of citric acid in the pastes may have brought about a more rapid loss in this compound. In the pastes, only 82.4, 76.4, 67.3 and 42.0% of the compound remained after 24 weeks storage at −10,° 10,° 25° and 37°C respectively, demonstrating the instability of this compound even under mild conditions.

These data point out the difficulties in preparing a stable processed ginger product. Losses of color, odor and the major pungency component were large for samples stored at 25°C, and while refrigeration did retard these changes, there was still considerable degradation of the product stored at 10°C over 24 wk. While a short shelf-life for the refrigerated paste is possible, it may be that the complex nature of fresh ginger is not adaptable to processing.

Table 2—Changes in (6)-gingerol concentration during storage of ginger pastes

Wk of Storage	Storage temperature (° C)			
	−10	10	25	37
0	992.3[a]	992.3	992.3	992.3
4	955.8	934.2	832.0	815.8
8	921.2	886.7	785.8	658.5
16	831.3	734.5	716.5	564.2
24	817.9	758.3	668.3	416.6

[a] ppm

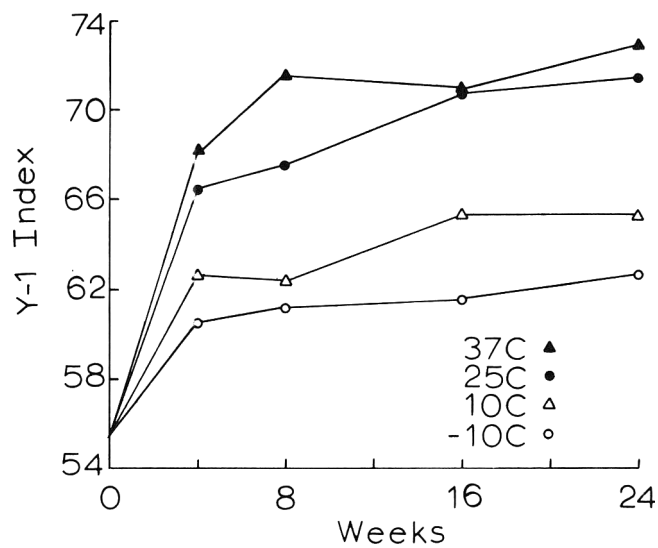


Fig. 1—Changes in YI color index of ginger pastes stored at four temperatures.

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Heat Recovery and Exergy Balance in a Tomato Paste Plant

D. FORCINITI, E. ROTSTEIN, and M. J. URBICAIN

ABSTRACT

Heat integration as a means of saving energy can be accomplished in a systematic manner using thermodynamically based methods. This paper examines the relationship between the exergy balance and the pinch point procedure to evaluate minimum utilities consumption. As a test case it is shown how these ideas are used to save energy in an existing tomato paste plant. Results indicate that energy savings correlate with decreasing productions of entropy. On the other hand, from a return on an original investment standpoint there is no correlation.

INTRODUCTION

AS ENERGY BECOMES an increasingly significant item in the unit cost of food products, energy conservation is regarded with more and more interest by food manufacturers, scientists and engineers. This interest was pinpointed by the First Symposium on Energy Management in the Food Industry, presented during the 1975 IFT Annual Meeting, which included an overview of energy utilization in the food processing industries (Unger, 1975). The work since then has covered mainly three areas: (a) Loss reduction by insulation, heat recovery or efficiency improvement (e.g.: Rao and Katz, 1976; Rao et al., 1976a, b); (b) Study of specific operations (e.g. Flink, 1977; Sokhansanj and Bakker-Arkema, 1981); and (c) Energy accounting (e.g.: Singh, 1978; Singh et al., 1980).

Two lines of work seem to be worthwhile exploring to pursue energy conservation further. One is the development of overall diagnostic tools, and this has been studied by Rotstein (1983), who discussed the exergy balance as the appropriate tool for this purpose. The other is the synthesis approach to improve an existing flowsheet by means of systematic procedures. This work intends to show how to accomplish the second and what is the relationship of the results obtained with the information provided by the diagnostic tools.

The basic idea is to take the whole plant as a thermodynamic system in which hot process streams must be cooled and cold ones must be heated. The trivial solution, and in fact the most expensive one, is to use cold and hot utilities to achieve both objectives. The alternative is to make use of the heat available in the hot streams to heat the cold ones, which play a symmetric role. It is apparent that the more this matching is achieved in a plant the less is the amount of utilities required to perform the required duty. This process is called heat integration and several methods have been published elsewhere (Linnhoff and Flower, 1978; Umeda et al., 1979; Cerdá et al., 1983; Linnhoff and Hindmarsh, 1983) to produce an optimal network of streams and heat exchangers, i.e. a system in which the maximum process streams energy is used before making use of utilities such as steam and cooling water.

In applying systematic heat integration methods, new plant structures are produced because new pieces of equipment are added (heat exchangers, heat pumps and the like). In each case, application of the exergy balance shows what the improvement was in energy usage and economic analysis shows the impact on economics. The objective of this work is to illustrate how heat integration can be accomplished in a systematic manner, showing that there is a thermodynamic logic that relates all alternative structures in a predictable manner. An additional aim is to show that this logic does not carry through the economic evaluation of the alternative structures.

Authors Forciniti, Rotstein, and Urbicain, are affiliated with PLAPIQUI, Univ. Nacional del Sur, 8000 Bahía Blanca, Argentina

THEORY

Superstreams

There is a feature which is common to all the above heat integration methods. Following Hohman (1971), a hot superstream is defined as the equivalent to the sum of all individual hot streams within the same temperature interval. When there are several hot streams, i = 1, 2, . . . , n, the heat content of the hot superstream in the particular temperature interval is:

$$Q = (\Sigma\, C_i)\, \Delta T$$

where C_i is the heat capacity flow rate. The cold superstream is built in an analogous manner. Use of superstreams reduces the problem to one of re-allocation of matches between process streams, letting aside the previous heat exchange configuration.

With this information a systematic procedure can be performed on the complete set of streams, to obtain the maximum extent of integration with a minimum of heat exchange units and a minimum usage of utilities, as explained below.

The pinch procedure and the exergy balance

Assume q process streams are to exchange heat among themselves or with j utility streams. Hot and cold superstreams may be built and represented as shown in Fig. 1, where the ordinate is the Carnot efficiency $\eta = [1 - (T_o/T)]$, the abscissa is Q and utilities are represented by cooling water and steam at a certain temperature. There is heat exchange between process streams which share a common Q and the balance is made up by using utilities. The $[1 - (T_o/T)]$ vs. Q plane is a meaningful representation. The exergy change of a stream can be estimated (Rotstein, 1983) as:

$$B_2 - B_1 = \int_{T_1}^{T_2} \left(1 - \frac{T_o}{T}\right) dQ \qquad (1)$$

The general expression for exergy balance is (Rotstein, 1983):

$$-\sum_{q}^{proc} w_q \hat{B}_q = \sum_{j}^{util} w_j \hat{B}_j + \dot{W}_u - T_o \dot{R}_s \qquad (2)$$

where $\sum_{q}^{proc}$ indicates summation over q = 1, 2, . . . , n process streams and $\sum_{j}^{util}$, summation over j = 1, 2, . . . , m utility streams. In a normal heat exchanger situation $\dot{W}_u = 0$. It follows from Eq. (1) and (2):

(1) The area under the lines in Fig. 1 represents the corresponding exergy change for the super streams. Thus, the area under line 1h – 3h represents $B_{3h} - B_{1h}$ and so on; (2) when streams exchange heat, i.e., they share the same abscissa interval, the net area between them (shaded in the figure) is the exergy change for the

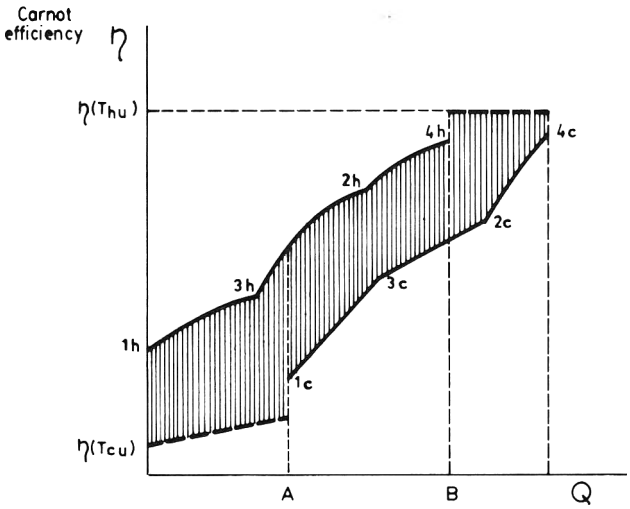


Fig. 1—Line 1h to 4h is the hot superstream. Line 1c to 4c is the cold superstream. The dotted line starting at η (T_{cu}) represents cooling water, the one corresponding to η (T_{hu}), steam. Heat exchanged between A and B represents exchange of heat between process streams.

corresponding heat exchangers; (3) this net area is, therefore, $\sum_{q}^{proc} w_q \hat{B}_q + \sum_{j}^{util} w_j \hat{B}_j$ and it follows from Eq. (2) that it represents $-T_o \dot{R}_s$ in usual heat exchange equipment where $\dot{W}_u = 0$.

The lines starting at T_{cu} and T_{hu} represent usage of cooling and heating utilities. (Q_R and Q_H, respectively). A desirable objective is to take full advantage of the process streams potential to exchange heat, thus minimizing the use of utilities. Hohmann (1971) presented a method to accomplish this objective, consisting in moving the cold superstream curve towards the hot superstream curve until a designer-specified minimum ΔT occurs at a certain Q. This is known as the "pinch point" for the problem. It represents the maximum heat exchange between process streams and, consequently, the minimum requirement for utilities. The case is illustrated in Fig. 2 for the same problem shown in Fig. 1. The procedure has accomplished the following results:

(a) the smallest distance between lines corresponds to ΔT_{min}, the minimum driving force, which will be accepted for the network design; (b) the hot and cold lines overlap further, which means a higher degree of energy integration; (c) that, in turn, provokes a smaller degree of irreversibility, represented by a smaller net shaded area between lines; (d) there is a smaller requirement of utilities (dotted lines are shorter) to complete the heat balance; and (e) production of entropy has decreased, as shown by a much shrunken shaded area.

There are several ways to calculate the location of a pinch point. Linnhoff and Flower (1978) suggested the use of a problem table. Umeda et al. (1979) used a graphical approach equivalent to drawing a figure like Fig. 2. Cerdá et al. (1983) proposed a linear programming method. In this study use was made of an analytical-numerical approach developed by Urbicain and Rotstein (1984). The corresponding computer program can be obtained from the authors.

Once the pinch point is located, two venues are open for optimization. One is to heat integrate the plant as it is, to attain the minimum utility usage Q_H and Q_R, bearing in mind that no heat must pass across the pinch (Townsend and Linnhoff, 1981). The other is to remove the pinch by changing the temperature of a superstream, which implies changing operating conditions in one or more pieces of equipment. This will result in a new pinch point and a lower utilities consumption (Umeda et al., 1979).

After the pinch temperatures and minimum utility requirements have been found, the actual design must be carried out. This is conveniently done (Linnhoff and Hindmarsh, 1983) by dividing the problem at the pinch and designing each part separately, starting the design at the pinch and moving away, obeying the constraint that immediately above the pinch it should be:

$$C_{hot} \leq C_{cold} \qquad (3)$$

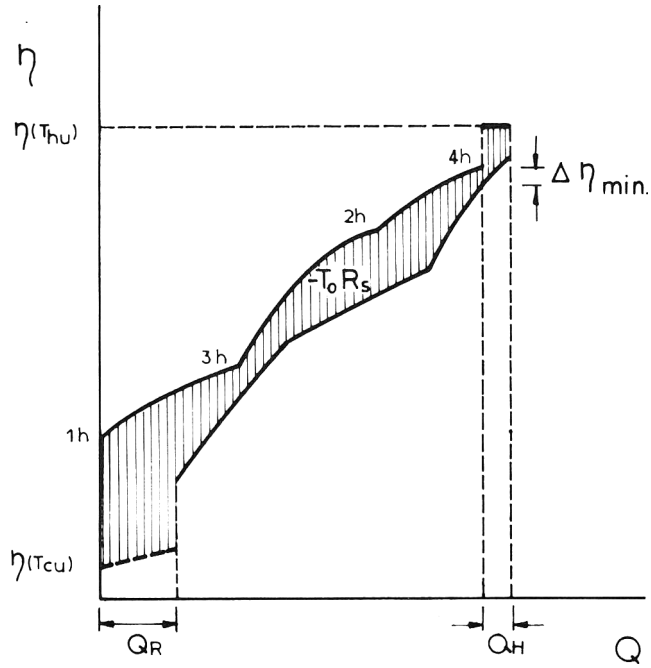


Fig. 2—The pinch point for the case of Fig. 1. Q_H and Q_R are minimum heating and cooling utilities consumption corresponding to the design value of ΔT_{min}. The shaded net area represents production of entropy as well as total exergy change. $\Delta\eta_{min}$ is the value corresponding to ΔT_{min}.

and the inverse constraint immediately below the pinch. These constraints are necessary so as not to violate ΔT_{min}. Once the temperatures go away from the pinch temperature, the constraints vanish.

In all cases the production of entropy for the new configuration is found from the exergy balance, Eq. (2), taking into account that the exergy change between any two streams can be estimated using Eq. (4) (Rotstein, 1983):

$$\hat{B}_2 - \hat{E}_1 = \hat{c}_p (T_2 - T_1) \left[1 - \frac{T_o}{(T_2 - T_1)_{m\ell}} \right] \qquad (4)$$

Case study for application of theory

To accomplish the stated objectives of this work a case study was made. For this purpose the results from an energy auditing of an existing tomato paste plant was used (Singh et al., 1980). The data reported in the reference were materials and energy. Since the above procedures require temperature data, all temperatures were back-calculated from the corresponding energy values and the results cross-checked at a similar local plant.

RESULTS & DISCUSSION

THE COMBINED back-calculation and cross-checks resulted in minor differences with respect to data reported by Singh et al. (1980) and by Rotstein (1983). The resulting base case is illustrated in Fig. 3. All relevant streams have been identified with a number. Streams 6 and 7, which are not shown in the figure, correspond to the 348°K recycle steam from the second to the first effect and concentrate leaving the second effect at 348°K. Stream numbers and properties are summarized in Table 1.

Before proceeding with heat integration, a heuristic rule is proposed: The final temperature of all hot streams which cool by losing heat to the atmosphere should be equal to the room temperature plus ΔT_{min}. This rule is justified in the Appendix.

Heat integration with the existing pinch

The algorithm described in Eq. (3) through (13) was

applied for ΔT_{min} of 5, 10, 15, 20, 25, 30 and 40°K. Table 2 shows the resulting hot and cold stream pinch temperatures and the corresponding minimum utility requirements. The result of the design procedure for ΔT_{min} = 25°K is shown, as an example, in Fig. 4. The matches are the same for all other ΔT_{min} although for ΔT_{min} = 5°K there is the option of matching streams 9 and 10 with streams 6 and 7. This would result in a 7% increase in fixed cost. The different solutions will be compared below, together with those resulting from removing the pinch point.

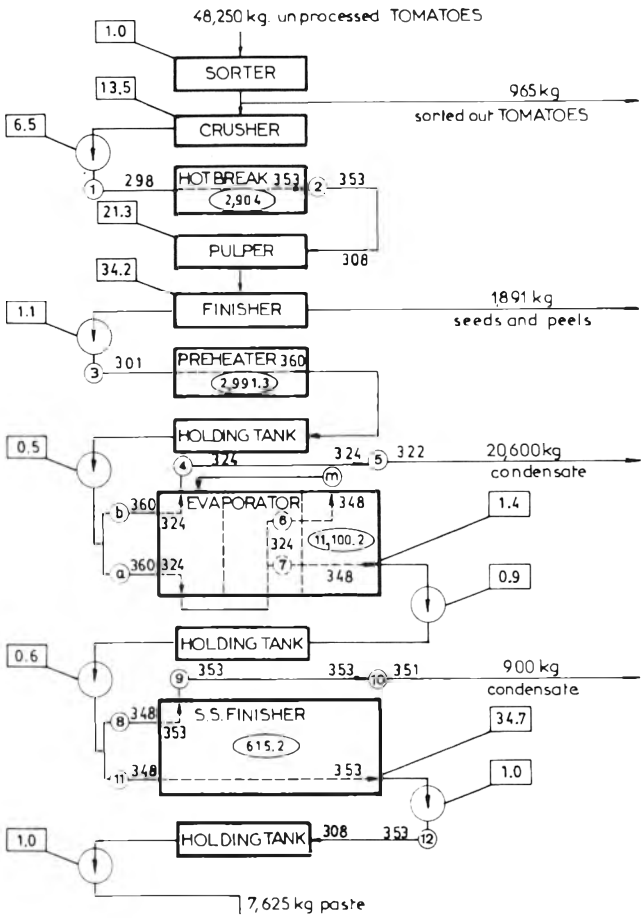


Fig. 3—Tomato paste processing plant. Basis: 1 hour. Ovals indicate heating loads. Rectangles indicate power consumption. Circles, stream number.

Heat integration modifying the existing pinch point

In the previous case streams 4 and 5 were not included in the analysis because they could not be used for heat integration in the existing configuration. If the pinch point is to be modified it means that changes in the configuration will be acceptable. Thus these streams are incorporated into the analysis. Use of the algorithm for ΔT_{min} = 10°K resulted in a pinch temperature of 324°K for the hot superstream. The location of the pinch is shown in Fig. 5. To replace this pinch it is required to go up in the temperatures of streams 4 and 5, vapor leaving the evaporator first effect, or down in those of streams 1 and 3. The latter involves lowering the temperatures of streams 6 and 7 in the first effect of the evaporator. Since these temperatures are tied to the temperature of the hot stream 4, which leaves the second effect, no change can be accomplished this way. On the other hand streams 4 and 5 can operate at a higher temperature using vapor recompression, a technologically feasible alternative (Worral, 1982). This modification will not change the temperatures of streams 6 and 7, which belong to the second effect, as it would occur with a straightforward increase of temperature of streams 4 + 5. Successive trials with increasing pressures using ΔT_{min} = 10°K lead to a condition of maximum recovery, corresponding to compressing stream 4 via a 3,104 HP compressor to 363°K. The new pinch point is 301°K for the cold stream, Q_R = 20.7 kWh and Q_H = 1,317.5 kWh. Heat integration was done as before, resulting in a network with six heat exchangers, two steam heaters and one cooling unit.

Due to the excessively large size of the compressor, two different alternatives were considered: One, using two smaller compressors (860 and 678 HP respectively) to compress the vapor from the evaporative effects, resulting in a pinch point at 324°K, Q_R = 1,980.1 kWh and Q_H = 2,961.5 kWh. The second one, imposing a ΔT_{min} = 5°K in the evaporator (Worrall, 1982, Dinnage, 1970), requiring compressors even smaller (766 HP and 567 HP, respectively), but, as expected, larger utilities: Q_R = 2,267.1 kWh and Q_H = 3,123.6 kWh, the pinch point being the same, at 324°K.

Table 1—Streams and heat loads in tomato paste plant

Stream	Thermal description	Heat capacity flow rate, C kW/K	Latent heat flow wλ, kW	T_s °K	T_t °K	Heat load kW
1 (cold)	Hot break heating	52.8	–	298	353	−2,904
2 (hot)	Cooling after hot break	52.8	–	353	308[a]	2,376
3 (cold)	Preheater heating	50.7	–	301[a]	360	−2,991.3
4 (hot)	Condensing water vapor from 2nd effect evaporation	–	13,615.1	324	324	13,615.1
5 (hot)	Condensate sub-cooling	24.0	–	324	322	48
6 (cold)	Heating and vaporization of water in first effect	16.2	10,509.8	324	348	−10,898.6
7 (cold)	Paste heating in evaporator first effect	8.4	–	324	348	−201.6
8 (cold)	Heating and vaporization of water in finisher	0.8	577.2	348	353	−581.2
9 (hot)	Condensing water vapor from finisher	–	577.2	353	353	577.2
10 (hot)	Condensate subcooling	1	–	353	351	2
11 (cold)	Paste heated in finisher	6.8	–	348	353	−34.0
12 (hot)	Cooling after finisher	6.9	–	353	308[a]	310.5

[a] Corresponds to ΔT_{min} = 10°C

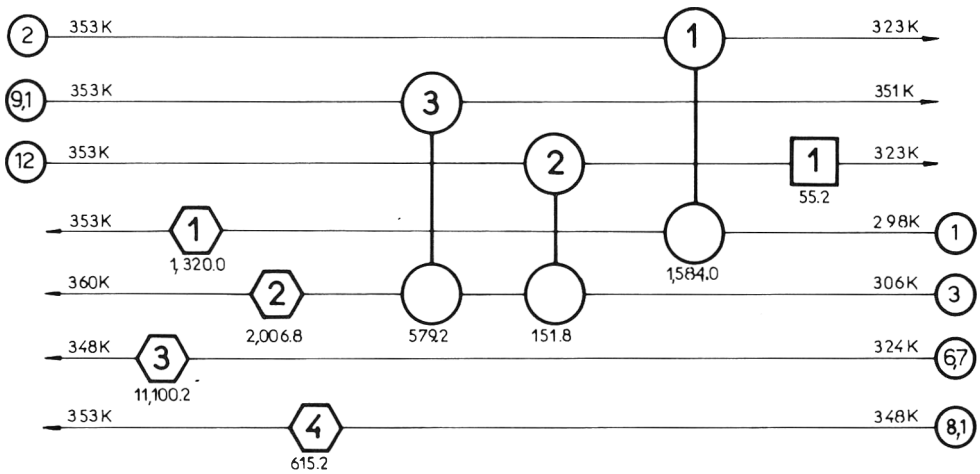


Fig. 4—Heat integrated structure for ΔT_{min} = 25°K. Squares are cooling units using cooling water, hexagons are steam heaters, tied circles represent heat exchange matches.

Table 2—Pinch temperatures and minimum utility requirements for different ΔT_{min}

ΔT_{min} °K	Pinch temperature Hot stream °K	Cold stream °K	Q_R kWh	Q_H kWh
5	305	300	13.8	14,111.0
10	311	301	20.7	14,365.7
15	318	303	34.5	14,576.6
20	324	304	41.4	14.831.3
25	331	306	55.2	15,042.2
30	337	307	62.1	15,296.9
40	353	313	581.3	16,210.3

Preliminary screening of alternatives

A preliminary screening of alternatives was made on the basis of estimating the cost of new equipment and comparing it with savings in utilities. This is customary procedure (Linnhoff and Flower, 1978), although before the actual investment is materialized a more detailed study is necessary, taking into account operability, start-up and shut-off procedures, etc. The basis for cost estimation is shown in Table 3. Heat transfer coefficients were obtained from Goose and Binsted (1973). Utility costs are from Duckham and Fleming (1976). Compressor costs are from Peters and Timmerhaus (1968). Values were adjusted for inflation following Kohn (1979).

Table 4 shows final results of the preliminary screening. Fixed cost is the depreciated cost of heat exchangers and other equipment. Total cost is fixed plus utilities cost. Savings refer to utilities which are not used because of heat integration. Preliminary profit estimate is the difference between savings and fixed cost. Return on original investment (ROI) is used to measure profitability (Thorn and Carlson, 1970). It is the percentage relationship of profit over fixed cost. If the pinch is not removed, the best alternative from a profitability standpoint corresponds to ΔT_{min} = 30°K. From a thermodynamic standpoint the best alternative, the one which produces less entropy, corresponds to ΔT_{min} = 5°K. When the pinch is removed (networks 9 to 11 in Table 4), the best alternative in both cases corresponds to vapor recompression using ΔT_{min} = 5°K. This may not be the ultimate answer in a particular situation where utilities are scarce. In that case the new pinch obtained by recompression of the vapor from the evaporator second effect would be more desirable. Fig. 6 shows the optimal flowsheet when the selection is based on return on original investment. It should be noted that the heat exchanger E2 in Fig. 5, where diluted paste is heated with full-strength paste, is subject to fouling and may require extensive piping to accomplish the match. Since elimination of E2 would only imply at the most an 8% decrease in net profit, it can be skipped without changing the conclusions.

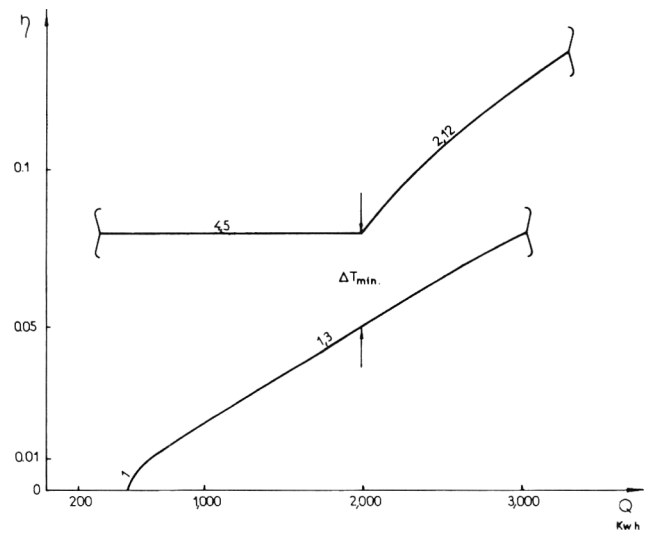


Fig. 5—Location of the pinch temperatures for ΔT_{min} = 10°K. Streams 4 and 5 are included in the analysis.

Relationship between alternative energy saving structures

When structural changes such as pinch-removing are allowed, the energy saving task becomes open ended. It seems relevant then to answer the questions posed in the introduction. Fig. 7 puts together the data for all cases, plotting both savings of utility and ROI as a function of production of entropy. It can be seen that there is a thermodynamic logic tying all cases: the less entropy is produced, the more is saved in utilities consumption. This logic does not extend to ROI because to save utilities there is a need to invest an increasing amount of fixed capital. Thus there is an optimum value which cannot be foretold. The issue is clearly shown when structures 1 through 8 are considered: the more energy-saving structure 8 corresponds to the lower production of entropy, while in terms of ROI there is an intermediate optimum. When the pinch is removed, the new structures performance is alligned with the previous ones in terms of utility savings, but in terms of ROI a new line appears.

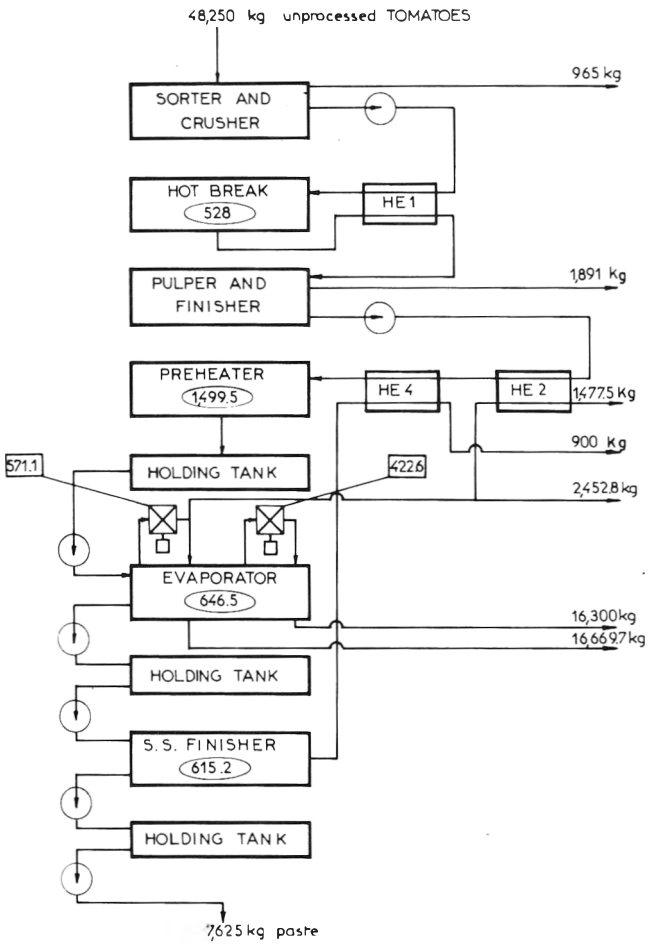


Fig. 6—Optimal flowsheet.

Table 3—Basis for cost estimation

Overall heat transfer coefficients, U	
steam – diluted paste	750 W/m^2 K
diluted paste – diluted paste	560 W/m^2 K
diluted paste – concentrated paste	400 W/m^2 K
Capital cost	6000 $A^{0.6}$
Depreciation	12 years
Compressors cost	\$ 4,112 $P^{0.62}$
Steam cost	\$ 0.006 /kg
Power	\$ 0.045 /kg
Cooling water	\$ 1.58 x 10^{-5} /kg

CONCLUSIONS

THIS WORK SHOWS that heat recovery in a food processing plant can be accomplished by systematic development of heat integration solutions, as opposed to inspection and local decision. In particular, it is shown that for an existing tomato paste plant savings can be accomplished in the order of a quarter of a million dollars per year, representing a return on original investment of 275%.

The different heat integration solutions involved changes in plant configuration: from minor ones, incorporating new heat exchangers, to major ones involving vapor recompression. It was shown that there is a thermodynamic logic that ties together all solutions: the less is the production of entropy, the more utilities are saved. This logic does not carry through to economics: in terms of return on original investment there are optimal solutions which cannot be predicted before hand.

NOTATION

A = heat exchange area, m^2
B = exergy, kWh
C = heat capacity flow rate, kW/K
P = power, kW
Q = heat flow, kW
R = property production, (if entropy, kWh/K)
T = temperature, °K
U = overall heat transfer coefficient, kW/m^{2}K
W = work, kWh
w = mass flow rate, kg/h
c = specific heat, kWh/kg K

Greek letters

η = Carnot efficiency

Subscripts

cu = cooling utility
hu = heating utility
H = heating
R = cooling
ℓ = losses
max = maximum value
min = minimum value
o = surroundings state
p = constant pressure
s = entropy
u = useful

Symbols

⁻ = per kg
• = derivative with respect to time

Table 4—Final results of preliminary screening

Network	Fixed cost, \$/year	Total cost, \$/year	Savings \$/year	Preliminary profit estimate \$/year	$T_o R_s$ kWh	Return on original Investment (%)
1) Initial	–	455,778.2	–	–	4,702.2	–
2) ΔT_{min} = 40 K	5,838.5	441,380.43	20,236.87	14,398.37	4,505.5	246.6
3) ΔT_{min} = 30 K	11,894.8	424,474.16	43,199.44	31,304.64	4,244.01	263.18
4) ΔT_{min} = 25 K	13,765	419,941.29	49,602.51	35,500.46	4,119.9	234.70
5) ΔT_{min} = 20 K	16,404	417,278.34	54,904.46	38,500.46	4,119.9	234.70
6) ΔT_{min} = 15 K	20,100	414,481.26	61,307.54	41,297.54	4,037.7	206.38
7) ΔT_{min} = 10 K	25,734	414,903.31	66,609.49	40,875.49	3,974.9	158.84
8) ΔT_{min} = 5 K	38,300	421,066.24	73,012.56	34,712.56	3,900.5	90.63
9) V.R.[a] stream 4	81,985	377,814.5	159,949.3	77,964.3	2,651.4	95.09
10) V.R.[a] streams 4 and 6	72,183	286,544.53	241,417.27	169,234.27	1,989.0	234.45
11) V.R.[a] streams 4 and 6	67,470	270,194.9	253,053.9	185,583.9	1,858.5	275.06

[a] V.R. = Vapor recompression

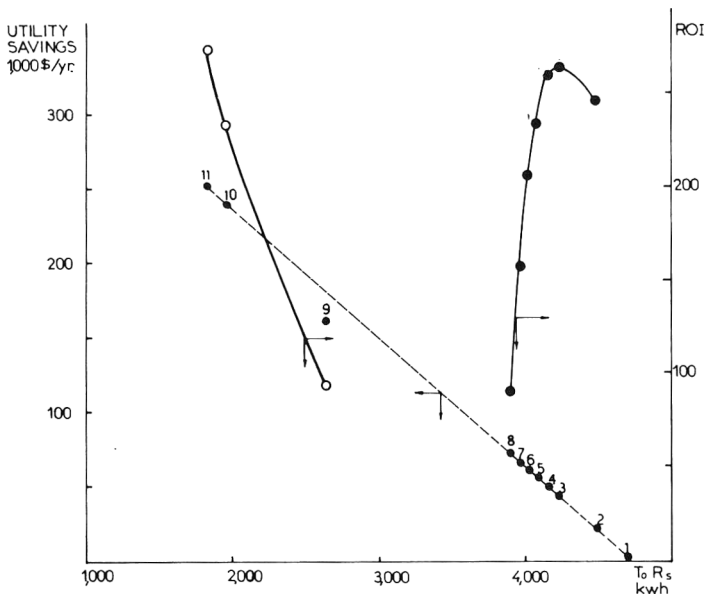


Fig. 7—Savings in utilities and return on original investment as a function of entropy production for all cases studied. Numbers on data points refer to the case as described in Table 4.

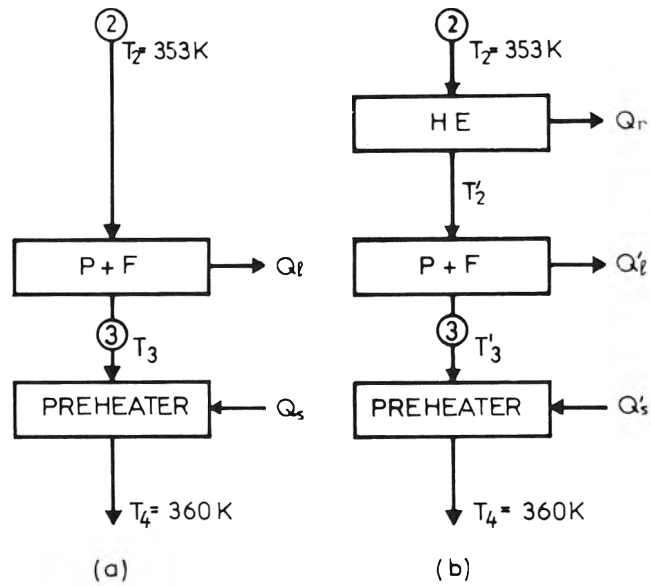


Fig. 8—Assigning temperatures for heat integration. P + F represents pulper and finisher, HE is the hot side of the heat exchanger to be incorporated. Case (a) is with no heat integration, case (b) corresponds to an eventual heat integration match with an unspecified stream.

APPENDIX

Heuristic rule for the final temperature of hot streams

The proposed rule states that the final temperature of all hot streams should be equal to $T_o + \Delta T_{min}$. Consider for example the stream leaving the hot break. It will lose heat until it reaches the preheater, where it will be heated again. Thus, it is a likely candidate for heat integration and its entering temperature to the preheater must take this fact into account. To do this the system as it is, from the hot break exit to the preheater exit must be compared with an analogous system where stream 2 releases Q_r kWh to an arbitrary stream in a suitable heat exchanger. The case is illustrated in Fig. 8. Say the heat loss and preheater heat load are Q_ℓ and Q_s in the first case and Q'_ℓ and Q'_s in the second case. The difference between heat recovered and the increment in preheater head load is:

$$\Delta Q_r = Q_r - (Q'_s - Q_s) \qquad (A1)$$

Replacing in terms of the corresponding heat capacity flow rates and temperatures:

$$\Delta Q_r = C_2 (T_2 - T'_2) - C_3 (T_4 - T'_3) - C_3 (T_4 - T_3) \qquad (A2)$$

Heat losses to the surroundings tie T_2 with T_3 and T'_2 with T'_3:

$$Q_\ell = (UA)_\ell (T_2 - T_3)_{m\ell} \qquad (A3)$$

$$Q'_\ell = (UA)_\ell (T'_2 - T'_3)_{m\ell} \qquad (A4)$$

Using Eq. (A3) and (A4) in Eq. (A2) and rearranging:

$$\Delta Q_r = a (T_2 - T'_2) \qquad (A5)$$

where:

$$a = C_2 - C_3 \exp [-(UA)_\ell / C_3] \qquad (A6)$$

Since $a > 0$ and T_2 is given it follows that to maximize the heat recovery Q_r requires minimizing T'_3. The lower boundary for T'_3 is:

$$T'_2 = T_o + \Delta T_{min} \qquad (A7)$$

since no match with cold streams will be feasible below this temperature. The same applies to the paste exit temperature.

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Ca, Fe and Zn Content of 'Jewel' Sweet Potato Greens as Affected by Harvesting Practices

R. D. PACE, T. E. SIBIYA, B. R. PHILLS, and G. G. DULL

ABSTRACT

A total of 64 "Jewel" sweet potato green samples collected at the end of the growing season, were analyzed to determine and compare the effects of multiple topping on dry matter, calcium, iron and zinc contents of "younger" 4-inch leaf and stem-petiole tips with "older" 4-inch portions of the same material. Older material contained the highest concentration of the minerals, and the concentration in both "young" and "old" plant material was unaffected by yearly planting. When the mineral content was compared to the Recommended Daily Dietary Allowance for adults it was relatively good.

INTRODUCTION

THE MOST POPULAR leafy green vegetables grown in home gardens of many Americans are turnips, kale, mustard and collards. However, a readily available supply of these vegetables during the hottest months (June, July, August and Stptember) of the year is limited due to climatic conditions. Alternately, sweet potato greens are available and could serve as a nutritious fresh vegetable supplement.

The practice of consuming sweet potato greens as a vegetable in the United States is not as popular as it is in foreign countries. Liberians consume the vegetable on a daily basis (As-Saqui, 1982), and in China, the leaves, stems and petioles are eaten separately to add dietary variety. Thailand, the Philippines, Indonesia and Malaysia are other countries where the vegetable is also used on a regular basis.

Reported values for Ca, Fe and Zn in sweet potato greens are higher than quantities contained in spinach and cabbage on a wet weight basis (Villareal et al., 1978). Harland et al. (1980) stated that Ca and Fe in the total diet of adults meet 100% of the U.S. RDA with Zn meeting 89% of it; and that leafy green vegetables in the total diet contribute a good portion of the requirement of each mineral, i.e. Ca at 21 mg/day, Fe at 0.3 mg/day and Zn at 0.2 mg/day. Murphy et al. (1975), reported that zinc content in green leafy vegetables ranged from 0.4 - 0.8 mg/100g. Additionally, mineral content of topped (multiple harvested) sweet potato greens tends to increase around the middle (77 - 84 days) and latter (112 - 119 days) parts of the growing season (Gonzales et al., 1977).

Results in the literature on nutrient content and the affect of harvesting practices are reported from countries where the environment differs from that in the United States; therefore, this research was conducted to determine the effect of multiple harvesting practices on dry matter, Ca, Fe and Zn contents in "younger" and "older" sections of the sweet potato green during two crop years.

Author Pace is Associate Professor of Human Nutrition, Dept. of Home Economics, Tuskegee Institute. Author Sibiya is affiliated with The Univ. of Swaziland in Luyengo, Swaziland (Southern Africa). Author Phills is Professor of Horticulture and Director of the George Washington Carver Agriculture Experiment Station, Tuskegee Institute. Authors Dull is affiliated with the USDA-ARS Richard B. Russell Agricultural Research Center, Athens, GA.

MATERIALS & METHODS

Experimental design

This research was conducted on the Tuskegee Institute Vegetable Farm on Franklin Road during 2 years and at two locations. During each year, the experimental field layout included two treatments (topped and nontopped) for "Jewel" sweet potato greens. Each treatment consisted of a 9m row replicated four times. Topped plants were cut back to within 0.6m from the base of the plant during three time periods (45, 90, 125 days). Only 20.32 cm portions ("younger" and "older") from the samples cutback during the last period were collected for the determination of dry matter, Ca, Fe and Zn. The two treatments including both "younger" and "older" greens separated into leaves and stems-petioles replicated four times produced a total of 64 samples. The "younger" portion refers to the initial 10 cm part of the tip while the "older" portion refers to the second 10 cm part from the tip.

Field care and treatments

Plants were kept free of weeds as much as possible and withered plants were replaced when needed with plants from the guard rows to assure that each row had 30 plants throughout the growth period.

Sample collection and preparation

At the end of 125 days, 20.32 cm portions from the tip (to include "younger" and "older" portions) of the 30 vines per row were cut and placed into pre-labelled bags according to treatments and replications. Sample aliquots of each replication were washed and divided into leaves and stems-petioles. Approximately 200g of each portion were homogenized for 5 min in a Hamilton-Beach Food Processor and placed into pre-labelled freezer bags.

Sample drying and storage

Frozen samples were freeze-dried in a Vacudyne Freeze Dryer Pilot plant size Model VPFD-CX/with a plate temperature of 27 - 30°C. Weights of the dried samples were recorded to determine their dry matter (DM) content. Dry weights were determined by standard AOAC methods (1980). The freeze-dried samples were then shelved and later analyzed for Ca, Fe and Zn by Atomic Absorption Spectrophotometry.

Mineral determinations

Standard machine setting conditions for Atomic Absorption Spectrophotometry of each element as described by Perkin Elmer (1982) were followed. A modified wet digestion method by McHarb et al. (1976) was used to digest all the organic plant material. A half gram of dried sample was placed in 100 mL Tecator brand constricted digestion tubes. Ten milliliters concentrated HNO_3 acid were added to each tube and the contents were allowed to

Table 1—Dry matter content of "younger" and "older" leaves and stems-petioles of "Jewel" sweet potato greens[a,b]

Source	Dry matter (g %)
"Younger" leaf tips	17.27 ± 1.15[c]
"Older" leaves	18.78 ± 1.12[c]
"Younger" stem-petioles tips	12.08 ± 0.66[d]
"Older" stems-petioles	12.82 ± 0.41[d]

[a] Means ± S.E. are based on eight observations.
[b] Any two means with the same superscript are not significantly different using Duncan's Multiple Range Test at the 5% level probability.

Table 2—Calcium, iron and zinc content in "Jewel" sweet potato sources[a,b]

Source	Calcium		Iron		Zinc	
	Dry Wt.	Wet Wt.	Dry Wt.	Wet Wt.	Dry Wt.	Wet Wt.
	mg/100g					
"Younger" leaf tips	836 ± 42[c]	150 ± 19[f]	10.88 ± 0.41[c]	1.78 ± 0.11[h]	2.88 ± 0.15[c]	0.49 ± 0.03[g]
"Older" leaves	1144 ± 43[d]	217 ± 18[g]	13.37 ± 0.73[d]	2.36 ± 0.32[g]	2.48 ± 0.13[d]	0.46 ± 0.03[g]
"Younger" stem-petiole tips	931 ± 94[c]	118 ± 14[e]	4.69 ± 0.18[e]	0.52 ± 0.11[i]	1.58 ± 0.10[f]	0.18 ± 0.01[h]
"Older" stems-petioles	852 ± 31[c]	109 ± 5[e]	4.57 ± 0.38[e]	0.55 ± 0.11[i]	1.23 ± 0.12[e]	0.16 ± 0.02[h]
RDA	800 mg		14 mg		15 mg	

[a] Means ± S.E. based on eight observations.
[b] Any two means in the same column with the same superscript are not significantly different using Duncan's Multiple Range test at $P < 0.05$.

digest in a Tecator DS 12 system at 160°C for 3 hr. Cooled samples were brought to 100 mL with deionized water.

RESULTS & DISCUSSION

Dry matter

Dry matter content from fields 1 and 2 in leaves and stems-petioles are shown in Table 1. The data from fields 1 and 2 are combined since there were no significant differences between fields. Leaves had a significantly higher DM content than did stems-petioles which suggests an increase in the quantity of organic and inorganic nutrients being deposited in the former.

Calcium, iron and zinc content in sweet potato leaves

Data on the mineral content in selected sources of "Jewel" sweet potato greens are contained in Table 2. Older leaves contained significantly more Ca than did leaf tips and stems-petioles. Zinc content in 10 cm samples of stems-petioles and leaves decreased as the plant aged, which was the opposite for Ca and Fe. The fact that leaves are the site for most photosynthetic reactions in plant cells could account for the high mineral concentrations present in them. Additionally, the older plant leaves contained the highest mineral concentration possibly because they were closest to the root where nutrient uptake from the soil occurred. The comparison between "younger" and "older" plant material is of significance since this information has not been previously reported. Although "older" plant material contained the highest mineral content, little is known concerning the tenderness of this plant material for human consumption.

During the second growing season, nontopping of the "older" plant material enhanced only Fe deposition significantly. Nontopping did not elicit this response in younger plant material. Ca and Zn were also not affected by treatment effects.

For food and nutrition scientists and consumers of interest also is the mineral contribution from sweet potato greens to the adult Recommended Daily Dietary Allowance (1980). For Ca the content ranged from 104 - 143% of the RDA on a dry weight basis and 13 - 27% on a wet weight basis. For iron the contribution ranged from 32 - 96% on a dry weight basis and from 3 - 17% on a wet weight basis. Zinc on the other hand, contributes much less (8 - 19% on a dry weight basis and 1 - 3% on a wet weight basis) than does Ca or Fe. These data support those reported by Harland et al. (1980) and Murphy et al. (1975) for green leafy vegetables. Both Ca and Fe content of sweet potato greens make them a relatively good source of these minerals.

CONCLUSIONS

SWEET POTATO GREENS are as nutritious in Ca and Fe as are other green leafy vegetables. The results also suggest that the "older" rather than "younger" plant material should be eaten since this contains more minerals. Collection of this vegetable over the growing season would not significantly alter the contribution of these minerals in the diet.

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Rapid Determination of Total and Solid Fat Contents in Chocolate Products by Pulsed Nuclear Magnetic Resonance

H. K. LEUNG, G. R. ANDERSON, and P. J. NORR

ABSTRACT

A rapid pulsed NMR method of fat analysis in chocolate without prior solvent extraction was developed. Total fat content of 43 chocolate products was determined at 60°C using sucrose-oil mixtures as standards. Solid fat content (SFC) was calculated from the liquid oil content at different temperatures. Total fat content agreed to 0.90% standard error of prediction as compared with the Soxhlet method. SFC as determined by NMR correlated well with the dilatometry (r = 0.98) and the DSC (r = 0.96) results. This technique is suitable for quality control of chocolate and other low-moisture, high-fat products.

INTRODUCTION

THE USE of nuclear magnetic resonance (NMR) in fat analysis has received considerable attention in recent years. It is rapid, nondestructive, requires no weighing, and is easily adaptable to on-line measurement. In the 60's and early 70's, continuous wave (cw) NMR was utilized in the determination of total fat content (Alexander et al., 1967; Wiggall et al., 1970; Shanbhag et al., 1971) and solid fat content (Walker and Bosin, 1971). Protons from liquid fat give a narrow absorption peak while solid protons yield a broad band. This technique has been replaced by pulsed NMR in which the decay of signal after a short radiofrequency (rf) pulse is monitored (Ferra and Becker, 1971). Solid protons have short relaxation times and therefore the signal decays rapidly. On the other hand, liquid proton signal decays at a much slower rate. By measuring the signal at two different times, the percentage of solid present can be determined. Using this principle, Praxis Corp. (San Antonio, TX) and Bruker/IBM Instruments, Inc. (Danbury, CT) have introduced low resolution pulsed NMR spectrometers designed for routine moisture and fat analysis.

Pulsed NMR has been used to determine oil content in seeds and other food products (Hester and Quine, 1977; Srinivasan, 1979; Lambelet, 1980; Tiwari and Burk, 1980; Brosio et al., 1981; Barry et al., 1983). However, the most important application of pulsed NMR in fat analysis is determination of solid fat content of shortening (Van Putte et al., 1975; Templeman et al., 1977; Desarzens et al., 1978; Madison and Hill, 1978; Van den Enden et al., 1978; Brosio et al., 1980; Van Boekel, 1981; Van den Enden et al., 1982; Suzuki and Murase, 1982; Lamblet, 1983a, b). The official AOCS method for solid fat is dilatometry (AOCS, 1974) which measures volume change of fat upon melting. It is time consuming and applicable only to fats with a solid fat index (SFI) of 50 or less at 10°C. Differential scanning calorimetry (DSC) has been suggested as a possible alternative to dilatometry. However, the DSC results are affected by heats of fusion, polymorphism, crystalization and melting rates, and sample size (Templeman et al., 1977; Van den Enden et al., 1982; Manning and Dimick, 1983; Sleeter, 1983). The small sample size required and the inherent difference in tempering procedure compared to dilatometry and NMR have also been cited as negative factors. Pulsed NMR provides a direct measure of solids in fat. It is generally considered as the most promising tool in solid fat determination.

Despite the extensive reserach on pulsed NMR measurement of fat in food systems, little or no attempt has been made to determine both total and solid fat of the same product. Also, previous studies on solid fat by pulsed NMR were conducted using pure oil or shortening. Little work has been done on direct solid fat determination of food products without solvent extraction.

Total and solid fat content at various temperatures are two of the critical parameters in chocolate products (Ninifie, 1980; Minson, 1984). They play important roles in controlling viscosity of the molten chocolate and texture of the finished product. Ideally, the fat should remain solid throughout the distribution and melt rapidly in the mouth.

Fat content of chocolate products is usually determined by Soxhlet extraction which takes about 2 days. This procedure is not suitable for quality control since the decision to accept or reject a shipment often needs to be made within an hour. The same is true for SFI which requires extraction of fat from the sample, followed by time-consuming dilatometry. Therefore, a rapid method for total and solid fat determination is needed for the quality control of chocolate as a product or ingredient. an early attempt to quantify chocolate fat using cw NMR was reported by Wiggall et al. (1970). They found good agreement between the NMR and extraction methods. The potential applications of cw NMR in the chocolate industry were discussed by Tuma and Sramkova (1982). Recently, Lambelet (1980) determined fat content of chocolate, cheese and dry milk with pulsed NMR. A similar pulsed NMR study was conducted by Desarzens and Michel (1982) on chocolates and related products.

The objectives of this study were: (1) to develop a rapid pulsed NMR method for total and solid fat analysis of chocolate without prior fat extraction; (2) to evaluate the NMR method for fat content against the standard solvent extraction method; and, (3) to compare solid fat by pulsed NMR with the results by dilatometry and DSC.

MATERIALS & METHODS

Materials

Forty-three chocolate, chocolate coating and chocolate creme samples were used in this study. The chocolate coatings were supplied by the Wilbur Chocolate Company (Lititz, PA) and the Shade Foods, Inc. (Belmont, CA). The chocolate cremes were prepared at the Pillsbury Research and Development Laboratory using chocolate coatings and other ingredients. Six chocolate products (Hershey's milk chocolate, Nestle milk chocolate, Nestle milk chocolate morsels, Baker's German's sweet chocolate, Guittard milk chocolate chips and Almond Bark chocolate coating) were purchased from a local grocery store.

The vegetable oil (Durkex 500) used to prepare standards for the NMR measurement was supplied by Durkee Industrial (Strongsville, OH). It has an A.O.M. stability rating of 350 hr maximum and is liquid at temperatures above 21°C. The powder sucrose (Pills-

Authors Anderson and Norr are with The Pillsbury Company, Research & Development Laboratories, 311 Second St., S.E., Minneapolis, MN 55414. Author Leung is with the Dept. of Food Science & Human Nutrition, Washington State Univ., Pullman, WA 99164-6330.

bury Ingredient Specification No. 19224) was obtained from California and Hawaiian Sugar Company. All other chemicals used were reagent grade.

Fat and moisture

Fat content of the chocolate samples was determined by the Soxhlet extraction using petroleum ether (AOAC, 1980). Solid fat index (SFI) of the fat extracted from the samples was determined by dilatometry (AOCS, 1974). Moisture content was determined by the vacuum oven method (AOAC, 1980).

Differential scanning calorimetry

A Perkin-Elmer DSC-2 Differential Scanning Calorimeter (DSC) equipped with a Model 3600 Thermal Analysis Data Station and an Intracooler I was used to quantify solid fat at different temperatures. About 20 mg of sample was placed in a tared aluminum DSC pan. The pan was hermetically sealed using a Perkin-Elmer Volatile Sample Sealer. An empty DSC pan was used as reference.

The sample was tempered in the DSC cell by: (1) cooling from 20°C to 0°C, and holding for 10 min; (2) warming from 0°C to 26.7°C and holding for 10 min; (3) cooling from 26.7°C to 0°C and holding for 10 min; (4) cooling from 0°C to −40°C and holding for 15 min. the tempering procedure was designed to simulate the sample conditioning used in dilatometry (AOCS, 1974). After tempering, the samples were scanned from −40 to 60°C at 5°/min. Solid fat content at various temperatures was determined using the Perkin-Elmer Partial Area Software Kit.

Nuclear magnetic resonance

The NMR measurement was performed using an IBM Minispec 20 Pulsed NMR Spectrometer operating at 20 MHz, with magnet temperature maintained at 40°C. The instrument is equipped with a microprocessor for analysis of the free induction decay (FID) curve of the proton at two points after a 90° pulse (Van den Enden et al., 1978; Van Putte, 1983). The signal measured at 12μ sec (A) and 70μ sec (B) after the pulse is proportional to the total protons and liquid protons present in the system, respectively. By incorporating a factor f to correct for the decay of signal from zero time to 12μ sec, the percent solid of the sample can be determined. On this basis, the Minispec 20 was programmed (EDM 1005) to give a direct readout of percent solid of the sample for each measurement.

$$\% \text{ Solid Phase} = f(A - kB)/[f(A - kB) + kB]$$

where f = solid correcton factor ≅ 1.4; and k = liquid correction factor ≅ 1.05.

The standard samples were prepared by mixing different proportions of powder sucrose and vegetable oil in beakers. Sucrose was used to simulate the high sugar content of the chocolate products. Percent oil of the standards were calculated from the weight of sucrose and oil in the mixtures. The samples were transferred to 10 mm NMR tubes, sealed with parafilm, and equilibrated to 21.1°, 33.3°, 37.8°, or 60°C in water baths before NMR measurement. Chocolate, coating and creme samples were melted at 60°C, transferred to NMR tubes and sealed. No weighing was required since the ratio of liquid protons to total protons was measured. The samples were tempered by holding at 60°C for 10 min, 0°C for 30 min and then at the desired temperature for 30 min before NMR measurement. This tempering procedure was proved satisfactory in our laboratory for a variety of shortenings. All of the samples were prepared in duplicate. The NMR measurement of each sample was repeated four times with a relaxation delay (trigger time) of 3 sec between pulses. The trigger time was selected to avoid saturation of the liquid oil (Farrar and Becker, 1971). Since the probe temperature was not controlled, the NMR measurement was performed immediately after the sample was removed from the water bath and wiped dry. The instrument was checked daily with calibraton standards supplied by IBM.

RESULTS & DISCUSSION

Total fat

Fig. 1 shows a typical standard curve relating percent solid by pulsed NMR at 60°C and fat content of sucrose-oil mixtures. The measurement was performed at 60°C because all of the fats in the chocolate products are in liquid form at this temperature. The standard curve may vary depending on the specific instrument, calibration standards, and instrument settings and adjustment.

The percent solid determined by NMR (y-axis) did not correspond exactly to the solid content (100-% fat) of the standards (Fig. 1) since the instrument was calibrated at room temperature using known polymer standards supplied by IBM. The discrepancy may be due to temperature effect on calibration and differences between the sucrose-oil mixtures and the polymer standards.

The fat content of 43 chocolate products determined by pulsed NMR are compared with the values by Soxhlet extraction (Fig. 2). The linear regression equation for the two sets of values was,

$$y = 0.935x + 2.09$$

where y = % fat by NMR; and x = % fat by Soxhlet. The correlation coefficient (r) was 0.977 and the standard error of prediction (SEP) was 0.90%.

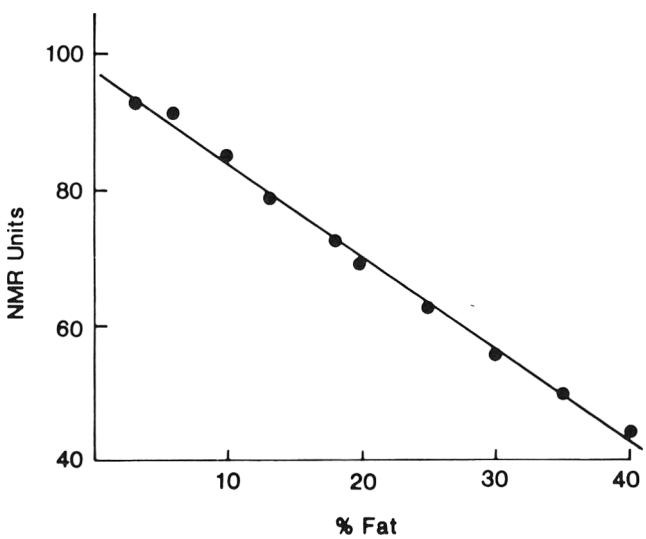


Fig. 1—A typical standard curve showing NMR readings at 60°C and oil content of sucrose-oil mixtures.

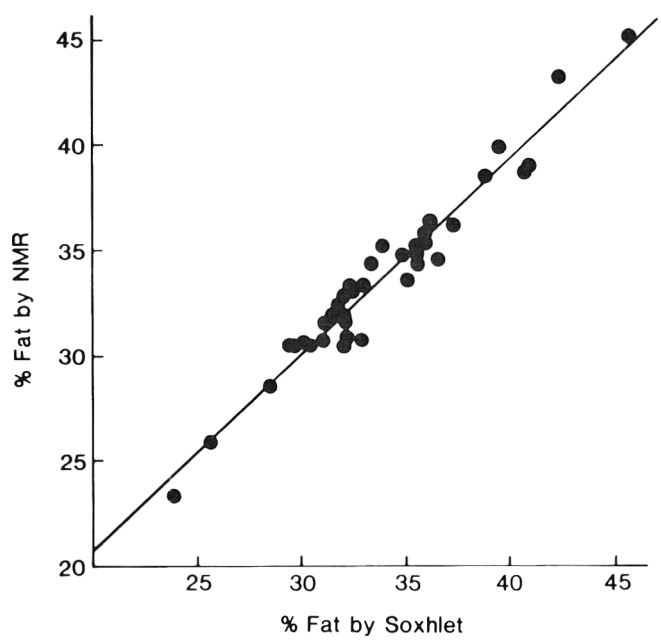


Fig. 2—Comparison of fat content determined by the pulsed NMR and Soxhlet methods for 43 chocolate samples.

The 43 samples used in total fat determination included milk chocolates, chocolate coatings and chocolate cremes with widely different composition of fat (Fig. 2). In previous studies, fat content of unknown chocolate samples were determined directly from a plot of NMR reading versus % fat by solvent extraction (McCarten, 1975; Lambelet, 1980; Desarzens and Michel, 1982; Jones, 1982). In the present study, the oil-sucrose mixtures were used as calibration standards. By not relying on the Soxhlet extraction as a primary standard, this procedure provides an independent method of measuring fat content.

In a pulsed NMR study of fat in cocoa products, Desarzens and Michel (1982) found that the spin echo technique yields better results than the free induction decay (FID) method. They attributed the difference to the presence of paramagnetic ions (iron, copper) in the samples. The spin echo method utilizes two pulses and requires pre-weighing of the sample. The FID method used in this study employs only one pulse and has been utilized as a standard technique in moisture, fat and solid fat determination. The rather large SEP obtained in the present study may be due in part to interference by paramagnetic ions. The spin echo technique for fat or moisture determination warrants further investigation.

Moisture content of the samples were less than 3%. At such a low moisture, water is in a tightly bound state and does not interfere with fat determinaton by pulsed NMR (Lambelet, 1980; Desarzens and Michel, 1982).

NMR and dilatometry

Pulsed NMR determination of liquid fat content at different temperatures was similar to that for total fat except that different standard curves were used. As temperature decreased, both the slope and intercept of the standard curve decreased. The correlation coefficients of the standard curves at 60°, 37.8°, 33.3°, 25.0°, and 21.1°C were 0.993 - 0.997. The overall standard deviation of NMR measurement was 0.28 NMR units.

Solid fat content (SFC) of the samples at each temperature was calculated as the difference between total fat and liquid fat at that particular temperature divided by the total fat content. Fig. 3 shows the relationship between the SFI of the extracted fat by dilatometry and the SFC of the chocolate products for 18 samples at 21.1°, 33.3°, and 37.8°C. The NMR and dilatometry values were linearly correlated (r = 0.980) according to the following equation:

$$y = 1.562x + 0.66$$

where y = SFC by NMR; and x = SFI by dilatometry.

Although NMR and dilatometry values correlated well with each other, there is considerable scattering of individual data points. In fact, the correlation is poor when only the data points of a single temperature are considered. Also, the NMR values were generally higher (slope = 1.56) than the dilatometry values. The differences between the two measurements may be due to two factors. First, pulsed NMR measures the relative amount of solid/liquid protons of the product, while dilatometry is a more indirect method based on volume change of fat during melting. Second, the tempering procedures used for pulsed NMR and dilatometry were different. Since cocoa and confectionery fats are subjected to polymorphism, different forms of fat crystal may exist in the samples tempered differently. Van den Enden and co-workers (1978; 1982) found that the relationship between pulsed NMR and dilatometry values vary depending on temperature, range of solid fat content, as well as the types of fat present. Thus the two techniques are inherently different and do not necessarily yield the same results. The slope (1.56), being larger than unity (1.0), suggests the NMR procedure enhanced the detectability of solids and provided added experiment leverage in measuring the solid/liquid ratio.

The procedure used in this study differed from the AOCS (1981) recommended method for SFC in two major aspects. The AOCS method calls for a Praxis Pulsed NMR Solid Fat Analyzer, whereas this study utilized an IBM Minispec-20 pulsed NMR spectrometer. The two instruments operate on the same principle and function similarly. Also, the AOCS method was deisgned to measure solid fat in fats and oils, whereas the present procedure determines liquid fat in chocolate products at different temperatures. Nevertheless, the two methods are expected to yield the same results since they are both based on the relative number of solid and liquid protons present in the sample.

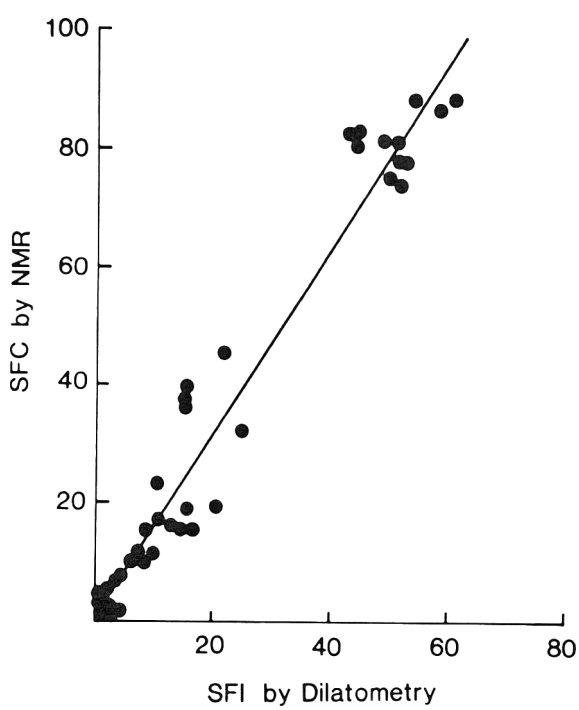


Fig. 3—Comparison of SFC by pulsed NMR and SFI by dilatometry for 18 chocolate samples at 21.1°, 33.3°, and 37.8°C.

NMR and DSC

The melting characteristics of 15 chocolate and chocolate coatings were determined using DSC. Fig. 4 shows the typical melting curves for a chocolate containing cocoa butter and a chocolate coating made with vegetable fat. The coating sample showed a second melting peak at about 34°C, which is absent in real chocolate. For most of the 15 samples, the fats were solid below 0°C and melted completely at 40 - 45°C. A distinct advantage of DSC over pulsed NMR and dilatometry is its ability to provide a "fingerprint" of melting behavior for each fat sample in a single scan.

When the DSC method is used, the SFC values at different temperatures are usually determined by measuring the partial area under the melting curve. This may lead to erroneous results since the heats of fusion of different fats may vary considerably. To test whether the area under the melting curve was proportional to the amount of fats present, fat content of the 15 samples was correlated with the total heat of fusion (peak area) per mg sample. Analysis of the data indicated no significant correlation between fat content and heat of fusion (r = 0.17). This casts doubt on the accuracy of the DSC method in solid fat determination.

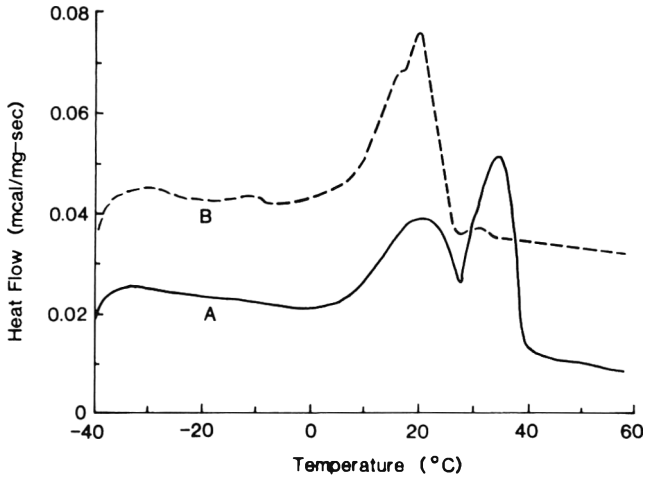


Fig. 4—DSC scans of (a) a chocolate coating and (b) a milk chocolate.

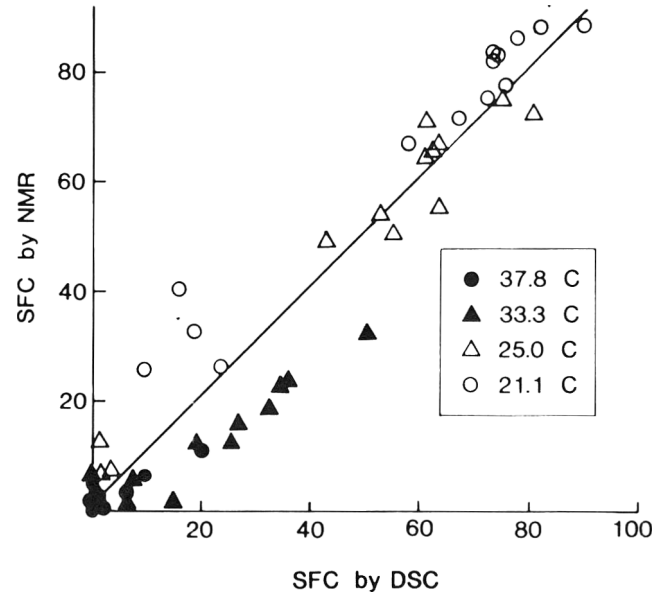


Fig. 5—Comparison of SFC by pulsed NMR and DSC for 15 chocolate samples at 21.1°, 25.0°, 33.3°, and 37.8°C.

Despite the shortcoming of the DSC method, SFC determined by pulsed NMR and DSC at 21.1°, 25.0°, 33.3°, and 37.8° were closely correlated (Fig. 5). Linear regression analysis (n = 60) yielded the following equation (r = 0.961);

$$y = 1.00x + 1.05$$

where y = SFC by NMR; and x = SFC by DSC. Although there were considerable variations between the NMR and DSC values, the regression equation showed a slope of 1.0 and an intercept of 1.0. This indicates a good overall agreement between the two methods.

Recently, Lambelet (1983a, b) compared the pulsed NMR and DSC methods for determining solid content of cocoa butter and milk fat. They found the DSC values to be consistently higher than the NMR values. This observation applied only to the 33.3°C readings in the present study (Fig. 5). It should be recognized that DSC is a "dynamic" method in which measurement is made at changing temperatures, whereas NMR is a "static" method in which sample was held at a constant temperature. Therefore, differences between the two method are expected.

The good correlation of the pulsed NMR results with the DSC and dilatometry values clearly demonstrates the potential of the new NMR procedure in quality control of chocolate and similar products.

CONCLUSIONS

A NEW PULSED NMR procedure for rapid determination of total and solid fat contents of chocolate products without prior fat extraction was developed. The method utilized sucrose-oil mixtures as standards, and measures liquid fat content of the samples at different temperatures. Standard error of predicton for total fat based on the standard Soxhlet extraction method was 0.90%. SFC determined by the new method correlated well with SFI by dilatometry (r = 0.98) and SFC by DSC (r = 0.96). Total fat determination required only 15 min and SFC about 50 min. The method is rapid, consumes no solvents, and is near ideal for quality control applications of chocolate and other low-mositure, high-fat products.

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Cocoa Seeds: Changes in Protein and Polysomal RNA During Development

PAUL J. FRITZ, KERI A. FRITZ, JOHN M. KAUFFMAN, GORDON R. PATTERSON, CHARLES A. ROBERTSON, DEAN A. STOESZ, and MELINDA R. WILSON

ABSTRACT

Amounts of soluble protein increased three- to four-fold while microsomal protein and polysomal RNA levels decreased by similar factors in cocoa seed extracts at different times during the developmental period of rapid cotyledon growth to fruit ripening. During early development, up to 90% of translatable mRNA was poly A(−). In mature seeds this decreased to about 75%. The radioactive polypeptides synthesized by a wheat germ *in vitro* translation system were the same whether the system was programmed with poly A(−) or poly (A+) RNA. Polypeptides synthesized in the *in vitro* translation system were electrophoretically similar to those extracted from intact seeds, testimony to the integrity of isolated messenger RNA.

INTRODUCTION

UNDERSTANDING the complexity of food sources, procurement, processing, preservation, and preparation have necessitated the use by food scientists of knowledge and techniques from many areas of basic and applied science. Thus, application of the methods of molecular biology to food science is a natural extension of the process of understanding this complexity. In this paper some molecular biological methods have been used in a beginning attempt to explain, at the molecular level, the nature and accumulation of substances in post harvest raw cocoa cotyledons. It is hoped that knowledge of this kind will be helpful in control of certain aspects of the manufacture of chocolate and cocoa products.

Developmental studies on cocoa beans have shown that 4 to 5 months after pollination, dramatic increases in seed lipid (Lehrian and Keeney, 1980), and methyl xanthine content (Senanayake and Wijesekera, 1971) occur at times when pod growth has ceased and seed protein concentrations seem to be decreasing (Zak and Keeney, 1976). Most of the lipid in ripening and mature cocoa seeds is triacylglycerol, comprising about 60% of seed weight and present in at least 100-fold excess over phospholipids, the next highest lipid component (Lehrian and Keeney, 1980). Protein constitutes only about 10% of mature cocoa seed weight (Zoumas and Finnegan, 1979).

The aim of the present work was to analyse quantitatively and qualitatively the cocoa seed protein and polysomal RNA at different stages of seed development to determine how protein concentrations changed during development and at what stage major proteins began to appear.

MATERIALS & METHODS

Plant material

Flowering cocoa trees in Hershey Foods Corporation's Chocolate World (Hershey, PA) were hand pollinated in April and May. The trees grow in a large greenhouse area with a natural photoperiod of 10 to 15 hr depending upon the season, and a minimum day temperature of 24°C. Pod growth was monitored by weekly measurements of length and circumference.

Some pods were from the Hershey Foods Corporation experimental research facility at Belize, Central America.

Protein extraction

Seeds were removed from pods and embryonic axes separated from cotyledons. Embryonic axes and cotyledons were weighed separately and homogenized in five volumes 60 mM Tris HCl, 14 mM β-mercaptoethanol, pH 7.8, using a glass homogenizer with Teflon pestle. The homogenizer was surrounded by crushed ice. Homogenates were centrifuged at 10,000 × *g* for 30 min at 4°C. Supernatants were centrifuged at 100,000 × *g* for 90 min at 4°C. Microsomal pellets were suspended in 1/2 volume 70 mM Tris HCl, pH 7.4, 0.25 M sucrose. Postmicrosomal supernatants were saved for protein analysis and electrophoresis. Microsomal suspensions in plastic tubes surrounded by ice, were sonicated using four 15-sec bursts in a Branson model 140 sonifier cell disruptor set at maximum intensity. Samples were analyzed quantitatively using the Pierce Chemical Company reagent and known amounts of bovine serum albumin as standards, and qualitatively by polyacrylamide gel electrophoresis (Laemmli, 1970). Twenty-five μL microsomal suspension and sufficient volume of post microsomal supernatant to give 100 μg protein were mixed with 10 μL sodium dodecyl sulfate (SDS) sample buffer, placed in a boiling water bath for 3 min, then placed in sample wells of a vertical electrophoresis apparatus. Electrophoresis was carried out for 3½ hr at 25 mA and 160 volts using 5% stacking gels and 12% separating gels. Following electrophoresis, the gel was stained overnight using 0.05% Coomassie Brilliant Blue R in 25% isopropanol, 10% acetic acid. Destaining was by diffusion in 7.5% acetic acid, 5% methanol.

mRNA isolation from developing cocoa seeds

Messenger RNA was isolated from polysome pellets using the procedure of Heinze et al. (1980) with modifications. After powdering with a mortar and pestle in liquid nitrogen, seeds were extracted in five volumes buffer A (400 mM KCl, 250 mM sucrose, 50 mM Tris pH 8.3, 10 mM Mg acetate, 7 mM β-mercaptoethanol, 2% Triton X-100) supplemented with 100 mM diethyldithiocarbamate (DDTC) and 1% polyvinyl pyrollidone (PVP) 10,000. After centrifuging through a 1.7M sucrose cushion, also supplemented with DDTC and PVP, polysome pellets were suspended in 1.5 mL buffer D (400 mM NaCl, 10 mM Tris pH 7.5, 1 mM EDTA, 0.5% SDS). After proteinase K (Beckman) digestion, samples were extracted once with an equal volume of phenol mixture (one part redistilled phenol saturated with a solution of Tris HCl 50 mM, pH 7.5; EDTA 1 mM; NaCl 400 mM; in one part chloroform-isopentanol 10:1). The aqueous phase was placed on a 1 × 3.5 cm oligo (dT) cellulose (Bethesda Research laboratories) column previously equilibrated at room temperature with buffer D. Unbound (poly A-) RNA fractions were combined and again placed on the column. Bound (poly A+) RNA was eluted with buffer D minus NaCl. Bound and unbound fractions were processed separately; 0.1 volume of 2M sodium acetate, pH 5.5, was added, followed by 2.5 volumes cold ethanol and samples were placed at −20°C overnight. When poly(A+) fractions were adjusted to 400 mM NaCl and chromatographed a second time, a negligible amount of poly(A−) RNA was detected. Hybridization experiments with [^{3}H]poly (dT) (Kauffman and Gross, 1974) verified the identity of the poly(A+) and poly(A−) RNAs.

RNA was recovered by centrifugation at 5,000 × *g* for 15 min. Pellets were dissolved in 3.0 mL water and RNA concentrations determined by 260 nm absorbance (A_{260} = 1 = 40 μg/mL). Samples were lyophilized and dissolved in a volume of water calculated to give concentrations of 4.4 and 0.18 μg/μL for poly(A−) and poly(A+) fractions, respectively.

Authors P.J. and K.A. Fritz, Kauffman, Robertson, Stoesz, and Wilson are with the Dept. of Pharmacology, The Milton S. Hershey Medical Center, The Pennsylvania State Univ. College of Medicine, Hershey, PA 17033. Author Patterson is affiliated with Hershey Foods Corp., Hershey, PA 17033.

Sucrose gradient sedimentation profile of cocoa seed polyribosomes

Polysome pellets were prepared from immature cocoa cotyledons (about 110 days after pollination), washed once with deionized water dissolved in 0.5 mL TKME buffer (40 mM Tris, pH 8.5; 200 mM KCl; 30 mM $MgCl_2$; 5 mM EDTA) and layered onto a 15 - 50% sucrose gradient prepared in TKM buffer (40 mM Tris, pH 8.5; 20 mM KCl; 10 mM $MgCl_2$). Centrifugation was for 15 hr at 4°C and 39,000 rpm in a Beckman SW40 rotor. Fractions (0.5 mL) were collected from the top with the aid of a Beckman fractionator and Gilson minipuls Rabbit peristaltic pump.

Sucrose gradient fractionation of poly(A+) RNA

Polysomal RNA from 115g immature cocoa cotyledons was processed through two oligo (dT) cellulose columns. Poly(A+) fractions were dialysed for 6 hr against 0.2M sodium acetate then precipitated overnight at −20°C with 2 volumes ethanol. The ethanol precipitate was dissolved in 0.5 mL FET (70% deionized formamide; 3 mM EDTA; 3 mM Tris pH 7.5), incubated for 15 min at 37°C then layered on a 5 - 20% sucrose gradient in FET. Centrifugation was for 16 hr at 39,000 rpm, 4°C in a Beckman SW40 rotor. Fractions (0.5 mL) were collected, precipitated with ethanol, dissolved in 5 mL water, lyophilized, dissolved in 100 μL H_2O, and translated in a wheat germ system using ^{35}S-labelled methionine as the tracer.

In vitro translation of cocoa seed mRNA

A nuclease-treated wheat germ in vitro translation kit with placental ribonuclease inhibitor (Bethesda Research Laboratories) was used. Reaction mixtures were 15 μL with 5 μCi[^{35}S] methionine (1500 Ci/mmol) or [^{3}H] leucine (132 Ci/nmol) (Amersham) and 1 μL of either poly(A+) or poly(A−) cocoa mRNA, globin mRNA, or water. Incubation was for 60 min at 25°C followed by treatment with 1.5 μL pancreatic RNAase (10 μg/μL) for 30 min at 30°C. Messenger RNA activity was determined by counting 2 μL of reaction mixtures on Whatman GFC filters after washing consecutively with 150 mL (a) ice cold 10% TCA for 10 min (b) 5% TCA, twice for 2 min each (c) 95% ethanol, twice for 2 min each. Filters were dried in air and counted in 5 mL Aquasol (New England Nuclear). [^{35}S]-methionine or [^{3}H]-leucine-labelled translation products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970) followed by fluorography using the En^{3}Hance procedure (New England Nuclear product information sheet).

RESULTS

THE COCOA POD growth curve (Fig. 1) is similar to growth curves of field grown pods (Schroeder, 1958) indicating that greenhouse grown pods were suitable as a model system. Although pod size was maximum about 110 days after pollination (DAP), seed maturation proceeded for an additional 60 to 70 days (Lehrian and Keeney, 1980). Beginning at 112 DAP, pods were harvested at weekly intervals, during August, September and October, up to 166 DAP. Up to 112 DAP, the seeds consisted of an inconspicuous embryo and a liquid endosperm surrounded by a thin white testa embedded in white pith (Lehrian and Keeney, 1980).

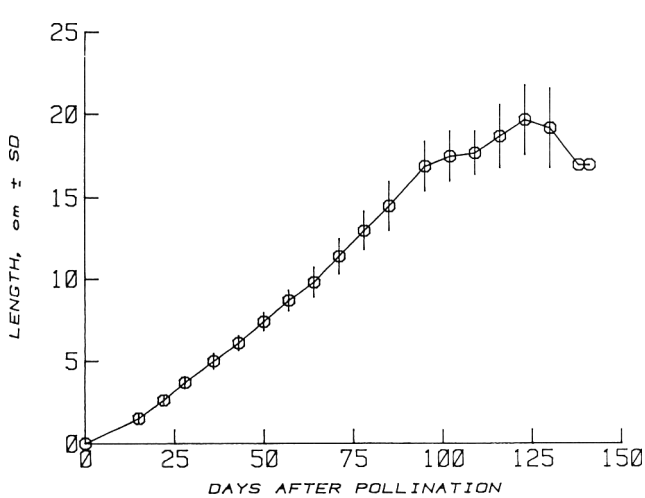


Fig. 1—Cocoa pod growth curve. Number of samples decreased from 20 at 15 DAP to 1 at 138 and 141 DAP because in early development some pods spontaneously aborted and in later stages some pods were picked for experimental purposes. Values are means ± standard deviation.

Protein

Quantitative. Cocoa seed soluble protein concentratons were low during early development, increased as development progressed, and reached a plateau as the seeds approached maturity (Table 1). Microsomal proteins were present in at least 100-fold lesser amounts than soluble proteins and, in contrast to the soluble proteins, decreased with seed development, as did the amounts of polysomal RNA (Table 1).

Qualitative. Seeds were analyzed 3 days after pod picking in Belize. Ages were estimated by pod length (Fig. 1) and by appearance of seeds. Very little protein could be detected at early developmental stages but, as is true of a number of higher plants, and indeed what appears to be a general principle of higher plant seed development (Yamagata et al., 1982), cocoa seed development was characterized by accumulation of relatively few proteins over a short, well-defined period. Beginning about 100 DAP, two prominent polypeptides around 20,000 and 28,000 daltons were present in the cytoplasmic fraction of cocoa seed extracts (Fig. 2C). At this early stage of development, high molecular weight proteins were prevalent in microsomal extracts, the most prominent, about 50,000 daltons (Fig. 2A). Mature seeds, in contrast, lacked high molecular weight microsomal proteins (Fig. 2B).

Cocoa seeds, like soybeans (Goldberg et al., 1981), are organized into two developmentally distinct systems, the embryonic axis and the cotyledons. The axis gives rise to the root, stem, and leaves of the plant after germination while the cotyledons are primarily responsible for synthesis and storage of food reserves (mostly cocoa butter) during seed development, and senesce after germination. There were no qualitative differences in protein patterns between cocoa embryonic axes and cotyledons but quantitatively there were higher amounts of soluble protein in embryonic axes and higher amounts of microsomal protein in cotyledons. Microsomal protein was greatly reduced in mature seeds while soluble protein increased (Table 1).

The gel electrophoresis patterns (Fig. 2) were obtained using sodium dodecyl sulfate denaturing gels which dissociate proteins into their component subunits. Thus the

Table 1—Protein and RNA content of developing cocoa seeds[a]

Days after Pollination	mg Protein per gram seed		μg Polysomal RNA per gram seed	
	Soluble	Microsomal	Poly (A+)	Poly (A−)
112	7.5	0.032	0.28	28.5
116	8.0	0.035	0.27	34.8
123	11.5	0.033	0.80	39.8
130	23.5	0.031	0.41	28.7
137	23.5	0.029	0.41	28.7
144	24.5	0.023	0.20	18.5
151	29.5	0.015	0.11	3.4
166	27.5	0.017	0.14	4.8

[a] Five seeds were used for protein determination; 40 - 45 seeds were used for RNA preparation.

observed polypeptide translation products gave no information about functional multi-subunit proteins that might be present in cocoa seeds during development.

RNA

Preparation from polysomes. Isolated polysomes of immature seeds (approximately 110 DAP) were of high integrity as seen by the sucrose gradient sedimentation profile (Fig. 3). There were only a few monomers and dimers but a wide range of heavier polysomes suggesting undegraded, intact mRNA-protein complexes. (Poly(A) rich mRNA from these polysomes was obtained by oligo (dT) cellulose chromatography. The size distribution of poly(A)-containing RNA was analyzed by sedimentation on 5 – 20% sucrose gradients (Fig. 4). Gradient fractions were used to program protein synthesis in a wheat germ *in vitro* translation system. The vast majority of active fractions were found in the 7 – 10S region of the gradient.

RNA quantitation. The amount of seed polysomal RNA was much lower at later stages of development, in contrast to the amount of soluble protein (Table 1). The concentration of soluble protein increased from 7.5 mg per gram seed at 112 DAP to about 24 mg per gram seed by 130 DAP and further changes did not occur. Thus, high soluble protein concentrations were maintained as the seed approached maturity at times when polysomal RNA levels were at their lowest.

In vitro translation products of cocoa seed messenger RNA. The validity of the wheat germ *in vitro* translation system, in terms of its ability to respond in a linear fashion to increasing amounts of cocoa seed mRNA, is shown in Fig. 5. Although the amount of mRNA decreased as the seed approached maturity, the specific activity, in terms of its ability to be translated in a wheat germ *in vitro* transla-

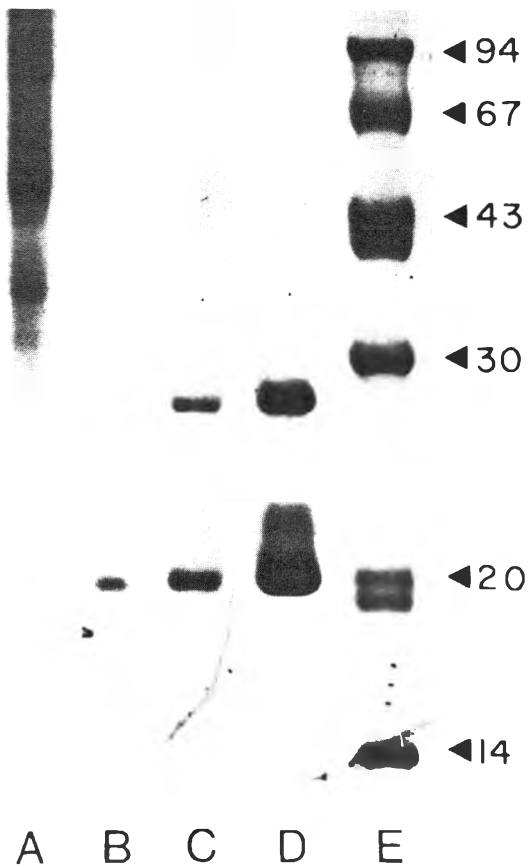


Fig. 2—Electrophoretic separation of proteins extracted from cocoa seeds: A – Sonicated cotyledonary microsomal pellet about 110 DAP, 35 µg protein; B – Sonicated cotyledonary microsomal pellet about 180 DAP, 33 µg protein; C – Embryonic axis post microsomal supernatant about 110 DAP, 100 µg protein; D – Embryonic axis post microsomal supernatant about 180 DAP, 100 µg protein; E – Molecular weight standards. Sizes in kilodaltons shown by arrows on right vertical axis.

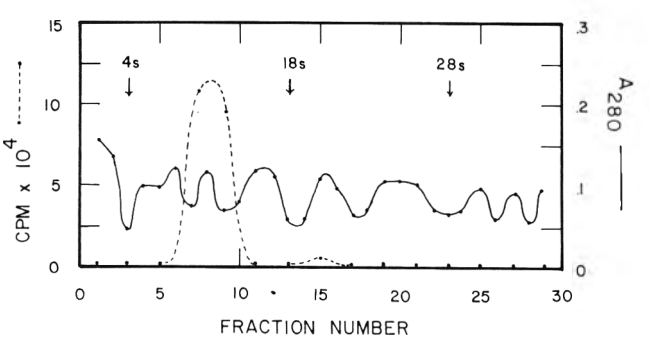


Fig. 4—Sucrose gradient fractionation of poly(A+) RNA.

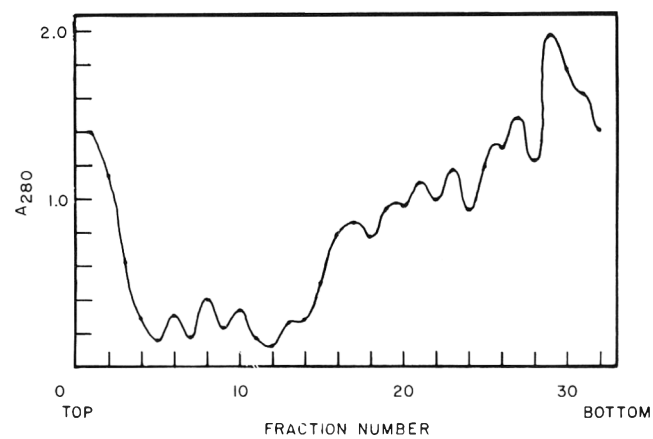


Fig. 3—Sucrose gradient sedimentation profile of cocoa seed polyribosomes.

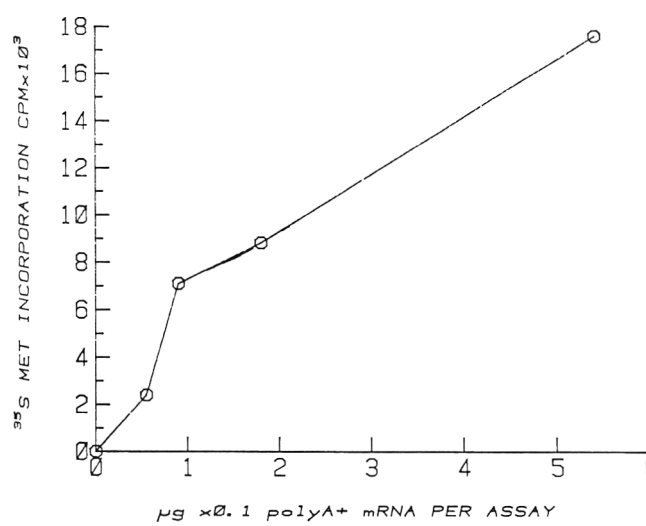


Fig. 5—In Vitro Translation of Cocoa Seed mRNA.
Poly(A+) mRNA isolated from cocoa seeds 130 DAP was added in varying amounts to the wheat germ in vitro translation system. Details of the protocol are given in Materials & Methods.

tion system, was essentially unchanged throughout the developmental period studied (Table 2). Poly(A+) mRNA was 10 to 20 times more active in the wheat germ system than poly(A−) RNA, since most of the poly(A−) RNA was ribosomal RNA. However, it should also be noted that the absolute amount of cocoa seed messenger RNA activity was greater in the RNA populations lacking polyadenylate tails. Moreover, examination by polyacrylamide gel electrophoresis of the proteins coded by cocoa seed messenger RNA demonstrated no differences between poly(A+) and poly(A−) samples (not shown).

Polypeptides synthesized by the *in vitro* translation system, programmed with poly(A+) messenger RNA from immature (Fig. 6A) and mature (Fig. 6B) cocoa beans, were examined by SDS-polyacrylamide gel electrophoresis. The major proteins found in the in vivo studies (Fig. 2) are also seen in these *in vitro* experiments, demonstrating the integrity of the isolated mRNA. Consistent with the increase in soluble protein during development (Table 1), greater amounts of protein were formed using messenger RNA from mature beans. Also, more high molecular weight proteins were seen when messenger RNA from mature beans was used to program the *in vitro* translation system.

DISCUSSION

SEED PROTEINS have been studied in an increasing number of higher plants for the purpose of achieving greater understanding of processes controlling gene expression. The most revealing studies have been done by measuring changes in protein and mRNA during seed development. Some general principles, based on comparison of results from a variety of plants, have emerged. Seed development is characterized by accumulation of relatively few proteins over a short, well-defined period. For example, bean seed globulin, representing well over 90% of the protein in a mature seed, is essentially absent in the seed 13 days after flowering, but by day 28 is present at maturity levels (Hall et al., 1980). Mature seeds of soybean contain two major storage proteins that begin to accumulate 18 - 20 days after pollination (Meinke et al., 1981). Cottonseeds contain two principal storage proteins (Dure and Chlan, 1981). Rice (Yamaguta et al., 1983) has one major storage protein. It is believed that most seed storage proteins are the products of multigene families resulting from gene duplication and divergence (Dure and Chlan, 1981; Okita and Greene, 1982).

Studies of gene expression have been facilitated because it has been possible to produce polypeptides of about the same size as authentic storage proteins using *in vitro* protein synthesizing systems programmed by polysomal messenger RNA. Such capability allows, for example, studies of the effects of plant hormones on translatable mRNA (Zurfluh and Guilfoyle, 1982). Also, questions regarding the effect of transcriptional and posttranscriptional processes on gene regulation can be addressed using these methods along with synthesis of cDNAs and recombinant DNA technology (Fischer and Goldberg, 1982; Evans et al., 1984).

In agreement with the developmental framework established in previous investigations on cocoa lipids (Lehrian and Keeney, 1980) and methyl xanthines (Senanayake and Wijesekara, 1971), we found that cocoa seed proteins increased in a similar fashion (Table 1). By contrast, we found that mRNA levels decreased in later stages of development (Table 1). Cocoa seed mRNA levels are notably low compared to other plants; bean seeds, for example, contain at least 20 times more of this component than cocoa seeds (Hall et al., 1980).

In spite of difficulties in comparing *in vivo* and *in vitro* protein patterns (Dure et al., 1981), we found that the major proteins seen in the intact seeds (Fig. 2) were closely matched by those coded by isolated mRNAs (Fig. 6), indicating that the isolated mRNAs could be translated into full length polypeptides. We also found that 75 - 90% of the total mRNA activity was associated with poly(A−) RNA at all stages of development (Table 2), and that poly(A+) and poly(A−) fractions stimulated synthesis of the same radioactive proteins in the wheat germ *in vitro* translation system (data not shown). It is, of course, possible that the poly(A−) fractions have short oligo(A) tails that are not retained on oligo (dT) cellulose columns; however, binding studies with ^{3}H poly(dT) gave no evidence for this. Poly(A) free messenger RNA is not unprecedented in plant cells; Ragg et al. (1977) reported that mRNA for phenylalanine ammonia-lyase, the first enzyme in the biosynthetic pathway leading to flavonoid glycoside production in parsley cells, was 60 - 80% poly(A) free. Wheeler and Hart-

Table 2—In vitro translation of developing cocoa seed messenger RNA

	Picomoles [^{35}S] MET incorporated/μg mRNA				
	A+		A−		
DAP[a]	Per μg RNA	Per g Seed	Per μg RNA	Per g Seed	% Total activity per g seed
112	1.92	0.54	0.174	4.9	90.0
116	1.77	0.48	0.160	5.6	92.0
123	2.62	2.1	0.136	5.4	72.0
130	2.19	0,89	0.142	4.1	82.0
137	1.97	0.81	0.172	4.9	85.8
144	2.54	0.51	0.139	2.6	83.6
151	1.97	0.22	0.187	0.63	74.1
166	1.69	0.24	0.153	0.73	75.2

[a] Days after pollination

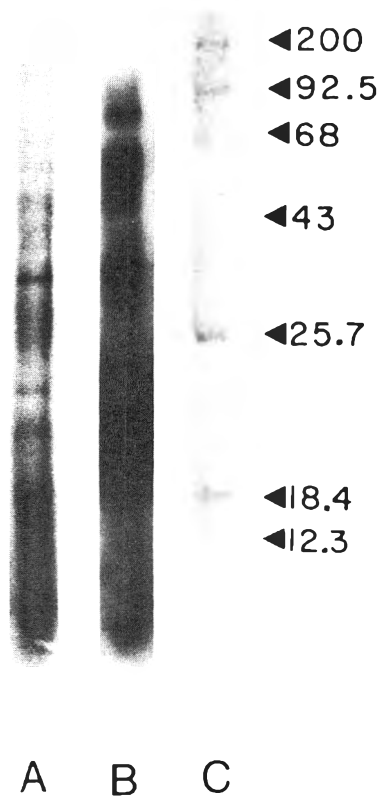


Fig. 6—Electrophoretic separation of proteins coded in vitro by cocoa seed mRNA: A — mRNA from 112 DAP seeds, 0.2 μg; B — mRNA from 166 DAP seeds, 0.2 μg; C — molecular weight standares. Sizes in kilodaltons shown by arrows on right vertical axis.

ley (1975) could find no evidence that spinach chloroplast mRNA contained any poly(A) tracts and suggested that this was yet another example of how chloroplasts resembled prokaryotes. Berry and Sachar (1983) studied the effect of plant hormones on poly(A) polymerase and poly(A+) messenger RNA levels in pea epicotyls and concluded that they were under a dual control, repression by auxin, and stimulation by gibberellic acid. The effect of these hormones on cocoa seed development is unknown.

In summary, we have quantitatively and qualitatively analyzed cocoa bean soluble and microsomal proteins and messenger RNA over the developmental period of seed solidification to maturity. The results demonstrate that cocoa messenger RNA can be isolated in a form capable of being translated *in vitro* into full length polypeptides similar to those found *in vivo*. These studies open the way to isolating and characterizing the cocoa bean genes for these polypeptides, as we have begun to do (Wilson and Fritz, 1984).

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Effect of Cleaning and Sanitizing on the Attachment of *Pseudomonas fragi* to Stainless Steel

LINDA S. STONE and EDMUND A. ZOTTOLA

ABSTRACT

The effects of two clean-in-place (CIP) procedures on attachment of *Pseudomonas fragi* ATCC 4973 to a stainless steel surface were studied using scanning electron microscopy (SEM). Under normal CIP conditions those cells that remained in the system did not exhibit attachment fibrils. Under conditions of lowered detergent water temperature and detergent and sanitizer concentrations, those cells that were present after CIP showed attachment fibrils. Organisms remaining after normal CIP were not viable, whereas those present after suboptimum cleaning were. These viable cells produced attachment fibrils within 24 hr at 21°C and 96 hr at 4°C when grown in milk.

INTRODUCTION

IN THE DAIRY INDUSTRY, cleaning and sanitizing is a major necessity. It is important in the preservation of the product and in protecting against the spread of disease. Clean-in-place (CIP) is the most common practice used for cleaning and sanitizing equipment without dismantling. The systems employ any one of several different cleaning regimens but usually include a pre-wash rinse to remove the bulk of the soil, a detergent wash, a post-detergent rinse, and a pre-soil sanitizer (Haverland, 1981; Mitchell, 1981; Dunsmore et al., 1981b). Types and concentrations of chemicals used are selected from a wide array of classes and formulations based on the type of soil, construction materials of equipment, method of cleaning, water hardness and USDA/FDA acceptance (Carlson and Gould, 1967). Milk soil contains both acid-soluble (calcium phosphate and other minerals) and alkali-soluble (fat and protein) fractions (Webb et al., 1974), thus an alkaline detergent followed by an acid step is usually recommended for CIP of dairy systems (Mitchell, 1981).

Though no detergency process is ever completely effective, when properly applied after each soiling of the equipment, CIP systems adequately clean and sanitize processing equipment to help maintain product quality (Dunsmore et al., 1981b). The period between cleanings can range from several hours to several days. Long periods between cleanings allow the buildup of organic material and microorganisms which are associated with the environment (Dunsmore et al., 1981b).

The microflora of raw milk consists predominantly of psychrotrophic gram-negative rods and gram-positive spore forming rods whose sources include water, soil, and improperly cleaned milk handling equipment (Mikolajcik, 1979; Cousin, 1982; Bigalke, 1984). The genus *Pseudomonas* is the predominate psychrotrophic gram-negative rod found in milk (Witter, 1961; Thomas, 1974). Some of the *Pseudomonas* found in raw milk have been shown to produce polysaccharide fibrils or fimbriae (Fuerst and Hayward, 1969; Zoltai et al., 1981) which enhance their attachment to surfaces. One such organism, *Pseudomonas fragi*, has demonstrated attachment to several inert surfaces, including stainless steel (Zoltai et al., 1981; Schwach and Zottola, 1982).

Many studies have been done on the efficiency of a CIP system in lowering microbial numbers (Maxcy, 1969; Bigalke, 1978; Dunsmore et al., 1980, 1981b; Dunsmore, 1981; Dunsmore and Thomas, 1981), but no research has been done on the effects of CIP systems on the attachment fibrils. The purpose of this study was to determine if *P. fragi* attaches to the walls of stainless steel equipment as reconstituted nonfat milk flows through the equipment and to observe the effects of two CIP procedures on the attached organisms through the use of scanning electron microscopic (SEM) techniques.

MATERIALS & METHODS

A SPECIALLY CONSTRUCTED stainless steel pipeline system filled with approximately 15L of heat-treated reconstituted 10% nonfat dry milk (NFDM) inoculated with *P. fragi* was used in all attachment and CIP studies. The system, shown in Fig. 1, consisted of 10 ft of 1½ and 2 in. stainless steel pipe, a varidrive positive pump (Creamery Package No. 2 with vented head and rubber impellers), and a surge tank. Precut 6 x 6 mm stainless steel chips of finish No. 4 from type 304 stainless steel were fixed with 3M Scotch Brand double stick tape to the inside of the elbow (Fig. 2) and on a plug in the T-section of the pipe (Fig. 3). To facilitate their retrieval, the stainless steel squares fixed to the inside of the elbow were drilled with holes in the corner and tied with Johnson & Johnson's unwaxed dental floss (Fig. 4) that was threaded through and taped to the inside of the sampling orifice.

The elbow and T in the system were selected as sample sites because of the differences in rate of and type of flow at these

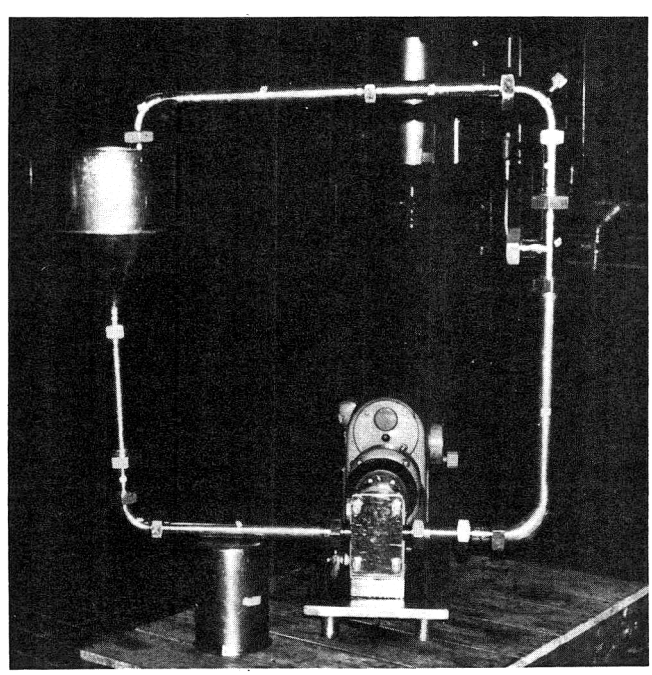


Fig. 1—Specially constructed stainless steel pipeline system consisting of 10 ft of pipe, a positive pump, and a surge tank.

Author Zottola is affiliated with the Dept. of Food Science & Nutrition, Univ. of Minnesota, 1334 Eckles Ave., St. Paul, MN 55108. Author Stone's present address is: The Pillsbury Co., Research & Development, 311 Second St. SE, Minneapolis, MN 55414.

points. These differences were not measured nor were Reynolds Numbers determined. It was assumed that at these two points surface shear stress would be different as shown by Duddridge et al. (1982). All sampling was done in the 2-in. sections of the line.

Before each experiment, the system was dismantled and hand-washed using a chlorinated alkaline cleanser. The gaskets, plug, elbow and stainless steel chips were sterilized by autoclaving for 15 min at 121°C before assembly into the system. The entire system was then sanitized with 200 ppm sodium hypochlorite for 3 min.

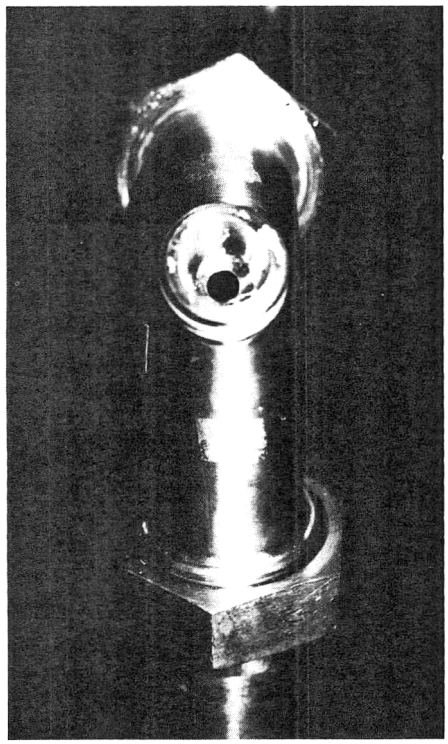


Fig. 2—Sampling orifice of elbow through which stainless steel chips were pulled at predetermined sampling times.



Fig. 3—Stainless steel chips fixed with double stick tape to the plug in the T-section of the system.

Preparation of inoculated milk

Grade A low heat nonfat dry milk (NFDM), obtained from the same lot from Mid-American Dairymen (St. Paul, MN) was rehydrated to 10% solids in distilled water and heat treated, to kill vegetative cells, by steaming at 100°C for 30 min.

Pseudomonas fragi ATCC 4973, cultured through two to three successive transfers in heat-treated 10% NFDM, was used to inoculate 15L of 10% NFDM to 10^3 to 10^4 CFU/mL.

Attachment study

Ten percent reconstituted NFDM inoculated with *P. fragi* to approximately 10^3 CFU/mL was pumped for 30 min at 1½ hr intervals over an 8 hr period at 21°C. Two stainless steel chips were removed from the plug and elbow at 0.5, 3.5, 4, 7.5 and 8 hr, fixed, and viewed for attachment in the SEM. The reconstituted nonfat milk was pumped at a flow rate of 30L/min.

Cleaning methods study

Two commonly used clean-in-place (CIP) procedures were used to determine the effect the different steps in the two procedures would have on the attachment of *P. fragi* to stainless steel in our pipeline system. Reconstituted, heated nonfat dry milk was inoculated with *P. fragi* and circulated through the system by the pump for 30 min at 1½ hr intervals over a 6 hr period at 21°C and at 3½ hr intervals over a 48 hr period at 4°C. At the end of these time periods the milk was drained from the pipeline and the system was cleaned by one of the two procedures. Stainless steel chips were removed from the sampling locations at each step of the cleaning process. These chips constituted the samples.

The method followed in Procedure A consisted of a cold water rinse for 3 min as soon as the milk had been drained from the pipeline. Chips were removed after the cold water rinse. The next step in the cleaning process consisted of a 7 min wash with 2500 ppm alkaline detergent at 68-71°C. The alkaline detergent contained potassium hydroxide and potassium hypochlorite as the active cleaners. After the 7 min wash the alkaline detergent was drained and sample chips were removed. The alkaline wash was followed by a 3 min wash with 1000 ppm acid detergent at a temperature of 11°C. Phosphoric acid was the active ingredient in the acid detergent. The acid detergent was drained from the system, sample chips removed and the system was left as is overnight (15-18 hr). The following morning sample chips were removed. The pipeline was then given a sanitizer rinse of 200 ppm sodium hypochlorite for 3 min. The sanitizing solution was drained from the system and the final sample was removed.

Procedure B was similar to Procedure A, except that an acid sanitizer was substituted for the acid detergent and the sodium

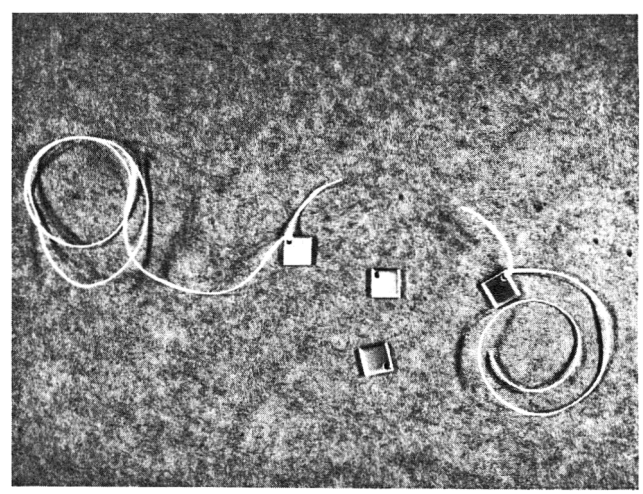


Fig. 4—Stainless steel chips tied with dental floss that were fixed to the inside of the elbow with double stick tape.

hypochlorite. The acid sanitizer was circulated through the pipeline following the alkaline detergent wash for 3 min at a concentration of 1300 ppm at a temperature of 11°C. The same concentration of sanitizer for the same time as above was used the following morning. The acid sanitizer contained phosphoric and citric acids. These cleaning procedures and sampling times are summarized in Table 1. The flow rate of the cleaning solutions was 30L/min with both procedures.

A variation of the normal cleaning procedures given above was used in the worst-case conditions studies. The concentrations of the alkaline detergent, acid detergent, sodium hypochlorite and acid sanitizer given previously and in Table 1 are the concentrations suggested by the manufacturer. In the worst-case conditions experiments one-half the recommended concentrations were used. In addition, the temperature of the alkaline detergent wash in both procedures was reduced to 38°C.

Viability study

The viability of cells remaining in the system after CIP was tested in a series of experiments where both CIP procedures were applied under normal and worst-case conditions after 24 hr of intermittent pumping at 21°C and 48 hr at 4°C.

Eight stainless steel chips were removed following the pre-soil sanitizing step (step 10 in Table 1) with four fixed for viewing in the SEM. The remaining four chips were used to determine the presence of viable bacteria by incubation in 10 mL heat-treated 10% NFDM for no more than 48 hr at 21°, or 96 hr at 4°C. The presence of viable cells was determined by plating 0.1 mL of the incubated reconstituted nonfat milk on plate count agar (Difco). This was done immediately after addition of the chips to the milk and after 24, 48, 72 or 96 hr of incubation. Growth on PCA indicated viability of cells remaining on the chips. Numbers of surviving bacteria were not determined. We were only interested in survival per se, not how many survived.

Increased milk contact time

The effect of increased milk contact time (96 hr versus 24-48 hr) and subsequent increased microbial population on CIP efficiency was tested in two 96 hr runs. The milk was pumped for 30 min every 8 hr during the 96 hr of storage at 4°C. To clean the system, clean-in-place (CIP) Procedure B (Table 1) was used at normal and 1/2 concentrations. Sampling protocol was as previously described.

Water temperature effect

The effect of alkaline detergent water temperature on CIP efficiency was tested in a 24 hr experimental run where the pipelines and inoculated milk were maintained at 21°C. The system was then cleaned using CIP-A (Table 1) at normal concentrations and an alkaline detergent water temperature of 11°C. Viability of remaining organisms was determined after 24 hr of incubation in milk as previously described.

The ability of these bacteria that were viable after this treatment to attach to stainless steel was determined by placing sterile stainless steel chips into the milk containing the cells that grew after the treatment. These chips were removed after 24, 48, 72 and 96 hr of incubation and fixed for SEM viewing.

SEM fixation procedure

Upon removal from the system, chips were given three 15 min rinses in 0.1M sodium cacodylate buffer (pH 7.2), and then fixed in a solution of 2.7% glutaraldehyde, 500 ppm ruthenium red and 0.1M sodium cacodylate at room temperature for at least 4 hr. Fixation was followed by three more 15 min rinses in 0.1M sodium cacodylate buffer. A 20-min second fixation was done in a freshly prepared solution of 2% osmium tetroxide, 500 ppm ruthenium red and 0.033M sodium cacodylate buffer (pH 7.2) at room temperature. Samples were given three more 15 min rinses in 0.033M sodium cacodylate buffer (pH 7.2) and then dehydrated at room temperature in an acetone series (10 min each in 25%, 50%, 75%, 99%, and three 15 to 30 min changes in 100%). Dehydration was followed by critical-point drying in a Bomar SPC/EX using CO_2 as the transition medium. The stainless steel chips were mounted on aluminum stubs with carbon paint and coated with gold-palladium in a Kinney vacuum evaporator, model KSE-2AM.

All observations were made using a Philips 500X scanning electron microscope (SEM) at an accelerating voltage of 12 kv.

RESULTS

Attachment in a piping system

Microbial attachment was observed in the first 1/2 hr of pumping in the plug area and at 4½ hr in the elbow. An example of the type and extent of attachment seen is shown in Fig. 5. The apparent large difference in the time that attachment was seen between the two locations may be due to the sampling times which were 4 hr apart or may be due to the difference in flow rates at the two locations. The plug area represents a dead end which would have the slowest rate of flow whereas the sample point at the elbow probably has the greatest flow rate. Failure to find organisms exhibiting attachment on 1/2 hr elbow sample does not mean that there were no organisms attaching elsewhere

Table 1—CIP procedures and sampling scheme used to determine their effect on attachment of P. fragi *to stainless steel chips*

Cleaning Procedure A	*Cleaning Procedure B*
1. Post-soiling rinse (cold water — 3 min)	1. Post-soiling rinse (cold water — 3 min)
2. Chips removed	2. Chips removed
3. Alkali detergent[a] (2500ppm in 68-71°C water for 7 min)	3. Alkali detergent[a] (2500ppm in 68-71°C water for 7 min)
4. Chips removed	4. Chips removed
5. Acid detergent[b] (1000ppm in 11°C water for 3 min)	5. Acid sanitizer[c] (1300ppm in 11°C water for 3 min)
6. Chips removed	6. Chips removed
7. Left overnight	7. Left overnight
8. Chips removed	8. Chips removed
9. Pre-soil sanitizer (sodium hypochlorite — 200ppm for 3 min)	9. Pre-soil sanitizer (acid sanitizer — 1300ppm for 3 min)
10. Chips removed	10. Chips removed

[a] Contained potassium hydroxide + potassium hypochlorite
[b] Contained phosphoric acid
[c] Contained phosphoric and citric acids

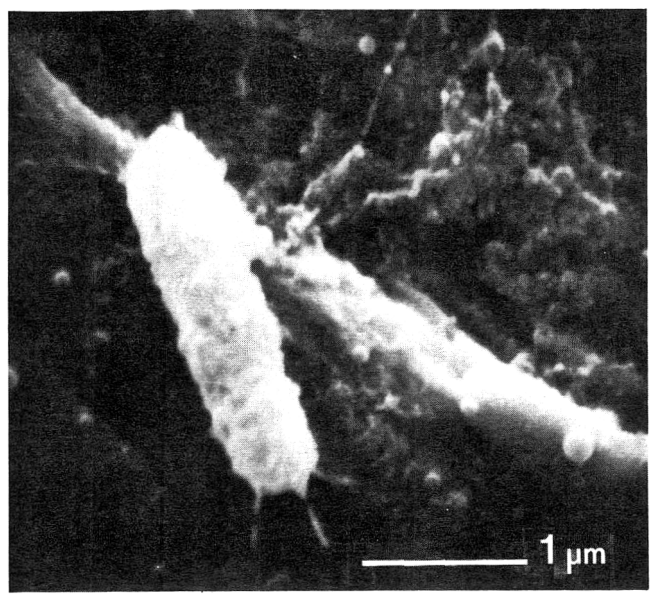


Fig. 5—SEM photomicrograph of cell attached to stainless steel chip removed from elbow after 4½ hr of contact time.

in the elbow or system. Low populations with most microorganisms in lag phase at this time decreased the chances of finding organisms with attachment fibrils in any particular spot.

Although actual determinations of numbers of cells seen in the samples upon SEM viewing were not done, it appeared to us that as contact time between the bacteria and surface increased the degree of attachment and attachment fimbrae increased between the bacterial cells and the stainless steel surface. This was observed at both sampling locations.

With current SEM techniques it is extremely difficult to accurately quantify what is observed. Thus, observations and conclusions given in this paper represent subjective analysis of what was seen rather than quantification.

CIP effects on attachment

Under normal cleaning conditions it appeared that most of the attached cells were removed in the post-soiling rinse (step 1 in Table 1). After each step in the cleaning process with both procedures it became extremely difficult to find attached cells on the stainless steel chips. Nevertheless, cells retaining attachment fibrils were observed through the acid detergent step in Procedure A and the alkaline detergent step of Procedure B. Attachment fibrils were not found on cells remaining beyond these steps in the cleaning procedures. There appeared to be no difference in the ability of the two CIP cleaning procedures to disrupt the attached microorganisms when normal concentrations of detergent and sanitizer were used at the temperatures recommended by the manufacturer.

The two CIP procedures proved to be less efficient under worst-case conditions than under normal cleaning conditions. Cells remained on the stainless steel chips after both CIP procedures. Among the cells remaining after each CIP step, attachment fibrils were observed only through the alkaline detergent wash in Procedure A and after almost every step of Procedure B. Procedure A appeared to be more effective at removing the attachment fibrils under lower temperature and chemical concentration than Procedure B. Fig. 6 shows the type of attachment seen on cells remaining after cleaning with Procedure B under worst-case conditions.

Viability of remaining cells

The results of a series of experiments to determine viability of cells remaining on the chips in the pipeline system after both CIP procedures under normal and worst-cases conditions are given in Tables 2 and 3. In all normal CIP treatments (Table 2), occasional cells remained in the system after CIP, but none exhibited attachment fibrils. With the exception of Procedure A at 21°C, none of the chips removed from the pipeline and incubated in milk showed growth from remaining cells. Growth on PCA from incubated chips cultured from the plug could indicate a lack in cleaning and sanitizing efficiency in the dead end section of the system.

Under worst-case conditions (Table 3), organisms with attachment fibrils were found in some part of the system in every treatment except Procedure A at 4°C. Remaining cells demonstrated viability when cultured on PCA with the microorganisms producing attachment fibrils and attaching to the stainless steel chips during the incubation period. The 21°C samples with cells remaining on the chips after CIP exhibited attachment fibrils within 24 hr of incubation for viability determination. The 4°C samples produced attachment fibrils after 96 hr of incubation.

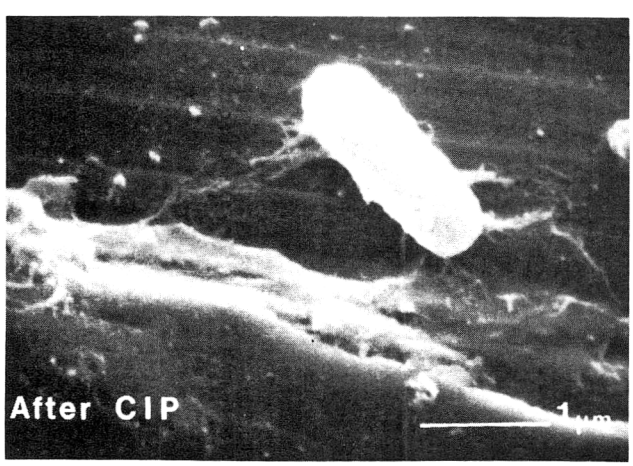


Fig. 6—SEM photomicrograph of a cell attached to stainless steel chip removed from plug after cleaning the pipeline with Procedure B worst-case condition. Note extensive attachment fibrils.

Increased milk contact time

Table 4 shows the effect of a 96 hr milk storage time on the efficiency of CIP-B. There appeared to be no difference between the viability of cells left on the chips after longer incubation and CIP (96 hr – Table 4) and viability of cells on the chips from shorter incubation times (24 and 48 hr – Table 3). Chips removed and incubated after CIP showed no growth of remaining cells when normal concentrations of B were used. However, when lower detergent water temperature and chemical concentrations were used growth with attachment was observed.

Water temperature effects

Water temperature appeared to play a role in the efficiency of CIP-A when the detergent water temperature was lowered to 11°C but normal detergent sanitizer concentrations were maintained. The data in Table 5 show no attachment fibrils present on cells remaining after CIP, but they do demonstrate viability and attachment after 24-hr incubation in sterile reconstituted nonfat milk after removal from the system.

DISCUSSION

THIS STUDY SHOWS that *P. fragi* is capable of attaching to stainless steel milk equipment soon after introduction into the system and remains attached as milk circulates in and through the equipment. As has been seen in other systems (Fletcher, 1977; Zoltai et al., 1981; Duddridge et al., 1982), the extent of attachment increased with time. Neither the sample area in the elbow nor in the plug seemed to be more conducive to microbial attachment than the other, though it would seem that a dead end area such as the plug on the T fitting would be a likely spot for attachment. This was a qualitative rather than a quantitative study, so no statements can be made in relation to numbers of organisms attaching between the two areas sampled. It sometimes appeared, however, that organisms were easier to find on the chips from the plug than on those from the elbow.

There are many theories as to the factors which influence attachment of an organism to any particular spot, ranging from nutrients (Sutherland, 1982), amount of soil accumulated (Dunsmore et al., 1981b), surface topography, including harborage sites (Maxcy, 1969), and shear rates (Sutherland, 1982; Duddridge et al., 1982). Thus, there is great variability in the system from site to site, and the bacterial flora will not be uniformly distributed (Dunsmore et al., 1981b). All of these factors are suspected of contributing to the variability observed in the organisms found on duplicate chips in the pipeline system.

Table 2—Growth and attachment of microorganisms remaining on stainless steel chips after cleaning the milk pipeline with normal CIP procedures

Temp[a]	Run[b] time (hr)	Population of *P. fragi* (CFU/mL) Initial	Final	Treatment[c]	Location of chip in system	Cells exhibiting[d] attachment fibrils on chip	Growth in remaining cells on chip on Plate Count Agar
21°C	24	2.3×10^4	1.9×10^7	A — Normal	Elbow	None	No
					Plug	None	Yes
21°C	24	8.2×10^4	1.7×10^7	B — Normal	Elbow	None	No
					Plug	None	No
4°C	48	5.8×10^4	7.0×10^7	A — Normal	Elbow	None	No
					Plug	None	No
4°C	48	3.4×10^4	2.6×10^7	B — Normal	Elbow	None	No
					Plug	No cells found	No

[a] Temperature of milk and pipeline during experiment
[b] Time milk was in pipeline system
[c] Treatment = cleaning procedures given in Table 1
[d] None indicates cells were seen on chips but attachment was not observed

Table 3—Growth and attachment of microorganisms remaining on stainless steel chips after cleaning the milk pipeline using CIP conditions of reduced temperature (38°C) and reduced concentrations of detergent and sanitizer

Temp[a]	Run[b] Time (hr)	Population of *P. fragi* (CFU/mL) Initial	Final	Treatment[c]	Location of chip in system	Cells exhibiting attachment fibrils on chip	Growth and attachment of microorganisms remaining on chips: Growth on Plate Count Agar	Growth and attachment of microorganisms remaining on chips: Attachment after incubation at 21°C for:
21°C	24	1.3×10^4	1.2×10^7	A	Elbow	Yes	Yes	SNT[d]
					Plug	Yes	Yes	SNT
21°C	24	1.6×10^4	5.3×10^7	B	Elbow	Yes	Yes	24 hr
					Plug	None	Yes	24 hr
4°C	48	7.3×10^4	1.1×10^7	A	Elbow	None	No	None
					Plug	None	Yes	96 hr
4°C	48	4.0×10^4	2.9×10^6	B	Elbow	Yes	Yes	96 hr
					Plug	None	Yes	96 hr

[a] Temperature of milk and pipeline during experiment
[b] Time milk was in pipeline system
[c] Treatment = cleaning procedures given in Table 1 except that concentrations of detergent and sanitizer were reduced 50% and water temperature reduced to 38°C
[d] SNT — Samples not taken

Table 4—Effect of additional milk contact time in the pipeline on the ability of CIP procedure B at normal and 50% concentration to disrupt and destroy attached microorganisms

Temp[a]	Run[b] time (hr)	Population of *P. fragi* (CFU/mL) Initial	Final	Treatment[c]	Location of chip in system	Cells exhibiting attachment fibrils on chip	Growth and attachment of microorganisms remaining on chips: Growth on Plate Count Agar	Growth and attachment of microorganisms remaining on chips: Attachment after incubation at 21°C for:
4°C	96	120(est)	1.6×10^7	B — Normal (temp 68-71°C)	Surge Tank	Possible	No	SNT[d]
					Plug	None	No	SNT
4°C	96	8.5×10^4	2.9×10^7	B — ½ con. (temp 38°C)	Surge Tank	None	Yes	48 hr
					Plug	Possible	Yes	96 hr

[a] Temperature of milk and pipeline during experiment
[b] Time milk was in pipeline system
[c] Treatment = cleaning procedures given in Table 1
[d] SNT — Samples not taken

The numbers of bacteria in the milk were determined at the beginning and at the end of each experiment. These populations are given in Tables 2, 3, 4, and 5. In each of the experiments a final population of *P. fragi* of 10^7 was obtained. This number of bacteria did not appear to affect the efficiency of the two cleaning methods when the recommended concentrations of chemicals and wash water temperature were used. Under lowered chemical concentrations and detergent water temperature, the two CIP procedures were not as effective and left viable cells on the chips. When worst-case conditions of cleaning were used, Procedure A seemed to remove the attached cells better than Procedure B when the milk contact time was 48 hr at 4°C (Table 3). It appears that the acid detergent step of A is a more effective tool than the acid sanitizer of B under lower environmental temperature, water temperature, and chemical

Table 5—Effect of reduced water temperature (11°C) on the ability of CIP procedure A at normal concentration to disrupt and destroy attached microorganisms

Temp[a]	Run[b] time (hr)	Population of *P. fragi* (CFU/mL) Initial	Final	Treatment[c]	Location of chip in system	Cells exhibiting attachment fibrils on chip	Growth and attachment of microorganisms remaining on chips: Growth on Plate Count Agar	Attachment after incubation at 21°C for:
21°C	24	4.4×10^4	2.1×10^7	A – Normal conc 11°C det. wash	Elbow	None	Yes	24 hr
					Plug	None	Yes	24 hr

[a] Temperature of milk and pipeline during experiment
[b] Time milk was in pipeline system
[c] Treatment = cleaning procedure given in Table 1

concentrations. The main purpose of an acid detergent is to remove mineral buildup. At the same time, it might be removing the mechanism necessary for the maintenance of the adhesive polymer. Fletcher and Floodgate (1976) found that when a fimbriated marine Pseudomonad was grown in the absence of Ca^{++} and Mg^{++}, the secondary polysaccharide material was greatly disrupted in a short period of time. Thus, the acid detergent could be more effective than the acid sanitizer at removing Ca^{++} ions needed to maintain the adhesive structure.

Chemical concentration may be a key in the destruction of the attachment fibril, with water temperature playing a major role in microbial kill. All cells found after a run using cold detergent water and normal chemical concentrations were devoid of attachment fibrils, but subsequent incubation in milk showed viable cells remained on the chips. When chemical concentrations were also lowered, attachment fibrils were found on several organisms remaining after CIP in most treatments (Table 3).

The lower environmental temperature of 4°C appeared to play a role in the production of the attachment fibril in the cells remaining after cleaning. Milk samples in which the stainless steel chips were placed after the cleaning procedures were plated at 0, 24, and 48 hr for 21°C (Table 3), and 0, 48, and 96 hr at 4°C (Table 4) to determine the presence of viable cells. Additional chips were fixed for viewing in the SEM. With the exception of one experiment done at 4°C for 96 hr (Table 4), production of the attachment fibril was not seen until after the chips had been in milk for 96 hr. Fimbriated cells were seen within 24 hr in the 21°C samples. The 4°C incubation temperature may slow down the metabolism of the cell to the point that fimbriae production is not seen until growth has occurred for some time. Thus, even at lower detergent and sanitizer concentrations, *P. fragi* may be inhibited from further fimbriae production after CIP through the combination of refrigerated temperature and an acid detergent in the CIP system.

CONCLUSIONS

IT IS CONCEIVABLE from these results that if the interim between cleanings exceeds 8 hr, considerable attachment of microorganisms may occur. If the cleaning procedure is inadequate, total disruption of attachment may not occur, viable cells may remain, and the product coming into contact with the surface could be contaminated with the attached microbes.

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Relationship Between the Growth Phase of *Pseudomonas fragi* and Its Attachment to Stainless Steel.

LINDA S. STONE and EDMUND A. ZOTTOLA

ABSTRACT

Attachment of *Pseudomonas fragi* ATCC 4973 to a sterile stainless steel surface was followed under stationary and dynamic growth conditions at 25°C and 4°C using scanning electron microscopy techniques. The organism was grown in 10% reconstituted nonfat dry milk for 28 hr at 25°C and 9 days at 4°C. Attachment was observed in both the stationary and dynamic environments at 1/2 hr at 25°C and at 2 hr at 4°C. There appeared to be no difference in the development of attachment fibrils on the organisms in the two environments or at the two temperatures. The extent of attachment increased as the microbial population increased over the growth cycle under both environments at both temperatures.

INTRODUCTION

THE PRESENCE of psychrotrophic bacteria in raw or pasteurized milk is a common occurrence because it is difficult to exclude them during production operations (Speck and Adams, 1976; Mikolajcik, 1979; Cousin, 1982; Bigalke, 1984). Increased low-temperature storage and extensive pumping or agitation increases the oxygen content of the milk, which favors the growth of the psychrotrophic microorganisms (Brandt and Ledford, 1981). The psychrotrophs that predominate have been characterized as aerobic, motile, gram-negative rods of the genus *Pseudomonas* (Mikolajcik, 1979; Cousin, 1982; Bigalke, 1984), and their increased growth has implications for product quality and sanitation.

Some of these microorganisms produce polysaccharide filaments which enhance their attachment to surfaces. Zoltai et al. (1981) and Schwach and Zottola (1982) have shown that *Pseudomonas fragi*, a common contaminant of milk, attaches to stainless steel, the material most widely used in the construction of milk processing equipment. Therefore, the effects of increased agitation on the attachment of microorganisms to these surfaces is important. The purposes of this study were to relate the growth phase of *P. fragi* to its attachment to stainless steel surfaces and to determine whether there was a difference in the attachment of *P. fragi* to these surfaces under stationary and dynamic environmental conditions using scanning electron microscopic (SEM) techniques.

MATERIALS & METHODS

Preparation of inoculated milk

Nonfat dry milk (NFDM) obtained from Galloway-West, Fond du Lac, WI, was rehydrated to 10% solids in distilled water and heat treated to kill vegetative cells by steaming at 100°C for 30 min.

Pseudomonas fragi ATCC 4973, cultured through two to three successive transfers in heat treated 10% NFDM, was used to inoculate larger volumes of the same 10% NFDM to population levels of 10^4 to 10^5 CFU/mL.

Author Zottola is affiliated with the Dept. of Food Science & Nutrition, Univ. of Minnesota, 1334 Eckles Ave., St. Paul, MN 55108. Author Stone's present address is The Pillsbury Co., Research & Development, 311 Second St. SE, Minneapolis, MN 55414.

Microbial population was determined at each sampling time using spread plates containing plate count agar (Difco) as described by Gilliland et al. (1978).

Dynamic growth conditions study

Precut 6 x 6 mm stainless steel chips of finish No. 4 from type 304 stainless steel were fixed to the bottom of an 8 qt stainless steel beaker with 3M Scotch Brand double stick tape. For easy removal of the stainless steel chips, holes were drilled in the corner and each chip was tied with Johnson & Johnson's unwaxed dental floss. The dental floss was draped over the top of the beaker and taped to the outside. The beaker containing the chips was covered with foil and sterilized by autoclaving at 121°C for 15 min. The dynamic environment was created by shaking the beaker containing the stainless steel chips plus 1.0L inoculated 10% NFDM in a New Brunswick Scientific gyrotory water bath shaker, model G76, for 28 hr at 25°C at a speed of 100 rpm. At 4°C, the dynamic environment was created using a New Brunswick Scientific G10 gyrotory shaker at a speed of 110 rpm. The shaker containing the beaker, milk and sample chips was maintained at 4°C in a walk-in refrigerated room.

At predetermined times during the growth cycles two stainless steel chips were removed for SEM fixation and 2 mL milk were taken for population determination.

Stationary growth conditions study

Precut stainless steel chips without dental floss were incubated in 100 mL of inoculated 10% NFDM in 150 x 25 mm sterile petri dishes in temperature controlled rooms for 28 hr at 25°C, and 9 days at 4°C. Two stainless steel chips were removed with sterile forceps at predetermined times during the growth cycles for SEM fixation. A 1 mL sample of milk was used to determine population in the milk. The procedure described by Gilliland et al. (1978) was used.

SEM fixation procedure

Upon removal from the test system, the chips were given three 15 min rinses in 0.1M sodium cacodylate buffer (pH 7.2), and then fixed in a solution of 2.7% glutaraldehyde, 500 ppm ruthenium red and 0.1M sodium cacodylate at room temperature for at least 4 hr. Fixation was followed by three more 15 min rinses in 0.1M sodium cacodylate buffer. A second fixation was done in a freshly prepared solution of 2% osmium tetroxide, 500 ppm ruthenium red and 0.033M sodium cacodylate buffer (pH 7.2) for 20 min. The chips were then dehydrated at room temperature in an acetone series (10 min each in 25%, 50%, 75%, 99%, and three 15 to 30 min changes in 100%). Dehydration was followed by critical point drying in a Bomar SPC/EX using CO_2 as the transition medium. The stainless steel chips were mounted on aluminum stubs with carbon paint and coated with gold-palladium in a Kinney vacuum evaporator, model KSE-2AM.

All observations were made using a Philips 500X scanning electron microscope (SEM) at an accelerating voltage of 12 kv.

RESULTS

THE GROWTH CURVES of *P. fragi* at 25°C and 4°C under stationary and dynamic conditions are shown in Fig. 1 and 2. Growth curves from the dynamic environments show an increased rate of growth over the stationary environments. Generation times for the dynamic environments at 25°C and 4°C were 67.8 min and 532.8 min, respectively, while stationary generation times were 85.8 min and 624.3 min at 25°C and 4°C, respectively.

P. fragi exhibited attachment early in the lag phase in

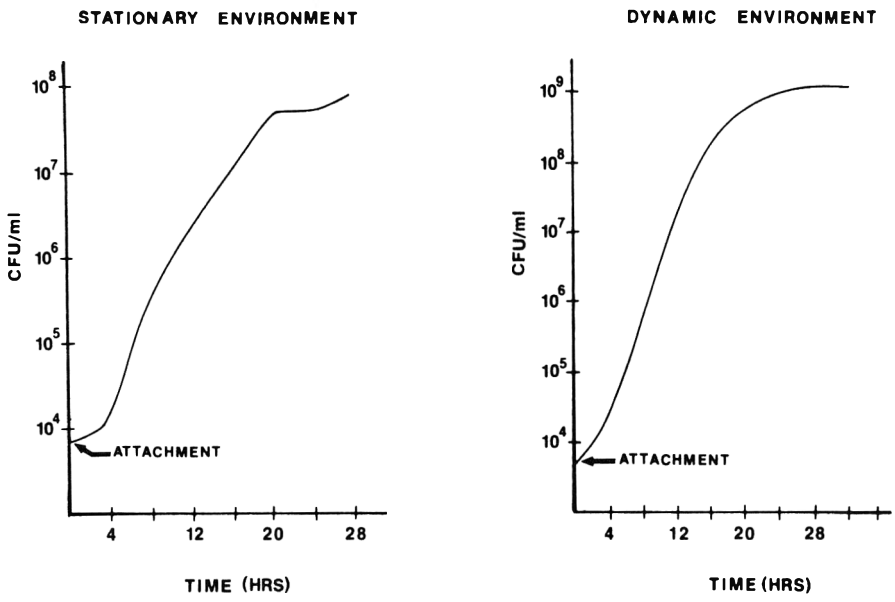


Fig. 1—Growth curves for P. fragi *under stationary and dynamic growth conditions at 25°C. Attachment was first observed at 1/2 hr in both environments.*

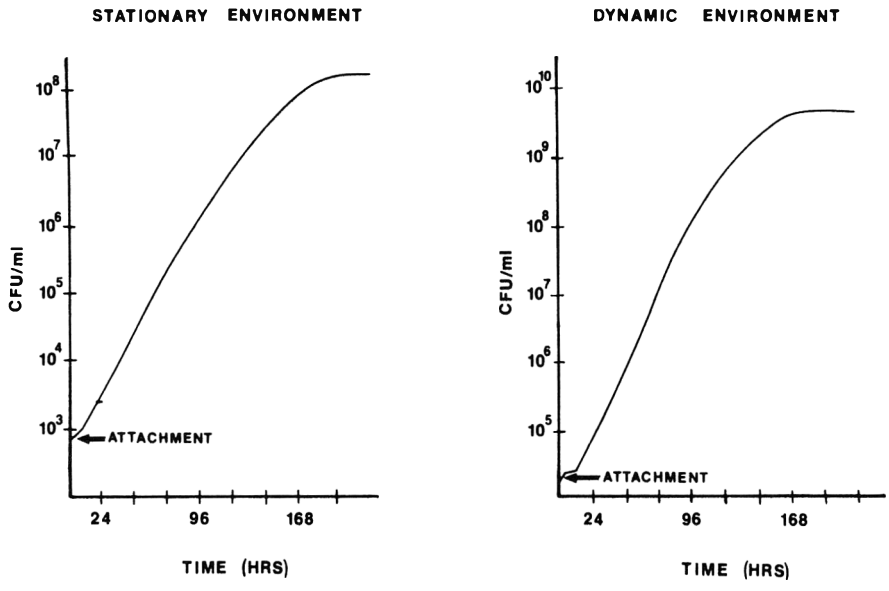


Fig. 2—Growth curves for P. fragi *under stationary and dynamic growth conditions at 4°C. Attachment was first observed at 2 hr in both environments.*

both environments at both temperatures. Attachment fibrils were seen on only a few cells within 1/2 hr of inoculation at 25°C, and 2 hr at 4°C.

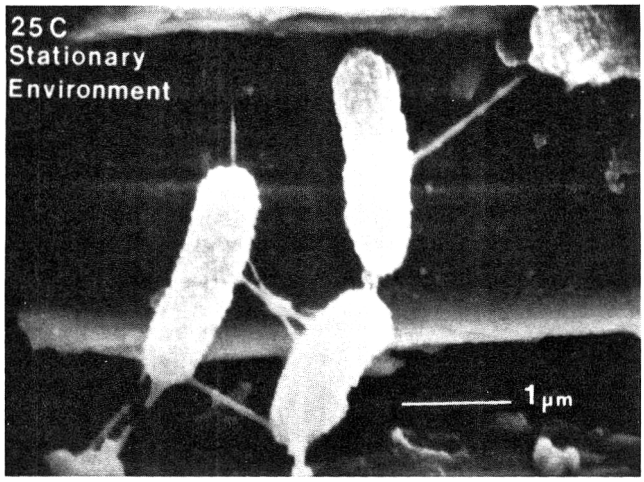
Fig. 3 and 4 show the attachment of *P. fragi* to stainless steel under stationary and dynamic growth conditions at 25°C (Fig. 3) and 4°C (Fig. 4). As can be seen from these two figures, neither growth conditions, dynamic and stationary, nor growth temperature, 25°C and 4°C, affected the formation of attachment fibrils by *P. fragi.*

As the numbers of *P. fragi* increased as shown in the growth curve, attachment to the stainless steel chip became more apparent. This occurred at 4°C and 25°C under both growth conditions. Early in the growth cycle, microcolonies and isolated single cells were usually observed without attachment fibrils, or exhibited only one or two fibrils which extended from one or both ends of the cell (Fig. 5). With increased time of growth the polar fimbriae observed were long and flexible, exhibiting extreme bending or curving (Fig. 6). The cells shown in Fig. 5 and 6 were grown at 25°C. Similar observations were noted with cells grown at 4°C.

DISCUSSION

BRANDT and LEDFORD (1981) found that a statistically significant reduction in growth rate of *Pseudomonas* species occurred in milk as oxygen levels were decreased. This reduction was even more pronounced as the environmental temperature was lowered from 9°C to 3°C. In this study, the growth rate of *P. fragi* did increase with agitation at both temperatures, as was expected, but there appeared to be no difference in the attachment fibrils produced under the stationary and dynamic environments. It appeared that oxygenation had no direct role in attachment fibril production but only in the rate at which the microorganism grew and consequently produced the fibril.

The dynamic environment had no effect on the ability of *P. fragi* to attach to the stainless steel surface when qualitative comparisons were made between samples from the two environments. Duddridge et al. (1982) showed that shear stress does affect initial attachment, with fewer numbers of microorganisms attaching as shear stress increases. The agitation used in this study may or may not mimic true environmental conditions; however, it did show



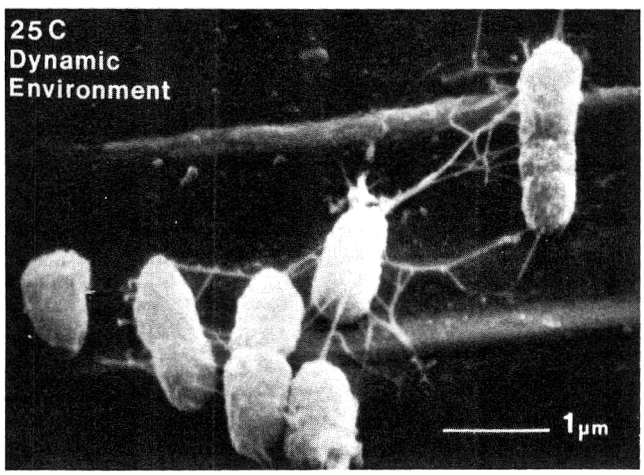
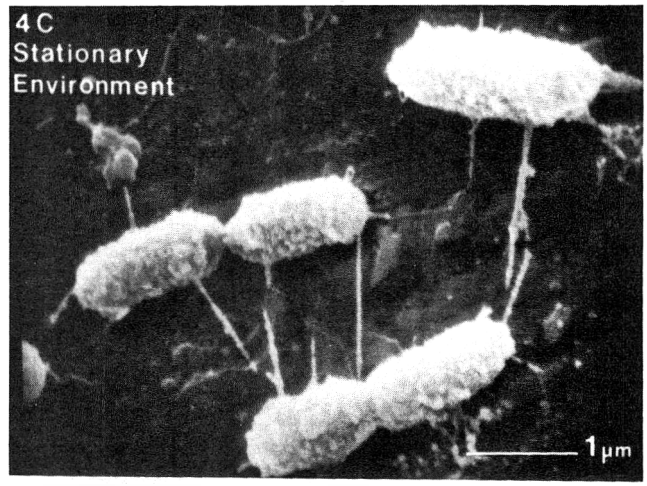


Fig. 3—SEM photomicrographs of attachment produced by P. fragi *at 16 hr under stationary conditions and 24 hr under dynamic growth conditions at 25°C.*



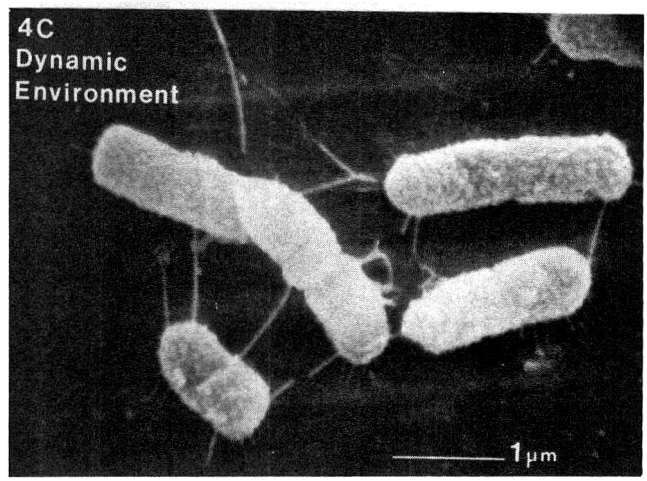


Fig. 4—SEM photomicrographs of attachment produced by P. fragi *at 96 hr under stationary and dynamic growth conditions at 4°C.*

that *P. fragi* attached to the surface during agitation of the system. The strength of the attachment under different agitation rates needs to be tested.

Fletcher (1977a) found that a low temperature of 3°C noticeably decreased the proportion of cells which attached when compared with attached cells at 20°C. This difference was not apparent in the attachment observed at 4°C and 25°C in the results given here. The results of this study were consistent with findings of previous researchers (Weiss, 1971; Fletcher, 1977a; Zoltai et al., 1981; Duddridge et al., 1982). Weiss (1971) and Fletcher (1977a) found that the number of attached cells was dependent on culture age, cell concentration, and the time allowed for attachment. Using a marine Pseudomonad, Fletcher (1977a) reported that the highest amount of attachment occurred in the lag phase followed by a decrease in the number of cells attaching through the stationary and death-phases. Weiss (1971) reported that 64% of *Pseudomonas aeruginosa* cells in log phase were piliated, but only 54% were in the stationary phase. No information was given for the lag phase. Though numbers were not determined in this study, visually there appeared to be a decrease in the number of attached cells in the later phases of the growth cycle. In addition, attachment of *P. fragi* was seen within the first 1/2 hr of incubation at 25°C and 2 hr at 4°C, which represents a very early time in the lag phase of both sets of growth curves. The cells which attached in early lag phase probably were more mature than those inoculated into the new environment.

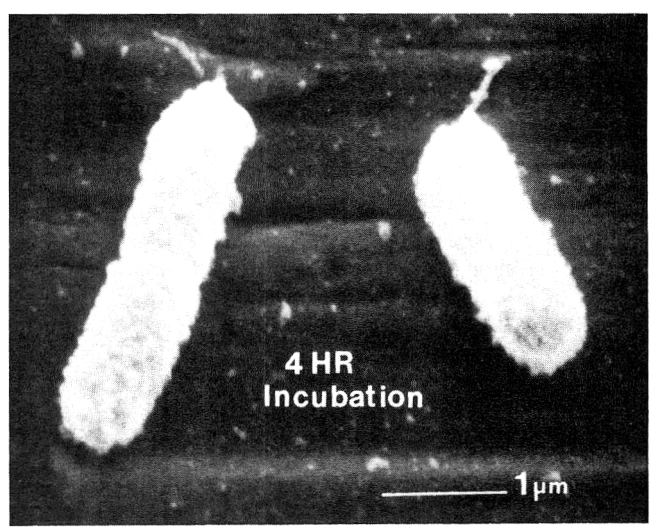


Fig. 5—SEM photomicrograph of attachment seen at 4 hr in the growth cycle of P. fragi *at 25°C.*

With an increase in attachment seen occurring over the log phase in both environments in this study, culture age, cell concentration, and time probably have an effect on the number of cells attached and amount of attachment produced by a cell. An increase in all of these parameters could

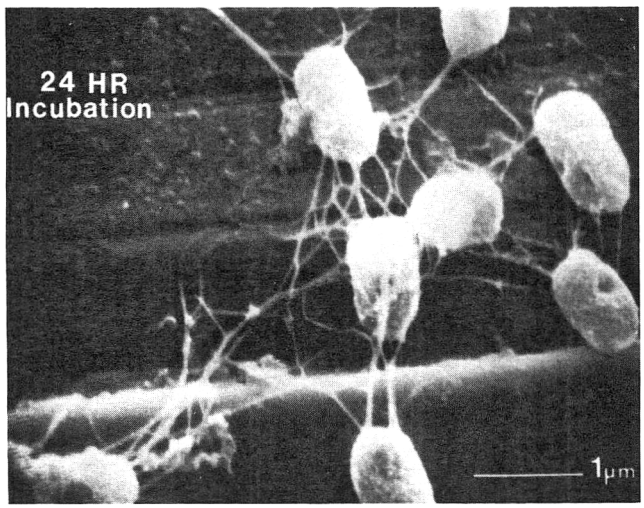


Fig. 6—SEM photomicrograph of extensive attachment produced by P. fragi *at 24 hr (late log phase) in the growth cycle under stationary growth conditions at 25°C.*

lead to an increase in the number of bacterial collisions with the substratum and, consequently, an increase in the opportunity for attachment (Fletcher, 1977a; Duddridge et al., 1982). The attaching microbes might also alter the substratum such that it is rendered more conducive to further bacterial attachment (Fletcher, 1977b). In addition, an increasing number of microbes is probably approaching the metabolic state needed for fibril production.

Long, curving, polar fibrils appear to be common in *Pseudomonas* species. These polar fibrils have been described by Fuerst and Hayward (1969) and specifically in *P. aeruginosa* by Weiss (1971). It is suggested that the polar filaments are flexible so that they do not interfere with flagellar motion (Weiss, 1971). In this study, the flexible polar fibrils were usually the first fibrils observed early in the growth cycle, with the more rigid peritrichous fibrils seen later.

This study has shown that *P. fragi* has the ability to attach to an inert stainless steel surface in either a stationary or dynamic environment. The turbidity of flow of a liquid as observed in our dynamic environment did not interfere with the attachment of the organism. These data suggest that attachment of the microbe to the stainless steel occurs shortly after the organism is introduced into the new environment. As growth continues, large microcolonies of the organism appear, and these are attached both to the surface and to other cells through the production of flexible polar and rigid peritrichous fibrils.

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Growth and Toxin Production by *Clostridium botulinum* in Model Acidified Systems

NORA TSANG, LAURIE S. POST, and MYRON SOLBERG

ABSTRACT

TPGY medium was acidified to pH 4.20, 4.60, 5.00 and 5.40 with acetic or citric acid. The media were inoculated with spores from three strains of *C. botulinum* type A, B or E. Growth, pH, and toxin production under anaerobic conditions were monitored for 8 wk. Spores of *C. botulinum* types A and B were incapable of outgrowth and toxin production at pH 4.60 or below when incubated at 35°C. Spores of *C. botulinum* type E were capable of growth and toxin production at 26°C in citric acid acidified systems at pH 4.20. Growth and toxin production were not detected below pH 5.00 when acetic acid was used.

INTRODUCTION

MANY INVESTIGATIONS have been conducted to determine the effect of pH on the growth and toxigenesis of *C. botulinum*. Data in the literature indicate that *C. botulinum* is not capable of growth and toxin production at pH levels below 4.6 (Ingram and Robinson, 1951; Townsend et al., 1954; Ohye and Christian, 1967; Baird Parker and Freame, 1967). Thus, it is generally agreed that canned foods with a pH level below 4.6 need not be heat processed in such a way that all *C. botulinum* spores are destroyed (Gardner, 1976).

Acidified foods are common in the U.S. food supply. They are normally given only a mild heat treatment since protection is assumed to be afforded by the artificially increased acidity. Several outbreaks of botulism have been traced to acidified foods including home prepared tomato products (CDC, 1973; CDC, 1974a,b; Dolman et al., 1950). Recent reports by Raatjes and Smelt (1979), Smelt et al. (1982) and Tanaka (1982) have demonstrated the ability of *C. botulinum* to grow and produce toxin at pH levels below 4.6.

The minimum pH required to inhibit growth and toxin production by *C. botulinum* varies from substrate to substrate (Townsend et al., 1954) and is dependent upon such factors as bacterial strain involved (Ingram and Robinson, 1951) and acidulant (Smelt et al., 1982). There is little information available concerning the maximum pH level at which different organic acids prevent the growth and toxigenesis of *C. botulinum*.

The purpose of this investigation was to construct model systems in laboratory media where growth and toxin production by *C. botulinum* types A, B and E could be studied at several pH levels established by the addition of acetic or citric acid. These acids were chosen on the basis of significance to the food industry. Acetic acid is a major acidulant in food products and citric acid is the major acidifying factor in tomatoes.

MATERIALS & METHODS

Strains selection

Spores of three strains each of *C. botulinum* producing type A, B or E toxin were used. The strains were: type A: 62, 69, 426; type B (proteolytic): 169, 642, 999; and type E: Beluga, D8, Minnesota. Spore stocks were obtained from the FDA, Washington, D.C.

Culture media

Trypticase Peptone Glucose Yeast Extract (TPGY) medium (BAM, 1978) without trypsin was used for culture and sporulation. TPGY (200 mL portions) was prepared and stored in screw cap bottles. Glacial acetic acid or citric acid was used to acidify the media to desired pH levels. The media were then autoclaved at 121°C for 20 min, cooled and stored in an anaerobic chamber at 35°C overnight prior to inoculation.

Plate counts were made on anaerobic egg agar (AEY), (BAM, 1978) with 0.05% cysteine-HCl. The plates were spread with 0.1 mL catalase solution (filter sterilized, 0.028%) containing 2000 units/mg protein approximately 1 hr prior to use and the plates were maintained in an anaerobic chamber at 35°C until required.

Preparation of spore stocks

Spore suspensions of type A and type B strains were heat shocked at 80°C for 15 min and cooled in an ice water bath for 45 sec. Each strain was inoculated into 10 mL TPGY medium (10% inoculation) and incubated at 35°C for 24 hr. Following at least two consecutive sub-cultures, 10% inoculations into 200 mL volumes TPGY were made. The cultures were incubated 3 - 4 wk at 35°C. Cultures were evaluated for spore development by examining wet mount slides using phase-contrast microscopy or by examining stained smears using brightfield microscopy.

Spores were harvested by centrifugation at 1500 x *g* for 20 min (5°C) when the spore concentration was approximately 80 - 90% of the population. Pellets were rinsed a minimum of five times with sterile distilled water. Stock suspensions of spores in sterile distilled water were subdivided into 1.0 mL units and stored at 4°C. The cultivation of *C. botulinum* type E followed the same procedures except that the spore suspensions were heat shocked at 60°C for 15 min, cultures were incubated at 26°C and spores were harvested after 7 - 14 days of incubation.

Stock spore suspensions were plated directly on AEY. Plate count data were used to calculate the concentration of each spore suspension for use in subsequent standardization procedures.

Preparation of the inoculum

A composite of spores consisting of the three selected strains of each *C. botulinum* type was made.

Stock suspensions of type A and B spores were heat shocked at 80°C for 15 min and cooled in an ice water bath for 45 sec. Type E spores were heat treated at 60°C for 15 min or, in later studies, treated with alcohol (1 part spore suspension to 3 parts 95% alcohol) for 1 hr at 26°C (BAM, 1978). Appropriate dilutions were made to obtain a standard concentration of spores for each strain. Equal volumes of each spore dilution thus obtained were combined to produce a mixed working suspension of known concentration for each *C. botulinum* type. Additional dilutions of the mixed working suspensions were made as required to obtain a concentration of 50 spores/mL after subsequent inoculation of the model media systems.

Model systems

TPGY was adjusted to pH levels of 4.20, 4.60, 5.00, and 5.40 (±0.05) with citric acid (5M solution anhydrous crystalline citric acid, Fisher, Inc.) or acetic acid (17.4M glacial acetic acid, Fisher, Inc.) (dropwise addition) using a Fisher Accumet pH meter model #291.

The authors are affiliated with the Dept. of Food Science, Cook College, New Jersey Agricultural Experiment Station, Rutgers Univ., New Brunswick, NJ 08903.

pH adjusted TPGY media (200 mL) in screw capped bottles were inoculated with approximately 50 spores per mL of a mixture of three type A strains, 50 spores per mL of a mixture of three type B strains or 50 spores per mL of a mixture of three type E strains. Media inoculated with *C. botulinum* type E were stored at 26°C in anaerobic jars and media inoculated with *C. botulinum* type A or type B spores were stored at 35°C inside an anaerobic chamber. Anaerobic jars were always opened and sealed within the anaerobic chamber. The chamber environment consisted of 5% hydrogen, 85% nitrogen, and 10% carbon dioxide.

The model system inoculated with *C. botulinum* type E was replicated and then studied over a tightened range of pH values. TPGY was acidified to pH 4.000, 4.100, 4.200, and 4.300 (±0.005) with citric acid and to pH 4.900, 5.000, 5.100, 5.200, 5.300, and 5.400 (±0.005) with acetic acid. The media were inoculated and incubated as before.

Commercially prepared buffers (Fisher, Inc.) were used to standardize the pH meter at every usage except where accuracy to the third decimal place was required (as with the study of *C. botulinum* type E over a tightened pH range). When such accuracy was called for, a standard phosphate buffer solution at pH 6.863 (25°C) and an acid potassium phthalate buffer solution at pH 4.004 (25°C) were used to standardize the pH meter to the third decimal place (AOAC, 1980).

Uninoculated controls were used to assure that the media were sterile and the pH did not change during the 8 wk period. These controls were also injected into mice to eliminate false results for botulinum toxin by the media. Inoculated media at pH 7.00 were used to assure the viability of the spores, suitability of the medium and the production of toxin by the inoculum.

Sampling

Sampling was scheduled on day 0 and at eight weekly intervals. The termination point of the study was taken as the third consecutive week in which growth and toxin production were confirmed. Inoculated bottles were shaken to mix thoroughly. Portions of each system were plated directly on AEY plates inside the anaerobic chamber to determine the total number of spores and heat sensitive spores or vegetative cells (8 plates, 0.25 mL/plate). Outside the chamber, one 3 mL aliquot of the type A or type B culture was heat shocked in an 80°C waterbath for 15 min, cooled immediately in an ice water bath for 45 sec and plated (8 plates, 0.25 mL/plate). One 3 mL aliquot of type E culture was treated with 9 mL 95% alcohol for 1 hr at 26°C and then plated (4 plates, 0.5 mL/plate). All plates were incubated at 35°C 36 - 48 hr in the anaerobic chamber. Counts after heating or alcohol treatment (plated aerobically) represented heat resistant spores. Initial (total) counts minus counts after either heat shocking or alcohol treatment represented numbers of vegetative cells and heat-sensitive spores. Total counts greater than the initial number of spores and cells represented growth. An increase in spore count represented sporulation.

The pH of each sample was measured as previously described.

Mouse assay for toxin

Samples of media were centrifuged at 1400 x *g* for 30 min and the sample supernatants were used to assay for toxin production. Supernatants of type A and B cultures were adjusted to pH 6.2 with gelatin phosphate buffer in a 1:1 ratio. These were not trypsin treated. The pH of type E culture supernatants was adjusted with gelatin phosphate buffer or 0.1N NaOH (BAM, 1978). Trypsin (1:250, Difco Laboratories, Detroit, MI) was added to 1.8 mL of the type E supernatant (0.2 mL of a fresh 10% solution) and incubated at 37°C for 1 hr. Each lot of trypsin used was tested for nonspecific toxicity by injecting two mice with 0.2 mL of a fresh 10% solution incubated at 37°C for 1 hr. The treated sample (0.5 mL) was injected intraperitoneally into a single female Swiss Webster mouse weighing 18 - 22g. If the mouse died from typical symptoms of botulism in this initial screening test, the screen was repeated and another mouse was injected with a heated (80°C, 15 min), trypsin treated sample of the supernatant. To confirm the presence of *C. botulinum* toxin, a mouse protected with type specific antiserum (0.1 IU/0.5 mL supernatant) was then challenged with the suspect toxin containing sample. An unprotected control mouse was also injected with culture supernate.

The complete assay of a toxin containing sample included the results from three mice injected with the active toxin containing sample and at least two mice injected with toxin containing sample subjected to two different toxin neutralizing procedures. All aberrant tests were subjected to additional replication to insure the accuracy of assay interpretation.

RESULTS

THE RECOVERABLE INOCULA from the various experiments ranged from 50 - 62 cfu/mL. These inocula concentrations were reasonably close to the target level of 50 cfu/mL. Immediately after inoculation almost all the model systems contained some heat sensitive or alcohol sensitive *C. botulinum* forms. These apparently germinated spores were considered as vegetative cells and comprised from 0 - 77% of the total count. Since some degree of germination was evident at all pH levels selected for the model system studies of *C. botulinum* types A, B and E, pH minima for germination apparently lie below the levels tested. The control TPGY media at pH 7.00 supported total cell counts of greater than 10^6 cfu/mL after 1 wk of incubation at 35°C. Toxins appropriate for each strain were produced. Trypsin controls did not result in any nonspecific deaths.

Neither growth nor toxin production were detected when *C. botulinum* type A spores served as the inoculum for acetic acid acidified model systems, when *C. botulinum* type B spores served as the inoculum for citric acid or acetic acid acidified model systems at targeted pH levels of 4.20, 4.60, 5.00 and 5.40, or when *C. botulinum* type A spores served as the inoculum for citric acid acidified model systems at targeted pH levels of 4.20, 4.60 and 5.00.

Heat resistant spores and heat sensitive cells of *C. botulinum* types A and B were present immediately after inoculation and throughout the incubation period. Combined spore and vegetative cell counts were variable but no significant increase occurred throughout the 8 wk of incubation at any pH level.

The pH readings of the acetic acid and citric acid acidified model systems obtained immediately after inoculation of spores were within ±0.02 units of the target pH values. The pH of the model systems did not vary by more than ±0.08 unit from initial pH levels throughout the incubation period.

Exponential growth by *C. botulinum* type A was observed in the pH 5.40 model system acidified with citric acid after the first week. A maximum combined spore and vegetative cell population of 6.5×10^4 cfu/mL was obtained at the end of the fifth week of incubation. Type A toxin was detected in this system at the end of the first week and thereafter. The combined spore and vegetative cell count at the time of first toxin detection was 51 cfu/mL. The heat resistant spore population in this model system declined during the first 4 wk of incubation and remained at a level below the initial population until the end of the study.

The system pH obtained immediately after inoculation was 5.39. pH and vegetative cell population increased simultaneously within the second and third weeks of incubation. pH levels stabilized and were maintained between 5.70 to 5.80.

The type A and type B model system studies were not replicated based on the distance of the results from any challenge to accepted limiting pH values for these strains.

Table 1 summarizes the results of the two investigations of growth and toxin production by *C. botulinum* type E spores in acetic acid acidified model systems. No significant increase in the total spore and vegetative cell population was observed and toxin was not detected in the replicate pH 4.20 and 4.60 systems nor in one pH 5.00 model system. Significant changes in either the spore or vegetative cell populations were not observed. A small amount of growth was detected in the replicate pH 5.00 model system at the end of the seventh week of incubation; however, toxin was not detected until the end of the tenth week of an ex-

tended incubation period. Growth and toxin were detected after the first week in both pH 5.40 model systems. When growth was detected in the pH 5.00 and 5.40 model systems, both the spore and vegetative cell populations increased dramatically.

The pH levels of both model system studies immediately after inoculation were within ±0.02 units of the target pH values. The pH 4.20 and 4.60 model systems did not vary by more than ±0.15 unit from the initial pH value during the eight week incubation period. The variation in pH of the 5.00 model systems did not exceed ±0.03 unit from the initial values despite the detection of growth in one investigation late in the study. A decrease in pH to approximately pH 5.20 was observed simultaneously with the detection of growth in both of the pH 5.40 model systems investigated.

Table 2 summarizes the results of two investigations of *C. botulinum* type E in citric acid acidified TPGY model systems. Toxin was detected in only one of the replicate pH 4.20 and 5.00 model systems, although an increase in the total spore and vegetative cell populations was observed in all systems. Toxin was detected in one pH 4.60 model system at week 4 following steady growth and at the end of week 3 in the replicate study following an initial 2 wk lag in growth. Toxin was detected at the end of the fourth week in one pH 5.40 model system study. In the replicate study, toxin was detected at the end of the first week, but a total spore and vegetative cell count was not obtained until 4 days later when the population had reached 1.1×10^3 cfu/mL. In those systems where growth was observed, both the spore and vegetative cell populations increased sharply.

The pH levels of the citric acid acidified model systems immediately after inoculation were within ±0.02 units of the target pH values. The pH of the 4.20 and 4.60 model systems did not vary by more than ±0.08 and ±0.05 unit, respectively, from initial pH levels. The pH of the nontoxic 5.00 model system decreased significantly after the third week and stabilized at approximately pH 4.65. The pH of the toxic 5.00 model system did not vary more than ±0.05 unit from the initial level. In both 5.40 model systems, pH decreased concurrently with the growth observed prior to the first detection of toxin. The pH of the first study stabilized at approximately 5.00 to 5.10 which was below the initial pH level. In the second study, the pH increased after the initial decline and reached approximately pH 6.00 by the end of the study.

Studies of *C. botulinum* type E growth and toxin production in acetic acid acidified media at target pH levels of 4.900, 5.000, 5.100, 5.200, 5.300 and 5.400 were conducted to more closely identify the limiting pH level (Table 3). Growth and toxin production were not detected in the pH 4.900, 5.000 or 5.300 model systems. The combined spore and vegetative cell population remained relatively unchanged. Growth and toxin production were detected in the pH 5.100 and 5.200 model systems at the end of the fifth week. The total spore and vegetative cell population of the pH 5.400 model system rose rapidly during the first week after inoculation, at which point toxin was detected. A decline in population followed. The pH levels of the systems immediately after inoculation were within ±0.008 unit of the target values and did not vary by more than 0.010 unit throughout the study in all cases except the pH 5.400 system. A steady decline from an initial pH of 5.399 to pH 5.187 (at the fourth week) was observed in this system.

The growth and toxin production of *C. botulinum* type E spores were studied in citric acid acidified model systems at target pH levels of 4.000, 4.100, 4.200 and 4.300 (Table 4). Growth and toxin were not detected in the pH 4.000 model system. The total spore and vegetative cell populations of the pH 4.100 and 4.200 model systems increased between the sixth and eighth week of the study which was extended to 10 weeks without toxin detection. The combined spore and vegetative cell populations decreased to a level below that of the initial inoculum by week 10. Growth and toxin production were detected in the pH 4.300 model system at the end of the sixth week and the total spore and vegetative cell population continued to increase.

The actual pH measurements of the TPGY model systems obtained immediately after inoculation of *C. botulinum* type E spores were within ±0.018 unit of the target values. The pH of the model systems did not vary more than ±0.025 unit from initial pH values throughout the study.

The type E model system study over a broad range of pH values was replicated. The type E model system study over a tightened range of pH values overlapped the two prior broad range studies to some extent and was not replicated.

DISCUSSION

THE ABSENCE of growth and toxin production by a mixture of three strains of *C. botulinum* type B spores in pH

Table 1—Growth and toxin productin of C. botulinum type E in duplicate acetic acid acidified TPGY model systems under anaerobic incubation at 26°C

Target pH[a]	Time to first toxin detection (weeks)	Population at time of first toxin detection[b] (cfu/mL)	Time to maximum cell count (weeks)	Maximum cell count[b] (cfu/mL)
4.20	–	–	2	0.3×10^2
	–	–	3	0.5×10^2
4.60	–	–	1	0.5×10^2
	–	–	0	0.5×10^2
5.00	–	–	7	0.5×10^2
	10	3.8×10^3	8	1.0×10^4
5.40	1	7.8×10^2[c]	3	6.3×10^6
	1	2.2×10^5	5	3.8×10^5

[a] pH measurements obtained immediately after inoculation of C. botulinum spores were within ±0.02 unit of the target pH values.
[b] Total combined spore and vegetative cell counts.
[c] Insufficient serial dilutions made to obtain valid count. Sample contained 6.3×10^6 cfu/mL at week 3.

Table 2—Growth and toxin production of C. botulinum type E in duplicate citric acid acidified TPGY model systems under anaerobic incubation at 26°C

Target pH[a]	Time to first toxin detection (weeks)	Population at time of first toxin detection[b] (cfu/mL)	Time to maximum cell count (weeks)	Maximum cell count[b] (cfu/mL)
4.20	–	–	8	2.5×10^2
	5	1.1×10^4	8	3.9×10^5
4.60	4	1.9×10^3	8	7.3×10^4
	3	7.6×10^2	8	2.6×10^4
5.00	–	–	8	1.0×10^5
	3	6.0×10^3	8	3.7×10^5
5.40	4	5.0×10^2	5	5.3×10^3
	1.5	1.1×10^3[c]	7	9.8×10^5

[a] pH measurements obtained immediately after inoculation of C. botulinum spores were within ±0.02 unit of the target pH values.
[b] Total combined spore and vegetative cell counts.
[c] Insufficient serial dilutions made to obtain valid count. Sample contained 1.1×10^3 cfu/mL on the 11th day.

5.40 model systems and by a mixture of three strains of *C. botulinum* type A spores in pH 5.00 model systems was independent of the acid used to adjust pH. These findings support the recommended maximum pH of 4.6 cited by the Food & Drug Administration (FDA) in the Good Manufacturing Practice (GMP) regulation for acidified food products (CFR, 1979). The results also support the statement by Gardner (1976) that pH 4.6 provides an ample margin of safety against the hazards of botulism in acidified food products.

Several outbreaks of botulism were attributed to *C. botulinum* types A and B in acidified food products with pH levels below 4.6 (CDC, 1973; 1974a, b). Growth and toxin production at low pH levels was proposed to be mediated by metabiosis (Huhtanan et al., 1976; Odlaug and Pflug, 1979; CDC, 1974a). Smelt et al. (1982) suggested that co-incubation with other microorganisms might reduce the E_h, remove residual oxygen and favor growth and toxin production by *C. botulinum* but they actually demonstrated that growth of non-*C. botulinum* microorganisms was not essential to growth and toxin production by *C. botulinum* types A and B at pH levels as low as 4.2. Tanaka (1982) postulated that the presence of protein particles might provide localized pockets of elevated pH that enabled growth and toxin production by *C. botulinum* types A and B in apparently low pH environments. The postulation was based upon observed toxin production at pH 4.30 in a medium containing a large amount of precipitated protein and absence of toxin in media prepared with hydrolyzed protein at pH below 4.6.

The observed growth and toxin production by *C. botulinum* type E in the TPGY model system at pH 4.20 to 4.30 was considerably below the 4.6 pH level recommended in the GMP regulation established by the FDA, but agrees with the results of Dolman and Iida (1963) that *C. botulinum* type E strain VH grew and produced toxin at pH 4.0 and 4.2 in pickled herring. Contradictory reports concerning the absence of growth and toxin production by *C. botulinum* type E at pH 4.6 were made by Segner et al. (1966), Ohye and Christian (1967) and Baird-Parker and Freame (1967).

Ingram and Robinson (1951) and Huhtanen et al. (1976) showed that the limiting pH varied among different strains of *C. botulinum*. The conflicting reports conerning the pH level required to inhibit growth and toxin production by *C. botulinum* may be attributed to the variability in acid tolerance exhibited by different strains of *C. botulinum* as well as differences in substrates and methodologies in different investigations.

Metabiosis was not a factor in the observed growth and toxin productin by *C. botulinum* type E in the TPGY model systems. Weekly platings ensured the absence of contaminating microorganisms. The pH profile of the model systems showed no change from the initial levels despite the detection of growth by *C. botulinum* type E in citric acid acidified cultures at pH 4.20, 4.60 and 5.00.

Growth and toxin production by *C. botulinum* type E at a pH below 4.6 in TPGY medium could not be attributed to the presence of any protein particles, since the medium did not contain any particulates. The liquid medium allowed uniform distribution of the acidulants and equilibrium pH was attained throughout the medium, thus precluding the existance of any localized regions of elevated pH.

Relatively low populations of *C. botulinum* type E cells and spores were enumerated in the TPGY model systems when toxin was detected in several experiments. Suboptimal pH conditions may have affected the physiological properties as well as the morphological characteristics of the organisms. Montville (1983b) observed aggregation of *C. botulinum* cells in low pH substrates. Cell aggregation may account for the relatively low counts observed and it may also offer an explanation for the rather large variability and fluctuation in the combined vegetative cell and spore counts within the model systems. It is also possible that under adverse pH conditions, proper cell division may have been impaired thereby affecting apparent cell numbers, but having no affect upon total metabolic activity by cells. The metabolic activities involved in toxin production may have been stimulated by adverse conditions to increase botulinum toxin production and secretion into the culture medium.

The botulinum assay used varied from the BAM procedure in an attempt to conserve the number of animals utilized. A degree of certainty was sacrificed since a positive result could have gone undetected in the first mouse screen. However, the dynamic nature of the study, which included toxin assays on successive weeks, would allow a missed positive in one week to be detected in the following week, although time of toxin production could be in error by as much as 7 days.

While growth and toxin productin by *C. botulinum* type E was observed in acetic acid acidified TPGY model systems at pH 5.000, 5.200, and 5.400, it was not observed at pH 5.300. The combined spore and vegetative cell count obtained immediately after inoculation was 63 cfu/mL and diminished to 17 cfu/mL during the eight week study. The possibility of this specific pH being inhibitory to the growth and toxin production by the organism is unlikely. The fact that this was a single experiment and that growth and toxin production were observed at two lower pH levels indicate that the failure of this particular culture to develop was due to an unexplicable experimental variable.

Table 3—Growth and toxin production of C. botulinum type E in an acetic acid acidified TPGY model system under anaerobic incubation at 26°C

Target pH[a]	Time to first toxin detection (weeks)	Population at time of first toxin detection[b] (cfu/mL)	Time to maximum cell count (weeks)	Maximum cell count[b] (cfu/mL)
4.900	–	–	0	0.5×10^2
5.000	–	–	4	0.7×10^2
5.100	5	6.3×10^2	7	1.1×10^3
5.200	5	3.2×10^3	5	3.2×10^3
5.300	–	–	6	0.4×10^2
5.400	1	4.1×10^6	1	4.1×10^6

[a] pH measurements obtained immediately after inoculation of C. botulinum were within ±0.008 unit of the target pH values.
[b] Total combined spore and vegetative cell counts.

Table 4—Growth and toxin production of C. botulinum type E in a citric acid acidified model system under anaerobic incubation at 26°C

Target pH[a]	Time to first toxin detection (weeks)	Population at time of first toxin detection[b] (cfu/mL)	Time to maximum cell count (weeks)	Maximum cell count[b] (cfu/mL)
4.000	–	–	5	0.4×10^2
4.100	–	–	8	0.9×10^2
4.200	–	–	7	1.3×10^2
4.300	6	9.8×10^2	7	1.0×10^4

[a] pH measurements obtained immediately after inoculation of C. botulinum were within ±0.018 unit of the target pH values.
[b] Total combined spore and vegetative cell counts.

It was not considered essential to repeat this experimental point since it had no bearing on the challenge to the established standard of pH 4.6 as the acceptable lower limit for *C. botulinum* growth.

Smoot and Pierson (1979) demonstrated the effect of substrate Eh on the limiting pH for the growth of *C. botulinum*. They showed that while a *C. botulinum* type A strain exhibited comparable growth in low E_h medium (−145mv) and in high E_h medium (−50mV) at pH 5.3, growth was greatly delayed and decreased in the higher E_h medium when the pH of the substrate was lowered to 5.2. Smelt et al. (1982) demonstrated that pure cultures of *C. botulinum* type A could produce toxin at pH 4.3 providing the growth medium was sufficiently anaerobic. They used anaerobic jars for the incubation of *C. botulinum* rather than overlaying cultures with vaseline, vaspar or other oxygen barriers. The use of strict anaerobiosis in the current TPGY model systems studies ensured a highly favorable growth environment for *C. botulinum* under low pH conditions.

Some differences in results between the TPGY model systems and those reported in the literature may be attributed to differences in testing procedures for growth and toxin production. Turbidity and/or gas formation in the culture substrates were used as primary indicators of growth by Townsend et al. (1954), Baird-Parker and Freame (1967), Emodi and Lechowich (1969), Ito et al. (1976) and Ito et al. (1978). In some of the cited studies, the primary indicators determined whether subsequent toxicity testing or confirmational testing for growth of the organism would be carried out. Montville (1983a) demonstrated that the accumulation of gas could be greatly delayed or absent in *C. botulinum* cultures at low pH. Therefore, some of the investigators may have reported nongas producing growth as negative growth.

The TPGY model systems were evaluated weekly for growth of *C. botulinum* and for toxin production. Increase in the vegetative cell and/or spore population, represented by the plate count data, indicated true growth in the systems. Although growth was accompanied by increased turbidity in some cases, it was not always apparent in low pH systems in the early stage of growth, even after toxin had been formed. Assays for botulinum toxin were carried out weekly, regardless of the degree of growth in the model system.

The TPGY model system studies demonstrated that growth and toxin production by *C. botulinum* is defined by both the pH of the system and the acidulant used in acidification. Acetic acid was a better inhibitor of *C. botulinum* than citric acid. A similar observation was made by Smelt et al., (1982). They demonstrated that soya suspensions which had been adjusted to pH 4.4 with acetic acid or citric acid supported the production of botulinum toxin in the presence of *B. subtilis* after 14 wk and 4 wk, respectively. Studies with *Salmonella* (Chung and Goepfert, 1970) or *Staphylococcus aureus* (Minor and Marth, 1972) showed that the type of acid determined the minimum pH at which growth was possible. Hydrochloric acid and citric acid were less inhibitory than lactic acid and acetic acid at the same pH. Juven (1976) reported that the minimum pH for the growth of *Lactobacillus brevis* varied from 3.0 in citric acid acidified media to 4.0 in acetic acid acidified media.

Townsend et al. (1954) reported that the inhibitory pH level for the growth of *C. botulinum* varies from food to food and from food to laboratory medium. Further research involving actual food products is needed. Outbreaks of type E botulism involving acidified marine food products have been rare and the hazard from such products minimal (Lerke, 1973). Nonetheless, the potential for growth and toxin productin by *C. botulinum* type E under acidified conditions must be recognized.

The TPGY model system investigation illustrates that where acidification is relied upon for the control of botulism in food products, consideration must be given to parameters other than the pH of the system. The parameters to be considered include the type and strain of *C. botulinum*, the type of acidulant used and the type of substrate involved. Better understanding of the mechanism by which an acidulant exerts its inhibitory effect is of great importance in establishing a valid regulatory approach to safety in acidified foods.

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Inhibition of *Clostridium botulinum* Type E in Model Acidified Food Systems

LAURIE S. POST, TERRY L. AMOROSO, and MYRON SOLBERG

ABSTRACT

The potential for growth and toxigenesis of a mixture of three strains of *C. botulinum* type E in model food systems at pH levels of 4.600 and 4.200 was investigated. Whole shrimp, shrimp puree, tomato puree, and tomato and shrimp puree were acidified to pH levels of 4.200 and 4.600 with acetic or citric acid. Inoculated and uninoculated control samples were tested during 8 wk incubation at 26°C. No significant *C. botulinum* growth or toxigenesis was detected in the food systems over the 8 wk test period and the pH of the various foods remained generally constant. This investigation affirms the safety of the generally accepted inhibitory pH level of 4.6 in the food systems studied.

INTRODUCTION

SEVERAL REPORTS have demonstrated the ability of *C. botulinum* types A and B to grow and produce toxin at pH levels below 4.6 (Raatjies and Smelt, 1979; Smelt et al., 1982; Tanaka, 1982). Tsang et al. (1985) reported that *C. botulinum* type E grew and produced toxin at pH 4.3 in citric acid acidified laboratory media. Food poisoning, attributed to *C. botulinum*, has occurred in home prepared products where the pH was below 4.6 (CDC, 1973; CDC 1974a, b). The generally accepted limiting pH of 4.6 is considered to provide an ample margin of safety against the hazards of botulism in acidified food products and such products are normally given only mild heat treatment (Gardner, 1976). One such commodity considered safe by virtue of pH alone is seafood cocktail. Most seafood is high in pH; however, the cocktail sauces used in packing reduce the pH below 4.6. Crab and shrimp provide a good growth medium for *C. botulinum* (Lerke, 1973). *C. botulinum* type E has been found in a number of raw seafoods including whitefish chubs, vacuum packaged flounder and several species caught off the Pacific Coast of the United States (Craig et al., 1968; Insalata et al., 1967; Pace et al., 1967). In one case, a small amount of toxin was detected in pickled herring at pH 4.2 and 4.0 (Dolman, 1964). Experimental evidence of the safety of seafood cocktails was sought by Lerke (1973). *C. botulinum* type E was not detected in a survey of 35 commercially prepared products.

The minimum pH required to inhibit growth and toxin production by *C. botulinum* varies from substrate to substrate (Townsend et al., 1954) and is dependent on such factors as bacterial strain involved (Ingram and Robinson, 1951) and acidulants used (Smelt et al., 1982). Acetic acid is a common acidulant in seafood cocktails and the major acidifying factor in tomatoes is citric acid. The acidification of shrimp in cocktail sauce is partly dependent upon the acidity of the tomatoes. This study was undertaken to determine the effects of citric and acetic acids upon germination, outgrowth, and toxin production by *C. botulinum* type E in model acidified food systems representing shrimp cocktail. Whole shrimp, shrimp puree, tomato puree and tomato and shrimp puree food systems were investigated at acidification levels of pH 4.200 and 4.600.

Authors Post, Amoroso, and Solberg are affiliated with the Dept. of Food Science, New Jersey Agricultural Experiment Station, Cook College, Rutgers Univ., New Brunswick, NJ 08903.

MATERIALS & METHODS

Preparation of shrimp and tomato stocks

Frozen shrimp (71/90 count) and fresh tomatoes were purchased from local suppliers. The frozen shrimp were cooked (100°C, 15 min), peeled and refrozen (−20°C) prior to use. Tomatoes were subjected to a hot break, peeled and passed through a pulper. The resulting tomato puree was pasteurized in a boiling water bath 100°C, 45 min) and frozen (−20° C) until use.

Spore inoculum

Spores of three strains of *C. botulinum* producing Type E toxin (Beluga, MN, and D8) were selected. The strains were obtained from the FDA, Washington, DC. Spore stocks were prepared and harvested by the method of Tsang et al. (1985).

Culture medium

Trypticase peptone glucose yeast extract (TPGY) medium (BAM, 1978) without trypsin was used for culture and sporulation. Plate counts were made on catalase treated anaerobic egg agar (AEY) as described by Tsang et al. (1985).

Calibration of spore stock suspensions

Stock spore suspensions were calibrated by direct plating of the stock suspensions on AEY. Plate count data were used to calculate dilutions of each spore suspension required to obtain a concentration of 50 spores/mL or g after subsequent inoculation of the model food systems.

Preparation of food purees and acid solutions

Shrimp puree was prepared by homogenizing thawed whole shrimp with water in a blender, 100g shrimp/146 mL water. The ratio was derived from examination of the solid to liquid ratio of commercially packed shrimp cocktail. Portions of tomato puree were thawed and set aside. A combined shrimp and tomato puree was prepared by blending tomato puree with whole shrimp in a blender, 100g shrimp to 146 mL tomato puree.

Citric or glacial acetic acids (Fisher, Pittsburgh, PA) were used to acidify the purees to pH 4.200 or 4.600. Due to the inherently low pH of tomatoes, the pH level of the tomato puree was first adjusted to a uniform 5.0 with 1N NaOH and then acidified to the desired pH level.

The purees were dispensed in 20 mL portions into individual sampling vials. The samples were autoclaved at 121°C for 30 min and allowed to equilibrate overnight in an anaerobic chamber prior to inoculation.

Solutions of each organic acid were prepared such that the pH of 100g of whole shrimp in 146 mL acidic solution would equilibrate at pre-selected pH levels of 4.200 or 4.600. This required the addition of 6.08 mL (pH 4.200) or 3.42 mL (pH 4.600) of a 17.4M solution of glacial acetic acid (Fisher, Pittsburgh, PA) to 146 mL of deionized distilled water or the addition of 2.85g (pH 4.200) or 1.90g (pH 4.600) anhydrous crystalline citric acid (Fisher, Pittsburgh, PA) to 146 mL of deionized distilled water.

Seven to eight shrimp were placed in individual sampling vials (vial totaling approximately 21g of shrimp). Appropriate aliquots of the acidic solutions were added. The samples were autoclaved at 121°C for 30 min and allowed to equilibrate approximately 18 hr in an anaerobic chamber prior to inoculation.

Inoculation of the samples

The spore inoculum consisted of a composite of the three selected strains of *C. botulinum*. Each individual stock suspension was alcohol treated for one hour (Tsang et al., 1985). The appropriate dilutions were made to obtain a concentration of 4×10^5 spores/mL. A 5 mL aliquot of each spore dilution thus obtained was combined to produce a mixed working suspension containing 4×10^5 spores/mL.

Each 20 mL aliquot of puree was inoculated with 25 μL of a 10-fold dilution of the working suspension to provide a final inoculum concentration of 50 spores/mL of puree. Each of seven shrimp (approximate weight 3g each) was aseptically inoculated in the center with 3.75 μL of a 10-fold dilution of the working suspension (using a 5 μL syringe, Hamilton, Inc. Reno, NV) and returned to the individual sampling vials containing the acid solutions. The final inoculum concentration was 50 spores/g shrimp. Control samples of purees and shrimp were inoculated with equal volumes of sterile distilled water. The food samples were incubated at 26°C in anaerobic jars.

Sampling

Sampling procedures were carried out within the anaerobic chamber. Required numbers of individual sampling vials were removed from storage. Food purees were directly plated on AEY agar plates inside the chamber without treatment to determine the total number of germinated cells, vegetative cells, and spores. The plates were incubated at 35°C for 36–48 hr inside the anaerobic chamber. The pH of each sample was measured using a Fisher Accumet pH meter model #291. A standard phosphate buffer solution at pH 6.863 (25°C) and an acid potassium phthalate buffer solution at pH 4.004 (25°C) were used to standardize the pH meter to the third decimal place (AOAC, 1980). An aliquot of puree was reserved for toxin testing.

Table 1—Effect of storage time on recovery of C. botulinum *type E from model food systems acidified with citric acid or acetic acid*

Sample	Storage time (wk)	Citric acid pH 4.200[a]	Citric acid pH 4.600[a]	Acetic acid pH 4.200[a]	Acetic acid pH 4.600[a]
		CFU/mL	CFU/mL	CFU/mL	CFU/mL
Tomato puree	0	49	45	43	48
	8	3	11	4	3
Shrimp puree	0	48	48	43	42
	8	21[b]	30[b]	25[c]	42[c]
Tomato and Shrimp puree	0	45	48	45	43
	8	3	3	3	3

[a] Targeted pH levels at time of inoculation.
[b] Counts after an additional sampling following 21 wk of incubation were 15 and 18 CFU/mL for systems at pH 4.200 and 4.600, respectively.
[c] Counts after an additional sampling following 21 wk of incubation were 0 and 12 CFU/mL for systems at pH 4.200 and 4.600, respectively.

An individual sampling vial of whole shrimp was selected. pH measurements were made of the shrimp and the acidifying solution. The shrimp were homogenized in a stomacher with an equal weight of gel phosphate buffer (pH 6.8) in preparation for toxin testing. Sampling was carried out weekly for an 8 wk period.

Mouse assay for toxin

Samples of purees were mixed with equal volumes of gel phosphate buffer. The food purees and homogenized whole shrimp were centrifuged 30 min at 12,000 rpm. Supernatants were decanted and the pH adjusted to 6.2 with 1N NaOH. Prior to injection, 0.2 mL of a fresh 10% trypsin solution (Trypsin 1:250, Difco Laboratories, Detroit, MI) was added to 1.8 mL of the puree and whole shrimp supernatants and incubated at 37°C for 1 hr. The bioassay procedure followed that of Tsang et al. (1985).

Controls

Uninoculated controls were used to assure that the purees and shrimp in acid solution were sterile and that the pH did not change during the 8 wk period. These controls also were injected into mice to eliminate the possibility of the food giving false results for botulism toxin. Inoculated media at pH 7.0 were used to assure the viability of the spores, suitability of the medium and the production of toxin by the inoculum.

RESULTS

CLOSTRIDIUM BOTULINUM spores either showed no growth or appeared to decline in numbers with time in citric acid acidified tomato puree, shrimp puree, and shrimp and tomato puree at either a target pH of 4.200 or 4.600 as shown in Table 1. Similar results were observed when acetic acid was used as the acidulant (Table 1). Tomato and shrimp and tomato puree systems showed approximately a ten-fold decline in cell numbers by week 8. A ten-fold decline in cell numbers was apparent in shrimp puree system after 21 wk of incubation. Uninoculated controls remained sterile. Toxin was not detected in any puree system or in whole shrimp in acidified solutions during the entire incubation period. Nonspecific toxic reactions occurred periodically during the bioassay for toxin and were eliminated by dilution of the sample with gel phosphate buffer.

The initial and final 8 wk pH values for each inoculated and uninoculated acidified food system are listed in Table 2. The pH of inoculated and uninoculated puree systems fluctuated throughout the 8 wk incubation period. Any overall changes in pH were not significant since such variations were within those attributable to experimental error. The pH of whole shrimp increased steadily until week 8 for systems acidified with citric acid to a target pH of 4.600 and for systems acidified with acetic acid to a target pH of 4 200 or 4.600.

DISCUSSION

ALTHOUGH SHRIMP was suggested as a good growth medium for *C. botulinum* (Lerke, 1973) and home canned

Table 2—Effect of storage time on pH of inoculated[a] and uninoculated model food systems acidified with citric acid or acetic acid

System	Citric acid Initial pH	Citric acid pH week 8 Inoculated	Citric acid pH week 8 Uninoculated	Acetic acid Initial pH	Acetic acid pH week 8 Inoculated	Acetic acid pH week 8 Uninoculated
Tomato puree	4.200 ± 0.014	4.182	4.175	4.200 ± 0.009	4.199	4.203
	4.600 ± 0.013	4.562	4.572	4.600 ± 0.027	4.585	4.603
Shrimp puree	4.200 ± 0.010	4.166	4.183	4.200 ± 0.018	4.108	4.238
	4.600 ± 0.017	4.565	4.580	4.600 ± 0.004	4.468	4.550
Shrimp and Tomato puree	4.200 ± 0.031	4.217	4.204	4.200 ± 0.091	4.172	4.206
	4.600 ± 0.055	4.500	4.487	4.600 ± 0.071	4.614	4.615
Whole Shrimp	4.200 ± 0.198	4.386	4.197	4.200 ± 0.186	4.713	4.654
	4.600 ± 0.145	4.992	4.858	4.600 ± 0.029	4.823	4.841

[a] Initial inoculum was approximately 50 spores/mL *C. botulinum* type E.

tomato products with pH values below 4.6 have supported *C. botulinum* growth and toxin production (CDC, 1974a, b; 1981; 1982), outgrowth and toxigenesis were prevented in the tomato puree, shrimp puree, shrimp and tomato puree, and whole shrimp food systems used in this study. The food systems were initially adjusted to a target pH of 4.200 or 4.600 with citric or acetic acids Inhibition occurred independently of acid used and despite slight increases in pH which took place during the 8wk incubation period in some of the food systems. In a survey of 35 samples of commercially prepared seafood cocktails, Lerke (1973) was unable to detect *C. botulinum* in any sample although pH values ranged from 4.10–4.85. When Lerke (1973) inoculated homogenates of crab and shrimp with *C. botulinum* type E, toxigenesis was prevented at pH 4.86 for crabmeat and 5.03 for shrimp acidified with a citric-acetic acid mixture. This current investigation provides additional confirmation of the safety of acidified shrimp products, specifically shrimp in tomato based cocktail sauce.

The pH of several food systems increased slightly above 4.6 during incubation, but the minimum pH for growth may not have been reached since the pH did not rise above 5.0. Tsang et al. (1985) observed a low level of growth and toxin production in one replicate of a complex laboratory medium model system acidified with citric acid to a target pH of 4.20 and detected growth and toxin in the same model system with a target pH of 4.300. Tsang et al. (1985) suggested that the strict anaerobiosis of their system could be a factor in the unexpected result. The same strict conditions of anaerobiosis were employed in the food systems studied yet growth was not detected. Tomatoes were alkalized to a uniform pH prior to acidification to the selected pH test levels. The procedure increased the total concentration of citrate ions present above the amount inherent in tomato products. The mode of action of organic acids has been hypothesized to involve the transport of undissociated acid molecules into a cell with subsequent dissociation due to the high physiological pH of the cell interior (Ramos et al., 1976). Formation of hydrogen ions and an accompanying decrease in physiological pH is one theory of cell inhibition (Baird-Parker, 1980). The increase in the citrate ion concentration would not be significant if hydrogen ion concentration was the basis of inhibition. However, if citrate ion concentration was also a factor, then greater inhibition would be exerted by the tomato puree systems than would normally be exerted by tomato products. Shrimp puree systems at week 8 seemed to allow greater recovery of spores, irrespective of acidulant used, than tomato puree systems. When both purees were combined, the tomato puree appeared to be the major influencing factor in spore recovery since counts at week 8 in the combined systems resembled those of the tomato puree systems.

The inhibition exerted by the food systems may be more than a function of pH. Certain nutritional requirements for growth may not have been present in the food systems or an environmental deficiency may have combined with the additional stress of unfavorable pH to exert an inhibitory effect. The laboratory media model system used by Tsang et al. (1985) held all parameters other than pH at optimal levels. The real food systems could not be defined or regulated to the same extent since tissue breakdown and subsequent changes in system composition occurred upon prolonged incubation at 26°C.

Although the potential exists for *C. botulinum* to grow and produce toxin at a pH below the commonly accepted limiting pH of 4.6, this current investigation affirms the safety of the 4.6 pH value in shrimp and tomato food systems. The results of these studies further support the suggestion of Tsang et al. (1985) that greater understanding of the factors involved in regulating growth of *C. botulinum* is essential and that reliance upon pH measurement alone is insufficient.

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Toxin Production in Hard-Cooked Eggs Experimentally Inoculated with *Clostridium botulinum*

L. B. LUBIN, R. D. MORTON, and D. T. BERNARD

ABSTRACT

Hard-cooked eggs were cooled for 30 min in water containing concentrations of spores of *Clostridium botulinum* ranging from 10^1 to 10^4 spores per mL. The eggs were then air dried and incubated either aerobically, anaerobically, or in sealed plastic containers and checked daily for toxin production using the mouse bioassay. Toxin was detected inside the eggs within 2 to 7 days depending on the method of incubation. Sensory changes in toxic eggs were also recorded, and although the proteolytic strains usually produced unacceptable odors and/or appearances, these changes were not always detected.

INTRODUCTION

RAW EGGS have serveral natural antimicrobial defenses. Some of these are physical while others are chemical in nature. The structure of the egg consists of four major components, the shell, membranes, albumen and yolk. The cuticle, which is the outside portion of the shell, protects the egg from bacterial invasion as long as this layer remains intact. The shell membranes are thought by some to provide an even more resistant physical barrier by acting as a bacterial filter (Haines and Moran, 1940; Williams and Whittemore, 1967). Chemically, antimicrobial defenses are provided predominantly by the lysozyme which is in the shell membranes and albumen (Korotova, 1957). While this enzyme will aid in cell lysis of some bacteria, others are resistant and may cause spoilage of raw eggs.

Although *Clostridium botulinum* is not among the normal microflora on the poultry egg shell (Bhargava, et al., 1975), research by Moats (1981) demonstrates that the bacterial load on the shells of commercially washed eggs is dependent on the sanitary condition of the washing equipment rather than the eggs. Improper or insufficient cleaning and sanitizing of equipment can provide a source for contamination of raw eggs with numerous types of microorganisms.

This paper presents the results of work undertaken to determine the ability of *C. botulinum* spores present in cooling water to penetrate the physical barriers of hard-cooked eggs, and to determine if the egg provides the necessary nutritional and environmental requirements for growth and toxin production.

MATERIALS & METHODS

Organism, inoculation, and incubation

Eggs purchased at a retail market were given a ten minute boiling water cook (100°C) and then cooled for 30 min in water inoculated with spores of *Clostridium botulinum*. Two separate spore suspensions were used in the study; type A strain 62A was used for the initial study and a composite of five (5) A-types and five (5) B-types was used in confirmatory testing. The *C. botulinum* strains used in the composite were: Type A – Clovis, 172A, 62A, 33A, 75-4A, 213B, 32B, 73-211B, 113B, and RH19B.

The authors are affiliated with the National Food Processors Association, 1401 New York Ave., NW, Washington, DC 20005.

Four inoculum levels ranging from approximately 1×10^1 to 1×10^4 spores per mL were used for each suspension. The spores were heat activated for 13 min at 80°C to inactivate any toxin that might be present and then added to the cooling water. To verify the inoculum levels, counts were performed on the cooling water at each target spore concentration using trypticase peptone agar (TPA) as suggested by Polvino and Bernard (1982).

After the cooling period, the eggs were removed from the water and air dried. Incubations were conducted at 30°C with the eggs held at one of three different conditions; aerobically, by storing in the original fiberboard containers, anaerobically in BBL Gas-Pak anaerobe jars, or in plastic food storage containers with tight fitting lids to simulate in-home storage conditions.

Toxin testing

Eggs were removed from incubation at predetermined intervals over a period of 1 wk and tested for toxicity using the mouse bioassay. The shell and shell membranes were removed from the egg and placed in a Whirl-Pak bag while the egg portion was placed in a separate Whirl-Pak bag. Gelatin phosphate buffer was mixed with the shell portion at a 1:3 shell to buffer weight/volume (w/v) ratio and this mixture was allowed to soak for approximately 10 min. The egg was mixed with buffer at a 1:2 w/v ratio and macerated for 1 min in a Stomacher 400. The resulting homogenates were centrifuged for 10 min at approximately 3000 rpm in a Clay-Adams safety head centrifuge. One-half mL of the supernatant was injected intraperitoneally into each of two Swiss Webster mice.

Toxin titer, organism count, pH and typing

The toxin titer was determined by the method of Reed and Muench (1938) using an egg that had been cooled in water containing both A and B type spores at a concentration of approximately 10^2 spores/mL and incubated aerobically. The pH's of the raw egg, toxic hard-cooked egg, and nontoxic hard-cooked egg were also determined.

An anaerobic plate count was performed on an egg which became toxic after it had been boiled and cooled in water containing the composite spore suspension at a concentration of about 10 spores/mL and then incubated aerobically for 10 days. In addition, the type of toxin present in an egg that had been cooled in water containing both A and B type spores at a concentration of approximately 10^2 spores per mL and incubated aerobically was determined by combining portions of homogenate with polyvalent (ABCDEF), Type A and Type B antitoxins. The toxin-antitoxin mixtures were incubated for 30 min at 30°C. A portion of the homogenate was also heated for 5 min at 100°C. The samples were tested for toxicity by injecting each of two Swiss Webster mice intraperitoneally with 0.5 mL of each treated homogenate.

RESULTS

RESULTS of the toxicity testing are summarized in Tables 1 and 2. These show the earliest detection times for a series of eight replicate experiments in which one egg from each condition was tested daily. Toxin was produced in the eggs cooled in water containing 62A, type A *C. botulinum* spores within 1 wk for all incubation conditions. Under anaerobic conditions toxin was produced by day 2 or 3 even when the inoculum level was approximately 10 spores per mL. The eggs stored in plastic containers required longer periods of time and/or a higher number of spores in the cooling water than the anaerobically stored eggs before toxin production was detected. They did become toxic, however, in less than 1 wk. Similarly, eggs

Table 1—Earliest detection time (days) for toxin production in eggs exposed to C. botulinum 62A

Approx. inoculum level	Anaerobic		Sealed plastic containers		Aerobic	
	Shell	Egg	Shell	Egg	Shell	Egg
10^4/mL	3	3	3	4	4	4
10^3	2	3	6	6	7	7
10^2	3	3	6	7	7	7
10^1	2	3	NT[a]	NT	NT	NT

[a] NT = Not Tested

Table 2—Earliest detection time (days) for toxin production in hard cooked eggs cooled in water containing a composite of 5 A and 5 B strains of C. botulinum

Approx. inoculum level	Anaerobic		Sealed plastic containers		Aerobic	
	Shell	Egg	Shell	Egg	Shell	Egg
10^4/mL	2	3	3	5	4	–[a]
10^3	2	3	6	6	4	5
10^2	3	5	5	–[a]	5	5
10^1	–[a]	–[a]	–[a]	–[a]	5	5

[a] Did not become toxic during the incubation period.

retained under aerobic conditions also became toxic within one week; either the same day or one day later than those incubated in sealed plastic containers.

Eggs cooled in water containing both A and B type spores showed similar results (Table 2). The eggs stored in sealed plastic containers and under aerobic conditions became toxic, however, 1 to 2 days earlier with this composite than those exposed to 62A. It should be noted that the results listed in Table 1 and 2 are the earliest detection times observed under the given conditions. In some cases detection took 1 to 5 days longer.

In most cases the shell fraction became toxic before the egg fraction. There were no instances when the egg was toxic and the shell was not. Apparently, toxin is being produced first either between the shell and the outer shell membrane or between the outer and inner shell membranes. The results of the counts performed on the cooling water, along with target inoculum levels, are as follows: (10^1), 7; (10^2), 62 - 97; (10^3) $7.0 \times 10^2 - 8.1 \times 10^2$; ($10^4$), $7.2 \times 10^3 - 1.0 \times 10^4$.

The toxin titers of both the egg portion and the shell portion were each determined to be 1×10^5 mouse LD_{50} per mL of homogenate. There appeared to be no difference between the pH of toxic hard-cooked eggs (pH 8.5), nontoxic hard-cooked eggs (pH 8.4) and raw eggs (pH 8.0). An anaerobic plate count of 2.7×10^5 organisms per mL of homogenate was determined after 10 days of aerobic incubation for the toxic egg that was cooled in water containing as few as 10 spores. Typing of the toxin demonstrated that both A and B-type *Clostridium botulinum* toxins were present.

DISCUSSION

RESULTS of this study indicate that in the proper conditions of the present tests, *C. botulinum* spores can penetrate the shell of hard cooked eggs. Although the cuticle layer of the shell normally prevents the penetration of bacteria into the pores of raw eggs, it appears that this may be damaged by handling conditions during the washing and/or cooking of the egg. Once the cuticle and debris in the pores has been removed, the shell becomes a porous matrix through which spores and bacteria may pass. Since the pore size ranges between 9 and 35μ, most microorganisms would have little problem in passing through the shell to the membrane. Additionally, cooking of the egg causes inactivation of the lysozyme in the membrane and albumen which is an important chemical defense against bacterial invasion.

Bean and MacLaury (1959) demonstrated that despite the natural defenses of the egg, organisms could be recovered from the inner surface of shell membranes within a few hours after cooling warm eggs in a chilled suspension of bacteria. The contraction of the eggs on cooling creates the air cell between the albumen and the shell membranes which apparently produces a vacuum which can draw in bacteria present in the cooling water. The phenomenon was further demosntrated by Gillespie and Scott (1965) who suggest that bacteria must penetrate to within 15 to 20μ of the shell membranes to reach sufficient water and an environment favorable for its growth. It is not surprising, therefore, that spores of *C. botulinum* may also be drawn into hard cooked eggs during cooling.

Those unfamiliar with clostridia may be misled by their description as strict anaerobes as an indication of sensitivity to oxygen toxicity, when it is actually more descriptive of overall biochemical function. Morris (1975) indicated that an anaerobe is an organism which "(i) generates energy and synthesizes its substance without recourse to molecular oxygen; and (ii) demonstrates a singular degree of adverse oxygen-sensitivity which renders it unable to grow under an atmosphere of air." As with other anaerobies, the clostridia display a spectrum of oxygen tolerance and response to redox potentials (Bernard, 1983). Thus, as has been shown with fresh mushrooms (Sugivama and Yang, 1975) and baked potatoes (Lubin et al., 1980; Sugiyama et al., (1981) *C. botulinum* can produce toxin even when products are not hermetically sealed in the conventional sense.

To date, no outbreaks of botulinum intoxication have been reported in association with the consumption of cooked eggs. Apparently, the water cooling of the eggs is important in transporting the organisms inside the shell, and therefore the presence of spores in the cooling water is important in producing toxicity. Once inside the shell a microenvironment apparently exists which allows the germination and outgrowth of *C. botulinum*. It may be that this microenvironment is formed initially between the albumen and the inner shell membrane. As Morris and O'Brien (1971) noted, once growth by *C. botulinum* commenced it reduced the Eh of the medium in which it was growing and thereby created even more widespread and favorable conditions. They also noted that Eh can be reduced by using large numbers of organisms in inoculations.

Botulinum toxin was produced in hard cooked eggs within 2 to 7 days after exposure to the spores in cooling water. Although in most cases the toxic eggs would be considered sensorily unacceptable, many eggs were not overtly spoiled even though they had detectable levels of toxin. Titers of toxic eggs were found to be 1×10^5 mouse LD_{50} which, when compared with the 3×10^3 mouse LD_{50} considered to be sufficient to produce symptoms in adult humans, indicates a possible hazard. Although *C. botulinum* spores normally may not be present on the egg surfaces, contamination of cooling water could cause the spores to pass through the egg shell where an environment exists which may be conducive to growth and toxin production. Boiled eggs, therefore, should be considered perishable and should be kept refrigerated or handled so as to assure against possible contamination with *C. botulinum* spores.

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Phenolic Spice Components Sporostatic to *Bacillus subtilis*

M. A. AL-KHAYAT and G. BLANK

ABSTRACT

Spores of *Bacillus subtilis* stored under different concentrations of clove infusion were microscopically observed to undergo germination. These spores, however, did not outgrow when subcultured on nutrient agar. Spores which remained refractile during storage in clove infusion at 37°C outgrew when transferred to nutrient agar. Clove ash was determined to contain substances which allowed complete germination or darkening of spores in distilled water. Eugenol, an essential oil found in cloves was determined to be sporostatic at concentrations of 0.05 - 0.06% (v/v). Several closely related compounds; isoeugenol, gingerol and zingerone also showed sporostatsis at levels of 0.05 - 0.6, 0.90 - 1.00, and 0.80 - 0.90% (v/v) respectively.

INTRODUCTION

MANY SPICE COMPONENTS are known to act as microbial antogonists (Pruthi, 1980). Eugenol, 4-allyl-2-methoxyphenol, an essential oil found in the volatile fraction of cloves is one of the best recognized of these natural antimicrobial compounds (Huhtanen, 1979; Azzouz and Bullerman, 1982; Kurita and Koike, 1982). Phenolic in nature, eugenol would also be expected to induce sporostatsis since many phenols and phenolic derivatives have been isolated and are known to act as sporostats (Loosemore and Russell, 1963; Sierra, 1970; Lewis and Jurd, 1972; Jurd et al.; 1971). Despite the chemical congruence of eugenol with other phenolics, there exists a paucity of literature pertaining to its possible sporostatic action.

The purpose of this study was to investigate the potential activity of cloves as a sporostat and its effect on the developmental process from spore to vegetative cell. In addition, several related spice derivatives of o-methoxyphenol were tested.

MATERIALS & METHODS

Culture and maintenance

Bacillus subtilis (*B. subtilis* spore suspension, Difco) was cultured in nutrient broth at 37°C for 24 hr and maintained on nutrient agar (NA) in screw-capped tube slants at 4°C.

Spice derivatives and cloves

Eugenol was obtained from Eastman Kodak Co. (Rochester, NY). Isoeugenol was obtained from Aldrich Chemical Co. (Montreal, Quebec). Gingeron, zingerone, and capsaicin were obtained from ICN Pharmaceuticals (Plainview, NY). Ground cloves were obtained from a local retail outlet.

Spore preparation

B. subtilis was grown in trypticase soy broth (TSB, BBL) for 24 hr at 37°C. The culture broth was then centrifuged for 30 min at 4000 x *g* and the cell pellet resuspended in sterile physiological saline, approximately 1/4 the original culture volume. Five mL portions of the cell suspension were evenly distributed over the surface of the sporulation medium which contained 200 mL NA (Gibco) in 1L Roux bottles. The bottles were incubated for 12 days at 37°C. Sporulation was monitored by phase microscopy. The resultant spore crops were harvested using cold distilled water and the pooled spores centrifuged for 3 hr at 750 x *g* at 4°C. This procedure was repeated five times. The final spore suspension was sonicated (while cooled in an ice bath) for 4 min (Braun-Sonic, 200 watt). The washing process with cold water was repeated four times as before. The final spore suspension was stored at 4°C until used.

Author Al-Khayat is with the Dept. of Food Science, College of Agriculture, Abu-Ghraib, Baghdad, Iraq. Author Blank is with the Dept. of Food Science, Univ. of Manitoba, Winnipeg, Manitoba, Canada, R3T 2N2.

Clove infusion

One mL portions of a heat activated spore suspension (10^5/mL) were incubated at 37°C in a series of 100 mL screw cap flasks each containing 50.0 mL of a gradually increasing clove infusion concentration (0 - 1%). The clove infusions were prepared by steam heating the appropriate dry weight of ground clove in distilled water for 15 min at 121°C. The infusions were cooled and centrifuged for 30 min at 1500 x *g*; the supernatant was then aseptically pipetted into previously sterilized flasks. The clove infusions had a pH of 4.20 ± 0.3 after heating and were used without further pH adjustment.

Samples were removed periodically and colony forming units (CFU) were determined by serial dilution using NA as the plating medium. Incubation was carried out at 37°C for 48 hr. Distilled water was used as the storage menstruum for the control; all treatments were performed in quadruplicate and results expressed as means.

Spore storage in 3% clove infusion, adjusted from pH 5.0 to 8.0, with 1N NaOH, was similarly evaluated. All treatments were performed using a respective pH adjusted control.

Germination of spores stored in 0 - 5% cloves for up to 4 wk at 37°C without pH adjustment, (4.2 ± 0.3) was monitored by phase contrast microscopy using a Petroff-Hauser counting chamber. Results are reported as percentage of remaining refractile spores.

Microcultures were prepared by layering ca. 1.0 mL of medium, consisting of nutrient broth and 1.5% noble agar (Difco) on the surface of sterile glass slides (25 x 75 mm). A spore suspension (0.01 mL) was pipetted onto the center of a sterile glass cover slip, allowed to air dry and then carefully pressed onto the agar slide. The edges of the cover slip were sealed with paraffin, after the excess agar had been trimmed away. The cultures were then placed in sterile petri plates contained in sealed plastic sleeves with moistened tissue and incubated at 37°C. Slide cultures were examined with a Zeiss phase contrast photomicroscope for outgrowth capability.

The ash content of the ground cloves were determined in triplicate as outlined in AOAC (1975). In order to determine the potential germinator effects of clove ash on spores, 0.17% (w/v) ash (corresponding to 3%, w/v clove infusion) was added to a series of culture flasks containing distilled water. Following sterilization, the flasks were adjusted to pH 5.0 - 8.0, inoculated with heat activated spores and incubated at 37°C. The spores were microscopically examined for phase-darkening.

Spice derivatives

Heat activated (10 min at 80°C) spore suspensions (0.1 mL) were pipetted into a series of culture plates with concentrations of 10^2 and 10^5 spores/mL. NA containing various concentrations (% v/v) of spice derivatives (Fig. 1) were autoclaved for 15 min at 121°C. Five plates were subcultured for each inoculum and incubated at 37°C for up to 72 hr. The presence or absence of colonies in the various treatments was recorded.

Since eugenol is practically insoluble in water, it was necessary to emulsify it prior to sporostatis evaluation. Eugenol (0.06%)

was prepared in steril 0.1% noble agar. A standardized spore inoculum was then added to the eugenol suspension and mixed. A control was similarly prepared without eugenol. Portions were removed periodically, microscopically examined and evaluated for CFU. The effects of eugenol on spores stored in a growth medium were determined by the direct addition of eugenol to nutrient broth. After mixing a turbid but stable suspension resulted.

The effect of pH on eugenol activity was investigated by incubating standardized spore inocula in NA containing 0.03% eugenol. Following steam sterilization, the NA-eugenol media were adjusted to pH 5.5 - 8.0 with 1N NaOH. Colony counts were performed after 48 hr incubation at 37°C. Control treatments contained no eugenol.

RESULTS & DISCUSSION

SURVIVAL of *B. subtilis* spores during clove infusion storage are shown in Fig. 2 and 3. Spores stored in clove infusion, 0 to 1% (Fig. 2) showed a decrease in CFU as both the concentration of clove infusion and the length of storage was increased. Increasing the initial pH of 3% clove infusions resulted in a progressive decrease in CFU during the first three days of storage (Fig. 3). During the latter three days, infusions initially adjusted to pH 6.0 and 7.0 gave the greatest decrease in CFU. The CFU in the controls remained relatively constant throughout the period of storage. Microscopic examination of spores stored in 3% clove infusion revealed an increasing number of phase-grey spores, indicating germination initiation, within the general spore population as the pH of the 3% clove infusion was increased. Phase-greying of spores in infusions adjusted to pH 7.0 and 8.0 was observed after 6 hr of storage. After 2 wk of storage, more than 95% of the spore population for all pH treatments was phase-grey. The spore population stored in 3% clove infusion, pH 7.0, for 2 wk was washed twice in fresh nutrient broth and subcultured on microslides. Microscopic examination revealed that those spores which became phase-grey while stored in clove infusion did not undergo further darkening; a complete cessation of the developmental process was evident. Washing of these spores with 2% Tween 80 and subsequent subculture on microculture slides also failed to reverse the blocking action. The spores which maintained their refractility while stored in 3% clove infusion, however, showed normal outgrowth (Fig. 4) upon subculture on microslides. Since the control spores remained refractile during storage, it seemed probable that clove infusion contained germinant(s).

Spores stored in 0 - 5% clove infusion without pH adjustment (pH 4.2 ± 0.3) at 37°C were examined periodically with the phase contrast microscope. During the first 2 wk of storage, loss of spore refractility with increasing clove infusion concentration was evident (Table 1). At 4 wk of storage, all spore populations examined except the control, consisted wholly of phase-grey spores.

The ash content of the ground cloves was obtained as a mean of triplicate determinations (5.89%, w/w). Clove ash (0.3%, w/v) was added to a series of culture flasks containing heat activated spores suspended in distilled water (pH 5.0 - 8.0). Phase contrast microscopy revealed that more than 95% of the spore populations underwent complete phase darkening within 72 hr when stored in ash suspensions adjusted to pH 6, 7, and 8. Spore populations stored in ash suspensions, pH 5.0, however, contained less than 10% completely darkened spores at 72 hr. The results obtained with spores stored in clove infusion (Fig. 3) would then indicate that clove infusion stimulated partial spore germination, especially under near neutral pH conditions. These germinated spores, however, did not outgrow upon subculture in NA. Since only partial spore germination in clove infusion was observed as compared to complete germination with clove ash, it would appear that residual eugenol in clove infusion exerted some blocking effect on spore outgrowth. The extent and rapidity of phase-greying spores in clove infusion would be expected to be dependent on the level of germinants, the residual eugenol content, and the pH.

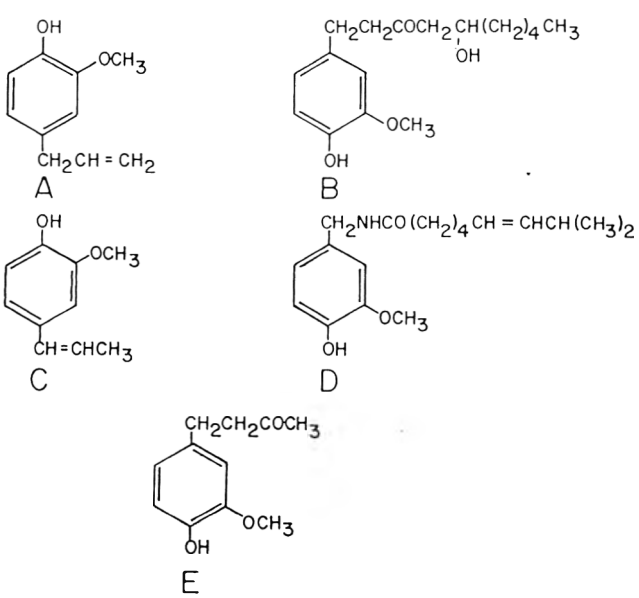


Fig. 1—Pungent spice derivatives of O - methoxyphenol. A - eugenol, B - gingerol, C - isoeugenol, D - capsaicin, E - zingerone.

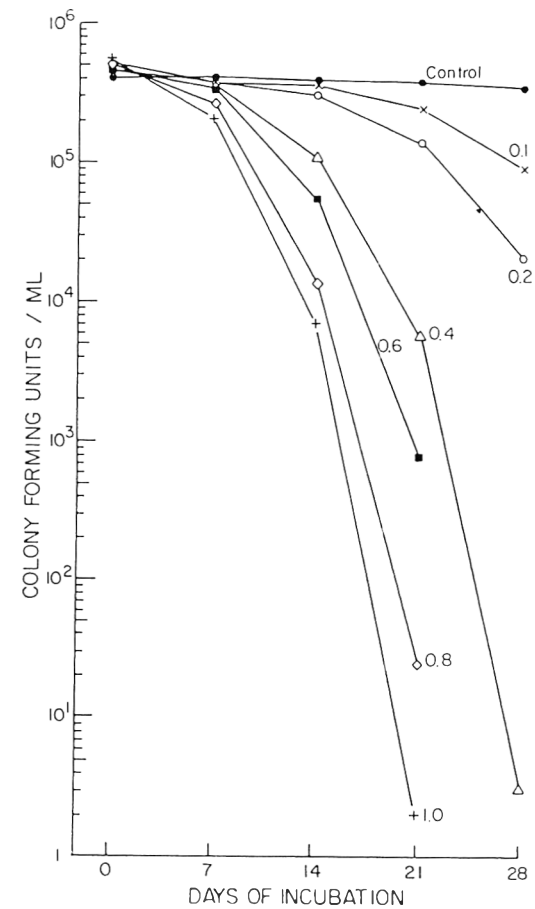


Fig. 2—Effect of 0, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0% clove infusions on the viabilityof B. subtilis spores at 37°C, subcultured in NA.

The ability of nonmetabolizable germinants to stimulate or serve as germination adjuncts for *B. subtilis* spores is well known (Fleming and Ordal, 1964). Foerster and Foster (1966) reported that *Bacillus* species showed increased germinative activity in the presence of various ions such as Na^+, Al^{3+}, and K^+. It is possible that the prepared spore suspension, although exhaustively washed, contained low concentrations or partial germinants of exogenous origin from the cultivation medium and/or nutrients released from sporangia during sonication, which became adsorbed on the spore surface. Alternatively, substances of endogenous origin, solubilized during heat activation, may have been present to promote germination in the presence of the ash constituents. Cloves are known to contain a wide variety of ion species, many of which have been cited to promote germination (Christensen et al., 1968). Fleming and Ordal (1964) also reported that *B. subtilis* spores germinate, albeit slowly, in the sole presence of certain inorganic ions.

The response of *B. subtilis* spores to temperature and pH during storage in clove infusion is given in Table 2. Control spores, regardless of the temperature or pH employed during storage, responded with little or no difference in CFU. However, spores stored at pH 8.0 in the 3% clove infusion did not appear to be influenced by the storage temperatures, since the rate and extent of outgrowth appeared similar at both temperatures. Spores stored at pH 5.0 - 3% clove infusion, however, responded differently to the storage temperature; 45°C was more deleterious to spore outgrowth than 30°C, particularly at 96 hr of storage. Spores stored in the pH 5.0 - 3% clove infusion were examined microscopically. The majority of the spores were wholly refractile at 24 hr of storage at both temperatures; at 96 hr, however, large populations of phase-grey spores occurred. Microscopic examination of spores in pH 8.0 - 3% clove infusion showed large numbers of phase-grey spores at 24 hr for both temperatures of storage. Since studies in this investigation have shown that spores which undergo germination in clove infusion did not develop further, it would appear that at an acid pH, elevated temperatures of incubation stimulate spore germination. This was not observed in pH 8.0 - 3% clove infusion despite reports citing increased phenolic activity due to increase in storage temperature (Leiguards et al., 1950).

Spore outgrowth in NA containing various spice derivatives is presented in Table 3. Eugenol and isoeugenol showed complete colony inhibition at 0.06%. Zingerone and gingerol prevented complete colony formation at 0.6, 0.9 and 1.0%, respectively. Colonies formed in all replicates with either high or low inocula at the highest concentration of capsaicin, (1.6%) assayed. The effectiveness of the methoxyphenol spice derivatives in preventing spore outgrowth appeared, in part, to be related to the inverse of the molecular weight of the derivative (Table 3). Differences in the molecular weight of the phenolic derivatives, due to the chemical composition of the side chains, have been reported to influence activity (Katayama and Nagai, 1960; Chakraborty et al., 1961; Jurd et al., 1971). Lewis and Jurd (1972) reported that the presence of additional groups, or the bulkier the group, on the phenolic ring was detrimental to sporostatic activity. Reduced solubility of the phenolics due to large side groups and/or chemical structure was reported by these investigators to influence the biological activity of the compounds.

Spores stored at 37°C for 1 month in 0.1% noble agar containing 0.06% eugenol remained wholly refractile. The spores, when enumerated in NA without any neutralizing agents, showed no difference in viable counts when compared to the control. The addition of 0.17%, w/v clove ash to spores stored in 0.06% eugenol at 37°C produced phase-grey spores after 72 hr. Although survivors were not enumerated, failure of these phase-grey spores to undergo further darkening and outgrowth upon subculture in microslide was observed through phase microscopy. This same effect was also observed with spores suspended in 0.1% and 0.2% eugenol-containing nutrient broth, indicating that eugenol did not interfere with events that initiate the ger-

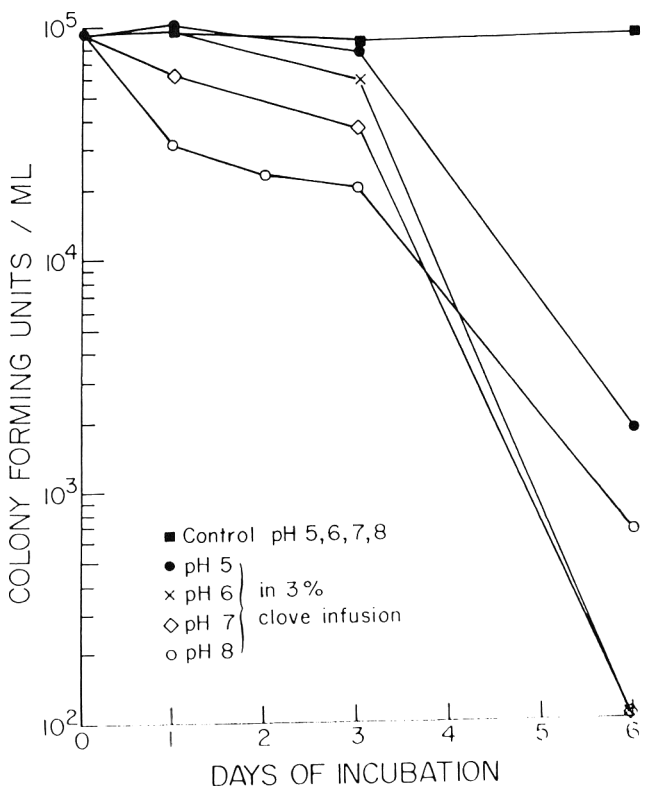


Fig. 3—Effect of storage at 37°C on B. subtilis spores in 3% clove infusion adjusted to different pH.

Table 1—Microscopic observation of spores stored in clove infusion

Storage (wk)	% Refractile spores in clove infusion (%)					
	0	1	2	3	4	5
1	99	99	99	99	95	90
2	99	40	40	30	10	1
3	99	1	1	1	1	0
4	95	0	0	0	0	0

Table 2—Effect of pH and temperature on B. subtilis *spores stored in clove infusion*

Clove infusion (%)	Storage temp (°C)	Colony forming units/mL[a] pH of Clove infusion			
		5.0		8.0	
		Storage time (hr)			
		24	96	24	96
0	30	1.55	1.27	1.75	1.73
	45	1.42	1.46	1.72	1.68
3	30	2.45	0.87	0.95	0.01
	45	1.97	0.04	0.45	0.04

[a] All counts are X 10^5/mL.

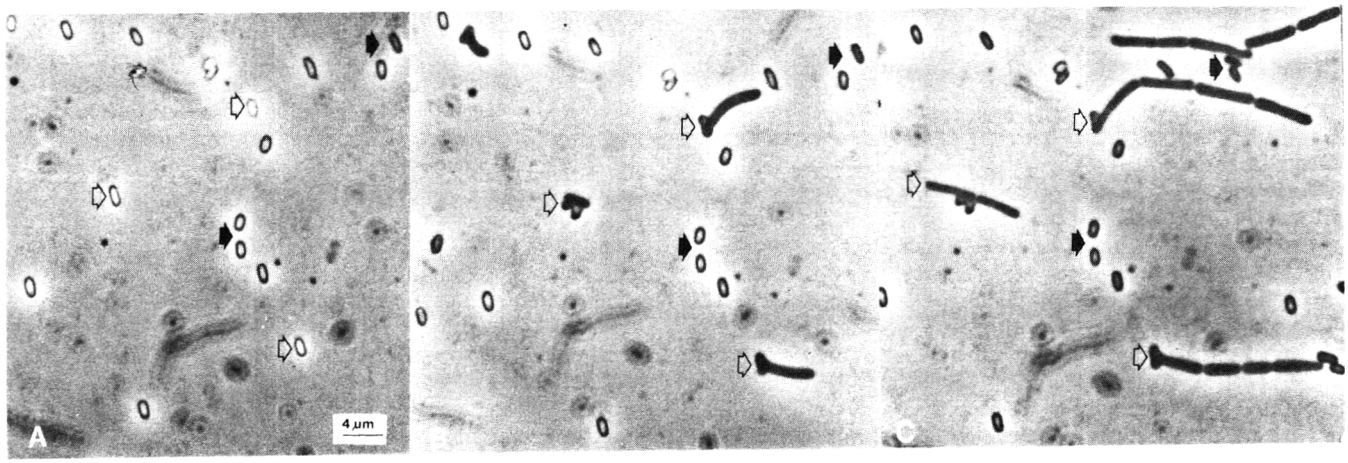


Fig. 4—Development of B. subtilis spores on microslide following storage in 3% clove infusion for 14 days at 37°C. (A) Spore population obtained after storage in clove infusion. Spores subcultured in nutrient agar, 0 hr; ⇒ refractile, → phase-grey. (B) At 4 hr refractile spores have progressed to outgrowth stage; phase-grey spores have remained unchanged. (C) At 8 hr refractile spores have progressed to cell division; phase-grey spores show no developmental change.

Table 3—Spore outgrowth in nutrient agar containing various spice derivatives

Spice derivative	Mol wt	Concentration (%,v/v)	Spore inoculum H[a]	Spore inoculum L[b]
Capsaicin	294.38	1.40	5[c]	5
		1.60	5	5
Eugenol	164.20	0.04	5	5
		0.05	3	0
		0.06	0	0
Gingerol	194.22	0.70	5	5
		0.80	5	3
		0.90	4	0
		1.00	0	0
Isoeugenol	164.20	0.03	5	5
		0.04	4	2
		0.05	1	0
		0.06	0	0
Zingerone	305.40	0.70	5	5
		0.80	3	1
		0.90	0	0

[a] High spore inoculum — 10^5/mL medium.
[b] Low spore inoculum — 10^2mL medium.
[c] Number of replicates out of five showing growth after 72 hr incubation at 37°C.

mination process. It would appear, therefore, that eugenol contained in a complete growth medium and/or in the presence of spore germinants, interfered with the completion of the germination process. Normally, spore germination is considered to be a rapid, irreversible process. The ability of eugenol to interfere with the process of complete darkening may suggest that germination in these spores occurs in various storages, of which at least two, partial darkening or phase-greying and complete darkening, may exhibit different sensitivities towards antagonistic agents.

The effect of pH on eugenol activity is presented in Fig. 5. In the range pH 6.0 - 8.0 control spores grew out in NA within 24 hr. All controls gave similar CFU. Spore outgrowth in NA containing 0.03% eugenol decreased with decreasing pH, suggesting that eugenol was more effective at acid pH. The tendency for increased activity of phenolics at acid pH has been reported (Sykes and Hooper, 1954); solubility and stability properties of the phenolic were thought to be involved.

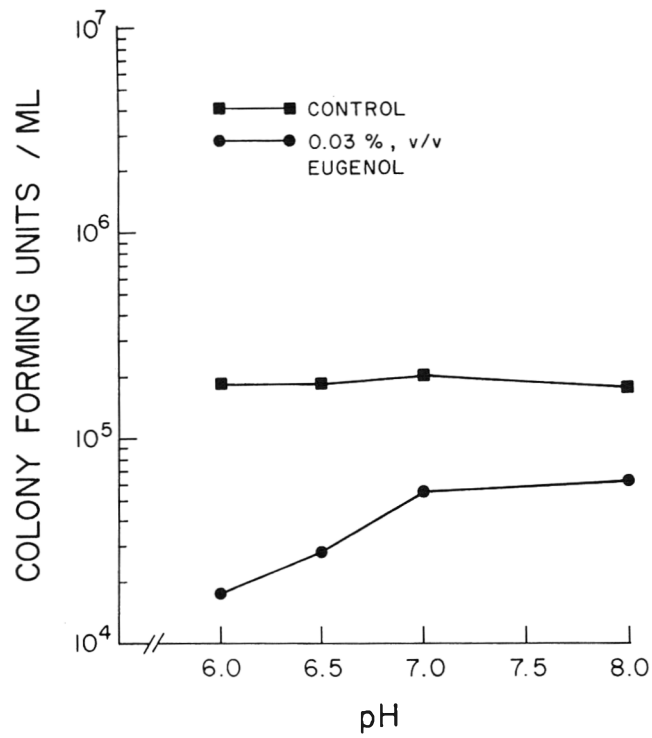


Fig. 5—The effect of pH on eugenol (0.03%, v/v) activity.

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Muscle Fiber—Connective Tissue Junctions in the Fish Blue Grenadier (*Macruronus novaezelandiae*). A Scanning Electron Microscope Study

H. ALLAN BREMNER and IAN C. HALLETT

ABSTRACT

The junctions between the muscle fibers and the connective tissue of blue grenadier (*Macruronus novaezelandiae* Hector) were studied using scanning electron microscopy. In fish before, during, and just after rigor mortis, the muscle fibers are attached to the myocommatal connective tissue sheets by fine collagenous fibrils. After chilled storage these fibrils deteriorate and the muscle fibers gradually detach from the myocommata. It appears that the fibrils are destroyed by endogenous collagenases and/or other proteinases.

INTRODUCTION

THE FLESH of teleost fish is constructed of adjacent muscle blocks called myotomes separated from each other by collagenous sheets called myocommata (Nursall, 1956). The myotomes on both sides of the axial skeleton each take the form of double cones which fit together along the long axis of the fish body to give the appearance at the surface of a series of W-shaped sections resting on their sides (Fig. 1). Within each myotome the muscle fibers (myomeres) run approximately parallel to each other but at varying angles to the myocommatal sheets to give the necessary moment for swimming during contraction. The myocommata are connected internally to the skin and the skeletal system and are also linked to the membrane dividing the fish into epaxial and hypaxial planes and to the median vertical septum. Further details of the structure and function of the system as a whole can be found in Nursall (1956) and Alexander (1969).

The junctions between the myomeres and the myocommata are reported to be formed by fine collagenous processes which have their origin in the myocomma and which then form the sheaths surrounding each muscle fiber (Love, 1970, Love et al., 1969). The microscopy of fish flesh has been reviewed by Howgate (1979). Most electron microscopy on fish muscle has been done on the fibers themselves using the transmission mode (TEM) (Jarenbäck and Liljemark, 1975; Liljemark, 1969). Relatively few reports have included scanning electron microscope (SEM) data. Almaas (1982) published SEM studies on isolated cod muscle cell envelopes and Schaller and Powrie (1971) published SEM micrographs of trout muscle fibers at various stages postmortem. Despite the importance of the myomere/myocommata interface both in vivo and postmortem, its study has been neglected. One reason for this could be the difficulty in preparing suitable samples.

Although there are differences in the gross organization between fish muscle and that of terrestrial animals, in which the muscles terminate in tendons which attach to the skeletal framework, the ultrastructures have been found to be similar. For this reason, and because no text describing fish muscle specifically could be found, the terminology applied to muscle by Gould (1973) is used in this paper. Thus the term plasmalemma (sarcolemma) applies to the cell membrane of the muscle fiber. External to this is the basal lamina (basement membrane) to which are attached the collagenous (reticular) fibrils of the endomysium that run into the surrounding perimysium which in fish is continguous to the myocomma.

Blue grenadier, known in New Zealand as hoki, is an increasingly important trawlfish (Patchell, 1982) related to the merluccid hakes (Bremner, 1980; Nelson, 1976). Freezing of fish in rigor is well known as a cause of gaping, the name given to the phenomenon when the connective tissues fail to hold the muscle blocks (and fibers) together (Love et al., 1969). In some species such as blue grenadier the incidence of gaping occurs progressively postrigor even during chilled storage. Fillets taken from these fish prerigor, or soon after rigor has resolved, generally do not gape whereas fillets from fish stored longer than about 4 to 5 days on ice, gape readily either when first cut or during handling. Blue grenadier is thus a suitable subject for the study of postmortem changes in the myomere/myocommata junction. This paper reports the development of techniques used to expose the myomere/myocommata interface for SEM and shows the results obtained with blue grenadier (*Macruronus novaezelandiae* Hector) muscle prerigor, during rigor, postrigor and after chilled storage.

MATERIALS & METHODS

BLUE GRENADIER from a number of sources were used in this study. Muscle samples were taken from the anterior dorsal area at approximately the 13th, 14th and 15th myotome, counting from the head.

Fish L were sampled immediately after catch on board the Tasmanian Fisheries Development Authority vessel FV 'Challenger', and were iced and used to study changes occurring during rigor mortis and subsequent storage in ice (Table 1). Samples from these fish were fixed in 3.2% glutaraldehyde in 0.16M phosphate buffer containing 2% NaCl and 0.5% sucrose to increase osmolarity (Kryvi, 1977). They were sent in fixative to the DSIR Mt. Albert Research Centre, Auckland, N.Z. After a short wash in buffer, samples were frozen, in dichlorodifluoromethane (CCl_2F_2) at −158°C and transferred to liquid nitrogen (LN_2) at −196°C. Frozen samples were fractured under liquid nitrogen by striking a cooled sharp blade strategically positioned over the sample. The frozen samples were then freeze dried in a modified vacuum evaporator with a copper block system cooled by LN_2 (Ladd Research Industries, Vermont, USA.)

Table 1—Sampling times for blue grenadier caught by F.V 'Challenger', April 1983

Sample	Time sampled (hr)	Time elapsed	Comments
L1	1909	0	Fresh prerigor fish straight out of the net.
L2	2045	66 min	Fish starting to go firm, packed in ice.
L3	2248	3 hr 40 min	Fish firm, in rigor.
L4	1100	4 day 16 hr	Post rigor
L5	1000	8 day 13 hr	Post rigor
L6	1000	10 day 13 hr	Fish starting to deteriorate

Author Bremner is with the CSIRO Division of Food Research, Tasmanian Food Research Unit, "Stowell," Stowell Ave., Hobart, Tasmania, Australia 7000. Author Hallett is with DSIR, Plant Diseases Division, Mt. Albert Research Centre, Private Bag, Auckland, New Zealand.

Fish M and Fish N, obtained from Sanfords Ltd., Auckland, N.Z. and from Sealords, Nelson, N.Z. respectively were held in ice. Samples from these fish were fixed, freeze-fractured then either freeze-dried or dehydrated in an ethanol series and then dried at the critical point from CO_2 (Samdri 780, Tousimis Research Corp., Maryland, USA.).

Fish O were frozen pre-rigor on board the N.Z. Ministry of Agriculture and Fish research vessel FV 'James Cook', and samples were held frozen at −30°C at the Mt. Albert Research Center. Samples were taken by allowing the fish to rise in temperature to the point where they could be cut, refreezing the sample in LN_2 or CCl_2F_2 before fracturing and subsequent freeze drying.

Solvent treatments

Samples from Fish O were placed in stainless steel mesh boats in glass vials and using gentle agitation on a rotating wheel (6 rpm) at 20°C were treated with various solvent solutions for either 1, 4 or 6 hr. These treatments were chosen to preferentially solubilize or disperse different flesh components in order to expose inner surfaces. The solutions used were: 1M calcium chloride to extract soluble components of the connective tissue (Robert and Comte, 1968); 6M urea to disrupt hydrogen and nonpolar bonds (Tsuchiya et al., 1980); 1% sodium dodecyl sulphate (SDS) as a secondary bond breaker to disrupt hydrogen and hydrophobic bonds (Connell, 1965); buffered 0.5M KCl to break ionic bonds (Anderson and Ravesi, 1968); or 1 mg/mL trypsin solution pH 7 in 0.14M NaCl to effect proteolysis. Treatment with distilled water was included as a comparison.

After fixation (Kryvi, 1977) these treated samples were dehydrated in an ethanol series then critical-point dried. None of these treated specimens was freeze fractured. After drying, all the prepared samples were mounted on stubs, sputter-coated with gold and examined with a Phillips 505 scanning electron microscope at the Mt. Albert Research Center.

RESULTS

CRITICAL-POINT DRIED SAMPLES were often too distorated to give good results; excessive folding of the samples could be seen by the naked eye. Attempts to restrain the critical point dried samples reduced visible folding but did not significantly reduce the distortion of muscle fibers and myocommata when viewed in the SEM. Freeze-dried samples remained similar in gross visual morphology to unprocessed samples and when viewed in the SEM showed little sign of folding or displacement of fibers or myocommata.

General description

The myomeres butt on to the myocomma (Fig. 2) to which they are attached by a fine network of collagenous processes that proceed from the myocomma to form the outer sheath on each myomere as noted by Love et al., (1969). The myomeres appear to fit into sockets on the myocomma and where the myomeres have been completely removed these sockets are evident (Fig. 3). The ends of

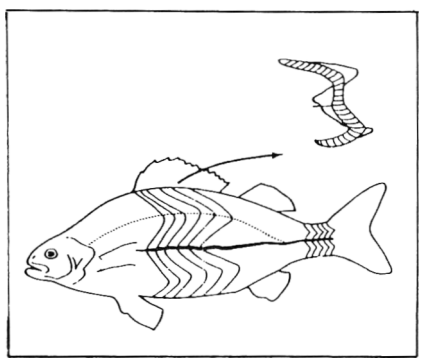


Fig. 1—Schematic diagram of the layout of fish muscle, showing the interlocking W-shaped myotomes separated by myocommata and a single myotome showing the complex double cone shape and the approximate orientation of the myomeres at the surface. Adapted from Ellis et al. (1978).

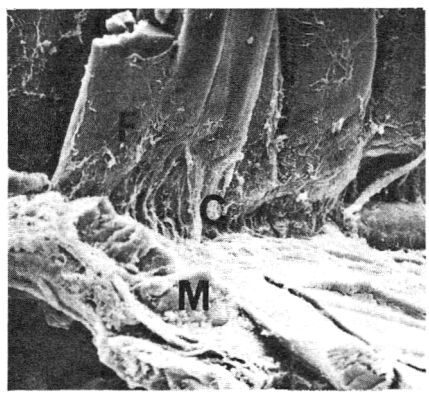


Fig. 2—Fine collagen fibrils C connect the myomeres F to the myocomma M. Fish L1 prerigor. Bar 0.1mm. Magnification: 269X.

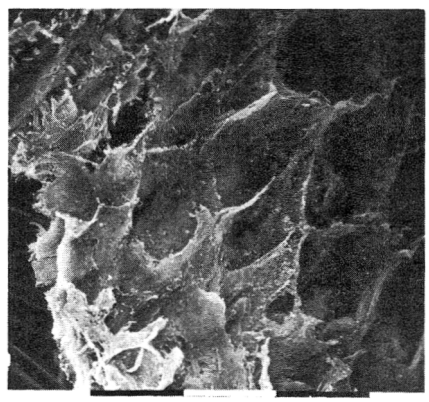


Fig. 3—Sockets on the myocomma. Fish M. Bar 0.1 mm. Magnification: 263X.

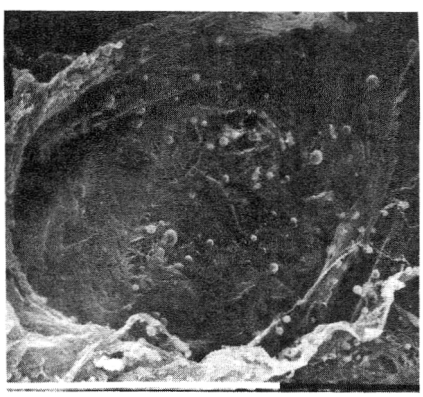


Fig. 4—Interior view of a socket on the myocomma. Spherical blobs may be remnants of former connections to the myomere. Fish M. Bar 0.1 mm. Magnification: 850X.

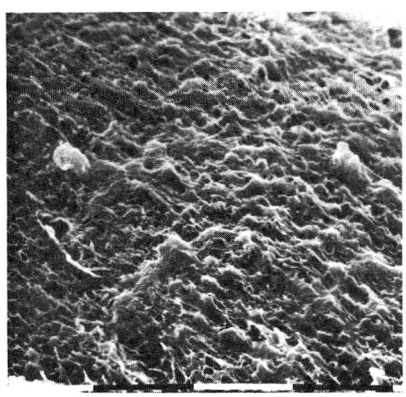


Fig. 5—The base of a fiber detached from the myocomma. Note raised points of former attachment. Fish N. Bar 0.01 mm. Magnification: 2880X.

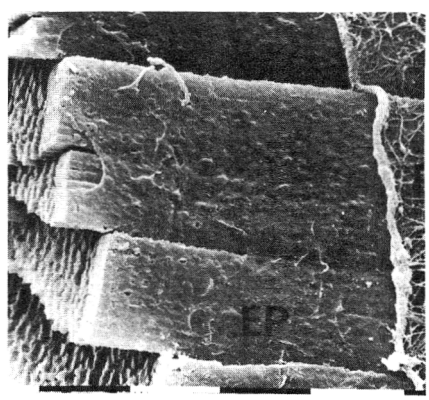


Fig. 6—Fractured fiber end with perimysium P peeled back and endomysium/plasmalemma EP intact. Fish L1. Bar 0.01 mm. Magnification: 2550X.

detached fibers (Fig. 5) and the inside of the sockets (Fig. 4) show either raised surfaces or small blobs of material that may previously have formed points of attachment.

Changes during rigor mortis

The structure of the pre-rigor muscle fiber with the typical elongated myofibrils near the periphery of the fiber and the more polygonal myofibrils in the interior is visible in Fig. 6. The connective tissue network of the perimysium has 'peeled' back from the fractured surface to reveal the plasmalemma and endomysium on the fiber surfaces. Viewed from different angles (not shown here) regular elevations running transversely across the fiber stood out in relief beneath the plasmalemma as observed in trout muscle by Schaller and Powrie (1971). A general view of typical fiber surface is shown (Fig. 7).

As the fish entered rigor the processes attaching the myomeres are still evident but in samples taken from fish in rigor, interfaces between the muscle fibers and myocommata are indistinct with the ends of fibers from adjacent myotomes jammed hard together (Fig. 8) and the transverse elevations are still evident (not shown here).

After 5 days storage in ice, when the fish are in the postrigor state the connections between fibers and connective tissue are less evident (Fig. 9) and at the fiber surface both the endomysium and plasmalemma layer beneath show signs of degradation (Fig. 10).

In samples taken after further storage in ice, muscle fibers free of connection to the myocommata occur (Fig. 11, 12) and the general area is often degraded and covered in debris (Fig. 12, 13, 14). The T system is still evident on the fibrils (not shown here) but the perimysium has generally deteriorated (Fig. 14, 15) although under the debris a fine reticular network is still evident.

Effects of solvent treatments

The nature and extent of dissolution or disruption by the solvents is fast and uncontrollable and some preparations are either badly disintegrated or amorphous or uninformative after treatment for only 1 hr.

The powerful action of urea causes nonspecific degradation noticeable in the myocommatal sheets from which 'tufts' of fibers readily break away (Fig. 16). Treatment for

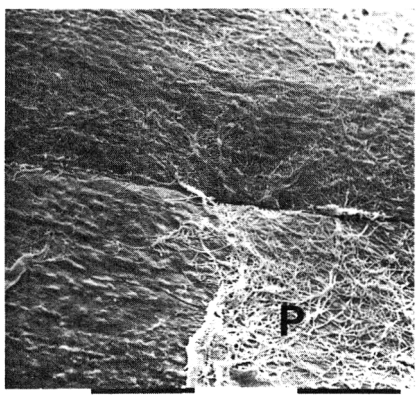


Fig. 7—Detail of muscle fiber surface of prerigor fish showing intact perimysium. Fish L1. Bar 0.01 mm. Magnification: 2550X.

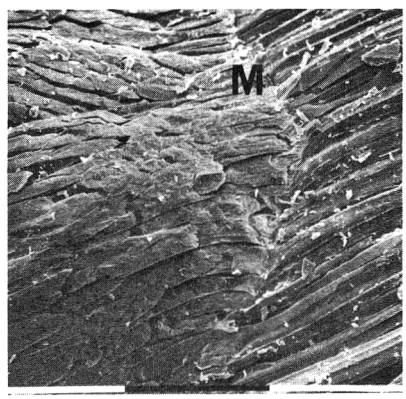


Fig. 8—Fish muscle in rigor. Interfacing myocomma M between adjacent myotomes runs from top to bottom. Fish L3. Bar 1 mm. Magnification: 41X.

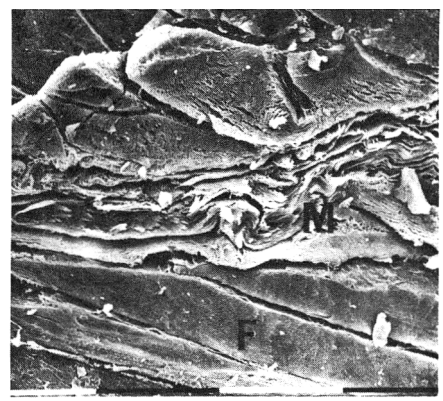


Fig. 9—Myofiber connective tissue interface in post rigor fish 5 days in ice after catch, no fibrous onnections between myofibers F which slant upwards from bottom right to myocomma M which runs across. The crumpled layers of myocommatal sheets are obvious. Fish L4. Bar 0.1 mm. Magnification: 348X.

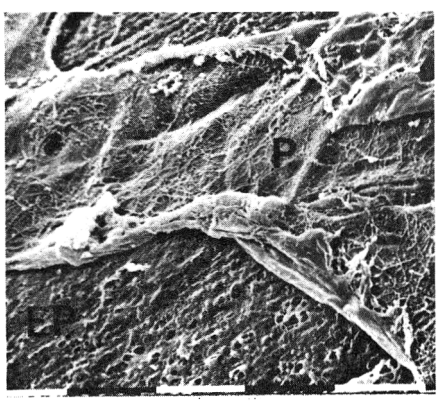


Fig. 10—Detail of muscle fiber surface of postrigor fish 5 days in ice after catch. Both perimysium P and endomysium/plasmalemma EP deteriorating. Fish L5. Bar 0.01 mm. Magnification: 2540X.

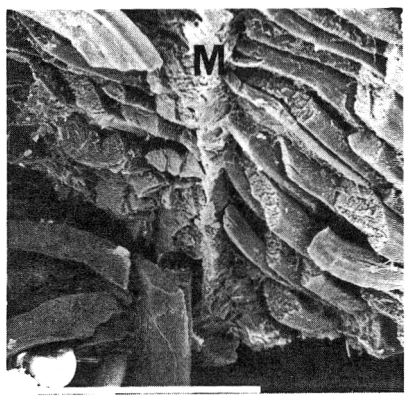


Fig. 11—General view of myofiber/myocomma junction 11 days in ice after catch. No connections between fibers F and myocomma M, which is situated vertically in centre. Fish L6. Bar 1 mm. Magnification: 79X.

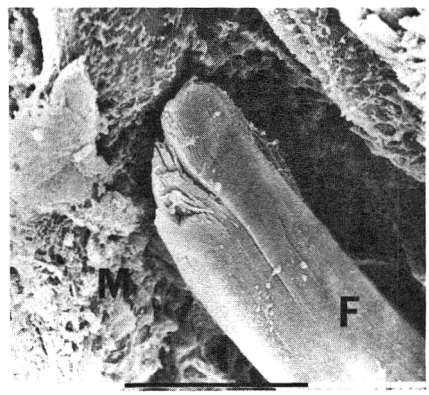


Fig. 12—Detail from Fig. 11. Muscle fiber F detached from myocomma M, no fine connections. Bar 0.1 mm. Magnification: 517X.

1 hr in a 1% SDS solution strips the outer layers of the myocommatal sheets leaving the larger, stronger fibers and the whole endomysium and plasmalemma, too, is attacked, apparently across the I band. Longer treatment with SDS (4 hr) accentuates this dissolution which runs transversely across the myofibers rather than between the fibrils (Fig. 17). It was also noted that after SDS treatment the samples did not turn yellow in the glutaraldehyde fixative. Yellowing normally occurs due to reaction of the glutaraldehyde with free amino groups.

Trypsin also causes general deterioration of the collagen fibrils and etches the collagen surfaces (Fig. 18). The overall degradation caused by 1M $CaCl_2$ gives no useful information. Muscle fibers dissolve readily in KCl but Fig. 19 shows the remnant of a fiber where it joins the myocomma. The fiber itself is swollen and hydrated and the perimysium is peeled back. With other preparations the endomysium and the plasmalemma disintegrates and apparent dissolution of the I band across the fibrils can be seen.

Exposure to water seemed also to result in disruption of the transverse system which collapsed below the surface of the myofibrils (Fig. 20). Sarcoplasmic proteins are water soluble and it is likely that they were extracted. Prolonged exposure to water for 6 hr caused more general disintegration in some fibers both across and between the myofibrils (Fig. 21).

DISCUSSION

FREEZE FRACTURING followed by freeze drying was the best of the methods tested for preparing samples to expose fiber/myocommata junctions. The use of solvents to preferentially remove some muscle components to expose underlying structures was hard to control.

The muscle fibers of blue grenadier bed into the myocommata and are attached to it by continuations of the collagenous fibrils (Fig. 2, 19) as reported for cod by Love et al. (1969). In terrestrial animals studies on the myotendonous junctions of the rectus abdominis muscle of the bull frog (Ajiri et al., 1978), the tongue of the guinea pig (Demmel et al., 1979), the mitral complex of the canine (Fenoglio et al. 1972), the papillary, tongue, diaphragm and gastrocnemius muscles of the guinea pig (Hanak and Böck, 1971) and the extensor carpi radialis longis and brevis muscles of the mouse (Trotter et al., 1981) have all shown the presence of small filaments, named microfibrils (Hanak and Böck, 1971), linking the muscle fibers to the connective tissue. Trotter et al., (1981) indicated that these microfibrils act as mechanical links at this junction. It seems clear from the present work that there are no connections along the course of the fiber and, in agreement with the observations reviewed by Gould (1973), the plas-

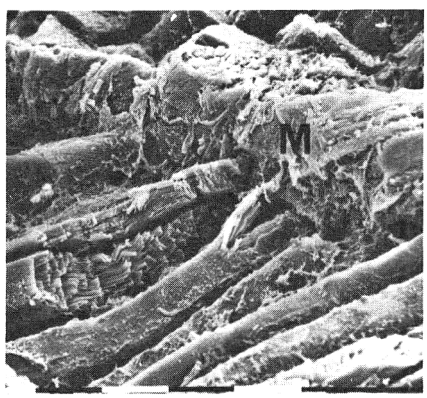


Fig. 13—A different area to that shown in Fig. 11, several fibers still show some attachment to the myocomma M and the area has degenerated. Fish L6, 11 days in ice after catch. Bar 0.1 mm. Magnification: 188X.

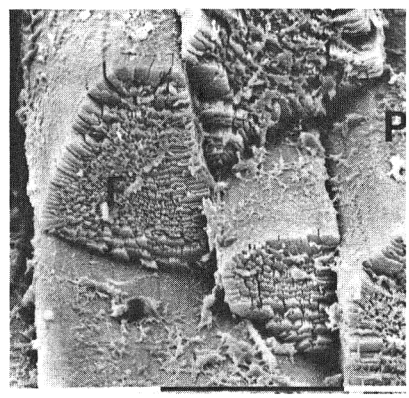


Fig. 14—General view of muscle fiber F ends at a fractured surface. Fish L6 stored 11 days in ice after catch. Note breakup of perimysium P cf. Fig. 6. Bar 0.1 mm. Magnification: 515X.

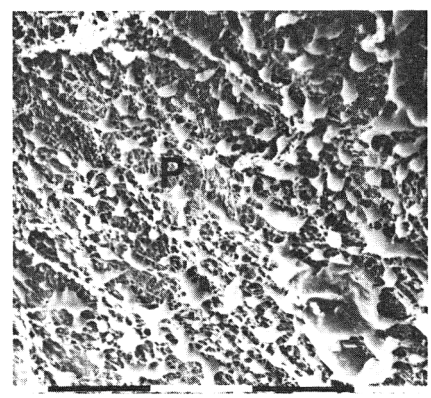


Fig. 15—Detail of perimysial P surface of a muscle fiber shown in Fig. 14 cf. Fig. 7. Bar 0.01 mm. Magnification: 2875X.

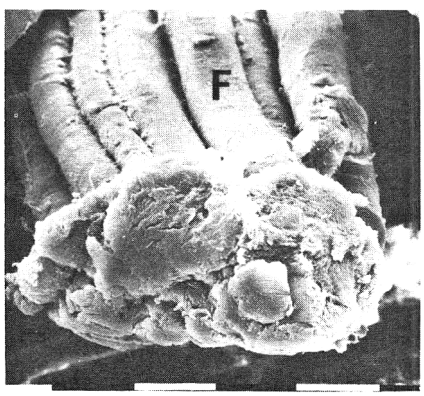


Fig. 16—Sample treated in 6M urea (1 hr). Tuft of fibres F loosened free of the myocomma. Fish O. Bar 0.1 mm. Magnification: 231X.

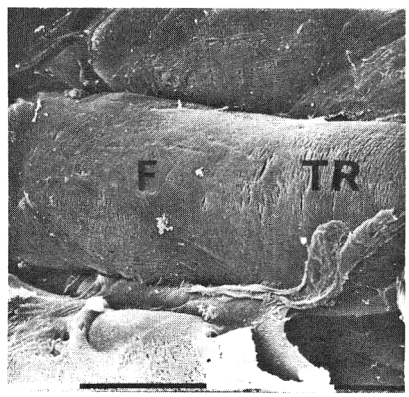


Fig. 17—Muscle fibers F treated 4 hr in 1% SDS. Note transverse fissures TR Fish O. Bar 0.1 m. Magnification: 359X.

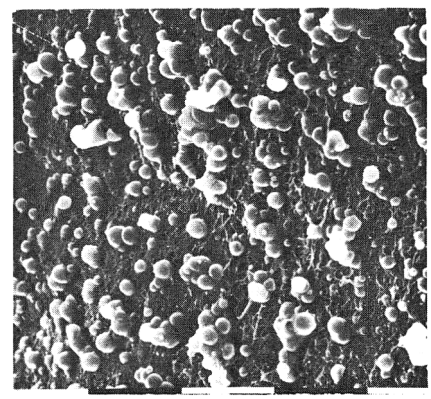


Fig. 18—Spherical blobs on the surface of myocomma from a sample treated with trypsin 1 mg/ml (1 hr). Fish O. Bar 0.01 mm. Magnification: 2620X.

malemma is independent of the perimysial (or reticuloendothelial) fibers (Fig. 6).

During chill storage the attachments between myomeres and the myocomma progressively deteriorate (Fig. 11, 12, 13) and indeed the whole sarcolemma deteriorates (Fig. 13, 14, 15). There are similarities to the degradative effects of cathepsins and collagenases on beef muscle reported by Eino and Stanley (1973).

The deterioration on the fibers (Fig. 5, 13) and the sockets (Fig. 4) is similar to that obtained with trypsin treatment (Fig. 18) and at times with KCl solutions (not shown). It is inferred from this that the progressive breakdown during storage is due to enzymic activity and that the enzyme(s) responsible must have powerful collagenolytic properties against the type of collagen of the perimysial fibrils. There are at least five genetically different types of collagen in connective tissues (Asghar and Henrickson, 1982; Eyre, 1980; Miller, 1982; Sims and Bailey, 1981) differentiated by the composition of the three helical chains which make up the molecule. Often more than one type of collagen is present in a tissue (von der Mark, 1981). Morphologically the collagen fibrils shown here resemble the perimysial felt work reported for rat, rabbit, cattle and sheep by Rowe (1981).

Almas (1982) isolated 'muscle cell envelopes' from cod muscle and concluded from the results of electrophoresis, chromatography and amino acid analysis that the collagen present was type I. This is in contrast to the results obtained on cow muscle by Bailey et al. (1979) who found that the perimysium was composed of a mixture of types I and III. The type III collagen appeared as a fine network. It seems obviously necessary to apply immunofluorescence or enzyme-linked immunoperoxidase techniques to fish muscle to ascertain the precise location and nature of the various collagen types present.

Different types of collagen have different susceptibilities to enzymic attack, for example types I and III are less resistant than type II (Harper, 1980). It is generally regarded that the initial attack on intact collagen is by specific collagenases and that once the initial cleavage of the helical polypeptide chains has been achieved other nonspecific proteinases can pursue the attack (Harper, 1980). Collagenases are ubiquitous and are closely associated with all types of collagen in a wide variety of tissues (Montfort and Perez-Tamayo, 1975) where they are probably bound to the collagen in the living animal (Pardo and Perez-Tamayo, 1975). Because of this association and their activity it is assumed that these collagenases function in the metabolism of the connective tissues. They are Zn^{2+} containing metalloenzymes that for full activity generally require Ca^{2+} (Cawston and Murphy, 1981); the collapse of the sarcoplasmic system would release the necessary calcium to stimulate the enzyme. Other collagenases and cathepsins with proteolytic activity are present in lysosomes in adjacent locations within the muscle fibers (Canonico and Bird, 1970; Reddi et al. 1972; Steiner et al., 1984). Fish muscle is known to have much greater catheptic activity than mammalian muscle (Siebert, 1958; Siebert et al., 1965).

Catheptic enzymes probably play a secondary role to the collagenases which are present as zymogens or proenzymes in the living tissue (Harper, 1980). Their activity is controlled by factors other than their levels alone (Montfort and Perez-Tamayo, 1975) and a number of inhibitory mechanisms have been found involving sarcoplasmic proteins (Hjelmeland and Raa, 1980), blood serum proteins and the plasma glycoprotein α_2-Macroglobulin (Starkey and Barrett 1977). Serum also contains inhibitors for cathepsins other than proteins; ATP also acts as an inactivator (Reddi et al., 1972). After death these control mechanisms would gradually cease to be effective.

The appearance of the myofibrils of blue grenadier is similar to that reported for trout (Schaller and Powrie, 1971) and cod (Almas, 1982). After 1 hr treatment with water (Fig. 20) they bear a striking resemblance to the dorsal muscle of rainbow trout stored 4 days postmortem at 3°C (Schaller and Powrie, 1971) where the transverse elements have collapsed below the surface of the fibrils. It is evident that water removes sarcoplasmic components (Fig. 20, 21) and thus some of the effects of the solvent systems used here may be due in part to the solvent effect of water alone. Water may have other effects such as removal of inhibitors and it is known that hypotonic solutions can rupture lysosomes (Dean and Barrett, 1976). This may have implications for the thawing in water of frozen fish and fillets of some species.

The T system and the I band seemed particularly susceptible to the action of SDS and breaks across the fibres rather than along their long axis occurred (Fig. 17). Similar transverse fissures have been noted in other preparations (not shown). These transverse fissures may be unique to the peripheral fibrils, with their elongated cross section, or they may indicate the presence of the connecting structures between myofibrils at the Z line level that are believed to be part of the organization of the overall muscle structure (Lazarides, 1980). It is not clear what to make of these observations but further research is warranted.

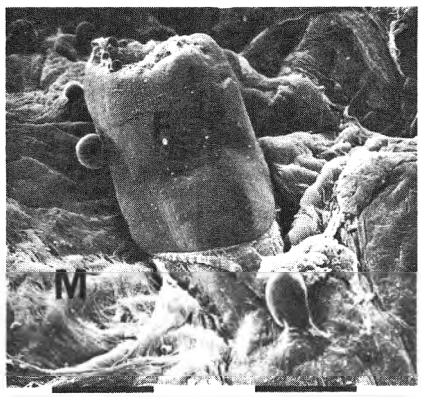


Fig. 19—Sample of muscle treated in 0.5M KCl (1 hr) showing remnants of a muscle fiber F attached to the myocomma M by a 'skirt' of perimysium P. Fish M. Bar 0.1 mm. Magnification: 289X.

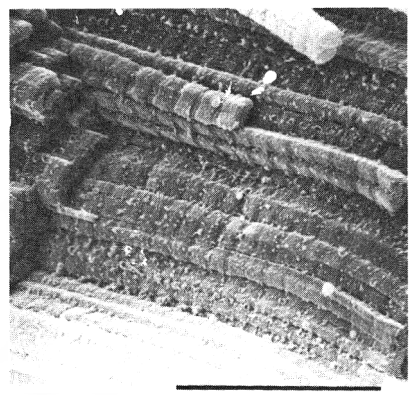


Fig. 20—Muscle fibrils from Fish O treated in water (1 hr). Note apparent collapse of T system below fibril surface. Bar 0.01 mm. Magnification: 5780X.

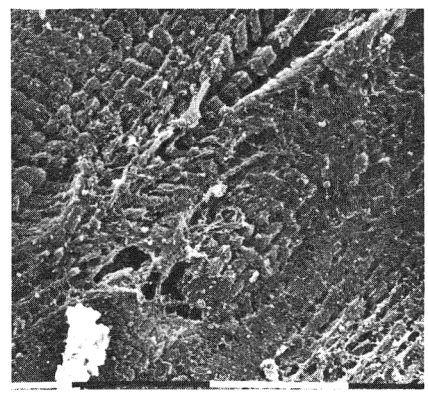


Fig. 21—Muscle fiber from Fish O treated in water (6 hr). Transverse and longitudinal disruption. Bar 0.01 mm. Magnification: 3890X.

Further work is also warranted on the detailed architecture of the myocomma and the myomere/myocommata junction using TEM and, on the types of collagen in fish tissue and on the collagenase(s) responsible for post mortem degradation.

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Heat-Induced Tendering of Turban Shell (*Batillus cornutus*) Muscle

YOSHIHIRO OCHIAI, YUTAKA KARIYA, SHUGO WATABE, and KANEHISA HASHIMOTO

ABSTRACT

The foot, opercular and visceral muscles were excised from the turban shell *Batillus cornutus*, and their heat-induced changes in toughness and ultrastructure were compared. When heated up to 30°C, the toughness of foot muscle decreased in the upper and middle regions, but increased in the lower region. The toughness of these three regions decreased at temperatures between 50–70°C, where collagen might be denatured. Some changes in toughness were also observed in the opercular muscle by heating up to 30°C, whereas the toughness of visceral muscle remained unchanged over a wide range of temperature. Transmission electron microscopy showed that the intrinsic structure of collagen fibrils in each muscle was lost at 60°C, with partial swelling.

INTRODUCTION

THE FOOT MUSCLE of some marine gastropods such as turban shell and abalone is extremely tough, although it is tendered when heated under certain conditions. Such heat-induced tendering has been associated with denaturation of stroma protein in the muscle (Takahashi and Tanaka, 1961). In fact, a large amount of collagen, which is generally related to toughness, is found in connective tissues such as skin and tendon (Cover et al., 1962; Dransfield, 1977; Moller et al., 1981). Moreover, Bailey et al. (1979) suggested that meat toughness has to do with age and cross-linkage of collagen.

On the other hand, paramyosin is contained in quantity in the adductor muscle of molluscs including turban shell (Lowey and Hanson, 1962; Szent-Györgyi et al., 1971; Pyeun et al., 1973). A relationship between paramyosin and the catch mechanism of bivalves has also been suggested (Rüegg, 1971; Twarog, 1976; Cohen, 1982). When the operculum is closed, turban shell muscle stiffens, and this muscular state continues even after the shell is removed. This phenomenon is similar to the catch mechanism, i.e., maintenance of large tension with little energy expenditure, although it is not evident in gastropods.

In the present study, three types of muscle – foot, opercular and visceral – were removed from the turban shell *Batillus cornutus*, heated at various temperatures, and then examined for their toughness and ultrastructure in order to elucidate the possible factor(s) involved in the heat-induced tendering of turban shell muscle.

MATERIALS & METHODS

Materials

Live specimens of the turban shell *Batillus cornutus* (average body weight, about 400g) were purchased at the Tokyo Central Wholesale Market, and transported to our laboratory. The muscle was removed and dissected into three parts of different appearances: the foot, opercular and visceral muscles (Fig. 1).

The authors are affiliated with the Laboratory of Marine Biochemistry, Faculty of Agriculture, Univ. of Tokyo, Bunkyo, Tokyo 113, Japan.

Toughness

Each muscle part was sectioned 1-mm thick at a right angle to the median line. The sections were placed in plastic cases, covered with a glass plate, and heated for 30 min in a water bath at temperatures ranging from 30° to 100°C at intervals of 10°C. After being left at 4°C overnight, each section was measured for toughness using a rheometer (R-UDJ-DM type, Sun Kagaku Co., Tokyo) equipped with a cylindrical plunger with a 1 mm diameter. Elevation speed of the sample table was maintained at 2 cm/min. The stress at puncture was recorded as a parameter of toughness. Preliminary experiments showed that the toughness scores of the foot muscle differed widely depending upon the measuring point, whereas those of the opercular and visceral muscles hardly differed. Therefore, the toughness of both latter muscles were measured at two arbitrary points in each section, and three regions (upper, middle and lower) were used in case of the foot muscle (Fig. 1). In addition, the samples for toughness measurement were left overnight only for convenience, since the toughness of the heated muscle was shown not to change with the lapse of cooling time.

Collagen content

Hydroxyproline content was determined on a 24-h hydrolyzate of each muscle. Estimation of collagen content was made on an assumption that the content of hydroxyproline in turban shell muscle corresponds to one-tenth of that of collagen (Kimura and Tanaka, 1983).

Myofibrillar protein content

Myofibrillar protein content was measured according to Hashimoto et al. (1979).

Protein concentration

Protein concentration was determined by a micro-Kjeldahl method.

Electron microscopy

Three types of turban shell muscle were heated at 30°C or 60°C for 30 min, cooled to room temperature, and then prefixed for 1 hr in 4% glutaraldehyde. The muscle specimens were cut into small pieces (approximately 0.5 x 0.5 x 5 mm) and stained for 20 min in 1% uranyl acetate. The pieces were dehydrated in a graded series

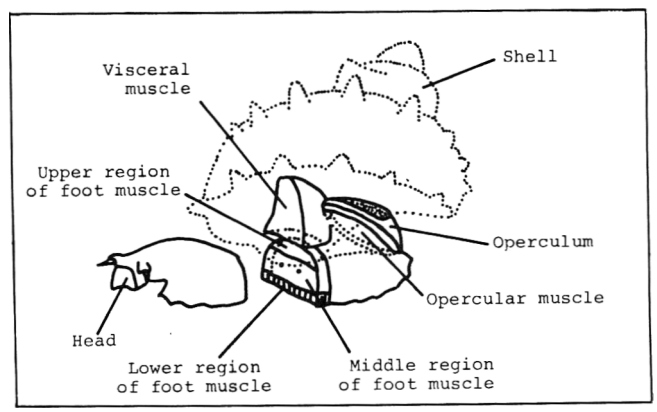


Fig. 1—A schematic diagram of the muscles sampled from turban shell.

of ethanol and acetone, and embedded in Poly/Bed 812 (Polysciences Inc.). Ultrathin sections (about 100 nm) cut on an MT-5000 ultramicrotome (Sorvall Co.) were stained with lead citrate (Reynolds, 1963). Micrographs were taken with a JEM 100-S type transmission electron microscope (JEOL Co.). The above procedure was also applied to each specimen with no heat treatment as the control. The diameters of filaments were measured directly from the electron micrographs of cross sections.

RESULTS & DISCUSSION

Heat-induced changes in toughness

Fig. 2 shows the changes in toughness of the foot, opercular and visceral muscles, as induced by heating at various temperatures. The toughness of the upper region of foot muscle was 2.9 kg in the control, decreasing to 1.1 kg after heating at 30°C for 30 min. Heat treatment of the middle region also caused a decrease in toughness from 1.4 kg to 1.2 kg. On the other hand, the toughness of the lower region increased from 0.2 kg to 0.9 kg, though the reason is not clear. The toughness of the opercular muscle changed from 0.8 kg in the control to 0.3 kg at 30°C, but remained almost constant at that level at temperatures above 30°C.

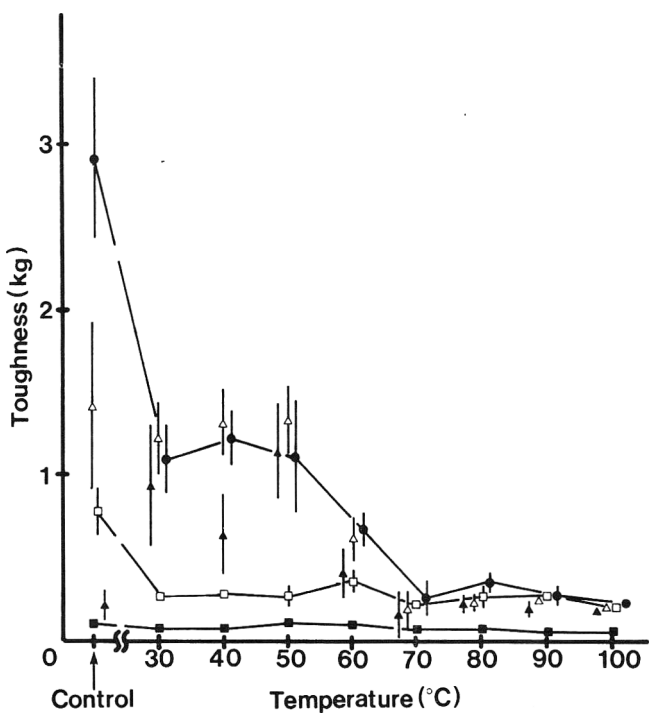


Fig. 2—Changes in toughness of the foot, opercular and visceral muscles of turban shell when heated at various temperatures for 30 min. The values are given with the standard error of 20 determinations. Symbols used: upper (●), middle (△) and lower (▲) regions of foot muscle; opercular muscle (□); visceral muscle (■).

The temperature-toughness curves of the three regions of foot muscle and opercular muscle converged at 70°C.

The visceral muscle showed a nearly constant toughness irrespective of heating temperature. This is inconsistent with the findings of Takahashi and Tanaka (1961) on toughening of the opercular and visceral muscles and tendering of foot muscle of turban shell. This discrepancy might be due partly to the freshness of sample and the conditions of heating followed by cooling. On the other hand, Moller et al. (1981) reported that the shearing force value of beef markedly increased on heating as a result of the denaturation of myofibrillar protein. The visceral muscle of turban shell showed the highest myofibrillar protein content and the lowest collagen content among the three types of muscle used (Table 1). Since the toughness of visceral muscle hardly changed on heating, myofibrillar protein might not play a major role in the toughening of the turban shell muscle.

Collagen content

Collagen contents were 7.7, 8.5 and 8.3% in the upper, middle and lower regions of foot muscle, the average being 8.2% (Table 1). Those of the opercular and visceral muscles were 5.5% and 0.9%, respectively. The collagen to crude protein ratios were 42, 47 and 46% in the upper, middle and lower regions of foot muscle, with the average of 45%. The opercular and visceral muscles had ratios of 34 and 5%, respectively. The collagen to crude protein ratios in the foot and opercular muscles were overwhelmingly higher than those of rabbit skeletal (2–10%) (Bárány et al., 1965; Bendall, 1967) and fish skeletal muscles (2–3%) (Kubota and Kimura, 1975).

Myofibrillar protein contents were 4.1, 5.0 and 7.9% in the foot, opercular and visceral muscles (Table 1). The myofibrillar to crude protein ratio was highest in the visceral muscle (45%), followed by the opercular (30%) and foot muscles (22%).

The differences of toughness in raw state among the muscle types seem to correlate positively with collagen content, but not with myofibrillar protein content.

Takahashi et al. (1957) found that there was a positive correlation between shrinkage temperature (Ts) and hydroxyproline content of collagen. From the hydroxyproline content (Table 1), Ts of turban shell collagen was estimated to be around 50°C using Takahashi correlation. Rigby (1971) mentioned that a positive correlation was also observed between Ts and the sum of proline and hydroxyproline in collagens from various sources. Based on his correlation, Ts of turban shell collagen was estimated to be around 50°C again using the sum of both amino acids in turban shell collagen (Kimura and Tanaka, 1983).

It is thus supposed that a greater decrease in the toughness of foot muscle above 50°C was caused principally by heat denaturation or gelation of collagen.

Electron microscopy

The diameter of collagen fibrils surrounding a myofibril was estimated to be 40–60 nm, and did not change, essen-

Table 1—Collagen contents and myofibrillar protein contents in the foot, opercular and visceral muscles of turban shell

Type of muscle	Content (g/100g wet tissue)[b] Crude protein (A)[c]	Collagen[d] (B)	Myofibrillar protein (C)	B/A (%)	C/A (%)
Foot[a]	18.2 ± 0.5	8.2 ± 0.3	4.1 ± 1.3	45	22
Opercular	16.1 ± 1.1	5.5 ± 0.9	5.0 ± 0.7	34	30
Visceral	17.4 ± 1.3	0.9 ± 0.1	7.9 ± 0.9	5	45

[a] Average value of the upper, middle and lower regions of foot muscle.
[b] Average value of three specimens with standard deviation.
[c] N content x 6.25.
[d] Hydroxyproline content x 10.

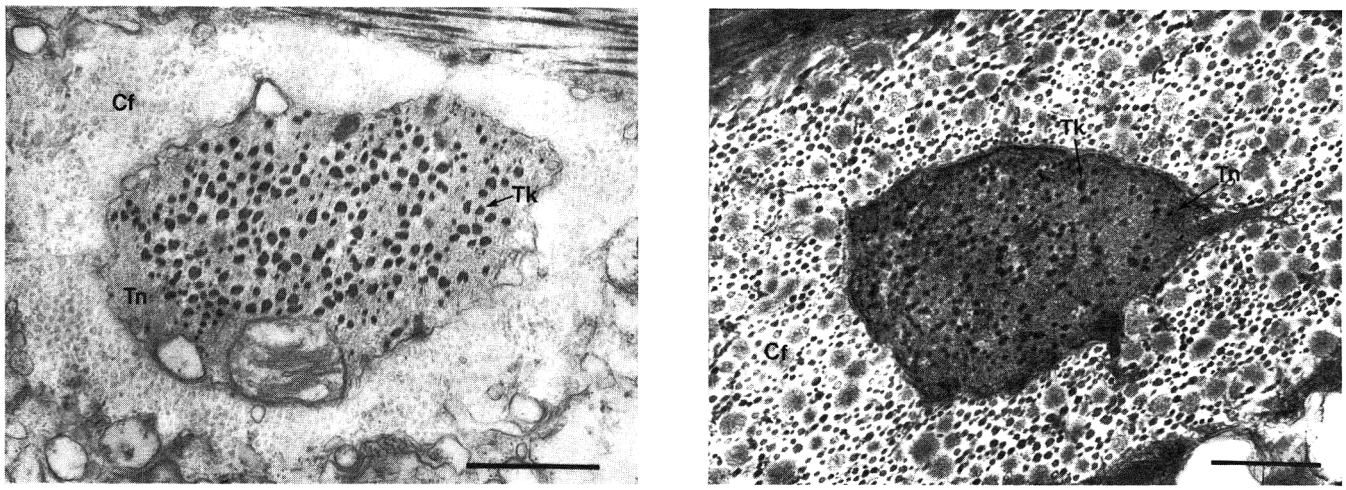


Fig. 3—Electron micrographs of turban shell foot muscle, unheated (left, 7,000X) and heated at 60°C (right, 10,000X), showing a cross-section of a myofibril surrounded by a layer of collagen fibrils. Note that some collagen fibrils of the heated sample are swollen. The bar represents 1 μm. Symbols used: Tn, thin filament; Tk, thick filament; Cf, collagen fibril.

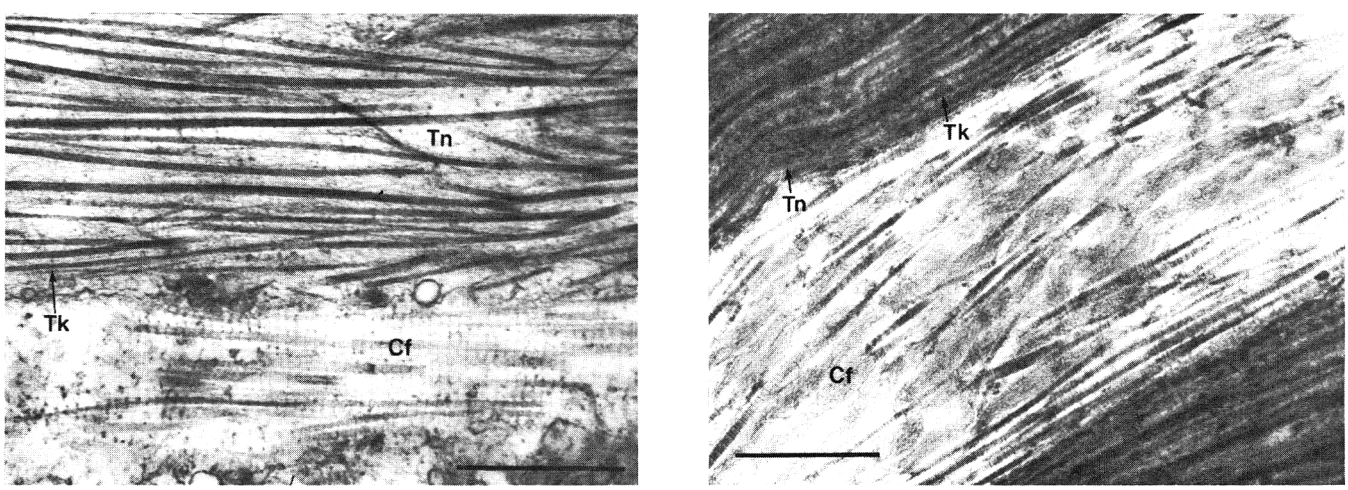


Fig. 4—Electron micrographs of turban shell foot muscle, unheated (left, 8,000X) and heated at 60°C (right, 10,000X), showing a longitudinal section of the collagen layer adjacent to a myofibril. The bar represents 1 μm. Symbols in micrographs are as in Fig. 3.

tially, at 30°C, irrespective of the type of muscle. When heated at 60°C, however, some fibrils of foot muscle showed a drastic increase in their diameters to 220 nm (Fig. 3). The swollen to nonswollen collagen fibril ratios differed widely among the test specimens, even from the same part of a muscle. In case of the opercular and visceral muscles, there was little difference between heated and unheated specimens (data not shown). The higher thermo-resistibility of these muscles might be due not only to a low collagen content, but also to a comparatively sparse musculature.

Electron microscopy of a longitudinal section of collagen fibril revealed that it did not swell all along its length at 60°C, partly retaining both the intrinsic periodicity (60 nm) and diameter (about 50 nm) (Fig. 4). The thickness of collagen layer in each muscle remained unchanged on heating at 60°C, although collagen fibrils swelled approximately five-fold. These results suggest that the intrinsic structure of collagen fibrils might be destroyed at around 50°C, causing some changes in the texture characteristic of the foot muscle of turban shell.

The thick filament diameters did not differ from each other through the three types of turban shell muscle, either before or after being heated at 60°C for 30 min (Table 2). However, the thick filaments became unclear on heating,

Table 2—Diameters of thick filaments in the three types of turban shell muscle before and after heat treatment (60°C, 30 min)

Type of muscle	Range of diameter (nm)	
	Before heating	After heating
Foot	22-70 (50)[a]	25-56 (40)
Opercular	24-81 (60)	43-65 (50)
Visceral	10-84 (50)	25-62 (40)

[a] Numbers in parenthesis represent the average values of 50 thick filaments. Five samples were used for each muscle.

some of them aggregating with each other. The thin filaments showed a stronger aggregation, resulting in more intense staining as compared with unheated ones (Fig. 3).

In conclusion, the heat-induced tendering of the foot muscle above 50°C may be ascribed to the partial swelling of collagen fibrils.

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Walleye Pollock (*Theragra chalcogramma*): Changes in Quality When Held in Ice, Slush-ice, Refrigerated Seawater, and CO_2-Modified Refrigerated Seawater Then Stored as Blocks of Fillets at −18°C

K. D. REPPOND, J. COLLINS, and D. MARKEY

ABSTRACT

Walleye pollock were held in ice, slush-ice, refrigerated seawater (RSW) and CO_2-modified RSW (MRSW) for up to 8 days, filleted and stored as blocks of fillets at −18°C. Good quality fillets were obtained from pollock held for 4 days in all systems. Uptake of salt presented a problem for pollock held more than 4 days in RSW at 1°C. Flavor scores remained essentially unchanged during frozen storage but nearly all samples became slightly tougher and slightly drier at 6 months. Freshness at time of freezing did not affect frozen storage characteristics. Protein functionality was generally adversely affected when fish were held in RSW or MRSW.

INTRODUCTION

TRADITIONALLY, domestic boats have used ice to chill fish while at sea but this method is labor intensive in view of the large catches that are typical of the pollock fishery. Furthermore, use of refrigerated seawater (RSW) or carbon dioxide modified refrigerated seawater (MRSW) has been shown to delay spoilage in shrimp or fish as compared to the use of ice (Bullard and Collins, 1978; Reppond et al., 1979; Reppond and Collins, 1983). Slush-ice has some of the same advantages as the RSW and MRSW systems; i.e., rapid chilling and less labor, plus the additional advantages of less cost of installation and probably less absorption of salt. Because much of the pollock resource is too small to fillet, processors of bottomfish will of economic necessity use mechanical flesh separators to produce minced flesh. Minced pollock flesh is currently used by Japanese processors to produce surimi, which in turn is used to form a variety of products (Okada et al., 1973). Use of minced pollock in such products will depend upon certain functional properties such as emulsifying capacity that may be as important as flavor and texture in determining the overall quality of a particular lot of pollock. The effects of holding pollock in RSW or slush-ice on these functional properties are not known. The objectives of this study were to compare the sensory and functional properties of pollock held in ice, slush-ice, RSW and MRSW using objective and subjective tests and to monitor changes in chemical and sensory attributes during frozen storage of the fillets.

MATERIALS & METHODS

Materials

Approximately 1,800 kg of pollock, averaging 700g, were caught August 20, 1981, by the R/V *Chapman* using bottom trawling gear in the vicinity of Cape Barnabas, Kodiak Island, AK. The fish were lightly iced either in an insulated tote or on deck until landed and transferred to the various holding systems about 10 hr after capture. The MRSW system, described in a previous report (Bullard and Collins, 1978), used half-strength seawater (actually a 1.75% NaCl solution) at −1.0°C. In this system, the pH of the brine was lowered to approximately 6.0 by addition of CO_2. The loading ratio by weight was 1.5 fish:1 brine. The RSW system was similar (1.75% NaCl, 1:5:1) to the MRSW system except that the temperature ranged between −0.3° and 1.8°C. Use of 1.75% NaCl at this ratio, however, provided a fish to salt ratio that was approximately that which would be found on a fishing boat using full strength seawater at a higher loading ratio of about 3:1.

The slush-ice system consisted of an insulated plastic tote equipped with perforated plastic piping connected to an air pump. Air was injected for 15 min every 3 hr to provide circulation. The ratio of fish:ice:1.75% brine was 8:3:1 initially. After 7 days, all the ice in this system had melted and enough was added to last one more day. The temperature of the slush-ice was −1.1°C for most of the experiment, rose slightly the last two days, but remained less than 0°C. The ice system consisted of an insulated box with a drain into which was placed a bed of ice followed by layers of fish and ice. The fish-to-ice ratio was 0:5:1.

Pollock for a control sample was iced overnight and filleted the next morning. About 70 fish were taken from each system at 2, 4, 6 and 8 days, hand filleted and trimmed of defects such as bloody spots and parasites. The material for sensory evaluation was frozen overnight as 18.5-pound (8.4 kg) blocks of fillets at −40°C in a plate freezer and transferred the next morning to another freezer where they were stored at −18°C. All blocks were sawed into six smaller blocks of equal size two days after the last sample was taken. These small blocks were glazed with water, wrapped in plastic coated freezer paper, packed into master cartons and stored at −18°C for the experimental samples and at −34°C for the controls. One small block from each of the storage treatments was also stored at −34°C for 2 - 3 wk until analyzed and used as zero frozen storage time samples.

The material for chemical analyses and functionality tests was prepared from the fillets left from preparing the blocks. Fillets were cut into several pieces, thoroughly mixed by hand to insure homogeneity, divided into subsamples which were placed in plastic bags and frozen in the plate freezer. The plastic bags protected the flesh from dehydration without interfering with the chemical analysis which would have been the case if the sample was stored as a water glazed block. As with the taste panel material, the bags of control and one experimental sample from each storage treatment were also stored at −34°C for several days until analyzed and are referred to as zero frozen storage time samples. The remaining samples were placed in master cartons and stored at −18°C. Changes due to frozen storage were assumed to be the same whether the pollock fillets were stored in blocks or in bags.

Chemical and functionality tests

The bags of pollock fillets were tempered overnight at 4°C and ground through a 6 mm plate while partially frozen. Analyses for NaCl, dimethylamine and trimethylamine were conducted as reported by Reppond et al. (1979) and Bullard and Collins (1980). Formaldehyde was determined by the method of Castell and Smith (1973) and volatile acid number by the AOAC (1980) method. Inosine monophosphate (IMP) was determined by the method of Spinelli and Kemp (1966) and hypoxanthine (Hx) by the Jones et al. (1964) modification of the Kalckar (1947) method. To determine functionality, a dispersion was prepared by blending 40g of ground flesh and 160g of chilled 2.0% NaCl solution in a jar previously chilled to 0°C. The dispersion was transferred to a beaker and stored overnight at 4°C. The dispersion was stirred with a spatula and the viscosity determined using a Brookfield viscometer. To determine the emulsifying capacity, 15g of the dispersion, 64g of cold water and 20g of vegetable oil were placed in a tared, chilled blender jar equipped with electrical leads connected to a Simpson meter set to measure resistance. The mixture was blended for 15 sec at a powerstat setting of 30 volts. Oil was added from a reservoir via tubing at a rate of 50g/min and blending continued at 100 volts. The addition of oil was stopped when the resistance abruptly

Authors Reppond, Collins (now retired), and Markey are with the Fisheries Utilization Research Laboratory, National Marine Fisheries Service, NOAA, P.O. Box 1638, Kodiak, AK 99615.

dropped and the blender and contents were reweighed to obtain the total weight of oil. Emulsifying capacity was calculated as grams of oil per gram of ground fish flesh. This procedure was modeled after that of Webb et al. (1970).

Sensory evaluation

Portions (75 x 50 x 20 mm) were cut from the small blocks of fillets and tempered at room temperature for 1 hr. The control and iced samples were lightly salted prior to cooking by steam for 20 min. At each seating of the panel, the panelists were served four experimental samples labeled with two-digit codes plus a control sample labeled "C". The experimental samples had the same time of fresh holding but came from different holding systems. The panel consisted of the staff of our laboratory and people employed in quality control laboratories of local seafood processors. The number of panelists varied between 10 and 12. The panelists were previously exposed to fish of varying quality and were instructed as to how samples should be scored. The panelists were instructed to compare the sample against the control for the presence of off-flavor, resistance to chewing (texture) and expression of fluid while chewing (moistness).

Flavor scores were rated on a 5-point scale, i.e., 5, no off-flavor (same as control); 4, slight; 3, moderate; 2, high; and 1, severe. The panelists were also requested to determine if the fish tasted too salty. If a panelist rated a sample as too salty, the flavor score from that panelist was set to 2 because too much salt is just as detrimental to quality as spoilage or cold storage flavor. Texture was rated on a 7-point scale, i.e., 7, very tough; 6, tough; 5, slightly tough; 4, firm; 3, slightly soft; 2, soft; and 1, very soft. Moistness was rated on a similar 7 point scale, i.e., 7, very dry; 4, moist; 1, very watery. The control was defined as having a score of 4 for both texture and moistness. The control sample was intended to serve as an example of the best quality pollock and our sensory test was designed primarily to detect departure from that ideal rather than overall acceptability. The scores of the judges were subjected to analysis of variance and other standard statistical methods (Sokal and Rohlf, 1979).

RESULTS & DISCUSSION

THE FILLETS from the different holding systems were the same in appearance and odor at 2 days, but as 4 days, fillets from fish held in MRSW seemed to be slightly softer and had slight gaping. Also, the fillets from RSW fish had the usual stickiness and drag on the filleting knife noted in previous RSW experiments. The appearance of the fish from all the systems was good both at 4 and 6 days. At 8 days, the fish held in RSW had a strong ammoniacal odor and the fish held in slush-ice had a similar odor but less intense. Fish held in MRSW and in ice had no particular off-odor, and visually appeared to be of acceptable quality. However, results from chemical and sensory analyses of cooked fillets indicated that at 8 days some spoilage was evident in fish held in all systems. With the normal processing delay between the time a boat arrives at the dock and the last fish are processed, products prepared from fish held even 6 days in any ofthe systems would be of borderline quality.

Chemical analysis

The increase in salt content was greatest for fish held in RSW which was probably caused by the warmer holding temperature (Fig. 1). Volatile acid number (VAN) remained largely unchanged from the control sample to 4 days for fish from all holding systems (Fig. 2). Between 4 and 6 days, the VAN changed little for fish held in ice or slush-ice, increased slightly for fish in MRSW but more than doubled in fish in RSW. At 8 days, the VAN values were substantially higher than their respective 6-day values, especially for the RSW sample. Flavor scores of the zero frozen storage samples were significantly correlated to VAN (correlation coefficient, $r = -0.798$, $P < 0.001$) which indicates VAN may prove useful as an indicator of spoilage.

Trimethylamine (TMA) content varied with time of holding and holding system in a similar manner except that the difference between the RSW values and the values for the rest of the samples was larger (Fig. 3) and the TMA content of fish in MRSW apparently decreased between 6 and 8 days. Loss of TMA during fresh storage of fish in MRSW never occurred in previous experiments at this laboratory. The substantial increase in TMA values between 6 and 8 days for fish held in ice and slush-ice indicated that some loss of quality occurred and that spoilage was imminent. TMA was significantly correlated to flavor scores ($r = -0.755$, $P < 0.001$) and may prove useful as an indicator of spoilage for pollock held in systems other than MRSW.

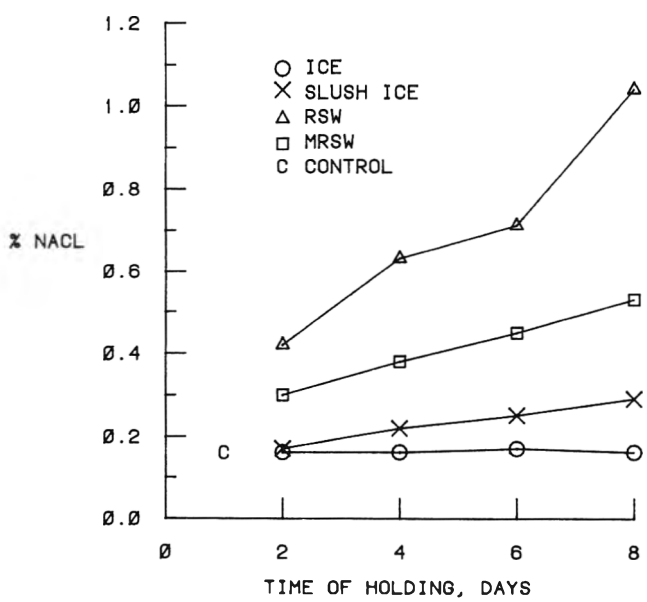


Fig. 1—Regression of salt content on time of holding walleye pollock in ice, slush-ice, RSW and MRSW.

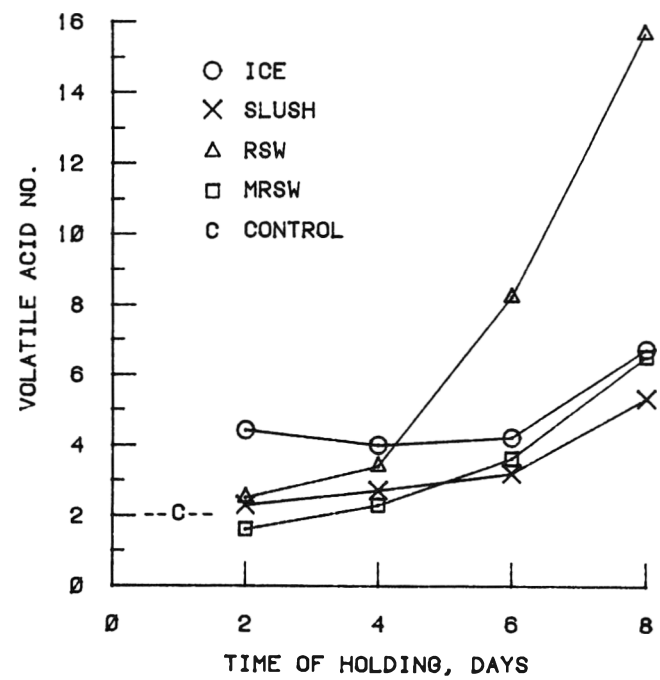


Fig. 2—Regression of volatile acid number (VAN) on time of holding walleye pollock in ice, slush-ice, RSW and MRSW.

Although the presence of IMP was linked with high quality in some species (Kuninaka et al., 1964), it formed and disappeared too quickly in pollock to be useful index of commercial quality (Table 1). Spinelli et al. (1964) showed that Hx content could be used to judge the length of time that several species of fish were held in chilled storage and in this study, the values for Hx increased linearly with time of holding pollock in most systems (Fig. 4).

During frozen storage of pollock and other members of the *gadoid* family, trimethylamine oxide (TMAO) reacts to give equal molar amounts of dimethylamine (DMA) and formaldehyde (FA) (Tokunaga, 1964; Amano and Yamada, 1964). Many researchers (Tokunaga, 1964; Castell et al., 1973; Yamada and Amano, 1965) suggest that the denaturation of the proteins of the muscle tissue by FA is the main cause of toughening of flesh from *gadoid* species during frozen storage. DMA also forms during fresh storage as noted by examining the zero frozen storage samples. DMA content increased linearly with time of fresh holding in the flesh of fish from all media except for that of fish held in MRSW which did not change (Fig. 5). Perhaps the acid pH (∿6) of the MRSW inhibited accumulation of DMA.

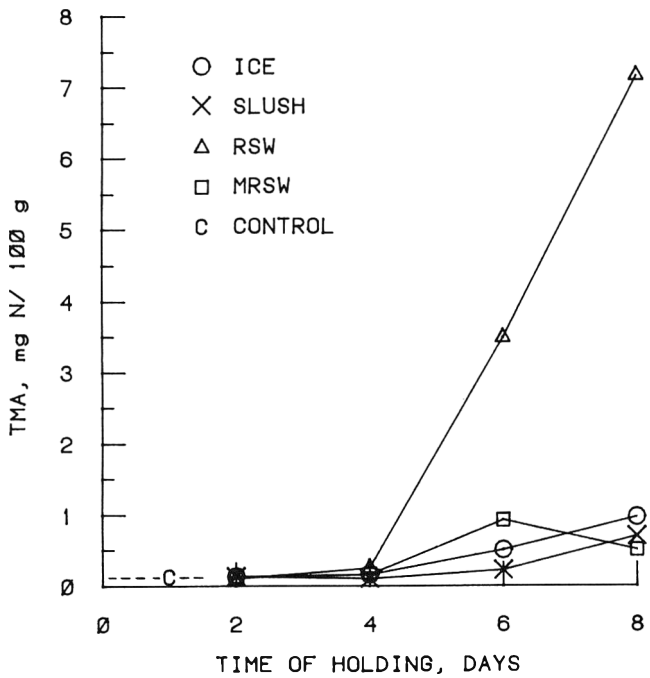


Fig. 3—Regression of trimethylamine (TMA) content on time of holding walleye pollock in ice, slush-ice, RSW and MRSW.

Table 1—Change in inosine monophosphate (IMP), formaldehyde (FA), emulsifying capacity (EC) and viscosity in the flesh of walleye pollock when held for 2, 4, 6 and 8 days in ice, slugh-ice, RSW and MRSW

Medium	Time days	IMP μM/g	FA ppm	EC g oil / g flesh	Viscosity centipoise
Ice	2	0.30	2.8	42.3	1940
	4	0.00	3.2	45.0	2685
	6	–	4.4	41.4	1973
	8	–	4.8	43.8	1973
Slush-ice	2	0.20	4.9	46.0	2367
	4	0.00	4.3	43.4	2295
	6	–	5.1	41.0	1877
	8	–	4.4	45.2	2100
RSW[a]	2	0.20	3.6	41.0	2108
	4	0.00	6.0	39.6	1793
	6	–	3.8	39.0	2125
	8	–	4.3	37.0	1580
MRSW[b]	2	0.70	5.5	40.1	1785
	4	0.00	4.4	35.6	1692
	6	–	2.3	31.6	1425
	8	–	1.5	35.9	1600
Control	–	1.67	6.1	40.2	1960

[a] RSW = Refrigerated seawater
[b] MRSW = CO_2-modified refrigerated seawater

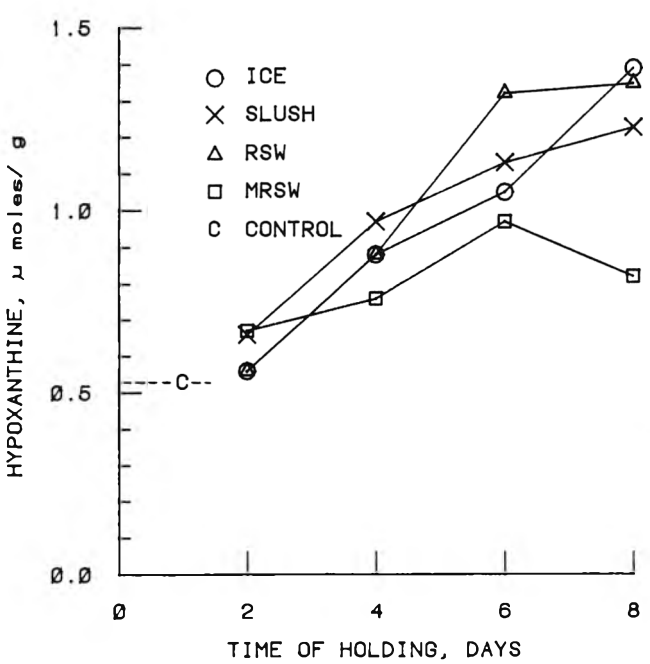


Fig. 4—Regression of hypoxanthine (Hx) content on time of holding walleye pollock in ice, slush-ice, RSW and MRSW.

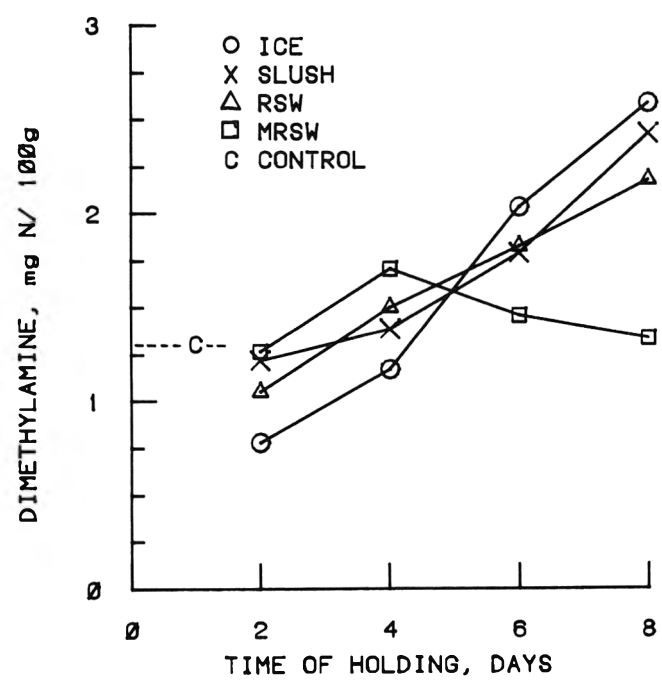


Fig. 5—Regression of dimethylamine (DMA) content on time of holding walleye pollocke in ice, slush-ice, RSW and MRSW.

During frozen storage, DMA content increased up to 9 months in all samples (Fig. 6). Between 9 and 12 months, some samples showed no change or even a decrease in DMA. Other researchers (Kramer et al., 1977; Kramer and Nordin, 1979) had similar results but no explanation was apparent. Analysis of covariance indicated that, with the exception of the 4-day RSW sample, the rate of DMA accumulation during frozen storage was the same for all samples, i.e., the rate was not affected by time of fresh holding prior to frozen storage. No explanation is apparent for the higher rate of formation for the 4-day RSW sample.

FA content changed little with time of fresh holding in any medium except in fish from MRSW which showed a slight decrease (Table 1). FA increased with time of frozen storage (data not shown) for all samples and in the same manner as DMA. The correlation coefficient between FA and DMA was highly significant ($r = 0.946$, $P < 0.001$).

Functionality

Emulsifying capacity (EC) was not affected by the time of holding in ice or in slush-ice, but decreased slightly in RSW and MRSW (Table 1). The acid pH of the MRSW may have caused the EC values of the flesh of fish from that medium to be lower than in the flesh of fish held in RSW. No pH measurements were made on the flesh itself. The viscosity values followed the same general trend as the EC values, i.e., unchanged with time of fresh holding in ice or slush-ice but decreased slightly in RSW and MRSW (Table 1).

Sensory evaluation

At zero months of frozen storage, flavor scores remained unchanged for 8 days for fish held in ice, slush-ice and MRSW (Table 2). The flavor scores of fish held in RSW remained unchanged for 6 days but decreased at 8 days because the flesh became too salty (1.08% NaCl). Saltiness was not a problem for fish held in MRSW as only a few panelists rated samples from MRSW as too salty. The quality of the pollock at the time of freezing had no influence on the accumulation of a "frozen storage flavor" during subsequent frozen storage. To facilitate the presentation of sensory results, the flavor, texture and moistness scores at 2, 4, 6 and 8 days of fresh holding were averaged for each holding medium. Such averaging was justified because time of fresh holding had little affect on flavor and no affect on texture or moistness scores. Although flavor scores decreased slightly during frozen storage, no sample

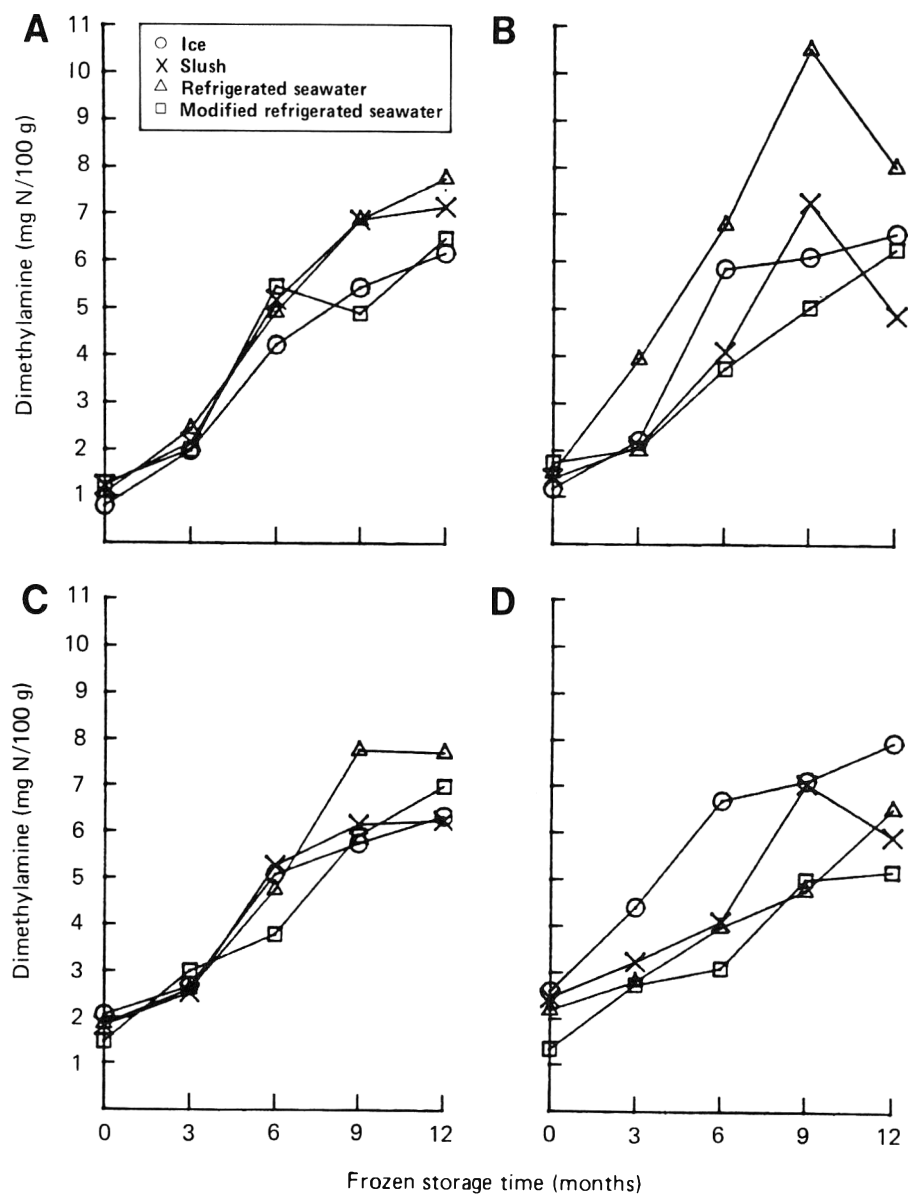


Fig. 6—Regression of dimethylamine content on time of frozen storage at −18°C of blocks of fillets of walleye pollock held in ice, slush-ice, RSW and MRSW for 2 (A), 4 (B), 6 (C), or 8 (D) days prior to frozen storage.

Table 2—Mean flavor, texture and moistness scores[c] of steamed portions of pollock held 2, 4, 6 or 8 days in ice, slush-ice, RSW and MRSW[d]

	Medium	Time, days 2	4	6	8
Flavor	Ice	4.33	4.58	4.30	3.91
	Slush	4.33	3.92	4.40	3.82
	RSW	4.67	4.08	4.30	2.09*
	MRSW	4.00	4.08	3.90	3.27
Texture	Ice	4.00[b]	4.00	4.20[ab]	3.73[a]
	Slush	3.75[b]	4.50	4.10[ab]	4.27[ab]
	RSW	3.67[b]	4.00	3.50[a]	4.00[ab]
	MRSW	4.78[a]	4.33	4.80[b]	5.00[b]
Moistness	Ice	4.38[ab]	4.33	4.80	4.10
	Slush	4.33[ab]	4.83	4.50	4.55
	RSW	3.77[a]	4.36	4.00	3.90
	MRSW	4.89[b]	4.58	4.90	4.91

[a,b] Means for same attribute within the same column with same or no superscript do not differ ($P < 0.05$).
[c] Flavor was evaluated on a 5-point scale (5 = no off-flavor, 1 = severe off-flavor). Texture and moistness were evaluated on 7-point scales (7 = very tough, dry; 1 = very soft, watery).
[d] Twelve panelists.
* Mean for same attribute is significantly different ($P < 0.05$) from rest of means within same row and column.

was rated unacceptable for reasons other than saltiness (Fig. 7A).

Texture scores were not affected by time of fresh holding in any medium nor by frozen storage for 3 months (Table 2, Fig. 7B). Thereafter, the flesh became slightly tougher in most samples. The texture of fish held in MRSW tended to be firmer than samples from fish held in ice or slush-ice. RSW samples were less firm than other samples. With the possible exception of the MRSW samples held 12 months at −18°C, no sample was so tough as to be rated unacceptable. Texture scores were significantly correlated with DMA ($r = 0.544$, $P < 0.01$) and FA content ($r = 0.474$, $P < 0.01$, $df = 78$) which adds to the body of evidence that the degradation of TMAO to DMA and FA affects the quality of frozen pollock. The time of fresh holding did not affect moistness, but the scores were higher (flesh was drier) for MRSW samples than for RSW samples (Table 2). As with texture, moistness scores increased (became drier) at 6 months of frozen storage (Fig. 7C). Only a few samples were rated by individual panelists as dry or very dry.

CONCLUSIONS

GOOD QUALITY fillets were obtained from pollock held for 4 days in all systems. Chemical tests indicated the onset of spoilage occurred between 6 and 8 days for fish held in ice, slush-ice and MRSW but even earlier for fish held in RSW. The presence of salt probably masked some of the spoilage flavor for fish held in RSW for 6 days but by 8 days the flesh was too salty. The lower temperature of the MRSW may have slowed the absorption of salt by fish in that medium. Pollock held in RSW or MRSW may not be suitable for processing into surimi or minced flesh because of the loss of protein functionality. Protein functionality remained largely unchanged for fish held in ice or slush-ice however. The quality of blocks of pollock fillets changed little during a year's storage at −18°C. Flavor scores decreased slightly and the flesh became slightly tougher and drier after 3 months. Formation of FA probably caused the change in texture and moistness. Changes in frozen storage were largely unaffected by treatment of the fish prior to freezing.

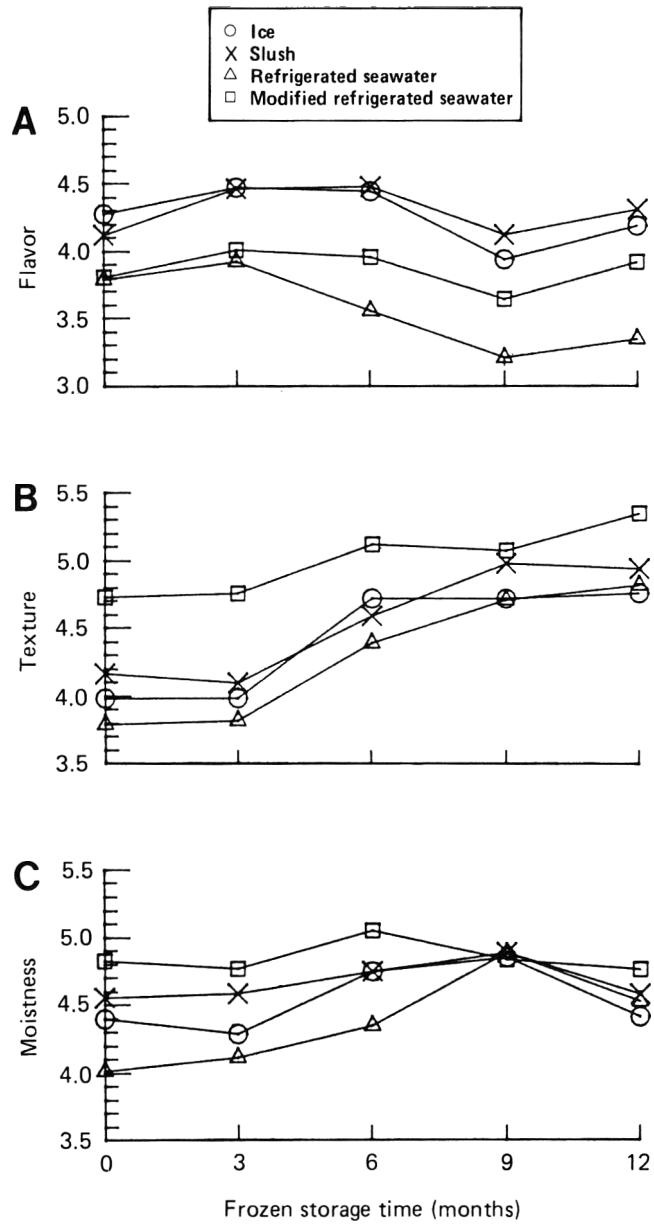


Fig. 7—Regression of mean flavor (A), texture (B), and moistness (C) scores on time of frozen storage at −18°C of blocks of fillets from walleye pollock held in ice (○), slush-ice (X), RSW (△) and MRSW (□). Flavor was evaluated on a 5-point scale (5 = no off-flavor, 1 = severe off-flavor). Texture and moistness were evaluated on 7-point scales (7 = very tough, dry; 1 = very soft, watery).

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Development of Botulinal Toxin and Sensory Deterioration During Storage of Vacuum and Modified Atmosphere Packaged Fish Fillets

LAURIE S. POST, DEBORAH A. LEE, MYRON SOLBERG, DAVID FURGANG, JOHN SPECCHIO, and CHARLES GRAHAM

ABSTRACT

Toxin production by *C. botulinum* type E was studied in cod, whiting, and flounder fillets packaged in air-permeable film, vacuum packages and packages flushed with N_2 or CO_2 during storage at 8°, 12°, or 26°C. Cod and whiting fillets were flushed with CO_2 and stored continuously at 4°C or cycled between 4° or 8° and 26°C. Cod and whiting fillets were flushed with gas mixtures and stored at 8°C or 26°C. Flounder deteriorated rapidly and was rejected by sensory evaluation prior to toxin detection during vacuum or modified atmosphere storage at 12°C and 8°C but after toxin detection at 26°C. Toxin was present either prior to or simultaneously with sensory rejection of cod and whiting fillets for all vacuum or modified atmosphere treatments and temperature regimens.

INTRODUCTION

THERE IS a large body of evidence which indicates that fresh fish may be contaminated with *Clostridium botulinum* organisms either as a result of the organisms being present in the microbiota of the fish ecosystem or as a result of post-catching contamination during processing (Bott et al., 1966; Foster et al., 1965; Fantasia and Duran, 1969; Craig et al., 1968; Eklund and Poysky, 1967; Insalta et al., 1967). Foodborne botulism outbreaks caused by fish were predominately due to nonproteolytic type E strains of *C. botulinum* in products which were consumed without further heat processing. Most fish are thoroughly cooked prior to consumption thus minimizing the hazard from heat labile botulinal toxin, if present. A potential for cross-contamination exists, however, from uncooked toxin containing fish through the hands of those preparing meals or from food contact surfaces to foods which would not be heated prior to consumption.

One of the current practices with respect to retail distribution of fish involves the storage of fish on ice without any packaging. Such practices, and their abuses, seem to result in sensory rejection of fresh fish based upon odor or appearance prior to the possible development of botulinal toxin. The desire of the fishery industry to extend the shelflife of fresh fish and increase market demand has led to the adaption of packaging techniques which have been successful in the preservation of other foods. One such approach is the use of either vacuum or modified atmosphere packaging. Modified atmospheres, including N_2 and CO_2 flushing, and vacuum packaging suppress the normal spoilage flora and thereby extend retail shelf life (Abrahamsson et al., 1965; Eyles and Warth, 1981; Stier et al., 1981; Fay and Regenstein, 1982. Cann et al., 1965, 1966; Eklund and Poysky, 1970).

The potential for *C. botulinum* outgrowth and toxin production during storage of fresh fish fillets in vacuum and modified atmospheres was evaluated using a comprehensive approach to provide a body of information on the influence of fish type, package atmosphere, and storage temperature. These studies were conducted to determine whether the fish fillets would become toxic prior to or after sensory rejection on the basis of odor or appearance.

The authors are affiliated with the Dept. of Food Science, New Jersey Agricultural Experiment Station, Cook College, Rutgers Univ., New Brunswick, NJ 08903.

MATERIALS & METHODS

Fish source

Fresh whole fish-cod (*Gadus morhua*), whiting (*Merluccius bilinearis*) or flounder (*Limanda ferrugina*) were purchased from the Fulton Fish Market (New York City) and were filleted by a local retailer. Fish were used on the day of purchase or stored overnight in crushed ice at 4°C for use within 24 hr.

Clostridium botulinum inoculum

Spores of five strains of *C. botulinum* Type E were obtained from the FDA, Washington, D.C. The strains were #070, Beluga, D8, Minnesota, and Birmingham. The spores were cultivated in Trypticase Peptone Glucose Yeast Extract (TPGY) medium (FDA, 1978) without trypsin. The spore suspensions were heat shocked for 15 min at 60°C (Tsang et al., 1985). After cooling under running tap water, each strain was inoculated into 10 mL TPGY medium and incubated for 24 hr at 26°C. Following at least two consecutive subcultures, 10% inoculations into 100 mL volumes TPGY were made. Sporulation was obtained after 7 to 10 days at 26°C. Cultures were evaluated for spore development by examination of stained smears through brightfield microscopy. At appropriate times, spores were harvested by centrifugation at 1500 x *g* for 20 min (5°C). Spore pellets were washed at least five times in sterile distilled water. Stock suspensions of spores were stored in distilled water at 4°C until calibrated by plate counts on Anaerobic Egg Yolk (AEY) agar with 0.5% cysteine-HCl (FDA, 1978).

Each of the five stock spore suspensions was diluted to obtain a concentration of 5×10^6 spores/mL and a composite was made by mixing equal volumes of the individual strains. The concentration of the composite mix was 5×10^6 spores/mL, confirmed by direct plating on AEY agar. Appropriate dilutions were made to inoculate the fish with ~ 50 spores of the composite mix per gram.

Packaging technique

The general procedure for packaging of fish was as follows: (1) Excess liquid from the surface of the fillets was removed by absorption with sterile paper towels prior to preparation for packaging; (2) Fillets were cut into approximate 100g portions at room temperature. When not being handled, fillets were maintained on aluminum foil over crushed ice. All handling was done with sterile gloves and was kept to a minimum; (3) A total volume of 100 μL inoculum containing 5000 spores or an equivalent volume of sterile distilled water (control) was delivered in 10 equal sized drops. The inocula were dispensed over the inside surface of the fish (versus the skin side) and spread with a sterile glass hockey stick. Physical uniformity of the 100g portions could not be regulated because of variation in fish size and shape. The distribution of the inoculum was determined by the inoculator.

Three packaging systems were employed: aerobic, obtained by wrapping in an air permeable film; vacuum, obtained by packaging in an evacuated pack; modified atmosphere, obtained by flushing vacuum packages with N_2, CO_2 or one of two gas mixes. The air-permeable packaging material was Choice Wrap Vitafilm (677-33-4018, Goodyear Co., Akron, OH) (O_2 transmission, 980 mL/100 in^2/atmosphere pressure/24 hr). A single layer of film covered the fish at all points. The remainder of the film on the side and ends of the package was rolled to the edge of the fish. Packages were spaced 3/4 in. apart with corrugated board strips in Z shapes to allow air contact from top and bottom. Vacuum and modified

atmosphere conditions were established in individual 5 in. x 8 in. bags (Gravureflex, South Hackensack, NJ) composed of 3/4 mil nylon and 3¼ mil ethylene vinyl acetate (O_2 transmission, 2.6 ± 0.1 mL/100 in^2/atmosphere pressure/24 hr; water permeability, 0.003 mL/100 in^2/24 hr at 95% relative humidity). Each bag was evacuated using a Royal-Vac packaging machine (Royal Packaging Equipment, Inc., Fort Lee, NJ) (vacuum 17 to 20 in Hg at time of sealing). Every fifth package was tested for actual vacuum. A desiccator jar was attached to a vacuum pump and a mercury manometer. A package was placed into the jar and the vacuum pump operated until the packaging film lifted from the fish surface. The desiccator was separated from the pump, air was fed in until the film just returned to the fish surface and the mercury manometer reading was recorded.

Bags used for modified-atmosphere packaging were prepared with septa composed of a silicone caulking material. After evacuation and sealing, a sterile 21 gauge syringe needle was inserted into the package through the septum and the package was filled to a soft inflated state with the appropriate gas. Bags were flushed with filtered CO_2, N_2 or one of two gas mixtures containing 90% CO_2: 8% N_2:2% O_2 or 65% CO_2:31% N_2:4% O_2. This was accomplished within 15 - 20 sec with a regulator gas pressure of ~ 3 psi. Every fifth package was tested for package atmosphere including H_2, O_2 and N_2 concentrations using a gas chromatograph (Carle Analytical Gas Chromatograph 311) equipped with a 1/8 in. stainless steel column (5 ft) (molecular seive 5A packing).

Storage conditions

Both control and inoculated packages of cod and whiting were stored at a refrigeration temperature (4°C). Control and inoculated packages of flounder, whiting and cod were stored at a mild abuse temperature (8°C), a moderate abuse temperature (12°C) or a severe abuse temperature (26°C). The 4°C storage room had a built-in defrost cycle which permitted a rise in the surface temperature of the stored fish, (measured with a copper constantan thermocouple) to approximately 6.5°C every 3 hr. The temperature rise and recovery was complete within approximately 1 hr. The 8°, 12° and 26°C storage rooms did not have defrost cycles and the temperature of fish stored within these rooms did not vary by more than ±0.25°C. Incubator temperatures were monitored by recording thermometers and daily checks of mercury thermometers inserted in vials of distilled water.

The varying parameters of fish type packaged, package atmosphere, and storage temperatures are listed in Table 1. Storage regimens provided either continuous temperatures or incorporated alternating periods at abusive and nonabusive temperatures. Cod and whiting fillets in CO_2 flushed packages were subjected to a 24 hr period at 26°C followed by continuous storage at 8°C or 4°C. Cod fillets in CO_2-flushed packages were subjected to cycled abuse between 26°C and 4°C according to two regimens: four cycles of 8 hr at 26°C and 16 hr at 4°C, followed by continuous storage at 4°C or three cycles of 16 hr at 26°C and 8 hr at 4°C, followed by continuous storage at 4°C.

Sensory evaluation

Duplicate samples were evaluated by a five to seven member panel. Panelists had been immunized against botulism prior to the study and were trained to evaluate the product for appearance and odor acceptability. Rating definitions are indicated in Table 2.

Samples were removed from the temperature controlled storage rooms and analyzed for package atmosphere using gas chromatography as previously described. Leaking packages were not used. The packages were allowed to equilibriate for approximately 30 min prior to evaluation. Appearance was evaluated and ratings were recorded. The packages were opened and exposed to air for 20 - 30 sec prior to presentation to panelists for odor evaluation. Panelists were not permitted to handle open packages and ratings were recorded by the presenter. Every attempt was made to maintain a 12 in. distance from the evaluator to minimize the hazard posed by a toxic package. When odors were minimal, the packages were brought within a closer proximity for evaluation. A rating of 4 was taken as the normal rejection point for most people despite any expense or inconvenience. A rating of 5 was taken as the absolute rejection point and indicated a product that had deteriorated to such a putrid condition, that no person would conceivably use it even if they had paid for it and were in a state of starvation.

C. botulinum toxin analysis

Each of the two samples subjected to sensory evaluation were analyzed for the presence of botulinal toxin. The ~100g samples were blended with 100 mL gel phosphate buffer at pH 6.2 (2X dilution) for 2 min in a Stomacher 400 (Dynatech Laboratories Inc., Alexandria, Va.). A 4X dilution was used if the fish flesh was too pasty. The slurry (25g) was centrifuged at 17,400 x *g* for 30 min at 5°C. The supernatant was decanted, trypsin treated (1.8 mL supernatant, 0.2 mL 10% trypsin solution, Difco, Detroit, MI) and tested for the presence of *C. botulinum* toxin by the BAM (FDA, 1978) assay procedure with some modification to reduce the number of mice used. A single mouse was utilized for the preliminary screening. If the mouse died, the screen was repeated and another mouse was injected with a heated (80°C, 15 min), trypsin treated sample. If the replicate mouse died and the mouse inoculated with the heated sample lived, two additional mice were inoculated to confirm the presence of *C. botulinum* toxin. A mouse was protected with type specific antiserum (0.1IU/0.5 mL sample) (Antitoxin E, CDC, Atlanta, GA) and then challenged with the suspect toxin con-

Table 1—Various combinations of package atmosphere and storage temperature to which cod, flounder and whiting were subjected

Atmosphere	Storage temperature[a]	Fish
Air	8°C	Flounder, cod, whiting
Air	12°C	Flounder, cod, whiting
Air	26°C	Flounder, cod, whiting
Vacuum	8°C	Flounder, cod, whiting
Vacuum	12°C	Flounder, cod, whiting
Vacuum	26°C	Flounder, cod, whiting
Nitrogen	8°C	Flounder, cod, whiting
Nitrogen	12°C	Flounder, cod, whiting
Nitrogen	26°C	Flounder, cod, whiting
Carbon dioxide	4°C	Cod, whiting
Carbon dioxide	8°C	Flounder, cod, whiting
Carbon dioxide	12°C	Flounder, cod, whiting
Carbon dioxide	26°C	Flounder, cod, whiting
Carbon dioxide	26°C (24 hr) → 4°C	Cod, whiting
Carbon dioxide	26°C (24 hr) → 8°C	Cod, whiting
Carbon dioxide	4 x [26°C (8 hr) → 4°C (16 hr)] → 4°C	Cod
Carbon dioxide	3 x [26°C (16 hr) → 4°C (8 hr)] → 4°C	Cod
90% CO_2: 8% N_2: 2% O_2	8°C	Cod, whiting
90% CO_2: 8% N_2: 2% O_2	26°C	Cod, whiting
65% O_2: 31% N_2: 4% O_2	8°C	Cod, whiting
65% O_2: 31% N_2: 4% O_2	26°C	Cod, whiting

[a] Continuous storage at this temperature unless otherwise indicated.

taining sample. An unprotected mouse was also injected. Death of the unprotected mouse and survival of the protected mouse was indicative of a toxin containing sample.

The large number of nonspecific deaths encountered led to the development of a procedure for their elimination. In later studies, nonspecific toxic reactions were eliminated by treatment with bovine serum (Solberg et al., 1985). A 1.8 mL aliquot of an inoculum implicated in a nonspecific death was incubated with 1 mL bovine serum for 1 hr at 37°C then subjected to usual trypsinization procedures. Gel phosphate buffer was used to dilute an aliquot of the inoculum to the same extent and was also injected. In early studies, nonspecific deaths were eliminated by dilution with gel phosphate buffer. The dilution procedure was less likely to detect low levels of toxin, if present, than was the bovine serum technique.

Sampling frequency and end point definition

Samples stored at 26°C were analyzed daily as were samples which were cycled several times between 26° and 4°C prior to storage at 4°C. Samples stored for 24 hr at 26°C and then moved to 8°C or 4°C storage were sampled three times during each week. Samples stored continuously at 4°C were sampled two or three times during each week. Sampling was terminated when on three consecutive sampling days ratings of 5 for either odor or appearance were recorded or the presence of toxin was confirmed, whichever occurred first. Data reported indicate the first day of the consecutive series on which a sensory rating of 5 was recorded or toxin was detected.

RESULTS

INOCULATED and uninoculated samples were not different with respect to sensory deterioration. Odor was consistently the quality attribute causing earliest sensory rejection. Toxin was detected in four of a total of 1,016 uninoculated samples incubated during the 2 yr study. Toxin was found after 14 days in a single uninoculated control sample of whiting flushed with CO_2 and stored for 24 hr at 26°C followed by continuous storage at 4°C. The product was marginally acceptable in odor and appearance. Toxin was detected in single samples of uninoculated cod after 10 days (acceptable in odor and appearance) and 29 days (rejected on the basis of odor alone) of storage, and in uninoculated whiting after 9 days (acceptable in odor and appearance) of storage at 8°C in a 90% CO_2:8% N_2:2% O_2 gas mixture.

The results obtained for inoculated cod, whiting, and flounder fillets in various package atmospheres and subjected to various storage temperature regimens are summarized in Tables 3, 4, and 5. Toxin was detected in all species after 1.0 - 3.0 days of storage at 26°C. Samples were generally either unacceptable (absolute rejection) or nearly unacceptable (normal rejection) by sensory evaluation at the time of toxin detection. Toxin was present prior to normal rejection of cod in CO_2-flushed packages in one of two experiments (Table 3), whiting in 90% CO_2:8% N_2:2% O_2-flushed packages (Table 4) and flounder in N_2-flushed packages (Table 5). Toxin was present prior to absolute rejection of cod fillets in vacuum packed, N_2-flushed, CO_2-flushed, and 65% CO_2:31% N_2:4% O_2-flushed packages (Table 3); whiting fillets in air-permeable, CO_2-flushed, 90% CO_2:8% N_2:2% O_2-flushed and 65% CO_2:31% N_2:4% O_2-flushed packages (Table 4) and flounder fillets in air-permeable, N_2-flushed and CO_2-flushed packages (Table 5). Vacuum- and modified-atmosphere packaging followed by abusive storage at 26°C afforded no extension of storage time relative to air permeable packaging except for cod in which samples remained in the region between normal rejection and absolute rejection for as long as 4 days more than samples in air permeable wrap (Table 3).

Samples stored at 12°C in air-permeable wrapped packages were unacceptable by sensory evaluation at least 2 days prior to toxin detection. Fillets became toxic after 6 - 15 days of storage (Tables 3, 4, and 5). Hazardous situations arose when toxin was detected in acceptable cod and whiting fillets which had been stored in vacuum packed, N_2-flushed and CO_2-flushed packages (Tables 3 and 4). Normal rejection of fillets stored at 12° demonstrated a hazardous situation only for cod in N_2-flushed packages (Table 3). Time to toxin detection was extended by vacuum and modified atmosphere treatment in all cases except for cod fillets in N_2-flushed packages (Table 3). Flounder spoiled more rapidly than either cod or whiting and was rejected at least 2 days prior to toxin detection (Table 5). The mean extension of storage time to absolute sensory rejection due to vacuum- and modified-atmosphere treatment was 10.3 days for cod, 12.3 days for whiting and 0 days for flounder.

Fillets stored at 8°C in air-permeable wrapped packages were rejected by sensory evaluation before toxin was detected (Tables 3, 4, 5). Vacuum- and modified-atmosphere treatment extended the sensory shelf life 17.4 days for cod, 13.2 days for whiting and 7.3 days for flounder. During the extended period, toxin was detected either prior to or on the day of absolute sensory rejection for all cod and whiting treatments (Tables 3 and 4). Flounder fillets deteriorated more rapidly and were subjected to normal and absolute sensory rejection before the detection of toxin during storage in all atmospheres tested (Table 5).

Continuous mild abuse of cod fillets at 8°C in packages flushed with 90% CO_2:8% N_2:2% O_2 or 65% CO_2:31% N_2:4% O_2 resulted in toxin detection prior to both normal and absolute sensory rejection (Table 3). Normal rejection

Table 2—Rating definitions for sensory evaluation of cod, whiting and flounder fillets

	DESCRIPTIVE TERMINOLOGY		
Rating	Appearance[a] Cod and Whiting	Appearance[a] Flounder	Odor
1[b]	Fish translucent and bright with definite striations	Flesh firm, totally translucent to totally opaque, color light to dark (varying shades of pink)	Little or no odor
2	Flesh translucent with slight opaqueness, striations clear	Flesh firm, slight darkening in color	Slight fishy odor
3	Flesh opaque with slight translucency, slight darkening of color, striations evident	Flesh firm, off colors evident (yellow, green, gray)	Fishy or slight off-odor, not objectionable
4	Flesh opaque with definite darkening, loss of striations	Tissue deterioration evident, definite darkening of color	Noticeable odor, strong fish or off odor
5	Definite tissue breakdown, soft appearance, flesh dark	Definite tissue breakdown flesh dark	Putrid, trash-like odor, strong musty odor

[a] Drip may or may not be present, but is ignored since fillets could be drained prior to market display.
[b] Highest quality.

was observed prior to or at the same time as toxin formation for vacuum packed or N_2-flushed packages (Table 3). The gas mixtures at 8°C greatly extended product shelf life but reduced the time to toxin detection as compared to the air permeable wrap. Whiting fillets in 90% CO_2:8% N_2:2% O_2-flushed packages stored at 8°C were first absolutely rejected after 15 days of storage but were not consistently rejected until after 22 days of storage (Table 4). Toxic fillets were first detected on day 8 and thereafter on days 10, 17, 22, and 24. Whiting fillets in 65% CO_2:31% N_2:4% O_2-flushed packages stored at 8°C were rejected after 14 days of storage but were toxic on days 5, 12, 14 and 16 (Table 4). Normal sensory rejection of whiting fillets were recorded prior to toxin detection in vacuum packed, N_2 flushed and CO_2 flushed packages but not in either of the gas mixtures.

Continuous refrigerated storage of cod fillets at 4°C resulted in toxin detection prior to sensory rejection (Table 3). Toxin was detected intermittently on days, 18, 22, and 39 in one study while the samples were not absolutely rejected throughout 60 days of storage. Toxic samples were present in an inconsistent pattern on days 21, 26, 30, 47, and 54 in a second study while the fillets were not consistently absolutely rejected throughout a 58 day storage period. Whiting fillets in CO_2 flushed packages, stored continuously at 4°C, were absolutely rejected on the basis of odor after 43 days of storage, but were toxic after 27 days of storage at which time the samples were 12 days beyond the normal rejection point (Table 4). Rejection on the basis of appearance did not occur until day 51.

A 24 hr severe abuse treatment at 26°C followed by continuous refrigerated storage (4°C) of cod fillets in CO_2 flushed packages resulted in the detection of toxin prior to either absolute or normal sensory rejection (Table 3). Similar results were observed with post abuse storage at 8°C. Whiting fillets in CO_2 flushed packages subjected to 24 hr at 26°C followed by continuous storage at 8°C were rejected (absolute) after 10 days of storage but were toxic on days 6, 10 and 11 (Table 4). Normal rejection occurred on day 6. Whiting fillets, in CO_2-flushed packages subjected to 24 hr at 26°C followed by continuous storage at 4°C, were rejected after 16 days of storage but were toxic on the seventh day after packaging (Table 4). Normal rejection occurred prior to the first detection of toxin.

Cycling abuse at 26°C and refrigerated storage at 4°C, resulted in toxin detection prior to absolute sensory rejection of cod fillets in CO_2-flushed packages (Table 3). Toxin was detected on the same day as normal rejection. Fig. 1 compares sensory rejection to toxin detection as a function of total hours spent at 26°C during cycling abuse. Packages were stored a total of 0, 24, 32 or 48 hr at 26°C intermittent with storage at 4°C. Cod fillets contained toxin after about 20 days of continuous storage at 4°C in CO_2-flushed packages. Time to toxin detection was reduced with increasing hours of abuse at 26°C. While time to absolut rejection was also reduced, outright rejection occurred only after toxin was detected. Time to normal rejection began to decline more rapidly, however, and normal rejection occurred simultaneously with toxin detection after 32 and 48 total hr of storage at 26°C.

Temperature versus time to toxin detection and sensory rejection in CO_2-flushed and vacuum packages of cod fillets were compared (Fig. 2). A CO_2 atmosphere extended time to both absolute and normal rejection at 4°C considerably beyond the time to toxin detection. At 8°C and 12°C, toxin was detected prior to or simultaneous with normal rejection of CO_2-flushed packages. Vacuum-packaged samples, however, were given a normal rejection prior to toxin detection at 8°C and 12°C, but, like CO_2-flushed packages,

Table 3—Days of storage under various parameters of atmosphere and temperature required for inoculated[a] cod fillets to become toxic or to be rejected by sensory evaluation

Temperature regimen	Atmosphere	Toxin detection (days)	Absolute[b] rejection (days)	Normal[c] rejection (days)
26°C	Air	2	2	2
	Vacuum	2	6	2
	N_2	2	4	2
	CO_2	2,2[d]	5,3[d]	3,2[d]
	90% CO_2[e]	3	2	2
	65% CO_2[f]	1	3	2
12°C	Air	>9[g]	6	6
	Vacuum	14	17	10
	N_2	6	17	13
	CO_2	11	15	11
8°C	Air	>10[g]	6	6
	Vacuum	20	28	16
	N_2	17	17	17
	CO_2	19	27	23
	90% CO_2[e]	8	29	17
	65% CO_2[f]	9	16	16
4°C	CO_2	18, 21[d]	>60[g], 58[d]	53, 40[d]
26°C (24 hr) → 8°C	CO_2	6	15	8
26°C (24 hr) → 4°C	CO_2	14	27	18
4 x [26°C (8 hr) → 4°C (16 hr)] → 4°C	CO_2	8	22	8
3 x [26°C (16 hr) → 4°C (8hr)] → 4°C	CO_2	3	>8[g]	3

[a] Inoculated with 50 spores C. botulinum type E/g.
[b] Absolute rejection point, 5.0 on sensory rating scale. Considers added expense and inconvenience.
[c] Normal rejection point, 4.0 on sensory rating scale.
[d] Study performed in duplicate.
[e] 90% CO_2: 8% N_2: 1% O_2.
[f] 65% CO_2: 31% N_2: 4% O_2.
[g] Termination of study preceded toxin detection or sensory rejection.

were given an absolute rejection after toxin detection. At 26°C, both vacuum packaged and CO_2-flushed packages were given a normal rejection simultaneous with toxin detection while absolute rejection followed toxin detection. Vacuum-packaged samples showed slightly extended times to absolute spoilage at 26°C in comparison to CO_2-flushed packages.

Toxin was present in cod and whiting fillets prior to or simultaneous with absolute sensory rejection in all vacuum- and modified-atmosphere treatments at all continuous storage temperatures. Toxin detection preceeded absolute rejection of flounder fillets only in N_2- and CO_2-flushed packages held at 26°C. It was further observed that in air-permeable packages, absolute sensory rejection preceeded toxin detection with both slight and moderate abuse treatments but was a hazard with extremely abusive treatment. Fig. 3 depicts the mean times to toxin detection and sensory rejection for samples in air-permeable film and modified atmosphere packages held at varying temperatures of abuse.

DISCUSSION

IT CAN BE CONCLUDED from these studies that storage in vacuum packed, N_2-flushed, CO_2-flushed, and mixed gas-flushed packages extends the storage life of fresh fish, but may permit the development of toxin, in many situations, before the fish would be rejected with absolute certainty. The hazard is even more acute in that the rejection was usually on the basis of odor rather than appearance. The fish could appear acceptable while containing toxin. The packaged fish could subsequently be opened and placed upon food contact surfaces so that the toxin could be transferred from food handler's hands or from food contact surfaces to other foods prepared without any heat process. Thus, while the fish would be considered inedible after it had been smelled, its toxin may have been transferred to an unsuspecting victim.

Abrahamsson et al. (1965) studied *C. botulinum* type E toxin development in herring fillets at a severe abuse temperature of 20°C. They reported no toxin detected at 1 day and detectable toxin at 2 days of storage with a large inoculum of spores. These results are comparable to the 1 to 3 days at 26°C required for toxin detection in the present study at a slightly higher temperature and with a much smaller inoculum. Abrahamsson et al. (1965) reported more rapid toxin development in vacuum-packaged fish than in fillets packaged in air-permeable wrap. The results of this study show the same trend, although proving that the small difference observed is real would be very difficult. The work of Stier et al. (1981) involving fresh salmon fillets inoculated with 4 x 10^2 spores/g also supports the present study. Samples stored at 22.2°C in air permeable packages and under a mixed gas modified atmosphere were toxic at 2 days. The present study showed slightly extended times to spoilage due to a difference in the guidelines for sensory evaluation. The panelists were instructed to consider themselves a person with little money who had already purchased the product and had nothing else to eat. The panel, based upon their own concepts of fish edibility, would have considered a score of 4.0 as rejected. The mean time to rejection at a 4.0 level was 1 day in air-permeable wrap and 2 days in CO_2-flushed, N_2-flushed and vacuum-packed samples. Values determined in this manner confirm the spoilage times indicated in the literature.

Eklund and Poysky (1970) inoculated haddock with 100 spores/g and during vacuum packed storage at 10°C, detected toxin and spoilage simultaneously at 11 days. Cann et al. (1966) inoculated herring, cod, and haddock with 100 spores/g and detected toxin at 5, 12, and 6 days, respectively, during vacuum packed storage at 10°C. Unfortunately, they did not test for spoilage. In the present study, toxin was detected in vacuum-packed cod, whiting, and flounder fillets stored at 12°C after 14, 12, and 15 days of storage, respectively. These values are similar to the 10°C storage results reported by Cann et al. (1966) and Eklund and Poysky (1970). The time to spoilage for flounder in this study agrees well with the data of Eklund and Poysky

Table 4—Days of storage under various parameters of atmosphere and temperature required for inoculated[a] whiting fillets to become toxic or to be rejected by sensory evaluation

Temperature regimen	Atmosphere	Toxin detection (days)	Absolute[b] rejection (days)	Normal[c] rejection (days)
26°C	Air	3	4	2
	Vacuum	2	2	2
	N_2	2	2	2
	CO_2	2,2[d]	3,3[d]	2,2[d]
	90% CO_2[e]	1	4	2
	65% CO_2[f]	2	3	2
12°C	Air	>8[g]	5	5
	Vacuum	12	19	9
	N_2	12	14	9
	CO_2	12	>19[g]	12
8°C	Air	>12[g]	6	4
	Vacuum	17	17	10
	N_2	17	17	10
	CO_2	20	26	15
	90% CO_2[e]	8	22	13
	65% CO_2[f]	5	14	7
4°C	CO_2	27	43	15
26°C (24 hr) → 8°C	CO_2	6	10	6
26°C (24 hr) → 4°C	CO_2	7	16	5

[a] Inoculated with 50 spores C. botulinum type E/g.
[b] Absolute rejection point, 5.0 on sensory rating scale. Considers added expense and inconvenience.
[c] Normal rejection point, 4.0 on sensory rating scale.
[d] Study performed in duplicate.
[e] 90% CO_2: 8% N_2: 2% O_2.
[f] 65% CO_2: 31% N_2: 4% O_2.
[g] Termination of study preceded toxin detection or sensory rejection.

Table 5—Days of storage under various parameters of atmosphere and temperature required for inoculated[a] flounder fillets to become toxic or to be rejected by sensory evaluation

Temperature regimen	Atmosphere	Toxin detection (days)	Absolute[b] rejection (days)	Normal[c] rejection (days)
26°C	Air	2	4	2
	Vacuum	2	2	2
	N_2	2	4	4
	CO_2	2	3	1
12°C	Air	11	9	5
	Vacuum	15	13	8
	N_2	14	7	7
	CO_2	10	8	8
8°C	Air	>12[d]	7	5
	Vacuum	>21[d]	14	7
	N_2	>21[d]	14	4
	CO_2	23	15	10

[a] Inoculated with 50 spores C. botulinum type E/g.
[b] Absolute rejection point, 5.0 on sensory rating scale. Considers added expense and inconvenience.
[c] Normal rejection point, 4.0 on sensory rating scale.
[d] Termination of study preceded toxin detection or sensory rejection.

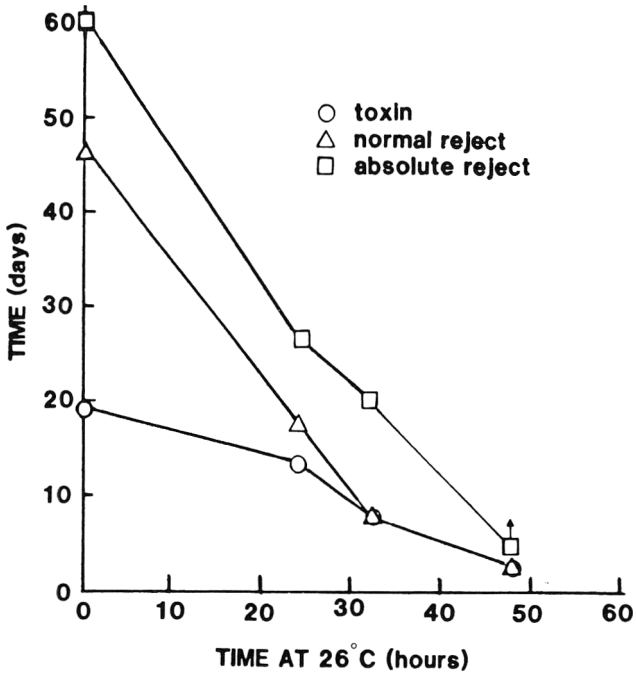


Fig. 1—Effect of abusive storage at 26°C combined with refrigerated storage at 4°C upon time required for toxin detection and sensory rejection of carbon dioxide flushed cod fillets inoculated with 50 spores C. botulinum type E/g. (⊥ termination of study preceded sensory rejection).

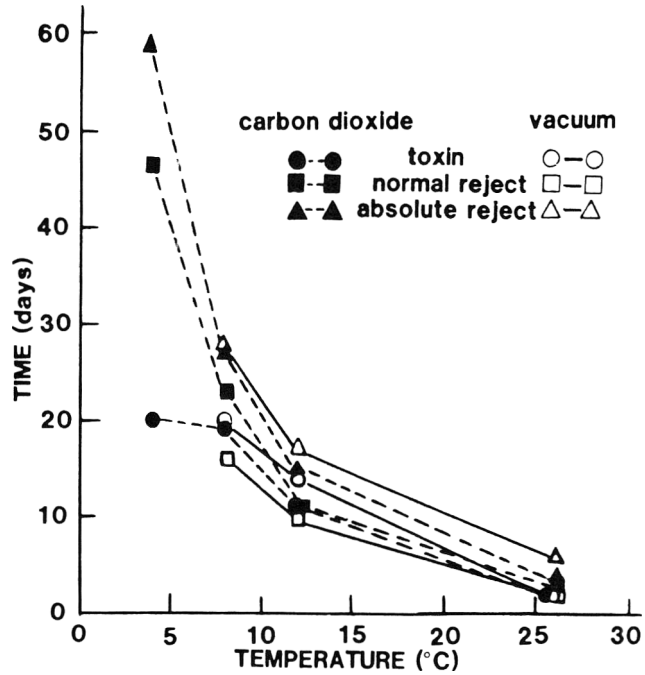


Fig. 2—Effect of storage temperature upon time to toxin detection and time to sensory rejection of carbon dioxide flushed and vacuum packaged cod fillets inoculated with 50 spores C. botulinum type E/g.

(1970) for haddock. Differences in inoculum strains and in fish species used may give rise to the minor discrepencies between these data and those of the other investigators.

Toxin was detected between 5 and 23 days in the various oxygen restricted packages stored at 8°C in this study. Eklund and Poysky (1970) reported first detection of toxin at 49 days in vacuum packed samples of haddock and Dover sole stored at 7.8°C. Better agreement is seen with the results of Cann et al. (1965) who reported toxin detection between 11 and 32 days in vacuum-packed herring at 5°C. Eklund and Poysky (1970) reported spoilage in vacuum packed fish at 7.8°C within 12 or 14 days. The mean time to sensory rejection of vacuum-packed samples using a score of 4.0 was 11 days in the present study which is in agreement with the previously published literature. The time to toxin detection at 8°C was decreased in comparison to other atmosphere modification techniques in packages of cod and whiting flushed with gas mixtures containing 2% or 4% oxygen. A possible explanation of this phenomenon may be that oxygen-requiring microorganisms were able to grow rapidly and establish reducing conditions which then favored the development of the anaerobic *C. botulinum*.

Storage in CO_2 flushed packages at 4°C extended shelf life significantly, but caused little delay in the development of botulinum toxin relative to 8°C storage. The delay may have been partly minimized by the defrost cycle in the 4°C storage area. Cod subjected to abuse at 26°C intermittent with storage at 4°C demonstrated results corresponding to the total storage time at 26°C. As total storage time at 26°C increased, time to toxin detection decreased. The fillets, however, were not absolutely rejected until after toxin detection. Thus, it is evident that fillets subjected to even slight temperature abuse show an increased hazard potential.

Two frequently employed atmosphere modification techniques are CO_2-flushing and vacuum packaging. In this study, normal rejection in vacuum packages occurred prior

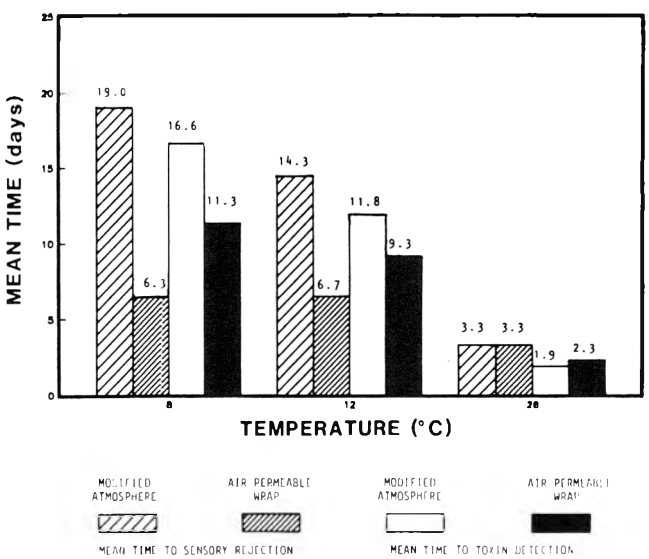


Fig. 3—Mean time to toxin detection and absolute sensory rejection in fish fillets packaged in an air permeable film or under modified atmospheres and stored at 8, 12 or 26°C.

to toxin detection, but in CO_2-flushed packages of cod, toxin was detected before normal rejection. Absolute rejection always occurred simultaneous with or after toxin detection for both techniques. The extreme safety factor used in the odor evaluation scheme is realistic and desirable if safety is to be assured, should the fillets be inadvertently held beyond the point of normal rejection.

These studies indicate that vacuuum and modified atmosphere packaging alone is not capable of providing the

safety required for extended storage of fish fillets with respect to outgrowth and toxin production by *C. botulinum* type E in the absence of a fail-safe mechanism by which storage temperature could be maintained at 0°C. In order to effectively utilize the extended shelf life aspect of vacuum- and modified-atmosphere storage, some intervention is needed to assure the delay of toxin production should even mild temperature abuse occur. Possible approaches include the use of low dose ionizing radiation which may injure the spore, and the use of anti-botulinal agents.

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Purification and Properties of β-Glucuronidase from the Muscle of Bombay Duck (*Harpodon Nehereus*)

S. B. WARRIER, V. NINJOOR, and G. B. NADKARNI

ABSTRACT

Bombay duck muscle β-glucuronidase purified 4200-fold had molecular weight of 160,000 as estimated by gel filtration on Sephadex G-200. The glycoprotein enzyme exhibited dimeric structure on SDS-PAGE and had a pI of 5.0. The enzyme was optimally active at pH 5.2 when phenolphthalein β-D-glucuronide was used as the substrate while with p-nitrophenyl glucuronide the pH optimum was 4.6. Saccharo-1,4-lactone was a potent competitive inhibitor of the enzyme. Heavy metalic ions such as Hg^{2+}, Cd^{2+} and Ag^{+} also proved to be inhibitory to the enzyme. Radiation inactivation of the enzyme could be protected in the presence of mercaptoethanol. Sodium chloride activated the enzyme while sodium tripolyphosphate inhibited it.

INTRODUCTION

HYDROLYTIC ENZYMES of fish skeletal muscle have been the subject of several investigations (Tappel, 1965; Bird, 1975; Warrier et al., 1972) in view of their reported participation in autolytic processes (Doke *et al.*, 1979; Makinodan et al., 1980) that lead to early spoilage. Among muscle hydrolases, β-glucuronidase seemed to be involved in the hydrolysis of glucose-galactose moieties present in collagen and the mucopolysaccharide-protein complexes of the connective tissue matrix (Blumenfield et al., 1963; Butler and Cunningham, 1966). The enzyme is also implicated in the cleavage of glucuronidic disaccharide linkages of the protein polysaccharide complex occurring in the connective tissue (Roden, 1965; Dutson and Lawrie, 1974). In meat foods such as in beef and lamb, the rapid tenderization following electrical stimulation has been attributed to the synergistic action of this enzyme with other hydrolases (Savell et al., 1978; Dutson et al., 1980). Lysosomal localization of β-glucuronidase in a variety of tissues including fish skeletal muscle is well established (Warrier et al., 1972; Barrett and Heath, 1977; Bird, 1975). However, unlike other lysosomal acid hydrolases, the enzyme displays a dual distribution in subcellular compartments, i.e., lysosomes and microsomes (Fishman et al., 1967). β-Glucuronidase of lysosomal origin has been studied extensively in organ tissues such as liver (Stahl and Touster, 1971; Tomino and Paigen, 1975), kidney (Kato et al., 1970) and preputial gland (Ohtsuka and Wakabayashi, 1970). Nevertheless, investigations on skeletal muscle β-glucuronidase especially from aquatic species are rather scarce.

The present study was undertaken to purify and determine the properties of β-glucuronidase from the muscle of Bombay duck, a fish amenable to rapid spoilage. Inhibitory characteristics of the enzyme were studied in view of their possible application in devising suitable preservation methods to minimize the enzyme mediated spoilage.

Authors Warrier, Ninjoor and Nadkarni are affiliated with the Biochemistry & Food Technology Division, Bhabha Atomic Research Centre, Trombay, Bombay 400 085, India.

MATERIALS & METHODS

Materials

Phenolphthalein β-D-glucuronide, p-nitrophenyl β-D-glucuronide, naphthol ASB1-β-D-glucuronide, saccharo-1,4-lactone, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC), Coomassie brilliant blue, hexokinase (type III), bovine serum albumin (BSA), human gamma-globulin, horse heart myoglobin, ovalbumin, p-chloromercuribenzoate (pCMB) and dithiothreitol (DTT) were obtained from Sigma Chemical Co. (St. Louis, MO). o-Aminophenyl-β-D-glucuronide was obtained from Koch Light (Buckingham, UK). Sephadex G-200, blue dextran, Concanavalin A (Con A) Sepharose 4B, cyanogen bromide (CNBr)-activated Sepharose 4B were the products of Pharmacia (Uppsala, Sweden). Reagents for electrophoresis were supplied by Eastman Kodak (Rochester, NY). 3,3′ Diamino-dipropylamine was from Buschs SG, Switzerland and alpha methyl mannoside was procured from Aldrich chemical Co., WI. All other chemicals were of analytical grade and used without further purification.

Fish sampling

Fresh off-shore Bombay duck (*Harpodon nehereus*) was obtained from a local market and brought to the laboratory in ice. Fish of the same size were selected and freed from external adherences by washing profusely with cold water. After cutting off heads and tails, they were eviscerated, cleaned and sliced. Fillets weighing 200g were packed in polyethylene bags and frozen in contact freezer for 4 hr at −25°C. The frozen fish were held at −20°C for 3 days, thawed, and the exudate (drip) was collected as described earlier (Warrier et al., 1972).

For preparing homogenate, fillets devoid of skin and bone were scissor minced and homogenized in 250 mM sucrose containing 1 mM ethylenediaminotetraacetic acid, disodium salt (EDTA) using a Sorvall omnimixer with the rheostat adjustment at 3 for 2 min. The suspension was diluted to a final concentration of 15% (w/v) with sucrose-EDTA and passed through two layers of cheesecloth to remove fibrous material.

Assay of β-glucuronidase

The enzyme in 100 mM acetate buffer pH 5.2 and 1.25 mM phenolphthalein β-glucuronide (sodium salt) in a total volume of 1 mL was incubated at 45°C. The reaction was stopped by the addition of 4 mL of pH 10.7 buffer (133 mM glycine, 67 mM sodium chloride and 83 mM sodium carbonate) (Stahl and Touster, 1971). After cooling and centrifugation at 1500 rpm for 15 min, the color intensity of liberated phenolphthalein was recorded at 540 nm. Phenolphthalein was used for standardization.

When p-nitrophenyl β-D-glucuronide was used as substrate (15 mM), at pH 4.6, the reaction was stopped with 1N NaOH (Barrett and Heath, 1977), and the released p-nitrophenol was quantitated at 420 nm. The unit of activity equals the release of nmoles of phenolphthalein or p-nitrophenol/hr and the specific activity is unit/mg protein.

Estimation of protein

Protein was determined by the method of Miller (1959) using bovine serum albumin as the standard.

Assay of other enzymes

Activities of aryl sulfatase, N-acetyl β-glucosaminidase and β-galactosidase were determined at various stages of purification by the methods described by Barrett and Heath (1977).

Estimation of molecular weight

The Sephadex G-200 column (2.5 x 82 cm) equilibrated with 20 mM Tris/HCl buffer, pH 7.3, and used earlier for the gel filtration of the enzyme was calibrated for molecular permeation. Void volume (Vo) was assessed by chromatographing blue dextran-2000. The elution volumes (Ve) for human gamma-globulin (MW = 160,000), yeast hexokinase (MW = 80,000), bovine serum albumin (MW = 67,000), ovalbumin (MW = 45,000) and horse heart myoglobin (MW = 16,700) were determined by gel filtration on Sephadex G-200 column under the identical conditions that were used for β-glucuronidase. The flow rate was 25 mL/hr with 3.8 mL/fraction.

Amino acid analysis

The acid hydrolysate of the purified enzyme was prepared by heating the enzyme solution in 6N HCl in a vacuum sealed tube at 110°C for 24 hr. After hydrolysis, the acid was evaporated and the residue dissolved in 200 mM citrate buffer, pH 2.2. The sample (1 mL) containing 0.5 mg protein was used for the amino acid analysis (Moore and Stein, 1963) which was carried out with a Beckman Unichrom amino acid analyser (Model 4231). Galactosamine and glucosamine were also determined on the hydrolysate.

Effect of compounds

The enzyme solution in 100 mM acetate buffer, pH 5.2, was preincubated with the compounds in a total volume of 0.5 mL at various concentrations for 30 min at 37°C and the assay was carried out as described earlier.

Exposure to gamma-radiation

The enzyme solution in 100 mM acetate buffer, pH 5.2, was subjected to varying doses of gamma-radiation in ice in the absence and presence of 2 mM mercaptoethanol. The irradiation was done at a dose rate of 2.2 Krad/min in a Gamma Cell-220 (Atomic Energy of Canada Ltd.) having a Co^{60} source.

Polyacrylamide gel electrophoresis (PAGE)

The procedure of Davis (1964) was employed for electrophoresis. The gels were stained for protein with 0.25% Coomassie blue and destained by 7% acetic acid. In another experiment electrophoresis was carried out at pH 4.5 using acetic acid/β-alanine buffer. Enzyme staining of the electrophoretic gel was carried out according to Hayashi et al. (1964) with naphthol ASBI-glucuronide as the substrate. Procedure of Weber and Osborn (1969) was used for electrophoresis under denaturing conditions (SDS-PAGE).

Purification of β-glucuronidase

Earlier studies (Warrier et al., 1972) had revealed that the skeletal muscle hydrolytic enzymes leached out and accumulated in the exudate of Bombay duck in concentrated form. Therefore, the drip from muscle was employed as one of the purification steps. Drip obtained from 1 kg of Bombay duck fillets stored frozen for 3 days served as the starting material.

Heat treatment

The centrifuged (10,000 rpm, 10 min), clear drip (200 mL) was heated at 55°C for 5 min and cooled in an ice bath. The resulting precipitate was discarded after centrifugation at 4°C. All further operations were carried out at 4°C unless stated otherwise.

Ammonium sulfate fractionation

Solid ammonium sulfate was added to the thermally treated drip to 30% saturation. After 2 hr the sample was centrifuged and the supernatant was brought to 70% saturation by further addition of solid ammonium sulfate. After 20 hr the precipitate was collected, dissolved in minimum volume of 50 mM Tris/HCl buffer, pH 7.2 (Tris, pH 7.2) and dialyzed against three changes of the same buffer. The dialyzed fraction was centrifuged to remove insoluble material.

Con A-Sepharose 4B chromatography

The ammonium sulfate fraction was applied to a Con A Sepharose 4B column (2 x 8 cm) preequilibrated with 50 mM Tris, pH 7.2, containing 150 mM NaCl. After eluting unadsorbed protein with the equilibration buffer, the bound enzyme was displaced by passing through 200 mM alpha-methyl mannoside dissolved in 50 mM Tris, pH 7.2 containing 150 mM NaCl. The fractions exhibiting enzyme activity were pooled, dialysed against distilled water and concentrated by ultrafiltration using Amicon ultrafiltration unit (Amicon Instruments, Lexington, MA) fitted with a PM-10 membrane.

Affinity chromatography on saccharo-1,4-lactone bound Sepharose

Binding of saccharo-1,4-lactone to Sepharose 4B was carried out as described by Harris et al. (1973). Briefly, the method consisted of coupling 3,3'diaminopropylamine to CNBr activated Sepharose 4B to yield a 'long arm' aminoalkyl derivative. Saccharo-1,4-lactone was then coupled to the aminoalkyl derivative through its free carboxyl group using a water soluble carbodiimide. The gel was packed in a column (2 x 15 cm) and equilibrated with 2 mM Tris, pH 7.2, containing 10 mM NaCl. The enzyme fraction from the Con A-Sepharose step was applied to the column and 5 mL fractions were collected at a flow rate of 120 mL/hr using the equilibration buffer for elution. After collecting 30 fractions (when E_{280} approached zero) the elution was changed to 50 mM acetic acid containing 20 mM NaCl and 30 more fractions were collected. The fractions possessing enzyme activity (37-42) were pooled and dialysed against 20 mM Tris, pH 7.3, containing10 mM NaCl and concentrated to a minimum volume. The method described by Harris et al. (1973) was used for carrying out substrate (o-aminophenyl β-D-glucuronide) bound affinity chromatography.

Gel filtration

The enzyme obtained from the previous step was applied to a Sephadex G-200 column (2.5 x 82 cm) pre-equilibrated with 20 mM Tris, pH 7.3, containing 10 mM NaCl. The elution was affected with the same buffer at a flow rate of 25 mL, and 3.8 mL fractions were collected. Active fractions were pooled and concentrated.

Isoelectric focusing

LKB column electrofocusing apparatus model LKB 8100 (LKB Products, Bromma, Sweden) was used. A linear 5–50% sucrose gradient containing 1% (v/v) Ampholine (pH 3.5–10.0) was formed in a 110 mL column. Approximately 100 μg of β-glucuronidase in 1.5 mL of 22% sucrose was applied in the middle of the gradient. Electrofocusing was carried out at 500v for 24 hr and 2 mL fractions were collected and analysed for enzyme activity and pH values.

RESULTS

BOMBAY DUCK β-glucuronidase was purified 4200-fold with 17% recovery by steps summarized in Table 1. The final preparation was free from aryl sulfatase, N-acetyl β-glucosaminidase and β-galactosidase. Affinity chromatography on saccharo-1,4-lactone bound Sepharose 4B was a useful step in the purification since the contaminating proteins were eliminated almost exclusively. β-Glucuronidase emerged as a major protein peak when eluted with 50 mM acetic acid containing 20 mM NaCl (Fig. 1). Following gel filtration on Sephadex G-200, the enzyme was purified to apparent homogeneity as revealed by the coinciding peaks of protein and enzyme activity (Fig. 2).

The enzyme displayed a single protein band on PAGE which corresponded to β-glucuronidase activity as depicted in Fig. 3. The movement of the enzyme was very slow in both acidic and alkaline PAGE systems. The homogeneity of the preparation was further ascertained by subjecting the purified enzyme to sucrose density gradient isoelectric focusing in 1% Ampholine with pH values ranging from 3.5–10.0. As shown in Fig. 4, the enzyme emerged as a single peak and migrated to a position corresponding to a pI value of 5.0 after 24 hr of isoelectric focusing.

The enzyme displayed a pH optimum of 4.6 with p-nitrophenyl β-D-glucuronide while with phenolphthalein β-D-glucuronide the corresponding pH was 5.2. The enzyme had a temperature optimum of 45°C. The Lineweaver-Burk plots gave a Km value of 42 x 10^{-5} M with phenolphthalein β-D-glucuronide and 111 x 10^{-5} M with p-nitrophenyl

Table 1—Purification of β-glucuronidase from Bombay duck skeletal muscle

Fraction	Total protein (mg)	Total activity (Units)	Specific activity (units/mg)	Recovery (%)	Purification ratio (fold)
Muscle	90706.00	138780	1.53	100	1
Drip	2195.00	115654	52.69	83	34
Heat treatment (55°C, 5 min)	1796.00	107275	59.73	77	39
Ammonium sulfate fraction (30-70%)	759.20	84271	111.00	61	72
Con A-Sepharose 4B chromatography	31.58	61265	1940.00	44	1268
Saccharo-1,4-lactone affinity chromatography	15.05	56814	3775.00	41	2467
Sephadex G-200 gel filtration	3.56	23300	6545.00	17	4277

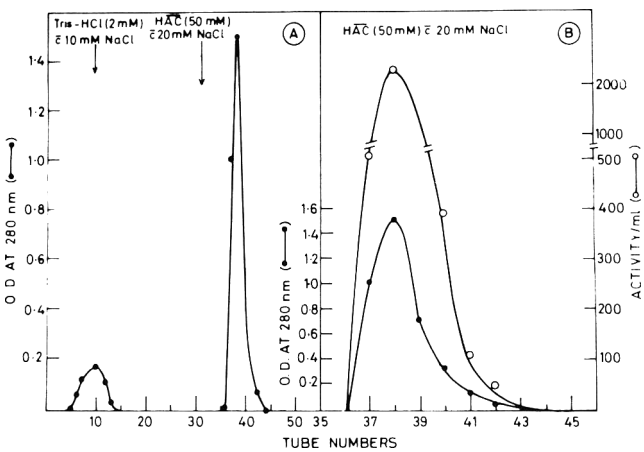


Fig. 1—Affinity chromatography of Bombay duck β-glucuronidase on saccharo-1,4-lactone bound Sepharose 4B. A, the elution pattern of protein with Tris, pH 7.2 and acetic acid containing NaCl; B, the second peak of protein in Fig. A corresponding to the enzyme activity is shown in magnified form.

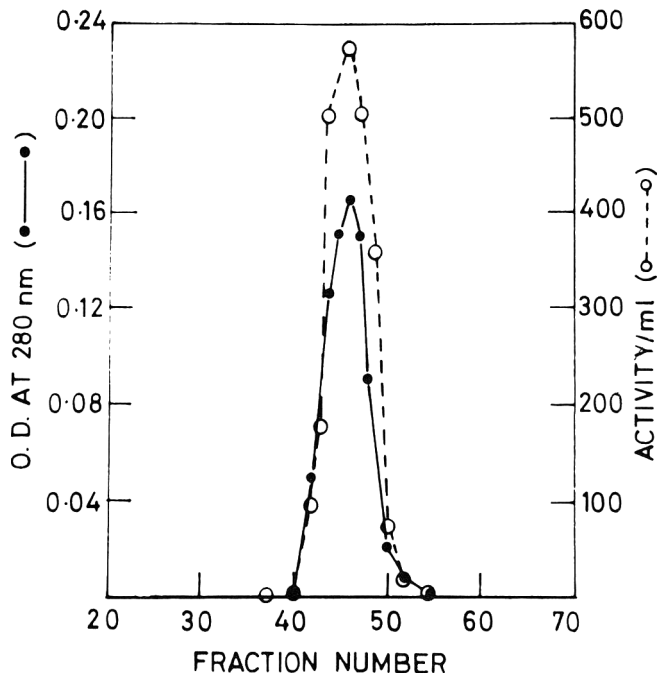


Fig. 2—Gel permeation chromatography of β-glucuronidase.

β-D-glucuronide respectively. The V_{max} values obtained were 29.40 and 58.80, respectively (Table 2).

Heat stability

The response of β-glucuronidase to thermal treatment is shown in Fig. 5. Preheating at 45°, 50°, and 55°C for 30 min was not deleterious to the enzyme. Incubation at 60°C for 30 min, however, caused 30% loss in the activity. In contrast, thermal treatment at 70°C for the same period resulted in 80% inactivation of the enzyme. Arrhenius plot of activity, measured between 30°C and 45°C, gave activation energy of 10.32 Kcal/mole of enzyme. The enzyme was relatively stable on storage both at refrigerated and frozen conditions. Only 10% of catalytic activity was lost after storage at −10°C or 0°C for 4 months.

Effect of compounds

The data on the modification of β-glucuronidase activity by several compounds are summarized in Table 3. Heavy metals such as Hg^{2+} and Cu^{2+} inhibited the enzyme strongly at 2 mM concentration. At the same concentration Cd^{2+}, Ag^{+} and Zn^{2+} showed only moderate inhibition. However, on increasing the concentration to 10 mM these metalic ions also inhibited the enzyme to the same extent as Hg^{2+} and Cu^{2+}. Among other metalic ions tested Mn^{2+} activated the enzyme at 10 mM concentration. pCMB inhibition was reversed by the addition of DTT as well as mercaptoethanol. Saccharo-1,4-lactone was found to be a competitive inhibitor of the enzyme with an apparent K_I of 1 x 10^{-6} M. Sodium tripolyphosphate (NaTPP) at 20 mM concentration completely inhibited the enzyme. Other compounds such as DNA, BSA, DTT and NaCN did not exert any influence on the enzyme activity.

Exposure to different doses of gamma-radiation rendered dose dependent inactivation of the enzyme. At a dose of 50 krad there was 40% inactivation, whereas at 100 krad the inactivation was 80%. Addition of mercaptoethanol (2 mM) prior to irradiation protected the enzyme from radiation damage suggesting the involvement of thiol group in the catalytic activity.

Estimation of molecular weight

The apparent molecular weight of β-glucuronidase was 160,000 as determined by gel filtration on Sephadex G-200 (Fig. 6). However, on SDS-PAGE the enzyme migrated to a region corresponding to a molecular weight of 80,000 suggesting that the enzyme is a dimer.

Amino acid and carbohydrate composition

The enzyme contained high concentration of glutamic and aspartic acids and only a trace of cysteine (Table 4). The enzyme was a glycoprotein yielding 1% hexosamine after 24 hr of acid hydrolysis.

DISCUSSION

PURIFIED β-glucuronidase of Bombay duck appeared homogeneous both by its elution pattern through Sephadex

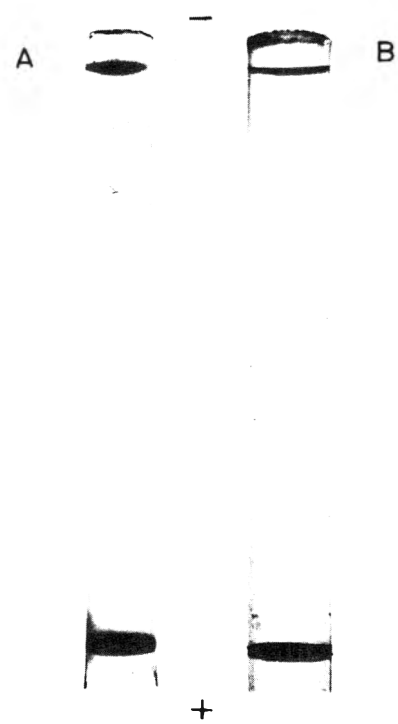


Fig. 3—PAGE of purified β-glucuronidase. A, gel stained for protein, B, gel stained for enzyme.

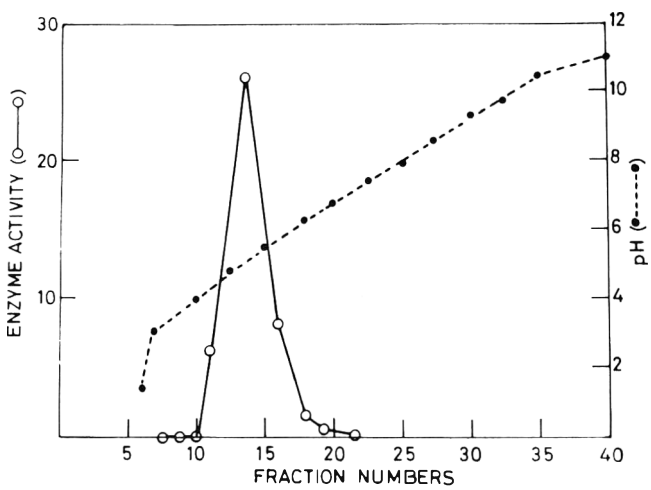


Fig. 4—Isoelectric focusing of β-glucuronidase. β-Glucuronidase (100 μg) was electrofocused in a pH gradient between 3.5-10 for 24 hr in LKB column electrofocusing apparatus model LKB 8100. The fractions (2 mL) collected after the electrofocusing were analysed for enzyme activity and pH.

G-200 and electrophoretic separation on polyacrylamide gel. Permeation chromatography on Sephadex G-200 gave a single protein peak which coincided with the activity peak. The enzyme activity across this region was constant (Fig. 2). PAGE of the native enzyme also yielded a single band which reacted to specific enzyme staining positively. Further, on isoelectric focusing, the enzyme displayed a single region of activity corresponding to a pI of 5.0.

Among the purification procedures, affinity chromatography involving Con A-Sepharose and saccharo-1,4-lactone bound Sepharose was found to be the most effective in eliminating contaminating proteins and increasing the purity of the enzyme to higher magnitude. Affinity chromatography on substrate/inhibitor bound Sepharose

Table 2—K_m and V_{max} values of Bombay duck β-glucuronidase[a]

Substrate	K_m (M)	V_{max} (μmoles/mg protein/hr)
Phenolphthalein β-D-glucuronide	42×10^{-5}	29.40
p-nitrophenyl β-D-glucuronide	111×10^{-5}	58.80

[a] The values were derived from Lineweaver-Burk plots.

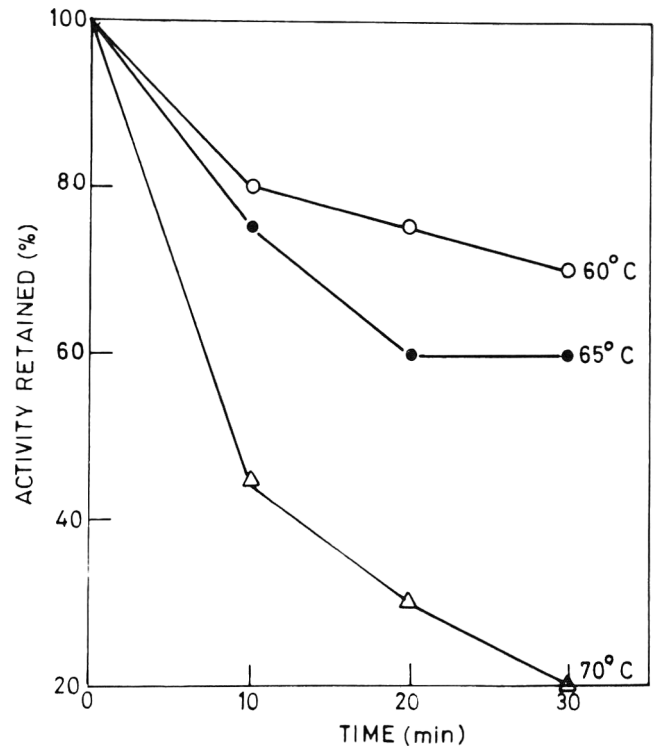


Fig. 5—Effect of thermal treatment on the stability of β-glucuronidase. The enzyme sample was incubated for different intervals of time at stated temperatures following which the residual activity was measured.

(Harris et al., 1972) or antibody bound Sepharose (Owens and Stahl, 1976; Alvares and Balasubramaniam, 1982) have been successfully used in the purification of β-glucuronidase from mammalian sources. In the present investigation, while the substrate bound affinity chromatography employing o-aminophenyl β-glucuronide as a ligand failed (results not shown), inhibitor bound affinity chromatography yielded 2400-fold purification of the enzyme.

The molecular weight of Bombay duck β-glucuronidase was 160,000 as estimated by gel filtration on calibrated Sephadex G-200 column. However, on SDS-PAGE the enzyme showed a single band and migrated to a region corresponding to a molecular weight of 78,000 ± 2000 indicating that the enzyme was a dimer. This would imply that fish β-glucuronidase is a polypeptide of 80,000 consisting of two identical subunits. There are many reports in the literature on the multiple forms of β-glucuronidase that behave distinctly differently on separation by ion exchange resins (Tulsiani et al., 1975), isoelectric focusing and SDS-PAGE (Owens et al., 1975; Glaser et al., 1975). Owens et al. (1975) have demonstrated the existence of multiple forms of β-glucuronidase in rat liver lysosomes and microsomes using isoelectric focusing on sucrose gradient and polyacrylamide gels. Three major molecular forms of rat

Table 3—Effect of various compounds on β-glucuronidase activity[a]

Addition	Concentration (mM)	Activity retained (%)
Nil	—	100
Hg^{2+}	2	0
Cd^{2+}	2	86
	10	15
Ag^{+}	2	80
	10	5
Cu^{2+}	2	16
	10	0
Zn^{2+}	2	76
	10	60
Mg^{2+}	2	100
	10	100
Mn^{2+}	2	100
	10	140
p-CMB	2	73
	10	0
p-CMB + DTT	2 + 10	100
p-CMB + Mercaptoethanol	2 + 10	96
Saccharo-1,4-lactone	0.001	50
	0.01	15
Sodium tripolyphosphate	2	90
(NaTPP)	20	0
Sodium chloride	2	100
	10	115

[a] The dialyzed enzyme solution treated with the compounds was incubated at 37°C for 30 min and the residual activity assayed. The metalic compounds were chlorides excepting Cu^{2+} and Ag^{+} where sulfate and nitrate were used respectively.

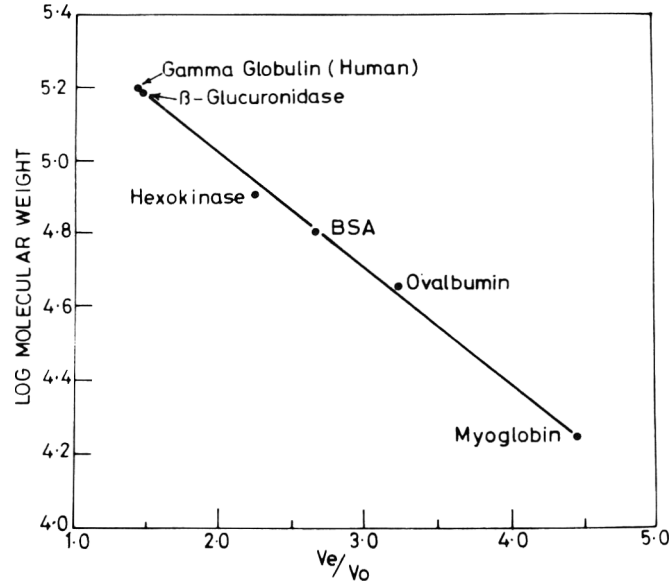


Fig. 6—Determination of molecular weight of Bombay duck muscle β-glucuronidase by Sephadex G-200 gel filtration. The marker proteins are indicated in the Figure.

Table 4—Amino acid composition of Bombay duck β-glucuronidase[a]

Amino acid	Moles/mole	Amino acid	Moles/mole
Aspartic acid	178.40	Methionine	11.15
Threonine	85.90	Isoleucine	69.43
Serine	98.71	Leucine	119.35
Glutamic acid	162.54	Tyrosine	14.90
Proline	38.24	Phenylalanine	57.43
Glycine	109.12	Lysine	55.20
Alanine	92.31	Histidine	30.10
Cysteine	Trace	Arginine	33.44

[a] Hexosamine (total) was 0.05 μmole/mg protein. Cysteine was found in trace quantities and therefore the amount was not calculated. The values are averages of duplicate determinations. Insufficient material precluded carrying out hydrolyses for various times.

preputial gland β-glucuronidase have been identified by chromatography on hydroxylapatite having very similar amino acid composition and kinetic properties but varying in their carbohydrate content (Tulsiani et al., 1975). The enzyme from marine mollusk reportedly exists in two molecular forms with molecular weight of about 250,000 (Diez and Cabezas, 1979). β-Glucuronidases from different sources such as rat liver lysosomes and microsomes, preputial gland and kidney are believed to be tetramers having molecular weights in the range of 240,000 - 320,000 (Stahl and Touster, 1971; Owens and Stahl, 1976; Keller and Touster, 1975; Lin et al., 1975). Bombay duck β-glucuronidase displayed a pI of 5.0. In the case of bovine liver β-glucuronidase the reported pI is between 4.9 and 5.2 (Himeno et al., 1974). Rat liver lysosomal and microsomal β-glucuronidase had different isoelectric points (Himeno et al., 1974). However, they exhibited several similarities in terms of pH optimum, molecular weight and Km values.

β-Glucuronidase from various sources has been shown to display double pH optima. Stahl and Touster (1971) have observed double pH optima of rat liver β-glucuronidase which disappeared at high salt concentrations. Levy et al. (1958) reported double pH optima for rat preputial gland β-glucuronidase with phenolphthalein glucuronide as the substrate and a single pH optimum with phenyl glucuronide. In contrast, Hetu and Gianetto (1970) showed that rat liver lysosomal β-glucuronidase exhibited a single pH optimum when tested with several glucuronides. In the case of Bombay duck β-glucuronidase, a single pH optimum was also obtained with both phenolphthalein glucuronide and nitrophenyl glucuronide. However, the values of pH optimum were not the same with the two substrates.

The high temperature optima and heat stability seem to be characteristic property of hydrolases associated with fish muscle (Doke et al., 1980). Although β-glucuronidase is routinely assayed at 37°C (Ohtsuka and Wakabayashi, 1970), a temperature of 56°C was also used for assaying the enzyme from various sources (Tomino and Paigen, 1975; Smith and Ganschow, 1978). The temperature stability recorded in the case of fish β-glucuronidase is in accordance with the reported observation (Lin et al., 1975) that mouse kidney β-glucuronidase is stable up to 10 min at 65°C. Also, among the two forms of mollusk β-glucuronidase one form remained unaffected up to 90 min at 60°C while the other form was completely inactivated (Diez and Cabezas, 1979). In addition to the comparative heat stability the Bombay duck enzyme was stable at cold storage with little or no effect on its activity.

No striking difference from the established inhibition pattern was observed with the fish enzyme. The potent competitive inhibitor of β-glucuronidase, saccharo-1,4-lactone, rendered the fish enzyme totally inactive at low concentrations. Similarly, heavy metals like Hg^{2+} and Ag^{+} inhibited the enzyme presumably by their interaction with the sulfhydryl groups located in the active site (Fernley, 1962). The observed inactivation of the enzyme on exposure to gamma radiation and protection offered by the addition of sulfydryl compounds lend further support to the likely presence of -SH group in the active site. Interestingly the importance of carboxyl and histidine groups in the elaboration of β-glucuronidase activity has also been proposed by Wang and Touster (1972).

Classically β-glucuronidase has been established as a glycoprotein enzyme (Himeno et al., 1974). Bombay duck

enzyme also exhibited glycoprotein nature since it adsorbed on Con A Sepharose and contained 1% hexosamine. The amino acid composition of fish β-glucuronidase compared favorably with that reported for the enzyme from other sources such as preputial gland (Ohtsuka and Wakabayashi, 1970), bovine (Himeno et al., 1974) and mollusk liver (Diez and Cabezas, 1979). Higher dicarboxylic amino acids and low cysteine have been the feature of the amino acid composition of β-glucuronidase.

Several hydrolytic enzymes including proteases (Doke et al., 1980) and β-glucuronidase (Warrier et al., 1982) have been recognized in the spoilage of stored fishery products and the need for arresting their activity for extending the shelf-life has been emphasized. In view of this, several methods such as dip treatment in NaCl and NaTPP as well as combination processes involving heat treatment have been developed to arrest the enzyme activity (Doke et al., 1980; Ninjoor et al., 1981). Among these, addition of NaCl and NaTPP which are commonly employed in fish processing for increasing water holding capacity (Kumta and Gore, 1970) has been found to be successful in inhibiting the activity of lysosomal hydrolases (Ninjoor et al., 1981). In the present study, Bombay duck β-glucuronidase responded differently towards these compounds. While the enzyme was slightly stimulated by NaCl, the addition of NaTPP was highly inhibitory at the concentration routinely used in commercial fish processing. Although the mechanism of inhibition is not known, the present observation on the NaTPP induced inhibition of β-glucuronidase should stimulate further work on developing suitable processing parameters for the preservation of Bombay duck.

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Modeling of Microbial Growth in Refrigerated Packaged Beef

M. C. ZAMORA and N. E. ZARITZKY

ABSTRACT

The effects of gaseous permeability of the film and storage temperature (0° and 4°C) on the microflora of refrigerated beef were analyzed; total aerobic counts, anaerobic counts, *Pseudomonas* sp., *Brochothrix thermosphacta*, *Lactobacillus* sp., *Enterobacteriaceae* and yeasts were determined as functions of time. Storage periods to reach a total count of 10^7 CFU/cm^2 were established for different conditions. Microbial growth was modeled by mathematical equations in terms of initial contamination, growth rate and length of the lag phase for each component of the mixed flora.

INTRODUCTION

MICROBIAL SPOILAGE is delayed, but not prevented, by storage of meat at temperatures between −1°C and 5°C (Gill and Newton, 1978). On meat stored aerobically at cooling temperatures, the flora is usually dominated by species of *Pseudomonas* (Ayres, 1960). When meat is vacuum packaged, the contaminating flora is exposed to an atmosphere containing 20–25% carbon dioxide and less than 1% oxygen (Grau, 1981). High carbon dioxide and low oxygen tension depress the growth of pseudomonads. Ingram (1962) and Johnson (1974), reported that the remaining oxygen in vacuum packages was converted to carbon dioxide by meat tissue respiration and bacterial activity. Anaerobic and facultative anaerobic strains such as *Lactobacillus*, *Brochothrix thermosphacta*, (previously termed *Microbacterium thermosphactum*, Sneath and Jones, 1976) and *Enterobacteriaceae*, constituted the dominant microbial flora (Sutherland et al., 1975; Seideman et al., 1976; Gill and Newton, 1978; Grau, 1978). However, several reports (Ingram, 1962; Pierson et al., 1970; Beebe et al., 1976; Christopher et al., 1979) showed that lactic acid bacteria became dominant in refrigerated vacuum-packaged beef.

There are various shelf life estimations of vacuum packaged primal cuts of beef stored at 0°C in bags of low gaseous permeability, ranging from 5–15 wk (Fournaud et al., 1973, Sutherland et al., 1975; Beebe et al., 1976; Newton and Rigg, 1979). The results of Egan and Shay (1982), suggested a shelf life of about 4–5 wk at 5°C. These values could be extrapolated to about 10–12 wk for large cuts (lower surface to volume ratio) at 0°C.

The purposes of this study were: to analyze the microbial growth on refrigerated beef, packaged in plastic films of different gaseous permeability and represent the results by mathematical equations that would permit the estimation of the time necessary to reach a bacterial density of 10^7 CFU/cm^2 at each storage condition.

MATERIALS & METHODS

STORAGE EXPERIMENTS with packaged refrigerated beef were performed at two temperature levels (0 ± 0.3°C and 4 ± 0.5°C), using two types of plastic films with different degrees of permeability to oxygen: low density polyethylene, 60 μm thick (water vapor permeability WVP = 12g vapor/m^2 day at 30°C and RH = 78%, oxygen transmission rate OTR = 6500 cm^3/m^2 atm day at 23°C), and for vacuum packaging a composite EVA/SARAN/EVA coextruded film, 60 μm thick, (trade name Super Cryovac, WVP = 7.2 g/m^2 day at 30°C and RH = 78%, OTR = 37.5 cm^3/m^2 atm day at 25°C and RH = 75%). SARAN is a polyvinyl and polyvinylidene chloride copolymer and EVA is ethylene vinyl acetate copolymer.

Oxygen transmission rate of the composite film decreases at low temperatures, following an Arrhenius relationship (Eustace, 1981); the OTR of these films at 3°C was generally less than 10% of the value at 25°C.

The beef samples were obtained from semitendinosus muscles removed from steers classified as U_2 grade A according to the Argentine National Meat Board Classification (carcass weight of 240 kg) with a postmortem time of 48 hr at 4°C. The muscles were cut in slices, transversally to the fibers, 1.5 cm thick and 10 cm in diameter (aprox. weight 117 g). Samples were packaged under vacuum using Minidual equipment with a single chamber at 4.5 mm Hg and heat sealing. The degree of vacuum inside the packages was measured according to the method of Seideman et al. (1976). The packaged samples were placed inside an anaerobic jar (Oxoid) which was gradually evacuated, determining by manometric reading the pressure at which the film was detached from the meat. The average pressure of the internal atmosphere in the packages was 40 mm Hg.

Heat shrinkage of the low permeability film was performed by dipping the packages in a water bath at 84°C for 1 sec.

The temperature of the storage cabinets was recorded by means of thermocouples, inserted within the beef slices, connected to a Data Logger Fluke, 2240-C; a meat electrode INGOLD LOT 405-M4 was used for pH determinations.

The storage experiments were carried out with at least four muscles from different animals to observe the influence of initial contamination, pH and biological variability.

Microbiological evaluation of the slices was performed by cutting a ribbon (20 cm^2 of surface and approximately 0.3 cm thick) along the lateral area of the muscle. The sample was then placed in 180 mL sterile 0.1% peptone broth, shaken 15 min at 250 rpm and at 30°C; appropriate dilutions were made with sterile 0.1% peptone broth.

Total aerobic counts were made on plate count agar (Oxoid) with incubation at 30°C for 2 days. Anaerobic counts were obtained on plate count agar (Oxoid) with incubation for 3 days at 30°C in anaerobic jars (Oxoid). For enumeration of *Pseudomonas* sp., Masurovsky agar (Noskowa, 1972) was used; Pseudomonas agar F (Difco) was employed to determine fluorescent pseudomonads with incubation at 30°C for 2 days. Lactobacilli were counted on MRS agar (Oxoid) following incubation at 30°C for 3 days. For the detection of *B. thermosphacta*, STAA agar (Gardner, 1966) was used, and the plates were incubated at 22°C for 2 days. Yeast counts were made on Wort agar (Oxoid) following incubation at 30°C for 2 days. *Enterobacteriaceae* counts were made on Violet Red Bile Dextrose agar (Oxoid) following incubation at 30°C for 2 days (Mossel et al., 1975). All the results were expressed as log CFU/cm^2 (CFU = colony forming units).

Storage periods were prolonged until the total microbial counts, incubated in aerobiosis, reached about 7.00 (log CFU/cm^2). Statistical analysis were performed on log counts of individual samples and reported data represent the mean of four experiments with different animals. To determine the distribution of the microflora, 20–30 colonies were picked at the beginning and during the storage period from countable plates used for the total aerobic counts; one plate of each selective medium was also retained at each sampling period. Diagnostic schemes and procedures used for identification of isolates were those presented by Vanderzant and Nickelson

Authors Zamora and Zaritzky are affiliated with Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA)–(CONICET–CIC–UNLP), Facultad de Ciencias Exactas, 47 y 116 La Plata (1900), Provincia de Buenos Aires, Argentina.

(1969). Standard biochemical tests were supplemented with Enteric 20 strips (Inolex) for *Enterobacteriaceae* identification.

The influence of heat-shrinking film on initial microbial counts was analyzed using sections from the same muscle packaged in EVA-SARAN-EVA; half of these samples were submitted to heat shrinkage and the other half were not treated.

The colorimetric measurements of the meat samples were carried out using a surface colorimeter (Hunter Lab D25-A3).

A Gas Chromatograph Shimadzu GC-6A with a thermal conductivity detector and a data processor Chromatopac C-R 1A were used to determine the gaseous microatmosphere composition inside the packages. The technique used by Karel et al. (1963) was modified by connecting a silica gel column (30–60 M) of 1m length, which operated at 115°C within the oven of the gas chromatograph and a molecular sieve column 5A (30–60 M) 2m length, placed on the outside of the equipment, which operated at 0°C. The flow of H_2 was maintained at 40 cm^3/min. A Precision Sampling A-2 Syringe with maximum volume of 25 μL and a retention valve was used for gaseous injections. Under the described experimental conditions the average retention times for the different tested gases were: CO_2 = 0.82 min; N_2 = 3.48 min; O_2 = 1.32 min.

RESULTS & DISCUSSION

THE MICROBIAL GROWTH in a mixed flora during the exponential phase can be represented by:

$$\frac{N}{N_o} = \sum_{i=1}^{n} x_{io}\, e^{\mu_i(t - \theta_i)} \qquad (1)$$

where: N = number of total CFU/cm^2 at a time t; N_o = initial number of total CFU/cm^2; i = 1, . . .n, components of the microbial population; x_{io} = initial fraction of i in the total flora ($x_{io} = N_{io}/N_o$); μ_i = growth rate of i, during exponential phase (1/day); θ_i = lag time of component i (days).

Eq. (1) estimates the number of microorganisms growing on beef as a function of storage time. Initial composition of the mixed flora (x_{io}), μ_i and θ_i values for each component were obtained from the performed experiments. The growth rate and lag time for each microorganism were functions of temperature, pH of the meat cut, and permeability of the packaging film.

Growth rate and lag time under different storage conditions

In polyethylene bags at 4°C the total bacterial counts rapidly reached values of 7.0 (log CFU/cm^2), with a very short lag phase. The range of initial pH in the samples was 5.5–5.7. After 8 days of storage all samples showed counts that exceeded 10^7, and spoilage signs such as odors and a brown color caused by metmyoglobin formation; pH values showed a slight increase of 0.15 unit. Initially, *Pseudomonas* sp., *Lactobacillus* sp. and *Brochothrix thermosphacta* constituted approximately 90% of total bacterial population, but at the end of the storage period *Pseudomonas* species predominated; fluorescent pseudomonads constituted only a small fraction of the microflora.

The influence of temperature was noticeable in polyethylene at 0°C, showing an increase in the lag phase and a decrease in the growth rate. The storage time doubled when temperature decreased from 4° to 0°C for meat of pH < 5.9. The final percentage of *Pseudomonas* sp. at 0°C was slightly greater to that at 4°C. These findings agree with the results of Gill and Newton (1977, 1978), who established that in meat stored under aerobic conditions, *Pseudomonas* species had a marked advantage in growth rate compared to other genera and that this advantage tended to increase at decreasing temperature.

In vacuum packaging at 4°C and pH < 5.9 an increase in the lag phase and a decrease in growth rate were observed. The storage period was extended for approximately 14 days, without showing any type of alteration. At the end of storage, *Lactobacillus* sp. represented approximately 90% of the microflora, and their counts were similar to those corresponding to total anaerobic counts.

At 0°C, the longest periods of storage were obtained in vacuum packaging, resulting from the combination of low temperature and low film permeability. The pH values showed a slight decrease of 0.10–0.15 unit during storage time. *Enterobacteriaceae* and *B. thermosphacta* grew during the first days but their growth stopped when the lactobacilli population constituted the dominant flora according to the results of Roth and Clark (1975).

The parameters μ_i, θ_i of Eq. (1) and the corresponding standard deviations $\sigma_{\mu i}$, $\sigma_{\theta i}$ were determined from the linear regression of experimental values expressed as ln Ni vs time. The values obtained for each microorganism on meat cuts of pH < 5.9, and total initial counts between $10^{3.9}$ and $10^{4.4}$ (CFU/cm^2) are shown in Tables 1 and 2 for aerobic and vacuum packaging conditions, respectively, together with values of the time necessary to reach the stationary phase of growth.

Table 1—Growth rate and lag time of microorganisms under aerobic conditions (Polyethylene)

	0°C					4°C				
Microorganisms	Growth rate μ(1/day)	σ_μ	Lag time θ_i(days)	σ_{θ_i}	Stationary phase (days)	Growth rate μ(1/day)	σ_μ	Lag time θ_i(days)	σ_{θ_i}	Stationary phase (days)
Pseudomonas sp.	0.795	0.055	5.0	0.6	18	1.070	0.025	0.3	0.2	> 7
B. thermosphacta	0.724	0.058	5.0	0.7	16	1.000	0.063	0.4	0.2	> 7
Lactobacillus sp.	0.450	0.018	3.0	0.4	12	0.805	0.078	0.4	0.2	4
Enterobacteriaceae	0.650	0.018	2.5	0.4	10	0.900	0.020	0.4	0.2	3
Yeast	0.450	0.028	3.0	0.6	10	0.750	0.105	0.5	0.3	4

Table 2—Growth rate and lag time of microorganisms under vacuum packaged beef (EVA-SARAN-EVA)

	0°C					4°C				
Microorganisms	Growth rate μ(1/day)	σ_μ	Lag time θ_i(days)	σ_{θ_i}	Stationary phase (days)	Growth rate μ(1/day)	σ_μ	Lag time θ_i(days)	σ_{θ_i}	Stationary phase (days)
Pseudomonas sp.	0.131	0.008	14.0	1.1	35	0.53	0.02	5.5	0.7	12.0
B. thermosphacta	0.264	0.017	12.0	0.6	24	0.60	0.03	3.5	0.5	10.0
Lactobacillus sp.	0.310	0.008	12.0	0.8	40	0.75	0.01	3.8	0.2	16.0
Enterobacteriaceae	0.258	0.010	9.0	0.9	33	0.62	0.02	2.5	0.3	12.0
Yeast	0.133	0.009	12.0	0.5	22	0.35	0.02	2.0	0.7	14.0

The estimation of σ_{θ_i} was obtained following the Inverse Regression analysis by Draper and Smith (1981); the "fiducial limits" of θ_i were determined by the intersection of the 95% confidence interval loci about the regression line, with the horizontal straight line of the lag phase of growth.

Experiments with muscles of pH = 6.10 at T = 0°C in vacuum packaging showed a different behavior pattern. In this case, *B. thermosphacta* grew rapidly (θ_i = 8 days) and were the predominant bacteria during the storage period. By comparing the counts obtained after 28 days of storage in samples at pH = 5.6 and pH = 6.10, all microorganisms reached higher levels when the pH increased; *B. thermosphacta* showed a faster growing rate (μ = 0.30/day), while *Lactobacillus* sp. was the least affected (μ = 0.32/day). Thus, the growth of *B. thermosphacta* was conditioned by the pH of the meat and the gaseous permeability of the film. These findings agree with studies of Campbell et al. (1979), who solved conflicting observations concerning the growth of that bacterium (Pierson et al., 1970; Roth and Clark, 1972; Sutherland et al., 1975; Seideman et al., 1976). Grau (1980), indicated that undissociated lactic acid was the effective inhibitor of *B. thermosphacta*. Moreover, a high number of lactobacilli inhibited the growth of other species in meat, (Newton and Gill, 1978) apparently due to the production of specific antibiotic substances produced by such bacteria (Gill and Newton, 1978; Smith and Palumbo, 1981). These substances have been shown to be more active at low pH (Collins-Thompson and Rodríguez López, 1982).

During the experiments with low permeability films, the gaseous composition inside the packages was measured to verify the degree of vacuum. The chromatograms showed a rapid evolution of CO_2; from an initial composition of 0.18% CO_2, concentration values were higher than 6% after 6 hr of being packaged. During a storage period of 5 days at 4°C the concentration of CO_2 increased to 18%.

Initial composition of microbial flora

Experimental mean values of initial composition of microbial flora (x_{io}), can be observed in Table 3. These values were obtained from the individual and total microbial counts and the simultaneous application of the identification schemes described above. Standard errors of microbial counts ($\sigma_{\log N}$) are also shown in the same table.

Initial microbial counts in vacuum packaged samples that had been submitted to heat shrinkage were compared to nontreated packages; heat shrinking had no significant effect on those counts ($P > 0.05$).

Table 3—Initial distribution of microbial flora in beef

Microbial type	x_{io}	Standard error $\sigma_{\log N}$
Pseudomonas sp.	0.350	0.075
Lactobacillus sp.	0.300	0.064
B. thermosphacta	0.240	0.061
Yeast	0.060	0.050
Enterobacteriaceae	0.050	0.035

Error analysis of the model

Propagation of errors in Eq. (1) permitted the estimation of the variance of log N (Himmelblau, 1970).

The function:

$$y = \log N = f(N_{io}, \mu_i, \theta_i)$$

was first linearized expanding in a Taylor series around a reference state indicated by the superscript (*). By propagation of errors the following result was obtained:

$$\sigma_y^2 = \frac{\alpha}{(N^*)^2} \sum_{i=1}^{n} \left[\left(\frac{\partial f^*}{\partial N_{io}}\right)^2 \sigma_{N_{io}}^2 + \left(\frac{\partial f^*}{\partial \mu_i}\right)^2 \sigma_{\mu_i}^2 + \left(\frac{\partial f^*}{\partial \theta_i}\right)^2 \sigma_{\theta_i}^2 \right] \quad (2)$$

where $\alpha = (\log e)^2 = (0.4343)^2$.

The reference state was selected as that corresponding to half of the storage time for each temperature and packaging condition. The final expression for the calculation of the propagated standard error was:

$$\sigma_{\log N} = \frac{0.4343}{N^*} \left[\sum_{i=1}^{n} N_i^{*2} \left(\frac{\sigma_{N_{io}}^2}{N_{io}^2} + (t^* - \theta)^2 \sigma_{\mu i}^2 + \mu_i^2 \sigma_{\theta_i}^2 \right) \right]^{1/2} \quad (3)$$

By considering the log normal distribution of microbial population $\sigma_{N_{io}}^2$ was calculated as follows:

$$\sigma_{N_{io}}^2 = \frac{\sigma_{\log N_{io}}^2 \cdot N_{io}^2}{(\log e)^2} \quad (4)$$

where $\sigma_{\log N_{io}}$ was obtained from Table 3.

Eq. (1) allowed for the prediction of microbial counts by using the information in Tables 1, 2, 3. Results that were evaluated at approximately half the storage period for each condition are shown in Table 4. Predicted values were compared to experimental data of total bacterial counts ($\log N_{exp}$) recovered on plate count agar (PCA); there was a low percentage difference between these results. The estimated standard error of the model ($\sigma_{\log N}$) was calculated from Eq. (3) and is also shown in Table 4.

Applications of the model

Vacuum aging periods have been recommended for beef (Larmond et al., 1969; McDougall, 1971; Effenberg and Schotte, 1972; de Man et al., 1976), based, in most cases, only on its tenderness, without considering microbial growth, which is the principal factor that limits the aging period.

Taking into account the studies relating to tenderness, and establishing 10^5 CFU/cm^2 as a limit of microbial counts in beef destined for direct commercialization or freezing processes, the application of the model leads to recommended vacuum aging periods of 14 days at 0°C and 6 days at 4°C. In both cases the pH must be lower than 6.0 and the initial cell density approximately 10^4 CFU/cm^2

Table 4—Comparison of predicted microbial population and total experimental microbial counts

Packaging film	Temp (°C)	Days of storage	Predicted values of log N Eq. (1)	$\sigma_{\log N}$ (Eq. 3)	Total microbial counts $\log N_{exp}$	% difference in log N
Polyethylene	0	8.0	5.34	0.09	5.40	1.11
Polyethylene	4	3.5	5.78	0.09	5.80	0.34
EVA-SARAN-EVA	0	20.0	5.17	0 06	5.20	0.58
EVA-SARAN-EVA	4	8.0	5.41	0 07	5.43	0.37

Table 5—Time necessary to reach a cell density of 10^7 CFU/cm^2 (t_{10^7}) (Initial contamination 10^4 CFU/cm^2 and pH < 6.0)

Storage conditions	t_{10^7} (days)
EVA-SARAN-EVA 0°C	38
EVA-SARAN-EVA 4°C	14
Polyethylene 0°C	14
Polyethylene 4°C	7

Table 6—Influence of gaseous film permeability on the color of packaged beef (Hunterlab tristimulus colorimetry)

	L	a	b
Initial	39.74	13.43	9.95
14 days storage in Polyethylene @ 0°C	42.10	9.36	9.74
14 days storage in EVA-SARAN-EVA @ 4°C			
Before reoxygenation	42.80	13.64	11.00
After 50 min of reoxygenation	38.16	20.35	12.35

Table 7—Percentage distribution of microbial flora on packaged beef at time t_{10^7}

	Vacuum		Polyethylene	
Microbial type	0°C 38 days	4°C 14 days	0°C 14 days	4°C 7 days
Pseudomonas sp.	1.3	1.9	69.2	67.6
Lactobacillus sp.	91.1	92.3	3.5	1.8
B. thermosphacta	1.4	2.1	26.1	29.5
Yeast	0.2	0.6	0.2	0.2
Enterobacteriaceae	5.9	3.1	1.0	0.1

or lower. If aging is carried out using high gaseous permeability films, the specified times would then exceed the established limits of contamination.

Microbial flora composition and shelf life predictions. The time necessary to reach 10^7 CFU/cm^2 (t_{10^7}) determinated the shelf life of beef in aerobic storage, but in vacuum packaging, total counts did not have the same significance as a measure of spoilage (Nottingham, 1982) that can only be detected several weeks after the bacteria have reached their maximum density (Gill, 1982).

Table 5 shows values of t_{10^7} corresponding to beef samples with initial contamination levels of 10^4 CFU/cm^2 and pH < 6.0.

Johnson (1974) showed that in properly processed vacuum-packaged beef stored at 0°C, the bacterial counts reach maximum numbers of 10^7 (CFU/cm^2) during the fifth week of storage. Seideman et al. (1976) found that counts for psychrotrophs, mesophiles and lactobacilli of vacuum-packaged beef were $10^{6.6}$ – $10^{7.1}$ per in^2 after 35 days at 1-3°C.

From a point of view of the microbial count the t_{10^7} of beef in polyethylene at 0°C and in EVA/SARAN/EVA at 4°C are the same (Table 5), but considering the appearance of the product, which was evaluated in terms of the redness chromaticity parameter "a", a better color was obtained with the lower permeability film, after reoxygenation (Table 6).

Percentage distribution of microbial flora was calculated at t_{10^7} (Table 7), using Eq. (1). These values were experimentally confirmed through the application of the identification schemes showing a good correlation between both results.

Thus, it can be concluded that the proposed model permitted a satisfactory prediction of microbial growth in packaged refrigerated beef in storage conditions that are common in the meat industry.

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Irradiation of Meat for the Production of Fermented Sausage

J. S. DICKSON and R. B. MAXCY

ABSTRACT

Fermented sausage was prepared from batter which had been processed with gamma radiation to reduce the microflora. Doses up to 500 Krad reduced the total aerobic bacteria in commercial batter up to 2.2 log cycles. Irradiation reduced the coliform and staphylococci counts to levels that minimized public health concern after fermentation. These reductions allowed the use of a lower inoculum and a longer fermentation. The reductions shifted the percent inoculum relative to the total population from 1% in the controls to 70% in the 500 Krad samples, and the shift in population resulted in a more uniform fermentation and final product.

INTRODUCTION

LOW DOSE gamma irradiation has a major impact on the microflora of foods. Most of the vegetative bacteria, including pathogens, are eliminated by a dose of 500 Krad (Maxcy, 1982). Furthermore, the post-irradiated microflora is quite different in composition from the pre-irradiated flora (Ingram and Farkas, 1977). Therefore, the nature and evolution of the spoilage flora may differ from that of the usual raw meats (Maxcy, 1983).

Wolin et al. (1957) reported that the spoilage flora of irradiated beef held at refrigeration temperatures consisted primarily of lactic acid bacteria, in contrast to that of fresh meat, which consisted primarily of gram-negative psychrotrophs. Tiwari and Maxcy (1971) reported similar results with irradiated, refrigerated ground beef, even though ground meat generally has a more diversified flora than fresh raw meat. The flora was predominantly gram-positive bacilli, which were probably lactobacilli (Urbain, 1983). Vacuum-packaged fish (Pelroy and Eklund, 1966) and beef (Niemand et al., 1981) reflected the same radiation-induced shift in spoilage microflora during refrigerated storage. The combined selective effects of irradiation and refrigeration caused the lactobacilli to dominate the flora. While there were bacteria which were more resistant to radiation than the lactobacilli (Tiwari and Maxcy, 1971), they were unable to compete at the low temperatures.

Irradiation has a potential use in the production of fermented sausage, which relies on the dominance of lactic acid bacteria in the microflora to produce the desired flavor and inhibit the growth of pathogens (Smith and Palumbo, 1983). While a massive inoculum is used to attain dominance over the inherent flora, irradiation of the sausage batter might increase the assurance of dominance and reduce the necessary inoculum level. Furthermore, irradiation would ensure the safety of the product by eliminating pathogenic bacteria in the vegetative state (Urbain, 1983) and would be similar in principle to pasteurizing milk prior to cheese making. The germination and/or outgrowth of any toxigenic spores would be prevented by the nitrites present in the batter.

The purpose of this work was to determine the potential of irradiation for reduction of pathogens and control of the process in production of fermented sausage.

Authors Dickson and Maxcy are affiliated with the Dept. of Food Science & Technology, Univ. of Nebraska-Lincoln, Lincoln, NE 68583-0919.

MATERIALS & METHODS

Enumeration of bacteria

Media and conditions of incubation are listed in Table 1. For certain selective enumerations, nutrient agar and Baird-Parker agar were used with the surface plating technique (AOAC, 1980). All other media were used with the standard pour plate count procedure (APHA, 1978).

Starter culture

Pediococcus cerevisiae was propagated by multiple transfers in m-Plate Count Broth (PCB, Difco) at 37°C. The inoculum was harvested by centrifugation (9000 x *g*) when the cells were in late logrithmic growth phase, with a cell density of approximately 7.5 x 10^6 cfu/mL. The pellets were resuspended in PCB and diluted in phosphate buffer (APHA, 1978) to attain the appropriate inoculum in the batter.

Sources of meat

A commercial meat batter for summer sausage containing all ingredients except the starter culture was obtained from a local packing house. A second batter was prepared in 1 kg quantities, using meat from both commercial and University sources with the final composition given in Table 2.

Production of sausage

Commercial batter was vacuum-packaged with a Busch Multivac AG 500 (Maulburg, GFR) with the pressure reduced to approximately 3-4 Torr, irradiated at different dose levels, and 100-g portions were aseptically transferred to sterile 500 ml beakers. The procedure was similar to the laboratory scale sausage production used by Daly et al. (1973). The irradiated batters were inoculated with approximately 1.8 x 10^4 cfu/g and allowed to ferment at 37°C for 24 hr. Samples were plated for total aerobic bacteria, coliforms, lactic acid bacteria and staphylococci before as well as after irradiation, and after fermentation. The pH of the samples was monitored every 8 hr by mixing 5g meat with 25 mL distilled, de-

Table 1—Media and conditions of incubation for enumeration of bacteria

Bacteria	Medium	Incubation Time (hr)	Incubation Temp (°C)
Total aerobic bacteria	Plate count agar (PCA; Difco)	48	32
Coliforms	Violet red bile agar (VRBA; Difco)	24	32
Lactic acid bacteria	LBS (BBL)	72	32
Staphylococci	Baird-Parker agar with egg yolk-tellurite supplement (B-P; Difco)	18	37
Aerobic, non-lactic bacteria	Nutrient agar (NA; Difco)	48	32

Table 2—Composition of sausage batter

Ingredient	Quantity (kg)
Beef (20:80, fat:lean)	9.0
Pork trim (20:80 fat:lean)	13.6
Sodium chloride	0.75
Water	0.5
Glucose	0.5
Sodium ascorbate	0.014
Sodium nitrite	0.00375
Seasonings	0.133
Total	24.5

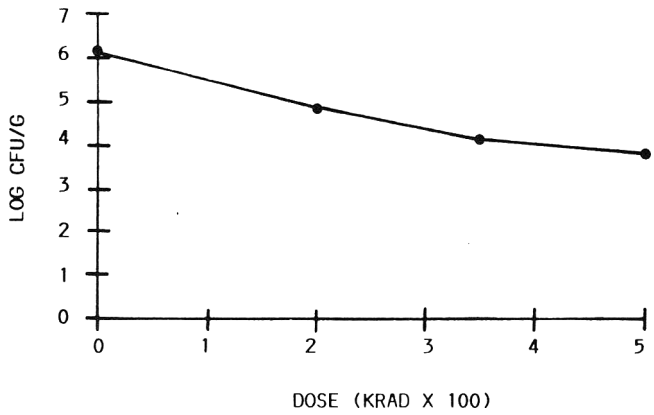


Fig. 1—Reduction of total aerobic bacteria counts by irradiation of commercial batter.

gassed water in a Stomacher 400 (Dynatech Laboratories, Inc., Alexandira, VA.) for 30 sec.

The meat batter prepared in the laboratory was irradiated in 500-g samples, inoculated with starter culture to approximately 10^6 cfu/g and allowed to ferment as outlined above. Samples were plated every 3 hr for total aerobic bacteria, aerobic nonlactic bacteria, coliforms, lactic acid bacteria and staphylococci. The pH was determined every 3 hr as outlined above.

Irradiation

Irradiation was carried out using a Cobalt-60 source similar to the one described by Teeny and Miyauchi (1970), delivering approximately 4 Krad/min. All samples were irradiated at 5°C ± 3°C.

RESULTS & DISCUSSION

Commercial batter

The effect of irradiation on commercial batter was dose dependent, with reductions in total aerobic counts ranging from 1.3 log cycles at 200 Krad to 2.2 log cycles at 500 Krad (Fig. 1, average of 6 reps). While the reduction in population at these dose levels was relatively low, it has been shown that highly radiation-resistant vegetative cells may constitute a considerable part of the inherent flora of some samples of ground meat (Tiwari and Maxcy, 1972). The highly resistant flora, however, is relatively inert in meat (Snyder and Maxcy, 1972). Reduction by irradiation of a more active spoilage flora would be on the order of 3 to 4 log cycles (Tiwari and Maxcy, 1971; Urbain and Giddings, 1972).

An average inoculum (1.8×10^4 cfu/g) was added to the batter after irradiation, and the relative proportions of the inoculum to the total microflora varied from 1% for the control to 70% for the 500 Krad treatment (Table 3). After fermentation, the counts reflected the growth of the starter culture as well as the residual microflora. The final

Table 3—Proportion of inoculum to total flora in irradiated commercial sausage batter

Irradiation dose	Inoculum as percent of total flora
Control (0 Krad)	1.2
200 Krad	19.2
350 Krad	53.3
500 Krad	70.3

total aerobic and lactic acid bacteria counts were approximately the same, with both irradiated and control samples containing approximately 7×10^8 cfu/g.

The average of the initial coliform counts on the non-irradiated samples was approximately 10^4 cfu/g (average of 6 reps). Irradiation with 200 Krad reduced the count to 4.2×10^1 cfu/g. The counts were reduced to <10 cfu/g by irradiation with 350 and 500 Krad. During fermentation there was a four-fold increase in coliform count in the control samples. The samples irradiated with 200 or 350 Krad showed an increase in counts either through growth or by recovery of injured cells. The maximum counts, however, were less than 10^3 cfu/g. This was less than 2.5% of the average count for the non-irradiated control. There was no detectable increase in coliforms in the 500 Krad samples.

Staphylococci were reduced to 1.6×10^2 by 200 Krad from an initial level of approximately 2.4×10^3 cfu/g (Fig. 2). Doses of 350 and 500 Krad reduced the counts to 6.9×10^1 cfu/g and 4.4×10^1 cfu/g, respectively. There were substantial increases in the numbers of these bacteria during fermentation, which demonstrated a major cause of concern in the event of a slow fermentation. The final counts in the control samples were above 10^6 cfu/g, which is the level at which it is generally assumed certain staphylococci pose a public health hazard (Banwart, 1979). The irradiated samples had final counts below this critical level therefore indicating a reduction in hazard with irradiated product.

Laboratory scale batter

An irradiation dose of 500 Krad reduced the initial total aerobic counts in the laboratory scale sausage batter in a manner similar to that reported with the commercial batter (Fig. 3). After inoculation, the total aerobic counts reflected the addition of the starter culture, while the nutrient agar counts remained essentially unchanged. As the fermentation progressed, the total aerobic counts closely paralleled the lactic acid bacteria counts. The population of the aerobic, nonlactic bacteria (indicated by the nutrient agar counts) increased slowly and then began a gradual decline as the pH of the sausage dropped below 5.00 after 18 hr (Fig. 3).

The nutrient agar counts were used to estimate the population of aerobic, nonlactic bacteria. The growth of the somewhat fastidious lactic acid bacteria was slowed, because nutrient agar lacked some growth factors and surface plating further delayed the growth of these microaerophilic bacteria. Some lactic acid bacteria were undoubtably enumerated by this technique and some nonlactics were not, but the counts gave a reasonable estimate of the aerobic, nonlactic population.

The starter culture remained in lag phase for approximately 3 hours in both the control and irradiated samples. Growth rates were approximately the same in both the control and irradiated samples for the duration of the fermentation, with final counts of 10^9 cfu/g. The change in pH was essentially the same for both samples until 15 hr, when the pH of the irradiated sample dropped below that of the control. The final pH values were 4.69 and 4.38 for the control and irradiated samples, respectively.

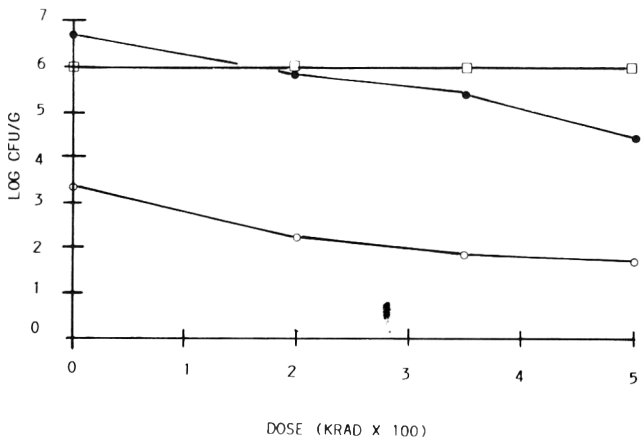


Fig. 2—Increases in staphylococcal counts during fermentation of irradiated commercial batter: ○—○ count before fermentation; ●—● count after fermentation; □—□ count presumed to represent a public health hazard.

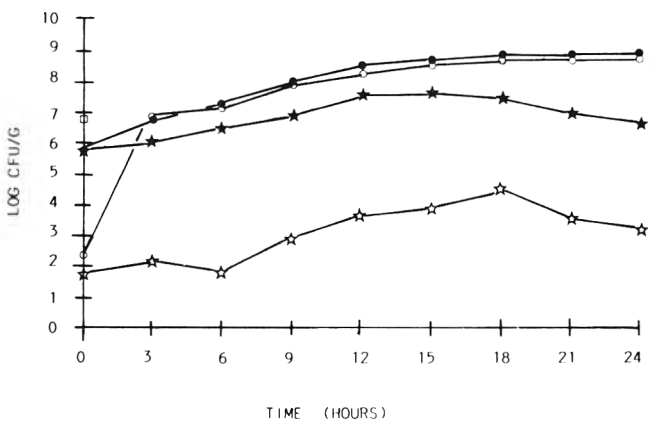


Fig. 3—Growth of total aerobic bacteria and aerobic nonlactic bacteria in irradiated laboratory scale sausage batter: ●—● control, total aerobic; ○—○ 500 Krad, total aerobic; ★—★ control, aerobic nonlactic; ☆—☆ 500 Krad, aerobic nonlactic; □ inoculum level.

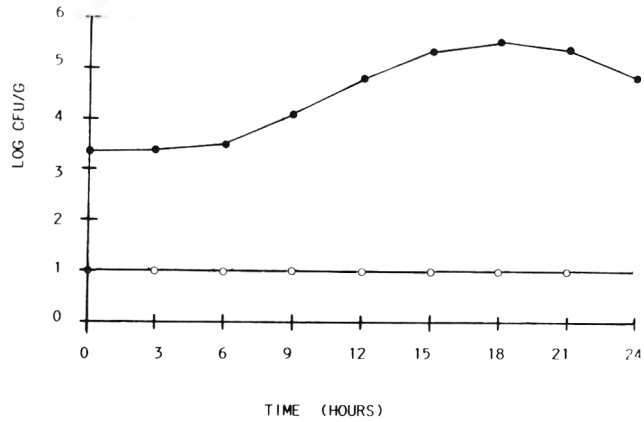


Fig. 4—Coiforms in irridiated laboratory scale sausage batter: ●—● control; ○—○ 500 Krad, no visible growth on any plate.

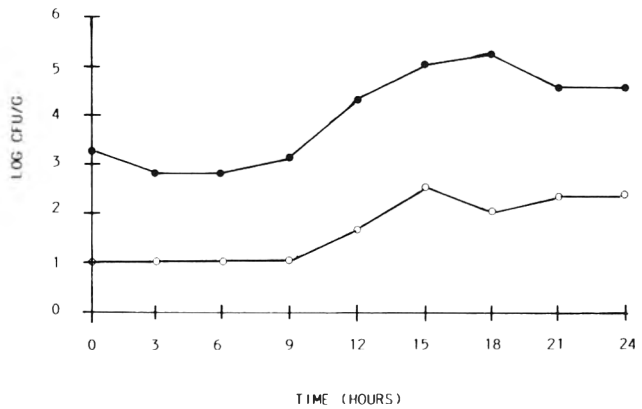


Fig. 5—Growth of staphylococci in irradiated laboratory scale sausage batter: ●—● control; ○—○ 500 Krad.

Irradiation reduced the coliforms in the batter below detectable limits. The initial coliform counts in the control samples were $>10^3$ cfu/g, peaked at 18 hr, and then declined during the rest of the fermentation (Fig. 4). The unfavorable environment of the batter (pH $<$ 5.00 at 18 hr) injured some of the bacteria to the point that they would not grow on selective media.

The population of staphylococci was reduced to undectable levels (reduction $>$ 2 log cycles) by irradiation. However, some growth was found 12 hr into the fermentation, indicating either growth of surviving cells or recovery of injured cells (Fig. 5). The maximum staphylococcal counts for the irradiated samples were less than 0.1% of the counts for the control samples, which were greater than 10^5.

In summary, irradiation lowered the counts of bacteria of public health significance as indicated by reductions in numbers of staphylococci and coliforms. The reductions in these groups of bacteria would be expected to provide a margin of safety under normal processing conditions and in the event of a starter failure. The results of a partial starter failure might be similar to the results of the very low (ca. 10^4 cfu/g) inoculum levels used with commercial batter. The population density of staphylococci and coliforms did not reach levels that indicated a health hazard after 24 hr incubation at 37°C. In addition to the safety benefits, reducing the levels of competing bacteria would produce a more uniform product by allowing better control of the fermentation process. The lower total counts made it possible to use a reduced inoculum level with the laboratory scale batter and still produce an acceptable sausage. The reduced inoculum level combined with increased product uniformity would provide an economic advantage to the processor.

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Effects of Selected Inorganic Phosphates, Phosphate Levels and Reduced Sodium Chloride Levels on Protein Solubility, Stability and pH of Meat Emulsions

C. L. KNIPE, D. G. OLSON, and R. E. RUST

ABSTRACT

The effects were determined of four selected inorganic phosphates at two levels combined with two reduced levels of sodium chloride on the stability, pH, solubilized protein and water-holding capacity of raw meat emulsions. Tetrasodium and tetrapotassium pyrophosphates resulted in higher raw pH, greater protein solubility and improved emulsion stability and water-holding capacity than sodium or potassium tripolyphosphate. Increasing the sodium chloride level from 0.75% to 1.50% decreased the relative effects of phosphate type and level on emulsion stability. The effect of doubling phosphate level on emulsion stability was much less than the effect of doubling the sodium chloride level; however, doubling phosphate level was more effective in increasing pH and soluble protein level of raw emulsions.

INTRODUCTION

SODIUM CHLORIDE has long been recognized as a key ingredient for stabilizing comminuted meat products. However, there has been much concern developing recently over the effects of high sodium intake from our diets (Kolari, 1980). In response to this concern, many attempts have been mace to replace sodium chloride with other salts, such as magnesium chloride and potassium chloride (Olson and Terrell, 1981).

It has been known that inorganic, alkaline sodium phosphates would enhance water-holding capacity (WHC) in ham and bacon (Brotsky and Everson, 1972), but much less was known about the effects of inorganic phosphates, particularly potassium phosphates, in comminuted meat products because the direct addition of inorganic phosphates to comminuted products had not been previously approved by the United States Department of Agriculture (USDA). The was changed in 1982 when the Food Safety and Inspection Service of the USDA approved the direct addition of selected sodium and potassium phosphates during the processing of cooked sausages (Anonymous, 1982).

This new ruling allowed the use of an ingredient that could potentially increase the stability of reduced-sodium meat products while not increasing sodium content, provided that potassium phosphates could substitute for sodium phosphates.

The relative effects of some of the previously approved phosphates have been compared (Shults et al., 1972; Shults and Wierbicki, 1973; Jauregui, 1981). The increase in water-holding capacity (WHC) caused by the addition of alkaline phosphates, with the exception of pyrophosphates, is claimed by some to be due to a nonspecific ionic strength effect (Bendall, 1954). The effect of pyrophosphate on the WHC of meat seems to be greater than would be expected due to ionic strength alone.

The effects of selected sodium phosphates on meat pH and protein solubility and ultimately the WHC and emulsifying capacity of meat have also been studied (Swift and Ellis, 1956; Puolanne and Matikkala, 1980; Sherman, 1961; Fukazawa, et al., 1961; Saffle and Galbreath, 1964; McCready and Cunningham, 1971).

The purpose of our study was to compare the effects of two sodium and two potassium inorganic phosphates on meat emulsion characteristics and to add to the understanding of inorganic phosphate action in meat products.

Authors Knipe, Olson, and Rust are affiliated with the Dept. of Animal Science, Iowa State Univ., Ames, IA 50011.

MATERIALS & METHODS

Formulation

All meat was purchased from a local supplier and processed in the Iowa State University Meat Laboratory. Lean beef trimmings (approximately 90% lean) and regular pork trimmings (approximately 50% lean) were ground through 4.8 mm (3/16") plates on a Weiler & Company Grinder (Model 6) and were mixed separately. The fat content of each meat type was determined by using a 5.9 kg (13.0 lb) sample by the Anyl-Ray method (Kartridg-Pak, Davenport, IA). The meat block was formulated to 34% fat (Table 1). The beef and pork for each treatment were weighed, bagged in polyethylene and stored in a −20°C (−4°F) freezer for a period no longer than 2 months. Twelve hours before processing, the meat was removed from the freezer and tempered in a −1.7°C (29°F) cooler.

The phosphates used included sodium tripolyphosphate and tetrasodium pyrophosphate, potassium tripolyphosphate and tetrapotassium pyrophosphate.

These emulsions also contained 0.0156% (156 ppm) sodium nitrite, 0.055% (550 ppm) sodium erythorbate and 0.5% spice (Table 1). These concentrations were calculated based on the final weight of the emulsion with a projected cooked yield of 93%.

Sample preparation

A meat emulsion was made in a Hobart food chopper (Model 8418ID) in a −1.7°C (29°F) cooler. The chopper bowl was chilled overnight in a −1.7°C (29°F) cooler and was placed in crushed ice between each batch. The lean beef was chopped for 30 sec. preceding the addition of salt, phosphate and ice, then chopped to 4.4°C (40°F), at which time the pork portion, sodium nitrite, sodium erythorbate and spice were added. Chopping continued until the emulsion reached a temperature of 12.7°C (55°F). This experiment was replicated three times.

Soluble protein

The (salt) solubilized protein content was determined by a modification of the method of Saffle and Galbreath (1964). Three 10g

Table 1—Emulsion ingredient formulations by experimental design

	% sodium chloride in emulsion			
	0.75		1.5	
	% phosphate in emulsion		% phosphate in emulsion	
Ingredient (g)	0.15	0.30	0.15	0.30
Beef (8.4% fat)	1542.2	1542.2	1542.2	1542.2
Pork (50.0% fat)	1360.8	1360.8	1360.8	1360.8
Ice	580.6	580.6	580.6	580.6
Sodium chloride	27.58	27.58	55.16	55.16
Inorganic phosphates	5.52	11.03	5.52	11.03
Sodium nitrite	0.57	0.57	0.57	0.57
Sodium erythorbate	2.02	2.02	2.02	2.02
Spice	18.39	18.39	18.39	18.39

samples of each raw emulsion were homogenized with 100 mL unbuffered 0.6M NaCl for 15 sec at 27,000 rpm with a Virtis "45" homogenizer. This homogenate was quantitatively transferred to 250-mL polycarbonate centrifuge bottles and centrifuged for 15 min. at 5,500 rpms (5,000 x *g*) and 5°C in a Beckman J-21C refrigerated centrifuge. After centrifugation, the fat layer floating on the surface was gently moved to one side with a stainless-steel spatula and 1 mL aliquots, in duplicate, were drawn from the clear salt and solubilized protein solution. To each 1 mL solution was added 5 mL Biuret reagent (a modification of the procedure of Gornall et al., 1949). This mixture was stirred and allowed to stand for 15 min. for optimum color development. Optical density was then determined with a Beckman Spec 20 at 540 nm, and converted by using Bovine Serum Albumin (BSA) standard curve to milligrams (mg) protein per mL solution. The total volume of solution in each centrifuge bottle was measured and multiplied by the mg/mL value resulting in "total mg protein." The 10g weight was divided into the "total mg protein" and recorded as mg soluble protein per g emulsion sample.

Emulsion stability

Emulsion stability was assessed by a modification of the method of Townsend et al. (1968). Thirty-four grams of raw emulsion were hand-stuffed in triplicate into 22.2 x 101.6 mm (.7 x 4 in) polycarbonate tubes (35 cc Monoinject syringe barrels) by using a modified plastic silicone cartridge and grease-gun ratchet. The small end of the tubes were stoppered. After the chopping of all emulsions was completed and all tubes were filled, the tubes were placed in a 48.9°C (120°F) water bath. The temperature of the water was raised so that the internal temperature (monitored at 3 cm below the surface of the emulsion and in the geometric center of the tube contents) reached 68.8°C (156°F) in 1.25 - 1.5 hr. The tubes were inverted over funnels, allowing the rendered fraction to be decanted into 15 mL graduated conical tubes. The tubes were centrifuged for 20 min about 1500 rpm by using an International (Universal Model UV) centrifuge. The volume of released fat, gel-liquids and solids per 34g emulsion were recorded.

pH

The pH of emulsion slurries was determined by using a standardized, Fischer Accumet Selective Ion Analyzer Model 750 (pH meter) fitted with a combination pH electrode.

Water-holding capacity

A water-holding capacity test was developed, which involved adding 12 mL physiological saline in 40g raw emulsion in 50 mL polycarbonate centrifuge tubes. The tube contents were mixed with a glass stirring rod, centrifuged at 10,000 x *g* for 15 min on a Beckman J-21C refrigerated centrifuge. Duplicate samples were centrifuged immediately while an additional set of duplicate samples was held for 24 hr at 4°C before centrifugation. After centrifugation, samples were inverted in funnels over 15 mL graduated conical tubes. The milliliters of expressed fluid were recorded.

Salt analysis

The salt (NaCl) content of the raw emulsions was measured with a Fischer Accumet Selective Ion Analyzer Model 750, fitted with an Orion chloride electrode (model 94-17) and a double-junction reference electrode. Salt determination was done according to procedures developed by Orion Research, Inc. (1980).

Phosphate analysis

A gravitimetric Quimociac Molybdophosphate method was used (AOAC, 1970) to measure the phosphorous pentoxide concentration of the raw emulsion.

Proximate analysis

Moisture, protein and fat contents of the raw emulsion were determined by AOAC methods (1975).

Statistical analysis

Statistical analysis of all data were performed by using the Statistical Analysis System's (Barr et al., 1970) analysis of variance (ANOVA). In addition, Duncan's Multiple Range Tests (Steel and Torrie, 1980) were performed when more than two treatments were being compared.

RESULTS

THE EFFECTS of four selected phosphates on emulsion stability are shown in Table 2. Tetrasodium pyrophosphate (NaPP) and tetrapotassium pyrophosphate (KPP) produced the most stable emulsions at added sodium chloride levels of 0.75% and 1.50%. At the opposite extreme, potassium tripolyphosphate (KTPP) resulted in significantly higher ($p < 0.05$) total cookout and gel-liquid released. Sodium tripolyphosphate (NaTPP) was not significantly different ($p > 0.05$) from the other three phosphates used for total cookout, but its effect was between those of KTPP and either NaPP or KPP. Although NaTPP showed the same relative effect upon milliliters of gel-liquid released, it was significantly different ($p < 0.05$) from KTPP. The results of released fat and solids indicated no significant difference ($p > 0.05$) among the four selected phosphates.

In the specific cation and anion effects (Table 2), the sodium cation and the pyrophosphate anion caused a significant reduction in gel-liquid released ($p < 0.05$ and $p < 0.01$, respectively) upon cooking. Only the phosphate

Table 2—Effects of selected inorganic phosphates[a] on stability, pH, soluble protein and the water-holding capacity of raw meat emulsions

Treatment	Total cookout (mL)[b]	Gel-liquid (mL)[b]	Fat (mL)[b]	Solids (mL)[b]	mg Soluble protein per g emulsion	pH	WHC-1 hr (mL released)	WHC-24 hr (mL released)
NATPP	2.85a,b[f]	2.47b	0.31	0.24	50.30b	6.25b	11.27a,b	8.19b
NaPP	2.25b	2.07b	0.19	0.05	49.96b	6.29a	10.55b	7.63b
KTPP	3.21a	2.92a	0.23	0.05	48.08b	6.22c	11.99a	8.95a
KPP	2.37b	2.19b	0.23	0.06	53.40	6.30a	10.84b	7.68b
S.E.	0.20	0.13	0.07	0.09	0.81	0.001	0.25	0.21
Na^{+}[d,e]	2.55	2.27*	0.25	0.14	50.13	6.28	10.91	7.91
K^{+}	2.79	2.55	0.23	0.06	50.74	6.27	11.42	8.32
S.E.	0.14	0.09	0.05	0.06	0.57	0.002	0.18	0.15
TPP[d,e]	3.03**	2.69**	0.27	0.15	49.19**	6.24**	11.63**	8.57**
PP	2.31	2.13	0.21	0.05	51.67	6.30	10.70	7.66
S.E.	0.14	0.09	0.05	0.06	0.57	0.002	0.18	0.15

[a] Sodium tripolyphosphate (NaTPP), tetrasodium pyrophosphate (NaPP), potassium tripolyphosphate (KTPP) and tetrapotassium pyrophosphate (KPP).
[b] mL per 34g emulsion sample.
[c] Mean values for total cookout, gel-liquid, fat and solids, mg soluble protein, pH, WHC-1 and WHC-2, N = 12 per treatment mean.
[d] Mean values for total cookout, gel-liquid, fat, solids, N = 12 per treatment mean.
[e] Mean values for mg soluble protein, pH, WHC-1 and WHC-24, N = 24 per treatment mean.
[f] Mean values in a column followed by different letters (a,b,c) are significantly different ($p < 0.05$).
*Significant ($p < 0.05$).
**Highly significant ($p < 0.01$).

anions were highly significantly different ($p < 0.01$) in their effect on total cookout.

Doubling the phosphate level (Table 3) decreased total cookout and amount of released gel-liquids by half ($p < 0.01$). In addition, doubling the sodium chloride level had an even greater effect on these two parameters. In terms of level of released fat, increasing sodium chloride level caused a significant ($p < 0.05$) reduction. The effect of sodium chloride level on fat release was observed to be at a lower significance level than on total cookout and gel-liquid released.

Tetrapotassium pyrophosphate (KPP) was significantly superior ($p < 0.05$) in solubilizing proteins to the other three phosphates tested in this experiment (Table 2). NaTPP and NaPP, while resulting in higher mean values, were not significantly different ($p > 0.05$) from KTPP in affecting soluble-protein values.

As to the specific phosphate cation and anion effects, PP significantly ($p < 0.01$) increased soluble protein. There was no significant difference ($p > 0.05$) between the effects of Na or K on soluble protein.

The effect of added phosphate and sodium chloride levels on soluble protein is presented in Table 3. Doubling the added phosphate level increased the soluble protein by approximately 7.5% ($P < 0.05$). On the other hand, doubling the added sodium chloride level had no significant effect ($p > 0.05$) on soluble protein.

The effect of the phosphates on raw emulsion pH is presented in Table 2. NaPP and KPP resulted in a significantly higher ($p < 0.05$) raw emulsion pH than either NaTPP or KTPP. NaTPP resulted in a significantly higher ($p < 0.05$) pH than KTPP. However, there was virtually no difference in pH (Table 2) due to cations (Na+ or K+) but a highly significant difference due to anions (TPP^{-} and PP^{-}).

Increasing the added phosphate level from 0.15% to 0.30% significantly ($p < 0.001$) increased raw emulsion pH while increasing added sodium chloride level from 0.75% to 1.50% had no effect on raw emulsion pH (Table 3).

The effects of the four selected phosphates on the water-holding capacity (WHC) of raw emulsion are presented in Table 2. NaPP and KPP resulted in significantly ($p < 0.05$) less expressed fluid than KTPP at 1 hr and 24 hr. NaTPP resulted in expressed fluid levels between NaPP or KPP and KTPP at 1 hr but was not significantly ($p > 0.05$) different from any of the other three phosphates. At 24 hr NaTPP resulted in a significant ($p < 0.05$) improvement in fluid retention compared to KTPP.

A significant ($p < 0.001$) positive correlation has been observed between WHC-1 hr. and gel-liquid released ($r = 0.72$) and between WHC-24 hr. and gel-liquid released ($r = 0.68$) when determined with the effects of all four phosphates combined.

The effect of cations and anions on WHC was determined. Sodium (Na^{+}) was more effective in increasing WHC than potassium (K^{+}) at 1 hr and 24 hr ($p = 0.057$ and 0.066, respectively). Comparing anions pyrophosphate significantly ($p < 0.01$) enhanced WHC at both time periods after chopping.

Doubling the added phosphate level in the range tested, decreased free moisture by 16.6% after 1 hr and by 36.4% after 24 hr (Table 3). At both time periods, the effect of increased phosphate level on WHC was highly significant ($p < 0.001$).

Similarly, doubling the sodium chloride level in the range observed caused a significant ($p < 0.001$) decrease in free fluid at both time periods.

DISCUSSION

THE RESULTS indicate that NaPP and KPP resulted in more stable emulsions than NaTPP and KTPP (Table 2). This effect is due to the anions (TPP- and PP-) compared. The relative effect of the two anions agrees with the results of Shults et al., (1972) and Shults and Wierbicki (1973). Jauregui (1981) reported similar results when NaPP and NaTPP were added on a percentage basis, as in this study.

The effect of phosphates and NaCl on the soluble-protein level of meat emulsions was investigated to help explain the effects of these same treatments on emulsion stability. The relative effect of KPP on soluble protein (Table 2) compares favorably to its effect on emulsion stability, however, NaPP seems to have a relatively smaller effect on soluble protein than it has on emulsion stability. This suggests that other factors are responsible for NaPP's influence on emulsion stability.

Doubling added phosphate level in the range tested significantly ($p < 0.05$) increased soluble protein (Table 3). This would be expected and probably is due to a combination of pH, ionic strength and actomyosin dissociation effects (Sherman, 1961; Ishioroshi et al., 1979; Bendall, 1954). Doubling NaCl level did not have a significant ($p < 0.05$) effect on soluble protein.

The effects of the phosphates tested on raw emulsion pH corresponded more closely to their effects on emulsion stability than did their effects on soluble protein (Table 2). This would suggest that the pH effect of phosphates was more important to emulsion stability than soluble protein level.

The effect of phosphates on the WHC of raw emulsions was monitored and compared with their effect on emulsion stability upon cooking. The relative effect of the four phosphates and the significance of the differences were identical when comparing the results (Table 2) of WHC-1 hr were compared to total cookout, and WHC-24 hr were compared to gel-liquid released. This would indicate that the effect

Table 3—Effects of added phosphate and sodium chloride (NaCl) levels on stability, pH, soluble protein and water-holding capacity (WHC) of raw meat emulsions

Treatment	Total cookout (mL)[a,b]	Gel-liquid (mL)[a,b]	Fat (mL)[a,b]	Solids (mL)[a,b]	mg Soluble protein per g emulsion[a]	pH[a]	WHC-1 hr (mL released)[a]	WHC-24 hr (mL released)[a]
0.15% phosphate	3.60**	3.23**	0.37	0.18	48.63*	6.20**	12.14**	9.92**
0.30% phosphate	1.74	1.60	0.12	0.02	52.23	6.34	10.19	6.31
S.E.	0.32	0.28	0.07	0.07	2.55	0.001	0.28	0.46
0.75% NaCl	4.84**	4.39**	0.38*	0.19	50.69	6.26	13.24**	10.76**
1.50% NaCl	0.50	0.44	0.11	0.01	50.17	6.28	9.09	5.47
S.E.	0.32	0.28	0.07	0.07	2.55	0.001	0.28	0.46

[a] Mean values, N = 24, per treatment mean.
[b] mL per 34g emulsion sample.
*Significant ($P < 0.05$).
**Highly significant ($p < 0.01$).

of phosphates on the WHC of the raw emulsion was very similar to its effect on emulsion stability upon cooking.

The data (Tables 2 and 3) show that WHC at 24 hr was always greater than at 1 hr. This agrees with the results of Bendall (1954) and supports his theory that some time is needed to reach the full potential of added phosphates on the WHC of meat.

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Effects of Magnesium Chloride Combined with Tetrasodium Pyrophosphate on Meat Emulsion Characteristics

C. L. KNIPE, D. G. OLSON, and R. E. RUST

ABSTRACT

The effects were determined of adding various combinations of magnesium and sodium chloride plus tetrasodium pyrophosphate on the stability, pH, solubilized protein, chopping times and textural characteristics of raw and cooked meat emulsions. The results of adding different combinations of magnesium and sodium chloride were quite similar whether ionic strength or chloride ion concentration was kept constant. Emulsion stability and raw emulsion pH were reduced with increased proportion of added magnesium chloride. However, the level of solubilized protein found in raw emulsions increased with increasing proportions of magnesium chloride.

INTRODUCTION

IN RESPONSE to the recent concern over sodium levels in our diets (Kolari, 1980), researchers have attempted to find a substitute for sodium chloride that would provide the water-holding and emulsion-stabilizing properties of sodium chloride but with less added sodium (Olson and Terrell, 1981).

The effects of sodium chloride on the water-binding capacity (WHC) and stability of meat emulsions are well known, and are explained by the fact that the negatively charged chloride ions screen the positively charged protein groups of meat (Schut, 1976). This screening phenomenon results in repulsion of the protein groups and an enlargement of the space available for water absorption.

The use of magnesium chloride ($MgCl_2$) in meat has been studied with some researchers reporting that $MgCl_2$ enhanced WHC of meat (Swift and Ellis, 1956; Wierbicki et al., 1957) while others reported $MgCl_2$ to be detrimental to WHC and emulsion stability of meat (Schut, 1976; Seman et al., 1980). Schut (1976) believed that divalent cations, such as magnesium, decreased WHC of meat by screening the negatively charged proteins groups.

In addition, $MgCl_2$ has been reported to be involved with the binding of pyrophosphate to myosin plus the dissociation of the actomyosin complex (Kiely and Martonosi, 1968; Yasui et al., 1964; Granicher-Frick, 1965; Tonomura, et al., 1967) and the combination of $MgCl_2$ and inorganic phosphate has been shown to be beneficial in the extraction of myosin from muscle (Penny, 1968).

This experiment was designed to determine the efficacy of using various combinations of sodium and magnesium chloride plus tetrasodium pyrophosphate to produce stable reduced-sodium meat emulsions.

MATERIALS & METHODS

Formulation and sample preparation

Meat emulsions were formulated and prepared according to procedures of Knipe et al., (1985), with the exceptions of nonmeat ingredients used which are formulated in Table 1. Also, magnesium chloride was added in the initial chopping step with the sodium chloride.

This experiment was designed to compare various combinations of sodium chloride and magnesium chloride. One set of comparisons was made by using combinations of sodium chloride and magnesium chloride in which their concentrations were of equal ionic strength (Table 1); the other comparisons involved combinations of sodium chloride and magnesium chloride with equal chloride concentrations (Table 1). The iso-ionic strength comparisons were repeated four times; the isochloride concentration comparisons were repeated three times.

Authors Knipe, Olson, and Rust are affiliated with the Dept. of Animal Science, Iowa State Univ., Ames, IA 50011.

Chemical and physical analysis

Soluble protein, emulsion stability, pH, salt analysis, phosphate analysis, proximate analysis and statistical analysis were all carried out as described by Knipe et al., (1985). In addition, cooked emulsion pH values were obtained from the cooked samples of the emulsion stability test.

Textural analysis

Three 12.5 mm (diam) x 12.5 mm (length) samples per treatment were used for textural analysis. Each sample was compressed twice to 50% of its length, by using an Instron 1122 with a 500-kg loadcell. The ratio of the second peak area to the first peak area was used to evaluate cohesiveness. Hardness was monitored with the height of the first peak. The Instron was set at a crosshead speed of 200 mm per min and a chart speed of 400 mm per min.

RESULTS

WHEN IONIC STRENGTH was kept constant, increasing the $MgCl_2$ proportion of added salts resulted in an increase in emulsion cookout (Table 2). The increase in cookout was not significant ($P > 0.05$)until the added NaCl concentration was reduced by half. One-half (0.5) percent $MgCl_2$ alone caused a further significant ($P < 0.05$) increase in cookout over the NaCl and $MgCl_2$ combinations. Similarly, when chloride concentration was kept constant, each decrease in NaCl level resulted in a significant ($P > 0.05$) increase in total cookout (Table 3). The other cookout components also were increased with decreasing NaCl content; however for fat, cookout was not significantly ($P > 0.05$) higher. With iso-ionic strength comparisons, soluble protein was significantly ($P < 0.05$) increased on addition of even the lowest level of $MgCl_2$ (Table 2). Increased $MgCl_2$ concentration did not result in a further significant ($P > 0.05$) increase in soluble protein. Again, comparable results were observed with the iso-chloride comparisons in that increasing $MgCl_2$ concentration resulted in increased soluble-protein levels (Table 3). A significant ($P < 0.05$) difference in soluble-protein level was observed only when comparing 0.75% NaCl with either of the two treatments which contain NaCl and $MgCl_2$.

Increasing the concentration of $MgCl_2$, when ionic strength was kept constant, caused a decrease in the raw emulsion pH (Table 2). This decrease was significant ($P < 0.05$) only for comparisons of NaCl treatments with NaCl-$MgCl_2$ combinations and for comparisons of NaCl-$MgCl_2$ combinations with $MgCl_2$ alone.

Chopping time required to reach an emulsion temperature of 4.4°C (40°F) was significantly ($P < 0.05$) reduced with the addition of any level of $MgCl_2$ (Table 4) when ionic strength was kept constant. On the other hand, for iso-chloride concentrations, the different combinations

resulted in no significant ($P > 0.05$) effect on chopping times (Table 5).

The addition of 0.5% $MgCl_2$ alone caused a statistically significant increase ($P < 0.05$) in hardness (Table 6). This increase is very likely due to the decrease in moisture retention caused by this treatment. This level of hardness was significantly ($P < 0.05$) different from only the 0.92% NaCl treatment. Cohesiveness values were not significantly different ($P > 0.05$) for any of the treatments.

DISCUSSION

AT CONSTANT IONIC STRENGTH, increasing $MgCl_2$ concentration significantly ($P < 0.05$) increased the cookout of all components of the emulsion stability test (Table 2). This could be attributed to the accepted detrimental effect of divalent cations on the water-holding capacity of meat (Schut, 1976). However, in keeping ionic strength constant over all treatments, the chloride ion concentration decreased as $MgCl_2$ concentration increased, which may have been one of the causes of the reduced emulsion stability.

Even when chloride ion concentration was kept constant each level of decreasing NaCl content resulted in a significant ($P < 0.05$) decrease in emulsion stability (Table 3). The magnesium content would be increasing to a greater extent in such combinations and is the apparent reason for the decrease in emulsion stability under those circumstances.

As expected, the presence of $MgCl_2$ significantly ($P < 0.05$) increased the soluble protein level of raw emulsions (Tables 2 and 3). This agrees with the results of Penny

Table 1—Emulsion ingredient formulation by experimental design

	Iso-ionic strength				Iso-chloride concentration		
	#1	#2	#3	#4	#1	#2	#3
	0.92% NaCl	0.75% NaCl 0.093% $MgCl_2$	0.46% NaCl 0.25% $MgCl_2$	0.5% $MgCl_2$	0.75% NaCl	0.50% NaCl 0.20% $MgCl_2$	0.25% NaCl 0.41% $MgCl_2$
Beef	1605.7	1605.7	1605.7	1605.7	1383.5	1383.5	1383.5
Pork	1360.8	1360.8	1360.8	1360.8	1519.6	1516.6	1519.6
Ice	580.6	580.6	580.6	580.6	580.6	580.6	580.6
Tetrosodium pyrophosphate (0.5%)	16.21	16.21	16.21	16.21	16.21	16.21	16.21
Sodium nitrite (0.0156%)	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Sodium erythorbate (0.055%)	1.78	1.78	1.78	1.78	1.78	1.78	1.78
Spice (0.50%)	16.21	16.21	16.21	16.21	16.21	16.21	16.21
Sodium chloride	29.82	24.31	14.91	–	24.31	16.21	8.10
Magnesium chloride (hexahydrate)	–	6.44	17.31	34.62	–	14.10	28.21

Table 2—Effect of various combinations[a] of sodium chloride (NaCl) and magnesium chloride ($MgCl_2$) plus tetrasodium pyrophosphate on stability, soluble protein and pH of raw meat emulsion

Treatment	Total Cookout (mL)[b,c]	Gel-liquid (mL)[b,c]	Fat (mL)[b,c]	Solids (mL)[b,c]	mg soluble protein/g emulsion[c]	pH[c]
0.92%	0.90c[d]	0.85c	0.01c	0.02b	57.16b	6.37a
0.75% NaCl/ 0.093% $MgCl_2$	1.57c	1.54c	0.00c	0.03b	70.36a	6.29b
0.45% NaCl/ 0.25% $MgCl_2$	6.57b	4.95b	1.38b	0.23b	71.05a	6.25b
0.50% $MgCl_2$	12.83a	8.11b	4.25a	0.47a	72.10a	6.19c
S.E.	1.40	0.94	0.42	0.07	3.60	0.005

[a] Iso-ionic strength.
[b] mL per 34g emulsion sample.
[c] Mean values, N = 4 per treatment mean.
[d] Mean values in a column followed by different letters (a,b,c) are significantly different ($P < 0.05$).

Table 3—Effect of various combinations[a] of sodium chloride (NaCl) and magnesium chloride ($MgCl_2$) plus tetrasodium pyrophosphate on stability, soluble protein and pH of raw and cooked meat emulsions

Treatment	Total Cookout (mL)[b,c]	Gel-liquid (mL)[b,c]	Fat (mL)[b]	Solid (mL)[b,c]	Mg soluble protein /g emulsion[b]	Raw pH[b]	Cooked pH[b]	pH change[b]
0.75% NaCl	0.59c	0.56c	0.01b	0.02c	49.33b	6.50	6.58a	+0.08
0.50% NaCl/ 0.20% $MgCl_2$	2.75b	2.42b	0.27b	0.06b	62.86a	6.45	6.46a,b	–0.04
0.25% NaCl/ 0.41% $MgCl_2$	10.57a	7.15a	3.22a	0.20a	67.12a	6.43	6.25b	–0.16
S.E.	0.51	0.26	0.32	0.009	1.34	0.003	0.01	0.13

[a] Iso-chloride concentration.
[b] Mean values, N = 3 per treatment mean.
[c] mL per 34g raw emulsion.
[d] Mean values in a column followed by different letters (a, b, c) are significantly different ($P > 0.05$).

Table 4—Effect of various combinations[a] of sodium chloride (NaCl) and magnesium chloride (MgCl) plus tetrasodium pyrophosphate on chopping times required to reach given temperatures

Treatment	Time to 4.4°C (40°F)[b,c]	Time to 12.8°C (55°F)[b,c]	Total time[b,c]
0.92% NaCl	14.15a	8.33	22.48
0.75% NaCl/ 0.093% $MgCl_2$	10.31b	7.21	20.02
0.46% NaCl/ 0.25% $MgCl_2$	10.27b	7.50	17.77
0.50% $MgCl_2$	10.46b	7.44	17.90
S.E.	0.61	0.71	1.61

[a] Iso-ionic strength.
[b] Mean values, N = 4 per treatment mean.
[c] Minutes.
[d] Mean values in a column followed by different letters (a, b) were significantly different ($P < 0.05$).

Table 6—Effect of various combinations[a] of sodium chloride (NaCl) and magnesium chloride ($MgCl_2$) plus tetrasodium pyrophosphate on the textural properties of cooked emulsion meat product.

Treatment[a]	Hardness[b,c]	Cohesiveness[b,d]
0.92% NaCl	46.65b[e]	0.58
0.75% NaCl/0.093% $MgCl_2$	49.78ab	0.49
0.46% NaCl/0.25% $MgCl_2$	50.85ab	0.51
0.50% $MgCl_2$	61.60a	0.59
S.E.	3.64	0.04

[a] Iso-ionic strength.
[b] Mean values, N = 4 per treatment mean.
[c] First peak height in mm.
[d] Ratio of second peak area to first peak area.
[e] Mean values in a column followed by differnt letters (a, b) were significantly different ($P < 0.05$).

(1968), who reported that pyrophosphate plus magnesium chloride extracted more myosin from prerigor meat than potassium chloride. The meat used in this experiment was not in the prerigor state; however, Mommaerts (1948) reported that the presence of magnesium enhanced the effect of pyrophosphate in reducing the viscosity of actomyosin solutions. This would suggest that magnexium chloride and pyrophosphate caused the dissociation of actomyosin and increased the solubility of myosin. This increase in soluble protein due to $MgCl_2$ was apparently not enough to counteract the detrimental effect of magnesium cations on emulsion stability (Tables 2 and 3). Higher concentrations of $MgCl_2$ did not further increase soluble protein significantly ($P < 0.05$).

$MgCl_2$ significantly ($P < 0.05$) decreased raw emulsion pH (Table 2) when ionic strength was kept constant. This could be due to the previously mentioned decrease in chloride ion concentration; just as the adition of NaCl increases chloride ion concentration and pH (Berman and Swift, 1964), so may decreasing chloride ion concentration decrease pH. When constant chloride concentration was maintained, $MgCl_2$ had no significant ($P > 0.05$) effect on pH. A decrease in protein pH due to $MgCl_2$ was previously reported by Ghosh and Mihalyi (1952).

The addition of $MgCl_2$ significantly ($P < 0.05$) decreased the chopping time to reach 4.4°C (40°F) (Table 4). If

Table 5—Effect of various combinations[a] of sodium chloride (NaCl) and magnesium chloride ($MgCl_2$), plus tetrasodium pyrophosphate on chopping times

Treatment[1]	Time to 4.4°C (40°F)[2,3]	Time from 4.4°C to 12.8°C (55°F)[b,c]	Total time[b,c]
0.75% NaCl	7.42	11.59	19.01
0.50 NaCl/ 0.20% $MgCl_2$	7.18	11.74	18.92
0.25% NaCl 0.41% $MgCl_2$	6.67	11.67	18.33
S.E.	0.22	0.26	0.41

[a] Isochloride concentration.
[b] Mean values, N = 3 per treatment mean.
[c] Minutes.

magnesium chloride and pyrophosphate were causing the dissociation of actomyosin, it would be expected that they would also decrease the viscosity of emulsions, as was reported to occur in actomyosin solutions by Mommaerts (1948). Such a decrease in emulsion viscosity should decrease frictional heat buildup during chopping and result in longer chopping times to reach specified temperatures. The presence of $MgCl_2$ did not significantly ($P > 0.05$) affect total chopping time or time to reach 12.8°C (55°F).

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Effects of Sodium Hydroxide and Selected Inorganic Phosphates on the Characteristics of Reduced Sodium Meat Emulsions

C. L. KNIPE, D. G. OLSON, and R. E. RUST

ABSTRACT

The effects of adding sodium hydroxide (NaOH), separately or in combinastion with various inorganic phosphates, on meat emulsion characteristics were studied. In comparing the addition of either NaPP or NaOH, 0.075% NaOH resulted in a higher raw emulsion pH and greater solubilized protein than 0.30% NaPP, but NaPP resulted in a more stable emulsion. The combination of 0.075% NaOH and 0.30% NaPP resulted in a soluble protein level less than when the ingredients were added individually and only slightly higher than if neither were present. The addition of NaOH (at 1 part NaOH to 4 parts phosphate) decreased cooked yields. With NaAPP and NaPP, NaOH reduced yields below that of the control.

INTRODUCTION

SODIUM HYDROXIDE (NaOH), in combination with inorganic sodium phosphates, has been approved for use in ham and bacon processing since 1952 (Anonymous, 1977). Very little published data are available on the use of NaOH in meat products. Terrell and Sair (1970) reported on the use of FOS-10 (a mixture of 1 part sodium hydroxide to 9 parts sodium tripolyphosphate) and stated that the addition of this commercial product resulted in a 0.6% increase in cooked yield of canned hams over the use of sodium tripolyphosphate alone.

Both NaOH and alkaline inorganic phosphates would be expected to raise the pH of meat emulsions. Water-holding capacity (WHC), emulsifying capacity, and protein solubility of meat emulsions are all reported to be enhanced as the pH is increased above the isoelectric pH (Hamm, 1971; Froning and Neelakantan, 1971; Fukazawa et al., 1961; Saffle and Galbreath, 1964).

The viscosity of meat emulsions has been shown by various researchers to decrease upon the addition of polyphosphates. Tóth and Hamm (1969) indicated that, although pyrophosphate had no effect on viscosity of emulsions made from prerigor meat, the viscosity of postrigor meat emulsions was reduced with the addition of 0.3 - 0.5% pyrophosphate and salt, suggesting that pyrophosphate is involved in the dissociation of the actomyosin complex.

Hargett et al. (1980) found sodium acid pyrophosphate to lower the viscosity of meat emulsions and went on to propose that the lowered viscosity resulted in a reduction in frictional heat buildup. Similarly, Swift and Ellis (1957) reported that the rate at which emulsion temperatures increased during chopping was significantly lower with the addition of phosphates.

The purpose of this study was to compare the effects of adding NaOH and selected inorganic phosphates, separatlely and in combination, to meat emulsions in an attempt to add to the understanding of the roles of phosphates and pH in meat emulsions.

MATERIALS & METHODS

MEAT EMULSIONS were formulated and prepared according to procedures of Knipe et al. (1985), with modifications. The first part of this experiment involved a comparison of the use of sodium hydroxide and tetrasodium pyrophosphate, individually and in combination, in meat emulsions. Ingredients used in these emulsions are formulated in Table 1.

The second part of this experiment compared the use of four phosphates (hexameta-, sodium acid pyro-, sodium tripoly- and tetrasodium pyrophosphate) with and without the addition of sodium hydroxide in meat emulsions made with industry-scale equipment. The emulsions were formulated to contain 34% fat using unfrozen beef (range 18.1 - 20.0% fat) and pork (range 48.4% - 51.8% fat). The meat portion of each batch weighed 11.34 kg. Nonmeat ingredients are listed in Table 2. The emulsions were made by using a Kramer-Grebe Model VSM 65 vacuum chopper (6 blade head). The emulsions were transferred to a vacuum stuffer (Vemag Robot 1000 S Type 116, Robert Reiser and Co., Inc., Boston, MA), pumped to a Townsend Frank-A-Matic DB-4 (Des Moines, IA), stuffed, and linked into number 21 Teepak type RP cellulose casings. After all nine batches were linked, the wieners were transferred to a H. Maurer and Sohne Allround System oven (Riechenaue, Federal Republic of Germany) and cooked to 68.3°C (155°F).

Both parts of this experiment were replicated four times.

Chemical and physical analysis

Soluble protein, emulsion stability, pH, texture, salt analysis, phosphate analysis, proximate analysis and statistical analysis were all carried out as described by Knipe et al. (1985).

Emulsion viscosity (μ) was measured during stuffing and linking on the Frank-A-Matic using the equation (Fox and McDonald, 1978):

$$\mu = \frac{\pi \cdot \Delta p \cdot D^4}{128 \cdot Q \cdot L} \frac{32.2\ lb_m\ ft}{lb/ft\ sec} = lb/ft\ sec$$

where: Δp = pressure differential between the metering pump of the Frank-A-Matic and the end of the stuffing tube; D = tube diameter = 0.024 ft (#14 stuffing horn); Q = volumetric flow rate = mass flow in

$$\frac{lb/sec}{Density\ (lb/ft)} = ft^3/sec$$

Density was determined by submerging five individually weighed links in 24°C water and the volume of water displaced was determined. Density was recorded as lb sample/ft of displaced water.

L = length of stuffing tube = 1.69 ft

Table 1—Emulsion ingredient formulations by experimental design

Ingredient	Treatments #1	#2	#3
Beef (19% fat)	1383.5	1383.5	1383.5
Pork (47.7% fat)	1519.6	1519.6	1519.6
Ice (distilled/deionized)	580.6	580.6	580.6
Sodium chloride (0.75%)	24.31	24.31	24.31
Tetrasodium pyrophosphate (0.30%)	9.73	–	9.73
0.5N Sodium hydroxide solution (0.075%)	–	81.10	81.10
Sodium nitrite (0.0156%)	0.51	0.51	0.51
Sodium erythorbate (0.055%)	1.78	1.78	1.78
Spice (0.50%)	16.21	16.21	16.21

Authors Knipe, Olson, and Rust are affiliated with the Dept. of Animal Science, Iowa State Univ., Ames, IA 50011.

The lb/ft sec units were converted to centipoise (cP) by the equation:

$$\frac{\text{lb/ft} \cdot \text{sec}}{0.672 \times 10^3} = \text{cP}$$

Chopper bowl revolutions for each treatment were recorded at 8.6°C (47.5°F) and 12.8°C (55°F).

Frankfurter cooked yields were determined by the equation:

$$\text{Cooked Yield \%} = \frac{\text{Cooked weight}}{\text{Raw weight}} \times 100$$

RESULTS & DISCUSSION

Emulsion stability

In comparing NaOH and NaPP, both significantly ($P < 0.01$) improved all components of emulsion stability (Table 3). When four phosphates (NaHMP, NaAPP, NaTPP and NaPP) were compared, all caused a significant ($P < 0.05$) improvement in emulsion stability (Table 4). Both NaTPP and NaPP were significantly ($P < 0.05$) better than either NaHMP or NaAPP in stabilizing meat emulsions. Again, the addition of NaOH improved ($P < 0.01$) emulsion stability (Table 4).

Cooking yields

The four phosphates tested here did not significantly ($P > 0.05$) improve cooked yields (Table 4). Also, the addition of NaOH significantly ($P < 0.01$) decreased cooked yields (Table 4).

Protein solubility

Neither NaOH or NaPP had any significant ($P > 0.05$) effect on soluble protein level (Table 3). However, this is misleading; treatment means reveal that NaOH and NaPP individually increased soluble protein considerably, but in combination they increased soluble protein only slightly. The significant ($P < 0.04$) interaction of NaOH with NaPP on soluble protein, presented in Fig. 1 explains the observed detrimental effect of the addition of both NaOH and NaPP on soluble protein of meat emulsions.

pH

Both NaOH and NaPP significantly ($P < 0.01$) increased raw emulsion pH (Table 3). While NaOH increased the pH more than NaPP it was less effective in enhancing emulsion stability (Table 5).

Table 2—Nonmeat ingredient formulations by treatments

Ingredient (g)	Treatments								
	#1	#2	#3	#4	#5	#6	#7	#8	#9
Sodium hexametaphosphate (0.50%)	–	63.33	63.33	–	–	–	–	–	–
Sodium acid pyrophosphate (0.50%)	–	–	–	63.33	63.33	–	–	–	–
Sodium tripolyphosphate (0.50%)	–	–	–	–	–	63.33	63.33	–	–
Tetrasodium pyrophosphate (0.50%)	–	–	–	–	–	–	–	63.33	63.33
1N Sodium hydroxide (0.125%)	–	–	264.00	–	264.00	–	264.00	–	264.00

Table 3—Effect of sodium hydroxide (NaOH) and tetrasodium pyrophosphate (NaPP) on emulsion stability, soluble protein and pH

Treatment[a]	Total Cookout (mL)[b]	Gel-liquid (mL)[b]	Fat (mL)[b]	Solids (mL)[b]	mg Soluble protein/g emulsion[a,c]	pH
No NaOH	8.82**	5.51**	3.08**	0.26**	52.04	6.12**
0.075%	2.12	1.95	0.12	0.08	56.42	6.73
S.E.	0.21	0.19	0.05	0.035	2.25	0.01
No NaPP	9.75**	6.34**	3.17**	0.24*	55.15	6.29**
0.30%	1.19	1.12	0.03	0.10	53.30	6.57
S.E.	0.21	0.19	0.05	0.035	2.25	0.01

[a] Mean values, N = 8 per treatment mean.
[b] mL per 34g sample.
[c] Significant NaPP by NaOH interaction ($P < 0.04$).
*Significant ($P < 0.05$).
**Highly significant ($P < 0.01$).

Table 4—Effect of selected phosphates[a] and sodium hydroxide (NaOH) on emulsion stability, cooked yields and raw and cooked emulsion pH

Treatment	Total cookout (mL)[b]	Gel-liquid (mL)[b]	Fat (mL)[b]	Cooked yields (%)	Raw pH	Cooked pH
Control[c]	1.51a[e]	1.45a	0.04	91.70	6.01d	6.23d
0.50% NaHMP	0.94b	0.87b	0.05	92.22	6.55c	6.57c
0.50% NaAPP	1.03b	0.99b	0.03	91.79	6.12d	6.23d
0.50% NaTPP	0.43c	0.41c	0.00	92.13	6.85b	6.71b
0.50% NaPP	0.41c	0.39c	0.04	91.90	6.91a	6.81a
S.E.	0.12	0.11	0.02	0.18	0.02	0.03
NaOH[d]	0.98**	0.94**	0.02	92.21**	6.18**	6.30**
0.125%	0.55	0.51	0.04	91.69	7.00	6.85
S.E.	0.08	0.07	0.01	0.11	0.01	0.02

[a] Sodium hexametaphosphate (NaHMP), sodium acid pyrophosphate (NaAPP) sodium tripolyphosphate (NaTPP) and tetrasodium pyrophosphate (NaPP).
[b] mL per 34g raw emulsion.
[c] Mean values, N = 8 per treatment mean.
[d] Mean values, N = 20 per treatment mean.
[e] Mean values in a column followed by different letters (a,b,c,d) are significantly different ($P < 0.05$).
** Highly significant ($P < 0.01$).

All four phosphates resulted in a significantly higher ($P < 0.05$) raw emulsion pH than the control, with NaTPP and NaPP increasing raw emulsion pH to the greatest extent (Table 4). Cooking the emulsions resulted in the same relative effects of the four phosphates on pH (Table 4). However, pH increase upon cooking was greater when raw emulsion pH was lower.

Textural analysis

NaOH significantly ($P < 0.01$) reduced hardness and cohesiveness (Table 6). NaPP also reduced hardness and cohesiveness but at a lower level of significance ($P < 0.05$). This probably occurred because NaOH and NaPP also enhanced emulsion stability or increased moisture retention (Table 3).

Table 5—Effect of sodium hydroxide (NaOH) and tetrasodium pyrophosphate (NAPP) on emulsion stability and raw emulsion pH treatment means

Treatment	Total cookout (mL)[a]	Gel-liquid (mL)[a]	Fat (mL)[a]	pH
Control	15.90	9.39	6.10	5.95
0.075%	3.61	3.30	0.24	6.63
0.30% NaPP	1.75	1.64	0.07	6.30
0.075% NaOH/0.30% NaPP	0.63	0.60	0.003	6.83

[a] mL cookout per 34g emulsion.

When four phosphates were compared, only NaPP resulted in significantly ($P < 0.05$) lower hardness than the control (Table 7). This is very likely due to increased moisture retention, which should result in a lower hardness score. NaOH also caused a significant ($P > 0.01$) reduction in hardness. None of the four phosphates resulted in cooked product cohesiveness significantly ($P < 0.05$) different from the control. However, NaTPP caused a significant ($P < 0.05$) reduction in cohesiveness when compared with the other three phosphates. NaOH significantly ($P < 0.05$) reduced coehsiveness.

Emulsion viscosity

Emulsion viscosity was significantly ($P < 0.001$) affected by the addition of phosphates (Table 7). NaHMP and NaAPP significantly ($P < 0.05$) reduced the viscosity when compared to the control. NaTPP and NaPP further ($P < 0.05$) reduced emulsion viscosity below that of NaHMP and NaAPP. The addition of NaOH also decreased emulsion viscosity significantly ($P < 0.05$).

Chopper bowl revolutions needed to reach 8.6°C (47.5°F) were significantly ($P < 0.05$) less when NaHMP, NaAPP and NaPP were added to the emulsion than for the control, but NaPP resulted in significantly ($P < 0.05$) more revolutions to raise the emulsion temperature from 8.6°C to 12.8°C (55°F) (Table 7). Since the fat portion of the meat was added at 8.6°C (47.5°F), it would seem that NaPP is exerting some influence on the fat portion, resulting in

Table 6—Effect of sodium hydroxide (NaOH) and tetrasodium pyrophosphate (NaPP) on chopping time to reach specific emulsion temperatures and textural properties of cooked comminuted product

Treatment[a]	Time to 4.4°C (40°F)[b]	Time from 4.4°C to 12.8°C (55°F)[b,c]	Total time[b]	Hardness[a,d]	Cohesiveness[a,e]
No NaOH	5.47	12.57	18.09	49.20**	0.68**
0.075% NaOH	5.95	12.06	18.01	35.10	0.46
S.E.	0.44	0.17	0.90	1.88	0.04
No NaPP	5.47	11.75	17.22	45.94*	0.64*
0.30% NaPP	5.95	12.92	18.99	38.34	0.51
S.E.	0.44	0.17	0.90	1.88	0.04

[a] Mean values, N = 8 per treatment mean.
[b] Time in minutes.
[c] Significant NaPP by NaOH interaction ($P < 0.03$).
[d] First peak height in mm.
[e] Ratio of second peak area to first peak area.

Table 7—Effects of selected phosphates[a] and sodium hydroxide (NaOH) on the textural properties of cooked emulsion meat product, the viscosity of meat emulsions and the chopper bowl revolutions required to reach specified temperatures

				Chopper Revolutions		
Treatment	Hardness[b]	Cohesiveness[c]	Viscosity[d]	to 8.6°C (47.5°F)	to 12.8°C (55°F)	Total
Control[e]	67.89a[g]	0.58ab	593.7a	22.98a	18.18b	41.12ab
0.05% NaHMP	63.57ab	0.62a	552.4b	20.75b	19.15b	39.90b
0.50% NaAPP	65.45a	0.63a	545.9b	19.92b	20.16ab	40.13b
0.50% NaTPP	61.36ab	0.57b	517.6c	21.26ab	19.14b	40.41b
0.50% NaPP	59.58b	0.63a	501.9c	20.35b	22.24a	42.59a
S.E.	1.76	0.02	7.46	0.53	0.93	0.66
No NaOH[f]	67.05**	0.63*	541.9*	22.76**	18.53**	41.29
0.125% NaOH	58.13	0.58	526.7	18.46	21.73	40.18
S.E.	1.11	0.01	4.72	0.33	0.59	0.42

[a] Sodium hexametaphosphate (NaHMP), sodium acid pyrophosphate (NaAPP), sodium tripolyphosphate (NaTPP) and tetrasodium pyrophosphate (NaPP).
[b] First peak height in mm.
[c] Ratio of second peak area to first peak area.
[d] Viscosity in centipoise (cP) units.
[e] Mean values, N = 8 per treatment mean.
[f] Mean values, N = 20 per treatment mean.
[g] Mean values in a column followed by different letters (a,b,c) are significantly different ($P < 0.05$).
*Significant ($P < 0.05$).
**Highly significant ($P < 0.01$).

more revolutions to reach the emulsion temperature of 12.8°C (55°F). In the total revolutions required to reach 12.8°C (55°F), only NaPP had any significant ($P < 0.05$) effect. NaPP's effect on total revolutions required to raise emulsion temperature was inversely proportional to its effect on viscosity.

DISCUSSION

THE ADDITION of NaOH alone improved emulsion stability, but to a lesser extent than NaPP alone (Tables 3 and 5). The combination of these two ingredients further increased emulsion stability.

NaOH added with various phosphates (NaHMP, NaAPP, NaTPP, or NaPP) resulted in improved emulsion stability over the control (Table 4). Using commercial size equipment, the order of increased stability was: NaAPP < NaHMP < NaTPP, NaPP (Table 4). NaOH, with phosphate effects absorbed, resulted in a highly significant ($P < 0.01$) decrease in total cookout.

On the other hand, NaOH reduced cooked yields of frankfurters when combined with phosphates (Table 4). This was particularly true when NaOH was added with NaAPP or NaPP. With NaOH effects absorbed, there was no significant ($P > 0.05$) difference among the four phosphates on cooked yield (Table 4).

Although neither NaOH or NaPP alone significantly ($P > 0.05$) increased the soluble protein level (Table 3), there was a significant ($P < 0.04$) interaction between NaPP and NaOH on solubilizing protein (Fig. 1), indicating that the addition of NaOH and NaPP in combination affected the meat proteins in some manner that decreased the solubilization of proteins compared with their addition individually. When NaOH and NaPP were added together, a heavy white precipitate was observed during the soluble protein determination. This suggests that such a treatment was causing precipitation of proteins.

NaOH, as well as NaPP, significantly ($P < 0.01$) increased emulsion pH (Table 3). NaOH increased pH to a greater extent than NaPP; however, NaPP enhanced emulsion stability more than NaOH. This would suggest that whereas increasing pH definitely has an effect on stabilizing meat emulsions, some other characteristic of NaPP is exerting an influence above and beyond that of the pH effect. Jauregui (1981) attributed 80% of NaPP's effect on WHC to a pH effect. The emulsion pH treatment means (Table 5) show that the mean pH value of emulsions treated with both NaOH and NaPP is approximately 6.8. Although this pH range would be near optimum for solubilization of salt-soluble proteins, the solubility of collagen would be decreased (Lowry et al., 1941). This high pH was not detrimental to emulsion stability (Table 5).

The pH values for the different phosphates (NaHMP, NaAPP, NaTPP, and NaPP) in meat emulsions (Table 4) relate well to their relative effect on the pH in water (Ellinger, 1972). The pH change of meat emulsions upon cooking seems to depend upon the raw emulsion pH. The higher the raw pH values, the less pH rises upon cooking.

The addition of NaOH and phosphates significantly ($P < 0.05$) reduced emulsion viscosity (Table 7). Absorbing the effects of NaOH, the treatments in order of significant ($P < 0.05$) viscosity decrease were: Control < NaHMP, NaAPP < NaTPP,NaPP. These results agree with Hamm (1975) who reported that NaPP reduced the viscosity of meat homogenates.

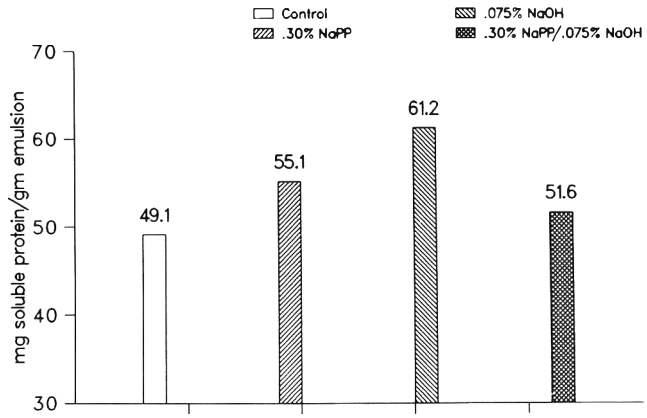


Fig. 1—Effect of addition of sodium hydroxide and tetrasodium pyrophosphate on soluble protein in meat emulsions.

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Effects of Low Temperature and Increased Oxygen Concentration on Color of Intact Pre-rigor Muscle

D. P. CORNFORTH, W. R. EGBERT, and D. V. SISSON

ABSTRACT

The effects of low temperature and increased oxygen concentration on color of intact pre-rigor muscle were determined by use of the Hunter color difference meter. Normally dark, intact, pre-rigor muscle turned bright red when held at low temperature and high oxygen concentration. The effect was reversible in that the muscle again turned dark when returned to air at room temperature. The bright red color observed in pre-rigor muscle at low temperature and in oxygen was due to lower mitochondrial oxygen consumption at low temperature, which allowed myoglobin to become oxygenated at high oxygen concentration.

INTRODUCTION

PRE-RIGOR MUSCLE homogenates will turn bright red (bloom) in the presence of rotenone, a mitochondrial inhibitor (Cornforth and Egbert, 1985), even though the pH remains high. Normally, muscle will not bloom (remains dark) until the pH falls below about 6.0 (Price and Schweigert, 1978). Electrical stimulation within 1 hr postmortem is the practical procedure for rapidly achieving bloom in intact muscle (Carse, 1973; Savell et al., 1979; Houlier et al., 1980), the mechanism being a rapid muscle pH decline, inhibiting mitochondrial oxygen consumption (Cornforth and Egbert, 1985). However, there is apparently no procedure available, either practical or experimental, for achieving bloom in intact pre-rigor muscle while maintaining a high pH.

The objective of this study was to determine the effects of low temperature and high oxygen concentration on the color of high pH, intact, pre-rigor muscle slices.

MATERIALS & METHODS

BEEF MUSCLE Longissimus dorsi samples were obtained 30-45 min after slaughter from two choice grade heifers and two commercial grade cows and held on ice during transport. Samples were obtained by cutting through the L. dorsi at the 10th and 12th ribs, removing an 8 cm thick cross-section of the muscle. Control post-rigor muscle samples were obtained from the same animal 96 hr postmortem. Slices 2 mm and 5 mm in thickness were obtained on a Commercial Food Processing Machine (Model 818, Berkel, Inc., LaPorte, IN). Slices (2 mm) were dipped in isotonic saline solution (0.9g NaCl per 100 mL water) to prevent dehydration, then treated for 30 min as follows: (1) in air, 3°C (on ice); (2) air, 20°C (room temperature); (3) oxygen, 3°C; (4) oxygen, 14°C; (5) oxygen, 3°C for 30 min then to air, 20°C; (6) air, 3°C for 30 min then to air 20°C; and (7) oxygen, 3°C (5 mm sample thickness). Samples were sliced and treated as described within 2 hr postmortem.

The oxygenation apparatus consisted of an oxygen tank connected through rubber tubing to a Gas Pak 150 large anaerobic jar, (BBL, Cockeysville, MD). Ice was placed in the chamber to a depth of about 5 cm. Samples were held at 3°C by placing them directly on ice overlayed with plastic wrap. Samples were held at 14°C by placing them in the chamber on the top of a 600 mL inverted beaker. For oxygenated samples, a slow flow of oxygen was used to flush the sample chamber throughout the experiment. Samples were held under these conditions for 30 min prior to initial Hunter color readings. In treatments 5 and 6, samples were treated as described for 30 min, then initial color readings were obtained. The samples were then transferred to air at 20°C, and further color readings were taken.

Hunter color measurements were obtained at 10, 20, 30, 40, and 60 min after initial readings, using a Hunter Lab Digital Color Difference Meter, (Model D25D2A Hunter Associates Lab, Inc., Reston, VA), standardized with the red (tomato color) standard plate (L=25.9, a=27.4, b=13.1).

Muscle samples were inverted in Hunter color sample containers before taking readings to insure that readings were measured on the surface directly exposed to air or oxygen. After each reading, the sample was returned to its original position until the next measurement. After the last color readings, a portion of the sample was minced, and sample pH was taken within 4 and 100 hr postmortem for pre-rigor and post-rigor samples, respectively, using an Orion pH meter (Model 601A Orion, Cambridge, MA). Surface pH was taken using a portable pH meter (Extech, Boston, MA). A split-split plot factorial analysis of variance and least significant difference (LSD) values were computed for color readings and pH values (Cochran and Cox, 1966).

RESULTS

THE ANALYSIS OF VARIANCE table for Hunter color values is presented in Table 1. The main effects grade, treatment, and time were significant for Hunter color 'L' values (Table 1). The main effects grade, rigor state, and treatment were significant for Hunter color 'a' values, while only the treatment main effect was significant for Hunter color 'b' values (Table 1).

All muscle samples were very dark colored prior to treatment, characteristic of pre-rigor beef muscle. Compared to all other treatments, a higher ($p < 0.05$) redness or "a" value was observed for 2 mm thick slices of both pre-rigor, choice and pre-rigor, commercial muscle samples treated with oxygen at 3°C for 30 min (Fig. 1). Pre-rigor samples treated with oxygen at 3°C for 30 min, but then transferred to air at 20°C had high "a" values initially, but the "a" value (redness) decreased rapidly after the sample was transferred to air. Both remaining oxygen treated, pre-rigor samples [oxygen, 14°C and the thick (5 mm) sample in oxygen at 3°C] tended to be more red than the air treated samples (Fig. 1). Pre-rigor air treated samples remained dark colored throughout the experiment. Dark colored samples corresponded to Hunter color "a" values of about 11 or less. Samples with "a" values above 15 were red, and samples with "a" values above 18 appeared bright red.

All post-rigor samples appeared red, but post-rigor samples in oxygen definitely had higher "a" or redness values than similar samples in air at 20°C. However, the post-rigor sample in air at 3°C did have consistently high redness values throughout the experiment, for both choice and commercial samples (Fig. 1).

Hunter color "L" values (lightness) and "b" values (yellowness) are presented in Tables 2 and 3, respectively. Lightness or "L" values, were lower (i.e., darker) for samples from the older, commercial grade animals (Table 2), compared to samples from choice grade animals. The "b" or yellowness values were higher for samples in oxygen at 3°C (Table 3), as was also the case for redness values. There

Authors Cornforth and Egbert are with the Dept. of Nutrition & Food Sciences, and Author Sisson is with the Dept. of Applied Statistics, Utah State Univ., Logan, UT 84332.

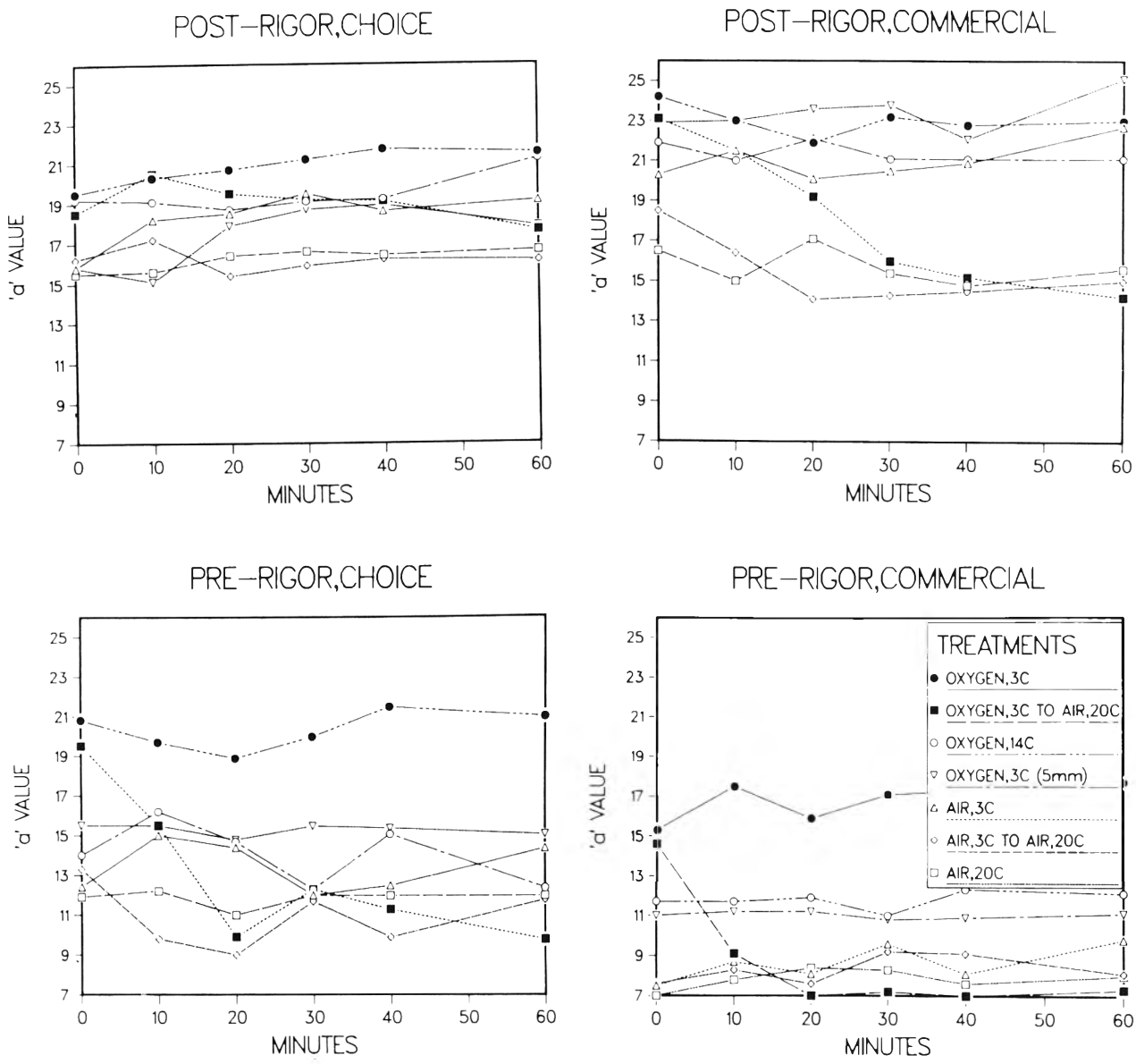


Fig. 1–Mean Hunter color 'a' or redness values of intact muscle slices with time. The 'a' value = red when positive, green when negative. Zero time equals 30 min after placing samples under the following treatment conditions: (1) oxygen, 3°C; (2) oxygen, 3°C to air, 20°C; (3) oxygen, 14°C; (4) oxygen, 3°C (5mm); (5) air, 3°C; (6) air, 3°C to air 20°C; and (7) air, 20°C. Each point is the mean of two samples done in duplicate. LSD values are as follows: (1) LSD = 2.84 for comparison of means by time within a given treatment and muscle group. (2) LSD = 3.84 for comparison of means between treatments for a given time within a muscle group. (3) LSD = 3.81 for comparison of means among muscle groups for a given treatment and time.

were no significant differences in pH among treatments (Table 4), but differences among groups were apparent. All post-rigor samples had pH values below 6.0, while all pre-rigor samples had pH values above 6.0.

DISCUSSION

INTACT, pre-rigor muscle slices held at low temperature in an oxygen atmosphere did turn bright red (bloom) but rapidly returned to the dark color characteristic of pre-rigor meat when the sample was transferred to air (approximately 20% oxygen) at room temperature. This observation may be explained as follows: low temperature slows mitochondrial respiration, while high oxygen concentration saturates myoglobin with oxygen, causing the sample to turn red. When the sample is returned to room temperature in air, the mitochondrial oxygen consumption increases, oxymyoglobin is deoxygenated and the sample turns dark again.

Pre-rigor muscle homogenates will bloom in the presence of rotenone, a mitochondrial inhibitor (Cornforth and Egbert, 1985). Preliminary attempts to obtain bloom in intact pre-rigor muscle by injection of mitochondrial inhibitors were not successful. Apparently, blending is needed to allow the inhibitors to come in direct contact with the muscle mitochondria.

The results of this study showed that intact pre-rigor muscle slices will bloom at low temperature in an oxygen atmosphere. These conditions would slow mitochondrial oxygen consumption and saturate myoglobin with oxygen. Thus the observation that the muscle turns red under these conditions is consistent with the conclusion that mitochondrial metabolism is indirectly related to muscle color. Both low temperature and high oxygen concentration are necessary to achieve bloom. Neither low temperature (air, 3°C treatment) or high oxygen concentration (oxygen, 14°C) alone was sufficient to attain bloom. The control sample

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TABLE 1—Analysis of variance of Hunter color lightness (L), redness (a), and yellowness (b) values

Source of variation	df[a]	'L' MS[b]	'L' F[c]	'a' MS	'a' F	'b' MS	'b' F
Grade (G)	1	2267.10	67.80*	240.96	46.8*	108.88	4.21
Animal within grade	2	36.68		5.14		25.84	
Rigor state (R)	1	7546.21	6.87	7222.03	430.46**	2472.88	37.00
GR	1	7.77	<1	1159.20	69.09*	2.20	<1
Error (a)	2	1097.52		16.77		66.81	
Treatment (T)	6	109.03	5.59**	631.97	30.23**	76.19	35.60**
RT	6	14.54	<1	68.57	3.28*	8.33	3.89**
GT	6	10.31	<1	29.36	1.40	3.18	1.48
GRT	6	7.33	<1	40.82	1.95	4.13	1.93
Error (b)	24	19.49		20.89		2.14	
Time in minutes (M)	5	14.00	3.34*	7.48	1.97	1.10	1.62
GM	5	2.31	<1	6.71	1.76	0.60	<1
Error (c)	10	4.18		3.79		0.68	
RM	5	8.39	1.90	6.77	1.65	0.82	1.19
TM	30	7.70	1.74*	22.21	5.41**	2.94	4.25**
RTM	30	4.20	<1	8.45	2.06**	0.92	1.33
GRM	5	4.55	1.03	24.13	5.87**	2.45	3.54**
GTM	30	3.13	<1	2.79	<1	0.87	1.27
GRTM	30	3.19	<1	4.63	1.12	0.76	1.11
Error (d)	130	4.41		4.10		0.69	
Sampling	336	2.53		0.80		0.17	
Total	671						

[a] Degrees of freedom
[b] Mean sum of squares
[c] Calculated F value
* significant at $p \leq 0.05$.
** significant at $p \leq 0.01$.

TABLE 2—Mean Hunter Color "L" values[a] for muscle samples with time

Muscle Group	Time (min) 0[b]	10	20	30	40	60
Pre-rigor, choice[d]						
Treatment						
Air, 3°C	23.8[c]	23.2	23.1	23.4	23.6	22.5
Air, 20°C	22.6	22.2	23.0	23.0	21.6	21.2
Oxygen, 3°C	27.1	26.7	26.3	26.8	26.4	25.8
Oxygen, 14°C	23.6	23.1	24.0	25.1	23.5	23.8
Oxygen, 3°C to Air, 20°C	27.5	24.3	25.1	23.4	23.0	25.2
Air, 3°C to 20°C	24.3	23.0	23.0	22.9	21.6	20.9
Oxygen, 3°C (5mm)	25.0	25.5	23.9	25.0	24.6	24.8
Pre-rigor, Commercial						
Air, 3°C	21.9	20.0	20.5	20.7	20.6	20.0
Air, 20°C	18.2	18.6	18.5	18.9	17.8	17.8
Oxygen, 3°C	22.4	22.1	22.0	22.0	22.2	22.8
Oxygen, 14°C	21.8	20.7	20.5	21.3	21.0	21.0
Oxygen, 3°C to Air, 20°C	21.4	18.9	18.0	18.4	18.5	18.5
Air, 3°C to 20°C	22.6	19.2	18.7	18.8	18.8	19.0
Oxygen, 3°C (5mm)	20.8	20.6	19.3	20.4	20.1	20.1
Post-rigor, Choice						
Air, 3°C	30.4	30.0	30.7	31.1	31.2	32.0
Air, 20°C	29.6	29.1	28.8	29.2	29.1	28.5
Oxygen, 3°C	31.2	31.5	31.9	31.9	31.9	32.1
Oxygen, 14°C	29.3	29.9	29.6	29.2	28.8	28.9
Oxygen, 3°C to Air, 20°C	31.9	30.9	30.0	29.8	29.6	29.0
Air, 3°C to 20°C	32.2	29.8	29.6	29.3	29.1	28.5
Oxygen, 3°C (5mm)	31.1	31.6	30.5	31.5	31.3	32.1
Post-rigor, Commercial						
Air, 3°C	27.8	28.2	28.5	28.5	29.3	29.0
Air, 20°C	24.7	25.2	25.1	25.0	24.9	26.7
Oxygen, 3°C	27.5	27.8	28.1	28.5	28.3	27.3
Oxygen, 14°C	26.1	26.0	26.9	26.5	27.3	26.4
Oxygen, 3°C to Air, 20°C	28.4	27.5	27.1	26.0	25.8	25.8
Air, 3°C to 20°C	28.1	26.5	26.3	26.1	25.9	26.1
Oxygen, 3°C (5mm)	28.3	28.8	29.1	29.0	29.3	29.0

[a] L = Lightness; 100 = white, 0 = black
[b] 0 Time = 30 min after placing sample under treatment conditions.
[c] Each value is the mean of two samples done in duplicate
[d] LSD values are as follows: (1) LSD = 1.17 for comparison of means by time within a given treatment and muscle group; (2) LSD = 1.41 for comparison of means between treatments for a given time within a muscle group. (3) LSD = 2.30 for comparison of means among muscle groups for a given treatment and time.

Table 3—Mean Hunter Color "b" values[a] for muscle samples with time

Muscle group	Time (min) 0[b]	10	20	30	40	60
Pre-rigor, Choice[d]						
Treatment						
Air, 3°C	6.2[c]	6.3	6.9	6.7	7.0	6.4
Air, 20°C	4.8	4.8	5.6	7.2	5.6	5.0
Oxygen, 3°C	9.6	8.7	9.7	8.8	8.9	9.2
Oxygen, 14°C	5.8	6.4	6.8	6.8	5.9	6.1
Oxygen, 3°C to Air, 20°C	9.0	6.4	6.1	5.3	5.1	5.2
Air, 3°C to 20°C	6.4	5.0	5.7	5.5	4.6	4.4
Oxygen, 3°C (5mm)	7.2	7.6	7.2	6.7	7.4	7.6
Pre-rigor, Commercial						
Air, 3°C	5.1	5.4	5.3	4.9	5.4	5.3
Air, 20°C	4.6	4.4	4.6	5.4	5.0	5.1
Oxygen, 3°C	7.0	7.4	7.0	7.9	7.5	7.8
Oxygen, 14°C	6.8	6.6	6.4	6.4	6.2	6.9
Oxygen, 3°C to Air, 20°C	6.5	4.6	4.6	4.9	4.8	4.9
Air, 3°C to 20°C	5.1	4.8	4.9	4.8	5.0	5.0
Oxygen, 3°C (5mm)	6.2	5.9	5.9	5.6	5.9	6.1
Post-rigor, Choice						
Air, 3°C	9.3	10.0	10.3	10.7	10.8	10.1
Air, 20°C	9.3	9.9	9.5	9.9	9.8	9.4
Oxygen, 3°C	10.7	11.0	11.7	11.5	11.6	11.8
Oxygen, 14°C	10.4	10.6	10.4	10.3	10.3	10.8
Oxygen, 3°C to Air, 20°C	11.2	11.3	10.3	10.0	10.0	10.0
Air, 3°C to 20°C	9.5	9.6	9.5	9.4	9.4	9.4
Oxygen, 3°C (5mm)	10.9	11.1	9.8	10.0	10.6	11.1
Post-rigor, Commercial						
Air, 3°C	10.0	10.1	10.3	10.5	10.8	10.8
Air, 20°C	8.7	8.7	8.2	8.5	8.1	8.6
Oxygen, 3°C	10.4	10.8	10.8	10.4	10.7	10.7
Oxygen, 14°C	10.4	10.0	10.4	9.9	9.7	10.0
Oxygen, 3°C to Air, 20°C	10.8	10.5	9.8	8.9	9.1	8.6
Air, 3°C to 20°C	9.9	8.9	8.1	7.8	7.8	8.1
Oxygen, 3°C (5mm)	10.0	10.6	10.5	10.4	10.2	10.8

[a] Yellow when positive, blue when negative.
[b] 0 Time = 30 after placing samples under treatment conditions.
[c] Each value is the mean of two samples done in duplicate.
[d] Least significant difference (LSD) values are as follows: (1) LSD = 2.95 for comparison of means by time within a given treatment and muscle group; (2) LSD = 3.84 for comparison of means between treatments for a given time within a muscle group; (3) LSD = 8.30 for comparison of means among muscle groups for a given treatment and time.

Table 4—pH of muscle samples[b]

Group	Treatment: Air, 3°C Surface	Air, 3°C Minced	Air, 20°C Surface	Air, 20°C Minced	Oxygen, 3°C Surface	Oxygen, 3°C Minced	Oxygen, 14°C Surface	Oxygen, 14°C Minced	Oxygen, 3°C to Air, 20°C Surface	Oxygen, 3°C to Air, 20°C Minced	Air, 3°C to 20°C Surface	Air, 3°C to 20°C Minced	Oxygen, 3°C (5mm) Surface	Oxygen, 3°C (5mm) Minced
Pre-rigor, Choice	6.6[a]	6.24	6.4	6.17	6.4	6.27	6.4	6.16	6.6	6.18	6.6	6.18	6.4	6.29
Pre-rigor, Commercial	6.8	6.52	6.7	6.42	6.8	6.50	6.8	6.44	6.9	6.34	6.8	6.32	6.9	6.57
Post-rigor, Choice	5.7	5.34	5.6	5.34	5.5	5.29	5.5	5.29	5.6	5.30	5.5	5.28	5.4	5.28
Post-rigor, Commercial	5.4	5.54	5.5	5.35	5.5	5.38	5.5	5.38	5.4	5.34	5.6	5.35	5.5	5.38

[a] Mean of two samples done in duplicate
[b] No significant differences ($p<0.01$) observed among treatments within a group.

in air at 20°C did not bloom as expected. The sample chilled in air, then transferred to room temperature in air also did not bloom.

There was a definite increase in redness of thick (5 mm), chilled samples in oxygen, but these samples did not achieve the redness of similarly treated thin (2 mm) samples. Sample thickness had no effect on sample temperature; both samples were at 3°C. Thus, mitochondrial metabolism was similarly slowed in both thick and thin samples. Thus differences in oxygen diffusion into these samples was probably responsible for the observed color differences, with thin samples being more fully oxygenated and thus more red.

Post-rigor samples did bloom under all treatments, consistent with low mitochondrial activity in samples at pH values below 6.0 (Ashmore et al., 1973). However, post-

—Continued on page 1028

Aging Index, Lysosomal Enzyme Activities, and Meat Tenderness in Muscles from Electrically Stimulated Bull and Steer Carcasses

F. Y. WU, T. R. DUTSON, C. VALIN, H. R. CROSS, and S. B. SMITH

ABSTRACT

Correlations among biochemical indexes and muscle tenderness were evaluated in longissimus samples from bull and steer carcasses. Electrical stimulation increased carcass temperature, which was significantly ($P<0.05$) correlated with shear force, aging index, carcass pH pH, and microsomal and percentage free lysosomal enzyme activities. Aging index was not correlated with the decrease in carcass pH elicited by electrical stimulation. Meat from steer carcasses was more tender, displayed more negative aging indexes, and had greater soluble activities of β-glucuronidase and cathepsin B than meat from bull carcasses.

INTRODUCTION

POSTMORTEM TENDERIZATION of muscle generally is considered to be due to the activity of two enzyme systems: calcium activated factor (CAF) and lysosomal enzymes. These two systems may work synergistically, since lysosomal enzymes may degrade the CAF inhibitor, and therefore promote CAF activity (Goll et al., 1983). Calcium activated factor, with pH optimum around 7, has been shown to degrade troponin T, troponin I, tropomyosin, C-protein, and desmin (Goll et al., 1983), cleave myosin heavy chain if myosin light chain 2 is unphosphorylated (Pemrick et al., 1980), and release α-actinin from Z-lines (Suzuki et al., 1975).

Lysosomal enzymes include proteases, glycosidases, and phosphatases (White et al., 1978). Electrical stimulation of carcasses accelerates glycolysis and ATP hydrolysis, which cause a rapid decline in pH and appear to maintain higher carcass temperatures due to the release of lactic acid, inorganic phosphate and heat (Bendall et al., 1976; White et al., 1978). It has been suggested that the combination of low pH and high temperature promotes the rupture of lysosomal membranes and releases those enzymes which act on muscle components (Dutson, 1983).

A potential indicator of postmortem proteolysis is the aging index. Ouali (1984) defined the biochemical aging index as the slope of the linear regression between the myofibrillar ATPase (Mg^{2+}:Ca^{2+}-dependent) activity and ionic strength. The aging index is affected by CAF, lysosomal enzyme activities and time postmortem (Ouali and Valin, 1980). α-Actinin, released from Z lines by CAF, activates Mg^{2+}:Ca^{2+}-dependent myofibrillar ATPase at 50 mM KCl, but the activating effect disappears when the KCl concentration reaches 130–150 mM (Yang et al., 1978). Thus, the slope of the regression of ATPase activity against ionic strength is dependent in part upon the extent of α-actinin release from Z-lines.

The objectives of this experiment were to determine the effects of electrical stimulation of carcasses from castrated and intact male cattle on the distribution of lysosomal enzyme activities, aging index, pH and carcass temperature, and to determine the relationships among the above characteristics as well as to meat tenderness.

Authors Wu, Cross and Smith are affiliated with the Meats & Muscle Biology Section of the Dept. of Animal Science, Texas A&M Univ., College Station, TX. Author Dutson is with the Dept. of Food Science & Human Nutrition, Michigan State Univ., East Lansing, MI. Author Valin is with Institute National de la Recherche Agronomique, Station de Recherches sur la Viande, Theix, France.

MATERIALS & METHODS

TWELVE A-maturity bulls and 12 A-maturity steers of similar breeding and management background were slaughtered at the U.S. Meat Animal Research Center abattoir at Clay Center, Nebraska. Care was taken not to stress the animals prior to slaughter. The right side of each carcass was electrically stimulated (400–500 V, 1.5 Amp, 60 Hz, 20 pulses/min for 2 min) within 45 min postmortem. All carcasses were subjected to the same cooler conditions. Temperature was measured using a probe-type electrode on the longissimus dorsi muscle 2 and 6 hr after electrical stimulation. The pH was measured at 2 hr after electrical stimulation in longissimus dorsi muscle homogenates containing iodoacetate (Bendall, 1973).

Samples to be used for taste panel and shear force measurements were obtained at 48 h, wrapped in polyethlylene-coated freezer paper, and frozen at −40°C. Subsequently, samples were thawed and cooked to 70°C internal temperature. Maximum shear force, required to shear a 1.3 cm diameter sample, was measured on an Instron Universal Testing machine. Taste panel tenderness was evaluated by a descriptive panel trained according to procedures outlined by Cross et al. (1978).

Lysosomal enzyme activities

Samples were removed at 2 h post-stimulation from the longissimus dorsi muscle. Ten grams minced muscle in 50 mL 0.25M sucrose:0.02M KCl solution were homogenized in an ice water bath using a Virtis Model "23" at full speed for 80 sec (50 sec on, 30 sec off and 30 sec on). After the homogenate was filtered through two layers of cheesecloth, the pH of the filtrate was recorded and then adjusted to 7.0 with KOH. Thirty-eight grams of the solution were centrifuged at 3,000 × *g* for 10 min. The supernate was decanted and the volume was recorded. The pellet was resuspended in 20 mL sucrose-KCl solution and the volume recorded. This was designated as the nuclear portion. A 20 mL volume of supernate was centrifuged at 105,000 × *g* at 2°C for 2 hr. The supernate was decanted (soluble portion). The pellet was resuspended in 10 mL sucrose-KCl solution and designated as the microsomal portion. Samples were frozen in liquid nitrogen and stored at −20°C until analysis. Frozen samples were thawed in running water and then kept at 0–4°C throughout the enzyme assay except during incubation. Protein content was measured by biuret (Gornall et al., 1949); samples were diluted to 0.1 mg/mL with 0.1% Brij 35 for the assays of cathepsin B, cathepsin H and β-glucuronidase. Cathepsin B and cathepsin H were measured using the procedures described by Barrett (1980). β-Glucuronidase was measured with modification of the procedure of Moeller et al. (1976). Five hundred μL diluted samples were preincubated with 250 μL buffer containing 300 mM sodium citrate:4 mM EDTA (pH 5.0) at 40°C for 6 min. The assay was started by adding 250 μL 200 μM 4-methylumbelliferyl-β-D-glucuronide. After 30 min, the incubation was stopped by adding 1 mL 500 mM Na_2CO_3 (pH 10.4) and placing the tubes in an ice water bath. Fluorescence was measured using a SPF-500 Spectrofluorometer (American Instrument Co.). Excitation and emission wavelengths were 360 and 448 nm, respectively, for β-glucuronidase, and 360 and 460 nm, respectively, for cathepsin B and cathepsin H. Supernatant, microsomal, and nuclear enzyme activities were expressed as unit per g muscle. Percentage free activities was calculated as supernatant activity divided by the sum of supernatant and microsomal activities. Total activities were the sum of supernatnat, microsomal, and nuclear activities.

Table 1—Sex and side effects for variables from electrically stimulated and control bull and steer carcasses

	Bull		Steer					
	E.S.[a] (n = 12)	Control (n = 12)	E.S. (n = 12)	Control (n = 12)	Standard dev.	Sex PR>F	Side PR>F	Sex X Side PR>F
Shear force (kg)	4.31	5.71	3.80	4.59	1.01	0.009	0.001	0.261
Taste panel tenderness[b]	5.10	4.85	5.37	5.12	0.37	0.014	0.024	0.959
Aging index[c]	−33.3	−23.4	−43.1	−37.0	13.80	0.005	0.048	0.629
Temperature (2 hr, °C)	29.3	25.7	29.9	26.5	2.0	0.230	0.001	0.831
Temperature (6 hr, °C)	16.8	12.2	16.6	12.5	1.5	0.924	0.001	0.508
pH 2 hr	5.93	6.36	5.79	6.53	0.22	0.785	0.001	0.017
Lysosomal pH	5.81	6.09	5.62	6.24	0.20	0.732	0.001	0.007
β-glucuronidase[d]								
soluble (U/g)	0.168	0.167	0.205	0.207	0.052	0.015	0.958	0.932
microsomal (U/g)	0.038	0.060	0.026	0.096	0.021	0.048	0.001	0.000
Total (U/g)	0.440	0.478	0.487	0.606	0.138	0.034	0.055	0.314
Free (%)	81.7	74.7	87.7	67.6	8.0	0.797	0.001	0.006
Cathepsin B								
soluble (U/g)	1.65	1.70	2.00	2.01	0.48	0.020	0.849	0.911
microsomal (U/g)	0.333	0.463	0.206	0.670	0.215	0.379	0.001	0.005
Total (U/g)	7.11	7.52	6.32	7.47	1.77	0.410	0.134	0.471
Free (%)	83.8	79.3	90.5	74.2	7.1	0.704	0.001	0.005
Cathepsin H								
soluble (U/g)	12.9	13.4	10.8	11.9	2.0	0.003	0.202	0.608
microsomal (U/g)	0.136	0.165	0.121	0.207	0.040	0.264	0.001	0.016
Total (U/g)	13.8	14.4	11.8	13.3	2.0	0.007	0.072	0.414
Free (%)	99.0	98.8	98.9	98.2	0.34	0.002	0.001	0.024

[a] E.S. = electrically stimulated.
[b] 8 = extremely tender; 5 = slightly tender; 1 = extremely tough.
[c] Aging index defined as slope of linear regression between ATPase activity and ionic strength.
[d] Enzyme activities expressed as units per g muscle. Total and percentage free activities as described in text.

Aging index (myofibrillar ATPase activity)

Myofibrils were prepared according to the method of Bendall (1961) from bovine longissimus dorsi muscle obtained 24 hr postmortem. Twenty grams well trimmed longissimus dorsi muscle were homogenized in 10 volumes 1 mM EDTA:20 mM imidazole:80 mM KCl:5 mM 2-mercaptoethanol (pH 7.4). After 1 hr extraction and subsequent filtration through cheese cloth, the myofibrils were centrifuged at 5,000 × *g* for 15 min. The pellets were washed three times. The Mg^{2+}:Ca^{2+}-dependent myofibrillar ATPase activity was determined as described by Ouali and Valin (1980). The ATPase activity was measured at 22°C at pH 7.4 with a 0.01N KOH titrate in the presence of 4 mM ATP, 4 mM $MgCl_2$, 0.2 mM $CaCl_2$, and 1–2 mg myofibrils at ionic strengths adjusted with KCl solution ranging from 0.1–0.3M. The biochemical aging index was defined as the slope of the linear regression between the ATPase activity (μl KOH/min/mg protein) and the ionic strength (Ouali, 1984).

Statistical analysis

Simple linear correlation was performed on all combinations of variables. Analysis of variance was used to test the main sex and side effects and interaction between sex and side (Helwig and Council, 1979).

RESULTS

TABLE 1 contains the mean values of each measurement for each group. Meat from control bulls had the highest shear force value and the lowest taste panel tenderness value as compared with the other groups. Pooling the electrical stimulation and control values, and bull and steer data, indicated that meat from steers was more tender than that from bulls, and meat from electrically stimulated sides was more tender than meat from control sides. This agreed with previous observations, as reviewed by Asghar and Henrickson (1982).

Control bull longissimus muscle also had the least aging effect, as measured by the biochemical aging index. Main effect means indicated that electrical stimulation increased ($P<0.05$) the aging effect, and meat from steers displayed a greater aging effect ($P<0.01$) than meat from bulls.

Control sides from both bulls and steers had a lower carcass temperature at 2 and 6 hr postmortem, and higher carcass pH value and pH measured in lysosomal enzyme preparations, as compared to the respective electrically stimulated sides (Table 1). Furthermore, electrical stimulation of steer carcasses resulted in a more rapid decline in pH than in bull carcasses. The pH of the muscle homogenates obtained at 2 h postmortem was consistently higher than the pH of the lysosomal preparation, because iodoacetate was not added to the latter.

Muscle from steers had higher β-glucuronidase and cathepsin B activities, but had lower cathepsin H activity, in the soluble portion than samples from bulls. Electrical stimulation did not affect soluble activities, but decreased the microsomal activities, especially in samples from steers, for all three enzymes. Dutson et al. (1980) also reported lower microsomal enzyme activities in samples from electrically stimulated carcasses relative to nonstimulated control carcasses. Largely because of the decrease in microsomal activities in all three enzymes, the percentage free activity was higher in electrically stimulated samples relative to control values. The majority of the total activity was located in the nuclear (low speed pellet) fraction. Since this fraction contains some contamination by microsomal and supernatant enzymes, it was not included in the calculation of percentage free enzymes.

As expected, simple correlations (Table 2) indicated that shear force and taste panel evaluation of tenderness were highly correlated ($P<0.01$, $r = -0.71$). Aging index was significantly correlated with shear force and taste panel tenderness, as well as with percentage free activities of β-glucuronidase and cathepsin B. Carcass temperatures at 2 and 6 hr postmoretm had the highest correlations with shear force, but were not correlated significantly with taste

Table 2—Simple correlation coefficients for measurements from beef (n=48)

	Shear force	Taste panel	Aging index	Tem 2 hr	Tem 6 hr	pH 2 hr	Lys pH
Taste panel	−0.71*						
Aging Index	0.46**	−0.34*					
Temperature (2 hr)	−0.59**	0.25	−0.25				
Temperature (6 hr)	−0.54**	0.26	−0.30*	0.89**			
pH 2 hr	0.37*	−0.22	0.25	−0.54**	−0.62**		
Lysosomal pH	0.38**	−0.28*	0.24	−0.56**	−0.62**	0.89**	
β-glucuronidase							
soluble	−0.14	0.04	−0.15	0.18	0.11	0.02	−0.07
microsomal	0.29*	−0.22	0.28	−0.42**	−0.54**	0.80**	0.81**
total	0.15	−0.08	0.18	−0.15	−0.21	0.34*	0.32*
% free	−0.38**	0.27	−0.39**	0.51**	0.58**	−0.74**	−0.79**
Cathepsin B							
soluble	0.01	−0.17	−0.07	0.13	0.02	0.03	−0.00
microsomal	0.23	−0.18	0.29*	−0.34*	−0.47**	0.66**	0.69**
total	0.32*	−0.35*	0.35*	−0.14	−0.21	0.32*	0.38**
% free	−0.29*	0.20	−0.37**	0.43**	0.54**	−0.67**	−0.72**
Cathepsin H							
soluble	0.17	−0.30*	−0.19	0.01	0.05	0.13	0.01
microsomal	0.29*	−0.18	0.18	−0.46**	−0.46**	0.64**	0.56**
total	0.17	−0.32*	−0.17	−0.02	0.01	0.22	0.09
% free	−0.17	0.01	−0.25	0.47**	0.49**	−0.57**	−0.57**

* Significant at 0.05 level.
** Significant at 0.01 level.

panel tenderness. The pH of the lysosomal enzyme preparations was more highly correlated with shear force and tenderness than was the pH of the muscle homogenates containing iodoacetate (Table 2). Temperatures were negatively correlated with pH values. Microsomal enzyme activities were negatively correlated with temperature and positively correlated with pH. Conversely, percentage free β-glucuronidase and cathepsin B were positively correlated with temperature and negatively correlated with pH.

DISCUSSION

TEMPERATURE and pH are well established as important factors in postmortem muscle tenderization. Higher temperatures generally will increase enzyme activities and are believed to increase tenderness whereas the basis for the pH effect is still controversial (Dutson, 1983; Marsh, 1983). Differences in pH between muscle from control and electrically stimulated carcasses were enhanced by omitting iodoacetate from the homogenates, thus allowing glycolysis to proceed. This increased the correlation between pH and meat tenderness, further supporting the role of carcass pH in the tenderization process. Since the slaughtering conditions in this study were kept relatively constant, the higher temperature maintained in electrically stimulated sides (about 4°C higher than control at 2 and 6 hr postmortem) was probably due to the heat produced by ATP hydrolysis (Bendall et al., 1976; White et al., 1978).

The decrease in microsomal lysosomal activities elicited by electrical stimulation did not result in increased supernatant activities. Once lysosomal enzymes are released from lysosomes, they are more susceptible to autolytic degradation (Dutson et al., 1980). At the time the activities were measured (2 hr postmortem, plus sample preparation time), some free enzymes could have been degraded; therefore, the decrease in microsomal activities would not have resulted in increased supernatant activities. This is supported by the observed reduction in total enzyme activities in homogenates of samples from electrically stimulated sides relative to those from control sides.

Schworer et al. (1981) reported that rat liver lysosomes in a state of accelerated deprivation fused with early autophagic vacuoles, and the autophagic vacuoles increased in number sigmoidally. Larger lysosomes (autophagic vacuoles) are more sensitive to disruption during homogenization. Therefore, the decrease in microsomal activities in the present study could have been due to disruption of lysosomes either before or during homogenization.

Aging index, the degree of decrease in Ca^{2+}:Mg^{2+} myofibrillar ATPase activity as ionic strength increases, was correlated both with shear force and with taste panel tenderness. According to Ouali and Valin (1980), the decrease in ATPase activity with increasing ionic strength was due to the solubilization of α-actinin released from the Z-lines by calcium activated factor. At low ionic strength, α-actinin activates ATPase activity. As ionic strength increases, α-actinin is solubilized, presumably resulting in the loss of its activating effect on ATPase activity. Incubation with lysosomal enzymes also decreases ATPase activity, but this effect is not dependent upon ionic strength. Thus, the more negative values for aging indexes from electrically stimulated samples relative to control samples suggest that electrical stimulation caused a more rapid release of α-actinin from the Z-lines, which in turn was associated with more tender meat. This relationship also was observed for samples from steers relative to those from bulls.

Another mechanism for the aging index might be related to the actin-myosin interaction. As ionic strength increased, the isometric tension generated by glycerinated muscle fibers decreased (Prochniewicz-Nakayama et al., 1983), as did the Ca^{+2}-binding constant of Ca^{2+}-binding proteins (Permyakov et al., 1983). Free myosin or fragmented myosin containing S-1 heads would be more susceptible to solubilization and loss of interaction with actin than would myosin in thick filament form. Since actin strongly activates myosin ATPase (Taylor, 1979), high ionic strengths may in part reduce ATPase activity by decreasing troponin Ca^{+2}-binding subunit affinity for Ca^{+2}, thus decreasing the actin-myosin interaction. The decrease of ATPase activity would be more apparent in samples containing more free or fragmented myosin, and could explain the association between aging index and meat tenderness.

Because greater percentage of free lysosomal enzyme activities for β-glucuronidase and cathepsin B were significantly correlated with more negative aging indexes, the data suggested that free lysosomal enzyme activities influenced the integrity of the Z-lines or thick filaments in post-

mortem muscle. However, there was no significant correlation between aging index and pH, which would appear to rule out any influence of lysosomal enzymes on aging index, hence tenderness. The effects of carcass pH and temperature on aging index and, ultimately, meat tnederness, thus warrant further investigation.

In summary, aging index, temperature, pH and percentage free activities of β-glucuronidase and cathepsin B were all significantly correlated with shear force. However, the more consistent significant correlation of aging index with both measurements of tenderness (shear force and taste panel) indicated that the aging index might be a superior means to estimate meat tenderness.

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COLOR OF INTACT PRE-RIGOR MUSCLE . . . From page 1024

rigor samples in oxygen were definitely more red at both 3°C and 14°C. Post-rigor samples transferred from oxygen at 3°C to air at 20°C did decrease in redness. Samples in air at 3°C were more red than samples in air at 20°C as is well known in the meat industry (Price and Schweigert, 1978).

In conclusion, intact pre-rigor muscle at high pH will bloom when held at low temperature and in high oxygen concentration. Neither low temperature nor high oxygen concentration alone is sufficient for development of bloom. The bloom achieved is reversible; the muscle turns dark when transferred from oxygen at 3°C to air at 20°C. These observations can be explained on the basis of differences in mitochondrial oxygen consumption, oxygen availability, and oxygen diffusion in the various samples.

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Selected Nutrient Content of Beef Longissimus Muscle Relative to Marbling Class, Fat Status, and Cooking Method

P. T. BERG, M. J. MARCHELLO, D. O. ERICKSON, and W. D. SLANGER

ABSTRACT

Five ribs (IMPS 103) within each marbling class (practically devoid, traces, slight, small, modest and moderate) were analyzed for nutritional and cooking effect. Subjective marbling classification was effective in categorizing beef for dry matter, fat, protein and caloric density. Steaks were cooked with fat removed or left on either by broiling or microwave exposure. Broiled samples generally had more protein, and less fat and calories. Within cooking method, fat on steaks generally resulted in greater sample weight retention, less protein, less calories, more cholesterol, and higher dry matter. Cholesterol content was greatest in steaks with practically devoid marbling and lowest in steaks with modest and moderate marbling. Each subsequent marbling class added less than 10 Kcal/100g ("as is basis"). There was a small loss in calories available for consumption as a result of the cooking process.

INTRODUCTION

AMERICANS have traditionally relied heavily upon red meats as a primary source of high quality protein. Concern over diet relative to perceived health hazards has jeopardized the status of meat as a source of nutrition. The concept that meat is high in fat and cholesterol is widespread. The effect of this perception has resulted in a slight decline in the consumption of red meats, but more importantly, has created an atmosphere of negative attitudes which continue to be reinforced by the usage of outdated data on the nutrient composition of meats. Data generated prior to the 1975 USDA official standards for establishing quality grades not only reflect emphasis on nonnutrient aspects such as conformation and age, but also generally list caloric values for meats based on "nonskeletal tissue." Thus the data are not applicable under the impact of recent changes in animal feeding regimes and genetic changes characterized by the use of more muscular Continental breeds. Grade standards for beef have changed periodically. One can assume that such standards will be changed in the future. The one constant ingredient in the grade standards since 1965 has been the specifications regarding marbling. Previous work at this station (Erickson et al., 1981) indicated a high relationship between visual marbling and sample dry matter, protein, fat and caloric content. Surprisingly, no refereed reference to nutritive value of beef over a contiguous, specific range of marbling could be found although Briskey and Bray (undated) reviewed many studies on the association between grade and/or marbling and eating quality of beef. These references have meaning only within a specific time frame because of the standards in effect at the time of publication. The commonly used grade references generally reflect exaggerated fat percentages, ostensibly due to increased levels of subcutaneous and seam fat associated with USDA choice and prime grades. A common dietary recommendation popular among dieticians and medical practitioners is to "trim all exterior fat." Documentation as to the nutritional effect of this and other recommendations appears to be limited to what can be found through careful study of USDA Handbook 8 or subsequent versions thereof. (Watt and Merrill, 1963). The purpose of this study was to document the effects of cooking method and removal of extraneous fat before or after cooking on selected nutrient composition of samples within contiguous marbling classes.

All authors are affiliated with the Dept. of Animal Science, North Dakota State Univ., Fargo, ND 58105.

MATERIALS & METHODS

FIVE WHOLESALE RIBS (IMPS 103) within each of six marbling classes and within the "A" maturity group (USDA, 1975) were selected at a large packing firm. A USDA grader, as well as two university meat science personnel, carefully evaluated marbling level for each of the ribs. In order for a particular rib to be selected, all evaluators were required to agree that it was "typical" for a specified marbling class. (Typical was defined as "10 marbling units either side of '50" – example: Small 40 - 60). The marbling classes were practically devoid (PD), traces (TR), slight (SL), small (SM), modest (MT) and moderate (MD). The marbling classes corresponding to the USDA prime grade (slightly abundant, moderately abundant and abundant) were excluded from our selection because of the relatively low incidence of prime carcasses within the industry and a general feeling that grading consistency is difficult within these heavily marbled carcasses (Wellington and Stouffer, 1959).

Steaks were cut 2.54 cm thick and allocated to one of four treatment groups: broil with the fat on (BFO), broil with the fat removed (BFR), microwave with the fat on (MFO), and microwave with the fat removed (MFR). Individual steaks were quick frozen and held at −20°C (a maximum of 60 days). Prior to preparation for cooking, steaks were thawed to an internal temperature of approximately 1°C. Prior to cooking steaks assigned to the "fat removed" treatments were reduced to longissimus muscle completely free of subcutaneous fat. Steaks assigned to the "fat on" treatment groups were trimmed to a fat cover of approximately 0.6 cm and this fat cover was removed after cooking. Assignment of contiguous steaks from a specific rib depended upon random assignment of the most caudal steak. The procedure followed for preparation prior to cooking was similar for all cooking methods.

The cold sample was weighed and the initial temperature recorded. Microwave samples were cooked in a Panasonic model NE-9900 700 watt. The power setting was approximately 360 watts with a temperature sensing probe set at 68°C. Cooking time was 7 min for MFR and 15 min for MFO. If the internal temperature at the end of the specified time was less than 65.5°C samples were cooked an additional minute.

Broiled samples were prepared on a Farberware Open Hearth Grill (model 450 A). Cooking time was approximately 10 min per side for BFR and 15 min per side for BFO. Broil samples were removed from the broiler at approximately 65.5°C. For all samples the final temperature was recorded and the sample allowed to cool for approximately ½ hr to a constant temperature of roughly 27°C. The cooked, cooled samples were reweighed, those samples cooked with the fat on were trimmed of all extraneous fat and the samples were lyophilyzed.

Samples were analyzed for dry matter by oven drying at 105°C until a constant weight was reached or approximately 18 hr. Protein was determined by the macro-Kjeldahl method (AOAC, 1980). Fat content determination was by the Foss-let procedure (AOAC, 1980) and sample gross energy was determined by bomb calorimetry as described in the Parr 1241 Oxygen Bomb Calorimeter Manual. Total lipids of tissues were extracted gravimetrically with a chloroform-methanol mixture (2:1) using the basic procedure described by Folch et al. (1957). Cholesterol from lipid extracts was analyzed by the acetic anhydride-sulfuric acid colorimetric method of Stadtman (1957).

Statistical analysis was primarily by use of Statistical Analysis Systems, 1982 (SAS, 1982). Graphic plots illustrate the various comparisons by both marbling class means and treatment method means. The test for significant differences between means was by the method of Tukey (1953). Probability levels for each of the comparisons are reported on the respective figures; marbling class comparisons are opposite the vertical axis. Superscript notation denotes a difference between means at $P < 0.05$ probability level when the superscripts are different among the means along either the vertical or horizontal axis. The probabilities for significant interaction are also noted on each of the figures.

A split-plot analysis (Gill and Hafs, 1971) was used because ribs were nested within marbling class with each rib providing a sample for each of the treatment methods. The rib within marbling class mean square was used as the error term for statistical tests associated with marbling class. Method of cooking (broil vs microwave) as well as fat status (fat removed prior to or after cooking) within cooking method, was analyzed for significance using orthogonal comparisons.

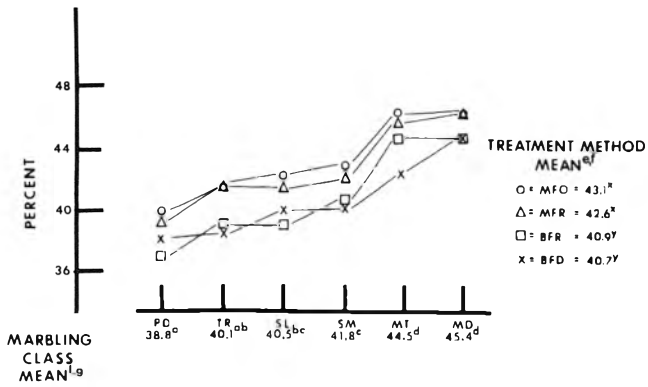


Fig. 1—Dry matter (percent) by marbling class and treatment method. PD = Practically devoid; TR = Traces; SL = Slight; SM = Small; MT = Modest; MD = Moderate; MFO = Microwave cooked, fat on; MFR = Microwave cooked, fat removed; BFO = Broiled, fat on; BFR = Broiled, fat removed.
a,b,c,x,yMeans with different superscript(s) differ according to Tukey's test ($P < 0.05$)
e,gF test from ANOVA significance ($P < 0.0001$)
fF test for interaction ($P < 0.57$)

RESULTS & DISCUSSION

THE PROGRESSION of marbling from PD to MD, with very few exceptions, resulted in progressively higher dry matter, fat and energy but lower protein. Fig. 1 shows the dry matter content of the samples. Except for dry matter content, all comparisons were on a dry weight basis in order that the variation and influence of moisture content of the sample would be eliminated. The assumption that "as is" would be "as eaten" is valid and references will be made to the "as is" basis where appropriate. Conversion to the "as is" basis for any marbling class or treatment may be obtained by multiplying by the appropriate dry matter mean.

As expected, as marbling class increased dry matter also increased (Fig. 1). This is similar to the results of Walter et al. (1965), although they reported moisture content and showed an increase between PD and, TR but then a steady decrease through slightly abundant (SA). Statistically significant differences among the marbling class means did not strictly follow USDA quality grade lines. The MT and MD classes showed higher dry matter content ($P < 0.05$) than the other classes but among the four remaining classes only PD and SM were significantly different. Conversion of the marbling class designations into their appropriate USDA quality grades may be accomplished easily because all samples were from carcasses within the "A" maturity group. PD and TR represent the Standard grade, SL is the only marbling class for the Good grade and SM, MT and MD are the marbling classes which make up the Choice grade (USDA, 1975).

Cooking method had an effect on moisture loss. Samples cooked by microwave had more ($P < 0.0001$) dry matter than those broiled. For the microwave, samples cooked with fat on averaged slightly more ($P < 0.09$) dry matter than those cooked with fat off. Fat status had no effect on broiling results ($P < 0.52$). No interaction between marbling class and cooking method was found ($P < 0.57$).

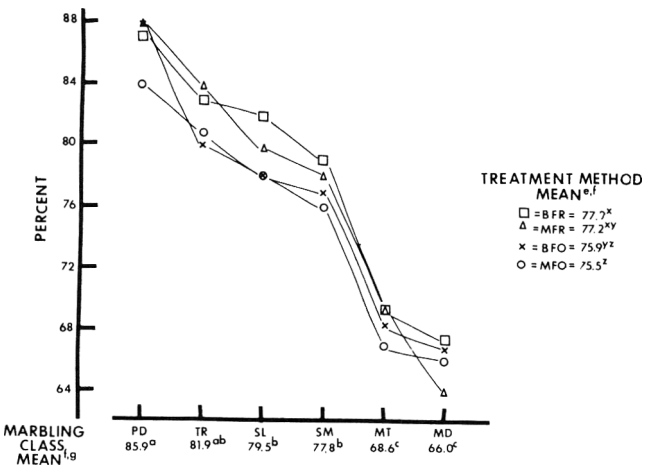


Fig. 2—Protein percent on a dry weight basis by marbling class and treatment method. PD = Practically devoid; TR = Trace; SL = Slight; SM = Small; MT = Modest; MD = Moderate; MFO = Microwave cooked, fat on; MFR = Microwave cooked, fat removed; BFO = Broiled, fat on; BFR = Broiled, fat removed.
a,b,c,x,y,zMeans with different superscript(s) differ according to Tukey's test ($P < 0.05$)
e,gF test from ANOVA significance at $P < 0.0001$
fF test for interaction ($P < 0.06$)

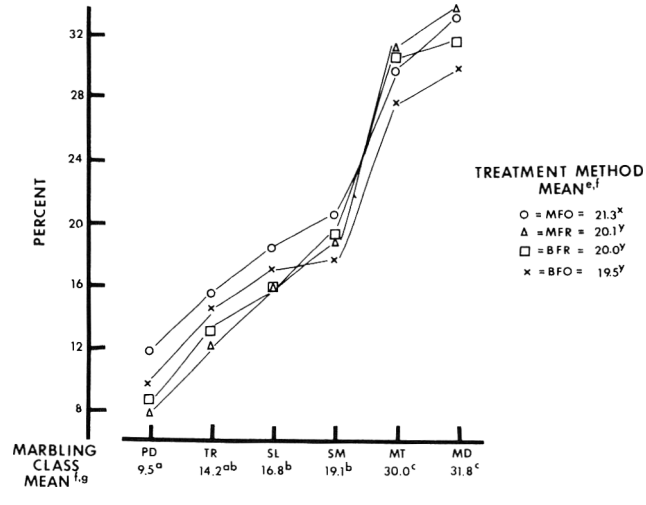


Fig. 3—Fat percent on a dry weight basis by marbling class and treatment method. PD = Practically devoid; TR = Traces; SL = Slight; SM = Small; MT = Modest; MD = Moderate; MFO = Microwave cooked, fat on; MFR = Microwave cooked, fat removed; BFO = Broiled, fat on; BFR = Broiled, fat removed.
a,b,c,x,yMeans with different superscript(s) differ according to Tukey's test ($P < 0.05$)
eF test from ANOVA significance $P < 0.0002$
fF test for interaction $P < 0.0001$
gF test from ANOVA significance $P < 0.0001$

Fig. 2 and 3 summarize the results for percent protein and fat, respectively. Tukey's comparison of the sample means by marbling class for protein and fat showed identical significance relationships. As expected, as the marbling increased, protein decreased and fat increased. PD samples had more ($P < 0.05$) protein and less fat than any of the marbling classes except TR. Furthermore, within the Choice grade, SM samples had greater protein and less fat than did MT and MD ($P < 0.05$). No differences were observed among the TR, SL and SM samples.

Samples broiled with the fat removed contained the highest average protein percentage. BFR had higher ($P < 0.05$) protein content than BFO and MFO samples; however, MFR was higher ($P < 0.05$) than only MFO. The trend was that samples cooked with the fat removed had higher protein values than samples cooked with fat on. The test for marbling class by treatment method interaction was $P < 0.06$.

Broiled samples contained less ($P < 0.001$) fat than those cooked by microwave. Fat status was not a significant factor among the broiled samples. The test for treatment method by marbling class interaction was $P < 0.0001$. MFO samples averaged higher ($P < 0.05$) for fat than all other treatment methods. Samples cooked with the fat removed tended toward lower fat content at the lower levels of marbling but averaged higher than those samples cooked fat on for the upper two marbling classes. The lower fat content of the broiled samples was especially evident in the MD marbling class.

It is difficult to compare our results with many previously published reports because most other results are on an "as is" basis and do not give the dry matter values necessary for comparison on a constant dry matter basis. Breidenstein et al. (1968) reported fat content for SL and MT to be 14.3 and 20.4, respectively. Their values are lower than ours, especially for the MT marbling class.

Assortment by marbling class is, in fact, visual grading for fat content and within our data is quite exact. The simple product-moment correlation coefficient between marbling class and fat content was 0.91 and between marbling class and protein content was −0.89. It is obvious from Fig. 2 and 3 that if our visual appraisal of typical SM had been slightly more lenient and the appraisal for typical MT slightly more restrictive, the graph lines would have been virtually linear with a correspondingly higher correlation coefficient. (More fat was selected in the MT class and less fat in the SM class than was consistent with caloric values found for the other marbling classes). The largest increase in fat percentage (correspondingly greatest decrease in protein) was between the SM and MT classes. This suggests that since both SM and MT are within the Choice quality grade that within this selection of samples more variation within a single grade exists than is desirable.

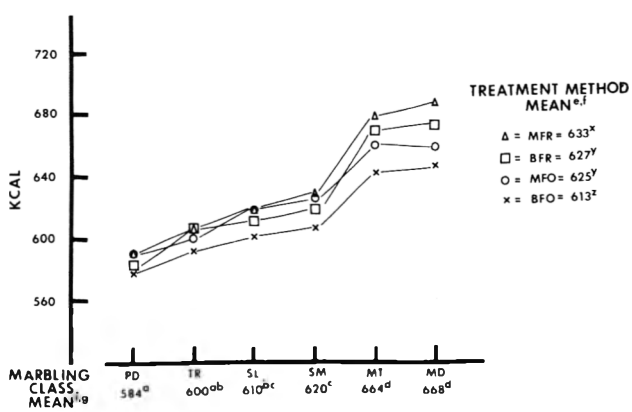


Fig. 4—Gross energy in kcal/100g dry weight basis by marbling class and treatment method. PD = Practically devoid; TR = Traces; SL = Slight; SM = Small; MT = Modest; MD = Moderate; MFO = Microwave cooked, fat on; MFR = Microwave cooked, fat removed; BFD = Broiled, fat on; BFR = Broiled, fat removed.
a,b,c,d,x,y,zMeans with different superscript differ according to Tukey's test ($P < 0.05$)
e,gF test from ANOVA significance $P < 0.0001$
fF test for interaction $P < .57$

Fig. 4 shows the gross energy values of the samples and emphasizes the difference in fat content between marbling classes. Again the largest increase relative to marbling was between SM and MT. Gross energy of beef results almost exclusively from fat and protein. The regresson coefficients of Atwater and Bryant (1900) of 4 Kcal/g of carbohydrate and protein and 9 Kcal/g for fat is commonly used to calculate energy content of various foods. (Although these values are really "physiological fuel values" not gross Kcal as such.) Our data suggest caution should be exercised before categorically accepting these regression factors (β-values) as absolutes. For example, regression models limited to fat and protein on an "as is" basis produced the results shown in Table 1 when analyzed within treatment method as compared to the combined data of all treatment methods.

All the R-square values indicate that a very high proportion of the variation within each treatment method is explained by the regression model; however, considerable differences exist in the magnitude of the regression coefficients, depending on treatment method. For the pooled data, the β-value for fat is very near the commonly used value of Atwater and Bryant (8.7 vs 9). Our value for protein was 5.2 Kcal/g which is uncorrected for caloric loss due to urea synthesis. Our data agrees with that of Atwater and Bryant at a value of 4 Kcal/g of protein when the commonly used value of 1.2 Kcal for urea synthesis is subtracted.

BFO samples had fewer ($P < 0.001$) calories than other treatment method samples. The obvious explanation would appear to be that inasmuch as BFO samples lost the least moisture in cooking, a sample of BFO cooked meat would be less calorically dense because of the additional water when compared to samples cooked by other means. Even though our procedure called for the entire lean sample to be lyophilized prior to being portioned for chemical or calorimetric analysis, differences in dry matter content of the original cooked sample may be reflected in the caloric density of the dried sample. The range in dry matter of the freeze dried samples was from 95 - 99% and variation appeared to be random rather than associated with a particular treatment method.

Moss et al. (1982) stated that samples which were portioned raw and then cooked had greater caloric density than an analogous weight of raw sample. They suggested

Table 1—Regression analysis summary by treatment method for a regression model limited to fat and protein content on the "as is" basis

	Treatment method				Ignoring cooking method
	MFR[a]	BFR[b]	MFO[c]	BFO[d]	
Intercept	119.0	15.9	31.5	10.3	22.0
β-value for fat	7.8	8.6	8.9	8.7	8.7
β-value for protein	2.5	5.3	4.8	5.5	5.2
R-square	98.2	98.7	97.7	99.1	99.5

[a] MRF = Microwave cooked, fat removed
[b] BFR = Broiled, fat removed
[c] MFO = Microwave cooked, fat on
[d] BFO = Broiled, fat on

the additional calories were due to infusion of fat during the cooking process. Our data do not support the contention of fat infusion due to cooking. We made our analysis by determining the calories left after cooking a steak compared to the calories found in a similar steak on the raw basis. The effect of drying during the cooking process as shown by retained sample weight is summarized in Fig. 5. No clearcut association with marbling class was apparent, although some significant differences among the marbling means were observed.

Microwave cooked samples retained less ($P < 0.0001$) weight than broiled regardless of presence or absence of fat during the cooking process. The test for interaction between marbling class and treatment method was $P < 0.14$. However, the graphic depiction for the microwave samples showed a distinct tendency toward an increase in retention of the original sample weight as marbling increased while with the broiled steaks a much less predictable trend was established. BFO samples retained a greater ($P < 0.04$) proportion of the original sample weight than fat removed samples. However, the graphic depiction of the various broiled marbling means revealed a highly erratic pattern.

Table 2–Caloric retention per 100g of raw portioned product by marbling class

Marbling class	KCal/100g raw[a] (A)	KCal available for consumption[b] (B)	Loss from cooking (Kcal/100g) (A – B)
Practically devoid	154	(227x0.66)=150	4
Traces	167	(240x0.66)=158	9
Slight	175	(247x0.67)=166	9
Small	173	(259x0.66)=172	1
Modest	207	(295x0.67)=198	9
Moderate	211	(304x0.69)=209	2

[a] Caloric values determined by Bomb calorimeter (Parr 1241).
[b] Average calories determined from cooked lean for each of the marbling classes multiplied by the average sample retention appropriate for that marbling class.

Table 2 shows the caloric content of beef lean on a raw, "as is" basis as compared to the calculated energy content of the retained weight of a corresponding sample within marbling class. The calculation was Kcal/100g of the cooked sample multiplied by the retained weight of that sample compared to the caloric content of a 100g sample raw. Harrold et al. (1981) showed, through in vitro studies, that beef protein was completely digested so a digestibility factor need not be added to the calculation of estimated consumable calories.

Obviously, if the steak was portioned after cooking, it would not be necessary to calculate consumable calories because the appropriate treatment method results could be used. In all cases, when group averages (either marbling or treatment means) were used to calculate consumable calories, a small loss in calories from the raw to cooked state was demonstrated. Apparently, on the average, more calories (fat) were lost as drip loss than were gained through infusion.

No consistent pattern was demonstrated for cholesterol levels relative to marbling class (Fig. 6). PD samples were highest (211 mg/100g while MT and MD were lowest (180 mg/100g each). Although there is visually little difference in the amount of marbling between PD and TR, the greatest difference in cholesterol content between adjacent marbling class means was demonstrated between these two ($P < 0.05$). The standard error of the mean for each of the marbling class means was low; the largest was for SL at 10.6 while MD and MT samples were the most uniform with standard errors of 4.2 and 5.3, respectively. Our mean values by marbling class were slightly lower than those reported by Rhee et al. (1982). Furthermore, our data do not agree with their indication of progression toward higher cholesterol levels with increased marbling.

Broiled samples had less ($P < 0.0001$) cholesterol than microwave cooked samples regardless of fat status. Within cooking method, those samples with the fat removed prior to cooking had less ($P < 0.0001$) cholesterol than samples cooked with the fat on. The MFO, BFO and MFR plots of mean values generally showed agreement of "line direc-

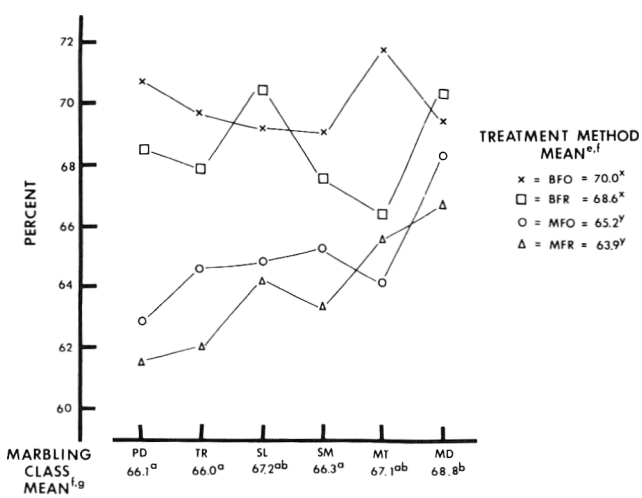


Fig. 5–Sample weight retention in percent by marbling class and treatment method. PD = Practically devoid; TR = Traces; SL = Slight; SM = Small; MT = Modest; MD = Moderate; MFO = Microwave cooked, fat on; MFR = Microwave cooked, fat removed; BFO = Broiled, fat on; BFR = Broiled, fat removed.
[a,b,x,y]Means with different superscript(s) differ according to Tukey's test ($P < 0.05$)
[e]F test from ANOVA significance $P < 0.0001$
[f]F test for interaction $P < 0.14$
[f]F test from ANOVA significance $P < 0.014$

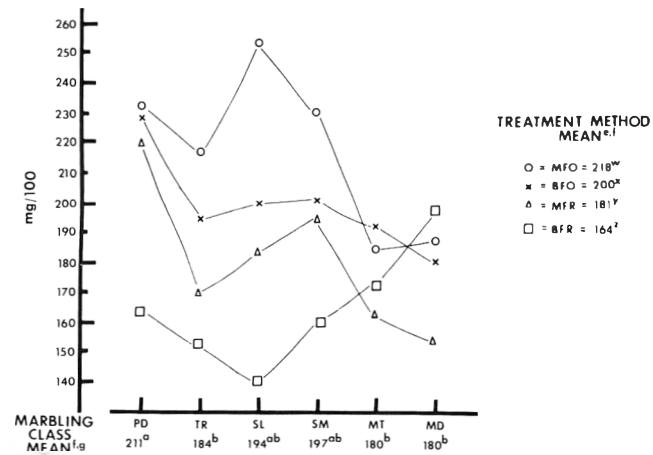


Fig. 6–Cholesterol (mg/100g) by marbling class and treatment method. PD = Practically devoid; TR = Traces; SL = Slight; SM = Small; MT = Modest; MD = Moderate; MFO = Microwave cooked, fat on; MFR = Microwave cooked, fat removed; BFO = Broiled, fat on; BFR = Broiled, fat removed.
[a,b,w,x,y,z]Means with different superscript(s) differ according to Tukey's test ($P < 0.05$)
[e,g]F test from ANOVA significance $P < 0.0001$
[f]F test for interaction $P < 0.0001$

tion" for each subsequent marbling class. The plot of samples cooked BFR showed agreement with the other cooking methods only between marbling classes PD and TR. This apparent inconsistency cannot be explained by sample retention nor through the behavior of this cooking method for other nutrient analysis. Drip loss was not saved for chemical analysis but it would be difficult to imagine that there would be a significant difference associated with treatment method for any nutrients found in drip loss. It is possible that the increase in fat due to marbling may in some way "protect" the cholesterol in the BFR cooking method; however, the opposite phenomenon would then be true for the MFR samples as the largest increase in sample fat was between SM and MT, but a steep decline in cholesterol was shown between these two marbling classes. Furthermore, Fig. 3 clearly shows an increase in sample fat content from PD through SL, but the cholesterol content of BFR samples declined from PD through SL.

SUMMARY

SUBJECTIVE MARBLING classification can be an effective means of categorizing beef for several nutritive components, most notably fat, protein and caloric density.

Although marbling class is highly associated with fat content of the lean tissue as well as with the caloric density of the sample, each subsequent marbling class added an average of less than 10 Kcal/100g ("as is" basis) regardless of whether the calories are calculated from the retained cooked weight or from the raw steak.

Cooking steaks with the fat on versus fat removed resulted in lean tissue samples with a higher level of cholesterol, although this increase was not consistent across all marbling classes. Additionally, cooking with the fat on generally revealed a lower caloric density in the lean than samples cooked with the fat removed.

Broiled samples had greater cooked weight retention, lower dry matter content, lower calories per unit of weight and less cholesterol than did microwave cooked samples.

No well defined trend in cholesterol content of the samples relative to marbling class could be demonstrated. There was a general trend toward lower cholesterol as marbling class increased for all treatment methods except BFR. Significant differences were shown among the treatment methods for choelsterol. Generally, samples cooked with fat removed showed lower cholesterol levels and within the fat status groups, those samples broiled had less cholesterol than the microwave cooked samples.

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Relationship Between Functional (Fat Binding, Emulsifying) and Physicochemical Properties of Muscle Proteins. Effects of Heating, Freezing, pH and Species

EUNICE LI-CHAN, S. NAKAI, and D. F. WOOD

ABSTRACT

Properties of proteins in salt extracts from beef top round and rockfish fillet changed upon heating and were dependent on pH, freezing, species and sample variability. Generally, heating to >45°C, freezing or lower pH (5.5) decreased dispersibility, increased surface hydrophobicity and slightly decreased sulfhydryl content. Emulsifying and fat binding properties followed parallel trends, being improved under conditions favoring combinations of high dispersibility, hydrophobicity and sulfhydryl content (i.e., heating at <45°C, higher pH, unfrozen). However, high temperature-low pH heating improved fat binding and favored gelation, which might be explained by hydrophobic interactions. Significant ($P<0.001$) multiple regression models were obtained describing relationships between physicochemical and functional properties of beef and fish proteins.

INTRODUCTION

FUNCTIONAL PROPERTIES of muscle proteins often dictate final quality of comminuted meat products. However, biological variability, ante- and post-mortem conditions and species differences can lead to difficulties in product formulation and quality control. Moreover, reliable and standardized methods for determining functionality of ingredients are lacking.

Our recent research has focussed on structure-function relationships of food proteins and emphasized the importance of protein hydrophobicity, charge and molecular structure on functional properties (Nakai, 1983; Nakai et al., 1984). These studies are now being applied to meat protein systems in response to the need to understand and enable prediction of functionality in comminuted products, based on simple and reproducible measurements of basic properties of muscle protein extracts, including hydrophobicity, charge and molecular structure relating parameters (e.g., sulfhydryl content). Emulsifying properties of beef top round proteins in salt extracts were more accurately described by multiple regression models involving both hydrophobicity and solubility parameters ($R^2 = 0.917$, $n = 26$, $P<0.001$), compared to regression with solubility alone ($r^2 = 0.730$, $n = 26$, $P<0.001$) (Li-Chan et al., 1984; Nakai et al., 1984). Studies on pre- and post-rigor beef neck muscle extracts demonstrated that as post-slaughter time before extraction increased, an increase in hydrophobicity was correlated with decrease in salt-extractable protein and apparent loss of brine holding capacity (Wood et al., 1984).

Heating is an integral part of comminuted meat manufacture. However, the effects of increasing temperature may depend on numerous factors, including: (1) pH of the muscle, which could range from 6.8–7.0 in pre-rigor processing to 5.4–6.0 in post-rigor processing (Ziegler and Acton, 1984); (2) frozen storage of the muscle prior to use; (3) species differences; and (4) variability between samples, which might be attributed to inherent biological differences, pre-slaughter stress-related factors or varying post-slaughter handling conditions.

The objective of the present study was to further investigate the relationships between functional and physicochemical properties of muscle proteins. Although solubility or dispersibility is usually classified as a functional property, it is often used as an indicator of other functional properties. In this study, importance of dispersibility, hydrophobicity, sulfhydryl content and net charge was investigated with respect to fat binding and emulsifying properties. Particular emphasis was centered on the effects of pH, heating, freezing and species differences on the structure-function relationships of the muscle proteins.

MATERIALS & METHODS

Materials

Beef top round was purchased fresh from a local supermarket. Live rockfish (*Sebastes*) was purchased from a local fish market. cis-Parinaric acid (CPA) was from Molecular Probes (Junction City, OR), while the magnesium salt of 1-anilinonaphthalene-8-sulfonic acid (ANS) was prepared from the technical grade sodium salft (Eastman Kodak Co., Rochester, NY), according to the method of Weber and Young (1964).

Preparation of meat extracts

Salt extracts of beef samples were prepared in 0.01M sodium phosphate buffer (pH 7), containing 0.6M NaCl, 1 mM $MgCl_2$ and 0.04% sodium azide, as previously described (Li-Chan et al., 1984). Salt extracts of fillets from the rockfish samples were similarly prepared, except that the rockfish tissue was passed only once through the grinder. Extracts were adjusted to pH 5.5-7.0 using 0.1N NaOH or HCl prior to heat treatment or freezing.

Heat treatment

The meat extracts after pH adjustment were heated by incubating 100 mL aliquots in Erlenmeyer flasks in water baths at the specified temperatures from 35° to 85°C for 30 min. After cooling, coagulated beef top round extracts were homogenized with a Brinkmann Polytron homogenizer (PT 20 probe generator, Brinkmann Instruments, Rexdale, Ont.) at approximately 2000 rpm for 15 sec. Rockfish samples did not yield a dense coagulum as did some beef samples; the gels produced on heating rockfish extracts could be dispersed by gentle stirring with a rod.

Freezing of rockfish extracts

One batch of pH-adjusted rockfish extracts, consisting of heated (as described above) and unheated samples, was frozen in individual Whirlpac polyethylene bags at −10°C for 11 days, then slowly thawed at 4°C. After thawing, samples which had been heated prior to freezing were observed to contain gel- or fibrous-like masses of protein. A Wheaton hand held homogenizer was used to gently disperse the insolubilized masses into more homogeneous suspensions to facilitate representative sampling.

A second batch of pH-adjusted extracts, consisting only of unheated samples, was frozen at −10°C for 19 days. After thawing at 4°C, weak gels were observed, which could be dispersed easily by gentle stirring with a rod. Aliquots of the dispersed, thawed extracts were then heated as described above.

Physicochemical properties

Dispersibility (D). Aliquots of extracts and heated samples were centrifuged at 1100 x g for 10 min. The supernatants and their

Authors Li-Chan and Nakai are affiliated with the Dept. of Food Science, Univ. of British Columbia, Vancouver, B.C. Canada. V6T 2A2. Author Wood is with the Food Research Institute, Agriculture Canada, Ottawa, Ontario, Canada, K1A 0C6.

uncentrifuged controls were analyzed for protein using the biuret-phenol spectrophotometric method (Brewer et al., 1974). The % dispersible protein was calculated as: 100 x (protein of supernatant)/(protein of uncentrifuged control). These values were defined in this paper as "dispersibility" to distinguish from "solubility," used previously (Li-Chan et al., 1984) for samples centrifuged at 27,000 x *g* for 30 min.

Hydrophobicity (S_0ANS, S_0CPA). Protein surface hydrophobicity (S_0) was determined essentially as previously described (Li-Chan et al., 1984; Kato and Nakai, 1980), using two fluorescent probes. Protein extracts were diluted to 0.001–0.020% protein using 0.01M phosphate buffer (pH 5.5 to 7.0) containing 0.6M NaCl, 1 mM $MgCl_2$ and 0.04% Na azide. Ten microliters of ANS (8.0 mM in 0.1M phosphate buffer, pH 7) or of CPA (3.6 mM in absolute ethanol containing butylated hydroxytoluene) was added to 2 mL of diluted protein. ANS-protein conjugates were excited at 390 nm and the relative fluorescence intensity values (RFI) were measured at emission wavelength of 468 nm on an Aminco Bowman spectrofluorometer, No. 4-8202 (American Instrument Co., Inc., Silver Spring, MD); RFI was adjusted to 30 in 100 full scale for the "standard" consisting of ANS in methanol. CPA-protein conjugates were excited at 325 nm and RFI measured at 420 nm; RFI was adjusted to 75 in 100 full scale for the "standard" consisting of CPA in decane. "Protein blank" RFI values were measured for each protein dilution in the absence of ANS and CPA. The initial slopes of net RFI versus % protein plots were calculated by linear regression analysis using a Monroe 1880 programmable calculator and were defined as S_0ANS or S_0CPA.

Sulfhydryl (SH, μM/g protein) was determined spectrophotometrically using Ellman's reagent, as described by Li-Chan (1983).

Net charge density (ZP). Protein dispersions were prepared by homogenizing 5 mL protein extract (approximately 0.1% protein) with 0.15 mL 3,3'-dimethylbiphenyl (Aldrich Chemical Co., Milwaukee, WI), using the Brinkmann Polytron at 2200 rpm for 20 sec. The resulting emulsions were diluted 50-fold with 0.01M phosphate buffer at the appropriate pH, with specific conductance of 2.5 mmhos/cm. Net charge density was measured as the zeta potential (ZP, mV) with a Lazer Zee model 501 meter (Pen Kem Inc., Bedford Hills, NY).

Functional properties

Emulsifying properties. Emulsifying capacity (EC, mL oil/0.2g) was measured by oil titration of salt extracts containing 30 mg protein/100 ml, as previously described by Li-Chan et al. (1984). Emulsifying activity index (EAI, m^2/g) was determined by the turbidimetric method of Pearce and Kinsella (1978) as modified by Li-Chan et al. (1984), except that emulsions were prepared using 3 mL of 0.5% protein and 1 mL of corn oil, homogenized at approximately 1750 rpm for 1 min in a Sorvall Omnimixer with microattachment assembly. Emulsifying stability index (ESI, min) of the emulsions prepared for EAI determination was defined as the time in min for A_{500} to decrease to one-half of that of the emulsion at zero time.

Fat binding capacity (FBC). The turbidimetric method for FBC (mL oil/g) determination of Voutsinas and Nakai (1983) was modified, as shown in Fig. 1. The major modifications were: (1) use of salt extracts (0.5% protein) instead of freeze-dried protein (preliminary experiments showed inferior functionality after freeze-drying); (2) vortex instead of sonication for mixing protein with oil (sonication produced highly viscous emulsions); (3) incorporation of an additional centrifugation at 17,300 x *g* for higher protein recovery and unbound oil removal. Standard curves of A_{600} versus oil were constructed for each protein extract, using known quantities of oil (from 0 to 120μL) homogenized with protein extract in digestion medium. Curve fitting analyses indicated that the standard curve data could be fit well to a quadratic model, $Y = a_0 + a_1X + a_2X^2$ (R^2 = 0.98–0.99, Y = A_{600}, X = microliters of oil). Except for extracts at pH 5.5 heated at >60°C, the majority of protein extracts yielded similar standard curves, with values in the range of 0.014 and −0.0001 for the regression coefficients a_1 and a_2, respectively, and values ranging from 0.02 for unheated to 0.10 for heated extracts for a_0, the term in the model which corresponds to A_{600} of the protein extract with no oil addition.

Statistical analysis

Backward stepwise multiple regression analyses were carried out on an Amdahl 470 V/8 computer by using the U.B.C. Triangular Regression Package. Only quadratic models were analyzed, i.e., potential independent variables to be considered in the regression equation included the individual protein parameters (S_0ANS, S_0CPA, D, SH, ZP), their squared terms, and their two-factor interactions.

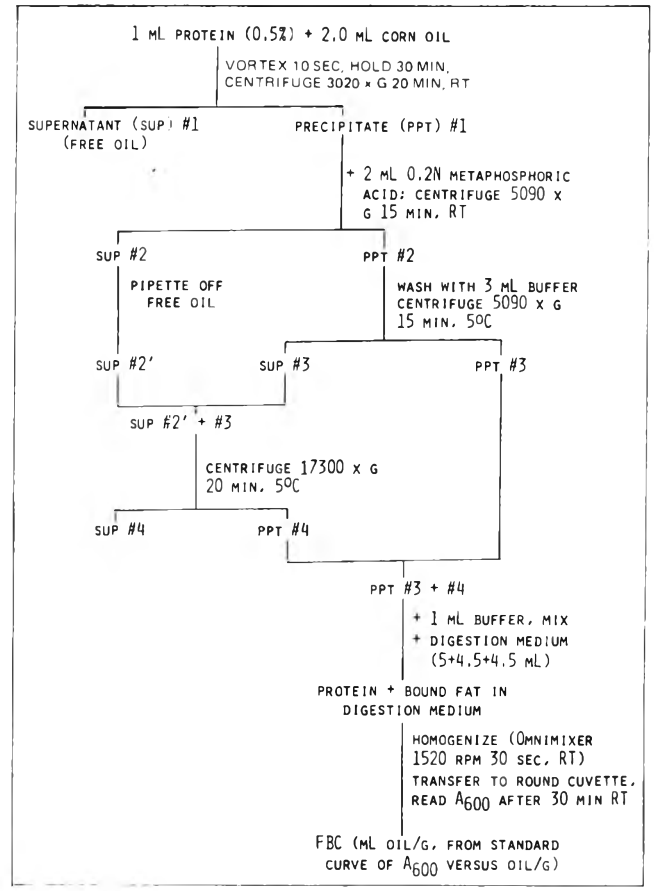


Fig. 1—Flow diagram of turbidimetric method for determination of fat binding capacity (FBC) of muscle protein extracts.

RESULTS

Beef top round—Effects of heating and pH

Salt extracts were prepared from five different samples of beef top round purchased from the same local supermarket over a period of 15 months; an additional sample was purchased from a local meat market. Considerable variability among samples was noted in their physicochemical and functional properties, as well as in the effects of heating and pH on these properties. The pH values of the samples suspended in 1% brine ranged from 5.4 to 6.3. Extracts prepared from samples with pH 6.0–6.3 generally had lower S_0CPA and higher emulsifying and fat binding properties than those extracts prepared from samples with pH 5.5–5.7. These results suggest that proteins extracted from muscle with a higher pH were less denatured and had better functional properties. These samples may not have reached as advanced stage of rigor mortis as did samples which had lower pH. It has been generally observed that pre-rigor muscle proteins have better functionality than post-rigor muscle proteins and that extractability of proteins decreases with post-mortem storage time. Even among samples with similar pH, however, some variability was observed, indicating that numerous factors other than stage of rigor and ultimate pH of the muscle play important roles in influencing protein properties and quality of the final product.

Fig. 2 and 3 represent data for the physicochemical and

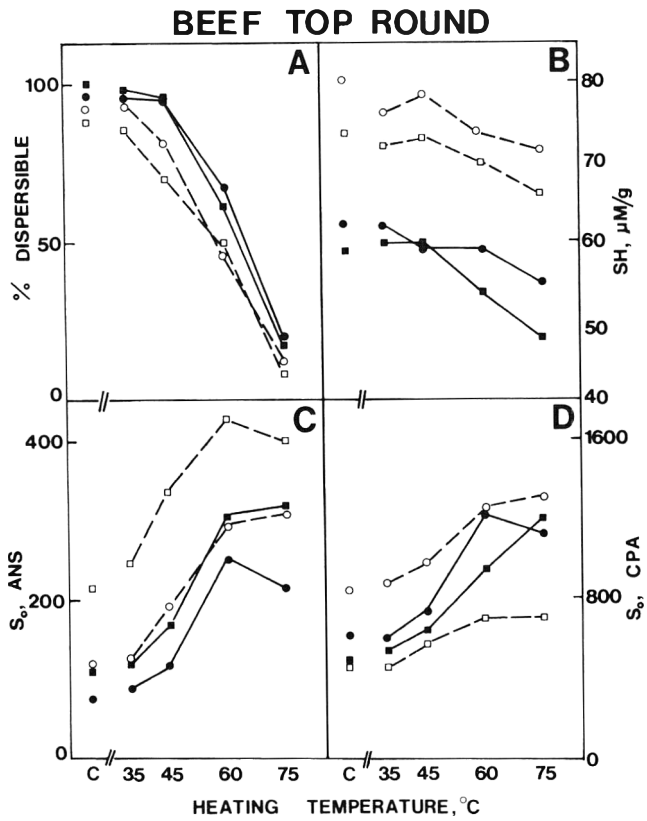


Fig. 2—Dispersibility (A), sulfhydryl content (B) and hydrophobicity (C,D) of beef top around extracts as a function of heating temperature. (Closed symbols, pH 7.0: ● sample 1, ■ sample 2. Open symbols, pH 5.5: ○ sample 1, □ sample 2).

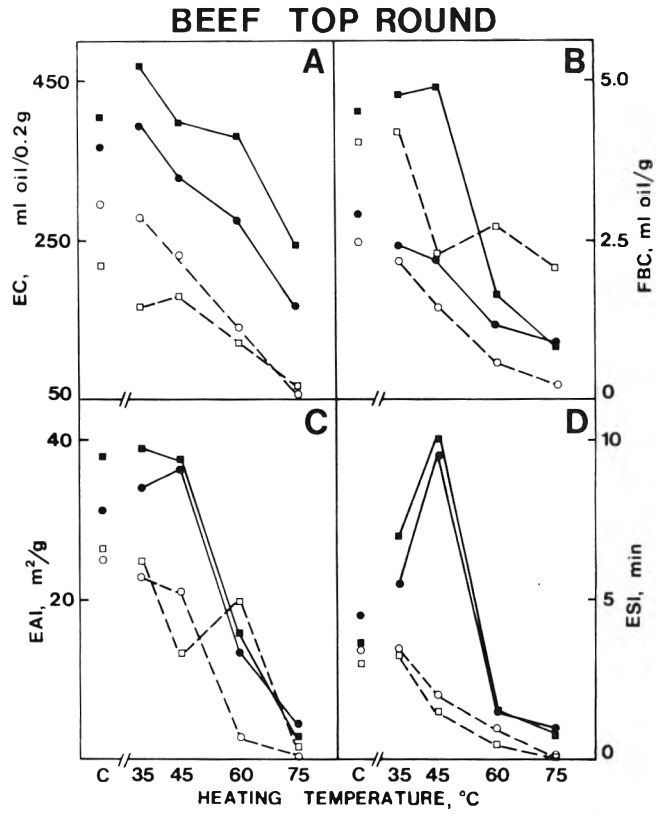


Fig. 3—Emulsifying (A,C,D) and fat binding (B) properties of beef top round extracts as a function of heating temperature (Symbols are as defined in Fig. 2).

functional properties of extracts from two samples of beef top round (pH in brine was 5.43 and 5.65 for samples 1 and 2, respectively). In spite of the variability between samples, some general trends of the effects of heating and pH were apparent. Specifically, upon heating to temperatures of 45°C or lower, hydrophobicity determined as S_0ANS or S_0CPA increased (Fig. 2C, 2D), while SH (Fig. 2B) and dispersibility (Fig. 2A) remained fairly constant, particularly at pH 7.0. Under these conditions, all of the functional properties investigated (EAI, ESI, EC, FBC) were generally high (Fig. 3A-D). Indeed, in some cases, functional properties after heating at 35°C and 45°C were improved over the unheated control, suggesting that controlled denaturation without accompanying coagulation or insolubilization, may allow the protein molecule to unfold thus exposing hydrophobic regions which may improve functionality. However, at temperatures above 45°C, marked decreases in dispersibility and SH and increases in S_0ANS and S_0CPA were observed, insolubilization was initiated and the functional properties generally deteriorated with increasing temperature.

With respect to pH, generally lower dispersibility, higher SH and higher S_0ANS and S_0CPA were observed in samples at the lower pH of 5.5. Functional properties were, on the whole, poorer at pH 5.5 than at higher pH. The isoelectric points of the salt-soluble myofibrillar proteins fall in the pH range 4.5–5.5. Thus it would be expected that as the pH of the extracts approached the isoelectric point of the proteins, the net charge density would approach zero, reducing electrostatic repulsion and facilitating insoluble aggregate formation. Absolute values of net charge density, measured as zeta potential (ZP, mV) were greater at higher pH, than at pH close to 5.5. However, little change in ZP values was observed on heating. It should be noted that ZP measurements were performed with protein suspensions diluted to low ionic strength (Specific conductance of 2.5 mmhos/cm). However, the other properties of the extracts were determined at high ionic strengths, in buffer containing 0.6M NaCl. Under high ionic strengths, the protein charge effects were minimized.

Backward stepwise multiple regression analyses were carried out on the data from beef top round extracts, and the results are shown in Table 1. Using D, S_0ANS, S_0CPA, SH and their quadratic terms as potential independent variables, highly significant ($P<0.001$) regression models were obtained to describe EC, FBC, EAI and ESI. Generally, it can be concluded that the emulsifying properties were positively correlated to D, SH and S_0. However, some square and interaction terms were also significant predictors in the regression equations, indicating that an optimum balance of dispersibility, hydrophobicity and structural parameters was needed for best functionality, rather than merely high values for each individual parameter. These results support the concept that optimal protein functionality is described by a balance such as the HLB (hydrophile-lipophile balance) system used to describe surfactant or emulsifier properties. FBC was negatively correlated to S_0ANS and S_0CPA but positively correlated to interaction between the two S_0 terms as well as the interaction between D and S_0ANS.

Rockfish—Effects of pH, heating and freezing

Fig. 4 and 5 show the physicochemical and functional properties of extracts prepared from rockfish upon heating at pH 5.5 or 6.8, compared to extracts subjected to freezing at −10°C for 19 days prior to heat treatment. Extracts from rockfish showed the same overall trends with heating temperature and pH as did beef top round extract. Thus, in

Table 1—Backward stepwise multiple regression quadratic models for predicting fat binding and emulsifying properties of control and heated salt extracts of beef top round muscle proteins

Dependent variable	Independent variable	Regression coefficient	F-Ratio	F-Prob.	Norm. coeff.
EC	D	18.77	46.48	0.0000	5.251
(R^2 = 0.7721, n = 74	CPA	1.396	20.93	0.0000	3.250
S.E. = 62.29	SH	24.39	21.12	0.0000	1.928
F-prob = 0.000000)	D^2	-0.4890×10^{-1}	8.457	0.0049	−1.602
	D•ANS	-0.9912×10^{-2}	15.97	0.0002	−0.4259
	D•SH	−0.1457	19.08	0.0000	−2.820
	CPA•SH	-0.2102×10^{-1}	20.65	0.0000	−3.101
	constant	−1578	21.76	0.0000	−12.72
FBC	ANS	-1.552×10^{-1}	9.504	0.0034	−1.106
(R^2 = 0.6647, n = 52	CPA	-0.7868×10^{-2}	24.25	0.0000	−1.538
S.E. = 0.91	D•ANS	0.5667×10^{-4}	5.676	0.0213	0.2250
F-prob. = 0.000000)	ANS•CPA	0.1929×10^{-4}	8.560	0.0053	1.567
	constant	7.755	56.53	0.0000	5.134
EAI	D	0.5820	47.45	0.0000	1.519
(R^2 = 0.8274, n = 52	D•SH	-0.3676×10^{-2}	8.693	0.0049	−0.6500
S.E. = 5.573	constant	−2.952	3.03	0.0878	−0.2245
F-prob. = 0.000000)					
ESI	CPA	0.2720×10^{-1}	10.04	0.0029	3.342
(R^2 = 0.7355, n = 52	SH	0.3034	5.186	0.0297	1.056
S.E. = 1.363	D^2	0.1712×10^{-2}	21.73	0.0000	2.814
F-prob. = 0.000000)	ANS^2	-0.5110×10^{-4}	6.830	0.0124	−1.012
	CPA^2	-0.6544×10^{-5}	5.525	0.0235	−1.315
	D•CPA	0.7406×10^{-4}	6.403	0.0152	0.6207
	D•SH	-0.2865×10^{-2}	12.88	0.0009	−2.780
	ANS•SH	0.3569×10^{-1}	5.427	0.0247	0.9689
	CPA•SH	-0.2793×10^{-3}	5.165	0.0282	−2.302
	constant	−21.79	7.544	0.0088	−9.064

both beef and rockfish extracts, moderate heating (45°C or below) generally improved functional properties. This may be the basis for the recommendation of a pre-heating or "setting" period at 35–40°C, prior to heating at higher temperatures during commercial manufacture of fish gel products using fish surimi. It has been suggested that the low temperature "setting" allows a slow denaturation and organization of the unfolded molecules, which allows formation of gels with better textural qualities than if random aggregation occurs simultaneously or precedes denaturation by directly heating to high temperatures (Lanier et al., 1982).

Although the overall trends were the same for rockfish and beef, some distinct differences between species were noted. At neutral pH, rockfish extracts were much less sensitive to loss of dispersibility by heating up to 85°C (Fig. 4A). S_0ANS and S_0CPA were generally higher for the unheated rockfish (Fig. 4C, D) than beef extracts (Fig. 2C, D). However, whereas beef proteins exhibited a 2.5–3.0 fold increase in S_0 on heating to 70–85°C at neutral pH, rockfish proteins showed only about 1.5-fold increase over this temperature range. SH of rockfish (Fig. 4B) was more intensively affected by heating temperature than beef (Fig. 2B). Rockfish proteins had generally better functionality than beef, especially at neutral pH (Fig. 5A-D). Except for ESI (Fig. 5D), the functional properties were less susceptible to heat deterioration at neutral pH.

With respect to pH, rockfish proteins were sensitive to decreasing pH to 5.5. At pH 5.5, rockfish proteins had much lower dispersibility (Fig. 4A) and SH (Fig. 4B) content after heating. While S_0ANS (Fig. 4C) was higher at pH 5.5 than at pH 6.8, the converse was true for S_0CPA (Fig. 4D). Although the emulsifying properties (EC, EAI and ESI) were poorer at pH 5.5 (Fig. 5A, C, D), fat binding was improved at pH 5.5 (Fig. 5B), particularly when pH 5.5

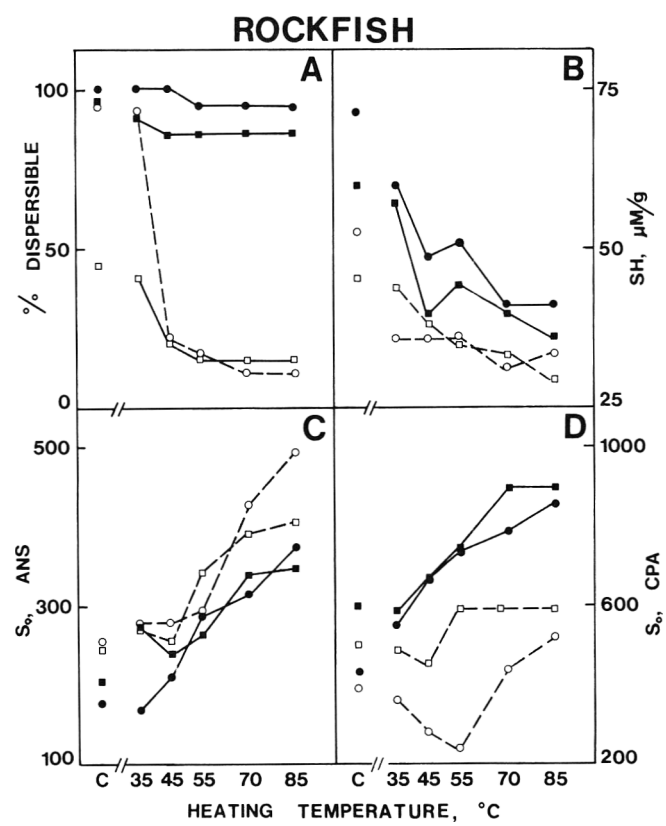


Fig. 4—Dispersibility (A), sulfhydryl content (B) and hydrophobicity (C, D) of rockfish extracts as a function of heating temperature. (Closed symbols, pH 6.8: ● fresh, ■ frozen. Open symbols, pH 5.5; ○ fresh, □ frozen).

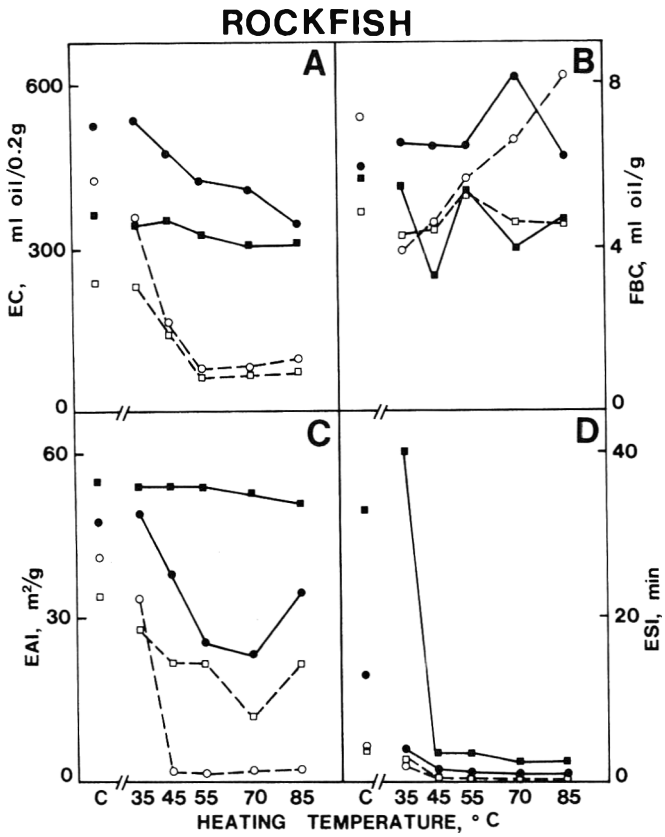


Fig. 5—Emulsifying (A, C, D) and fat binding (B) properties of rockfish extracts as a function of heating temperature. (Symbols are as defined in Fig. 4).

extracts were heated. Under these conditions, it was also observed that the rockfish extracts formed quite stable gels. Rockfish extracts which were frozen at −10°C, prior to subjecting to heat treatment, showed lower D and SH but higher S_0ANS and S_0CPA, which suggest protein denaturation and aggregation during the freeze and thaw cycle.

It was observed that even upon storage at 4°C, the rockfish proteins exhibited a tendency to aggregate into filamentous masses, which could not be disrupted easily by physical means such as homogenization or sonication. The nonhomogeneity made representative sampling for analyses difficult, and thus triplicate and even quadruplicate analyses were necessary for some samples. Different dissociating and denaturing agents were used to test for their ability to disperse the protein aggregates. Whereas rockfish extracts heated at 85°C at pH 6.8 could be solubilized in the presence of 8M urea and 1% mercaptoethanol, the extract heated at 85°C at pH 5.5 could only be dispersed in the presence of the combination of 8M urea, 0.6M NaCl, 1% mercaptoethanol and 2% SDS.

The results of backward stepwise multiple regression analyses of the rockfish data are shown in Table 2. The results of analyses which included ZP parameters as additional potential variables did not differ significantly from those in Table 2. Table 2 shows that a large proportion of the variability in the sample functionality can be described by the predictor variables, highly significant ($P<0.001$) regression models being obtained to describe all four functional properties investigated. As found for beef top round, emulsifying properties of rockfish were positively correlated with S_0, D and SH; several interaction terms and square terms were also significant. However, rockfish FBC was positively correlated to S_0ANS and negatively correlated to D, contrary to the trends observed for beef FBC. These results suggest that for rockfish proteins, lower dispersibility and higher hydrophobicity favored fat binding; under these conditions, the rockfish extracts formed gels, in contrast to the coagulum or weak gels resulting from beef extracts under similar conditions.

DISCUSSION

THE DENATURATION of proteins has traditionally been associated with loss of solubility and other functional properties. The results of the work presented here suggest that a limited amount of denaturation can improve functionality and that high solubility or dispersibility is not the only criterion which dictates muscle protein functionality. Highly significant prediction equations were obtained by backward stepwise multiple regression analysis when hydrophobicity and sulfhydryl parameters were considered in conjunction with dispersibility in explaining the emulsifying and fat binding properties of the salt extractable proteins from beef and rockfish. An optimum balance of these parameters was shown to be necessary for good functionality. The effects of sample variability, heating, pH, freezing and species differences were investigated, and it was demonstrated that changes in physicochemical descriptor parameters reflected changes in functional properties. Specifically, it was noted that moderate heating to 45°C or lower temperatures often improved functional properties, compared to the unheated controls. In the case of rockfish, heating to even higher temperatures at pH 5.5 also improved fat binding capacity.

These practical findings may be explained in the light of what is currently known of the profile of heat denaturation of muscle proteins. Ziegler and Acton (1984) have summarized conformational changes occurring for natural actomyosin with increasing temperature. In the 30-35°C temperature range, native tropomyosin is dissociated from the F-actin backbone, while at 38°C, the F-actin superhelix dissociates into single chains. The myosin light chain subunits dissociate from the heavy chains at approximately 40°C, followed by conformational changes in the head and hinge regions of the myosin molecules. The actin-myosin complex is dissociated in the 45-50°C range, then a major helix-coil transformation in the light meromyosin chains occurs at 50-55°C, followed by rapid aggregation of protein molecules.

Thus it appears that at temperatures below 50°C, aggregation is minimal and the major changes involve protein complex dissociation and molecule unfolding; rapid aggregation only commences at 55°C or higher temperatures, being brought about by changes in the myosin rod. Differential scanning calorimetry (Findlay and Stanley, 1984a, b) has indicated that thermal transitions of beef muscle can be localized in mainly three temperature ranges, which are above 45°C: T_1 (45-60°C, T_{max} = 54°C) is attributed to myosin; T_2 (60-76°C, T_{max} = 65°C) is attributed to sarcoplasmic proteins, actomyosin and connective tissue proteins; and T_3 (76-92°C, T_{max} = 80-85°C) is attributed to actin. Samejima et al. (1976, 1981) concluded, from optical rotatory dispersion and circular dichroism studies, that during thermal treatment of myosin, aromatic amino acids are transferred from a moderately hydrophobic environment to a more polar environment. Helix-coil transitions of the myosin rod were attributed to disruption of noncovalent rather than covalent forces. However, aggregation of the globular head regions of myosin was thought to be associated with oxidation of sulfhydryl groups (Samejima et al., 1981).

The dissociation of the muscle protein complexes and protein denaturation without accompanying aggregation which occur at temperatures below 50°C were reflected in the present work by changes in physicochemical properties,

Table 2—Backward stepwise multiple regression quadratic models for predicting fat binding and emulsifying properties of salt extracts of rockfish muscle proteins, including heated and frozen samples.

Dependent variable	Independent variable	Regression coefficient	F-Ratio	F-Prob.	Norm. coeff.
EC	CPA	0.4290	11.67	0.0011	0.9180
(R^2 = 0.8145, n = 70	D	3.793	62.78	0.0000	1.167
S.E. = 53.03	SH	4.942	23.46	0.0000	1.139
F-prob = 0.000000)	CPA^2	-0.2601×10^{-3}	11.18	0.0014	−0.8965
	ANS•SH	-0.5362×10^{-2}	20.53	0.0000	−0.6937
	D•SH	-0.3614×10^{-1}	19.56	0.0001	−1.045
	constant	−137.9	6.254	0.0150	−1.172
FBC	ANS	0.1328×10^{-1}	24.39	0.0000	1.1483
(R^2 = 0.7591, n = 70	D	−0.2194	19.87	0.0000	−2.855
S.E. = 1.440	D^2	0.1547×10^{-2}	18.20	0.0001	2.337
F-prob. = 0.000000)	ANS•CPA	-0.2557×10^{-4}	70.45	0.0000	−2.168
	ANS•D	0.1676×10^{-3}	13.11	0.0006	0.7043
	ANS•SH	-0.7540×10^{-4}	7.235	0.0092	−0.4475
	CPA•SH	0.1450×10^{-3}	51.83	0.0000	1.481
	constant	6.472	31.10	0.0000	2.237
EAI	CPA	0.8525×10^{-1}	14.71	0.0003	1.353
(R^2 = 0.5472, n = 70	D	0.5270	22.84	0.0000	1.203
S.E. = 11.00	ANS•CPA	-0.8344×10^{-4}	17.36	0.0001	−0.9476
F-prob. = 0.000000)	CPA•D	-0.5936×10^{-3}	9.957	0.0024	−1.131
	constant	−6.495	0.5027	0.4809	−0.4095
ESI	D	0.3801	35.52	0.0000	1.327
(R^2 = 0.4296, n = 56	ANS•D	-0.1219×10^{-1}	24.00	0.0000	−1.215
S.E. = 8.079	CPA•SH	0.1517×10^{-3}	3.626	0.0624	0.2693
F-prob. = 0.000002)	constant	−0.2820	0.0131	0.9094	−0.0271

namely, increase in surface hydrophobicity (both S_0ANS and S_0CPA) with little change in D and SH. Under these conditions, emulsifying and fat binding properties were improved compared to unheated controls. At higher temperatures, aggregation (reflected by decrease in D and SH and, to some extent at temperatures above 70°C, also by decrease in S_0) resulted in decrease in emulsifying properties. Similar trends were noted by Lim and Botts (1967) with respect to the ATP-ase enzyme activity of myosin. Conditions favoring moderate interaction of myosin with ANS corresponded to conditions favorable for ATP-ase activity, but conditions leading to extensive ANS-binding corresponded to loss of enzymatic activity. Slow aging at 4°C or brief exposure to temperatures from 25–50°C were also found to be accompanied by increase in ANS binding and decrease in enzymatic activity. Niwa et al. (1981a, b; 1975) also stressed the role of hydrophobicity in gelation of fish flesh paste and sol, using arylation and ethylsulfonation to introduce aromatic and apolar aliphatic groups. They suggested that differences between species with regard to ease of setting (i.e., gelation) could be related to the abundance of hydrophobic interactions existing on the surface of the myosin molecule.

With respect to pH, the transition temperature (T_m) of actomyosin has been observed to be shifted to lower values as pH is lowered from 6.0 to 5.5; at the same time, the calculated energy of activation from Arrhenius-type plots indicates that less thermal energy is required for the transition at pH 5.5 (Ziegler and Acton, 1984). Thus, the changes leading to aggregate formation and loss of dispersibility would occur at lower temperatures of heating at pH 5.5 than at higher pH, as illustrated in Fig. 2A and 4A.

The measurement of hydrophobicity was performed with two different fluorescent probes. Hayakawa and Nakai (1985) suggested that protein hydrophobicity might be classified into aliphatic and aromatic hydrophobicities, contributed by aliphatic and aromatic amino acid residues, respectively. CPA is composed of an unsaturated aliphatic hydrocarbon chain while ANS is composed of aromatic rings. Thus it is possible that CPA and ANS are bound at protein sites differing in aliphatic and aromatic hydrophobicities.

Generally both S_0ANS and S_0CPA followed the same trends upon heating of extracts at neutral pH, i.e., S_0 increased gradually upon heating to 45°C, then showed a larger increase on heating from 50–70°C, and finally reached a plateau level or slightly decreased upon further heating to temperatures above 70°C. These results suggest protein unfolding with increasing temperature to expose both aliphatic and aromatic hydrophobic regions (detected by CPA and ANS respectively). At temperatures above 70°C, little further change or slight decrease in S_0 suggests no further exposure of hydrophobic regions or even decrease in accessibility of hydrophobic groups which may be involved in aggregation.

The effect of pH on S_0 was more complicated. S_0ANS was usually greater for extracts at pH 5.5 than at higher pH, suggesting greater unfolding to expose aromatic groups as the pH approached the isoelectric point. The quantum yield of the ANS probe has been documented to be insensitive to pH in the range 2–8 (Gibrat and Grignon, 1982), so it appears that the effects of pH on S_0ANS in the present work are due mainly to protein or protein-probe interaction changes with different pH. However, upon using CPA at lower pH values, it was observed that S_0CPA of beef extracts measured at pH 5.5 were generally but not always greater at pH 7.0, but for rockfish, S_0CPA was less at pH 5.5. These results suggest that for rockfish, although aromatic hydrophobic groups (determined as S_0ANS) were more accessible at pH 5.5, aliphatic hydrophobic groups (measured as S_0CPA) were less accessible. Hamm (1966) pointed out that coagulation of proteins may occur without prior unfolding, especially in the isoelectric range of pH, because at that pH the lack of intramolecular electrostatic repulsion impedes the unfolding of the helix, whereas the lack of intermolecular electrostatic repulsion promotes

coagulation. S_0CPA values at low pH must be interpreted with caution due to the influence of pH on several factors, including protein conformation and charge, as well as probe charge and solubility, since CPA was found to be less soluble at pH below 5.0.

CONCLUSIONS

RESULTS of this study have demonstrated the importance of considering hydrophobicity and structural (sulfhydryl content) parameters in conjunction with dispersibility in explaining functional properties of the salt extractable proteins from beef and rockfish samples. An optimal balance of S_0, D and SH rather than high values of the individual parameters was essential for good functionality. Changes in the proteins under different conditions (pH, heating, freezing) and due to sample and species differences were observed in physicochemical descriptor parameters as well as functional properties. Highly significant regression models were obtained which demonstrated that much of the variability in the functional properties could be described in terms of S_0, D, SH and/or their quadratic terms. More refined models must await the accumulation of larger banks of data covering different species, muscle tissue, pre- and post-slaughter handling etc., and other functional properties such as gelation. It is feasible then that the important components of the descriptor parameters may be defined using multivariate analysis of data, to differentiate, predict and classify samples with varying degrees and types of functional properties, based on simple and reproducible determination of physicochemical properties which describe hydrophobic, structural and electric parameters of the proteins.

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A Scanning Electron Microscopic Study of Heat-Induced Alterations in Bovine Connective Tissue

F. Y. WU, T. R. DUTSON, and S. B. SMITH

ABSTRACT

Bovine sternomandibularis muscles were excised immediately following animal exsanguination for the study of collagen fibers and connective tissue network. Samples were cooked at 60°C or 80°C for 1 hr and were observed using the scanning electron microscope. An ordered array of collagen fibers was seen in the uncooked sample. The epimysium did not show large alterations after cooking; however, the perimysium and endomysium became granular at 60°C and gelatinized at 80°C. These differences may be related to the type of collagen or degree of crosslinking present in each location.

INTRODUCTION

THE NATURE of the relationship between muscle collagen and the tenderness of cooked meat is not well-established (Herring et al., 1967; Hunsley et al., 1971; Cross et al., 1973); however, it is believed that both the quantity and the quality of collagen are important factors (Bailey, 1972). Crosslinks, which increase in amount and strength with animal age within collagen fibers, stabilize the collagen fiber and increase its thermal stability (Shimokomaki et al., 1982). Collagen fibers undergo thermal contraction when heated above 60°C and the presence of crosslinks increases the amount of thermal contracture (Sims and Bailey, 1981). Since the different collagen types have different amounts and types of crosslinkages (Sims and Bailey, 1981), it is likely that collagen from these different tissue locations will react differently to heating.

Several studies have been conducted on the comparison of cooked and uncooked muscles by scanning electron microscopy (Schaller and Powrie, 1972; Cheng and Parrish, 1976; Jones et al., 1977; Voyle, 1981), but no report has been made of tissues examined immediately after slaughter as an uncooked control and no comparison was made of all three connective tissue types at different heating temperatures. This study was designed to demonstrate the native structure of collagen fibers in the epimysium, perimysium and endomysium and to indicate alterations in the native structure caused by different heating temperatures.

MATERIALS & METHODS

BOVINE STERNOMANDIBULARIS muscles were excised from two A maturity (approx. 18 month-old) steers immediately following exsanguination of the animal. Small pieces of muscle containing epimysium and perimysium were placed in 10 x 75 mm test tubes and heated at either 60°C or 80°C in water baths for 1 hr. Connective tissue strength fails between 60°C and 80°C (Locker and Carse, 1976). Additionally, 60°C corresponds to rare, and 80°C corresponds to well done in standard cooking. After heating, the tubes were cooled in tap water and the tissue was fixed at room temperature in 2.5% glutaraldehyde:0.1M phosphate (pH 7.4). Control samples were fixed immediately after excision. Samples in fixative were trimmed further to less than 2 mm in thickness. After 2 hr fixation, the samples were moved to a 4°C cooler for an additional 4 hr fixation and then washed three times with 0.1M phosphate buffer (pH 7.4). Samples were fixed with 1% OsO_4 in 0.1M phosphate buffer (pH 7.4) for 4 hr, washed three times with double distilled water and were dehydrated conventionally with ethanol. Samples impregnated with pure ethanol were placed in a petri dish containing liquid nitrogen and were freeze fractured at the desired location with a scalpel. The fractured samples were returned to pure ethanol to thaw and were critical point dried using amyl acetate as a transition fluid. Forty minutes were allowed for CO_2 to exchange in the bomb.

After critical point drying, all samples were stored in a dessicator. Dried tissue was attached to specimen stubs with conductive silver paint and was painted on all sides except the top to minimize charging in the microscope. Samples were coated with approximately 150 nm gold-palladium and were examined under Jeol JSM-25SII Scanning Microscope.

RESULTS

ALTHOUGH SAMPLES were obtained from two animals, the results from only one will be reported. However, similar results were obtained from both animals. The native structure of muscle tissue before cooking is shown in a cross section of muscle fibers (Fig. 1a). The myofibers are packed together closely prior to cooking. The endomysium and perimysium also are tightly packed and are just visible (Fig. 1b). Upon heating to 60°C (Fig. 2) and 80°C (Fig. 3), the perimysium and endomysium were more visible, due to shrinkage of the myofibers (as indicated by their more rounded appearance). At 60°C, the perimysium retained much of its fibrous appearance (Fig. 2b), but appeared coagulated at 80°C (Fig. 3a and 3b). Myofibers were polygonal in cross section before heating (Fig. 1b) but were circular in shape after heating to 60°C (Fig. 2b) and 80°C (Fig. 3b). Heating also caused a separation of the endomysium from the remainder of the myofiber (Fig. 2b and 3b), which may have been caused by the shrinkage of myofibers observed during heating.

The epimysium appeared to be formed by layers of collagen fibers oriented at specific angles for each layer (Fig. 4a, 5a, and 6a). Higher magnification revealed a fine network of collagen fibers in each layer (Fig. 4b, 5b, and 6b). Coagulation of the collagen fibers, indicated by their granular appearance, was caused by heating and more coagulation was observed at 80°C than at 60°C (Fig. 5 and 6). However, the fibrous structure of epimysium was still apparent after heating at either temperature (Fig. 5 and 6).

The network of collagen fibers was less dense and the fiber diameter smaller in the perimysium (Fig. 7, 8 and 9) than in the epimysium (Fig. 4, 5 and 6). Heating at 60°C caused extensive coagulation of the perimysium (Fig. 8); however, some collagen fibers were still identifiable after heating at 60°C. Heating at 80°C caused complete dissolution of the collagen fibers, so that the fibrous structure was no longer visible (Fig. 9).

Heating alterations also were observed when the perimysium was removed so that the endomysium could be viewed longitudinally. The fibrous network between the myofibers, hidden by the juxtaposition of fibers in cross

Authors Wu and Smith are with the Meats and Muscle Biology Section of the Animal Science Dept., Texas A&M Univ., College Station, TX 77843. Author Dutson, formerly with Texas A&M Univ., is now with the Dept. of Food Science & Human Nutrition, Michigan State Univ., East Lansing, MI.

sections of uncooked samples (Fig. 10a), is apparent in these micrographs (Fig. 11 and 12). Granular collagen was present in the 60°C heated samples (Fig. 11), while amorphous masses were observed along the myofibers of 80°C cooked samples (Fig. 12). When the appearance of cooked muscle (Fig. 11 and 12) was compared with similarly prepared uncooked muscle (Fig. 10a and 10b), the disappearance of blood vessels was apparent. This indicates proteins such as elastin were susceptible to heat denaturation as well.

DISCUSSION

INTRAMUSCULAR COLLAGEN has been classified histologically as the epimysium, perimysium and endomysium. However, there is no clear cut delineation between these three categories, because transition stages are present between the epimysium and perimysium and between the perimysium and endomysium. Bailey et al. (1979) used immunofluorescence to show that type I collagen is present in the epimysium, types I and III collagen are present in the perimysium, and Types III, IV, and V collagen are present in the endomysium. The type III collagen appeared to be present in greater proportions in the perimysium of less tender muscles relative to levels observed in tender muscles. This may be one reason for the transition from densely-packed, ordered collagen fibers in the epimysium to diffuse, random fibers in the endomysium. Collagen from older animals has inter- or intramolecular crosslinks, especially the nonreducible heat-stable crosslinks, which increase the thermal stability of collage fibers (Shimokomaki et al., 1972; Light and Bailey, 1980). Thus, it is expected that the collagen of muscles from different animals, particularly animals of different ages, will respond differently to heating. Judge and Aberle (1982) determined the thermal shrinkage of collagen and observed that intramuscular collagen of relatively old animals shrank at slightly higher temperatures than that of younger animals; furthermore, the thermal shrinkage temperature declined during postmortem aging.

Jones et al. (1977) showed that endomysial collagen had no consistent evidence of change at 50°C, but at 60°C it was no longer fibrous and might have begun to gelatinize. In the present study, heating at 60°C caused granulation of collagen fibers and heating at 80°C caused dissolution, particularly in the perimysium and endomysium. The coagulation of collagen at 60°C and its gelatinization at 80°C are similar to the results of Davey and Gilbert (1974) who demonstrated that collagen was denatured at 60°C and solubilized at 80°C. These researchers demonstrated more tenderness at 80°C than at 60°C, which may be related to the structural alterations demonstrated in the present study.

The amount of nonreducible heat-stable crosslinks in collagen influences the toughness of meat from mature animals (Light and Bailey, 1980), and appears to determine directly the degree of dissolution of connective tissues with heating. Hence, the combination of heating and scanning electron microscopy may be used to demonstrate changes with age of collagen crosslinking. If a correlation among collagen crosslinking, connective tissue solubilization and meat toughness can be established, then scanning electron microscopy will provide a direct means by which the

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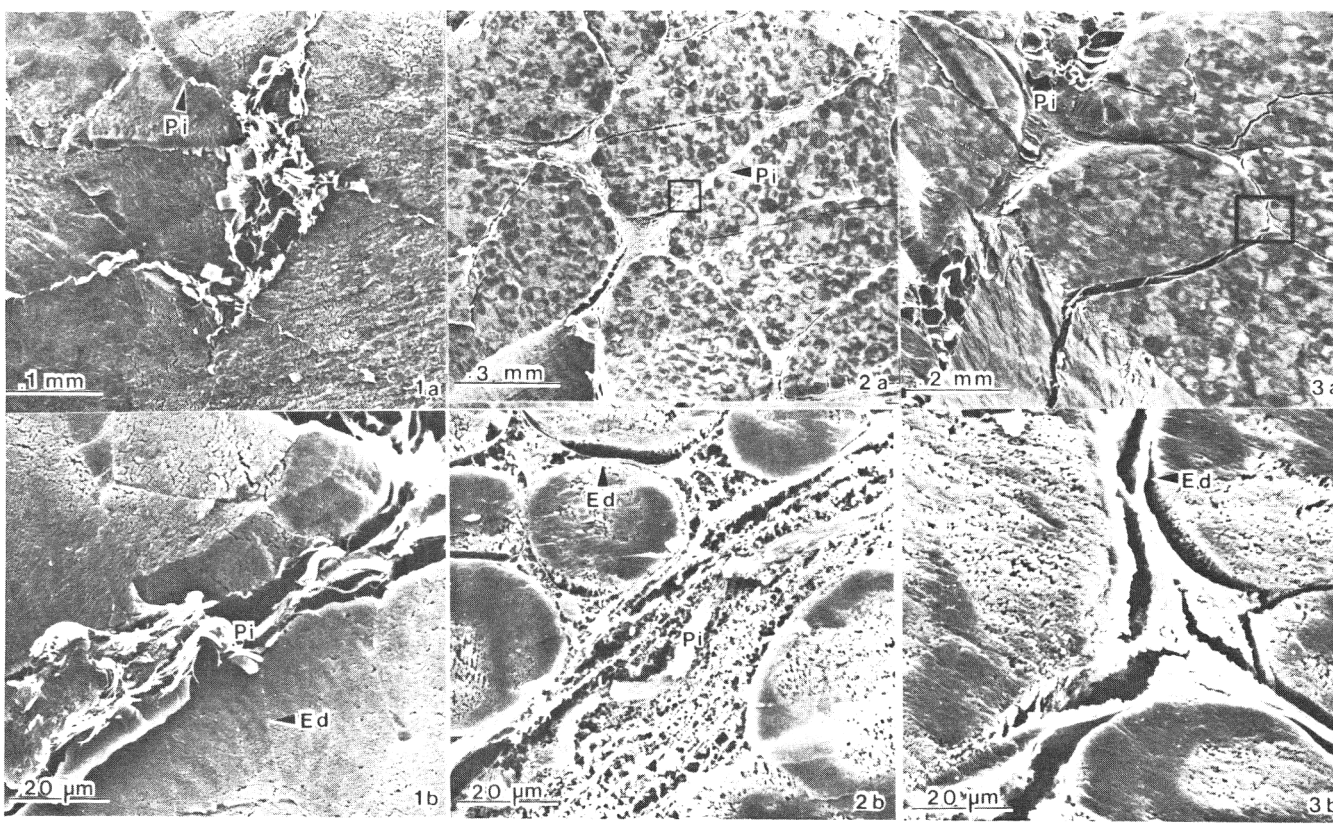


Fig. 1—Scanning electron micrograph showing a cross section of Sternomandibularis muscle sampled immediately following exsanguination and fixed and dried by the critical point method. (a) Perimysium (Pi) is clearly visible. x 180; (b) Endomysium (Ed) is tightly attached to myofibers. x 900.

Fig. 2—Cross section of muscle cooked at 60°C for 1 hr. (a) Perimysium (Pi) shows loss of integrity. x 63; (b) Enlargement of the square in "a" showing some coagulation of collagen fibers. Some endomysium (Ed) has separated from myofibers. x 900.

Fig. 3—Cross section of muscle cooked at 80°C for 1 hr. (a) Some gelatinized collagen is evident in the perimysium (Pi). x 90; (b) Enlargement of the square in "a" showing the separation of endomysium (Ed) from myofibers. x 900.

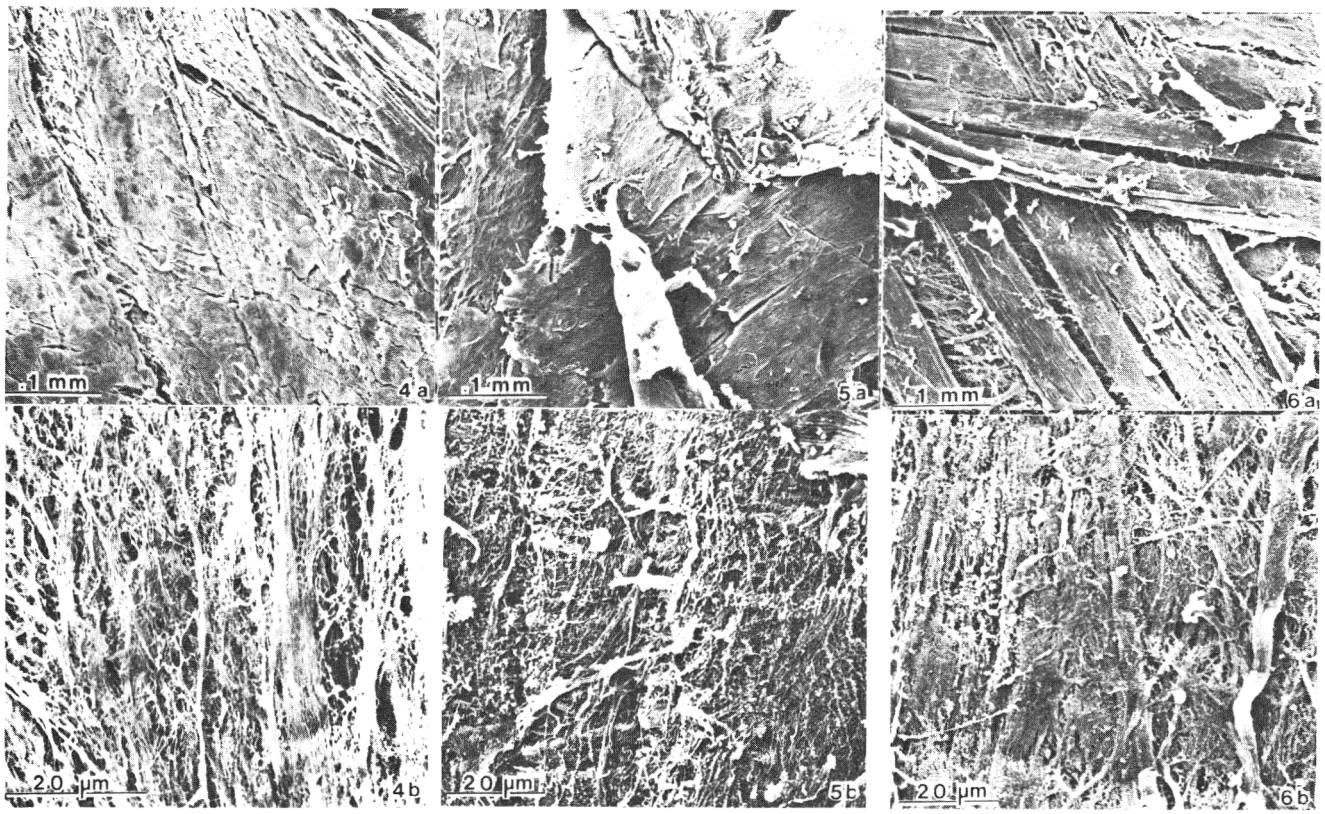


Fig. 4—Epimysium of control muscle. (a) Layers of collagen fibers are oriented at different angles. x 180; (b) A network of fine collagen fibers is evident. x 1350.

Fig. 5—Epimysium of muscle cooked at 60°C for 1 hr. (a) x 180; (b) Some coagulation of the collagen fibers is evident. x 900.

Fig. 6—Epimysium of muscle cooked at 80°C for 1 hr. (a) x 180; (b) More extensive denaturation of the collagen fibers is observed. x 900.

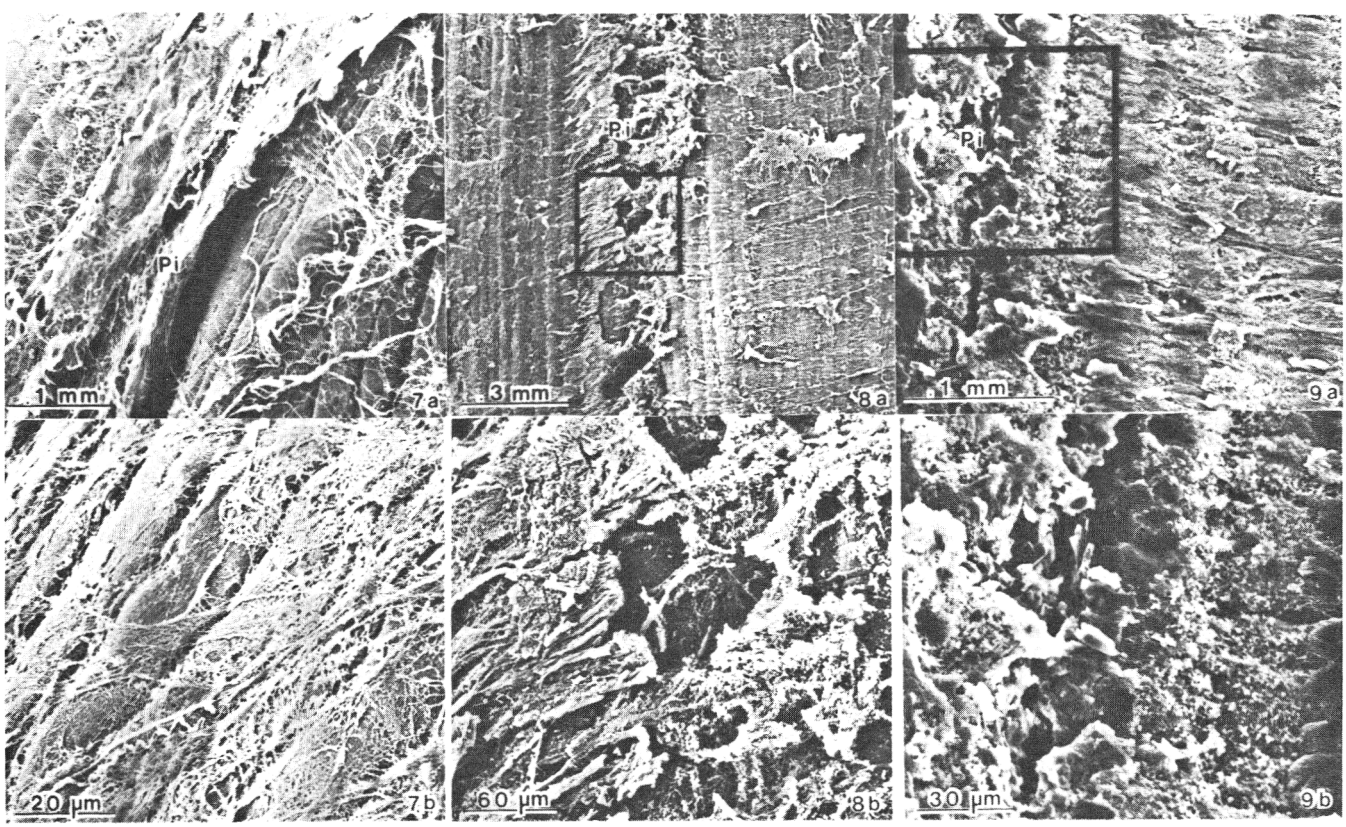


Fig. 7—Perimysium of control muscle (Pi). (a) x 180; (b) The fine network of perimysial fibers is evident on the surface of the myofibers. x 900.

Fig. 8—Perimysium of muscle cooked at 60°C for 1 hr. (a) Fibrous network now appears granular. x 63; (b) Enlargement of the square in "a" showing the coagulation of collagen fibers. x 270.

Fig. 9—Perimysium of muscle cooked at 80°C for 1 hr. (a) x 270; (b) Enlargement of the square in "a" showing that the fibrous structure is no longer identifiable. x 570.

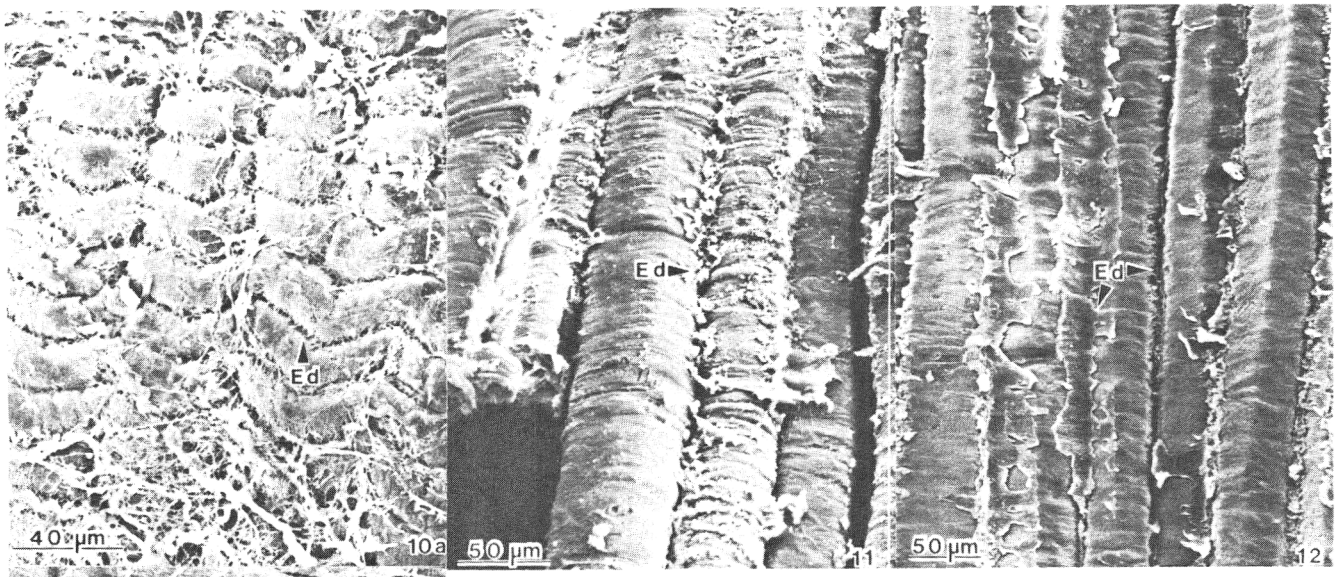


Fig. 11—Endomysium of muscle cooked at 60°C for 1 hr. Granular endomysium (Ed) is clearly visible. x 330.

Fig. 12—Endomysium of muscle cooked at 80°C for 1 hr. Chunks of coagulated endomysium (Ed) have accumulated along the myofibers. x 270.

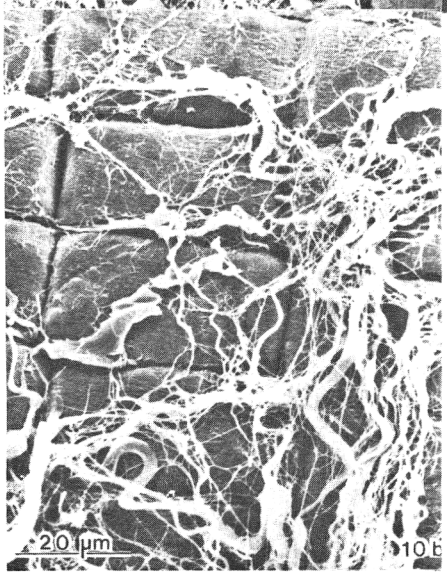


Fig. 10—Endomysium of control muscle. (a) Collagen fibers overlay the endomysium (Ed) of the myofibers x 480. (b) Collagen fibers on the surface of wavy myofibers; blood vessels (smooth surface) also are evident. x 1050.

contribution of collagen to meat toughness can be determined.

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Cholesteryl Linoleate Oxidation in Freeze-Dried Model and Beef Systems

C. W. SEO and JAMES T. KING JR.

ABSTRACT

An attempt was made to determine lipid oxidation by measuring the oxidative loss of cholesteryl linoleate in a freeze-dried model system and in a meat system. In the model system containing cholesteryl linoleate (1g), cobalt chloride (10 ppm) and cellulose (24g), there was a direct relationship between the loss of cholesteryl linoleate and pentane production, an index of lipid oxidation during storage. As the pentane production increased rapidly, the cholesteryl linoleate concentration decreased accordingly. However, in the meat system, the loss of cholesteryl linoleate extracted by isopropanol was not evident throughout the storage period while a significant amount of pentane was produced. Therefore, this method was suitable for following the oxidative rancidity for the model system but not for the meat system under the experimental conditions of the present study.

INTRODUCTION

IT IS A WELL KNOWN FACT that pentane is the major product from oxidation of linoleic acid and ethane from linolenic acid when headspace hydrocarbons are analyzed by gas chromatography. This is the basis for the use of headspace pentane determination to follow lipid oxidation in oils and dried foods. It has been reported that it was relatively simple and useful to determine the degree of oxidative rancidity in vegetable oils and potato chips (Warner et al., 1974) and in freeze-dried meats (Seo, 1976; Seo and Joel, 1980).

The mechanism of cholesterol esters oxidation is explained by a free radical chain reaction as in the case of oxidation of polyunsaturated fatty acids. Smith (1981) has reported that the course of autoxidation of cholesterol esters is probably the same as that of cholesterol, but the nature of the fatty acyl moiety determines the position to be attacked on its moiety and the rate of oxidation. Conceivably, oxidation of the fatty acid moiety of cholesterol esters should yield short-chain hydrocarbons. It was reported that cholesterol esters as well as cholesterol can be easily determined by liquid chromatography without elaborate sample manipulation (Duncan et al., 1979). From these facts, it was assummed that oxidative rancidity in the freeze-dried meat could be followed by measuring disappearance of cholesterol esters.

The purpose of this study was to establish a relationship between the disappearance of cholesteryl linoleate and pentane production, an index of lipid oxidation in freeze-dried model and meat system. If this relationship is established, it can be employed to determine the degree of lipid oxidation in freeze-dried meats.

MATERIALS & METHODS

Preparation of freeze-dried model and meat samples

A model system was prepared by combining and homogenizing 1.0g cholesteryl linoleate, 24g microcrystalline cellulose, 135 mL phosphate buffer (pH 6.4) and $CoCl_2 \cdot 6H_2O$ (10 ppm cobalt on a cholesteryl linoleate basis) with a Virtis homogenizer. The mixture was placed in stainless steel pans and dried by a Thermovac FDE-4 laboratory freeze-dryer. The temperature of the heating plate was set at 26.7°C and the range of vaccum was 80–100 μ Hg. It took about 20–24 hr to dry 200g of wet sample.

Top round beef was purchased from the local market. The meat was ground twice with an electric grinder and packed into short No. 2 C-enamel cans. The cans were heated in boiling water until the internal temperature of the meat reached 70°C. Then the meat was dried by a Thermovac FDC-4 laboratory freeze-dryer. The preparation of samples, sample storage, and gas chromatographic experimental conditions were adopted from the report by Seo and Joel (1980).

Sample storage

The bottles were sealed with aluminum caps containing silicon rubber septa which had been covered with an inert self-adhesive teflon tape. The samples were then stored at room temperature.

Gas chromatography

For hydrocarbon analysis a 4.0 mL sample of headspace gas was taken from each bottle by a gas-tight syringe and injected into a Hewlett Packard 5730A gas chromatograph equipped with a flame ionization detector. For quantitative analysis of pentane production, a standard curve was prepared for each day of analysis by injecting varying concentrations of the standard gas mixture containing C_1–C_6 (100 ppm each) saturated hydrocarbons into the gas chromatograph under the same experimental conditions.

High pressure liquid chromatography

High pressure liquid chromatography (HPLC) was performed with a Waters Associates chromatograph equipped with a 6000 A pump, Wisp sampling system, Waters system controller and data system, a Model 450 variable UV detector, and a C18 raidal pak compression column. The mobile phase was 50:50 isopropanol: acetonitrile at a flow rate of 0.8 mL/min and pressure of 50–80 psi. For quantitative analysis of cholesteryl linoleate, standards were prepared to contain 5 μg cholesteryl linoleate per 25 μL injection on the Wisp injection system. At least three standard injections were made to establish reproducible areas for the cholesteryl linoleate peak. Response factors were developed by the data system to quantitate results of sample analysis.

Sample preparation for high pressure liquid chromatography

To analyze the model samples for cholesteryl linoleate, 0.5g of sample and 10 mL of 2-propanol (HPLC grade) were shaken for 20 min with a wrist action shaker. The sample mixture was filtered into a beaker and washed with ca. 40–60 mL HPLC grade isopropanol. This extract was then diluted to 100 mL and subjected to analysis. Samples were taken in triplicate.

For meat samples, 5g of the freeze-dried sample with 20 mL of isopropanol was shaken by a wrist action shaker for 30 min and the final volume was made to 50 mL and the rest of the procedures were the same as the method described for the model sample.

RESULTS & DISCUSSION

PENTANE PRODUCTION increased as the storage time increased in model systems containing cholesteryl linoleate and cobalt chloride. After 6 days of storage, there was a linear relationship between pentane production and storage time (Fig. 1). This showed that the fatty acid moiety of the ester was oxidized rapidly in the presence of cobalt in the

Authors Seo and King are affiliated with the Dept. of Home Economics/Food Science, North Carolina A&T State Univ., Greensboro, NC 27411.

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J. Food Sci. 50(4): 1045–1047 (1985). Cholesterol linoleate oxidation in freeze-dried model and beef systems — C.W. Seo and J.T. King. On open 1046, pentane production in the legend to Fig. 1 should read: μL/0.5g rather than μL/5g. In Fig. 2, the points of cholesterol concentration on the Y-axis are incorrect. From top to bottom they should read: 4.8, 4.0, 3.2, 2.4, 1.6, 0.8, and 0 at the intersect of the x and y axis. Please make these corrections accordingly.

freeze-dried system. At the 20th day of storage the production peaked and leveled off thereafter.

The production of pentane from linoleic acid has been well documented. Autoxidized methyl linoleate (Horvat et al., 1964) and thermal decomposition of linoleic acid (Evans et al., 1967) produced pentane as the major hydrocarbon. Johns et al. (1973) also reported pentane production from linoleic acid oxidation catalyzed by lipoxygenase. The fact that cholesteryl linoleate also produced pentane might indicate that the presence of cholesterol linkage did not matter in terms of production of major hydrocarbon from linoleic acid. Seo (1976) reported that hydrocarbon production was very distinctive between the linoleic acid family (CH_3-CH_2-CH_2-CH_2-CH_2-CH=) and linolenic acid family (CH_3-CH_2-CH=). The configurations of the methyl end in fatty acids determine the major hydrocarbon being produced.

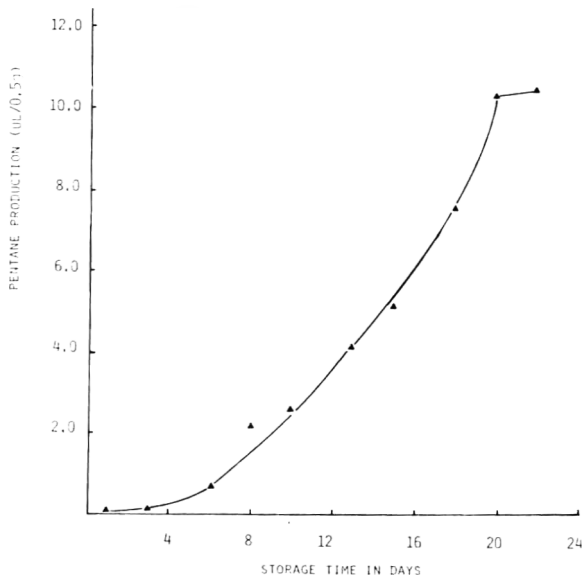


Fig. 1—Pentane production (μL/5 g) from freeze-dried model system containing cholesteryl linoleate, cobalt chloride and cellulose (lg:10 ppm:24g).

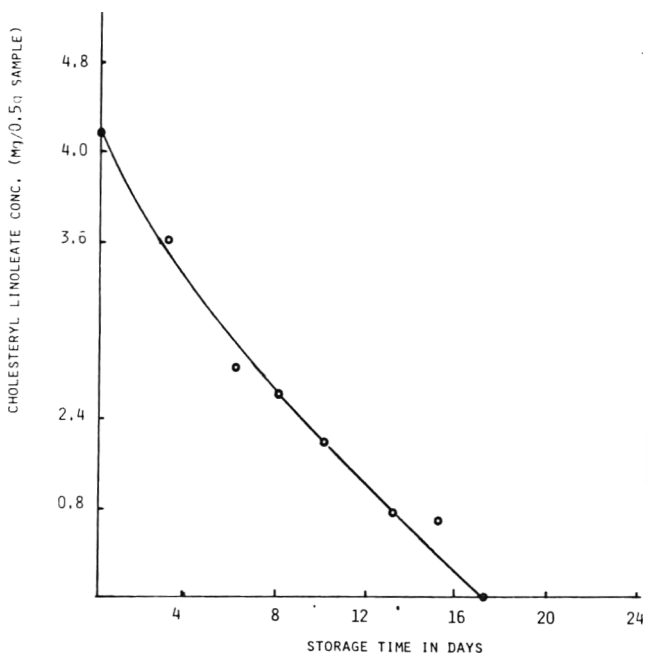


Fig. 2—Cholesteryl linoleate loss in freeze-dried model system containing cholesterol linoleate, cobalt chloride and cellulose (lg:10 ppm:24g) during storage.

Cholesteryl linoleate loss as the oxidation of fatty acid moiety progressed was determined on the same batch of samples. There was a rapid decrease in the level of the cholesteryl linoleate through oxidation during storage (Fig. 2). By the 17th day no detectable cholesteryl linoleate was present in the sample. However, the chromatogram of HPLC did not show any by-products of its oxidation. It would be very interesting to study the by-products of its oxidation. From these results, it was concluded that oxidation of cholesterol esters could be followed by measuring headspace hydrocarbon production and disappearance of cholesterol esters in freeze-dried model systems.

From the freeze-dried beef, free cholesterol, cholesteryl linoleate, and two other unknown esters were isolated with a liquid chromatograph. From the same meat, it was found that pentane production increased with increased storage time as expected (Fig. 3), but the cholesteryl linoleate loss was not evident throughout the storage period (Fig. 4).

Little change in cholesteryl linoleate levels in the meat might indicate that the linoleic acid moiety of cholesteryl linoleate was quite stable to oxidation. In regard to the stability of cholesteryl linoleate in meat against oxidation, it was speculated that the unsaturated fatty acyl moiety of cholesterol was much less susceptible to oxidation than free unsaturated fatty acids based on the following reports. Smith (1981) suggested that unsaturated fatty acid was a likely source of organic peroxides acting as initiators of cholesterol autoxidation. Thus, autoxidative attack in the polyunsaturated fatty acyl moiety might precede cholesterol oxidation. Smith (1981) also indicated that sterol esters were less sensitive to air oxidation than the cor-

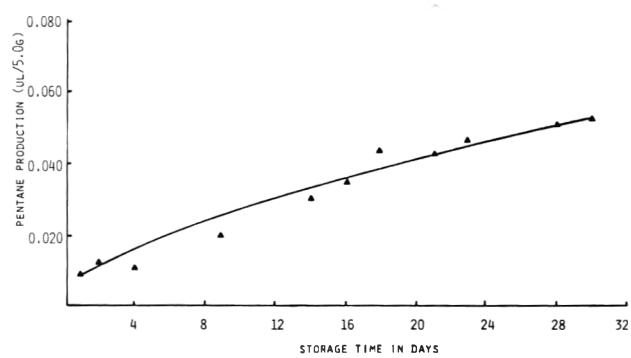


Fig. 3—Pentane production (μL/5g) from freeze-dried beef during storage.

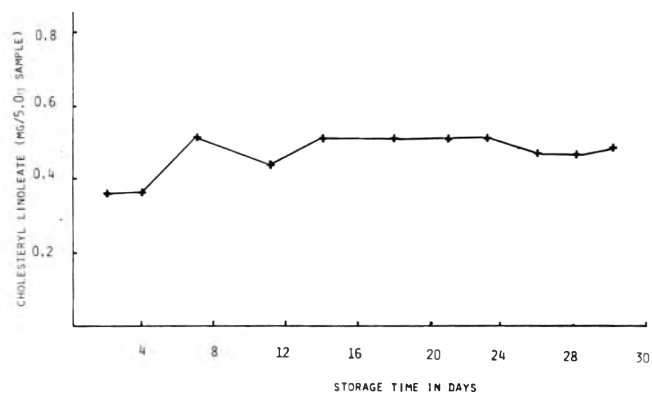


Fig. 4—Cholesteryl linoleate loss in freeze-dried beef during storage.

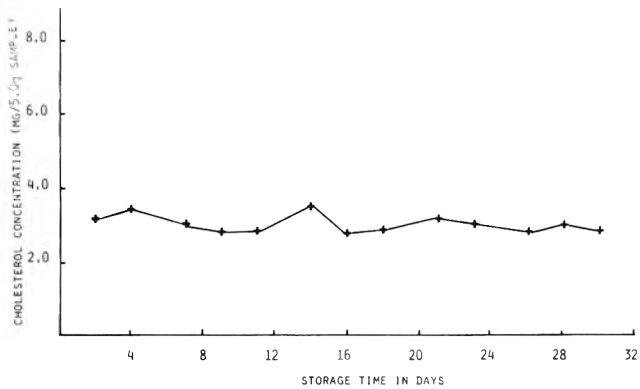


Fig. 5—Free cholesterol concentration in freeze-dried beef during storage.

responding free sterol. Pikul et al. (1984) reported that approximately 90% of the malonaldehyde measured was from the phospholipid fraction in total fat from chicken meat. Further studies are necessary to verify the stable nature of cholesteryl linoleate in freeze-dried meat. Free cholesterol which was also monitored in the same meat during storage showed no significant change during the 30-day storage period (Fig. 5).

It was concluded that the oxidation of cholesteryl linoleate could be followed by measuring its dissappearance and headspace pentane production in model systems during storage. However, cholesteryl linoleate loss was not evident in freeze-dried meat systems. This method was not applicable in following lipid oxidation in freeze-dried meat under the experimental conditions of the present study.

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Initial Sensory Evaluation of Bing Cherries Treated with Low Doses of Gamma-Radiation

M. O'MAHONY, S-Y. WONG, and N. ODBERT

ABSTRACT

Bing cherries from California were given low postharvest doses of gamma radiation (60–80 Krads) and were compared with nonirradiated controls during a period 3–11 days after harvest. Irradiated cherries showed a greater degree of shrivelling, a trend to be softer on chewing and also exhibited flavor differences. Trends for consistent differences in firmness tested manually, darkness of skin color and degree of brown blemishing were not noted.

INTRODUCTION

SINCE Balock et al. (1956) first introduced the idea of postharvest irradiation of fruit, its potential for disinfestation and prolongation of shelf life has been reviewed (Anon., 1982; Burditt, 1982; Maxie and Abdel-Kader, 1966; Moy, 1977). For cherries in particular, the effects of irradiation on the sensory properties have not been studied by formal evaluation of their sensory characteristics, although reports have been made based on casual observation (Anon., 1964; Jona and Arzone, 1979), quality scaling (Johnson et al., 1965) and hedonic scaling (Salunkhe, 1961). The present study provides an initial formal sensory analysis for bing cherries irradiated at doses measured as 60–80 Krad, approximately a week after harvest.

MATERIALS & METHODS

Specification of fruit: Transport, irradiation and storage

Size 11-1/2 bing cherries were harvested at the beginning of June 1983, from Winters, CA. On the day of harvesting, they were packed in open calex lugs (guaranteed minimum weight 18 lbs) and transported immediately by road during the morning to the irradiation facility (International Nutronics Inc.) at Palo Alto, CA: transport time 3 hr, ambient temperature 35°C outside, 18–21°C in vehicle, relative humidity 50–55%. Fruit were kept at the irradiation facility for 2 days: ambient temperature 16–33°C, relative humidity 50–55%. During this time, half the fruit were irradiated in their lugs at ambient temperature, using a cobalt-60 source, giving dosimer values on 60–80 Krad (0.6–0.8 KGy).

On the morning of the second day (approx 11 a.m.) the cherries were transported by road to Davis: transport time approx. 3 hr, ambient temperature 24–29°C outside, 18–21°C in vehicle, relative humidity 50–60%. At Davis, the fruit were stored prior to testing: ambient temperature 1°C, relative humidity 75%. Thus, storage at Davis commenced 2 days after harvest, with 10 lugs from each treatment.

Judges

Practiced judges were used as analytical instruments for testing samples of fruit, with minimal cross-sensory interference (O'Mahony et al., 1983); more than one judge was used as a failsafe rather than to constitute a statistical sample. Their purpose was to indicate suitable later instrumental analysis. Seven students at U.C.D. (6F, 1M, ages 20–29 yr) were selected after one to two orientation and practice sessions, in which they learned the appropriate behavioral procedures and sensory cues detailed later. They were also screened during testing sessions for inconsistency due to adaptation and fatigue.

Authors O'Mahony, Wong, and Odbert are affiliated with the Dept. of Food Science & Technology, Univ. of California, Davis, CA 95616.

Evaluation of flavor by mouth

The seven practiced judges determined, at their own speed, the difference in flavor between single cherries (irradiated vs control), each using ten successive dual standard tests (Peryam, 1958; Peryam and Swartz, 1950). Single cherries, with stem attached, were sampled randomly from each treatment and presented to the judge in 1 oz unwaxed paper cups (Portion Control Cups, American Convenience Products, Milwaukee, WI), coded with 3-digit random numbers for written response. The judge picked up the cherry by the stem, placed it in the mouth and removed the stem (pulling the stem from the cherry held behind the teeth). He/she then chewed the cherry until ready to swallow, whereupon he/she expectorated; this allowed experience of flavor-by-mouth (taste + smell). He/she then proceeded to the next cherry, after rinsing three times to reduce taste adaptation effects (O'Mahony, 1979).

Color cues were eliminated by wearing green goggles (3 layers of Roscolene #871 light green gel: Rosco Laboratories Inc., Port Chester, NY). Firmness cues for the whole cherry that might be detected manually, were eliminated by holding the stem. However, any brief cue from the resistance of the stem to removal or texture of the cherry by mouth, could not be eliminated. Testing took place 3–4 days after harvest; session lengths ranged 13–28 min, including brief rest periods.

Evaluation of firmness by chewing

The seven practiced judges determined, at their own speed, which of the two cherries (irradiated vs control) was firmer by chewing, using ten successive paired comparison tests. The procedure for sampling and testing was as in the previous test. In this case, firmness was judged while flavor could not be eliminated. Testing took place 4–8 days after harvest; session lengths ranged 6–10 min.

Evaluation of firmness by manual squeezing

The seven practiced judges determined, at their own speed, which of two whole cherries (irradiated vs control) was firmer, by squeezing between index finger and thumb; each judge performed ten paired comparisons.

Judges were presented with a single randomly sampled cherry from each treatment as in the prior test. They picked up the cherries by the stems in their nondominant hand. They then alternately squeezed each cherry along its longer lateral axis between the index finger and thumb of their dominant hand. This was continued until the judge had determined which cherry was firmer. Ten such pairs were evaluated. Color cues were eliminated as before by wearing green goggles. Preliminary experimentation indicated that odor cues could not be detected; taste and oral texture cues were avoided by not placing the cherry in the mouth. Testing was performed 3–4 days after harvest; session lengths ranged 4–19 min.

Evaluation of darkness of red color

The seven practiced judges ranked six plates of cherries (three irradiated, three nonirradiated controls) along a dimension 'light' to 'dark', using a procedure detailed elsewhere (O'Mahony et al., 1985a). Random samples of 10 cherries without stems, from each treatment, were placed on the plates for ranking. Judges were given examples of such cherries prior to testing, the more yellow being designated 'lighter' and the designations of 'darker' being attributed more as the cherries became a darker red. Testing took place 4–8 days after harvest; session lengths ranged 3–14 min.

Evaluation of whole cherry shrivelling

A random sample of 450 whole cherries was taken from each treatment. A judge was required to sort through each sample, sepa-

rate and count the number of cherries that exhibited any shrivelling. Three practiced judges performed this task separately for the same two samples of cherries, to obtain an estimate of sorting error and response bias (Green and Swets, 1966).

Shrivelling was defined as any wrinkling of the skin; the judges were given practice and orientation beforehand with extreme cases, to define shrivelling. Each sample of cherries was presented to the judge on a white matte tray, placed on a white matte surface, under the standard lighting conditions used in the prior measure; the order of presentation of the samples was varied over judges. Judges sorted the cherries into two further trays labelled 'wrinkled' and 'non-wrinkled', and counted the number in each; the counts were checked by the experimenters for reliability.

Judges did not place the cherries in the mouth, thus excluding taste and oral texture cues. Prior experimentation had shown that odor cues were not effective for this procedure. Because sorting was carried out manually to allow proper examination of the fruit, manual cues (firmness, weight, etc.) could not be excluded. Testing took place 11 days after harvest; session lengths ranged 48–60 min.

Evaluation of brown blemishes

Three practiced judges evaluated whole cherries for occurrence of brown blemishing; such blemishing developed from prior abuse in handling or from insects. The procedure was as for the prior test. Testing took place 11 days after harvest; session lengths ranged 35–50 min.

RESULTS & DISCUSSION

THE RANKED DATA for darkness of skin color were used to compute R-index values for each judge, indicating the degree of difference between irradiated and nonirradiated control cherry treatments, in terms of the probability of distinguishing between the two (O'Mahony, 1983; O'Mahony et al., 1979; 1980). Because the judges did not constitute a statistical sample, analysis was performed for each judge individually. The significance of each R-index value was determined using the Rank Sums test (Leach, 1979). Binomial tests (Roessler et al., 1978; Siegel, 1956) were used to analyze data from individual judges for the dual standard (one-tailed), and paired-comparison tests (two-tailed), and for proportional changes in counting tests (two-tailed).

Evaluation of flavor by mouth

The number of correct dual standard tests, out of ten, along with the significance level for each judge, was as follows: 9 (0.011), 8, 8 (0.055), 7 (0.172), 6, 6 (0.377), 3 (0.945).

Three judges detected significant differences in flavor; their subjective comments, though unreliable as such, indicated greater sweetness for the irradiated fruit. The flavor differences noted here were not found by casual observation in prior studies (Anon., 1964: 150–350 Krad; Jona and Arzone, 1979: 10–100 Krad), although Johnson et al. (1965) reported decreased quality ratings for 200–400 Krad doses.

The present result suggests that it is worthwhile performing initial chemical analyses for volatiles and taste components (e.g., sugars, acids). Further sensory analysis for odor changes was not attempted owing to difficulty in sample preparation, because of rapid odor change with time due to enzymatic reactions. This, and analysis for taste per se, would be the next logical step.

Evaluation of firmness by chewing

The number of times, out of 10, that the irradiated fruit was judged as less firm than the control, along with the significance level for each judge, was as follows: 9, 9 (0.021), 8, 8, 8 (0.109), 5 (>0.754), 4 (0.754).

Two judges discerned the irradiated fruit to be softer ($p \leq 0.021$). although at a less stringent significance level ($p \leq 0.109$), the number is increased to five. The detection of a softer fruit after irradiation corresponds with instrumental measures at higher dose levels of 100–500 Krad (Eaton et al, 1970; Johnson et al., 1968). It is difficult to compare the present data with casual reports of irradiation producing a 'crisper' cherry at 150–350 Krad (Anon., 1964) or a poorer quality of texture at 200–400 Krad (Johnson et al., 1965). The present results indicate the suitability of initiating appropriate physical analyses (e.g., shear press, etc.) and the possibility of cellular breakdown.

Evaluation of firmness by manual squeezing

The number of times, out of 10, that the irradiated fruit was judged as less firm than the control, along with the significance level for each judge, was as follows: 9 (0.021), 8 (0.109), 7 (0.344), 6, 6, (0.754), 5 (>0.754), 2 (0.109).

The results are not as clear as for the previous test, with trends going in both directions, albeit more in favor of a softer irradiated fruit. This may be due to inhomogeneity of the fruit sample or the nature of the test; subjective reports indicated that it was more difficult than evaluation of firmness by chewing. Any trend for firmness is slight.

Evaluation of darkness of red color

R-indices with their respective significance levels for each judge, were as follows: 91.1 (0.001), 77.8 (0.012), 60.0 (0.401), 53.3 (0.834), 40.0 (0.401), 31.1 (0.093), 24.4 (0.021). The greater the R-index (above 50) the darker the irradiated fruit, the smaller the R-index (below 50) the lighter the irradiated fruit.

It can be seen that some judges assessed the irradiated fruit as being significantly darker, while others gave the reverse result. With no trend appearing, this would seem to be an effect of sample inhomogeneity. Jona and Arzone (1979) and Anon (1964) did not notice color differences by casual observation, at doses ranging from 10–250 Krad; although the latter reported skin discoloration from 300–350 Krad. Eaton et al. (1970), using a color difference meter, noted increased skin darkness at doses higher than those used here (100–500 Krad), the darkness increasing with dose. Should there be any effect for the current sample of cherries, it would seem to be slight compared to ordinarily occurring variation.

Evaluation of whole cherry shrivelling

The ratios of the numbers of shrivelled fruit, out of 450, were, for each judge: control/irradiated, 31/101, 31/90, 27/114. The proporation of shrivelled fruit in the irradiated sample was significantly higher for all judges ($p \leq 0.001$). This confirms, for lower levels of irradiation, Johnson et al's (1965) casual observation for levels of 200–400 Krad. Instrumental analysis should be guided towards this structural deterioration.

Evaluation of brown blemishes

The ratios of the numbers of cherries exhibiting brown blemishing, out of 450, were for each judge: control/irradiated, 37/38, 43/47, 52/38. The proportion of blemished fruit in the irradiated sample was not significantly different from the proportion in the control sample, for all judges ($p \geq 0.347$). It would seem that the irradiation treatment did not accelerate the development of brown blemishing so soon after harvest; reported acceleration in blemishing found for other fruits (O'Mahony et al., 1985a) occurred after longer storage periods.

CONCLUSION

CHERRIES irradiated with low doses of gamma radiation showed a higher degree of shrivelling than nonirradiated controls, 11 days after harvest. There was also a trend for

some differences in flavor, 3–4 days after harvest, as well as a tendency for the irradiated fruit to feel softer when chewed, 4–8 days after harvest. However, manual judgments of firmness did not detect significant trends, nor did measures of darkness of red color or degree of brown blemishing, during testing 3–11 days following harvest. It should be borne in mind, however, that the testing took place soon after harvest, owing to the short shelf-life of cherries. Greater differences may have occurred after longer storage. Instrumental analysis should be directed initially to flavor, texture and the acceleration of shrivelling.

Subjective reports and observations indicated that the test methods were simple to perform. Where the sensory dimension was definable, the R-index ranking procedure was used to allow a wide sampling of cherries and obtain measures susceptible to parametric analysis. Where ranking was limited by memory difficulties, paired comparisons were used. Simple counting measures, although open to response bias, gave a high degree of agreement between judges; had they not, response bias could have been circumvented by the introduction of sureness judgements and the calculation of R-indices (O'Mahony, 1983). In this study, as in others (O'Mahony et al., 1985a,b) sensory evaluation was used as an analytical tool to enable screening to guide later instrumental analysis.

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Sensory Evaluation of Regina Freestone Peaches Treated with Low Doses of Gamma Radiation

M. O'MAHONY, S-Y. WONG, and N. ODBERT

ABSTRACT

Regina freestone peaches from California were given low postharvest doses of gamma radiation (65-75 Krads) and were compared with non-irradiated controls approximately one month after harvest. Practiced judges were used, with a technique of minimal cross-sensory interference, as an analytical tool to guide subsequent instrumental analysis. Strong differences were found in odor and components of taste not associated with sweetness. Lesser trends were noted for firmness and appearance. Untrained subjects also detected gross sensory differences.

INTRODUCTION

FOR DISINFESTATION and prolongation of shelf life of fruits, the use of postharvest irradiation treatments has been reviewed (Anon, 1982; Burditt, 1982; Maxie and Abdel-Kader, 1966; Moy, 1977), since Balock et al. (1956) first introduced the idea. For peaches, the effects of irradiation on the sensory characteristics have not been studied extensively. Differences between irradiated and nonirradiated fruits have been determined for color, flavor and aroma at 30-100 Krad (Moy et al., 1982) for 'quality' at 100-200 Krad (Maxie et al., 1965) and texture at 30-250 Krad (Larmond and Hamilton, 1968; Maxie et al., 1965; Moy et al., 1982). Ahmed and Dennison (1971) and Ahmed et al. (1972), using 150-300 Krad, also scaled peaches for degree of firmness, while Maxie et al. (1965), using 100-200 Krad, scaled degree of liking of firmness. This study examines a wide range of sensory characteristics for a single batch of peaches irradiated at lower doses, measured as 65-75 Krad.

MATERIALS & METHODS

Specification of fruit: Transport, irradiation and storage

Size 60 Regina freestone peaches were harvested at the end of June, 1983 from Sanger, CA. The peaches were packed in cardboard boxes (wt 22 lb) and then stored at Sanger for 13 days following harvest day: ambient temperature 1°C, humidity uncontrolled. On the 14th day after harvest the peaches were transported to Sacramento by refrigerated truck (temperature and humidity unspecified, estimated travel time 3 hr), then stored for 5 days: ambient temperature 7°C, relative humidity 80%.

On the 19th day after harvest the fruit were transported by road to the irradiation facility (International Nutronics, Inc.) at Palo Alto: transport time 3 hr, ambient temperature 26°C, relative humidity 61%. Fruit were kept at the irradiation facility until the afternoon of the following day: ambient temperature 22-26°C, relative humidity 63%. During this time, half the fruit were irradiated in their boxes using a cobalt-60 source; giving dosimeter values of 65-75 Krad (0.65 - 0.75 KGy).

The peaches were then transported by road to Davis: transport time 3 hr, ambient temperature 24-30°C, relative humidity 53%. At Davis the fruit were stored prior to testing: ambient temperature 0.5-3°C, relative humidity 82-95%. Thus, storage at Davis commenced 20 days after harvest.

Authors O'Mahony, Wong, and Odbert are affiliated with the Dept. of Food Science & Technology, Univ. of California, Davis, CA 95616.

Judges

Practiced judges were used as analytical instruments for testing samples of fruit with minimal cross-sensory interference (O'Mahony et al., 1983); more than one judge was used as a failsafe rather than to constitute a statistical sample. Their purpose was to indicate suitable later instrumental analysis. Students at U.C.D. (ages 20-29) were selected after one to two orientation and practice sessions, learning the appropriate behavioral procedures and sensory cues with sample peaches. They were also screened during testing sessions for inconsistency due to adaptation and fatigue. Untrained subjects, being more like consumers, were also tested. They were used to make inferences about the population. Students and staff at U.C.D. (ages 19-36 yr) were selected at random and merely required to understand the task at hand before commencing the simple behavioral procedures detailed later. The number of subjects was limited by the amount of fruit available.

Evaluation of degree of brown blemishing

Seven female practiced judges ranked six whole peaches, three irradiated and three nonirradiated controls, along a dimension 'least blemished' to 'most blemished'. Peaches were placed on white paper plates and ranked by arranging the plates in order, using a procedure detailed elsewhere (O'Mahony et al., 1983). This procedure avoided taste, odor and texture cues, although other visual or mass cues could not be eliminated. Each judge performed five ranking trials, giving a total of 15 peaches per irradiation treatment (irradiated vs control), per judge. Peaches were randomly sampled from 6 boxes (60 peaches per box) per treatment, with the modification that no judge examine the same peach twice. Testing took place during one day, 26 days after harvest; session lengths ranged from 5-10 min.

Evaluation of firmness of the whole peach by manual squeezing

The same judges used for the prior measure ranked six whole peaches, three irradiated and three nonirradiated controls, for degree of firmness, using a procedure detailed elsewhere (O'Mahony et al., 1985). This was performed immediately after the prior measure; session lengths ranged from 4-12 min.

Evaluation of firmness of peach slices during chewing

Six female practiced judges evaluated differences in firmness of peach slices, during chewing, each performing ten paired comparison tests. Judges used a procedure detailed elsewhere (O'Mahony et al., 1985) with the modification that noseclips were worn to eliminate odor (but not taste) cues. Slices were placed in the mouth by spoon, to avoid manual tactile cues. Each slice was chewed four times (at approx. 0.5 sec intervals) and expectorated before tasting the next slice. The slices were prepared by peeling each peach, cutting it longitudinally in half, removing the stone, and cutting each half longitudinally into three slices. Each peach thus produced six peeled longitudinal sections. Approx. 300 slices from each treatment were pooled and sampled randomly for testing. Testing took place during one day, 33 days after harvest; session lengths ranged from 5-18 min.

Evaluation of darkness of flesh color

The same six female practiced judges used in the prior measure, evaluated differences in the darkness of color of the flesh of peach slices, each using ten paired comparison tests. They were presented with two whole peaches (one irradiated, one nonirradiated control), each in a transparent glass bowl; the bowls were presented on a white matte surface under the standard lighting conditions used in the evaluation of brown blemishing. The judge cut a longitudinal slice from the peach (approx. 1/6th of peach) with a sharp knife

to evaluate the color of the flesh. She then repeated the same procedure for the second peach and indicated verbally which peach had the darker flesh. This was repeated for ten such paired comparisons. Judges did not place the peach slices in the mouth, so avoiding taste cues, while prior testing established that the judge's head was sufficiently distant to eliminate odor effects. The cutting of the peach slice introduced a firmness cue but avoided the effects of enzymatic browning.

Peaches were sampled randomly from each treatment with the constraint that no judge sampled the same peach more than once. Once one slice had been removed, the peach was returned to the pool for possible re-use. Any second slice was taken from the opposite side of the peach to avoid interference by perception of the color of the flesh at the original cut. No peach was sampled more then twice. Testing took place during one day, 29 days after harvest; session lengths ranged from 5–10 min.

Evaluation of flavor by mouth

Six female practiced judges determined at their own speed the difference in flavor by mouth between samples of chopped peaches (irradiated vs control), each using ten successive dual standard tests (Peryam, 1958; Peryam and Swartz, 1950). The tests were performed under red light to mask color differences; the peaches were chopped in small pieces to minimize texture cues. To minimize build up of peach residuals in the mouth and thus adaptation effects, judges rinsed two to three times between dual standard tests (O'Mahony, 1979).

Peaches were selected at random from each treatment, peeled, sliced and chopped into small pieces (approx. ½" × ½" × ½"), immediately prior to testing. They were then presented to the judge, filling by two-thirds 3 oz waxed paper cups coded with 3-digit random numbers for written response. The test stimuli were tasted once, the standards could be tasted ad-libitum. Testing took place during one day, 28 days after harvest; session lengths ranged from 8–25 min.

Evaluation of odor

Six female practiced judges (four from the panel for the prior evaluation) evaluated, at their own speed, odor differences between samples of chopped peaches (irradiated vs control), using ten successive duo-trio tests. Chopped peaches were prepared and presented as in the prior measure, except watch glasses were placed over the paper cups; these were removed immediately prior to sniffing. Judges smelled an empty paper cup immediately before each sample, to adapt to the odor of the cup (O'Mahony et al., 1983). Other aspects of the test were as for the prior measure, except that the peaches were not tasted. Testing took place during one day, 30 days after harvest; session lengths ranged from 12–30 min.

Evaluation of taste

Five female practiced judges (three from the panels for the prior two measures) evaluated taste differences in the same way as 'flavor by mouth', except that noseclips were worn to eliminate odor cues and duo-trio tests were used. Testing took place 30-33 days after harvest; session lengths ranged from 10–26 min.

Evaluation of 'sourness'

Four female practiced judges (three used from the prior test) evaluated taste differences in the same manner as before, except that the testing was performed after a rinse with gymnema sylvestre tea; this blocked the sweet taste receptors leaving the judges free to evaluate 'sourness', or more precisely: taste without 'sweetness' (O'Mahony et al., 1983). The gymnema sylvestre rinse prior to testing lasted 2 min. On expectoration of the tea, judges rinsed ad lib with water to eliminate the bitter aftertaste. They then tasted granulated sugar to ensure that sweetness could not be tasted. After every two to three duo-trio tests, sugar was re-tasted to ensure that the effect of the gymnema sylvestre had not worn off. If it had, a further 2 min rinse was used. Through the series of ten duo-trio tests, judges used three rinses. Testing took place during one day, 34 days after harvest; session lengths ranged from 10–25 min.

Evaluation by untrained subjects of gross sensory differences

Twenty-five untrained subjects (8M, 17F) each performed a single triangle test, to determine gross sensory differences between irradiated and control peaches. Although judges were familiar with and consumers of peaches, none had been trained for a peach tasting panel.

Subjects were presented with the whole peaches on a white matte tray, under incandescent light. They were told that the difference was due to the irradiation post harvest treatment, but not told which treatment the odd peach had received. The actual treatment of the odd peach was counterbalanced over subjects. Subjects were allowed to consume as much of each peach as desired and test the peaches in any way they wished; they responded verbally. Testing was performed 34–35 days after harvest; session lengths ranged from 3–5 min.

RESULTS & DISCUSSION

RANKED DATA from practiced judges for the first two evaluations were used to compute R-index values for each judge, indicating the degree of difference between irradiated and non-irradiated control peach treatments, in terms of the probability of distinguishing between the two (O'Mahony, 1983; O'Mahony et al., 1979; 1980). Because these practiced judges did not constitute a statistical sample, analysis was performed for each judge individually. The significance of each R-index value was determined using the Rank Sums test (Leach, 1979). Binomial tests (Roessler et al., 1978; Siegel, 1956) were used to analyze the data for individual practiced judges for dual standard, duo-trio (one tailed), and paired comparison (two tailed) tests. The sample of untrained subjects was analyzed for majority trend and inference about the population, using a two tailed binomial test.

Evaluation of degree of brown blemishing

R-indices with their respective significance levels (in parentheses) for each judge, were as follows: 66.7 (0.142), 58.0 (0.529), 56.0 (0.674), 42.0 (0.529), 41.4 (0.194), 29.0 (0.059), 22.0 (0.012). The greater the value above 50, the more blemished the irradiated peaches; the lesser the value below 50, the less blemished the irradiated peaches.

It can be seen that two trained judges indicated that the irriadiated peaches had a lesser degree of blemishing ($p \leqslant 0.059$). Considering the simplicity of the task, it is more likely that failure to detect significant differences by the other judges was more likely due to sampling than skill. Thus, irradiation may produce a slight reduction in blemishing. There have been no prior systematic studies on blemishing; the present finding suggests the need for further research.

Evaluation of firmness of the whole peach by manual squeezing

R-indices with their respective significance levels (in parenthesis) for each judge, were as follows: 60.0 (0.294), 54.0 (0.834), 51.0, 49.0 (> 0.999), 33.0 (0.142), 31.0 (0.093), 27.0 (0.036). Values above 50 indicate that irradiated peaches were judged as less firm, below 50 more firm.

It can be seen that one trained judge indicated that irradiated peaches were significantly firmer ($p < 0.05$). At a nearer 10% significance level, three judges would indicate this ($p \leqslant 0.142$). Failure of other judges to detect this trend may be due to lack of skill or to sampling effects. There have been no prior systematic studies of whole peach firmness; the possibility of a slight effect of radiation in maintaining firmness deserves more attention.

Evaluation of firmness of peach slices during chewing

The number of paired-comparisons, out of ten, on which the irradiated peaches were judged as more firm, along with significance levels for each judge, were as follows: 9 (0.021) 8, 8 (0.109), 7 (0.344), 6 (0.754), 5 (> 0.754). Only one judge indicated that the irradiated peaches were significantly

firmer ($p = 0.021$), while three indicated this at higher significance levels ($p \leq 0.109$). Failure of other judges to detect differences could be due to lack of skill or sampling of the peaches.

The slight trend for irradiated fruit to be firmer deserves further attention, especially in the light of contradictory results in the literature, for higher levels of irradiation. Maxie et al. (1965) found irradiated fruit to be softer at 100 Krad, the trend being less at 200 Krad. Ahmed et al., (1972) noted softer fruit at higher levels of irradiation (150–300 Krad), an effect confirmed instrumentally (Braddock et al., 1966). Larmond and Hamilton (1968) and Ahmed and Dennison (1971) noted no firmness differences at comparable levels, while Maxie et al. (1965) noted that peaches were firmer if irradiated while green. Moy et al. (1982) noted differences in firmness at 100 Krad but not 30 or 50 Krad; he omitted to report the direction of these differences. It is possible that irradiation has an effect on firmness but there would appear to be many variables involved, pertaining to the timing and manner of radiation, storage, and peach variety.

Evaluation of darkness of flesh color

The number of paired comparisons out of ten, on which the irradiated peach slice was judged as lighter in color, along with significance levels for each judge, were as follows: 8, 8 (0.109), 7 (0.344), 6 (0.754), 4 (0.754), 3 (0.344).

None of the judges exhibited a significant majority trend; any tendency was slightly in favor of a darker control, albeit with contrary results. The question of whether sensory differences can be detected deserves further attention, especially as Moy et al. (1982) reported significant differences in flesh color at 30 and 50 Krad but not at 100 Krad. Johnson et al. (1967) noted that irradiated (100 Krad) peaches exhibited a darker flesh color with a color reflectance meter, while Maxie et al. (1966) noted a higher anthocyanin content after irradiation (100–400 Krad).

Evaluation of flavor by mouth

The number of correct dual standard tests, out of ten, along with the significance level for each judge, were as follows: 10, 10, 10, 10 (0.001), 9 (0.011), 8 (0.055).

All judges found significant differences between the irradiated peaches and the nonirradiated controls. Subjective comments are unreliable but indicated either a loss of flavor or an 'off-flavor'. The strong differences found for flavor by mouth are interesting in that Moy et al. (1982) found no differences for comparable irradiation levels, although they did detect a slight trend at 30 Krad. Other studies at higher doses (100–250 Krad) also detected no significant differences (Larmond and Hamilton, 1968; Maxie et al., 1965). Further testing was performed to identify the sensory components of the flavor difference.

Evaluation of odor

The number of correct duo-trio tests out of ten, along with the significance level for each judge, were as follows: 10, 10, 10, 10, 10 (0.001), 9 (0.011).

All judges found significant differences between the irradiated peaches and the nonirradiated controls. Subjective comments are unreliable but indicated 'off-odors' for the irradiated samples. The strong differences found here do not agree with the finding of Moy et al. (1982) of no difference between irradiated peaches (30–100 Krad) and controls. The strong odor differences in this study suggest that odor is a major component in the difference found for flavor by mouth and that a prime area for instrumental analysis should be an examination of volatiles.

Evaluation of taste

The number of correct duo-trio tests, out of ten, along with the significance level for each judge, were as follows: 7, (0.172), 6, 6, (0.377), 4, 4 (0.828).

The lack of significant differences indicates that overall taste would not seem to be a significant component in the differences noted for 'flavor by mouth'. This is further reinforced by the fact that three judges on this panel had been used for the prior two measures, where they discerned strong differences in flavor and odor.

Evaluation of 'sourness'

The number of correct duo-trio tests, out of ten, along with the significance level for each judge, were as follows: 10, 10, 10 (0.001), 9 (0.011).

All judges significantly detected differences in the 'sourness' between irradiated and nonirradiated fruit. The lack of difference noted in the prior evaluation suggests that the sweetness of the peaches was masking taste differences detected here. This is reinforced by the finding that three of the present panel were used for the prior measure, where they did not detect differences. Although judges reported the peaches as tasting 'sour', this does not rule out the presence of other tastes. Strictly, this is a measure of total taste without sweetness. Although the present measure did not indicate which peach had the most sour taste, it did indicate that instrumental analysis should initially consider the effect of irradiation on acid content of the peaches.

Evaluation of gross sensory differences by untrained subjects

Of the 25 triangle tests performed, 16 were correct; this is a significant majority ($p = 0.002$). From this, it can be concluded that the untrained subjects could distinguish between irradiated and nonirradiated fruits. Subjective comments, although unreliable and inconsistent, indicated differences in flavor or texture. However, data from practiced judges would suggest that the differences were generally detected by 'flavor by mouth'. It should be remembered that this is only a small sample of untrained subjects, limited to the peaches remaining after the major tests had been completed; the sample was sufficiently small to render the distinguishing majority nonsignificant ($p = 0.23$). However, despite the small size of the sample and despite the fact that it was not necessarily a representative cross-section of consumers, the result is suggestive that the differences in odor and taste detected by practiced judges, may well be detected by consumers.

CONCLUSIONS

PEACHES IRRADIATED with low doses of gamma radiation showed consistent differences from nonirradiated controls in flavor-by-mouth, approximately one month after harvest. These flavor differences were seen to be due to changes in odor and also taste, although the taste changes were not associated with, and were masked by, the sweetness of the peach. These were strong differences and untrained subjects were also able to distinguish between the peach treatments. Thus, an initial step in instrumental analysis would be to consider changes in peach volatiles and the nonsweet constituents (e.g. acids) of the fruits.

There was a slight trend for irradiated peaches to appear firmer, both in chewing and in squeezing, as well as having less brown blemishes. Despite expectations, differences in flesh color were not detected. These latter effects do deserve further analysis because the data, although inconclusive, is suggestive. The effects may simply be weaker than for flavor.

Subjective report and observation indicated that generally the test methods were simple to perform. Tests where the judge was required to slice the peach or use gymnema sylvestre needed personal attention because they were more complicated; an experimenter sat with each judge, to ensure that the procedure was followed correctly. The dual standard test was found very satisfactory for flavor-by-mouth but for the longer and less comfortable odor and taste tests, the shorter duo-trio was chosen. Where the dimension of the difference was simple to explain, the R-index ranking procedure was used, to allow a wide sampling of the peaches and obtain more precise measures. Where the testing task became susceptible to memory difficulties, paired comparisons were used. The use of judges both as analytical tools and also as a sample from the population of untrained subjects follows previous procedures (O'Mahony et al., 1985), as does the use of minimal cross-sensory interference procedures and gymnema sylvestre as analytical tools (O'Mahony et al., 1983)

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A Comparison of Sensory Difference Testing Procedures: Sequential Sensitivity Analysis and Aspects of Taste Adaptation

M. A. P. D. E. O'MAHONY and N. ODBERT

ABSTRACT

The greater sensitivity of the 'one strong, two weak stimuli' over the 'one weak, two strong stimuli' versions of the triangle and duo-trio tests was confirmed using a model system of salt and water stimuli. The relative distinguishability of salt and water, in adaptation states varied by prerinses, was measured using an R-index procedure. The relative distinguishabilities were used as the basis for a predictive model, 'sequential sensitivity analysis', to explain the relative sensitivity of versions of the triangular and duo-trio protocols. Adaptation did not furnish a complete explanation of their relative sensitivity.

INTRODUCTION

VARIOUS PROCEDURES for sensory difference tests have been compared in terms of their sensory sensitivity (Byer and Abrams, 1953; Dawson et al., 1963; Filipello, 1956; Frijters, 1981; Gridgeman, 1955, 1956; Hogue and Briant, 1957; Hopkins and Gridgeman, 1955; Pokorný et al., 1981; Spencer, 1979). For the triangle test (Helm and Trolle, 1946) variations within the triangular procedure have also been examined. Strictly for the triangle test, such variations have different names. For a classical triangle test, both stimuli should occur on half the occasions as the odd sample (orders AAB, ABA, BAA and BBA, BAB, ABB). Should the procedure only have one stimulus as the odd sample (orders AAB, ABA, BAA or orders BBA, BAB, ABB), the test is called a three alternative forced choice (3-AFC) test (Frijters, 1980, 1981; Frijters et al., 1980), being one of several signal detection procedures (Green and Swets, 1966). This nomenclature will be adopted here.

Wasserman and Talley (1969) working with smoked and unsmoked frankfurters noted that judges differentiated the two better in a 3-AFC design where the odd stimulus was the stronger smoked sample. Filipello (1956) and Frijters et al. (1982), noted the same for model systems. Frijters (1980) hypothesized that adaptation was heavily involved in this process, there being greater adaptation in the case where two stimuli are stronger. Yet adaptation cannot furnish a complete explanation. Hopkins (1954), working with milk, noted idiosyncratic differences in sensitivity of judges to the two 3-AFC designs. Helm and Trolle (1946) with beers, and Grim and Goldblith (1965) with egg magma, noted a greater sensitivity when the odd sample in the 3-AFC was 'abnormal', 'modified' or 'off-flavored.' The former invoked an explanation in terms of greater variability in "modified" samples, while the latter invoked an explanation in terms of adaptation to longer remaining residual stimuli from prior tastings. Pfaffmann et al. (1954) also invoked explanation in terms of a greater simplicity in searching for one stimulus difference rather than two, on noting that both 3-AFC designs were more sensitive than the regular triangle design. Mitchell (1956) noted, for whiskies, a similar effect for the duo-trio test (Peryam and Swartz, 1950; Peryam, 1958). If one stimulus of the three was stronger or a more unusual flavor, the test was more sensitive.

The purpose of the present study was to develop a model to predict differences in sensitivity among the two 3-AFC and triangle designs and among alternative duo-trio designs.

Authors O'Mahony and Odbert are affiliated with the Dept. Food Science & Technology, Univ. of California, Davis, CA 95616.

MATERIALS & METHODS

Stimuli

Stimuli were NaCl solutions and samples of purified water. They were dispensed in 10 mL volumes by Repipet Adjustable Dispenser (Labindustries, Berkeley, CA) and presented in 10 mL pyrex beakers, at constant room temperature (21 - 27°C). Solutions were made up with reagent grade NaCl (Mallinkrodt Inc., St. Louis) in Millipore purified water, which also furnished the water samples (Milli-RO 4/5 Filtration and Reverse Osmosis System in series with a Milli-Q system ion exchange and activated charcoal; Millipore Corp., Bedford, MA).

Judges

The same 13 judges (5M, 8F, age 20 - 24 yr), chosen randomly from students at U.C.D., performed all three experiments. They were naive to the aims of the study and had not participated before in gustatory experiments with these stimuli.

Experiment I

Immediately prior to each of the six experimental protocols used in this experiment, judges had an orientation session to become familiar with the protocol in question. Judges then practised by tasting (sip and expectorate) alternately 3 mM NaCl and water stimuli, knowing their identity, to learn the taste difference. Judges continued until they felt they could perceive a difference, requiring 8 - 16 tastes of each stimulus, and then intimated their readiness for the experiment to begin.

Each judge was next required to perform ten difference tests to distinguish between 3 mM NaCl and water, for each of the six following protocols: 3-AFC test, with NaCl as the odd stimulus; 3-AFC test with water as the odd stimulus; Triangle test, with both as the odd stimulus; Duo-trio test, with NaCl as the standard; Duo-trio test with water as the standard; Duo-trio test with both used as the standard. For the triangle test or the duo-trio test with both standards, it was arranged that the NaCl or water stimulus should occur as the majority sample for half the presentations; they were alternated randomly. Judges tasted the stimuli as rapidly as possible taking no interstimulus rinses; this allowed any adaptation effects to occur (O'Mahony, 1974, 1979b). Each protocol was performed during a different experimental session (session lengths 5 - 12 min); the order of protocol presentation was counterbalanced over judges.

Experiment II

Judges first practiced, as in Experiment I, by tasting alternate samples of 3 mM NaCl and water. Next, judges learned the behavioral procedure, practised by making four judgements, and intimated their readiness for the experiment to begin. Each judge then tasted the samples of 3 mM NaCl and water after they had altered their adaptation state by tasting a pre-rinse of 10 mL of either 3 mM NaCl or water. Each judge tasted 20 NaCl stimuli after water and 20 after NaCl, 20 water stimuli after water and 20 after NaCl. The total 80 stimuli were tasted in random order as rapidly as possible, taking no interstimulus rinses, so as to allow differences in adaptation state to affect subsequently tasted stimuli (O'Mahony, 1974, 1979b). During the experiment, reference pairs of NaCl and water

were available for tasting; six judges found it necessary to use them, needing two to six pairs to refamiliarise themselves with the tastes of the stimuli.

Judges were required to rate the stimuli as 'salt', 'salt not sure', 'water', 'water not sure', according to the signal detection protocol from which the R-index is computed (O'Mahony 1979a, 1983). The R-index represents the probability of a judge distinguishing between the least distinguishable stimulus (NaCl tasted after NaCl, designated as the noise level) and the other stimuli (designated as signals). The higher the R-index (maximum value 100%), the more distinguishable the stimulus from the NaCl-after-NaCl noise level. The R-index value for the noise level is by definition 50%, the chance probability level.

Experiment III

The procedure for this experiment was the same as for Experiment I. Judges did thus not require orientation sessions to learn the behavioral procedures; they merely tasted alternate stimuli as before, followed by one difference test as a reminder of the specific protocol. They then performed the six behavioral protocols as before except that the general level of adaptation was raised by increasing the stimulus concentrations to 100 mM and 110 mM NaCl. On completion, the experiment was repeated using even stronger stimuli: 800 mM and 860 mM NaCl. Session lengths for each protocol ranged 7 - 13 min for the first set of stimuli and 8 - 20 min for the second set of stronger stimuli.

RESULTS & DISCUSSION

Experiments I and III

Table 1 indicates the mean numbers of correct difference tests out of ten for the various duo-trio and triangular protocols and stimuli. Significant differences between mean numbers correct for a given protocol and a given pair of stimuli were analyzed by Analysis of Variance followed by LSD tests: these are indicated in Table 1. Because the possible range of scores was small, introducing the possibility of distortion of normal distributions, nonparametric analyses were also used: the Kramer test (Kramer et al., 1974), and the Friedman test followed by Sign test multiple comparisons at adjusted levels of significance (Leach, 1979).

In Experiment I, the low adaptation condition (water vs 3 mM NaCl), the same trend (not always significant, $p < 0.05$, Table 1) occurred for triangular and duo-trio procedures. The protocol with one strong and two weak stimuli was the most sensitive, two strong and one weak the least sensitive, and the mixed design with both protocols had intermediate sensitivity. For the triangular design the Kramer test indicated that all conditions were significantly different ($p < 0.05$), the Friedman analysis also ($p \leq 0.065$). For the duo-trio condition the trend was less strong. The Kramer analysis indicated only the weak standard condition to be significantly different ($p < 0.05$), the Friedman analysis indicating a similar trend ($p = 0.11$). These results confirm prior findings in model systems (Filipello, 1956; Frijters et al., 1982) and trends noted in some food systems (Wasserman and Talley, 1969; Mitchell, 1956); they do not support Pfaffmann's (1954) prediction that both 3-AFC designs would be more sensitive than the triangle design.

As the general level of adaptation was raised, by raising the stimulus NaCl concentrations (Experiment III), the predicted differences between the protocols vanished. Only at the intermediate adaptation level for the triangular procedures did any significant differences remain (not in nonparametric analysis). Thus, adaptation although heavily involved (Frijters, 1980), had no simple causal relationship to the differences between the protocols, because as the general level of adaptation increased, the differences between protocols did not. In fact, they were obliterated.

Experiment II

Table 2 indicates the mean R-index values obtained in Experiment II for the 3 mM NaCl and water stimuli tasted after NaCl or water prerinses. Significant differences were analyzed by Analysis of Variance followed by L.S.D. tests and are indicated in the table. Student t-tests were used to determine that all values were significantly greater than 50% ($p < 0.01$), which indicated that distinguishing NaCl after an NaCl rinse was significantly more difficult than any of the other conditions.

Hypotheses were generated to explain the order of distinguishability of the stimuli. The NaCl stimulus tasted

Table 2—Mean percent R-index values indicating the degree of differentiation between NaCl tasted after NaCl (noise level) and NaCl or water variously tasted (signals)[c]

Adapting rinse	Stimulus	Mean R-index values
Water	Salt	76.9 ± 17.9[a]
Salt	Water	72.2 ± 21.8[a,b]
Water	Water	68.8 ± 18.1[b]
Salt	Salt	50.0 by definition

[a-b] Means with the same superscripts are not significantly different ($p < 0.05$).
[c] Each mean ± standard deviation represents R-indices from 13 judges.

Table 1—Mean number of correct triangle, 3-AFC and duo-trio difference tests from ten, for protocols in which the majority stimulus varied[g]

Stimuli		Mean number of correct tests		
Strong	Weak	3-AFC odd stimulus strong	Triangle both stimuli odd	3-AFC odd stimulus weak
3 mM NaCl	water	7.5 ± 2.8[a]	5.5 ± 1.7[b]	3.9 ± 3.0[b]
110 mM NaCl	100 mM NaCl	7.3 ± 1.7[c]	7.0 ± 1.4[c]	5.8 ± 2.1[d]
860 mM NaCl	800 mM NaCl	3.6 ± 1.8	3.9 ± 1.2	3.9 ± 2.6
		Duo-trio with weak standard	Duo-trio with both standards	Duo-trio with strong standard
3 mM NaCl	water	8.5 ± 2.5[e]	7.7 ± 1.7[ef]	6.4 ± 2.6[f]
110 mM NaCl	100 mM NaCl	7.4 ± 2.4	7.8 ± 1.1	8.6 ± 2.3
860 mM NaCl	800 mM NaCl	5.6 ± 1.6	5.6 ± 1.6	6.3 ± 1.8

[a-f] Comparisons between means were made only for a given protocol-stimulus combination. Means with the same superscripts were not significantly different ($p < 0.05$); where no superscripts appear, differences were not significant ($p > 0.3$).
[g] Each mean ± standard deviation represents scores from 13 judges.

after a water rinse was the most distinguishable stimulus, while it was the least distinguishable if tasted after NaCl. It may be hypothesized that the NaCl rinse adapted the taste receptors to the taste of NaCl (O'Mahony, 1974, 1979b), so that subsequent NaCl samples would be perceived as tasteless (or near tasteless) and often be reported incorrectly as water. A prior water rinse would lower the adapting concentration so that the NaCl stimulus would taste stronger and be correctly identified more often as NaCl. The incorrect reporting of NaCl stimuli as water, at or near the adapting concentration, is a common bias especially when contrasted with the stronger taste of NaCl above the adapting concentration. Subjects need more training than given in the present study to learn that the tastelessness of NaCl at the adapting concentration is not the same as the taste elicited by water stimuli (O'Mahony, 1972, 1973, 1979b). Other results cannot be explained by a simple consideration of adaptation alone.

Water tasted after NaCl was less distinguishable than NaCl after a water rinse; this confirms earlier observations (O'Mahony et al., 1974). Two explanations present themselves. Firstly, it may be hypothesized that the NaCl taste system is more sensitive to supra- than sub-adapting taste stimuli in that it responds more to a given increase in concentration from the adapting concentration than a corresponding decrease. There is still theoretical uncertainty, however, about the exact nature of the sub- and supra-adapting NaCl transduction processes (Beidler, 1971; De Simone and Price, 1976; De Simone and Heck, 1980; De Simone et al., 1981). The second hypothesis is more simple. Subjects may have believed the tastes elicited by water to be signalling the presence of NaCl rather than water. Water stimuli elicit definite taste sensations as do weak supra-adapting NaCl stimuli. Both are difficult to perceive or describe and they are easily confused (O'Mahony, 1973; O'Mahony et al., 1976). They could both be mistakenly categorized as indicating the presence of NaCl, especially when presented in context with tasteless stimuli like NaCl after NaCl, which were wrongly believed to be water.

Water tasted after the NaCl rinse was more distinguishable than after the water rinse. From a consideration of adaptation alone, water after NaCl would be hypothesized as having had a sub-adapting taste quality while water after water would have been tasteless. However, O'Mahony (1972) noted that it was difficult to adapt to no sensation with distilled water. Thus, it may be hypothesized that water tasted after water also produced a sensation that was confused with the taste of low concentration supra-adapting NaCl. Subjective reports also confirmed that water in the two stimulus conditions, elicited sensations that were different; they gave no clue, however, as to why one was more confused with the taste of low concentration NaCl.

Development of a model: Sequential sensitivity analysis

The relative sensitivities of the two 3-AFC designs, noted in Experiment I, can be explained in terms of the distinguishability of the stimuli in Experiment II. Such a model is detailed in Fig. 1. In the upper portion of the figure, the distinguishabilities (signal strengths taken from Table 2) of the stimuli tasted after various rinses, are represented by dashed and solid lines. Also detailed are the six possible sequences of presentation for the two 3-AFC protocols along with the signal strengths of each stimulus in these sequences, represented by the same dashed and dotted lines. The frequencies of occurrence of each signal strength are also given for each protocol. It can be seen that the frequencies in the upper 'one strong stimulus' 3-AFC protocol are weighted more to stronger signal strengths than in the lower protocol, the differences occurring in the frequencies of the two weakest signal strengths. Thus, it would be predicted from this sequential sensitivity analysis that the upper 'one strong stimulus' 3-AFC protocol would be more sensitive to differences than the lower 'one weak stimulus' 3-AFC protocol. It would also be predicted that the regular triangle test protocol would have intermediate sensitivity, being a mixture of both 3-AFC protocols. This is what was found in Experiment I.

This analysis is an oversimplification; it omits any consideration of the distinguishability of the first stimulus in each triad. This itself depends on the final stimulus of the priorly presented triad. For the upper 3-AFC protocol, this final stimulus will be water twice as often as salt, while in the lower protocol, the ratio will be reversed. Taking this into account, the same analysis can be repeated. For the comparative sensitivites of the two 3-AFC protocols, the same result is obtained, the 'one strong stimulus: 3-AFC is more sensitive. The totals obtained for the upper protocol in Fig. 2 become, from strongest to weakest signal strength: 2-2/3, 2-2/3, 3-1/3, 1/3; for the lower protocol they become: 2-2/3, 2-2/3, 2/3, 3-1/3.

Exactly the same sequential sensitivity analysis can be applied to the four possible orders of presentation for the duo-trio test, two orders with the water stimulus as the priorly presented standard and two with the salt stimulus as the standard. Fig. 1 can be used for this analysis. The first two orders of presentation for the upper 3-AFC protocol are those that occur for the duo-trio test with water as the standard. The first two orders of presentation for the

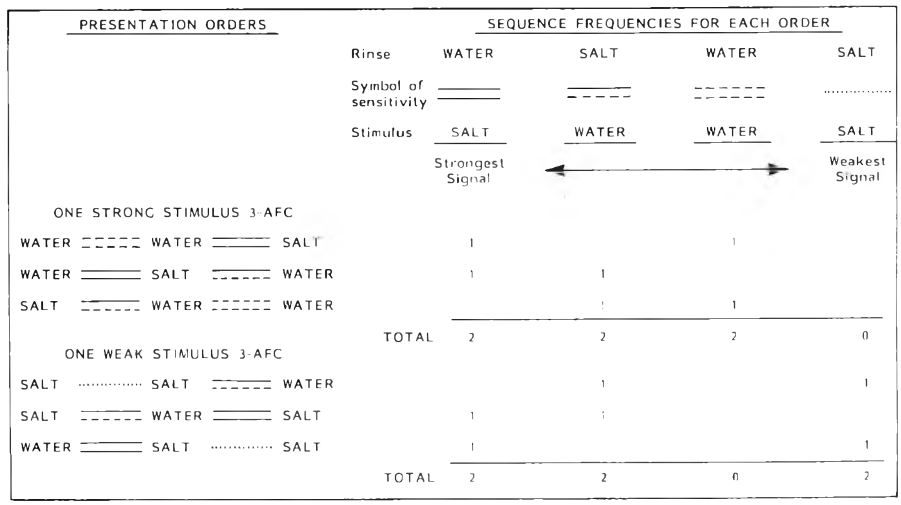


Fig. 1—Representation of the distinguishability of NaCl and water stimuli in the three possible orders of presentation for each of the two 3-AFC procedures making up the triangle test.

lower 3-AFC protocol are those that occur for the duo-trio test with salt as the standard. It can be seen that the duo-trio test with water as the standard has its frequencies weighted more to greater signal strengths and so would be predicted to be the more sensitive protocol. The regular duo-trio protocol having both salt and water as standards would be predicted to have intermediate sensitivity. These predictions were confirmed in Experiment I.

Comparisons between triangular and duo-trio protocols will not be made in this study, although an analysis of the percentage frequencies of the sequences in each protocol would predict that the duo-trio protocol would be more sensitive than respective triangular protocols and this was indeed observed in Experiment I. Such predictions are complicated, however, by insufficient knowledge of the relative weight to be given to each stimulus sequence, as well as of the psychological judgement aspects of the tests. Such comparisons will be appropriate when this model is developed further.

CONCLUSION

DIFFERENCES in the distinguishability of water and salt stimuli in different adaptation states, elicited by water and salt rinses, can be measured conveniently by the R-index rating procedure. Hypotheses invoked to explain such differences, considered more than a simple change in the level of adaptation; adaptation effects were more subtle. Using these data, a sequential sensitivity analysis can be used to predict successfully the relative sensitivity within various duo-trio or triangular procedures, merely by a consideration of signal strengths. Such an analysis can provide a basis for understanding such differences reported in the literature. That these differences were not merely the result of adaptation, was demonstrated by their obliteration when the general level of adaptation was raised by increasing the strength of the stimuli. The sequential sensitivity analysis developed for the low concentration stimuli would not apply for strong stimuli because the effect of the immediately preceding stimuli would seem to be disturbed by residuals from even earlier tastings (O'Mahony 1979b). The results of the sequential sensitivity analysis are specific to the particular stimuli under consideration. It invites generalisation to other stimuli by using the R-index procedure to measure the relative distinguishability of such stimuli in their respective sequences. The model has the potential for further development to allow prediction of differences in sensitivity between various test protocols and so contribute to the appropriate choice of difference test.

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Clinically Recorded Masticatory Patterns as Related to the Sensory Evaluation of Meat and Meat Products

EVA TORNBERG, STINA FJELKNER-MODIG, HÅKAN RUDERUS, PER-OLOF GLANTZ, KJELL RANDOW and DEREK STAFFORD

ABSTRACT

The masticatory pattern of meat and meat products, varying from red meat to sausages of frankfurter type, was registered by ten strain gauges attached to a prosthetic appliance. The deformation rates measured could be as high as 200 - 400 cm/min, which should be compared to the deformation rates of 20 cm/min usually used in instrumental analysis of meat texture. Among the instrumental records of the masticatory pattern the number of chewing cycles was the best single indicator of the sensory impression of meat texture. A highly significant correlation with the sensory evaluations was achieved when a toughness index was formed as a product of the loading rate and the number of chewing cycles. The relevance of this is discussed in the light of failure mechanics of food and a hypothetical neuromuscular control of mastication.

INTRODUCTION

MEAT is considered to be a high quality food by most people. From the sensory point of view the texture properties play an important role.

Sensory evaluation is, at present, the best way of recording the textural properties of meat (Brennan, 1980), but most sensory methods are time- and labor-consuming and therefore costly. To overcome the drawbacks of these methods several instrumental methods of texture analysis have been used (Voisey, 1979; Bourne, 1982). Szczesniak and Torgeson (1965) found that the Warner-Bratzler shear test instrument is the most widely used device for measuring meat toughness. In summarizing studies where correlations have been made between the Warner-Bratzler (W-B) shear tests and different methods for sensory testing, Szczesniak and Torgeson (1965) found correlation coefficients (r) ranging from −0.001 to −0.942.

There may be many reasons for the poor correlations and great variabilities. This has among others been discussed by Szczesniak (1968). The variations in the sensory evaluations might be too great because of erroneous panels or methodology (Bourne, 1982). Our concept of sensory perception of textural properties is a complex set of oral stimuli. The term "tender texture" of meat does not only imply ease of penetration through the meat but can embrace such characteristics as juiciness and the amount of residue after chewing. This multiple dimension of the sensory texture of meat was first pointed out by Cover et al. (1962).

It is also known that there is a great variability in meat itself. Meat tenderness can thus vary from one animal to another, from muscle to muscle within the same animal, and from one part to another in the same muscle.

A number of instrumental deficiencies have also been identified in the existing laboratory testing methods, (Voisey, 1976, 1979), particularly regarding the deformation rate of the tests (Peleg and Normand, 1982; Bourne, 1977; Voisey and Larmond, 1977). In deformation testing of foods the deformation rate is generally kept constant at a level of about 20 cm/min (Bourne, 1982), whilst the average deformation rates imposed on foods during mastication are believed to be in the order of 100 - 150 cm/min (Boyd and Sherman, 1975). Peleg and Normand (1982) have demonstrated theoretically, and Shama and Sherman (1973) experimentally, that both the deformation levels and the deformation rates affect the comparability of instrumental and sensory evaluation. However, Voisey and Larmond (1977) found that the relationship between W-B readings and sensory perception of beef tenderness was not improved by increasing the deformation rate from 25 to 155 cm/min.

In spite of the poor correlation between instrumental and sensory methods only a few studies have, however, been performed on masticating patterns (Yeatman and Drake, 1973; Bourne, 1982). The validity of these studies is also questionable as the force or load during mastication was recorded by a strain gauge inserted in a special inlay on the teeth. The inlay did not have precise retention and uncontrollable deformation could therefore appear between the strain gauged inlay and the teeth during mastication. It is believed to be possible to overcome these difficulties by placing multiple linear strain gauges and rosette gauges on the surface of a fixed prosthetic appliance using the techniques described and tested by Glantz et al. (1984a).

The aim of this investigation was to study the masticatory patterns for meat and some meat products using an intraoral prosthetic device with fixed strain gauges, compare these patterns with a simple sensory evaluation, and to discuss the implications for instrumental testing of eating quality of meat.

MATERIAL & METHODS

Samples of meat and meat products

Fourteen different samples of meat and meat products were evaluated. As shown in Table 1 nine of the samples were red meat, one was restructured beef, two were hamburgers and two were sausages of frankfurter type.

Samples 1 to 4 were from a crossbred gilt of Swedish Landrace and Swedish Yorkshire, slaughtered, graded and chilled according to routine methods. The carcass was of normal grade and the slaughter weight was 84.8 kg. The ultimate pH was 5.5 for M. longissimus dorsi (LD), 5.7 for M. semimembranosus (SM) and 6.0 for M. semitendinosus (ST). The pig meat was aged at +4°C for 7 days then sliced, vacuum packed and stored for 2 to 3 wk at −20°C.

Samples 5 to 9 and 11 were from a young bull of Swedish lowland breed (1.5 yr old) which was slaughtered, graded and chilled according to routine methods. The carcass was of normal grade and the slaughter weight was 212 kg. The ultimate pH was 5.5 for all muscles used. The meat was aged at +4°C for 12 days. LD and the middle part of ST were sliced, vacuum packed and stored for 1 to 2 wk at −20°C. SM was cut in two pieces. One was vacuum packed and stored at −20°C for 1 wk. The other was minced in a grinder (hole diameter: 3 mm), mixed with some salt and spices and formed into hamburgers. These were packed individually and stored at −20°C for 1 to 2 wk (sample 11).

Samples 10 and 12 were commercial products.

Authors Tornberg, Fjelkner-Modig and Ruderus are affiliated with the Swedish Meat Research Institute, P.O. Box 504, S-244 00 Kävlinge, Sweden. Authors Glantz and Randow are with the Dept. of Prosthetic Dentistry, Faculty of Odontology, Univ. of Lund, S-214 21 Malmö, Sweden. Author Stafford is affiliated with the Dept. of Restorative Dentistry, Dental School, Welsh National School of Medicine, Cardiff CF4 4XY, United Kingdom.

Samples 13 and 14 were sausages of frankfurter type. They were made from M. biceps femoris, back fat tissue, salt, water and potato flour. Water, fat and protein content was 64, 24 and 9%, respectively. Sausage A was made without vacuum in the bowl-chopper, whilst sausage B was made at an absolute pressure of 0.10 atm. Sensory tests evaluated the latter to be significantly firmer and less juicy, when unfried (unpublished results).

Cooking methods

Cooking conditions are given in Table 1. Samples 1 and 2 were chilled and served at room temperature. All other samples were served hot.

Warner-Bratzler force measurements

Force measurement was made with a Warner-Bratzler shearing device attached to an Instron Universal Testing Machine. The samples were prepared in the same manner as given in Table 1. Cores (diameter: 1.14 cm) were bored along the fibers perpendicular to the sample surface. The temperature of the sample was about 25°C. The cores were sheared in the Warner-Bratzler device and the shear force was registered as peak shear force. The measurement was repeated 4 to 11 times depending upon sample availability.

Clinical evaluations

The clinical evaluations consisted of an instrumental record of the chewing pattern and a verbal description of the sensory impression of the samples.

One partially dentate male, 63 yr of age, was used as the test subject. Thirteen natural teeth in the mandible were present and one conventionally designed three unit fixed prosthetic appliance served as a replacement for the lost natural left first molar tooth. From a functional point of view he had a dental situation equal to a whole set of natural healthy teeth extending from the left second molar to the corresponding tooth on the right hand side of the mandible.

He had a fixed prosthetic appliance in the maxilla with the following extension; abutment teeth: left and right first molars, left first premolar, right second premolar, and right canine. The following teeth were missing and thus replaced with pontics: right first premolar, left second premolar, left canine, left and right laterals, and left and right centrals.

The intermaxillary and functional contacts were found to be optimal and thus free from for example interferences and abnormal relations when judged independently by two senior consultants in prosthetic dentistry.

The subject considered himself to be in good general health and was found to be in good oral health on oral examination.

Table 1—Description of samples, raw materials, and cooking methods

Sample No.	Primal cut	Raw material		Cooking
1	Ham	Pig	M. semimembranosus	20 mm slice, vac. pac., heated with a velocity of 1°C/min in a water bath to a temperature of 58°C
2	Ham	Pig	M. semimembranosus	as sample no. 1 but to an ultimate temperature of 85°C
3	Cutlet	Pig	M. longissimus dorsi (12th - 15th vertebra thoracica)	20 mm slice fried for 3 min at 180°C on a double sided griddle
4	Ham	Pig	M. semitendinosus	as sample no. 3
5	Silverside	Beef	M. semimembranosus	as sample no. 1
6	Silverside	Beef	M. semimembranosus	as sample no. 2
7	Silverside	Beef	M. semimembranosus	20 mm slice fried for 2 min on a double sided griddle
8	Sirloin	Beef	M. longissimus dorsi (13th vertebra thoracica – 4th vertebra lumbalis)	as sample no. 7
9	Silverside	Beef	M. semitendinosus	as sample no. 7
10	Beef, restructured	Beef	beef trimmings, no salt and spices (fat content 10%)	5.5 mm slice fried for 1 min at 180°C on a double sided griddle
11	Hamburger	Beef	M. semimembranosus, minced (98.8%) salt and spices (1.2%) (fat content 2%)	10 mm, fried for 3 min at 180°C on a double sided griddle
12	Hamburger	Beef	beef trimmings, minced (98.8%) salt and spices (1.2%) (fat content 20%)	as sample no. 8
13	Sausage A	Pig & Beef	frankfurter type meat (32%) salt and spices (2%) (fat content 24%) made without vacuum	10 mm slice fried for 1 min at 180°C on a double sided griddle
14	Sausage B	Pig & Beef	frankfurter type meat (32%) salt and spices (2%) (fat content 24%) made with vacuum	as sample no. 13

Ten strain gauges were attached to various parts of the maxillary bridge with an adhesive type M-bond 4 3 b Adhesive (Micro-Measurements. Romulus, MI). Gauges 1, 2, 3, 7, 8, 9 and 10 were linear gauges type EA-09-031-MF-120 (Micro-Measurements) whilst gauges 4, 5 and 6 were the components of a rosette gauge type WA-09-031-WR-120 (Micro-Measurements). The resistance was 120.0 Ω for all the gauges. The gauge factor was 2.08 for the components of the rosette and 2.135 for the rest of the gauges. These factors were used to convert the actually measured apparent strain into true strain values. In order to protect the gauges from moisture contamination in the oral cavity they were covered with a silicone material type ORTV (Dow Corning Co., Midland, MI). The positions of the gauges are given in Fig. 1.

The gauges were connected to a strain gauge conditioner and amplifier and a UV-oscillograph also using the coupling systems described by Glantz et al. (1984b). The most suitable positions for the gauges were determined according to the laboratory analysis with strain laquers described by these authors.

The strain gauged maxillary bridge was attached to its abutment teeth, conditioned, allowed to equilibrate to the oral conditions, and then pretested during sets of maximum bites and empty mouth chewing cycles according to the methods given by Glantz et al. (1984a). The maxillary bridge was retained with a temporary cement type Nobetec (Astra, Södertälje, Sweden), which was handled according to the manufacturers instructions.

The patient was given standardized samples (20 x 20 x 15 mm) of the meat and the chewing pattern was recorded. When the patient considered the sample ready to swallow he spat out the sample residue and rinsed the mouth with water.

A verbal description of various sensory impressions was made in accordance to the test form given in Table 2. As the patient was not a trained sensory panelist the sensory evaluation was made on a scale of only three points to facilitate for the patient. "Resistance to initial chewing" refers to the ease of penetration through the sample at the start of chewing. "Elasticity" is used to describe the rubbery character of the sample. "Chewiness" refers to the amount of chewing that has to be applied to get the sample ready for swallowing, while "ease of disintegration" indicates the fracturability of the sample during chewing. A sum of these scores for every sample was formed, denoted "total toughness impression." "Amount of residue" and "juiciness" are self-explaining. The samples were tested in random order and the evaluation was repeated within one week.

RESULTS & DISCUSSION

Sensory evaluation

The results of the sensory evaluation during session 2 are given in Table 3. The initial and the masticatory sensory scores seem to run more or less in parallell for most of the samples. Total toughness impression together with scores for amount of residue make the cutlet (3), ST from both pork (4) and beef (9) and the restructured beef (10) the least tender samples. When comparing the tenderness of SM of pork (1, 2) and beef (5, 6) the former was judged to be more tender. The least juicy products were the cutlet (3) and the hamburger produced without additives (11).

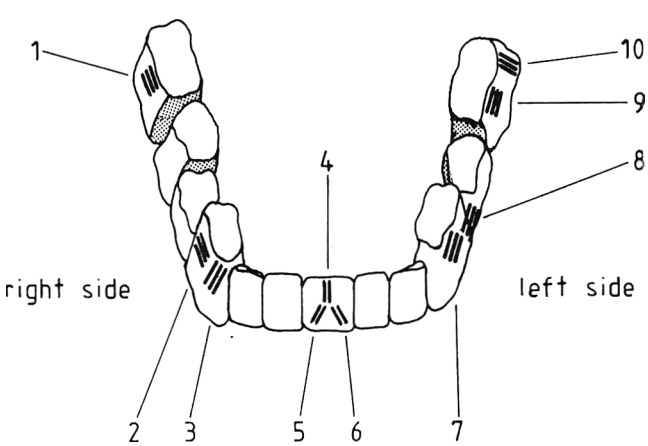


Fig. 1—Position of the gauges on the maxillary prosthetic appliance.

Warner-Bratzler force values

Table 3 also gives the results of the Warner-Bratzler force tests performed at session 2. The comminuted samples (10, 11, 13 and 14) had much lower values than the whole meat samples. Although the sensory scores in this investigation are crude, the following observations are interesting. The sensory impressions for the pork samples are not very well reflected by the shear force measurements. For the beef samples the agreement is much better. Again when the meat products are included there is no agreement, indicating that the measuring principle of the Warner-Bratzler device is not very relevant at least for pork and different meat products.

Masticatory pattern

Fig. 2 shows an example of the clinical recordings that were obtained from the ten gauges. Generally the course of mastication was relatively even throughout the individual experiments. This observation is not in accordance with Andersson (1953), who observed that mastication of meat was performed at relatively higher force levels at the beginning of the mastication process.

Both the linear gauges and the rosette gauge gave signals reflecting the type of complex clinical straining pattern previously reported for many types of prosthetic appliances (Glantz and Stafford, 1973, 1980; Glantz et al., 1984a). In general terms the three components of the rosette gauge showed equivalent quotients for the recordings from different meat samples and sessions. Thus this enables direct comparisons to be made between the recordings from the individual gauges.

The signals from the different strain gauges measure the deformation (elongation or compression) of the appliance at individual sites. This in turn is assumed to be an indirect measure of the force or load that is applied by the jaws. A detailed analysis of the signals from all individual gauges during the chewing of individual samples is not meaningful for the purpose of this paper. We have merely chosen to study the signal pattern from a few typical gauges comparing these between the different samples. Earlier experience shows that the fixing of the strains gauges is reliable and we have no reason to believe that the geometry and mechanical properties of the dental appliance change during a session of the type used here. Thus we assume that the strain gauge signals are proportional to and give a true and reproducible measure of the force used to chew the sample. The absolute value of this force is difficult to calibrate in a meaningful way (Glantz et al., 1984a, b). We have therefore chosen to express the *force or load exerted by the teeth on the food* as the strain (corrected to true $\mu\epsilon$) experienced by the strain gauges.

Table 2—Test form used for the sensory evaluation

	Low		High
Initial impression			
Resistance to initial chewing	0	1	2
Elasticity	0	1	2
Mastication impression			
Chewiness	0	1	2
Ease of disintegration	2	1	0
Residual impression			
Amount of residue	0	1	2
Juiciness	2	1	0

The type of deformation (elongation or compression) recorded from the individual gauges had little to offer with respect to the aim of this study. It has therefore not been considered when evaluating the recordings.

The total chewing times and the total number of chewing cycles were determined from the sets of recorder traces for every experiment. These data were then used for the calculation of the time per chewing cycle (= chew cycle time). As each chewing cycle was found to consist of one active (chew stroke) and one passive component, the times for both these were determined.

Finally, individual peak heights (maximum strain, $\mu\epsilon$) as well as the front slopes of the peaks (strain rate, $d\epsilon/dt$) were measured.

Maximum load

The maximum force or load exerted by the teeth on the food, expressed as mean maximum strain in gauges 1, 8 and 9, was calculated for each sample. The mean maximum load values and their corresponding standard deviations for gauge no. 1 are plotted in Fig. 3. No statistically significant differences were observed between the maximum load values recorded at the two sessions, even though most of the higher load values were noted during session 1 for gauge 1 and during session 2 for gauges 8 and 9.

As no systematic differences were observed the noted differences for individual gauges were probably either due to tooth movements appearing between the sessions or to a slight variation in the relations between the strain gauged bridge and its abutment teeth created at the recementation of the bridge.

In order to compare the signals from the different gauges the mean maximum load of both sessions was calculated for each sample and plotted in Fig. 4.

The maximum load was found to be significantly lower for the sausages than for the whole meat samples. Further, it was observed that for samples with decreasing amount of comminution, i.e., the two types of hamburgers and the restructured beef, the maximum load increased. For all the whole meat samples (1 to 9) higher load values were observed than for the comminuted samples (10 to 14). An exception to this pattern was, however, noted for gauge 9, where the readings for the two hamburgers were the highest. This could indicate variations in the manner of mastication between food samples of different degrees of comminution.

Table 3—Sensory evaluation and Warner-Bratzler force values of the different samples of whole meat and meat products from session 2. Total toughness impression is the sum of initial and masticatory scores. The samples are numbered according to Table 1.

	Initial		Masticatory					Warner-Bratzler force value (kg/cm^2)		
Sample	Resistance to initial chewing	Elasticity	Ease of disintegration	Chewiness	Total toughness impression	Amt. of residue	Juiciness	$\bar{x}$	s.d.	n
1	1	0	1	0	2	1	0	2.54	0.66	8
2	0	0	0	0	0	0	1	3.54	0.41	9
3	2	2	2	2	8	2	2	2.69	0.41	6
4	2	2	1	2	7	2	1	2.93	0.26	9
5	1	2	2	2	7	0	1	3.31	0.62	11
6	2	2	2	2	8	0	1	3.20	0.52	11
7:1	2	2	2	2	8	0	0			
7:2	2	2	2	1	7	1	1			
8	0	0.5	0	0	0.5	2	0	2.11	0.50	6
9	2	2	2	2	8	2	1	3.06	0.50	9
10	2	2	2	2	8	2	1	0.49	0.10	4
11	0	1	0	2	3	2	2	0.37	0.13	9
12	0	1	0	2	3	2	0			
13								0.17	0.04	9
14	0	0	0	0	0	0	0	0.19	0.04	12

$\bar{x}$ = mean value; s.d. = standard deviation; n = number of recordings.

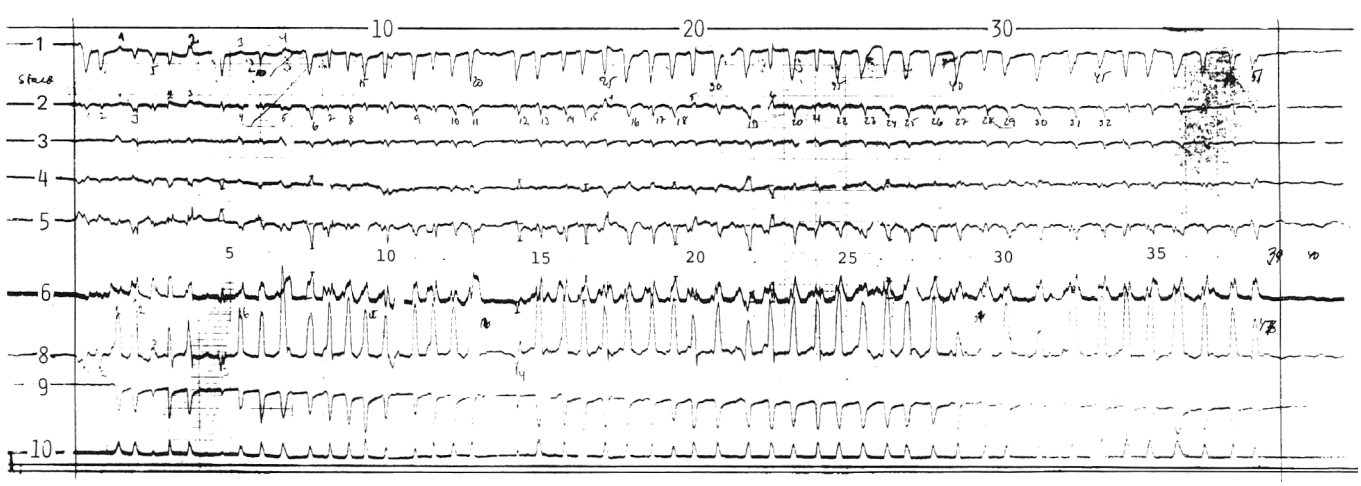


Fig. 2—UV recorder trace showing the type of masticatory pattern that is recorded by strain gauges fixed to a prosthetic appliance (Sample 8, session 2).

Number of chewing cycles

The total number of chewing cycles for every sample is given in Fig. 5. For all samples the total number of chewing cycles required was higher at session 2 than at session 1. This could be due to the fact that at the second session the test subject had been chewing other types of test foods before the beginning of the experiments. Irrespective of this fact, however, at both sessions the relative number of chewing cycles between different samples was very similar.

The number of chewing cycles was rather high and also varied widely – from about 20 to about 70. This can be compared to the results of Harrington and Pearson (1962) who obtained chew counts for loins ranging from 36 to 47 with one exceptional value at 60. The sausages were found to require only about 20 cycles, while the hamburgers and the most tender whole meat samples, sirloin steak (8) and SM of pork and beef (1, 2, 5 and 6), all required about 35 cycles each. Restructured beef (10) was chewed for 45 cycles at session 1 and 59 cycles at session 2. This was close to the number of chewing cycles required for the pieces of meat that were chewed the most, such as ST (4 and 9), which is particularly rich in connective tissue, especially in beef. The latter sample also required the highest number of chewing cycles.

Chew cycle time

Fig. 6 gives the chew cycle time for the different samples. The chew cycle time was found to vary between 0.7 and 0.95 sec and had a mean duration of 0.8 sec. No significant difference could be observed either between the two sessions or the different meat samples. Bourne (1977) has shown that a wide interindividual range of chew cycle times exists and that amongst groups of students chewing sticks of gum these times varied from 0.93 to 1.10 sec. Voisey and Larmond (1977) observed chew cycle times between 0.55 and 1.11 sec during the eating of spaghetti and beef with a mean value of 0.73 sec. Bearing this in mind, the chew cycle times observed in this study seem to be reasonably representative for a normal dentate population.

Chew stroke time

The chew stroke times varied between 0.15 and 0.34 sec (Fig. 6) corresponding to between 15 and 42% of the chew cycle time.

In comparison with the deformation rates of imitative, instrumental testing, the stroke time and not the chew cycle time should be considered. As can be seen in Fig. 6 the whole meat samples required mean stroke times of 0.27 sec (range: 0.23 – 0.32 sec). The corresponding values for comminuted meat products were 0.18 sec (range: 0.15 – 0.20 sec) and for restructured beef 0.23 sec.

As the length of one side of the tested pieces of meat was 1 – 2 cm, the actual stroke speed for whole meat could be as high as 200 – 400 cm/min. This value should be compared with the rate used at the Instron test in this

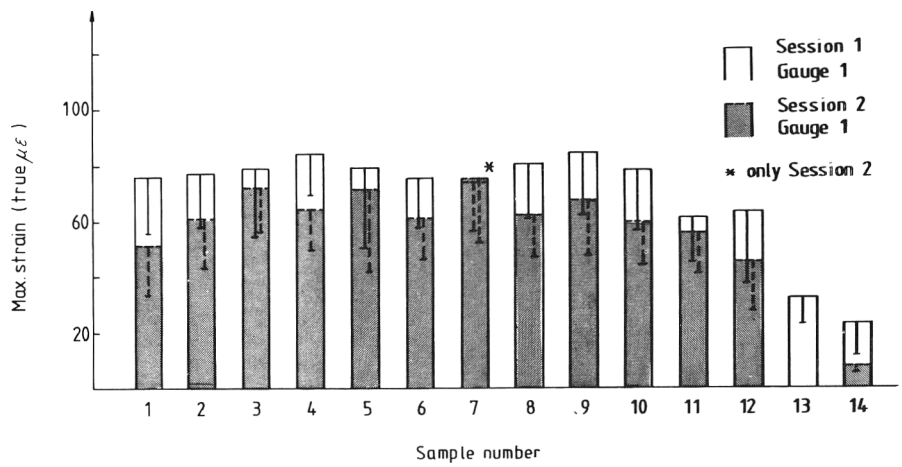


Fig. 3—Mean maximum load measured as maximum strain recorded in gauge 1 for individual samples at both sessions. For description of the samples see Table 1.

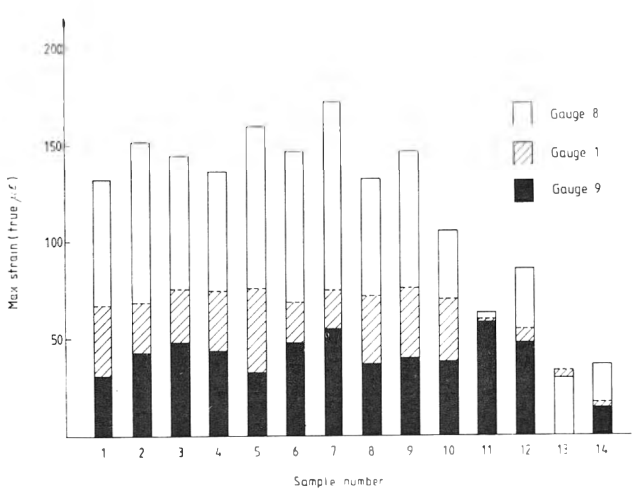


Fig. 4—Mean maximum load of the two sessions (measured as maximum strain recorded in gauges 1, 8 and 9) for every sample. For description of the samples see Table 1.

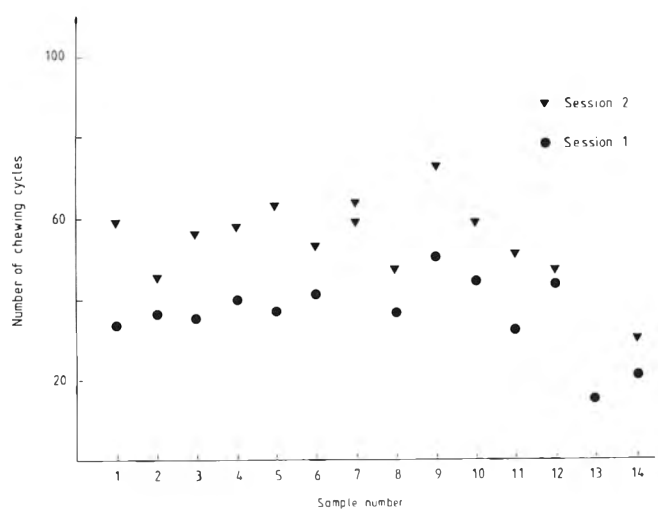


Fig. 5—Number of chewing cycles for every sample at the two sessions. For description of the samples see Table 1.

investigation, i.e. 9.6 cm/min. In instrumental evaluations of meat products Bourne (1982) recommends the deformation rate to be 20 cm/min. It has recently been shown that the deformation rate affects the rheological properties and rupture characteristics of the tested samples (Peleg and Normand, 1982). Therefore, in instrumental tests of meat texture, it is suggested that clinically relevant deformation rates should be used to improve the correlation between objective and subjective tests. There is, however, no general agreement on this point since Voisey and Larmond (1977) were unable to improve the correlation between sensory evaluation and shear force measurement by increasing the deformation rate from 2.5 to 155 cm/min. It should also be noted that the use of higher deformation rates so far has been limited by recorder pen response time in the standard instruments used. The use of micro-computers and electronic read-outs could possibly solve this problem.

Loading rate

The slope value ($d\epsilon/dt$) of the strain peaks has not been measured. This would have involved a rather detailed analysis of individual peaks. Instead, a mean slope value has been calculated from the peak height and the chew stroke time. These mean slope values for gauges 1, 8 and 9 have been plotted in Fig. 7.

In accordance with the view that the recorded strain values represent the (mastication) force or load on the food, the peak slope obviously represents the loading rate i.e. the increase in force or load with time.

If we assume that the resulting rate of movement of the teeth on the food material is constant, the front part of the peak will also approximate a force-deformation curve for the food material.

A concept for meat texture analysis

During mastication of food samples, deformation and failure of the samples take place. The deformations can either be recoverable (elastic) or non-recoverable (viscous or plastic). The failures may in turn be either ductile or brittle; they are considered ductile when accompanied by appreciable viscous deformation and brittle when not (Jowitt, 1979).

The basic principles of the current theories of failure appearance are based on the likelihood of propagation of cracks or discontinuities in the sample (Jowitt, 1979). When a meat sample is subjected to a tensile force normal to a crack, stress (σ_t) concentrates at the edge of the crack, in contrast to what happens in shear parallell to the crack when stress concentration is much less. Therefore tensile forces are the most significant during failure of meat products as they cause most crack propagation. It can be argued that no pure tension is likely to appear in food during mastication. If, however, a food sample has a flaky or filamentous structure, tensile force components will arise by stress transfer when it is sheared or compressed (Jowitt, 1979). Thus, in meat compression or shear is likely to result in direct or resolved tension when it is masticated. That tensile stresses can be important when meat ruptures during mastication and in the W-B test cell has also been discussed by Voisey (1976).

The smallest tensile stress, f_t, needed to extend a crack, is (Knott, 1973):

$$f_t = \left(\frac{2E\gamma}{\pi c}\right)^{1/2}$$

where E = the elastic modulus of the material during tension; γ = the surface energy of the fractured surfaces; c = the half length of the crack. This equation tells us that the longer the crack the smaller the force, f_t, required to propagate the crack. It also says that the larger the elastic modulus of the material the greater the force needed for crack propagation. There are many restrictions to this equation in practice, but nevertheless it gives the main principles of failure mechanics.

Firstly, the equation given above implies a fully brittle fracture in a linearly elastic material. Of course this is an oversimplication with regard to meat and meat products. Meat and meat products are viscoelastic materials characterised by a complex modulus, G*, in shear and complex modulus, E*, in tension. These moduli are in turn built up of two moduli, the storage and the loss modulus, where the former is the elastic modulus. The complex modulus of linearly viscoelastic materials can be evaluated from the slope of a force-deformation curve if performed non-destructively. According to the earlier discussion the strain-time-curves obtained in these studies can be looked upon as force-deformation curves. Mastication is, however, by no means a non-destructive process, and meat is not linearly viscoelastic under the strains in concern. Therefore the slope of the strain-time-curve (loading rate) does not directly give the complex modulus of the undestroyed meat but rather a mean of complex moduli obtained during deformation and fracturing of the meat product.

The elastic modulus of the meat at the point of the crack is governed not only by the viscoelastic properties of the meat but also by the way the applied stress is relaxed or transferred within the material. If the stress transferred in the meat product is reduced by viscous dissipation, the tensile stress at the crack is very much reduced. Hence crack propagation is prevented.

The viscoelastic properties of meat are also influenced by the deformation rate, the higher the rate of deformation the lower the amount of viscous dissipation during stress relaxation. It is therefore understandable that in order to create brittle fractures the loading rate must be high during mastication.

There are also other equally important mechanisms that hinder crack propagation. Stress concentration at the crack can be blunted by the edge being non-sharp, i.e. having a spheroidal form. As a special case of this type of crack blunting Purslow (1984) has recently shown for cooked meat that by the opening of weak interfaces (perimysial tracts) perpendicualr to the crack path (the Cook-Gordon mechanism), whole meat is almost totally insensitive to the presence of cracks.

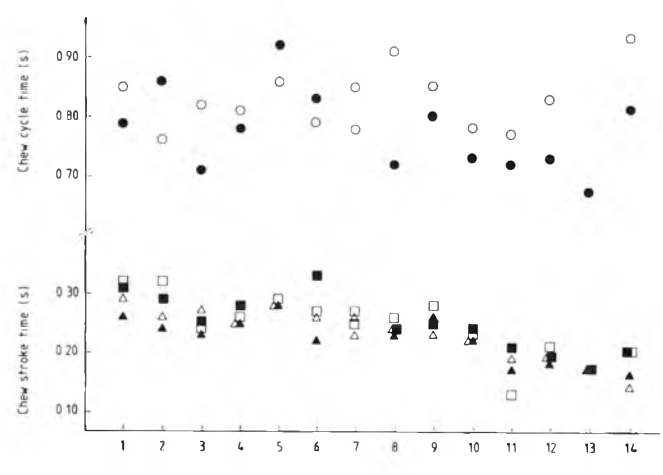


Fig. 6—Chew cycle time (● Session 1; ○ Session 2) and the chew stroke time for every sample at the two sessions. The chew stroke time of gauge 1 (▲, △, Sessions 1 and 2) and 8 (■, □, Sessions 1 and 2) are given. For description of the samples see Table 1.

It is interesting to compare the results of Fig. 4, 5 and 7 with regard to the different samples. The sausages had the lowest values in every figure. This is probably due to their high degree of comminution giving rise to a network on heating, which is capable of stress transfer (no Cook-Gordon mechanism). Moreover, there are more initial cracks and relatively low loading rates (Fig. 7), which results in a small f_t.

The hamburgers required higher maximum loads and higher number of chew cycles than the sausages. This could be due to the lower degree of comminution of the hamburgers, which gives rise to a smaller initial number of cracks and a looser network which binds the whole meat pieces together (less capable of stress transfer). Further, as can be seen in Fig. 7, hamburgers had comparatively high loading rates probably reflecting relatively large moduli. In Fig. 7 it can also be observed that the loading rates of the hamburgers are almost as high as those of the whole meat. However, for most of the gauges the mean maximum load was lower for the hamburgers than for the whole meat (Fig. 4). This could be due to the Cook-Gordon mechanism being much more effective in preventing crack propagation in whole meat as compared to hamburgers, because in the latter product the fibrillar structure has been broken down to a certain degree.

For the restructured beef (10) the load rate (Fig. 7) and the number of chewing cycles (Fig. 5) were within the same range as those for the least tender whole meat. The difference compared to the two hamburgers could be due to a higher degree of fibrillar structure (more crack blunting) and fewer initial cracks in the restructured beef, as a result of a lower degree of comminution. The similarity to the less tender whole meat could be caused by the raw material in restructured beef being relatively rich in connective tissue.

When comparing the different samples of whole meat the difference in tenderness observed could be due to variations in the modulus and to more or less adhesion between the denatured muscle fibers, giving rise to varying degrees of crackblunting. The most extensive chewing was observed for ST of beef (9), which is known to be one of the least tender muscles (Table 4). Samples 1, 2 and 8 were considered the most tender (Table 4) and needed relatively low numbers of chewing cycles during mastication.

Correlation between masticatory pattern and sensory evaluation

Multiple linear regression analysis was performed between the sensory attributes (total toughness, amount of residue and juiciness) and the clinical recordings (maximum load, loading rate recorded in gauges 1, 8 and 9) and the number of chewing cycles. Maximum load from the Warner-Bratzler test was also included in the regression analysis. The results are shown in Table 4.

The number of chewing cycles gave a statistically significant relationship with all combinations of the sensory attributes. Juiciness was of little relevance and total toughness is the main contributor to the variation in number of chewing cycles. The loading rate gave a significant relationship with all the sensory attributes only for gauge 8. For total toughness alone there was a significant relationship with loading rate also in gauge 1.

For the Warner-Bratzler values no significant relationship could be observed for any combination of the sensory attributes. The maximum load registered in gauges 1 and 8 correlates significantly only when total toughness is considered and with a lower correlation coefficient than for the number of chews and for the loading rate. The low correlation of the Warner-Bratzler readings can, to a certain extent, be explained by the low deformation rate as compared with true mastication. That, however, can hardly be the only reason as rather low correlation coefficients were found also for the maximum load during mastication.

To get a measure of meat texture that correlates better with sensory evaluations, toughness indices were formed by different combinations of the loading rate, the number of chewing cycles, and the maximum load. Such indices were calculated for gauges 1, 8, and 9 for all tested products and have been correlated as above with the sensory attributes. The results in Table 5 indicate that the best and most significant correlation is obtained for the toughness index that is made up of loading rate and number of chews, which proably is reflecting both the modulus of the food sample during mastication and the resistance to disintegration.

A simplified hypothesis for the neuromuscular control of mastication of meat products

Some of the observations made in this study can be summarised as follows: The course of mastication is relatively even throughout individual experiments. Number of chews explain most of the sensory impression. A product between the number of chews and the loading rate increases the degree of explanation for the sensory impression, whereas the contribution from the maximum load is relatively small.

It can be assumed that the magnitude of deformation of a meat sample during every stroke should be the same

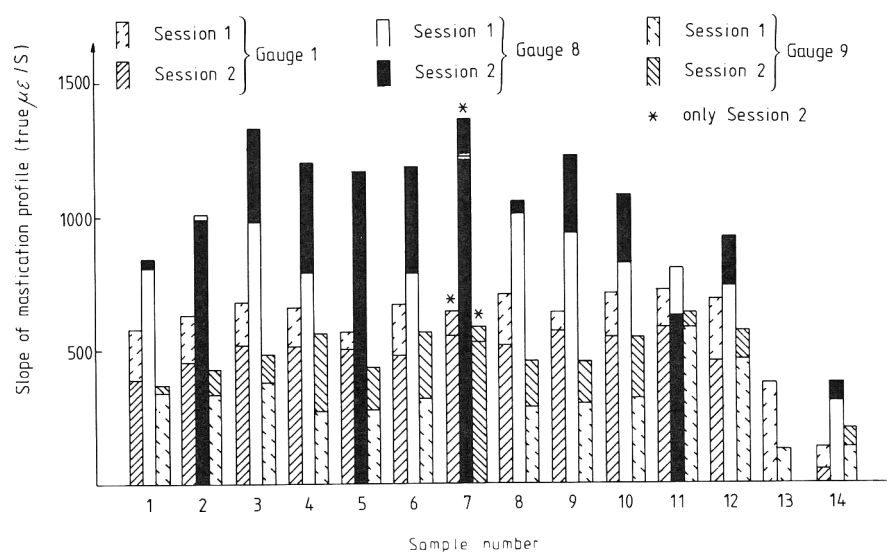


Fig. 7—Mean loading rate measured as the slope of the mastication profile for every sample at the two sessions and recorded in gauges 1, 8 and 9. For description of the samples see Table 1.

Table 4—Multiple linear regression analysis of instrumental clinical recordings and sensory attributes

Instrumental recordings	Sensory attributes: Total toughness impression	Toughness impr. + residual	Toughness + residual + juiciness
Number of chewing cycles	0.74**	0.77**	0.77*
Loading rate			
gauge 1	0.56*	0.68*	0.70 ns
gauge 8	0.75**	0.76*	0.76*
gauge 9	0.47 ns	0.60 ns	0.63 ns
Maximum load			
gauge 1	0.61*	0.62 ns	0.63 ns
gauge 8	0.56*	0.59 ns	0.60 ns
gauge 9	0.52 ns	0.61 ns	0.64 ns
Warner-Bratzler maximum load	0.27 ns	0.43 ns	0.43 ns

ns = not significant * = $p < 0.05$ ** = $p < 0.01$

Table 5—Multiple regression analysis of clinical toughness indices and sensory attributes

Toughness index	Gauge number	Total toughness impression	Toughness impression + residual	Toughness impr. + residual + juiciness
Loading rate × Number of chews	1	0.74**	0.79**	0.80**
	8	0.71**	0.86***	0.88**
	9	0.68**	0.85***	0.88**
Number of chews × Maximum load	1	0.75**	0.75*	0.75*
	8	0.69**	0.70*	0.71 ns
	9	0.76**	0.82**	0.82**
Loading rate × Number of chews × Maximum load	1	0.80***	0.80**	0.81*
	8	0.74**	0.75*	0.75*
	9	0.63*	0.77**	0.81*

* = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$

(if no control mechanism comes into play), zero deformation being defined by the size of the meat piece and the upper limit of deformation being determined by the teeth hitting each other. A prerequisite is that the different samples should be relatively similar in size. If as a first approximation it is assumed that the rate of the teeth movement is constant, the following control mechanism of the masticatory pattern can be suggested.

If the clinical record traces are viewed as force-deformation curves of the food material (as argued earlier), a higher modulus in the meat sample would give rise to a higher maximum load applied by the jaws at the same degree of deformation. The larger the modulus the bigger will be the maximum load. The observed even course of mastication throughout an individual experiment suggests that there exists an upper limit for the load that will be applied by the jaws. This postulated "upper limit" is not to be confused with the absolute maximum loads reported in the literature. It is rather a (much lower) trigger value at which a control mechanism sets in. (This trigger value may well be different for different food materials and could in fact be influenced by the visco-elastic properties of the specific food material.) Thus, if a meat sample has such a high modulus that this control mechanism comes into play, the loading will stop before the maximum deformation has been achieved. This means that for food samples with low moduli the difference in maximum load will be a measure of the difference in modulus. For samples with high moduli there will be no difference in maximum load. There will, however, be a difference in the loading rate, which will be larger for the meat with higher modulus. There will also be a difference in the degree of deformation being lowest for a high modulus sample. This smaller deformation and, therefore, lower probability of fracture can be compensated by a higher number of chewing cycles. According to this postulated mechanism tough meat should be characterised by a high number of chewing cycles having a steep slope of the force-deformation curve (high loading rate). This is also suggested by our results.

Problems will occur if a meat sample has such a high modulus that the resulting deformation will cause no failure at all. An increase in number of chews will then be of no value. Therefore, there probably exists a control mechanism that can adjust the rate of teeth movement so that the best combination of brittle and ductile failure is achieved.

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Effect of Storage Conditions of Dry Bean Seeds (*Phaseolus vulgaris* L.) on Texture Profile Parameters After Cooking

RUTH DOS SANTOS GARRUTI and M. C. BOURNE

ABSTRACT

Red kidney beans were stored at constant moisture and high and low temperature for 6 months. Instrumentally measured parameters of hardness, fracturability, gumminess, chewiness, springiness and cohesiveness were higher in samples stored at elevated temperatures than the control (2°C). The sensory panel found the stored beans had higher hardness, fracturability, lumpiness, chewiness, and skin toughness and less starchiness, gumminess, pastiness and moisture absorption than the control. Puncture forces followed approximately a normal distribution curve, and there was always some overlap between stored and control beans even when the stored beans had a much higher mean puncture force than the control. All textural parameters of cooked bean cotyledons changed substantially during storage of the seeds at elevated temperatures and high relative humidity.

INTRODUCTION

IT IS WELL KNOWN that dry legume seeds stored under conditions of elevated temperature and humidity progressively lose the ability to soften during cooking (Morris, 1963). This is a nutritional problem for those populations living in humid tropical and subtropical areas who depend on beans for supplying a large part of their dietary protein, an economic problem because of the loss of an important functional property of the beans and an energy problem for those areas where fuel is costly because of the long cooking times needed to soften the stored beans.

The term cookability refers to the condition in which the dry legume seeds achieve a degree of tenderness acceptable to the consumer during cooking. The loss in cookability of stored dry beans has been studied by several researchers, each one emphasizing different aspects: hydration rate (Rockland and Jones, 1974); cooking rate (Kon, 1980; Molina et al., 1975, 1976); storage time-temperature and moisture content (Burr et al., 1968; Mattson, 1946; Morris, 1963; Sefa-Dedeh and Stanley, 1979; Sefa-Dedeh et al., 1979); bean cell wall components (Rozo, 1982); changes in microstructure (Rockland and Jones, 1974; Varriano-Marston and Jackson, 1981); chemical and/or enzymatic changes that occur in the seedcoat and the cotyledon during storage (Gloyer, 1921; Jackson and Varriano-Marston, 1981; Moscoso et al., 1984).

In all the above studies the hardness of the cooked beans was measured either by cooking time required to reach an arbitrary degree of softening or by the firmness of the beans after a standard cooking time. No measurements were made of textural properties other than firmness.

The objective of this study was to fully characterize the changes in textural properties that occur after bean seeds have been stored for extended periods under warm humid conditions. Texture Profile Analysis (TPA) by instrument and trained sensory panel were used to accomplish this objective.

Author Bourne is affiliated with the NYS Agricultural Experiment Station, Cornell Univ., Geneva, NY 14456. Author Dos Santos Garruti is with the Universidade Estadual de Campinas, Campinas - 13100, SP Brasil.

MATERIALS & METHODS

DRY light-red kidney beans (*Phaseolus vulgaris* L.) of the Redkloud variety with 16% moisture content from the 1982 New York crop were used. Moisture content was measured by drying powdered samples in a hot air oven to constant weight. A lot of 100 kg of beans was cleaned and size-graded using sieves with 3/4 in long slits with parallel sides and semicircular ends. The fraction passing through 17/64 in. and retained by 12/64 in. slits representing approximately 95% of the beans was used in the experiment. The rejected 5% fraction consisted mostly of small and split beans. This fraction was rejected because it has been shown that small size bean seeds are prone to suffer from poor water imbibition (hardshell) after storage (Bourne, 1967). The beans were sprayed with a solution of potassium sorbate in absolute methanol in the proportion of 1:8 w/v per kg to control mold growth and then spread out in a single layer overnight to allow the methanol to evaporate before putting the beans into storage.

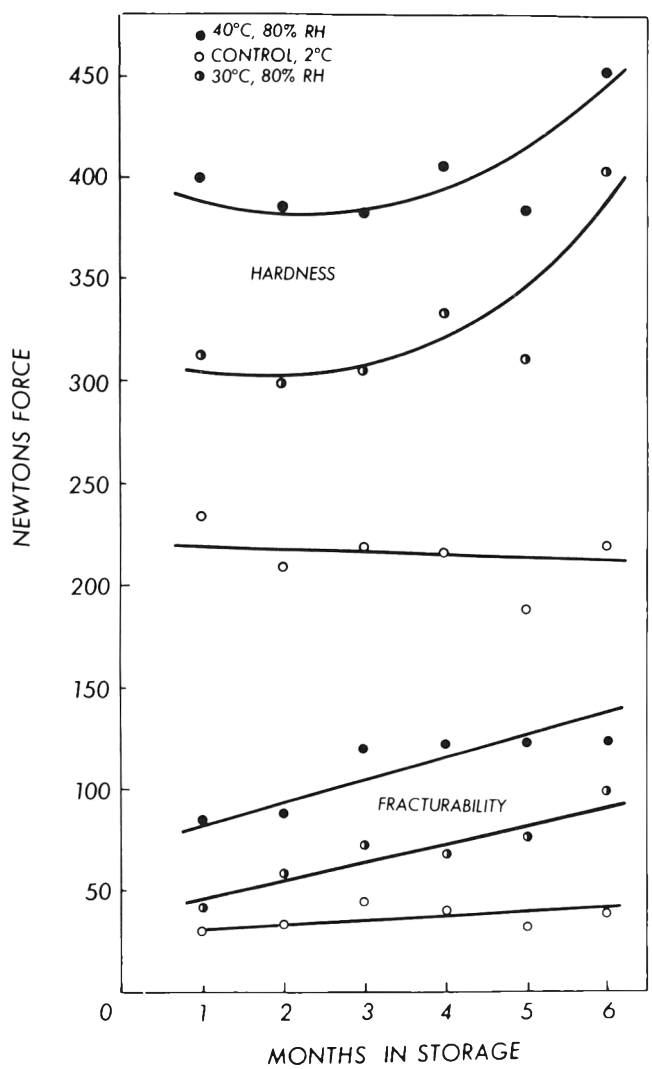


Fig. 1—Changes in instrumental hardness and fracturability of red kidney beans cooked 30 min after storage.

Table 1—Sensory texture profile procedure for cooked red kidney beans. Panel technique and definition of terms

Stage 1—Place one bean in mouth, manipulate gently with tongue without breaking the bean. Evaluate bean surface for:

Smoothness of skin - feel of surface on tongue
Moistness of skin – degree of moistness of surface

Stage 2—Chew the bean once between molars. Evaluate:

Hardness - force required to crush bean with teeth
Fracturability - degree to which bean crumbles, cracks or shatters
Starchiness - feeling of free starch grains
Lumpiness – presence of large particles harder than surrounding medium
Moistness - amount of moisture in crushed cotyledon

Stage 3—Place one bean in mouth and chew until ready to swallow. Evaluate for:

Chewiness - number of chews to reach final swallowing point
Gumminess - denseness and cohesion persisting during mastication
Starchiness – as above
Lumpiness – as above
Grittiness – presence of small harder particles that stand out in the medium
Skin toughness - force to cut skin with incisors
Moistness – as above

Stage 4—Place one bean in mouth, chew and swallow. Evaluate:

Rate of breakdown of cotyledon –
Rate of breakdown of skin –
Pastiness of bolus – viscous somewhat sticky sensation
Lumpiness of bulus (number of lumps) –
Lumpiness of bolus (hardness of lumps) –
Grittiness – as above
Toughness of skin pieces – as above
Moisture absorption by cotyledon –
Moisture absorption by skin –
Particles around gums and teeth - degree of coating remaining in mouth
Irritation in throat - degree of unpleasant coating in throat
Burning on tongue - sense of chemical irritation

Three storage conditions were used: (1) 2°C (control); (2) 30°C, 80% RH; (3) 40°C, 80% RH. Previous experiments have shown that dry beans stored at 2°C undergo almost no change in cooking properties in 1 yr. The dry beans were stored in tightly sealed 5 gal. plastic drums to prevent change in moisture content. The moisture content showed no change during storage. About every 4 wk the beans were mixed and approximately 2 kg were drawn off for soaking and cooking. The remaining beans were returned to storage. The 2 kg sample was shaken on bean sieves with 16/64 in. and 15/64 in. wide slits. The –16/64 in. + 15/64 in. fraction (fraction 1) was reserved for instrumental TPA because TPA requires close tolerance of sample dimensions. The +16/64 in. and –15/64 in. fractions together with that part of fraction 1 not needed for TPA were mixed to make fraction 2 which was used for the other tests.

Both fractions were soaked 15 hr in distilled water using a 1:4 bean/water ratio, then drained, rinsed in distilled water and drained again. One hundred gram aliquots of soaked beans were placed in 303 x 406 cans which were filled with distilled water at 95–100°C, sealed, and processed 30 min in boiling water then quickly cooled by immersion in cold water. The cooked beans were stored 20-48 hr at 2°C, opened, drained of free liquid and brought to room temperature before performing the texture tests.

Instrumental texture profile analyses were performed in the Instron Universal Testing Machine by compressing single whole beans between two extensive flat surfaces twice to 1.0 mm height at a speed of 5 cm per min. Instrumental TPA is based on the procedures developed by Friedman et al. (1963) and Szczesniak et al. (1963) as modified for the Instron by Bourne (1968). A computer-assisted procedure for extracting relevant data from the force-time plots was used (Bourne et al., 1978). The mean values obtained from replicate tests on 22 beans were calculated.

The single puncture test was performed in the Instron using a 1/8 in. diam. flat faced steel punch operated at 30 cm/min (Bourne, 1972) with a sample size of 200-300 beans. Cooked beans that showed no signs of splitting or rupture were selected for this test.

Table 2—Basic texture profile ballot

Product: Fresh red kidney beans, soaked and cooked 30 min at 100°C

I. **INITIAL (perceived on first bite)**

a) Mechanical

Hardness	2.4
Fracturability	0
Viscosity	not applicable

b) Geometrical — Unbroken skin is smooth and moist, the cotyledon is lumpy after one bite.

c) Other characteristics - Moist skin, moist cotyledon.

II. **MASTICATORY (perceived during chewing)**

a) Mechanical

Gumminess	1.0
Chewiness	26 chews
Adhesiveness	0.5

b) Geometrical — Cotyledon is lumpy, skin breaks into tough pieces.

c) Other characteristics – Moist

III. **RESIDUAL (just before, during and after swallowing)**

Rate of breakdown -	Cotyledon breaks down moderately fast, skin breaks down at a slower rate.
Type of breakdown -	Cotyledons break into a mixture of lumps and a starch paste, the lumps steadily decrease in size to cellular dimensions. Beans with dark skin have harder lumps than beans with light skin. The skin breaks into tough thin pieces that mix poorly with crushed cotyledon. Incisors are used to cut residual skin pieces.
Moisture absorption –	Cotyledon absorbs moisture slowly at first, then faster as mastication proceeds, but some slightly dry particles are felt. Skin absorbs no moisture.
Mouthcoating -	Pieces of skin and cotyledon stick around the teeth, gums, and throat. There is some astringency.

A trained panel of four judges was used for sensory texture profile analysis using the procedures developed by Szczesniak et al. (1963), Brandt et al. (1963), and Civille and Szczesniak (1973). The procedure used for cooked beans is shown in Table 1 and the basic texture profile ballot is shown in Table 2. Beans stored at 2°C were used as control and were assigned a score of zero for the comparative texture profile ballot shown in Table 3. Skin color of the beans was measured in a Hunter Colorimeter model D25-3 using color plate C20-923 for calibration.

Results

Instrumental TPA

The changes in texture profile parameters are shown in Fig. 1 to 4. Hardness of beans stored at 2°C (control) showed no change during storage for 6 months while beans stored at 30° and 40°C showed a large increase in hardness (Fig. 1). The 30°C beans were 1.8 times and the 40°C beans 2.1 times harder than the control sample after 6 months storage. This result is consistent with the findings of other researchers who measured hardness by extrusion (Mejia, 1979; Antune and Sgarbieri, 1979); and puncture (Moscoso et al., 1984; Rozo, 1982). The other TPA parameters given below have not previously been reported for changes in texture of stored bean seeds.

Fracturability greatly increased during storage. Beans stored at 30°C and 40°C were respectively 2.7 times and 3.3 times more fracturable than the control after 6 months

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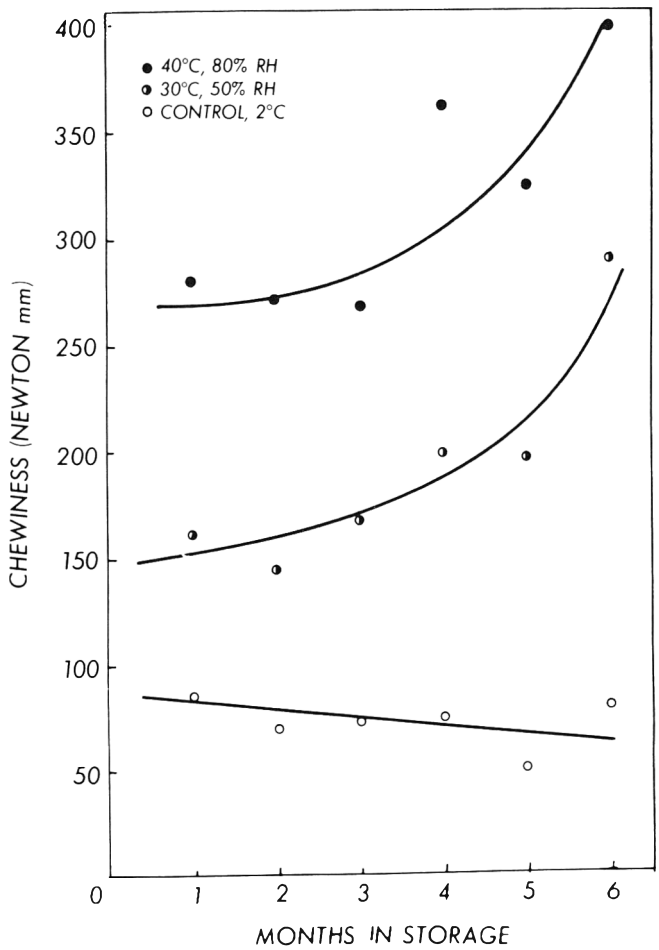


Fig. 2—Changes in instrumental chewiness of red kidney beans cooked for 30 min after storage.

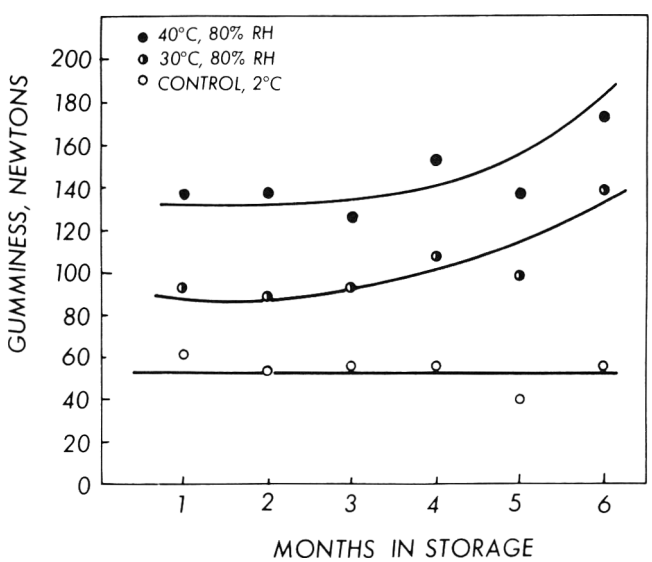


Fig. 3—Changes in instrumental gumminess of red kidney beans cooked 30 min after storage.

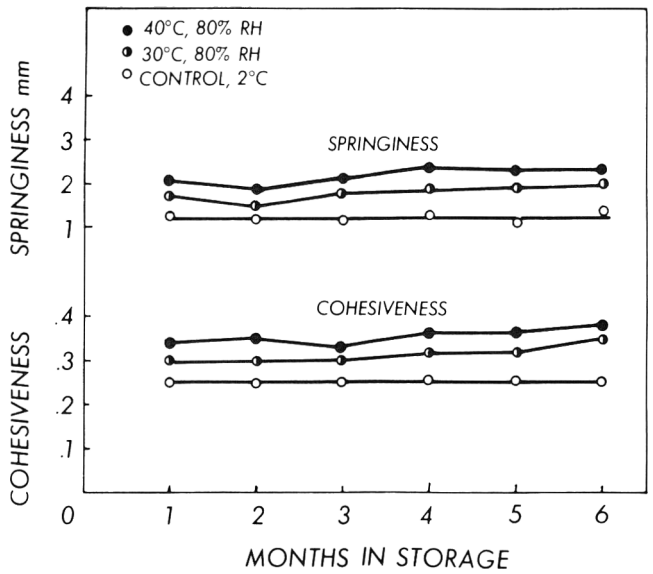


Fig. 4—Changes in instrumental cohesiveness and springiness of red kidney beans cooked 30 min after storage.

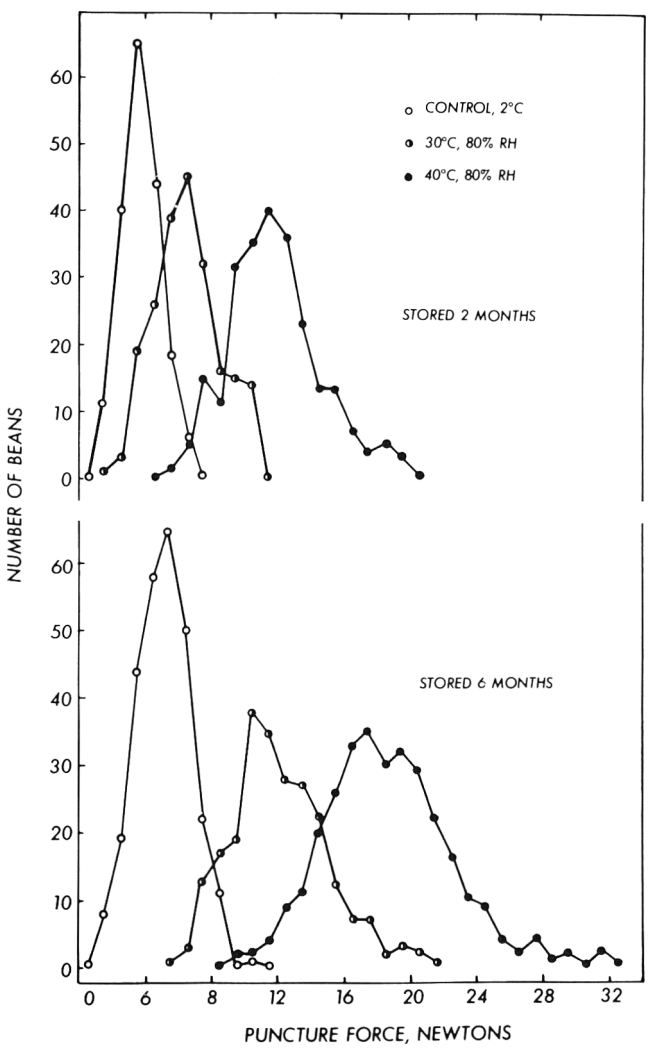


Fig. 5—Distribution of forces to puncture individual red kidney beans cooked 30 min after storage. The mean puncture force values are 5.9, 7.3 and 12.4 Newtons at 2 months, and 6.2, 12.4 and 19.0 Newtons at 6 months storage.

Table 3—Comparative sensory texture profile ballot for red kidney beans cooked 30 min after storage[a]

	1 month		3 months		5 months	
	30°C	40°C	30°C	40°C	30°C	40°C
Initial sensations						
Smoothness of skin	0	0	0	0	0	0
Moistness of skin	0	0	0	0	0	0
Hardness	3.3	3.7	3.7	5.3	4.7	7.0
Fracturability	2.0	2.0	2.0	4.7	3.7	5.3
Starchiness of crushed cotyledon	−2.7	−2.0	−2.0	−3.7	−4.3	−4.3
Lumpiness of crushed cotyledon	2.3	4.0	2.7	4.3	3.0	5.0
Moistness of crushed cotyledon	−1.3	−1.3	−1.3	−1.3	−1.7	−1.7
Masticatory sensations						
Chewiness	1.0	2.0	2.3	3.3	3.3	3.7
Gumminess	−3	−3	−3	−3	−3	−3
Starchiness of cotyledon	−2.0	−2.0	−2.7	−3.7	−3.3	−4.7
Lumpiness of cotyledon	2.7	2.7	2.7	3.3	4.0	5.3
Grittiness of cotyledon	0	0.7	2.0	2.0	1.0	1.7
Toughness of skin	2.3	1.7	1.3	3.3	2.7	4.0
Moistness	−1.0	−1.7	−1.3	−1.7	−1.7	−2.0
Final sensations						
Rate of breakdown of cotyledon	−2.0	−3.0	−2.0	−3.7	−3.3	−4.2
Rate of breakdown of skin	−2.0	−2.0	−1.0	−2.3	−2.3	−2.3
Pastiness of bolus	−2.0	−2.7	−2.3	−3.7	−2.3	−3.3
Lumpiness of bolus (number of lumps)	1.7	2.7	3.0	4.0	3.0	4.0
Hard pieces in bolus (hardness of lumps)	3.0	3.0	2.0	4.0	3.3	5.0
Grittiness	2.0	1.3	2.0	1.3	1.3	2.3
Toughness of skin pieces	2.7	1.3	1.7	3.0	2.0	3.0
Moisture absorption by cotyledon	−1.7	−2.0	−1.7	−2.0	−2.0	−2.3
Moisture absorption by skin	0	0	0	0	0	0
Particles around gums and teeth	1.7	1.7	1.7	3.0	2.3	3.0
Irritation of throat	0.7	1.7	1.0	3.0	2.0	3.3
Burning on tongue	1.0	2.0	2.0	3.3	2.3	3.7

[a] In this comparative sensory texture profile ballot, all beans were compared against the control sample which was given a score of zero for all testure parameters. A positive score indicates that the sample has more of that textural parameter than the control. A negative score incicates that the sample has less of that textural parameter than the control. A core of ± 1 means the sample is slightly different from the control while a score of ± 5 means the sample is much different from the control.

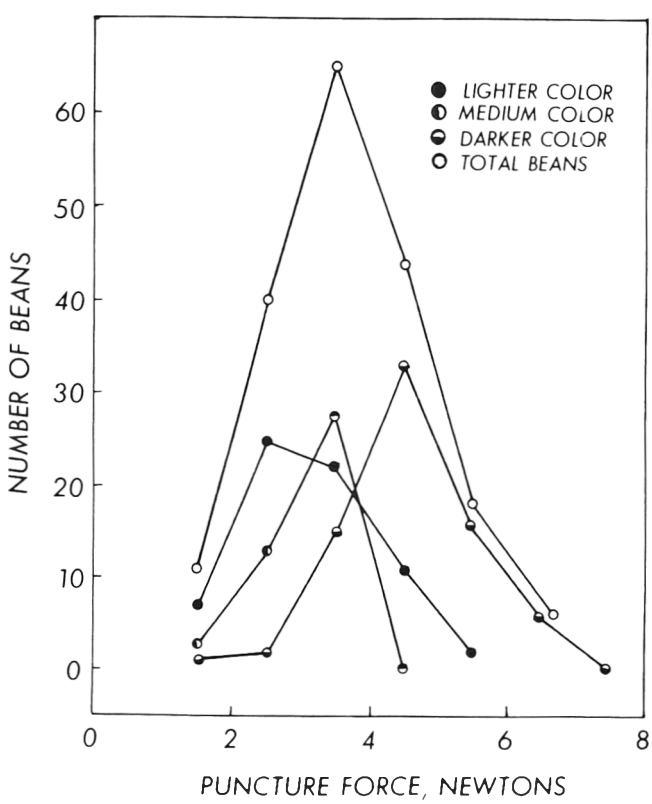


Fig. 6—Distribution of forces to puncture individual red kidney beans. cooked 30 min after storage for 1 month at 40°C. The Hunter Colorimeter L values for light, medium, and dark-skinned beans are respectively 20.7, 17.4, and 15.5.

storage (Fig. 1). Chewiness showed the greatest increase of TPA parameters. After 6 months, chewiness of 30°C beans was 3.6 times and 40°C beans 5.0 times that of the control (Fig. 2). Gumminess also showed a large increase during storage; the ratios for the samples to the control were respectively 2.5 and 3.1 for the 30°C and 40°C beans (Fig. 3). Springiness and cohesiveness showed a modest increase during storage and most of the increase occurred during the first month of storage (Fig. 4). After 6 months the ratios for springiness were 1.5 at 30°C and 1.7 at 40°C; and for cohesiveness 1.4 at 30°C and 1.5 at 40°C. These results show that not only hardness but all the TPA parameters changed substantially during storage at elevated temperatures.

Puncture test

The distribution of hardness among beans within a sample lot as measured by puncture tests on single beans is shown in Fig. 5 for beans stored 2 months and 6 months before cooking. In every case there is approximately a normal distribution curve for puncture forces and the control sample curve exhibits a narrower base than the samples stored at 30°C and 40°C. A comparison of the 2 months versus 6 months storage curves clearly shows both an increase in the mean puncture force and an increasing spread in the range of puncture forces for beans stored at 30°C and 40°C.

The distribution curves also show the extent of overlap between treatments. After 2 months storage there was still a large overlap between 2°C and 30°C beans and a small overlap between 2°C and 40°C beans. The amount of overlap declined during storage; but nevertheless, there were still a few 40°C beans that were less firm than a few 2°C beans after 6 months.

The beans stored at 30°C and 40°C became noticeably darker in color as storage study progressed. This phenomenon has previously been reported by Gloyer (1921), Snyder (1936) and Sada (1980). However, marked color differences developing between beans in the same sample during storage at 30°C and 40°C were noticed even though the color of the beans was almost uniform at the beginning of the experiment. Some of the beans were segregated by eye into three groups (light, medium, and dark colored) and subjected to the standard cooking procedure and puncture test.

An example of the results is shown in Fig. 6. The distribution curve for the light beans had a lower force range than the medium or dark colored beans.

Sensory texture profile analysis

The basic sensory texture profile ballot for red kidney beans is shown in Table 2. It described the textural sensations perceived during mastication of freshly harvested beans after cooking for 30 min. The textural parameters in the comparative sensory texture ballot shown in Table 3 were derived from the basic ballot shown in Table 2. In the initial sensations there was no change in smoothness of skin and moistness of skin during storage. There was a large increase in hardness, fracturability and lumpiness, and a decrease in starchiness and moistness of the beans stored at 30°C and 40°C compared with the control sample stored at 2°C.

In the masticatory sensations there was a large increase in chewiness, lumpiness of cotyledon and skin toughness, a small increase in grittiness of cotyledon, a large decrease in starchiness of cotyledon and a small decrease in moistness.

In final sensations there was a large decrease in rate of breakdown of cotyledon and pastiness of bolus and a moderate decrease in rate of breakdown of skin and moisture absorption by cotyledon. There was a large increase in the number and hardness of the lumps in the bolus and a moderate increase in toughness of skin, moisture absorption by the cotyledon and the number of residual particles that stick around the gums and teeth. There was also an increase of irritation in the throat and burning sensation on the tongue.

The overall picture that emerges from the sensory TPA is that after storage at elevated temperatures the texture of cooked beans changes from a soft, moist, pasty, starchy type of texture into a hard, fracturable, lumpy, nonpasty, somewhat dry texture similar to that of roasted peanuts and quite unlike the texture associated with normal beans. Storage at 30°C and 40°C changes not only hardness but all textural parameters except skin smoothness and moistness, and moisture absorption by the skin. The sensory TPA shows 23 different textural parameters changing during storage at elevated temperature.

CONCLUSIONS

THIS STUDY confirms the finding of earlier reports that storage of bean seeds at elevated temperatures results in beans with a harder texture after cooking. In addition, it shows for the first time that all of the other instrumental TPA parameters increase along with hardness. This study also shows for the first time the sensory texture profile of cooked beans and that most of the sensory parameters change profoundly during storage at 30°C and 40°C. Much more than changes in hardness is involved when bean seeds are stored at high temperatures.

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Effect of Partial Phytate Removal and Heat upon Iron Bioavailability from Soy Protein-Based Diets

C. J. RODRIGUEZ, C. V. MORR, and M. E. KUNKEL

ABSTRACT

The effect of heat and partial phytate removal upon in vivo iron bioavailability from soy protein was studied by chick hemoglobin repletion. Iron repletion diets were formulated with unheated and heated soy protein isolates and phytate-reduced soy protein isolates, with and without iron supplementation. Heating at 120°C for 20 min, 75% phytate reduction and 20 mg/kg iron supplementation improved iron bioavailability by 65–77%, 6–11% and 10–17%, respectively. These beneficial effects were interpreted in terms of promoting protein and protein-Fe-phytate complex digestion and release of endogenous and added iron.

INTRODUCTION

A MAJOR PORTION of the phytate from the soybean is coextracted and recovered with the protein fraction during the manufacture of soy protein isolate (Brooks and Morr, 1982). The phytate exists in the isolate as a complex with the proteins and polyvalent cations including Ca, Mg and other ions such as Fe and Zn, which has the effect of inhibiting their bioavailability (Cheryan, 1980; Maga, 1982; Honig et al., 1984).

This study was conducted to determine the effect of phytate removal and heat treatment upon the bioavailability of endogenous and added iron from soy protein-based diets by the chick hemoglobin (Hgb) repletion method (Pla and Fritz, 1971). The chick was chosen as animal model even though it is generally recognized that it provides similar iron bioavailability results as the rat (Pla and Fritz, 1971) or man (Kies, 1982).

A pilot-scale ion exchange process was developed by modifying the laboratory-scale process of Brooks and Morr (1982) and used to prepare sufficient phytate-reduced isolate for the study. Unfortunately, this process was not as effective as the laboratory-scale process for removing phytate from soy protein isolate.

MATERIALS & METHODS

Protein isolates

Control soy protein isolate-based diets were formulated with commercial soy protein isolate purchased from ICN Nutritional Biochemicals (Cleveland, OH). A pilot-scale ion exchange process was developed to prepare sufficient phytate-reduced soy protein isolate for the study. Low temperature processed, defatted soy flakes, furnished by Ralston Purina Company (St. Louis, MO), were sequentially extracted with 10 and 5 volumes of distilled water (v/w) adjusted to pH 8.5–9 with 1.5N NaOH (Brooks and Morr, 1982). The combined extracts were subjected to the cation-anion exchange process of Brooks and Morr (1982), except that a 3L bedvolume cation exchanger and a 12L bedvolume anion exchanger were used in place of the smaller capacity laboratory reactor columns. Flow rates for regeneration and treatment of soy extract were maintained at 100 mL/min for the cation exchanger and 400 mL/min for the anion exchanger to provide complete exchange. Treated extract was adjusted to pH 4.8–5.0 to precipitate the proteins, centrifuged and freeze dried as phytate-reduced soy protein isolate. Freeze-dried isolate was spread in a thin layer on the bottom of a flat container, loosely covered with aluminum foil and autoclaved at 120°C for 20 min.

Authors Rodriguez, Morr, and Kunkel are affiliated with the Food Science Dept., Clemson Univ., Clemson, SC 29631.

Experimental diets

Iron depletion and repletion diets conformed to NAS/NRC (1977) requirements for all components except for iron content. The iron depletion diet contained 20% commercial casein (U.S. Biochemical Corp., Cleveland, OH) and 5% commercial gelatin (The American Agricultural Chemical Company, Detroit, MI). Iron repletion diets contained 20% commercial soy protein isolate (see above) or phytate-reduced soy protein isolate. Four of the repletion diets were supplemented with 20 mg/kg of ferrous iron (analytical grade $FeSO_4 \cdot H_2O$ from Fisher Scientific, Atlanta, GA), which is adequate for complete hemoglobin (Hgb) repletion of chicks fed a nonfat dry milk and corn meal-based diet for 2 wk (Pla and Fritz, 1971).

The eight iron repletion diets were formulated to contain: (1) soy isolate without added iron; (2) soy isolate with added iron; (3) heated soy isolate without added iron; (4) heated soy isolate with added iron; (5) phytate-reduced soy isolate without added iron; (6) phytate-reduced soy isolate with added iron; (7) heated, phytate-reduced soy isolate without added iron; and (8) heated, phytate-reduced soy isolate with added iron.

Hemoglobin repletion

The Hgb repletion experiment was designed as a 2 x 2 x 2 factorial to compare: (1) control soy isolate vs phytate-reduced soy isolate; (2) unheated soy isolate vs heated soy isolate; and (3) isolates with and without iron supplementation.

Ninety-six one-day old Leghorn cockerel chicks from ISI America Hatchery (Newberry, SC) were fed the iron depletion diet ad libitum for 2 wk to deplete their Hgb concentrations to ≤5.0 g/dL (Pla and Fritz, 1971). The chicks were then separated into 24 groups of four chicks each with equivalent mean Hgb concentration values. These 24 groups were assigned to one of three replicate blocks with eight groups each. One group within each block was randomly selected and fed one of the eight iron repletion diets ad libitum for 10 days. This blocking arrangement was used to minimize possible variations in lighting, temperature and other extrinsic factors. The chicks were housed in a stainless steel Petersime brooder with raised-wire mesh floor. Feed and distilled water were replenished daily or oftener as needed. Use of the wire mesh floor brooder design made it impossible to prevent feed wastage or to determine feed consumption for the different groups. Chicks were banded and individually weighed and sampled for blood on alternate days throughout the test period.

Analytical

Iron was determined by atomic absorption spectrophotometry (AOAC, 1980). Phosphorus was by the method of Allen (1940) as modified by Brooks and Morr (1982), phytate by the method of Earley (1944) with modifications from Makower (1970) and Wheeler and Ferrell (1971) as reported by Brooks and Morr (1982). A factor of 28.2% was used to convert phytate phosphorus to phytate as recommended by deBoland et al. (1975) and Hgb was by the spectrophotometric method of Bjorn-Rasmussen et al. (1973).

Statistical treatment of data

Individual chick body weight and Hgb concentration data were subjected to analysis of variance to test for differences in response to the eight dietary treatments according to Steele and Torre (1980). The sums of squares of the primary treatment effects and the various levels of interactions were partitioned to permit ranking of the treatment effects. Mean body weight and Hgb concentration

values for the 12 chicks in each of the groups were graphed using a cubic equation to curve the data. Analysis of variance for a completely random, repeated measure design was used to determine least significant difference (LSD) for comparison of treatment means within days ($P<0.05$).

RESULTS

Phytate and iron content of soy protein isolates

Control soy protein isolate contained an average of 2.05 ± 0.10% phytate and phytate-reduced isolate prepared by the pilot-scale process contained an average of 0.51 ± 0.10% phytate. Thus, the pilot-scale ion exchange process removed only about 75% of the phytate from soy protein isolate compared to ⩾95% phytate removal by the laboratory ion exchange process of Brooks and Morr (1982). It is believed that the relatively incomplete phytate removal by the pilot-scale process was due to the use of larger bedvolume reactor columns that did not provide adequate contact of soy extract with the ion exchanger media. This pilot-scale ion exchange process was completely ineffective for removing endogenous iron from soy extract, e.g., both control and phytate-reduced soy isolate produced by the pilot-scale process contained 130 mg/kg endogenous iron. The laboratory-scale ion exchange process was only able to remove about 60% of the endogenous iron from soy protein isolate, indicating that this component is strongly bound by soy protein or soy protein-phytate complex.

Iron content of experimental diets

The casein and gelatin-based iron depletion diet contained an average of 22 mg/kg endogenous iron and the iron repletion diets contained: soy isolate without added iron (treatment groups 1 and 3), 41.5 ± 2.1 mg/kg iron; soy isolate with added iron (treatment groups 2 and 4), 58.0 ± 2.8 mg/kg iron; phytate-reduced soy isolate without added iron (treatment groups 5 and 7), 38.2 ± 3.9 mg/kg iron; and phytate-reduced soy isolate with added iron (treatment groups 6 and 8) 51.8 ± 2.2 mg/kg iron. About 73% of the total iron in iron supplemented repletion diets was from endogenous sources and the remainder of about 27% was added iron.

Body weight gain and hemoglobin repletion

Mean initial body weight values for the 12 chicks in each of the eight dietary treatment groups at day 0 ranged from 110–120g and their mean Hgb values ranged from 4.36–4.38 g/dL. Changes in mean body weight and Hgb concentration values for chicks in each treatment group for the 10 day repletion period are given in Fig. 1 and 2. Although mean body weight gains were nearly linear for all groups during the 10 day repletion period, treatment groups 5 (phytate-reduced soy isolate without added iron) and 6 (phytate-reduced soy isolate with added iron) resulted in smaller body weight gains than those from the other treatment groups. Since feed consumption was not determined, it was not possible to compute Hgb repletion in terms of endogenous or added iron ingested by the chicks.

Mean Hgb repletion values for each of the eight treatment groups exhibited three different responses during the 10 day iron repletion period (Fig. 2). Treatment groups 1 (soy isolate without added iron), 2 (soy isolate with added iron), and 5 (phytate-reduced soy isolate without added iron) provided least Hgb repletion; treatments 3 (heated soy isolate without added iron) and 6 (phytate-reduced soy isolate with added iron) provided intermediate Hgb repletion, and treatment groups 4 (heated soy isolate with added iron), 7 (heated, phytate-reduced soy isolate without added iron), and 8 (heated, phytate-reduced soy isolate with added iron) provided greatest Hgb repletion.

Analysis of variance ranking of these dietary treatment effects (Table 1) indicate that all three dietary and processing treatments used in this study, e.g., heat, phytate-removal and iron supplementation, improved Hgb repletion from soy protein isolate-based diets. Heating provided the largest improvement of 64–76%, iron supplementation provided a 13–17% improvement and phytate reduction provided a 6–11% improvement in Hgb repletion.

A more direct comparison of mean Hgb repletion data for day 10 treatment groups is made in Table 2. Although phytate-reduction provided only small improvement in Hgb repletion for soy isolate and phytate-reduced soy isolate without added iron (treatment groups 5 vs 1), heated soy isolate and phytate-reduced soy isolate without added iron (treatment groups 7 vs 3) and heated soy isolate and phytate-reduced soy isolate with added iron (treatment groups 8 vs 4), it provided a 26% improvement for soy isolate and phytate-reduced soy isolate with added iron

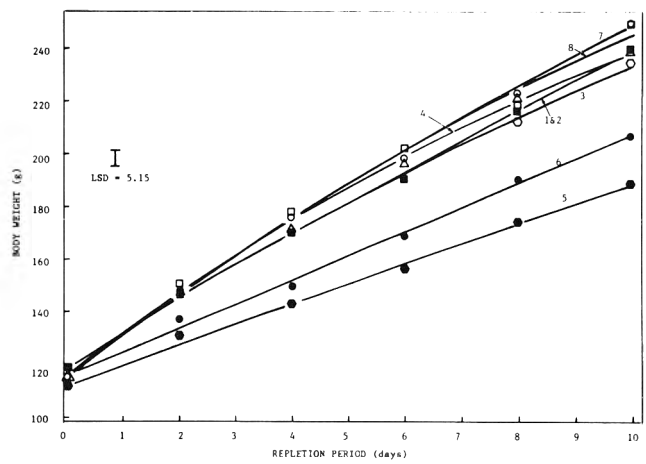


Fig. 1—Mean body weight of chicks during repletion period as a function of soy protein treatment: 1-soy isolate without added iron; 2-soy isolated with added iron; 3-heated soy isolate without added iron; 4-heated soy isolate with added iron; 5-phytate-reduced soy isolate without added iron; 6-phytate-reduced soy isolate with added iron; 7-heated, phytate-reduced soy isolate without added iron; and 8-heated, phytate-reduced soy isolate with added iron.

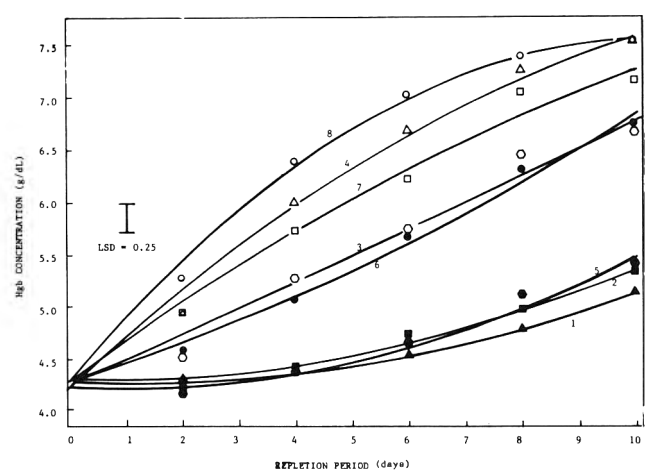


Fig. 2—Mean chick blood Hgb concentration during repletion period as a function of soy protein treatment: 1-soy isolate without added iron; 2-soy isolate with added iron; 3-heated soy isolate without added iron; 4-heated soy isolate with added iron; 5-phytate-reduced soy isolate without added iron; 6-phytate-reduced soy isolate with added iron; 7-heated, phytate-reduced isolate without added iron; and 8-heated, phytate-reduced isolate with added iron.

(treatment groups 6 vs 2). Iron supplementation provided greatest improvement in Hgb repletion for heated soy isolate (treatment groups 4 vs 3) and phytate-reduced soy isolate (treatment groups 6 vs 5). Heat treatment provided major improvement in Hgb repletion for all treatment group comparisons, but was least effective for phytate-reduced soy isolate with added iron (treatment groups 8 vs 6). The combination phytate-reduction and heat treatment was almost equally effective for improving Hgb repletion from soy isolate-based diets without added iron (39% improvement) as for those diets containing added iron (46% improvement).

DISCUSSION

THE LITERATURE CONTAINS numerous references dealing with phytate and its inhibition of iron and other trace mineral bioavailability from soy protein-based and other food products. This subject is quite controversial and evidence is presented that either supports or discounts the ability of phytate to alter iron bioavailability from soy protein-based diets. Much of the confusion associated with this topic is due to the highly reactive nature of the protein, phytate and iron moieties, the complex nature of the different food products in terms of their composition and physical structural properties, the influence of different processing treatments upon the chemical and physicochemical properties of the food that alter the susceptibility of the proteins and phytate to hydrolysis and utilization by the test animal and other extraneous factors (Cook, 1983; Clydesdale, 1983; Erdman, 1981; Reddy et al., 1982; Abdul-Kadir, 1980).

The present study was conducted to investigate the simultaneous effect of partial phytate removal, heat treatment and iron supplementation upon iron bioavailability from soy protein-based diets. Iron supplementation of soy protein isolate-based-diet (treatment groups 2 vs 1) provided only slight improvement in Hgb repletion, suggesting that added iron may be irreversibly bound by the protein or protein-phytate complex. However, iron supplementation of phytate-reduced soy protein-based diet (treatment groups 6 vs 5) resulted in a major improvement in Hgb repletion. Comparison of these two observations suggests that phytate may influence the bioavailability of added iron. However, the ion exchange process also removes Ca and Mg from soy protein isolate (Brooks and Morr, 1982), which results in smaller soy protein molecular complexes (Brooks and Morr, 1985) that would likely be more susceptible to protein and phytate hydrolysis and would thus release more of the iron than the larger sized protein-phytate complexes in control soy protein isolate. This conclusion is in agreement with the findings of Abdul-Kadir (1980) who found that phytate removal (about 78–80% phytate removal) improved the digestibility of soy protein isolate by the rat. The reduction of endogenous Ca content of phytate-reduced soy isolate should also improve the hydrolysis and utilization of phytate (Nahapetian and Young, 1984), which might in turn improve iron bioavailability. Iron supplementation was more effective for improving iron bioavailability from heated soy isolate-based diet (treatment groups 4 vs 3) than from unheated soy isolate-based diet (treatment groups 2 vs 1), which is probably due to heat-induced structural alteration of the proteins that render the protein-Fe-phytate complex more susceptible to protein and phytate hydrolysis and utilization and could thus improve iron bioavailability. Iron supplementation provided only minor improvement in Hgb repletion from heated, phytate-reduced soy isolate-based diet (treatment groups 8 vs 7), indicating that the endogenous iron in heated, phytate-reduced soy isolate is sufficiently available to provide a maximum Hgb repletion rate.

TABLE 1—Overall Hgb repletion due to various treatment effects[a]

Treatment effect	Repletion period (days)					
	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10
	(%)					
Heating	0.00	64.34	76.79	72.38	75.56	69.20
Phytate removal	0.00	11.05	6.09	6.72	8.91	8.96
Fe addition	0.00	13.17	13.20	17.44	10.20	13.56
Interactions (combined)	0.00	11.44	3.29	3.46	5.33	8.28

[a] Computed from sums of squares values for Hgb by analysis of variance

TABLE 2—Treatment effects upon Hgb repletion[a]

Treatment	Treatment[b] group	Δ Hgb (g/dL)	% difference
Iron supplementation			
soy isolate	2 vs 1	0.20	+ 3.9
heated soy isolate	4 vs 3	1.33	+24.5
phytate-reduced soy isolate	6 vs 5	1.33	+24.5
heated, phytate-reduced soy isolate	8 vs 7	0.37	+ 5.2
Phytate removal			
soy isolate without added iron	5 vs 1	0.28	+ 5.4
soy isolate with added iron	6 vs 2	1.41	+26.4
heated soy isolate without added iron	7 vs 3	0.49	+ 7.3
heated soy isolate with added iron	8 vs 4	0.05	+ 0.7
Heat treatment			
soy isolate without added iron	3 vs 1	1.53	+29.8
soy isolate with added iron	4 vs 2	2.14	+40.1
phytate-reduced soy isolate without added iron	7 vs 5	1.74	+32.1
phytate-reduced soy isolate with added iron	8 vs 6	0.78	+11.6
Phytate removal plus heat treatment, without added iron	7 vs 1	2.02	+39.3
Phytate removal plus heat treatment, with added iron	8 vs 1	2.39	+46.4

[a] Day 10 data.
[b] 12 chicks/group.

The beneficial effects of heat treatment upon iron bioavailability from soy protein and phytate-reduced soy protein-based diets were probably due to inactivation of trypsin inhibitors and unfolding of the protein molecular structures to increase the susceptibility of the proteins and phytate to digestion by proteolytic enzymes and phytase, respectively. These latter processes would then facilitate release of iron from the protein-Fe-phytate complex with concomitant improvement in bioavailability. The ability of the heat treatment to improve iron bioavailability from control and phytate-reduced soy isolates and the apparent inability of the laboratory-scale ion exchange process to remove endogenous iron from soy protein isolate suggests that iron may be bound directly to the protein, as is the case with zinc (Rao and Rao, 1976). It is also possible that the relatively small amount of residual phytate in the phytate-reduced soy isolate (about 25% residual phytate in this study) may be able to bind the endogenous iron by formation of protein-Fe-phytate complexes. Indeed, this latter mechanism may be responsible for the small amount of phytate (about 5% residual phytate in laboratory-scale ion exchanged soy protein isolate, Brooks and Morr, 1982) that is not removed from soy protein isolate by the laboratory-scale process. Additional work is needed to elucidate the mechanism responsible for the strong retention of endogenous iron by soy protein isolates.

The relatively small improvement in Hgb bioavailability from phytate-reduced soy isolate-based diet compared to control isolate-based diet (treatment groups 5 vs 1) indicates that phytate probably does not inhibit iron absorption by forming insoluble Fe-phytate complexes (Evans and Pierce, 1981) in the neutral to mildly alkaline conditions of the small intestine. Rather, phytate probably functions by complexing endogenous iron in the form of protein-Fe-phytate complexes as mentioned above. Since iron supplementation of phytate-reduced soy isolate-based diet (treatment groups 6 vs 2) exerted a much greater improvement (+26%) in Hgb repletion than was obtained by supplementation of control isolate-based diet, it is apparent that phytate inhibits the bioavailability of added iron more than that of endogenous iron when dealing with unheated soy protein isolate-based diet. Phytate removal has very little beneficial effect upon the bioavailability of either endogenous or added iron in heated soy protein-based diet (treatment groups 7 vs 3 and 8 vs 4). As indicated above, the reason for this latter relationship is probably the increased susceptibility of heated proteins to proteolytic and phytase digestion.

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Composition of Undigested Peptide Fragments from Gluten with Regard to Bioavailability of Essential Amino Acids

WENDELL A. LANDMANN, DHANAKOTI PADMANABHAN, and CHARLES R. YOUNG

ABSTRACT

Our purpose was to isolate and characterize the high molecular weight peptides resulting from the in vitro digestion of wheat gluten in a system simulating actual in vivo conditions, and to determine to what extent essential amino acids were included in these peptides. Large peptides were separated from the smaller products by Cu-Sephadex G-25, TLE and TLC, treated with dansyl chloride for end group determination, and re-dansylated to identify the remaining amino acids. Fifteen peptides, hexapeptides or larger, were isolated and were found to contain about 16.1% of the threonine, 15.2% of the methionine and 12.3% of the lysine originally present in wheat gluten.

INTRODUCTION

ADEQUATE KNOWLEDGE of food proteins and their amino acid availability is fundamental to the rational development of approaches for improving and subsequently maintaining the nutritional adequacy of human diets. Even though the amino acid profile is important in evaluating the nutritive quality of a protein, the digestibility of that protein is the primary determinant of the bioavailability of its amino acids. The absence of cleavage sites, or the blocking of cleavage sites for interactions of amino acid side chains with other substances, can play a major role in the length of peptides resulting from the digestion of the protein by the enzymes of the digestive tract. Large polypeptides that are produced would not be broken down further in the intestinal mucosa because they are not the proper substrates for the enzymes present there (Hansen and Millington, 1979). Studies by Matthews and Adibi (1976), Tagari and Bergman (1978) and Adibi (1976) showed that free amino acids appear in the portal blood at a faster rate when proteins rather than free amino acids in the same concentration are fed. The mechanism of absorption from the intestinal lumen appears to be rapid transport of di- and tripeptides across the intestinal wall, followed by final enzymatic hydrolysis in the brush borders or cell to free amino acids. Enzymes in the brush borders have been found to hydrolyze hexapeptides, although carriers seem to exist only for di- or tripeptides (Matthews and Adibi, 1976). Large polypeptides, tetrapeptides or larger, may be capable of being transported by the carriers, but may also act as inhibitors of the carriers.

Efforts to relate the chemical score (or essential amino acid profile) of a protein to the PER (protein efficiency ratio) have involved the development of equations which include digestibility factors. The most notable among these perhaps is that for the determination of c-PER (computed PER) (Satterlee et al., 1977).

The presence of undigested protein fragments has been suggested in protein evaluation studies (Parmer et al., 1978; McCurdy et al., 1978; Fillette et al., 1978). Products of digestion of egg albumin (Greenberg and Shipe, 1979), wheat gliadin and glutenin (Bietz and Fothfus, 1970) and soybean protein (Sklan and Hurwitz, 1980) have been shown to contain peptides varying in size from 300 to 15000 daltons.

Several in vitro methods for determining digestibility have been developed. Horn et al. (1952) used pepsin, trypsin and hog mucosa for a period of 24 hr each at 37°C. Akeson and Stahman (1964) used a pepsin-pancreatin system for 24 hours. Saunders et al. (1973) employed pepsin for 3 hr, followed by pancreatin for 24 hr. Hsu et al. (1977) used a multienzyme system comprised of trypsin, chymotrypsin and peptidase for ten minutes, and Marshall et al. (1979) used pancreatin for a 20-min digestion period. Satterlee et al. (1979) also measued in vitro digestibility by a two-step procedure which utilizes a bacterial protease for 9 min at 53°C after the multienzyme treatment. Gauthier et al. (1982) devised an in vitro procedure utilizing a 30 min pepsin digestion at pH 1.9, followed by a 24-hr pancreatin digestion at pH 8.0, carried out with simultaneous dialysis to remove digestion products which might be inhibitory to the enzymes. With the possible exception of the latter, none of the procedures approximate in vivo digestion conditions.

The purpose of this investigation was to isolate and characterize the high molecular weight peptides resulting from the in vitro digestion of wheat gluten in a system simulating actual in vivo conditions, and to determine to what extent essential amino acids were included in these peptides, thus affecting their bioavailability as well as the chemical score.

MATERIALS & METHODS

WHEAT GLUTEN had been well-studied in our laboratory; its PER and amino acid composition were known. Because of these facts and this protein's low chemical score, we selected it for this study. The digestion system utilized consisted of pepsin treatment for 3 hr, followed by pancreatin treatment for 2 hr, to approximate the times of passage through the stomach and duodenum. All mixtures were stirred continuously during digestion.

Wheat gluten (150 mg) was incubated with 2.25 mg pepsin (from hog stomach mucosa, 3900 U/mg protein using hemoglobin as substrate, Sigma Chemicals, St. Louis, MO) in 22.5 mL 0.1 HCl at 37°C for 3 hr. The solution was brought to pH 8.0 with 0.2N sodium hydroxide, and 6 mg pancreatin (from porcine pancreas, Grade IV, Sigma Chemicals, St. Louis, MO) in 11.25 mL pH 8.0 phosphate buffer was added. The digestion mixture was incubated for an additional 2 hr at 37°C. An enzyme control was prepared by incubating the enzymes under the described conditions with the gluten omitted. The enzymatic reaction was stopped by heating the digest in a boiling water bath for 5 min. Sodium azide (1 mg) was added to all enzyme hydrolyzates to prevent the growth of micro-organisms. The digestion mixture was centrifuged at 48000 × *g* for 30 min at 5°C. The supernatant was decanted and evaporated to dryness under vacuum and the dry residue dissolved in 2 mL deionized water.

The isolation and recovery of peptides was carried out using the Cu-Sephadex procedure of Rothenbuhler et al. (1979). Sephadex G-25, Fine (Pharmacia Fine Chemicals, Piscataway, NJ) was treated with cupric sulfate, washed twice with NaOH and borate buffer, pH 11, as described, and packed into a 1.5 × 25.5 cm column. After the column was washed with the borate buffer, 2 mL of the sample

Authors Landmann and Young are affiliated with the Dept. of Animal Science, Texas Agricultural Experiment Station, Texas A&M Univ., College Station, TX 77843. Author Padmanabhan, formerly at Texas A&M, is now with IBM, Austin, TX 78767.

solution above was applied and eluted with the borate buffer at a flow rate of 30 ± 2 mL/hr. Fractions of 3 mL each were collected and their absorptions measured at 230 and 280 nm and the fractions containing the peak pooled. After the pooled Cu-peptide fractions were dried in vacuum, and the residue reconstituted in 2 mL borate buffer, the copper was removed by applying the sample to a 1 x 5.5 cm column of Dowex Chelating Resin (No. 1-4751, mesh 50-100, Sigma Chemical Co., St. Louis, MO) and eluting with pH 11 borate buffer at a flow rate of 20 mL/hr. After elution was complete (30 mL) the solution was evaporated under vacuum and the residue dissolved in 1 mL deionized water. The sample was desalted by passing it through a 0.9 X 45 cm column of G-10 Sephadex Fine, eluting with deionized water. The desalted solution was dried under vacuum and taken up in a 1.0 mL deionized water.

Peptide mapping

Cellulose (Machery-Nagel M.N.300) purified according to the method of Heathcote et al. (1973), was coated on clean glass plates (20 X 20 cm) to a thickness of 0.5 mm and allowed to dry overnight in air free of ammonia. The sample (25 μL desalted solution) was applied 3 cm from the bottom and right edges of the plate at the anodic side, and thin-layer electrophoresis was carried out in a Desaga-Brinkmann unit (Brinkmann Instuments, Westbury, NY) operated at 400 volts for 1 hr and 15 min using formic acid-gl. acetic acid-water (1:3.4:56, by volume) at pH 2.1. After the plate was removed, dried for 15 min at 60°C and cooled, thin-layer chromatography was carried out in the second dimension, employing t-butyl alcohol-methyl ethyl ketone-acetone-methanol-water-ammonia (10:4:2:1:3:1, by volume), according to Heathcote and Howorth (1969). When the solvent front had reached a height of 13 cm above the origin, the plate was removed and dried. Peptides were detected by spraying the plate with a solution of Fluorescamine (Sigma Chemicals, St. Louis, MO) in acetone (0.05% w/v) and viewing it under U.V. light.

Each spot was carefully removed and extracted with 1 mL 0.1N HCl. The extract was passed through a 0.2 μm Millipore membrane (Millipore Corp., Bedford, MA) and dried in vacuo over solid KOH. The residue was taken up in 100 μl 0.2M $NaHCO_3$ and evaporated to dryness in vacuo over concentrated H_2SO_4.

Determination of end group and amino acid composition

A stock solution of dansyl chloride (100 mg/mL in acetone; Pierce Chemicals, Rockford, IL) was diluted 1:20 with acetone to give a few milliliters of working solution containing 5 mg dansyl chloride/mL. The dry peptide residue above was taken up in 20 μL deionized water, 100 μL dansyl chloride working solution added, the pH adjusted to 8.5 if necessary, and the tube sealed with Parafilm. After 30 min in a water bath at 37°C, the reaction was terminated by the addition of 10 μL 88% formic acid. The reaction mixture was then evaporated to dryness over silica gel in a vacuum desiccator. The residue was hydrolyzed with 100 μL 6N HCl containing 0.02% mercaptoethanol in a sealed tube at 105°C for 20 hr. Five μL of this hydrolysate was applied in 1 μL portions to a polyamide sheet for chromatography to identify the N-terminal amino acid, as described below. The remainder of the hydrolysate was again evaporated to dryness and the procedure for dansylation repeated, using 150 μL dansyl chloride working solution to react with the free amino acids. The re-dansylated solution was applied to polyamide sheets for chromatographic identification of the amino acids. The procedure was that of Woods and Wang (1967) as modified by Hartley (1970), using double-coated polyamide sheets (Cheng Chin Co., Taipei, Taiwan). Dansylated amino acid standards were applied on the back of the sheet to aid in the identification of the derivatives. Spots were visualized by viewing the chromatograms under long wavelength UV light.

Amino acid analysis of high molecular weight peptides

Five-tenths mL of the solution containing the peptides separated by the Cu-Sephadex procedure and desalted with Sephadex G-10 was dried in vacuo and the residue taken up in 100 μL 6N HCl containing 0.02% mercaptoethanol. The tube was sealed and heated at 110°C for 20 hr to hydrolyze the peptides. The hydrolysate was evaporated to dryness and taken up in 2.5 mL 0.1N HCl and analyzed by means of a Beckman automatic amino acid analyzer Model 121-C (Beckman Instruments, Fullerton, CA).

Nitrogen was determined on the pooled peptide fraction by the semimicro Kjeldahl procedure (AOAC, 1980).

In vitro digestibility test

The method of Hsu et al. (1977) was used for determining the in vitro digestibility of wheat gluten, gliadin and glutenin, with casein as the standard. The enzymes utilized were trypsin (Grade IX, hog pancreas, 14350 U/mg protein), chymotrypsin (Grade II, bovine pancreas, 68 U/mg protein) and peptidase (Grade III, hog intestinal mucosa, 50 U/g), all from Sigma Chemicals, St. Louis, MO.

Gliadin and glutenin were prepared from the wheat gluten used in this study according to the method of Ewart (1967).

RESULTS & DISCUSSION

THE ELUTION PATTERN of the enzyme digests of wheat gluten is given in Fig. 1. Under the applied conditions, all the larger peptides were eluted between 15 mL and 45 mL, the exclusion volume (tubes 5 through 15), and were followed by a small amount of low molecular weight peptides. The peptide map of the high molecular weight material is shown in Fig. 2.

The results of the in vitro multienzyme digestibility study on wheat gluten, gliadin and glutenin are given in Table 1. It can be seen that under the conditions employed here, glutenin is more digestible than gliadin and gluten. This may be due in part to its denaturation caused by the ethanol used in the isolation procedure for gliadin. The lower digestibility of gliadin may be ascribed to the larger number of lengthy peptides formed during the digestion, and implies that fewer sites for enzyme attack exist. Although the Hsu multienzyme system does not include all the enzymes of the digestive tract, it closely approximates the action of pancreatic enzymes. The apparent digestibility values from the Hsu method correlated well (R = 0.96) with the nitrogen values determined by the microKjeldahl method for the gluten, gliadin, and glutenin digests. A conversion factor of 5.85 was used for gluten, determined by Landmann et al. (1980) on the basis of amino acid composition. The amounts of the undigested peptides of gluten, gliadin and glutenin as percent of total protein are also given in Table 1. Comparison of the gluten peptide map (Fig. 2) with the general patterns seen in maps of gliadin and glutenin (not shown) also suggests that most of the undigested peptides are derived from gliadin.

The amounts of essential amino acids in the original protein were reduced by the amounts found in the undigested peptides and the resulting value used to calculate the corrected amino acid score, as follows: (% of essential amino

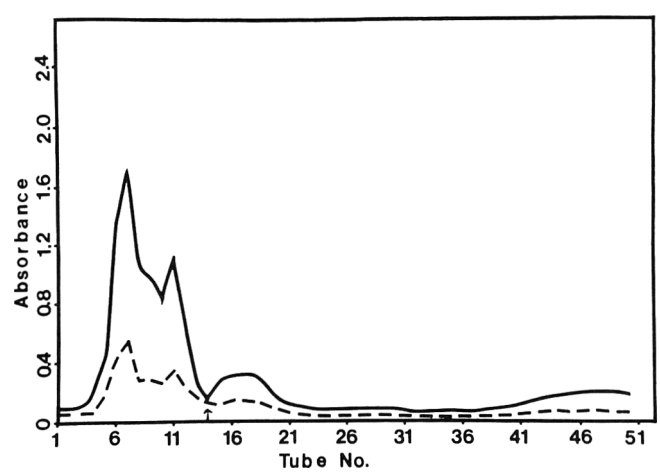


Fig. 1—Elution pattern of pepsin-pancreatin digest of wheat gluten from a copper-Sephadex G-25 column (25.5 X 1.5 cm). 0.3 mmol Cu/g Sephadex; eluting buffer: $Na_2B_4O_7$ pH 11.0; fraction size 3.0 mL. (——) absorbance at 230 nm; (- - - - -) absorbance at 280 nm. Void volume: 45 mL.

acid remaining in gluten)/(% of that amino acid in egg protein) according to Block and Mitchell (1946). The values for the amounts of essential amino acids contained in the undigested fraction are likely to be lower than is actually the case, since recoveries from the Cu-Sephadex and Sephadex G-10 columns, as well as other treatment steps, were assumed to be 100%, a highly unlikely circumstance. Values obtained and corrected chemical scores are shown in Table 2.

A comparison of chemical score based on the amino acid composition of gluten and the chemical score after adjusting for losses due to incomplete digestion shows a reduction of about 0.03 in the chemical score, based on lysine, the first limiting amino acid. Percentage loss of threonine, the second limiting amino acid, is even greater than that of lysine (16% of the amount of threonine is lost compared to 12% for lysine), resulting in a reduction of 0.07 in the chemical score for threonine. Percentages of other essential amino acids are also reduced in the range 6% to 15%. For example, 15% of the methionine is not available; thus its chemical score is reduced by 0.08. Any loss of methionine may seriously interfere with sulfur metabolism if cysteine is also low. Since the loss of methionine and threonine is greater than that of the lysine, the impact on the overall nutritional value of the protein may actually be more severe than indicated by the lysine score alone.

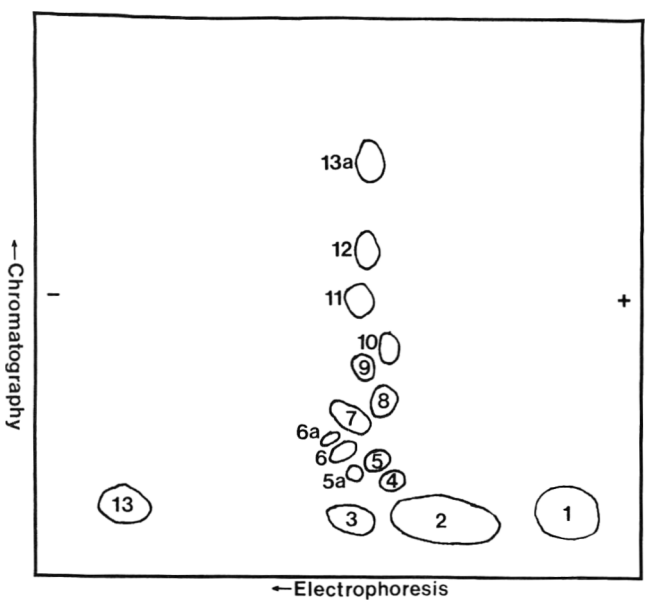


Fig. 2—Peptide map of wheat gluten digested with pepsin-pancreatin (pepsin: 3 hr, 37°C, pH 1-2, pancreatin: 2 hr 37°, pH 8.0). Thin layer electrophoresis on cellulose in formic acid-gl. acetic acid-water (1:3.4:56) pH 2.1, sample applied at anodic end run 75 min at 400 volts. Ascending chromatography in t-butyl alcohol-methyl ethyl ketone-acetone-methanol-water-ammonia (10:4:2:1:3:1). N-terminal groups: Met – spots 1, 4, 8; Gly – spots 2, 9, 11; Glu – spots 3, 5a, 7, 13; Tyr – spots 5, 6, 6a, 12; Ala – spots 10, 13a.

The various amino acids present in the undigested peptides as identified by dansylation and thin layer chromatography are given in Table 3. It is apparent that all peptides, except perhaps peptide 6A, are larger than hexapeptides. It is likely that peptide 6A, because of its behavior on the Cu-Sephadex column, is also larger than a hexapeptide, since more than one residue of each or any of the amino acids comprising it could be present.

The peptides obtained could be classified into five groups according to their N-terminal amino acids: methionine (3 peptides), glycine (3 peptides), glutamic acid (4 peptides), tyrosine (4 peptides) and alanine (2 peptides). The appearance of these N-terminal groups is not inconsistent with the specificities of the enzymes and the relative rates of cleavage of certain linkages between amino acids. It would be of interest to determine the sequence of amino acids in these peptides to ascertain the configuration of amino acids near the cleavage sites of a mixture of enzymes such as this, and to determine which of these peptides may have been derived from others.

While the digestion method used in this study approximates pH conditions and time of passage of food through the stomach, duodenum and jejunum, where almost all of the digestion of proteins occurs, it does not account for the effects of peptidases in sloughed mucosal cells. The continuous removal of end-products which may be inhibitory would also bring about a more complete digestion than was seen here. Still the large peptides remaining after the extensive action of all the endo- and exo-peptidases in the pancreatin appear to be further attacked slowly, if at all, suggesting that the mucosal enzymes would cause only slightly more degradation. This assumption is supported by the close correlation of the Hsu digestibility, which utilizes mucosal enzymes, with the digestibility as determined by our system.

In adjusting the pH to 8.0 after pepsin digestion, titration with NaOH was used to accomplish the neutralization

Table 1—Protein digestibility by the in vitro method of Hsu[a] and estimation by semi-micro Kjeldahl method[b]

Wheat protein	Hsu method %	Kjeldahl method % undigested
Gluten	80.69	13.46
Gliadin	82.64	10.78
Glutenin	88.10	8.14
Casein	88.70	–

[a] Values are calculated using the regression equation of Hsu et al. (1977): Y = 210.46 – 18.10 X where X = pH at 10 min.
[b] Conversion factor of 5.85 used to determine protein content (Landmann et al., 1980).

Table 2—Decrease in essential amino acids and in amino acid scores due to incomplete digestion

Essential amino acid	Wheat gluten[a] (g EAA/100g protein)	Undigested peptides[a] from 100g protein (g EAA)	% Not available	Amino acid score	
				Original	Corrected[b]
His	2.16	0.14	6.67	1.26	1.10
Ile	4.39	0.26	5.82	0.65	0.62
Leu	7.71	0.47	6.03	0.82	0.77
Thr	2.37	0.38	16.14	0.43	0.36
Lys	1.98	0.24	12.25	0.26	0.23
Met	1.99	0.30	15.23	0.55	0.47
Val	4.49	0.33	7.30	0.61	0.57
Phe	5.81	0.37	6.39	0.95	0.89

[a] Values are based on molecular weights rather than residue weights of the amino acids. Amino acids determined by automatic amino acid analyzer.
[b] Calculated by reducing the amounts of amino acids in the protein by the amount found in the undigested peptides.

Table 3—Amino acid composition of high molecular weight peptides

N-Terminal	Spot#[a]	Remaining Amino Acids	Min.M.W.
Met	1	Ala,Gly,Lys,Ser,Thr,Phe,Leu, Pro,Tyr	1400
Met	4	Ala,Gly,Ser,Val,Pro,Leu,Ile, Glu	1260
Met	8	Glu,Asp,Lys,Gly,Ala,Ser,Tyr, His,Ile,Phe,Thr	1680
Gly	2	Glu,Asp,Thr,Ser,Ala,Met,Tyr, Leu	1400
Gly	9	Glu,Asp,Ser,Thr,Val,Ile	980
Gly	11	Glu,Ser,Met,Thr,Tyr,Ala	980
Glu	3	Thr,Ser,Ala,Gly,Asp,Lys,Tyr, Leu,Ile,Met	1540
Glu	5A	Ala,Gly,Ser,Tyr,Thr,Lys,Ile	1120
Glu	7	Ala,Asp,Gly,Ser,Thr,Val,Try, His,Ile,Met,Phe,Pro,Lys	1960
Glu	13	Lys,Ala,Gly,Ser,Met,Phe,Ile	1120
Tyr	5	Arg,Lys,Ser,Glu,Ala,Gly,Met, Val	1260
Tyr	6	Lys,Phe,Ile,Leu,Val,Pro,Ala, Gly,Glu,Thr,Ser	1820
Tyr	6A	Ala,Gly,Ser,Thr	700
Tyr	12	Glu,Ala,Leu,Ile,Lys,Met,Ser, Gly	1260
Ala	10	Glu,Asp,Ser,Thr,Val,Ile	980
Ala	13A	Tyr,Val,Ile,Phe,Ser,Pro,Glu, Gly,Lys,Met	1540

[a] Numbers refer to peptide map, Fig. 2.

of the acid rapidly, with the final adjustment and maintenance of pH being carried out by the phosphate buffer. While $NaHCO_3$ is utilized in vivo, with a more gradual change in pH, we feel that the effect of the NaOH during the short time of exposure (about 2 min) is minimal in the overall hydrolysis.

CONCLUSIONS

THIS WORK clearly demonstrates that wheat gluten is incompletely digested by the enzymes of the gastrointestinal tract, and that at least 13.5% of the protein is lost, or is biologically not available, because of the large molecular weight peptides with minimal molecular weights ranging from 700 to 2000 daltons which are produced. These peptides have been shown to contain significant amounts of essential amino acids, which result in a 12% reduction of the chemical score based on lysine the first limiting amino acid. However, the greater losses of threonine and methionine may have a more severe impact on the quality of wheat gluten than is indicated by the reduction of the chemical score based on lysine alone.

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Effect of Phytate and Partially Hydrolyzed Phytate on *in vitro* Protein Digestibility

B. E. KNUCKLES, D. D. KUZMICKY, and A. A. BETSCHART

ABSTRACT

The effects of sodium phytate and partially hydrolyzed sodium phytate (0 - 82% hydrolyzed) on pepsin digestion of casein and bovine serum albumin were evaluated by an *in vitro* procedure using dialysates of pepsin digestion over a period of 0 - 23 hr. The inhibitory effect of phytate differed with substrate and increased with dose level. At the highest phytate level, the digestion of casein and bovine serum albumin was reduced by 14% and 7%, respectively. The inhibitory effect of the phytate was inversely correlated with the degree of phytate hydrolysis. Hydrolysis for 16 hr almost eliminated the inhibitory effect of phytate.

INTRODUCTION

PHYTATE (phytic acid or its salts) is present in many cereal grains and oilseeds. It is known to react with various nutrients causing, in many instances, decreased bioavailability. When bound to protein, it causes decreased solubility and functionality of the proteins (Bourdillon, 1951; Saio et al., 1967, 1968). Phytate may also cause proteins to be more resistant to proteolytic digestion (Kratzer, 1965). The extensive reviews on phytate by Cheryan (1980), Maga (1982), and Reddy et al. (1982) indicate the need for a better understanding of the interactions of phytate and protein. The objective of this study was to examine the effect of phytate and partially hydrolyzed phytate on *in vitro* digestibility of casein and bovine serum albumin (BSA).

MATERIALS & METHODS

Materials

Casein (Animal Nutrition Research Council standard, U.S. Biochemical Corp., Cleveland, OH) containing 13.7% N and 7.2% moisture was ball milled for 16 hr before use. Bovine serum albumin (Fraction V powder, 15.0% N, 5.8% moisture), sodium phytate (crystalline, from corn), pepsin (porcine stomach mucosa, 1:60,000) and Folin & Ciocalteu reagent (2N) were obtained from the Sigma Chemical Co. (St. Louis, MO). Chemicals were reagent grade. The dialysis tubing (size #27, 12,000 MW cutoff cut to 21.6 cm in length) was from VWR Scientific, Inc. (San Francisco, CA).

Phytate hydrolysis

Sodium phytate was hydrolyzed under conditions similar to those reported by de Boland et al. (1975). Phytate solutions containing 2.80g sodium phytate in 100 mL were adjusted to pH 5.2 with HCL and refluxed. The extent of hydrolysis was determined by analyzing the hydrolysates for free and total phosphate (APHA, 1965, 1975).

Phytate-protein binding

The following procedure was used to determine whether phytate was binding to the protein substrate: Casein (0.5g) was mixed with sodium phytate solutions (35.0 and 70.0 mg in 20 mL 0.1N HCl) at room temperature for 1 hr. (These conditions are the same as those used for *in vitro* digestibility determinations). The mixtures were then centrifuged and the supernatants analyzed for phytate by the method of Knuckles et al. (1982).

Digestion procedure

A modification of the method of Mauron et al. (1955) was used for the pepsin digestions. It was designed to simplify separation of the digestion products and simulate the "in vivo" process by minimizing product inhibition. The digestions at pH of about 1 are comparable to digestion in the stomach. The procedure was as follows: Casein (0.500g) or BSA (0.456g) providing 68.4 mg N was put into each of 4 dialysis tubes. (Other protein sources could be used and up to six samples could be run at one time in this system). The tubes were clamped at the bottom where a magnetic stirring bar was attached. Four levels of phytate or phytate hydrolysate, each in 20 mL of 0.1N HCl, were added 5 mL at a time with mixing. After 45 min, 25 mg pepsin in 1.0 mL water was added to each tube. The tubes were closed, mixed for 1 min, and then placed in 1-L Erlenmeyer flasks containing 1.0L of 0.1N HCl at 25°C. The flasks were in a circulating water bath (25°C) constructed so that the dialysis tube in each flask could be stirred continuously by magnetic stirrers underneath the bath constructed of clear plastic. At hourly intervals between 1 and 6 hr and 23 and 24 hr, an aliquot (1.0 mL) of dialysate was taken from each flask and analyzed by the method of Lowry et al. (1951). Nitrogen values used to calculate per cent protein digested were computed from absorbance and a previously prepared standard curve. Under the conditions of this study, a highly significant ($P < 0.01$, $r = 0.995$) linear regression of Kjeldahl nitrogen vs absorbance by the Lowry protein method was obtained for dialysates resulting from the pepsin digestion of casein. The Lowry protein method was used because it is easier, faster, and more sensitive then Kjeldahl nitrogen determination. Blank runs showed that phytate and its hydrolysis products, inositol, and phosphate, do not interfere with the Lowry protein method.

Experimental design

Two series of experiments were conducted. In the first series, the effect of sodium phytate on pepsin digestion of casein and BSA was determined. In the second series, the effect from each of the five hydrolysates was similarly determined. The sodium phytate was added to digestion mixtures at levels to give 0, 12.5, 25.0, and 50.0 mg phytate (not counting Na) per 68.4 mg N from casein or BSA. In the second series, phytate hydrolysates were added at levels that, prior to hydrolysis, contained phytate levels as mentioned above. Each set of digestions which included the 4 levels of phytate was done in triplicate on different days. Since the digestibility of the controls (0-level phytate) varied, comparisons among the hydrolysates were made after indexing the digestability to the control at 100%. Relative digestibilities for each treatment were obtained by indexing the mean response (absorbance from Lowry protein assay) over the six hour digestion period to the mean response of the 0-level phytate control as 100%. Analysis of variance and determination of 95% confidence limits were calculated (Steel and Torrie, 1960). The statistical significance was at the $P < 0.05$ probability level.

The phytate levels used in this study were selected as representative of a range that could exist in the diet; however, the ratio of phytate to protein is high. Thus, the results can only suggest possible effects that must be confirmed in controlled *in vivo* studies.

RESULTS & DISCUSSION

THE EFFECT of phytate on pepsin digestion of casein and BSA over 0 - 6 hr is shown in Fig. 1 and 2. Analysis of variance indicated that phytate significantly decreased pepsin digestion of both casein and BSA and that the effect of phytate level was linear and independent of digestion time.

Authors Knuckles, Kuzmicky, and Betschart are affiliated with the USDA-ARS, Western Regional Research Center, 800 Buchanan St., Albany, CA 94710.

After 22 and 23 hr, the decrease in protein digestion due to phytate was still significant, but there was no significant difference in digestion due to phytate level. The decrease in pepsin digestion of casein and BSA may be the result of the formation of a phytate-protein complex in which sites on the protein are less susceptible to enzymatic attack. When casein, insoluble under the conditions of this experiment, was incubated with two levels of phytate, no phytate was detected in the supernatant following centrifugation. Also, incubation of the pepsin with phytate prior to mixing with the substrate did not significantly affect protein digestion. These observations could be interpreted as an indication that phytate was bound to the casein. Our data support reports in the literature indicating that phytate-protein complexes are more resistant to proteolytic digestion (Barre, 1956; Kratzer, 1965).

The effect of phytate on digestion of BSA was appreciably less than its effect on digestion of casein. At the phytate levels of 12.5, 25.0, and 50.0 mg, relative digestibility of casein was reduced an average of 7, 9 and 14%, respectively, during the initial 6 hr digestion period. The corresponding reduction in mean values for digestibility of BSA was 1, 3 and 7%, respectively. The smaller decrease in digestion of BSA compared to casein could be attributed to differences in protein configuration and/or the ability of the two proteins to bind phytate. Some proteins, such as soluble corn germ protein, do not bind phytate while others, such as soy protein, strongly bind phytate (O'Dell and de Boland, 1976). Consequently, it would not be unreasonable to expect that the effect of phytate on pepsin digestion varies with protein source.

The digestion of casein and BSA varied from one set of digestions to another. This was demonstrated by the amount of protein digested in the control (0 mg phytate) for each of the 5 digestion sets (0, 4, 8, 16 and 24 hr hydrolysates). With casein as the substrate, protein digestion in these sets at the 0 mg phytate level was 39.3, 38.7, 38.6, 39.5 and 39.2%, respectively. With BSA as the substrate, protein digestion was 48.6, 46.2, 47.1, 45.6 and 46.5%, respectively. Since the digestion in the control varied between sets, the inhibitory effect of phytate hydrolysates on pepsin digestion of casein and BSA is indicated by relative *in vitro* digestibility (Tables 1 and 2). This inhibitory effect was reduced by hydrolyzing the phytate. Analysis of variance showed that the inhibitory response to levels of phytate hydrolysates was significant and linear. A similar relationship also existed between the inhibitory response and the degree of phytate hydrolysis.

Further examination of Tables 1 and 2 reveals that: (1) as with phytate, the phytate hydrolysates affect pepsin digestion of BSA less than that of casein, (2) hydrolyzing phytate beyond 65% release of phosphate causes little additional decrease in inhibitory effect, and (3) all hydrolysates, to some extent, decrease pepsin digestion.

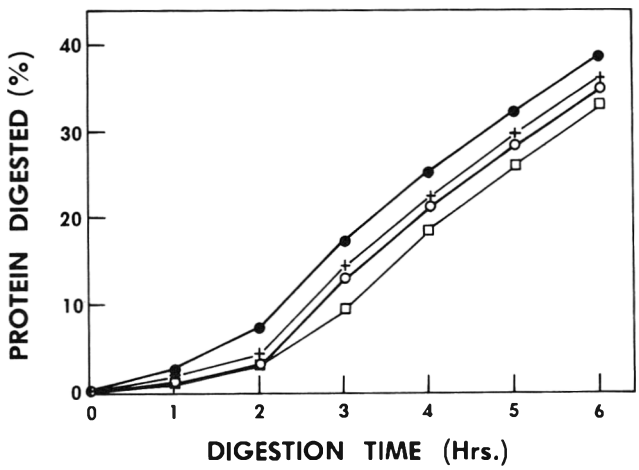


Fig. 1—Effect of phytate on pepsin digestion of casein. Phytate levels are 0 (•), 12.5 (+), 25.0 (○), and 50.0 (□) mg per 0.5g casein.

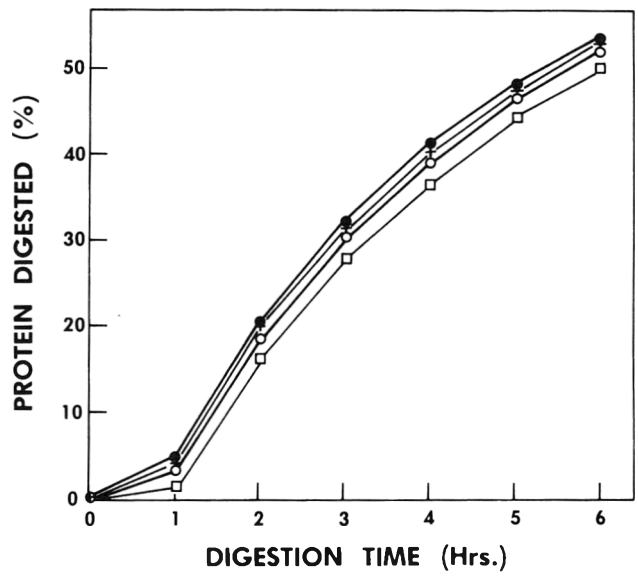


Fig. 2—Effect of phytate on pepsin digestion of bovine serum albumin (BSA). Phytate levels originally in the hydrolysates were 0 (•), 12.5 (+), 25.0 (○), and 50.0 (□) mg per 0.456g BSA.

Table 1—Effect of hydrolysis of phytate on pepsin digestion of casein

Phytate hydrolyzed (%)[a]	Hydrolysis time (hr)	Relative *in vitro* digestibility (%)[b] Phytate level per 68.4 mg N[c] 12.5 mg	25 mg	50 mg
0	0	96.2 ± 2.4	90.7 ± 2.3	85.6 ± 2.2
16	4	98.4 ± 2.4	95.2 ± 2.4	90.0 ± 2.3
37	8	97.1 ± 2.4	96.7 ± 2.4	93.0 ± 2.4
65	16	100.1 ± 2.4	98.4 ± 2.3	96.3 ± 2.3
82	24	98.0 ± 2.4	98.8 ± 2.4	96.0 ± 2.3

[a] Phosphate released as % of total phytate phosphate.
[b] Mean values from three digestions ± 95% confidence limits. Values (see text) were indexed to control (0 mg phytate) set at 100%.
[c] Added as sodium phytate.

Table 2—Effect of hydrolysis of phytate on pepsin digestion of bovine serum albumin (BSA)

Phytate hydrolyzed (%)[a]	Hydrolysis time (hr)	Relative *in vitro* digestibility (%)[b] Phytate level per 68.4 mg N[c] 12.5 mg	25 mg	50 mg
0	0	101.6 ± 2.1	96.9 ± 2.0	94.1 ± 2.0
16	4	98.8 ± 2.1	96.3 ± 2.1	94.6 ± 2.0
37	8	99.4 ± 2.1	99.0 ± 2.1	97.5 ± 2.1
65	16	99.9 ± 2.2	98.5 ± 2.1	98.2 ± 2.1
82	24	99.5 ± 2.1	100.7 ± 2.1	100.4 ± 2.1

[a] Phosphate released as % of total phytate phosphate.
[b] Mean values from three digestions ± 95% confidence limits. Values (see text) were indexed to control (0 mg phytate) set at 100%.
[c] Added as sodium phytate.

An explanation for the variation in inhibitory effect of phytate hydrolysates is the same as that described in the discussion of phytate. Although some foods such as bread and beans have significant quantities of phytate, the inhibitory effect of phytate is minimized as a result of changes which occur during preparation. In making bread (Ranhotra, 1972, 1973; Harland and Harland, 1980) and soaking beans (Kon et al., 1973; Reinhold, 1975), more than 65% of the phytate is hydrolyzed. It is assumed that most of this hydrolysis is due to the action of phytase.

Phytate was not detected in the 24-hr hydrolysate (82% hydrolyzed). This hydrolysate at the 50 mg level caused a decrease (4 ± 2.3%) in pepsin digestion of casein. These data suggested that inositol phosphate esters other than phytate also affected pepsin digestion. Inference regarding the effect of specific inositol phosphate esters (mono to penta phosphate) on casein digestion can only be made indirectly because no analysis was made of the amounts of each ester present in the phytate hydrolysates. However, the literature (de Boland et al., 1975) suggests that the 4, 8, and 16 hr hydrolysates may contain varying amounts of all the esters, whereas the 24 hr hydrolysate contains only the mono-, di-, and tri-phosphate esters (Arnold, 1956). Further investigation on the influence of specific phosphate esters on digestibility of protein as well as other macromolecules would enhance our understanding of mechanisms involved.

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Reference to a company and/or product named by the Department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

Effects of Free and Bound Chlorogenic Acid on the In Vitro Digestibility of Ribulose Bisphosphate Carboxylase from Spinach

WILLIAM E. BARBEAU and JOHN E. KINSELLA

ABSTRACT

The effect of free and bound chlorogenic acid on the *in vitro* digestibility of spinach ribulose bisphosphate carboxylase from spinach or fraction 1 (F-1) protein was investigated using a pH stat method. The pancreatin digestibility of F-1 protein was less than that of casein and BSA but greater than 11S soy protein. Noncovalently bound chlorogenic acid increased the initial rate of hydrolysis using both pancreatin and trypsin. Viscosity measurements suggested noncovalent binding of chlorogenic acid caused partial unfolding of F-1 protein making it more susceptible to proteolysis. Covalently bound chlorogenic acid decreased the rates of hydrolysis of F-1 by both trypsin and pancreatin, and this was correlated with changes in available lysine.

INTRODUCTION

THE NUTRITIVE VALUE of novel proteins is extremely important to their ultimate utilization by animals and/or man. Isolated leaf proteins possess a favorable balance of essential amino acids, (Betschart and Kinsella, 1974). However, biological evaluation of leaf protein nutritive value has often yielded values lower than predicted from their amino acid patterns (Saunders et al., 1973). This discrepancy may be related to some unidentified factors that limit the *in vivo* digestibility of leaf proteins and/or availability of selected amino acids.

The digestibility of crude leaf protein preparations has been determined using both *in vivo* and *in vitro* methods. The digestibility of cytoplasmic leaf protein was consistently superior to that of chloroplastic leaf proteins (Henry and Ford, 1965; Saunders et al., 1973; Byers, 1971). Significant variability in the digestibility of leaf protein preparations have been reported (Byers, 1971; Saunders et al., 1973). These discrepancies may be related to such factors as age and species of plant, method of leaf protein fractionation, processing conditions and the presence of antinutritional components. There is some evidence that the presence of phenolic compounds may reduce the digestibility of leaf protein concentrates. Lahiry et al. (1977) reported that bound chlorogenic acid reduced the *in vitro* digestibility of casein and the true digestibility of casein in rat bioassays was significantly lowered by the presence of bound phenolic compounds (Horigome and Kandatsu, 1968; Hurrel et al., 1982). Tannins, nonhydrolyzable polyphenolic compounds abundant in some plant foods, inhibit digestive enzymes *in vitro* (Goldstein and Swain, 1965; Tamir and Alumot, 1969, Milic et al., 1972). The use of polyvinylpyrolidone (PVP) and ultrafiltration to remove phenolic compounds from forage crops and leaf protein concentrates improved *in vitro* protein digestibility (Jung and Fahey, 1981; Lahiry et al., 1977).

We earlier reported both non-covalent and covalent binding of chlorogenic acid (CA) to purified ribulose bisphosphate carboxylase or fraction 1 (F-1) protein under controlled conditions (Barbeau and Kinsella, 1983). In the present study, we assessed the effects of chlorogenic acid binding on the *in vitro* digestibility of spinach F-1 protein using the pH stat method (Richardson and Catsimpoolas, 1979; Stinson and Snyder, 1980; Rothenbuhler and Kinsella, 1985).

Authors Barbeau and Kinsella are affiliated with the Institute of Food Science, Stocking Hall, Cornell Univ., Ithaca, NY 14853.

MATERIALS & METHODS

Casein (Hammerstein) was purchased from ICN Pharmaceuticals (Cleveland, OH) and trypsin (crystalline salt free, grade A) from Cal Biochem. (San Diego, CA). The bovine serum albumin (Fraction V), tyrosinase, pancreatin (procine pancreas grade III) and chlorogenic acid were supplied by Sigma Chemical Co. (St. Louis, MO). Ribulose bisphosphate carboxylase (98% pure) or F-1 protein was isolated and purified from spinach leaves by the method of Jones and Mangan (1976) as modified by Barbeau and Kinsella (1983). Soy 11S protein (>97% purity) was isolated from defatted soy flour by the method of Thanh and Shibaski (1976) using Sepharose 6B gel chromatography. All other chemicals used in this study were reagent grade. Distilled and deionized water was used in all experiments.

Apparatus

A London Radiometer pH stat instrument was used. This consisted of: a PHM 64 research pH meter, TTT 60 titrator assembly, ABU 12 autoburette and REC 61 servograph recorder. A digital/analog electronics board was interfaced between the autoburette and recorder to facilitate continuous plotting of the amount of base titrated vs time on the servograph recorder. Separate glass and calomel electrodes were used in the 5 ml reaction mixtures. The temperature and endpoint pH were maintained at 37°C and 8.000, respectively, and the autoburette speed was set at 20. The reaction chamber was continuously flushed with a gentle stream of N_2 previously saturated with water vapor. This prevented a drop in the pH of the reaction mixture due to formation of carbonic acid from dissolved CO_2. After continuous use for 24 hr, the glass electrode was soaked overnight in 1N HCl, to remove protein film from the surface, and stored in (pH 7.00) buffer until used.

Enzyme preparation

Pancreatin (150 mg) was added to 40 mL deionized distilled water (pH 7.5) and dissolved by stirring for 10 min at 40°C. The enzyme solution was centrifuged (10,000 × *g* for 10 min), the supernatant was decanted, and the pH of the enzyme solution adjusted to pH 8.00-8.02 with 0.1N NaOH. Aliquots of the enzyme (0.02-0.3 mL) were stabilized by freezing in liquid nitrogen and stored in the freezer at −70°C until needed. Prior to use, the enzyme aliquots were thawed by submersion in warm water, at 50°C. The trypsin solutions were prepared and handled in a similar manner except 100 mg enzyme was dissolved in 100 mL water giving a final enzyme concentration of 1.0 mg/mL. The pancreatin experiments were performed with a final protein:enzyme ratio of 40:1 while in the experiments using trypsin, the ratio was 150:1.

Protein solution(s)

Protein (3.0 mg/mL) was dissolved in deionized distilled water at 25°C and centrifuged at 10,00 × *g* for 10 min and 4.9 mL of the protein supernatant solution carefully pipetted into a pH stat reaction vessel. The pH of the protein solution was adjusted to approximately pH 8.00 with 0.1N NaOH just prior to equilibration in the pH stat. The protein concentrations were determined by the method of Lowry et al. (1951).

In vitro digestion of protein samples

The pH of the protein solutions was allowed to reach equilibrium for one hour in the pH stat vessel and then was titrated to an end-point of pH 8.000 prior to addition of enzyme. When the pH was stabilized and baseline set, enzyme (100 μL) was added to the protein solution with a microsyringe. The recorder was started simultaneously with addition of enzyme and the proteolysis reaction was monitored by continuously measuring the amount of NaOH (0.02N) required to maintain pH at 8.0. The results presented are an average of duplicate experimental runs performed with F-1 samples treated in an identical manner. Replicate runs were always within 5% error margin.

Quantitation of chlorogenic acid binding to F-1

Chlorogenic acid was bound to the protein and the amount bound to F-1 protein was determined as described previously (Barbeau and Kinsella, 1983).

Viscosity measurements

To determine if CA binding caused conformational changes in the protein, the effect of chlorogenic acid binding on the kinematic viscosity of F-1 protein solutions was determined. Varying concentrations of chlorogenic acid were dialyzed against F-1 protein (0.5% w/v) in 0.02M phosphate buffer, pH 7.0 at 25°C for 24 hr using dialysis cells as described previously (Barbeau and Kinsella, 1983), and the amount of chlorogenic acid bound to F-1 per mole of protein was determined. Fraction 1 protein solutions were then dialyzed against deionized water (4L) to remove unbound chlorogenic acid and buffer salts. The viscosity of F-1 solutions was measured using an Oswald viscometer held upright in circulating water bath at 25°C ± 0.1°C. Outflow times for deionized water and protein solutions were measured and a kinematic viscosity was calculated using the equation:

$$n / n_o = t / t_o - 1$$

where t and t_o are the outflow times for the unknown and deionized water, respectively.

Lysine determination

The available lysine content of the F-1 protein samples was determined in triplicate by the method of Fields (1969) using trinitrobenzene sulfonic acid (TNBS).

RESULTS & DISCUSSION

MOZERSKY and Panettieri (1983) and O'Hare et al. (1984) discussed factors, especially different buffering capacities of proteins, that could lead to discrepancies in results obtained for comparison of protein digestibility by the pH drop method. Differences in protein buffering capacities could be caused by the presence of salts, phenolic acids, etc., and/or exposure of previously buried unprotonated basic groups as proteins are hydrolyzed. This is less of a problem with the pH stat method especially at low protein concentrations. Histidine is the only amino acid with significant buffering capacity at pH 6–8. The epsilon amino group of lysine and the guanidinium group of arginine have pKa values above pH 9 (Lehninger, 1975). Thus at pH 8.0, (the pH of *in vivo* digestibility experiments performed in this study), the rupture of peptide bonds was the preponderant source of protons released and of the associated pH change during enzymatic hydrolysis of proteins.

A comparison of the initial rates of pancreatin hydrolysis of sodium caseinate, bovine serum albumin (BSA), F-1 and 11S soy proteins using the pH stat method is shown in Fig. 1. The rate of hydrolysis of F-1 protein was less than sodium caseinate and BSA but faster than 11S soy protein.

The differences in relative rates of pancreatin hydrolysis (Fig. 1) may, among other factors, reflect the three dimensional structure of these four proteins. Sodium caseinate with little ordered structure was hydrolyzed more rapidly than the other proteins which possess more ordered tertiary and quaternary structures. BSA is a rather compact globular protein consisting of six parallel alpha-helices centered

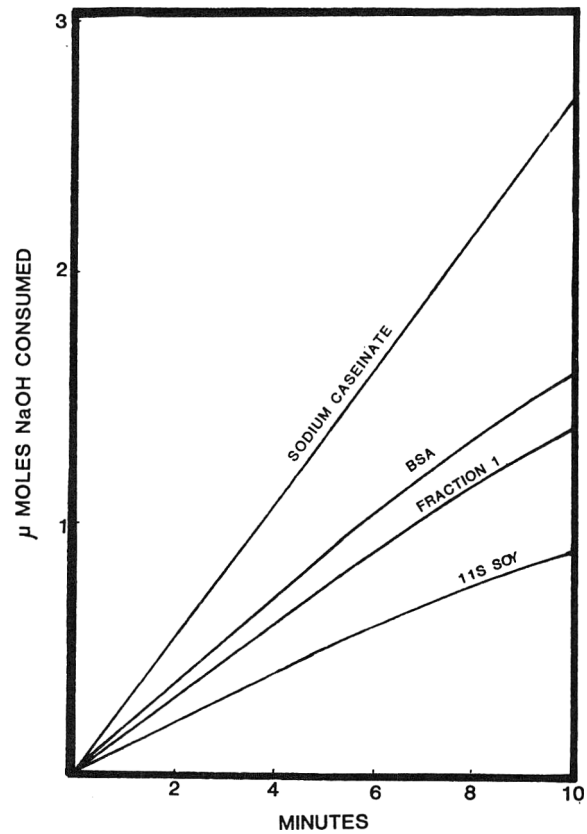


Fig. 1—Comparison of the relative rates of pancreatin digestibility of sodium caseinate, BSA, fraction 1 and 11S soy.

around a hydrophobic core (Walton and Menapa, 1979). This flexibility in the structure of BSA may account for the faster rate of pancreatin hydrolysis compared to F-1 and 11S soy proteins.

There is evidence that F-1 protein like BSA is not a rigid inflexible molecule but may undergo conformational changes or transitions (Kawashima and Ayabe, 1972; Badger and Andrews, 1974). However, the slower rate of pancreatin hydrolysis of F-1 protein, suggests that the "native" F1 enzyme is somewhat refractory to attack by pancreatic enzymes. The quarternary 11S protein molecule contains 21 disulfide bridges and has a tightly folded, very compact three dimensional structure (Saio et al., 1971). The apparent low digestibility of some soy products in which trypsin inhibitor has been destroyed may be related to the inherent resistance of soy proteins to proteolytic hydrolysis (Liener, 1981). Modification of the quarternary and tertiary structure of 11S soy protein by reduction of the disulfide bonds and disruption of hydrophobic interactions enhances digestibility (Lynch et al., 1977; Katama et al., 1979; Rothenbuhler and Kinsella, 1985).

Effect of chlorogenic acid

Because phenolic compounds occur in isolated leaf proteins we studied the effects of chlorogenic acid on pancreatin digestibility.

Chlorogenic acid when added directly to the pancreatin solution (0-4 mM) caused a small (<5%) concentration dependent decrease in the pancreatin hydrolysis rates of F-1 protein (data not shown). This observation is consistent with Delic et al. (1975) who reported that chlorogenic acid (2%) added to the diet inhibited growth rate and retention of dietary nitrogen by experimental mice. In contrast, Dryden and Satterlee (1978) reported that free chlorogenic acid had no effect on casein digestibility, PER, or growth rates of Tetrahymena pyriformis.

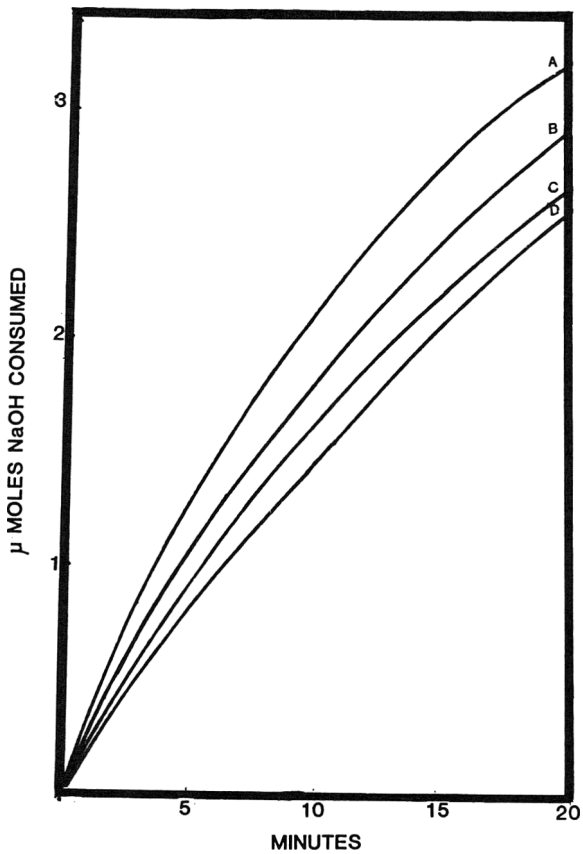


Fig. 2—Effect of noncovalently bound chlorogenic acid on the in vitro *digestibility of fraction 1 protein using pancreatin. (A) fraction 1 with 12 moles noncovalently bound chlorogenic acid; (B) fraction 1 with 10 moles noncovalently bound chlorogenic acid; (C) fraction 1 with 4 moles noncovalently bound chlorogenic acid; (D) fraction 1 control.*

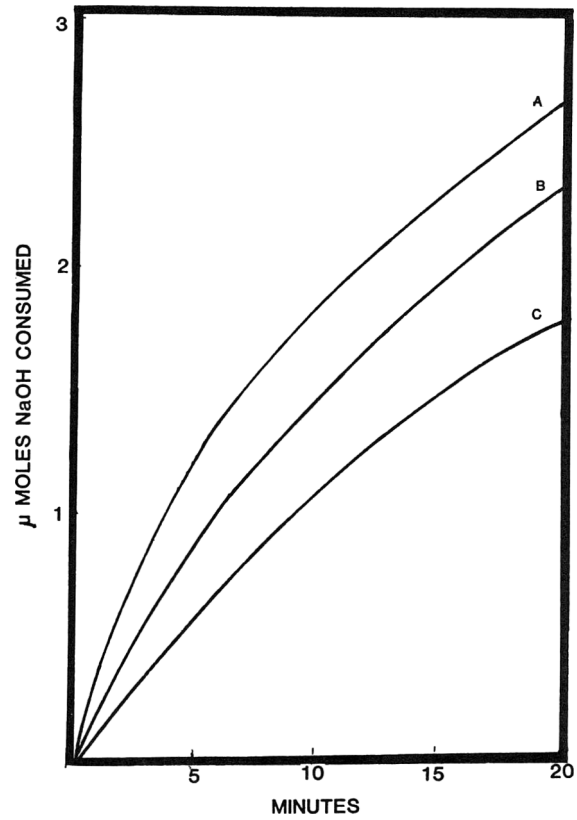


Fig. 3—Effect of noncovalently bound chlorogenic acid on the in vitro *digestibility of fraction 1 using trypsin. (A) fraction 1 with 10 moles noncovalently bound chlorogenic acid; (B) fraction 1 with 4 moles noncovalently bound chlorogenic acid; (C) fraction 1 control.*

Effect of noncoavlently bound chlorogenic acid

Both noncovalent and covalent interactions may occur between chlorogenic acid and F-1 protein (Barbeau and Kinsella, 1983). Therefore, in separate experiments, we studied how these interactions affected the pancreatin and trypsin digestibility of F-1 protein.

The initial rates of pancreatin hydrolysis of F-1 protein with noncovalently bound chlorogenic acid (CA) are presented in Fig. 2. The rates of hydrolysis progressively increased with the amount of CA noncovalently bound to F-1.

Noncovalently bound chlorogenic acid dramatically increased the initial rate of trypsin hydrolysis, but the total rates in all cases were less than those obtained with pancreatin (Fig. 3). Trypsin hydrolyzes peptide bonds primarily adjacent to lysine or arginine residues in proteins and thus may be sensitive to net charge on the substrate. If non-covalent interactions between CA and F-1 involve primarily lysine or arginine side chains on the protein, one would expect a decrease in the rate of trypsin hydrolysis. Earlier data indicated that electrostatic interactions between the ionized carboxyl group of CA and the positively charged side chain groups on F-1 protein possibly lysine are partly involved in non-covalent protein-ligand association (Barbeau and Kinsella, 1983). Hence, these results suggested that non-covalent interactions between CA and F-1 may have caused partial unfolding of the protein making it more susceptible to hydrolysis by trypsin.

Because viscosity is a sensitive indicator of conformational changes in protein molecules (Yang, 1961), the effect

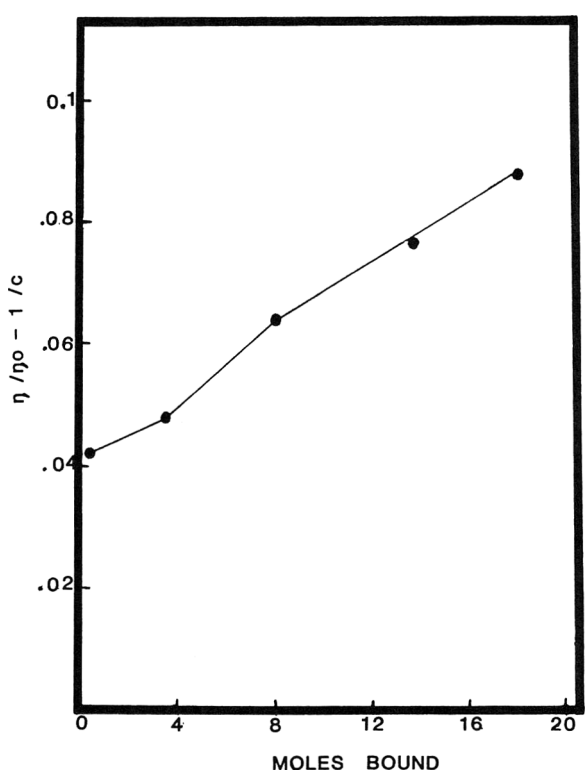


Fig. 4—Effect of chlorogenic acid binding on the reduced kinematic viscosity of fraction 1 protein. Viscosity measurements were made in an Oswald viscometer at 25°C using protein solutions (0.5% w/v) in deionized water at pH 7.0.

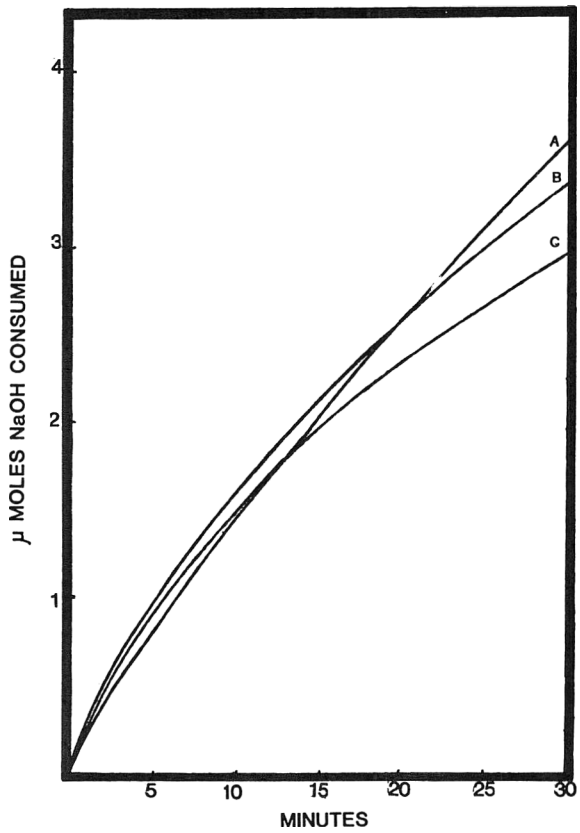


Fig. 5—Effect of covalently bound chlorogenic acid on the in vitro *digestibility of fraction 1 using pancreatin. (A) fraction 1 control; (B) fraction 1 with 12 moles covalently bound chlorogenic acid; (C) fraction 1 with 36 moles covalently bound chlorogenic acid.*

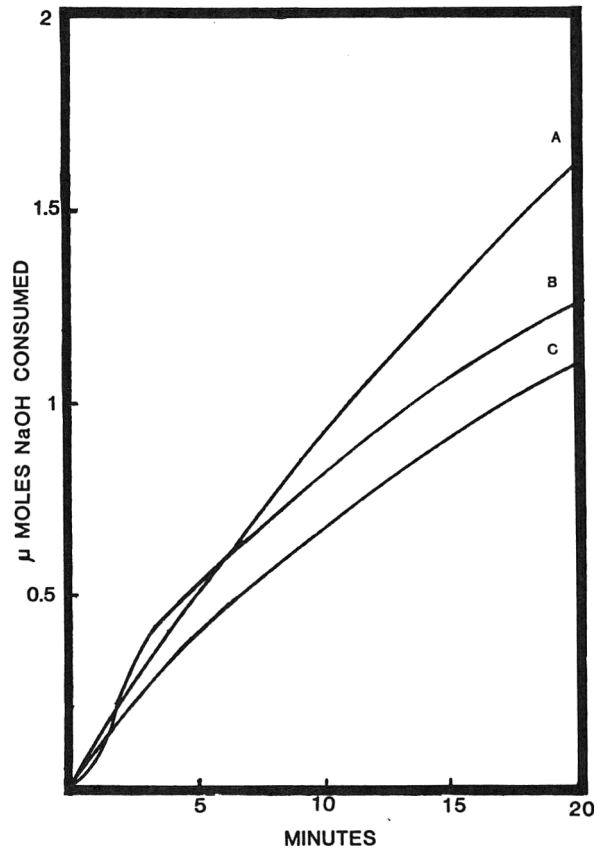


Fig. 6—Effect of covalently bound chlorogenic acid on the in vitro *digestibility of fraction 1 using trypsin. (A) fraction 1 control; (B) fraction 1 with 16 moles covalently bound chlorogenic acid; (C) fraction 1 with 36 moles covalently bound chlorogenic acid.*

of CA binding on the viscosity of F-1 protein solutions was determined (Fig. 4). There was an increase in viscosity with an increase in the number of moles of CA noncovalently bound to the protein. These results indicate that chlorogenic acid binding affected the hydrodynamic volume and/or shape of the protein molecule. The viscosity of protein solutions increases with binding of small molecular weight ligands such as sodium dodecyl sulfate (SDS) (Shetty and Rao, 1974). The increase in viscosity of F-1 protein indicates that chlorogenic acid binding cause change(s) in the three dimensional structure of F-1 which facilitate proteolysis.

Effect of covalent bound chlorogenic acid

In order to determine if covalent bonding of CA to F-1 affects digestibility, CA was covalently attached to F-1 protein using polyphenol oxidase. In the previous study, we estimated that in the presence of polyphenol oxidase, approximately 50% of the bound CA associated with F-1 protein was covalently linked. The remaining 50% was bound to the protein via noncovalent interactions (Barbeau and Kinsella, 1983). After dialysis to remove the noncovalently bound free CA, the *in vitro* digestibility assays revealed a small increase in the initial rate of pancreatin hydrolysis when chlorogenic acid was covalently bound to the protein. However, when protein hydrolysis was monitored beyond 20 min, changes in the relative rates of pancreatin hydrolysis of the F-1 were observed. The rates of hydrolysis of F-1 with covalently bound CA decreased after 10 min (Fig. 5).

Using the pH stat method, the amount of base consumed with time is an estimate of the relative *in vitro* digestibility of the protein. After 30 min hydrolysis, the F-1 control consumed 3.60 μmoles of base compared with 3.38 and 2.96 μmoles of base consumed for F-1 samples with 16 and 36 moles of bound chlorogenic acid, respectively. Hence, the *in vitro* digestibility of samples with covalently bound chlorogenic acid were 94% and 83% of the F-1 control.

In contrast to the results with pancreatin the covalently bound CA had a pronounced and immediate effect on the rates of trypsin hydrolysis (Fig. 6). During the first five minutes the sample with 16 moles of covalently bound CA was rapidly hydrolyzed. However, after 5 min, the hydrolysis rate gradually decreased. Comparison of the *in vitro* trypsin digestibility, at 20 minutes, of the F-1 samples with 16 and 36 moles of chlorogenic acid covalently bound were 81% and 74%, respectively, compared to the control.

Proteolytic enzymes such as trypsin initially attack and hydrolyze the most accessible peptide linkages in substrate protein (Mihalyi, 1978). The proteolysis patterns observed may reflect the heterogeneous population of F-1 molecules in the mixture. The noncovalent interactions of CA with F-1 cause partial unfolding of some of the F-1 molecules making them more susceptible to attack by proteolytic enzymes. Therefore, the initial increase observed in the rate of hydrolysis of covalent bound F-1 samples (Fig. 6) may be due in part to the presence of some F-1 molecules partially unfolded by noncovalently bound chlorogenic acid. As hydrolysis of these F-1 protein samples proceeded, the rate decreased compared to the control because fewer susceptible peptide bonds were available and the covalently linked CA may have limited the access of proteolytic enzymes by steric hindrance.

Table 1—Available lysine content of fraction 1 samples[a]

Protein sample	Available lysine Content (mg/100 mg protein)	Relative % digestibility
Fraction 1 Control	6.06 ± 0.40	100
Noncovalently bound chlorogenic acid		
Fraction 1 with 4 moles bound	5.89 ± 0.27	97
Fraction 1 with 10 moles bound	6.66 ± 0.47 *	110
Fraction 1 with 12 moles bound	6.94 ± 0.33 **	115
Covalently bound chlorogenic acid		
Fraction 1 with 12 moles bound	5.56 ± 0.27 *	92
Fraction 1 with 16 moles bound	4.86 ± 0.50 **	80
Fraction 1 with 36 moles bound	4.10 ± 0.70 **	68

[a] Mean values and standard deviations of triplicate determinations
* Significantly different from Fraction 1 Control ($p<0.05$)
** Significantly different from Fraction 1 Control ($p<0.01$)

Stinson and Snyder (1980) concluded that the initial rate of trypsin hydrolysis of proteins using the pH stat could be reliably determined within one or two minutes. The results of this study indicate that this may not always be true. When CA was bound to F-1, definite changes were observed in the rate of enzymatic hydrolysis of the protein with time.

Quinones are believed to crosslink with proteins by covalent interaction with free sulhydryl or amino groups (Mason, 1955; Hurrel et al., 1982). Such cross-linking could effectively shield some susceptible peptide bonds in proteins from the action of enzyme(s). Pierpont et al. (1977) reported cross-linking reactions between protein subunits of potato X virus (PVX) after incubation with chlorogenic acid and polyphenol oxidase. They postulated that formation of crosslinks between protein subunits of PVX occurred via covalent attachment of neighboring lysine residues to a single chlorogenic acid molecule. There is also evidence of covalence coupling reactions between the oxidative-condensation products of chlorogenic acid and tobacco leaf protein (Eagles et al., 1980). Thus, in our studies F-1 containing covalently crosslinked quinone molecules would be expected to be more resistant to attack by pancreatin enzymes than the native protein due to crosslinking of the protein by quinones. Pepsin hydrolysis may also be impaired by covalent linkage of chlorogenic acid to free E-NH_2 groups of lysine which are normally required for enzyme binding (Milhalyi, 1978).

In support of this we observed that the available lysine content of samples with covalently bound chlorogenic acid was considerably less than in the F-1 controls (Table 1). Available lysine decreased as the number of moles of CA covalently bound to the protein increased. However, in samples with noncovalent bound CA the available lysine content of F-1 increased slightly. Lysine residues in proteins are believed to be normally situated close to the protein surface. However, it is possible that some lysine groups in F-1 participate internally in ionic interactions (salt bridges) with aspartic or glutamic acid residues. It is known that F-1 dissociates into small and large subunits at alkaline pH>11 (Kawashima and Wildman, 1970). This suggests that lysine groups may be involved in the association of F-1 subunits. Non-covalent chlorogenic acid binding by inducing conformation changes may expose previously buried or unaccessible lysine residues in F-1 protein.

The pH stat method has advantages for measuring protein digestibility. The results are obtained quickly and the system is not costly to operate or maintain (Stinson and Snyder, 1980). Individual factors affecting protein digestibility such as the trypsin inhibitor in soybeans or effects of tannins are easily investigated using the pH stat method (Rothenbuhler and Kinsella, 1985). Finally, the pH stat method is useful for studing relationships between protein structure and *in vitro* digestibility. The pH stat method has some limitations. Like other *in vitro* methods, the pH stat method may not accurately reflect protein digestion in the human gastrointestinal tract as discussed by Milhalyi (1978). Since the pH stat method depends upon the titration of protons released from ruptured peptide bonds, high ionic strength (>0.04M) cannot be used with this system. Therefore, investigation of the digestibility of proteins soluble only in high ionic strength solvents is not possible.

The results presented here may have practical significance. Chlorogenic acid is only one of the compounds present in leaves or plant materials. Covalent reactions between plant phenolic compounds and proteins should be avoided or minimized during protein extraction. Inhibition of polyphenol oxidase activity in plant tissue extracts using SO_2 or metabisulfite may be necessary. Research to further characterize the usefulness of the pH-state method for evaluating factors affecting plant protein digestibility are in progress (Rothenbuehler and Kinsella, 1985).

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Effects of Baking and Boiling on the Ability of Selected Organic Acids to Solubilize Iron from a Corn-Soy-Milk Food Blend Fortified with Exogenous Iron Sources

S. W. RIZK and F. M. CLYDESDALE

ABSTRACT

Combinations of selected organic acids (ligands) and iron sources were evaluated for their ability to improve iron solubility in a corn-soy-,milk (CSM) food blend. Iron solubility of baked CSM was primarily affected by exogenous iron source at endogenous pH (5.2 - 6.3), and by organic acid at pH 2.0. Boiled CSM samples were directly influenced by pH (range 5.2 - 6.4), and by both organic acid and exogenous iron source at pH 2. Supplementation of baked CSM with hydrogen reduced elemental iron and either ascorbic or citric acid; or supplementation of boiled CSM with ferric orthophosphate and either cysteine, ascorbic, citric, or malic acid, was shown to provide greatest potential for promoting iron availability in man as measured by chemical means.

INTRODUCTION

FOR MANY YEARS the U.S. government has been involved in domestic and foreign assistance programs which provide food to the nutritionally deprived people of the world (Senti, 1974). In many developing countries, cereal and legume products provide a major portion of the diet, and those same populations have exhibited the greatest prevalence and severity of iron deficiency (Bothwell et al., 1982).

In the overseas food donation program, cereal-soy blends serve as a major source of protein and calories. In addition, they provide supplemental iron to populations at high risk of iron deficiency, such as weaning infants, preschool children, and lactating mothers (Bookwalter, 1983; Langsdorf, 1981).

The first formulated food product to be widely distributed was a blend of precooked cornmeal, defatted soy flour, nonfat dry milk, and soy oil (Senti, 1974). The product was designated corn-soy-milk mix, but is commonly known as CSM. Unfortunately, the iron needs of individuals consuming this cereal-soy blend are unlikely to be met since the bioavailability of iron from CSM is very low (Bothwell et al., 1982).

To date, the most reliable means of improving iron bioavailability from cereals and legumes is via the addition of ascorbic acid or meat (Bothwell et al., 1982). Ascorbic acid, in particular, has demonstrated an ability to reverse the inhibitory effect on iron absorption of soy protein products (Morck et al., 1982), and this reversal has been attributed to ascorbate's solubilizing capacity in vitro (Rizk and Clydesdale, 1983). However, use of ascorbic acid as an enhancer has been limited due to its poor stability under conditions of processing and storage (Bothwell et al., 1982).

The intent of this study was to evaluate the effectiveness of selected organic acids and heat treatments (boiling and baking) as potential promoters of iron bioavailability in a CSM food blend as estimated by chemical analyses.

Author Clydesdale is affiliated with the Dept. of Food Science & Nutrition, Massachusetts Agricultural Experiment Station, Univ. of Massachusetts, Amherst, MA 01003. Author Rizk is presently affiliated with the Research & Development Laboratories, The Pillsbury Company, 311 Second St. S.E., Minneapolis, MN 55414.

MATERIALS & METHODS

Chemical iron analysis

In 1979, Lee and Clydesdale (1979) developed a technique for quantitatively determining the elemental, ferrous, ferric, soluble, and complexed iron in foods. This procedure required blending a portion of a solid food with water to produce a slurry. The total iron analysis was performed on the slurry. A modification of this procedure was used in this study and has been outlined in detail elsewhere (Rizk and Clydesdale, 1983).

CSM food blend

The CSM dry mix was obtained courtesy of ADM Milling Company, Shawnee Mission, KS. This mix has been considered an inexpensive, nutritious, and convenient food source which can be served in porridges, beverages, soups, cookies, and fried cakes (Bookwalter, 1983).

Iron sources

Four exogenous iron sources were used for this study. Ferrous sulfate heptahydrate ($FeSO_4$) contained 20.1% iron (Fisher Scientific Company, MA); electrolytic reduced iron (ERFe) consisted of 98.3% iron with 98% less than 44 microns; hydrogen reduced iron (HRFe) consisted of 96.9% iron with 97% less than 44 microns (Glidden-Durkee, NY); and ferric orthophosphate (FOP) contained 29.1% iron (Joseph Turner & Company, NJ).

All iron sources were added in sufficient amounts to each sample to provide a 1:1 ratio of exogenous (iron source):endogenous (food) iron. These amounts were chosen in keeping with similar ratios employed by Cook et al. (1981) in human iron absorption studies, as well as for comparison to previous investigations performed in this lab (Rizk and Clydesdale, 1984). It should be noted that since the exogenous iron slurries contained twice the total iron as the endogenous iron samples, then unless the percent soluble iron was increased by a factor of 0.5, the total amount of soluble iron was actually increased.

Ligands

Five ligands were chosen for evaluation based on thermodynamic and kinetic stability constant data previously determined in this laboratory (Flynn et al., 1984; Gorman and Clydesdale, 1983; 1984). These included L-ascorbic acid (ASC), citric acid monohydrate (CIT), malic acid (MAL) (Fisher Scientific Company, MA), L-cysteine hydrochloride monhydrate (CYS) (J.T. Baker Chemical Company, NJ), and calcium lactate pentahydrate (LAC) (Eastman Kodak Company, NY).

Ligands were added in an 8:1 molar ratio (ligand:iron), since similar levels of ASC added to soy-based products increased iron absorption in humans five- to six-fold (Morck et al., 1982).

Sample preparation

In accordance with the manufacturers' suggested proportions, 20g CSM (3.85 mg endogenous iron) were blended with 200 mL double distilled deionized water until free from lumps. The appropriate amounts of ligands and/or iron sources were added to this blend. The pH of each slurry was recorded prior to further treatment, i.e., lowering the pH to 2.0, and throughout the text is referred to as the "endogenous" pH. This sample simulated a typical cold beverage and served as a control for the heat-treated samples.

Boiled CSM

The same proportions and mixing procedure were employed for both the uncooked and boiled samples. However, the latter samples

were heated approximately 8 min on an electric range and allowed to boil for 5 min. Samples were then immediately cooled to room temperature (20°C) in an ice water bath before analysis and brought back to volume with double distilled deionized water. This recipe corresponded to that of a hot beverage. Ligands and/or iron sources were added to the slurries prior to boiling

Baked CSM

The CSM mix was baked without other ingredients which typically constitute a bread, i.e., shortening, eggs, etc., in an attempt to observe the effects of baking on CSM alone. After addition of an exogenous iron source and/or organic acid, 8 mL double distilled deionized water were added to 20g CSM in a canning jar and mixed with a glass rod. This amount of water was approximately equivalent to the moisture content of the ingredients which would have normally been added and was sufficient to produce a doughy consistency.

This dough was placed into a paper cupcake holder (sample size = 3/4 in. height x 2½ in. width) in an aluminum tray and baked at 425°F (218°C) for 20 min. All sample were allowed to cool to room temperature, removed from the paper holders, and returned to the original canning jar. After addition of 100 mL double distilled deionized water, the sample was blended on the liquefy setting for 1 min using an Osterizer (Oster Corporation, Milwaukee, WI). This slurry was poured into a 250 mL beaker, along with another 100 mL aliquot of distilled water, which was used to rinse the jar.

Dual pH incubation procedure

Kojima et al. (1981) analyzed several ligands for their solubilizing effect on iron in a pinto bean suspension. They concluded that citric acid optimally solubilized the iron of this suspension at approximately pH 6, while ascorbic acid was most effective at pH values of 1 - 2. Therefore, it was decided to examine all samples at both the endogenous pH (range 5.2 - 6.4) and at the stomach pH (2.0) in order to cover a range of pH values where ligands could have a potential effect on iron solubility.

Selection of best iron-ligand combinations

The exogenous iron source which provided both the largest enhancing factor (the ratio of the percent soluble iron in a treated sample to an untreated sample) and percent soluble iron values in the presence of each ligand-CSM blend was examined further. Those iron-ligand combinations which exhibited the greatest potential were reevaluated using an in vitro method developed in this laboratory. This method simulated the pH, time and temperature conditions of the gastrointestinal tract and has been described in detail elsewhere (Rizk and Clydesdale, 1983).

Statistical analysis

Data were analyzed using t-tests to determine significant differences between two means (Snedecor and Cochran, 1976).

RESULTS & DISCUSSION

Baked CSM

Enhancing ability of organic acids. No significant differences in percent soluble iron were noted as a result of supplementation with an organic acid or exogenous iron source at pH E (Table 1). As expected, ASC was the predominant enhancer at pH 2 in the baked CSM system. Significant increases in percent soluble iron over the control were noted for all iron systems (Table 1) when ASC was added. However, soluble iron was either decreased or remained the same as the control for all other added ligands and iron sources at pH 2, with the exception of the CYS-ERFe combination. Unfortunately, supplementation of CYS in the baked CSM food blend at the present levels would not be recommended due to observed sensory problems, i.e., sulfide odor.

Effect of baking on iron solubility. While no consistent trends in iron solubilization due to baking were observed, enhancing factors were dependent upon a combination of exogenous iron source, pH, and organic acid present.

Ranhotra et al. (1979) reported an inhibitory effect of baking on available iron from bread fortified with $FeSO_4$. Processing techniques resembling commercial sterilization have also produced substantial improvements in bioavailable iron to animals from infant formulas (Theuer et al., 1971; 1973) and corn-milk based diets (Wood et al., 1978; Rizk et al., 1979; 1981) when fortified with iron phosphate salts, but not with $FeSO_4$.

Similar results were observed in the present investigation where samples containing $FeSO_4$ exhibited the greatest decrease in percent soluble iron at pH E due to baking. In fact, only combinations of HRFe-LAC, HRFe-CIT, FOP-ASC, and FOP-LAC produced significant increases in percent soluble iron in the baked CSM system as compared to the uncooked controls.

Effect of iron fortification on soluble iron. It was already realized that $FeSO_4$ is a very soluble and available iron source. Unfortunately, its usefulness as a fortificant in cereal-soy blends may be limited, due to its tendency to catalyze oxidative reactions resulting in undesirable odors and flavors (Cook and Reusser, 1983). It has also been suggested that the more reactive and bioavailable iron sources, such as $FeSO_4$, may be converted to insoluble hydroxides when stored at the pH of cereals and become irreversibly insoluble even if the pH is lowered to 2.0 (Clydesdale, 1983).

In contrast, elemental iron powders have exhibited a much lower potential for rancidity effects, even when stored at elevated temperatures for extended periods of time (Anderson et al., 1976; Harrison et al., 1976; Ranum and Loewe, 1978). This is particularly important since supplemental foods, such as CSM blend, may be stored for extended periods of time and under adverse conditions.

Of the three remaining iron sources, both HRFe and ERFe demonstrated good solubility characteristics. However, HRFe resulted in more significant increases in percent soluble iron due to baking, as compared to the control.

Effects of the simulated G.I. test on the selected iron-ligand combinations. The majority of iron absorption occurs in the duodenum where the lumenal pH ranges from 4.5 - 6.5, depending upon the meal, and only gradually rises to 7.3 in the ileum (Bell et al., 1961; Langley et al., 1969). Therefore, the simulated gastrointestinal (G.I.) test which involved sequentially incubating each sample at pH E, 2, 4, and 6, for 30, 30, 10, and 10 minutes, respec-

Table 1—Average[a] percent soluble iron in the baked CSM samples

Iron added[c]	pH	Control	Organic acid[b]				
			ASC	LAC	CIT	MAL	CYS
None	E[d]	11.4	10.0	10.3	13.0	12.0	13.5
	2	64.3	84.4**[e]	61.5	60.4	55.9	68.7
$FeSO_4$	E	17.3	12.9	13.5	13.5	15.4	15.8
	2	51.2	77.3**	48.2	43.0	45.4	55.9
HRFe	E	6.3	6.2	5.6	8.7	6.8	6.6
	2	43.1	60.1**	43.6	41.7	33.2	41.6
ERFe	E	8.0	6.3	5.9	9.4	9.0	9.9
	2	44.5	72.7**	41.5	42.4	40.1	50.7*
FOP	E	6.3	7.6	3.7	5.0	5.4	4.9
	2	47.6	67.3**	33.8	30.9	24.9	29.4

[a] Average of duplicates.
[b] ASC = ascorbic acid; LAC = lactic acid; CIT = citric acid; MAL = malic acid; CYS = cysteine.
[c] None = endogenous iron; $FeSO_4$ = ferrous sulfate heptahydrate; HRFe = hydrogen reduced elemental iron; ERFe = electrolytic reduced elemental iron; FOP = ferric orthophosphate.
[d] E = endogenous pH.
[e] Values marked with an asterisk show significant increases over the control at either the 5% (*) or 1% (**) level.

tively, at 37°C, was employed in an attempt to mimic the approximate temperature and pH conditions of this intestinal segment.

The results from the simulated G.I. test (Table 2) suggest that LAC and MAL would be ineffective as enhancers in this foodstuff. CYS showed moderate and ASC excellent enhancing properties at pH 2 and 4. The influence of CYS on iron solubility was anticipated and agrees with previous reports of its effect on iron absorption (Van Campen, 1973; Martinez-Torres et al., 1981; Layrisse et al., 1984). While CIT was superior at the more neutral pH values of E and 6, it was unexpectedly promising at pH 2 and 4, which is contrary to its effects in other food systems (Kojima et al., 1981; Rizk and Clydesdale, 1984).

At pH E and 6, the percent soluble iron in each of the above samples existed predominantly in the complexed form, whereas at pH 2, ionic iron was the major form. The only exception was the CIT-HRFe sample where a major proportion of the soluble iron at each pH was complexed.

However, the quantitation of ionic iron in this study using the bathophenanthroline compound must be interpreted with caution and, in fact, the term bathophenanthroline-reactive iron might be more appropriate than ionic iron. Since bathophenanthroline has a larger binding constant for iron than ASC (Gorman and Clydesdale, 1983) and can remove iron from ASC-iron complexes, it was possible that the ionic iron analyzed in this study was originally in an iron-ASC complex. Since all other ligands employed in this investigation have smaller thermodynamic stability constants for iron than ASC, then similar limitations in interpretation may exist for these samples as well (Flynn et al., 1984; Gorman and Clydesdale, 1984).

Boiled CSM

Enhancing ability of organic acids. At pH E, boiled samples without added ligands exhibited much greater soluble iron (26 - 54%) than the baked CSM samples (6 - 17%). Addition of an organic acid directly affected the endogenous pH (range = 5.2 - 6.4).

In order to determine if this initial pH had any effect on the iron profiles a separate experiment was performed where controls were developed to replace each organic acid with sufficient HCl to produce equivalent pH values. While no differences in chemical iron profile were observed between HCl and organic acid samples at pH 2, the total percent soluble iron, within the endogenous pH range, was more directly influenced by pH, and not ligand. For example, addition of HCl to the boiled CSM samples decreased the percent soluble iron from 50% (pH 6.4 – CON) to approximately 43% at pH 5.7 to 6.2 (LAC, ASC, and CYS), and further to 21% at pH 5.2 (MAL and CIT).

Table 2—A comparison of enhancing factors[a] at four pH values for baked CSM supplemented with selected organic acids and hydrogen reduced elemental iron

Slurry pH	ASC	Organic acid[b] LAC	CIT	MAL	CYS
E[c]	0.81	0.74	2.25**	0.77	0.80
2	1.77**[d]	1.13	1.78**	0.99	1.18*
4	2.88**	1.02	1.95**	0.95	1.35*
6	1.18	0.92	4.15**	1.00	1.00

[a] Enhancing factor is the ratio of the percent soluble iron detected with organic acid present to the percent soluble iron without organic acid present.
[b] ASC = ascorbic acid; LAC = lactic acid; CIT = citric acid; MAL = malic acid; CYS = cysteine.
[c] E = endogenous pH.
[d] Values marked with an asterisk show significant increases over baked samples without an organic acid at either the 5% (*) or 1% (**) level.

Recent chemical analyses performed in this laboratory on a soy protein isolate demonstrated substantial decreases in percent soluble iron when the pH was lowered to 2.0 (Rizk and Clydesdale, 1983). This is in contrast to the results obtained from the baked, boiled, and uncooked CSM systems where lowering the solution pH from E (endogenous) to 2 produced increases in soluble iron up to eleven-fold. While recent studies (Cook et al., 1981; Morck et al., 1982; Thompson and Erdman, 1984) have suggested an overwhelming ability of soy protein to repress nonheme iron absorption from several foods, the present data indicate that the soy component of CSM does not adversely affect iron solubility when pH is increased from 5.2 to 6.4.

Comparison of the percent soluble iron (Table 3) for each of the boiled CSM samples at pH 2 illustrates the overwhelming ability of ASC to improve iron solubility in CSM as compared to other ligands. In fact, FOP-fortified boiled CSM samples supplemented with either MAL or CYS were the only other combinations which improved iron solubility.

Effect of boiling on iron solubility. In general, boiling the CSM dry mix substantially augmented the percent soluble iron at pH E by two- to five-fold, and decreased it at pH 2. At pH E, the influence of each organic acid was dependent upon its pH-lowering ability. As previously described, lower endogenous pH values generated a smaller percent soluble iron. Therefore, at the more neutral pH values (pH E), iron solubility was influenced predominantly by heat treatment and pH.

Addition of $FeSO_4$ to the boiled CSM produced much smaller increases in percent soluble iron at pH E than for any other exogenous iron source. This phenomena is explained simply by considering that the boiling treatment does little for improving the already soluble $FeSO_4$, while causing significant increases in iron solubility when either HRFe or FOP are added. Interestingly, boiling CSM in the presence of FOP produced the greatest enhancement of soluble iron at the endogenous pH. Since incubation at the endogenous range did not influence the chemical iron profiles at pH 2, the decrease in soluble iron at this acidic pH must have been due to boiling alone.

Solubility effects resulting from iron fortification. The inappropriateness of $FeSO_4$ as a suitable iron fortificant for the CSM food blend has been outlined in detail elsewhere in this paper. Of the remaining iron sources, FOP offered the greatest potential for enhancement of iron bioavailability as determined by both percent soluble iron and capacity to promote iron solubility when supplemented in combination with organic acids. When added to the boiled CSM system, FOP was 99% (range = 83 - 137%) and 96% (range = 80 - 123%) as soluble as HRFe, and 96% (range = 80 - 133%) and 84% (range = 65 - 113%) as soluble as ERFe, at pH E and 2, respectively. In addition, the FOP-fortified boiled CSM was the only system where organic acids (MAL and CYS), other than ASC, caused significant improvements in soluble iron at pH 2 (Table 3). For these reasons, FOP was considered the exogenous iron source of choice for further study in the boiled CSM food blend.

Effects of the simulated G.I. test on the selected iron-ligand combinations. Table 4 contains the calculated enhancing factors for the simulated G.I. incubation method. Addition of organic acids did not significantly alter the percent soluble iron at pH 6, though addition of CIT produced a slight improvement. However, small, but significant, increases were observed at pH 4 for CIT, MAL, and CYS, with larger increases caused by ASC. If solubility could be directly related to bioavailability, then supplementation of CSM with these ligands may offer hope for

Table 3—Average[a] percent soluble iron in the boiled CSM samples

Iron added[c]	pH	Control	Organic acid[b]				
			ASC	LAC	CIT	MAL	CYS
None	E[d]	50.3	31.6	42.6	20.6	19.9	28.4
	2	54.5	80.0**[e]	48.3	44.6	38.7	44.5
$FeSO_4$	E	53.7	22.8	36.4	19.2	12.0	19.5
	2	45.5	85.9**	34.3	25.2	23.4	31.3
HRFe	E	28.2	12.8	20.2	14.7	8.0	11.4
	2	34.1	54.8**	27.4	26.3	31.5	30.7
ERFe	E	28.2	11.8	19.7	16.5	9.7	11.7
	2	41.5	68.1**	30.2	33.3	32.0	33.6
FOP	E	26.3	10.6	17.7	13.2	8.4	15.6
	2	27.6	43.9**	22.1	24.4	36.3*	37.7*

[a] Average of duplicates.
[b] ASC = ascorbic acid; LAC = lactic acid; CIT = citric acid; MAL = malic acid; CYS = cysteine.
[c] None = endogenous iron; $FeSO_4$ = ferrous sulfate heptahydrate; HRFe = hydrogen reduced elemental iron; ERFe = electrolytic reduced elemental iron; FOP = ferric orthophosphate.
[d] E = endogenous pH.
[e] Values marked with an asterisk show significant increases over the control at either the 5% (*) or 1% (**) level.

promoting iron absorption in populations consuming this product.

Since it has been estimated that infants with borderline iron deficiency would absorb only between 1.7% and 2.8% of the iron contained in uncooked CSM (Bothwell et al., 1982), the potential for promoting iron absorption from this food blend by chemical and/or physical means is enormous. The present investigation has illustrated that specific combinations of organic acids, exogenous iron sources, pH, and heat treatments can enhance soluble iron in a CSM blend. However, further elucidation of these parameters is required before optimal conditions can be recommended.

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Table 4—A comparison of enhancing factors[a] at four pH values for boiled CSM supplemented with selected organic acids and ferric orthophosphate

Slurry pH	Organic acid[b]				
	ASC	LAC	CIT	MAL	CYS
E[c]	0.38	0.73	0.36	0.27	0.38
2	1.58**[d]	0.77	0.77	0.93	0.91
4	3.99**	1.22	1.36*	1.36*	1.67*
6	0.77	0.75	1.14	0.71	0.93

[a] Enhancing factor is the ratio of the percent soluble iron detected with organic acid present to the percent soluble iron without organic acid present.
[b] ASC = ascorbic acid; LAC = lactic acid; CIT = citric acid; MAL = malic acid. CYS = cysteine.
[c] E = endogenous pH.
[d] Values marked with an asterisk show significant increases over boiled samples without an organic acid at either the 5% (*) or 1% (**) level.

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Changes in Fatty Acid Composition of Papaya Lipids (*Carica papaya*) During Ripening

HARVEY T. CHAN JR. and MYLES H. TANIGUCHI

ABSTRACT

Lipids from papayas (*Carica papaya* L.) Solo cultivar at various stages of ripeness were extracted and fractionated into neutral lipids (NL), glycolipids (GL), and phospholipids (PL). The major fatty acids of the three lipid classes at all levels of ripeness were C16:0, C18:1, C18:2, C18:3. NL increased in unsaturation with maturity as indicated in the decrease of the various saturated/unsaturated fatty acid ratios. GL decreased in the ratio of C16:0/C16:1 due to an increase in C16:1 fatty acid with increasing fruit maturity. The remaining fatty acids in GL remained relatively unchanged. The phospholipid fraction increased in unsaturation with maturity due to increases in C16:1 and C18:3 fatty acids.

INTRODUCTION

THE PAPAYA (*Carica papaya* L.) native to tropical America is widely distributed throughout the tropics. It is consumed mainly as fresh fruit and considerable amounts are shipped for export. Refrigeration temperatures required for the transport and storage of fresh papayas range from 10°C to 13°C. Lower storage temperatures induce chilling injury, symptoms which are uneven ripening, production of off-flavors and odors, and extreme susceptibility to decay fungi. The fruit has been noted to become less susceptible to chill injury as it ripens (Couey, 1982).

The susceptibility of chilling-sensitive plants has been postulated to be related to the degree of unsaturation of the plants' lipid components particularly the phospholipid fraction (Lyons et al., 1979; Raison and Wright, 1983). Although changes in fatty acid composition in nonoleaginous fruits during ripening has been reported for bananas (Wade and Bishop, 1978; Wade et al., 1980), apples (Galliard, 1968), mangoes (Bandyopadhyay and Gholap, 1973; Kane et al., 1978), and grapes (Bauman et al., 1977), such studies have not been reported for papayas.

Information on the changes in fatty acid composition of papayas during maturation would provide basic information on the possible role of fatty acids in chilling sensitivity at various levels of maturity. Reported herein are the results of a study on the changes of fatty acid composition in the neutral, glyco-, and phospholipid fractions of papayas at various levels of ripeness.

MATERIALS & METHODS

Source and preparation of fruit

Twenty papayas were harvested at each of the maturity stages of mature green (MG), colorbreak (CB), 1/4- and 1/2-ripe. The fruit were washed, quartered, deseeded and frozen at -40°C. The frozen fruits were lyophilized in a freeze-dryer then pulverized to a fine powder with mortar and pestle. The powder was stored at -40°C. The fruit sampling was replicated 4 times.

Lipid extraction

Twenty grams of lyophilized papaya powder were blended with 250 ml of Folch's reagent (2:1, v/v $CHCl_3$:MeOH) for 5 min in an explosion-proof Waring Blendor and the homogenate filtered in vacuo through a layer of Celite (Manville Products Corp.) on Whatman No. 2 filter paper. The residue and filter pad were rcextracted by blending with 250 mL of Folch's reagent, filtering again through Whatman No. 2 followed by two rinses of 50 mL Folch's reagent with a final rinse of 30 mL $CHCl_3$. The filtrate was partitioned in a 1L separatory flask with 0.2 of the total filtrate volume being 0.9% aq. NaCl. The lower layer containing the lipophilic compounds was then concentrated to near dryness at reduced pressure in a Buchi rotary evaporator at 37°C.

Authors Chan and Taniguchi are with the U.S. Dept. of Agriculture, Agricultural Research Service, Tropical Fruit & Vegetable Research Laboratory, P.O. Box 4459, Hilo, HI 96720

Chromatographic separation of total lipids

The crude lipid extracts were chromatographed into neutral-(NL), glyco-(GL), and phospholipid (PL) fractions by a silicic acid column (11 mm i.d.). A loading ratio of 2g lipid extract/100g Silicar CC-7 (Mallinkrodt) was used. Before packing the Silicar was prepared by first washing with methanol, then with H_2O to remove fines. The Silicar was then activated overnight in an oven at 120°C, mixed with the first elution solvent ($CHCl_3$) and packed in the column as a gel. The NL was eluted with 12 mL $CHCl_3$/g Silicar, the GL with 48 mL acetone/g Silicar, and the PL with 12 mL CH_3OH/g Silicar at flow rates of 1, 0.5, and 1 mL/min, respectively (Higgins and Peng, 1976). These solvents were removed in a rotary evaporator under reduced pressure at 37°C.

Thin-layer chromatography

The chromatographic separation of the lipid fractions was monitored for completeness of separation by thin-layer chromatography (TLC). Precoated TLC plates (Brinkmann Polygram SIL N-HR) were spotted (1 μL) with the lipid fractions dissolved in 3 mL $CHCl_3$. For the separation of NL a triple development system of hexane:benzene (3:1, v/v), followed by petroleum ether:ethyl ether:acetic acid (90:10:4, v/v), and then petroleum ether:ethyl ether:acetic acid (90:20:1, v/v) was used (Kozukue and Kozukue, 1981). The polar lipids were developed with chloroform:methanol: water (65:24:4, v/v). PL were detected by spraying with 0.2% ninhydrin in n-butanol:10% aqueous acetic acid (95:5, v/v) or by spraying with 10% phosphomolybdic acid in ethanol (Sigma Chemical Co.) and heating in an oven at 110°C. Purple spots with the ninhydrin spray indicated presence of PL (Johnston, 1971). GL were detected as blue-gray spots when sprayed with 10% diphenylamine in ethanol:12N HCl:17N acetic acid (1:5:4, v/v) and heated for 5-10 min at 110°C. A Rhodamine B (0.05% in 95% ethanol) spray was used to detect all classes of lipids. The lipids appeared as fluorescent spots under ultraviolet light.

Methylation

Each lipid fraction was dissolved in 5 mL benzene. Half milliliter aliquots of the lipids were methylated with 0.5 mL BF_3-methanol (14%) in 2 mL micro-reaction vessels (Supelco). The vessels were capped tightly and heated at 100°C for 60 min (Morrison and Smith, 1964). The solution was then cooled for 30 min, mixed well with 0.5 mL water and 0.5 mL hexane and centrifuged at 1200 x *g* for 10 min. The upper hexane layer was pipetted into another centrifuge tube, washed three times with water, dried over anhydrous Na_2SO_4, and stored under N_2 in a glass vial at -40°C (Pelick and Mahadevan, 1975).

Gas-liquid chromatography (GLC)

A Hewlett-Packard HP-5830A Reporting Gas Chromatograph with flame ionization detectors was used. The carrier gas (nitrogen) flow rate was 21 mL/min and the hydrogen flow rate was 20 mL/min. The methylated fatty acids were tentatively identified by GLC on 2.75 m x 2.1 mm i.d. stainless steel columns packed with 10%

DEGS on Chromosorb W 60/80. The column was run isothermally at 190°C. The injector temperature was 220°C and detector, 250°C.

For the separation, identification and quantitation of the methyl esters of the fatty acids, a 25m x 0.25 mm i.d. Quadrex glass capillary column coated with Silar 10c (Applied Science) was used. Linear velocity of the carrier gas (He) was 21 cm/sec. The split ratio was 100:1. The column was run isothermally at 190°C. The detector and injector temperatures were 220°C and 250°C, respectively. The retention times for the methyl esters were compared to those of known methyl esters (Supelco, Inc. and Applied Science) on the two columns used in this study. Peak areas and retentions of the methyl esters were calculated and reported by the digital processor using methyl pentadecanoate as an internal standard.

Statistical analysis

Data were analyzed on a Hewlett-Packard computer, HP-86, using the standard Hewlett-Packard programs, "Basic Statistics and Data Manipulation Pac" and "Regression Analysis Pac." Analysis of variance (ANOVA) was used to determine significance between ratios of saturated/unsaturated fatty acids of various lipid classes at each level of fruit maturity. The statistical methods used in these softwares can be found in a standard text (Graybill, 1976). Simple linear correlation coefficients, r, were computed for the correlation of changes in each fatty acid with changes in fruit maturity. The significance of correlation was determined as described by Little and Hills (1978). For the correlations the various maturities were assigned ranking values of MG=1, CB=2, 1/4-ripe=3, and 1/2-ripe=4.

RESULTS & DISCUSSION

Changes in papaya lipids with maturity

The lipids of papaya at each level of maturity were not significantly different from each other (Table 1). Differences in NL, GL, and PL between each level of maturity were not significantly different. Hence, papayas do not undergo any significant changes in lipid composition with maturity.

Changes in neutral lipid fatty acid composition with maturity

The changes in NL fatty acid composition with maturity are shown in Fig. 1. The dominant fatty acid of the NL at all levels of maturity was palmitic (about 30%) which remained relatively unchanged. Only linoleic acid showed a significant ($p \leq 0.05$) decrease which correlated ($r=-0.88$) with maturity. The cumulative conspicuous change occurring in NL during maturation is the relative gain in unsaturated fatty acids as indicated in the decrease in the various ratios of saturated to unsaturated fatty acids (Table 2). The ratio of C16:0/C16:1 showed the largest decrease which can be attributed to the large increase in palmitoleic acid with increasing fruit maturity.

Changes in glycolipid fatty acid composition with maturity

The dominant fatty acids in the GL were palmitic (about 21-25%), oleic (about 17%), linoleic (about 20-27%) and linolenic (about 18-27%) acids which remained relatively unchanged throughout the various maturities (Fig. 1).

Table 1—Changes in papaya lipid composition with maturity[a]

Fruit maturity	Total lipids g/100g dry wt.	% Neutral lipid	% Glycolipid	% Phospholipid
Mature green	2.01a	28.9a	45.5a	25.6a
Colorbreak	2.54a	32.6a	53.9a	13.5a
1/4-ripe	1.77a	34.1a	44.1a	21.8a
1/2-ripe	2.07a	29.1a	41.8a	34.0a

[a] Means separated in columns by lipid type, tested by ANOVA. Means followed by the same letter are not significantly different at the 5% level.

Simple correlation coefficients between the various fatty acids and maturity showed no significant changes during fruit maturation. However, the ratio of C16:0/C16:1 showed a slight but significant ($p=<0.05$)(Table 2) decrease with increasing fruit maturity due to the rise in palmitoleic acid with increasing fruit ripeness (Fig. 1).

Changes in phospholipid fatty acid composition with maturity

The dominant fatty acids in the PL fraction were palmitic (about 25-26%), oleic (about 21-25%) and linoleic (about 21-36%) (Fig. 1). Fatty acids showing positive significant ($p \leq 0.05$) correlations with maturity were palmitoleic ($r=0.88$) and linolenic acids ($r=0.92$) which reflects the increase in both palmitoleic and linolenic acids with increasing fruit ripeness as shown in Fig. 1. Linoleic acid was significantly ($p \leq 0.05$) negatively correlated ($r=-0.89$)

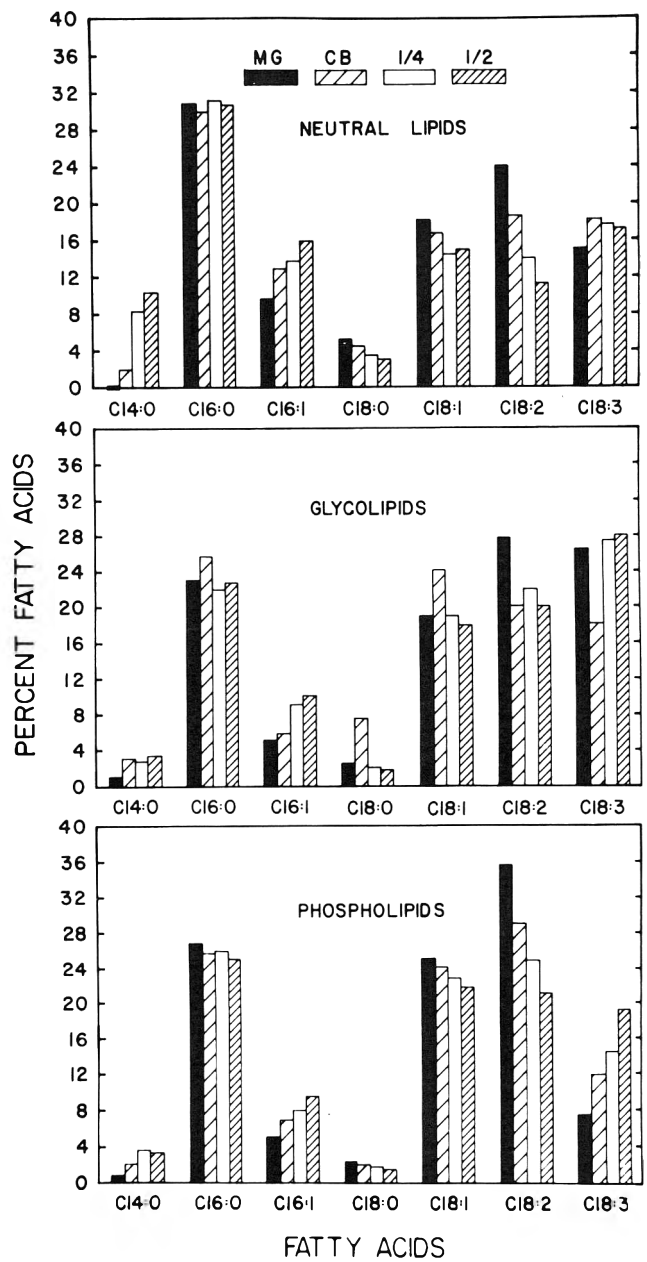


Fig. 1—Fatty acid distribution in papaya lipids at various maturities. Maturities are designated as mature green (MG), color breaking (CB), quarter-ripe (1/4), half-ripe (1/2).

Table 2—Ratio of saturated to unsaturated fatty acids in papaya lipid fractions at various levels of fruit maturity

Lipid fraction and ratio type	Maturity				F ratio
	MG[a]	CB[a]	1/4[a]	1/2[a]	
NL[b] C18:0/(C18:1+C18:2+C18:3)	0.085	0.063	0.061	0.061	4.23*
NL C18:0/C18:1	0.280	0.203	0.203	0.186	8.00**
NL C16:0/C16:1	3.255	2.507	2.355	1.931	38.31**
GL[b] C16:0/C16:1	4.710	6.164	2.540	2.320	6.18**
PL[b] C16:0/C16:1	5.400	3.713	3.227	2.647	25.79**
PL C18:0/C18:2	0.063	0.073	0.077	0.093	18.60**
PL C18:0/C18:3	0.300	0.177	0.140	0.103	30.60**

[a] MG = mature green, CB = colorbreaking, 1/4 = 1/4 ripe, 1/2 = 1/2 ripe.
[b] NL, GL, and PL denote neutral lipid, glycolipid, and phospholipid, respectively.
* Significantly different ($p \leq 0.05$).
** Significantly different ($p \leq 0.01$).

with fruit maturity reflecting the decrease in linoleic acid with increasing fruit maturity as shown in Fig. 1. Simple correlation coefficients between the fatty acid palmitoleic and both linolenic (r=0.87) and linoleic acids (r= -0.98) indicated a concomitant increase in both palmitoleic and linolenic acids along with a concomitant decrease in linoleic acid with fruit maturity (Fig. 1).

Perhaps the most conspicuous indicator of lipid changes occurring within the phospholipid fraction was the change in the various ratios of saturated to unsaturated fatty acids (Table 2). The ratios of C16:0/C16:1 decreased significantly ($p \leq 0.05$) with increase in fruit maturity reflecting the slight loss in palmitic acid and large increase in palmitoleic acid (Fig. 1). The ratio C18:0/C18:3 also decreased significantly ($p \leq 0.05$) with maturity reflecting a slight loss in oleic and a large increase in linolenic (Fig. 1). In contrast the loss of linoleic acid with increasing fruit maturity was reflected in an increase in the C18:0/C18:2) ratio. The decrease in linoleic acid with increases in palmitoleic and linolenic acids has also been observed in total lipid extracts of ripening mangoes by Bandyopadyay and Gholap (1973) who also reported an increase in triglycerides with ripening.

Another change in fatty acid composition occurring in mangoes reported by Kane et al. (1978) that was found with papayas in this study was the increase in the ratio of palmitoleic to palmitic acids with increase in ripening. As shown in Fig. 1 for papaya lipids, there was an increase with ripening in palmitoleic acids in all the lipid fractions. The changes occurring in banana lipids during ripening are also similar to those occurring in papayas. Wade et al., (1978) found the major composition changes of banana membrane lipids occurring in the PL fraction which increased in linolenic acid whilst linoleic acid decreased as the fruit ripened. The net result was a 10% increase in total unsaturated acids and a large increase in the double bond index.

Further evidence that the fruits' lipid composition may determine its susceptibility to chill injury was recently demonstrated by Kimura et al. (1982) who found the mitochondrial lipid of unsaturated fatty acids higher in a chilling resistant variety of apples than in a chilling sensitive variety. They postulated that the differences in unsaturated fatty acid content could cause the difference in resistance to chilling injury by playing a role in lowering the phase transition temperature.

Raison and Wright (1983) have recently demonstrated that relatively small amounts (1–2%) of high melting point PL can effectively influence thermal phase transition temperatures of the polar lipids of plant membranes. They also showed that high melting point PL containing disaturated lipids such as dipalmitoylphosphatidyl glycerol are present in membrane lipids of chilling sensitive plants and postulated the absence or low proportions in those of the chilling insensitive plants. Hence, the increase in the relative amounts of the lower melting point unsaturated fatty acids, palmitoleic and linoleic acids, in papaya PL may explain the increase in resistance to chill injury with concomitant ripening in papayas.

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Pyrolysis-GC Analysis as an Identification Method of Fats and Oils

JAMIL M. A. NAZER, CLYDE T. YOUNG, and F. G. GIESBRECHT

ABSTRACT

Pyrolysis at 700°C for 10 sec under N_2 gas and 360 mm Hg partial pressure of 18 fats and oils (including oils of the same type but from different sources or varieties), and subsequent GC analysis of the pyrolyzates, revealed differences in both the number and quantities of compounds produced. Statistical analysis of the data showed that all of these fats and oils were distinguishable from one another; moreover, varieties of the same lipid could also be differentiated. This technique requires only microgram quantities of the oil or fat to be analyzed, and the direct pyrolysis and GC analysis gives a "fingerprint" program that differentiates the lipid from others.

INTRODUCTION

FATS AND OILS which are commonly used in cooking, frying, or as an additive ingredient in most processed foods, have been extensively studied. This is partly due to the concern that modern-day diseases such as cardiovascular ailments which account for about 50% of U.S. mortality (U.S. Government, 1969) are related to lipid intake. In addition, there is concern with the determination of purity and quality of fats and oils by the processing industry, government, and other institutions.

The identification and characterization of authentic pure oils and fats as well as the detection of adulteration (Kaufmann and Wessels, 1967), has been heavily dependent upon identifying the presence of fatty acids specific to either the authentic or adultrant oil (Kuzdale and Paquot, 1962; Sanchez and Colmenarez, 1974). Similarly, the identification of sterols has been found helpful in identifying the sources of some oils and fats (Eisner et al., 1962; Recort and Beerthuis, 1963). For example, the use of methylated or silylated esters of fatty acids or sterols was used to distinguish animal fat from vegetable oils (Hubbard et al., 1977). However, the extent of such a classification has always been limited to the general source of the lipid material (animal or vegetable), and occasionally a further subclassification (such as soybean oil from all the other possible oils) can be deduced (Slover et al., 1980). This difficulty is due to similarity in composition for most of these natural materials as well as "induced" similarlity caused by interesterification, partial hydrogenation, or simple mixing of one variety by less expensive varieties.

Pyrolysis (thermal degradation) on the other hand, combined with infrared (IR), gas liquid chromatography (GLC), or mass spectroscopy (MS), has long been used in the polymer industry to identify new products and detect contamination and also study structures and stability (Perry, 1967). For example, Burns et al. (1976) used pyrolysis-GLC to differentiate 11 *Penicillia* and two other related species. Similarly, 16 mushroom species were studied through pyrolysis-GLC (Benoit-Guyod et al., 1980). In these studies, the pyrograms were produced and used to distinguish one material from the others. Pure triglycerides were pyrolyzed under various conditions (190 - 300°C), and the fragments thus produced were then identified (Crossley et al., 1962; Lien and Nawar, 1973).

The objective of this study was to differentiate fats and oils using a pyrolysis-GC technique.

Authors Nazer, Young, and Giesbrecht are affiliated with the Dept. of Food Science, North Carolina State Univ., Raleigh, NC 27695-7624.

MATERIALS & METHODS

THE LIPIDS analyzed in this study (Table 1) are commercial types, except for peanut oil. The peanut oil was extracted with diethyl ether from peanuts which were obtained from the Peanut Belt Research Station at Lewiston, NC. The 18 samples of fats and oils were stored at 4°C until analyzed.

Samples (10 μL) were pyrolyzed (Barnes PY-2 pyrolyzer, Barnes Eng. Co., Stamford, CT) at 700°C for 10 sec under nitrogen at 360 mm Hg pressure. The pyrolyzate (1.75 mL) was injected with a gas-tight syringe into a gas chromatograph (F&M Model 810) equipped with a flame ionization detector and a 1.8 m x 2 mm i.d. column packed with 80 - 100 mesh Porapak P. Operating conditions were: He 20 mL/min, H_2 46 mL/min, and air 440 mL/min. The chromatograph was programmed from 40°C to 190°C/min, then held for 5 min. The injector temperature was 155°C and the detector 270°C (Nazer and Young, 1984). Samples were analyzed in duplicate except for beef fat (#1), corn oil (#4), and olive oil (#8) which were analyzed in triplicate. The pyrolyzates were compared via retention times and quantitated by a computer data system. The data were analyzed statistically by using the General Linear Models Procedure (Barr et al., 1979), and the Waller-Duncan (Waller and Duncan, 1969) test. The lipid samples were saponified and methylated according to the method described by Metcalfe et al. (1966). The methyl esters were separated on a 1.8 m x 2 mm i.d. column packed with 10% Altech CS-10 on 110/120 mesh Chrom W-AW. The chromatograph was held at 160°C for 5 min and then programmed to 250°C at 10°C/min, then held for 2 min. The injector temperature was 170°C and the detector 270°C.

Table 1—Fats and oils analyzed

Type	Trade Names[a]	Source[b] and prod. date
A. Beef fat	From shoulder trimmings[a]	Raleigh, NC,[b] Dec. 80
B. Coconut oil	Palm tree	Singapore, Nov. 78
C. Ghee (butter fat)	Cow brand	Australia, Nov. 78
D. Corn oil	Gloria	Cyprus, Aug. 78
E. Corn oil	Vicky	Singapore, Aug. 78
F. Lard (part. hydr'd.)	Armour	Raleigh, NC[b] (unknown)
G. Linseed oil	Golden Harvest	Raleigh, NC,[b] Dec. 80
H. Olive oil	Sasso	Italy, Dec. 78
I. Olive oil	Sensat	Spain, Oct. 78
J. Palm oil	Bintuljazirah	Malaysia, Dec. 78
K. Peanut oil	Florunner	Lewiston, NC, Sept. 75
L. Peanut oil	Argentine	Lewiston, NC, Sept. 75
M. Peanut oil	Tennessee Red	Lewiston, NC, Sept. 75
N. Safflower oil	Hollywood	Raleigh, NC,[b] Jan. 79
O. Sesame oil	Kadoya	Japan (unknown)
P. Sesame oil	From bulk[a]	S. Korea, 78
Q. Soybean oil	Golden Harvest	Pennsylvania, 78
R. Sunflower oil	Sunlite	Raleigh, NC[b] (unknown)

[a] Discriptive terms are given where trade names are not available.
[b] Place of purchase is given when source is unknown.

RESULTS & DISCUSSION

TO COMPARE and understand the patterns of pyrolysis of the various fats and oils studied, fatty acid compositions were determined, and the means are listed in Table 2. These means are in general agreement with published data (Durkee, 1970), when one takes location and environmental differences into consideration.

Upon pyrolysis and subsequent GC analysis, a characteristic "fingerprint" pyrogram was produced for each fat or oil. Although many peaks appear identical (indicating they may consist of the same fragments based upon their retention times), the relative amounts and the ratios between peaks differed, both between the types of fats or oils and for each variety of the same type. To quantitate these differences, 20 peaks were each assigned an identifying number based on the mean retention time. Although peak heights could be used in comparisons, peak areas were found more reproducible. Table 3 lists the mean peak areas for the 18 lipids pyrolyzed, with mean squares, and significant differences between lipids and between runs. Greatly significant differences in lipid compositions were found for all of the lipids. For the 18 lipids analyzed, there are 21 possible factors for differentiation. Overall, the variability among replicate runs for all of the lipids was highly significant ($P < 0.01$) for peaks 6 and 12 and the total volatiles. Variability was significant ($P < 0.05$) for peaks 2, 4, 9, and 13 but was statistically not significant for the other 14 peaks. Reproducibility and the ability to differentiate one oil from another would probably be greatly improved if an in-line pyrolyzer was used since most of the error was probably due to the difficulty of transferring an aliquot from the pyrolysis chamber to the GLC.

–Text continued on page 1098

Table 2–Percent fatty acid compositions of the fats and oils used in the pyrolysis[a]

	Butyric	Caproic	Caprylic	Capric	Lauric	Myristic	Myristoleic	Pentadecanoic	Palmitic	Palmitoleic	Margaric	Stearic	Oleic	Linoleic	Linolenic	Arachidic	11-Eicosenic	Arachidonic	Behenic	Lignoceric
	4:0	6:0	8:0	10:0	12:0	14:0	14:1	15:0	16:0	16:1	17:0	18:0	18:1	18:2	18:3	20:0	20:1	20:4	22:0	24:0
A. Beef fat						5.3		2.5	30.0	5.2		10.5	43.6	2.3		0.7				
B. Coconut oil		0.4	6.6	5.5	48.3	17.4			8.7			2.4	7.5	3.1						
C. Corn oil									12.7			2.7	25.7	51.9	6.9					
D. Corn oil									19.0			2.4	32.6	40.6	4.4					
E. Ghee (butterfat)	2.3	1.9	0.6	1.8	2.4	9.8	1.3	1.5	25.9	2.8	1.4	13.1	28.6	3.6	2.8			0.1		
F. Lard						1.1			24.9	2.2		15.2	43.3	10.5		0.4	1.5	0.8		
G. Linseed oil									6.5			2.4	23.4	23.2	44.4					
H. Olive oil									17.5	1.0		1.7	67.1	10.8	0.8	1.0				
I. Olive oil									10.6	0.5		2.4	73.0	10.8	0.8	0.9				
J. Palm oil					0.5	1.3			43.6			3.5	38.6	12.0	0.3	0.2				
K. Peanut oil									11.1			1.8	51.9	29.4		1.3	1.7		1.6	1.0
L. Peanut oil									13.7			2.5	44.4	33.7		1.8	1.5		1.7	0.7
M. Peanut oil									12.8			2.5	44.2	34.6		1.7	1.6		1.9	0.8
N. Safflower oil									5.9			1.7	11.1	79.8	0.6	0.4			0.2	0.2
O. Sesame oil									10.8			4.3	36.7	44.7	1.3	2.3				
P. Sesame oil									12.2			3.0	36.1	46.0	1.6	1.5				
Q. Soybean oil									12.6			2.8	22.3	53.6	8.5	0.2				
R. Sunflower oil									6.8			3.8	17.8	70.9	0.5	0.2				

[a] Values reported here are the means of duplicate runs on the GC. No special effort was made to separate and evaluate some of the trace (0.1%) fatty acids if not apparent in the chromatograms produced under the prescribed conditions.

Table 3–Peak areas x 10 of pyrolysis of fats and oils[a]

Sample	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20	Total
	Retention time, in min																				Total volatiles
	(0.30)	(0.80)	(0.90)	(3.1)	(7.0)	(8.0)	(11.0)	(12.1)	(12.8)	(15.0)	(16.5)	(17.5)	(18.0)	(19.1)	(19.6)	(20.7)	(22.0)	(23.0)	(24.0)	(25.2)	
A. Beef	294	1307	246	810	567	358	1916	0	176	877	0	75	658	33	0	178	0	80	0	30	8451
B. Coconut oil	254	1195	192	622	404	138	992	0	47	536	0	18	240	0	0	148	0	57	0	38	4881
C. Corn oil	297	1514	323	916	1419	262	2492	86	438	864	58	191	432	51	35	322	77	63	12	0	9921
D. Corn oil	300	1480	300	894	1079	312	1952	73	322	782	52	169	477	43	22	244	54	53	11	0	8591
E. Ghee	206	999	217	625	422	234	1325	0	130	666	10	69	381	23	0	182	0	78	0	41	5608
F. Lard	242	1170	210	668	513	310	1504	0	158	682	0	68	411	0	0	146	0	56	0	0	6138
G. Linseed oil	274	1492	401	1042	970	780	2174	548	1002	872	185	394	362	68	162	299	49	148	0	0	11389
H. Olive oil	292	1400	264	895	728	478	1857	201	246	992	23	110	792	24	0	226	20	62	0	0	8599
I. Olive oil	237	1358	275	928	791	467	2111	73	240	1102	57	118	923	66	0	270	17	93	24	0	9126
J. Palm oil	283	1259	258	846	719	317	2031	51	165	992	0	77	710	28	0	241	0	98	0	29	8091
K. Peanut oil	194	1147	242	742	751	385	2134	20	220	835	13	118	629	48	0	217	35	70	29	0	7829
L. Peanut oil	270	1496	267	879	1071	265	2129	0	283	862	22	142	580	27	0	222	42	63	32	0	8642
M. Peanut oil	235	1482	284	892	899	470	2243	0	275	901	50	154	664	56	19	248	48	82	37	0	9009
N. Safflower oil	255	1560	322	939	1532	259	2223	31	387	756	62	217	445	72	33	344	103	38	28	0	9558
O. Sesame oil	200	1228	255	771	864	450	1996	68	301	757	36	159	555	57	25	272	71	71	32	0	8158
P. Sesame oil	220	1349	273	825	904	474	1901	55	298	780	55	160	539	53	27	260	65	53	49	0	7948
Q. Soybean oil	249	1510	336	965	1374	297	2340	107	475	860	86	243	564	77	31	371	103	99	36	0	10160
R. Sunflower oil	225	1418	319	941	1605	210	2543	61	364	865	72	215	615	83	29	420	132	68	50	0	10263
	Mean squares of the above analysis x 10^3																				
Lipids (df = 17)	4.2**	51*	5.3**	29.9**	279**	43.6**	306.8**	34.2**	85.3**	36**	4.1**	15**	59.5**	1.2**	2.0**	11.4**	3.5**	1.2**	0.7**	0.5**	5423**
Duplicate (df = 1)	2.9	106.5*	0.86	15.6*	35.2	11.3**	32.3	0.17	3.9*	4.8	0.08	1.7**	12.0*	0.03	0.01	0.72	0.04	0.02	0.05	0.02	2959**
	(Mean area, minimum and maximum) x 10 of peaks for all 18 lipids above																				
Mean area	257	1354	277	848	923	359	1992	76	307	832	43	150	554	45	21	256	45	74	19	8	8404
Minimum	194	999	192	622	404	138	992	0	47	536	0	18	240	0	0	146	0	38	0	0	4881
Maximum	374	1560	401	1042	1065	780	2543	548	1002	1102	185	394	923	83	162	420	132	148	50	41	11388

[a] These are means of two values for all sample except for beef fat, corn oil (#D) and olive oil (#H) which are for three values. Each number was rounded off.

*,**: Nonsignificant and significant at 5 and 1% levels of probability, respectively.

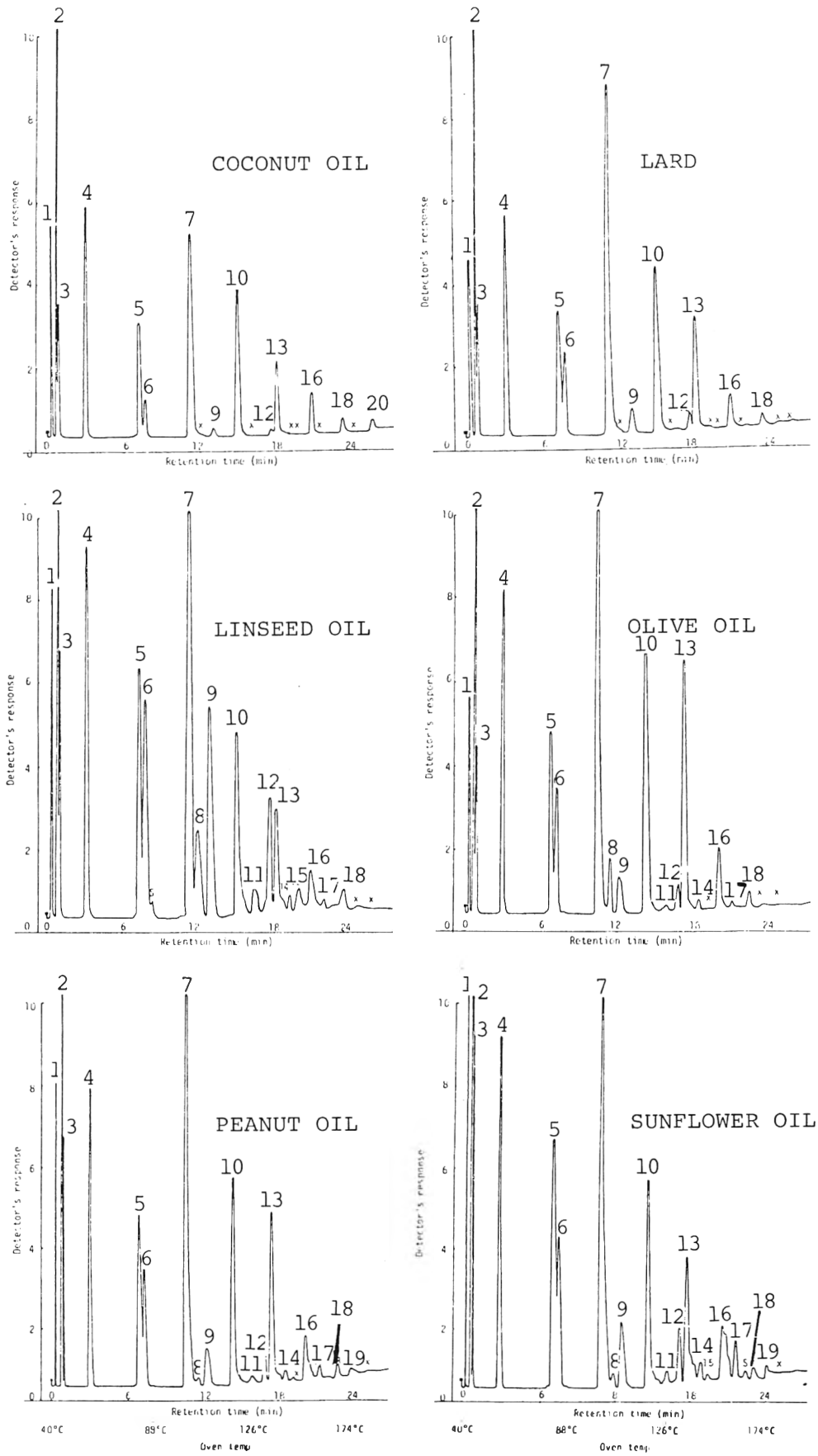


Fig. 1—Pyrogram of coconut oil, lard, linseed oil, olive oil (Spanish), peanut oil (Florunner), and sunflower oil (x indicates a position for a missing peak that appears in other pyrograms and is used for comparison of the 20 peaks; S is a special peak appearing in this pyrogram and is not used for comparison.

Fig. 1 shows six pyrograms of coconut oil, lard, linseed oil, olive oil (Spanish variety), peanut oil (Florunner variety), and sunflower oil to illustrate the contrasts among various lipids. Most of the peaks were common to all the lipids but in varying concentrations. This similarity can be expected since these natural lipids are relatively pure materials. Many of the fatty acids that make up the triglycerides generally have the same structures, differing mainly in the degree of unsaturation and chain length.

Table 4 summarizes the Waller-Duncan multiple comparison on the means of the peak areas (Table 3) for all 18 lipids. For example, using peak number 9 for comparison, the lipids, beef fat, ghee, lard, and palm oil were not significantly different in their conent of that component, therefore, this peak can be used to distinguish any one of this group of four lipids from the remaining lipids, but cannot be used to distinguish the four from one another. However, the individual lipids in this group can be distinguished from one another using other peaks for comparison (see peaks, 3, 4, 5, 7, 8, 10, 11, 13, 14, 16, and 18). Similarily, beef fat can be best distinguished from lard using peak 13; peaks 7, 10, and 14 also differ.

Discriminant analysis (Fisher 1936, 1938; Rao, 1952) was used to compare more readily the pyrograms (Gnandesikan, 1977). A three dimensional scatter plot (Fig. 2) was constructed using three discriminant functions (Gnandesikan, 1977) or canonical variates (Rao, 1952) as coordinates. The original variables used in this study are peak areas from the pyrograms. Each fat or oil (A to R, see Table 2) assumed a specific position on this 3-D diagram. Linseed oil (G), which had an exceptionally high amount of C18:3 (44%), was widely separated from the remainder of the samples and is located on the left of the diagram. In contrast, safflower (N) and sunflower (R) oils had high C18:2 content (80% and 71%, respectively) with little C18:3, so they were located up on the right of the diagram. Corn (C) and soybean (Q) oils, having about 52% and 54% of C18:2, and 6.9% and 8.5% of C18:3, respectively, were located closer to the middle of the diagram. Coconut oil (B), ghee (E), and lard (F), which had 3.1%, 3.6%, and 10.5% of C18:2, respectively, were located in the lower left of the diagram. It should be noted that Fig. 2 was created from the reduced un-normalized peak areas of pyrograms and the results cannot be completely correlated with the percent fatty acids data in Table 2.

Stepwise discriminant analysis (Barr et al., 1979) was used to examine the relationships between the dependent fatty acid variable and the independent variables consisting of pyrolysis peak areas (Table 5). Both C16:0 and C18:0 (two of the four more common saturated fatty acids) gave low R^2 values of 0.37 and 0.33, respectively. This indicates that many fragments in rather small quantities were associated with these fatty acids. On the other hand, both C18:1 and C18:2 (two of the more common unsatur-

*Table 4—Waller-Duncan test on the peak areas of all the 18 lipids**

	P_1	P_2	P_3	P_4	P_5	P_6	P_7	P_8	P_9	P_{10}	P_{11}	P_{12}	P_{13}	P_{14}	P_{15}	P_{16}	P_{17}	P_{18}	P_{19}	P_{20}	Total volatiles
Beef fat	bcdef	abcde	fg	efg	j	cd	g	i	g	def	i	g	bc	e	f	kl	i	de	e	c	efgh
Coconut oil	cdefg	cde	b	h	k	h	i	i	h	h	i	h	i	f	f	l	i	ef	e	ab	j
Corn oil (Cyp.)	bc	abc	b	ab	c	efg	ab	cd	b	cd	de	d	fgh	d	b	cd	c	de	de	d	bc
Corn oil (Sign.)	ab	a	b	ab	d	def	defg	de	c	de	de	d	defg	d	de	fgh	de	de	cde	d	cde
Ghee (butterfat)	gh	e	g	h	k	fg	h	i	g	g	h	g	h	e	f	k	i	cd	e	a	ij
Lard	cdefgh	cde	gh	gh	j	de	h	i	g	g	i	g	gh	e	f	l	i	ef	e	d	i
Linseed oil	a	abcd	a	a	f	a	cde	a	a	cd	a	a	h	b	a	de	ef	a	e	d	a
Olive oil (Spa.)	bed	abcd	cd	bcd	hi	b	efg	b	ef	b	g	f	a	e	f	hij	h	dc	e	d	ef
Olive oil (Ita.)	defgh	abcd	cd	bc	h	b	cdefg	def	ef	a	de	f	a	bc	f	efg	h	bc	bcd	d	de
Palm oil	bcde	abcde	de	cde	i	de	defg	fg	g	b	i	g	b	e	f	ghij	i	b	e	bc	fgh
Peanut oil (Flo.)	h	de	ef	fg	hi	c	cdef	hi	f	cde	h	f	bcd	d	f	J	g	de	bc	d	h
Peanut oil (Arg.)	bcdef	abc	cd	bcd	e	efg	cdefg	i	de	cd	g	e	cd	e	f	ij	fg	de	b	d	efg
Peanut oil (Tenn.)	efgh	abcd	c	bcd	g	b	bcd	i	de	c	e	e	bc	cd	e	fghi	ef	bcd	ab	d	de
Safflower oil	cdefg	ab	b	bc	b	efg	cd	gh	c	f	d	c	efgh	b	b	bc	b	f	bcd	d	bcd
Sesame oil (Jap.)	gh	bcde	de	efg	g	b	defg	def	d	f	f	e	cde	cd	cd	ef	cd	de	bc	d	fgh
Sesame oil (Kor.)	fgh	abcd	cd	def	g	b	fg	ef	d	ef	de	e	def	d	cd	fgh	cd	ef	a	d	gh
Soybean oil	cdefgh	abc	b	ab	c	def	abc	c	b	cd	b	a	cd	ab	bc	b	b	b	ab	d	b
Sunflower oil	efgh	abcd	b	abc	a	g	a	def	c	cd	c	c	bcd	a	bc	a	a	de	a	d	b

* Peaks with the same letter are not significantly different.

Table 5—Stepwise discriminant analysis correlation[a] between dependent fatty acid and pyrolysis peak area(s) (P)

C4:0[b] (1)[c]	C6 : 0 (3)	C8: 0 (3)	C10 : 0 (3)	C12 : 0 (5)	C14 : 0 (9)	C15 : 0 (3)
Total (1.00)[d] peak area	P9 (1.00)	P9 (1.00)	P9 (1.00)	P14 (0.94) P1,14 (0.98)	P9 (0.86) P9,20 (0.92)	P9 (1.00)
C16: 0 (35)	C18: 0 (35)		C20 : 0 (25)	C22 : 0 (7)	C24 : 0 (7)	
P17 (0.37) P11,18 (0.54) P1,11,18 (0.64) P3,11,18,19 (0.69)	P4 (0.33) P4,19 (0.40) P4,19,T (0.42) P4,8,9,19 (0.48)		P6 (0.31) P16,19 (0.52) P6,11,19 (0.67) P6,9,11,19 (0.70)	P17 (0.91) P10,17 (0.99)	P5 (0.93) P5,7 (0.96)	
C16: 1 (9)	C18: 1 (35)	C20 : 1 (7)	C18 : 2 (35)	C18 : 3 (23)		C20 : 4 (3)
P12 (0.60) P3,12 (0.90)	P13 (0.62) P13,16 (0.80) P13,17,20 (0.92)	P1 (0.80) P1,7 (0.98)	P17 (0.91) P17,18 (0.94) P5,17,18 (0.95)	P15 (0.90) P15,18 (0.96) P15,18,19 (0.980		P14 (0.98) P14,20 (1.00)

[a] Negative or positive correlation; [b]Dependent fatty acid; [c]Degrees of freedom; [d]Maximum R^2 improvement for dependent fatty acid.

ated fatty acids) gave high R^2 values indicating a strong relationship with peaks 13 and 17, respectively. It was not possible to establish many clear relationships using each fatty acid as the dependent variable.

Therefore, the stepwise discriminant analysis was used on the same data using the pyrolysis peak areas as the dependent variable and the fatty acids as the independent variables (Table 6). Peak 7, which made up about 22% of the total concentration (Table 3), had an R^2 of 0.65 (Table 6) with C14:0. The R^2 improved to 0.83 with the addition of C20:4 and C18:1 to the model. Peak 2, which made up about 16% of the total concentration of volatiles, gave a maximum R^2 of only 0.45 and had no strong correlation with any specific fatty acid. Peak 5, which made up about 11% of the total volatile concentration, gave a maximum R^2 of 0.84 with C18:2. This would suggest that most of the P5 fragment originated from C18:2. Data in Table 6 indicate that some of the fatty acids present (e.g. C14:0,

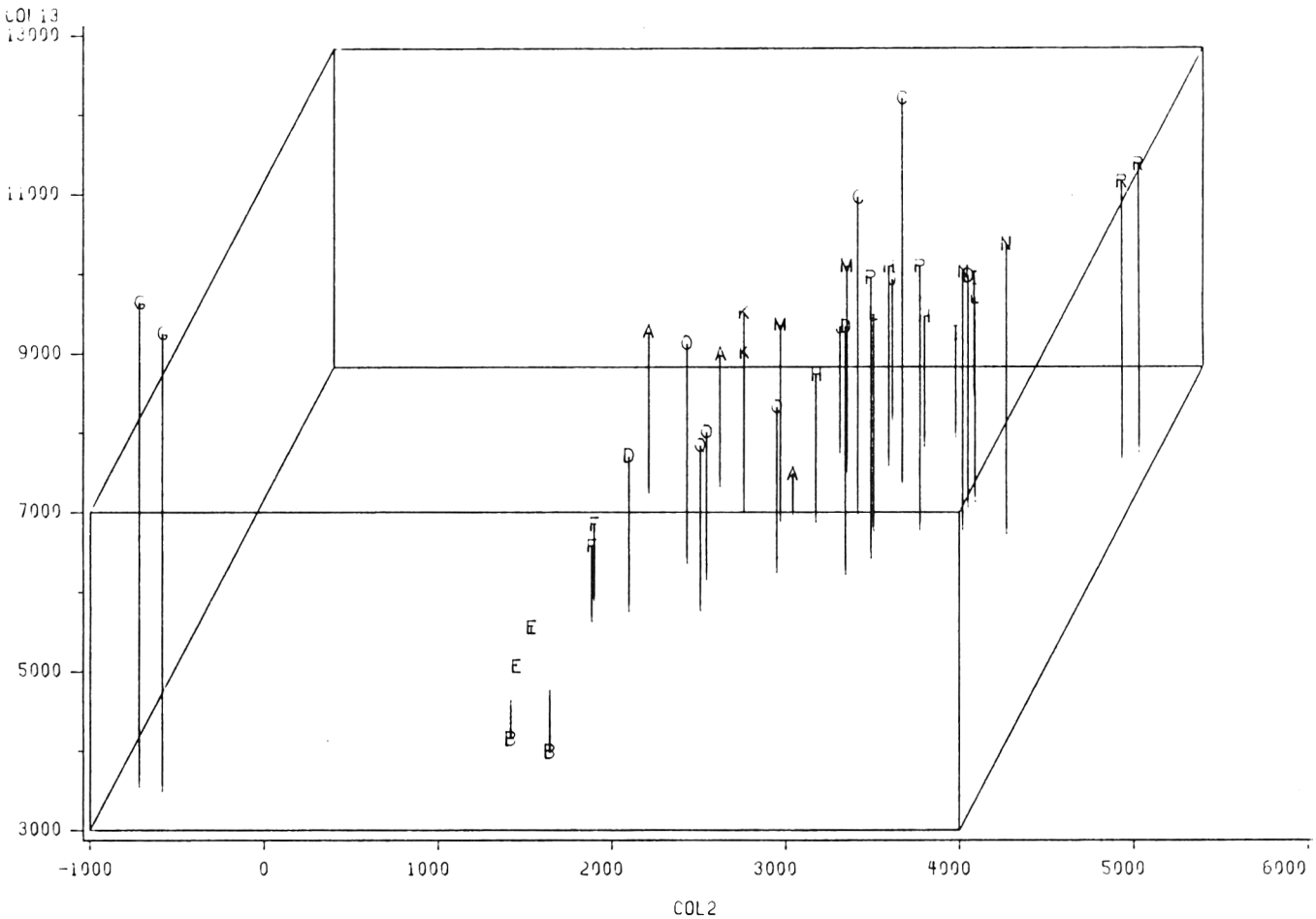


Fig. 2—Scatter diagram of reduced peak areas to 3 discriminant coordinates (Gnanadesikan, 1977), of 18 fats and oils (A to R, see Table 3). The numbers are arbitrary integration units of peak areas.

Table 6—Stepwise discriminant analysis correlation[a] between dependent pyrolysis peak areas (P) and fatty acids

P_1 (35)[b]	P_2	P_3	P_4	P_5	P_6
18:3[c] (0.36)[d] 18:3, 16:0 (0.42) 18:3, 16:0, 4:0 (0.51) 18:3, 16:0, 4:0, 20:0 (0.57) 18:3, 16:0, 4:0, 20:0, 18:0 (0.59)	weak correlation (Max. R-square = 0.45)	19:3 (0.45) 18:3, 18:2 (0.80) 18:3, 18:2, 18:1 (0.85)	14:0 (0.42) 14:0, 18:0 (0.58) 10:0, 18:0 (0.60) 10:0, 18:0, 20:0 (0.74)	18:2 (0.84) 18:0, 20:0 (0.88) 18:0, 20:0, 18:1 (0.92)	18:3 (0.43) 18:3, 18:1 (0.73) 18:3, 18:1, 20:0 (0.84)

P_7	P_8	P_9	P_{10}	P_{11}
14:0 (0.65) 14:0, 20:4 (0.78) 14:0, 20:4, 18:1 (0.83)	18:3 (0.86) 18:3, 18:1 (0.89) 18:3, 18:1, 20:1 (0.92)	18:3 (0.80) 18:3, 18:2 (0.95) 18:3, 18:2, 18:1 (0.98)	14:0 (0.42) 14:0, 18:1 (0.61) 14:0, 18:1, 20:4 (0.74)	18:3 (0.71) 18:3, 18:2 (0.89) 18:3, 18:0, 18:1 (0.92)

P_{12}	P_{13}	P_{14}	P_{15}	P_{16}
18:3 (0.59) 18:3, 18:2 (0.93) 18:3, 18:2, 18:1 (0.96)	18:1 (0.62) 18:1, 20:4 (0.72) 18:1, 20:4, 18:2 (0.76) 18:1, 20:4, 18:2, 16:0 (0.81)	18:2 (0.54) 18:2, 18:3 (0.63) 18:2, 18:3, 18:1 (0.72)	18:3 (0.90) 18:3, 18:2 (0.96)	18:2 (0.68) 18:2, 20:1 (0.75) 18:2, 20:1, 14:0 (0.80)

P_{17}	P_{18}	P_{19}	P_{20}	Total
18:2 (0.91) 18:2, 18:3 (0.92)	18:3 (0.57) 18:3, 18:1 (0.65) 18:3, 18:1, 16:0 (0.69)	18:2 (0.50) 18:2, 20:0 (0.68) 18:2, 20:0, 18:3 (0.69)	14:0 (0.60) 14:0, 16:0 (0.87) 14:0, 16:0, 20:4 (0.90) 14:0, 16:0, 20:4, 14:1 (0.92)	14:0 (0.50) 14:0, 20:4 (0.65) 14:0, 20:4, 18:3 (0.77) 14:0, 20:4, 18:3, 20:0 (0.82)

[a] Negative or positive correlation; [b] Degrees of freedom; [c] Symbol of fatty acid; [d] Maximum R^2 improvement for dependent pyrolysis peak areas.

C18:1, and C18:2, and mostly C18:3) dominated the fragmentation process (pyrolysis) and had the greatest influence on the pyrogram patterns produced.

The peaks produced upon pyrolysis of oils and fats were probably due to fragmentation of the triglyceride molecules. Based on findings of other workers (Crossley et al., 1962; Levy and Paul, 1967; Lien and Nawar, 1973; Perry, 1967), thermal decomposition in the absence of oxygen of such complex triglyceride mixtures as natural commercial oils and fats would result in the formation of olefinic groups; aldehydes, ketones, acids, esters, etc. Small molecular weight alkanes, alkenes, and CO_2 would also be produced with concentrations proportional to the pyrolytic severity. Analysis of the pyrolysis products (Tables 3, 5, and 6) indicates that oils with higher unsaturated fatty acid contents produced more fragments in higher ratios than fats or oils with higher levels of saturation. This agrees with the findings of studies using both model fatty acid methyl esters and model triglycerides (Crossley et al., 1962, Levy and Paul, 1976). It should be noted that the results obtained were from the analysis of the volatile portion of pyrolysis. The residual portion was possibly due to polymerization products (Sen gupta, 1967) and other compounds (Lien and Nawar, 1973) that results from interactions of the fragments produced.

The simplicity of this technique, its small sample requirement, speed, and wide variation in the number of peaks produced and peak concentrations among the pyrograms of the various oils and fats indicate that pyrolysis-GC is a potential technique for identification purposes.

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Bioavailability of Calcium in Heat-Processed Milk

C. E. WEEKS and R. L. KING

ABSTRACT

Bioavailability to rats of calcium in milk subjected to the DASI FreeFallingFilm system (UHT) of pasteurization was compared with the bioavailability of the mineral in raw, high temperature-short time pasteurized, and stored UHT milks. In two other studies, the calcium in the milks was equilibrated with ^{47}Ca. Intestinal absorption (both uncorrected and corrected for endogenous fecal excretion) and femoral deposition of calcium were measured. In another study, femur calcium was determined in rats deriving calcium and phosphorus exclusively from the milks. No significant effects were attributable to heat treatment.

INTRODUCTION

CALCIUM is an essential nutrient for humans. In 1973, dairy products, excluding butter, supplied 76% of the 970 mg calcium that is the average daily per capita intake in the U.S. No other food category provided more than 5% (Phillips and Briggs, 1975). The per capita consumption of fluid milk steadily declined from 1966 to 1980 (USDA, 1981). A projected continuation of this trend, with a resulting decrease in calcium intake, makes it imperative to ascertain that technological advances in milk processing are compatible with mineral availability.

The purpose of these studies was to compare the bioavailability of calcium in milk subjected to the DASI FreeFallingFilm system (hereafter referred to as UHT) with the bioavailability of the mineral in raw and high temperature-short time (HTST) pasteurized milks. The UHT system, which is based on steam infusion, has been described by Nahra (1979), Slater (1973), and Kosaric et al. (1981).

MATERIALS & METHODS

Experimental design

In Study 1, the arrangement of treatments was a 2^4 factorial design with calcium adaptation level (high and low), milk (HTST and UHT), volume consumed (5 and 10 mL), and sex as sources of variation. There were three animals per group. Intestinal absorption, corrected for endogenous fecal excretion, of a calcium radionuclide equilibrated with the test milks was measured.

Study 2 was a 2 × 4 × 2 factorial design with calcium adaptation level (high and low), milk (raw, HTST, UHT, and stored UHT), and sex as sources of variation. Five milliliters milk were given to each animal. The test animals, which had been used in Study 1 and maintained on the test diets, were redistributed into four weight-matched groups of three males and three females. Intestinal absorption, uncorrected for endogenous fecal excretion, and bone and intestinal yields of ingested calcium radionuclide equilibrated with the test milks were measured.

Study 3 was a 2 × 3 × 2 factorial design with period of milk consumption (6 and 8 wk), milk (raw, HTST, and UHT), and sex as sources of variation. There were five animals per group. Deposition of calcium in the femur was determined.

Author Weeks is affiliated with the Div. of Nutrition, Food & Drug Administration, Washington, DC 20204. Author King is affiliated with the Food Science Program, Univ. of Maryland, College Park, MD 20740.

Animals

Equal numbers of male and female weanling Sprague-Dawley rats were obtained from Harlan Sprague-Dawley, Inc., Indianapolis, IN. Upon receipt, the weights of the animals used in Studies 1 and 2 were 44–65g and in Study 3, 44–55g. The animals were identified with ear tags. After they were received, the rats used in Studies 1 and 2 were fed *ad libitum* ground Purina Certified Rodent Chow for 2 days and those used in Study 3 were fed this chow for 10 days before the experiments were begun.

Housing

The animals were housed in individual suspended stainless steel cages, except that immediately before administration of the radionuclide in Studies 1 and 2 they were transferred to metabolism cages. They remained in these cages until excreta were no longer radioactive or until the experiment was ended.

The stainless steel metabolism cages were designed to separate urine and feces. The cages were modified by the addition of a removable stainless steel plate cover over the feeding tunnel opening. Thus, feed was supplied inthe main chamber of the cage. Cages were randomly assigned location on 3- or 5-tier racks, which were placed in a 22–24°C room with 45–50% relative humidity and equal periods of light and dark.

Diets

High- and low-calcium adaptation diets were prepared by adding calcium lactate to powdered Low Calcium Test Diet (No. 170120, Teklad Test Diets, Madison, WI 53711). The diets contained 0.5 or 0.2% calcium, respectively, by analysis. Low-calcium low-phosphorus diet was prepared by mixing 240g egg white solids, 10g AIN-76 vitamin premix (American Institute of Nutrition, 1977), 1g choline chloride, 50g corn oil, 10g Teklad calcium-phosphorus-free mineral mix, and 689g dextrose.

Deionized distilled water was offered *ad libitum* except that during Study 3 water was withheld until the milk was consumed.

Each rat received its assigned diet during the entire experimental period: 11 wk for Studies 1 and 2 and 6 or 8 wk for Study 3. Food consumption was recorded. In Studies 1 and 2, the animals were fasted for 12 hr before and at least 3 hr after administration of the radionuclide. In Study 3, the rats were fed a weight of test milk 5 days per week based on solid diet consumption during the preceding 24 hr. The milk:diet ratio was constant for all animals on a given day. The milk was weighed into weighed inverted glass liquid diet containers. The containers were weighed after consumption and the amount consumed was determined by difference.

Test milks

Raw whole, HTST pasteurized and homogenized (71°C, 15 sec), and UHT homogenized (71°C, 15 sec; 141°C, 3 sec) milks were processed at the University of Maryland. These milks were always obtained from the same batch, processed 1 day before use, and held at 4°C until fed. Stored UHT milk was aseptically bottled and held for 58 days at 4°C. Milks fed in Study 3 were obtained weekly.

Diet and milk analyses

The mineral content of the diets and test milks was determined using a Jarrell-Ash Model 975 Plasma Atomcomp. Ionic calcium of the milks was determined using an Orion Model 901 Ionalyzer, an Orion Model 93-20 Calcium Selective Ion Electrode, and Calcium Standard Solution 92-20-06 (Orion Research Inc., Cambridge, MA 02138). Osmolality of the milks was determined on a Fiske OS osmometer (Fiske Associates, Burlington, MA 01803).

Radionuclide procedures

The calcium radionuclide was administered to the rats on days 24 and 66 of the experimental period for Studies 1 and 2, respectively.

Administration of calcium radionuclide. ^{47}Ca as $CaCl_2$ in HCl, specific activity about 1500 mCi per g Ca, was obtained from Union Carbide Corp. (Oak Ridge, TN 37530). This solution was designated stock ^{47}Ca solution. The activity at time of anticipated dosing was determined using the activity and time of determination supplied with the radionuclide and the standard decay table. ^{47}Ca was obtained for each study and was diluted as follows: Calcium radionuclide solutions 1 and 2 were prepared by diluting stock ^{47}Ca solution with deionized distilled water so that when 1 mL of the diluted solution was added to 100 mL milk, 5 and 10 mL aliquots, respectively, of the milk contained about 1 μCi ^{47}Ca.

In Study 1, radionuclide solutions 1 and 2 were added to HTST and UHT milks. In Study 2, radionuclide solution 1 was added to raw, HTST, UHT, and stored UHT milks. The milks were allowed to equilibrate at 4°C overnight and were brought to room temperature before feeding. Test doses of the milks were transferred to stainless steel feeding cups. Each cup was placed in a reproducibly identical position in the chamber of a Packard Model 446 Armac whole body scintillation detector [NaI(Tl) crystals] with channels set to read gamma radiation from ^{47}Ca and to exclude lower radiation of the immediate decay product, ^{47}Sc. The cups were counted for at least 0.5 min and the counts were displayed on a Packard Model 282A Dual Precision Ratemeter. Time of day was recorded. One cup had no radioactive sample and was used in making background determinations.

Counted cups were affixed to the feeding tunnel of the metabolism cages and the covering plates removed from all cages at the same time. The cover plates were replaced after 1 hr and the rats were denied access to the tunnel during their remaining time in the metabolism cages. The cups were removed from the cages and a volume of water equal to that of the milk which they had contained was used to rinse the walls of the cups. These cups and the cup used for background determinations were counted until at least 10,000 counts were obtained. Time of day was recorded. In Study 2, activity was determined at two energy levels, which constituted duplicate readings.

Samples. In Studies 1 and 2, feces were collected daily from the bottom portion of the cages beginning on the day of administration of the calcium radionuclide and continuing for 9 days. All feces from one animal were combined in a counting vial, slurried in hot water, and diluted to a constant volume with water.

In Studies 2 and 3, both femurs of each animal were excised immediately after death by decapitation and the bones were cleaned of adhering soft tissue. In Study 2, each bone was placed in a scintillation vial and dissolved overnight in concentrated nitric acid. In Study 3, one femur from each animal was air-dried and the calcium content was determined by neutron activation analysis. The epiphysis of the other femur were removed and the marrow was removed from the medullary area. All segments of each bone were defatted for 5 days in chloroform:methanol (1:3) and dried in a vacuum oven at 95-100°C. Dry fat-free weight was determined. The intestinal tract distal to the pylorus was removed from each rat in Study 2 and placed in a counting vial. Activity was determined on the intact tissue.

^{47}Ca activity standards. Five standards with concentrations of 1-10% or 10-100% of the administered dose, depending on the sample activity range, were prepared. The standards were diluted to the same volume as that of the samples and were placed in the scintillation chamber in reproducibly identical positions.

^{47}Ca activity calculations. *Percent of dose ingested.* The percent of dose ingested was calculated by using the formula:

$$\% \text{ of dose ingested} = (A_i - A_r)/A_i \times 100$$

where A_i = initial count of milk + ^{47}Ca radionuclide at time t_i corrected for background and A_r (residual activity of cup after consumption of contents) = $A_{fe}^{-0.693t/t_{1/2}}$, where A_f = observed residual count of cup after consumption of contents at time t_r corrected for background, $t_{1/2}$ = half life of ^{47}Ca = 108.72 min, and t = elapsed time between t_i and t_r in min.

Percent of dose absorbed corrected for endogenous fecal excretion (Study 1). Standards and samples were corrected for background counts and physical decay to counts at t = 0. The standards were expressed as percent of dose. Fecal sample activity as percent of dose absorbed at intervals from 100-200 hr after dosing was calculated by interpolation from the least squares regression line. The linear regression of percent of dose absorbed vs time was calculated for each animal. The x-intercept is absorption at t = 0 and the rate of excretion, as percent of ingested dose, is the slope of the line.

Fecal activity uncorrected for endogenous excretion, bone and intestinal activities (Study 2). The activities of the fecal, bone, and intestinal sample corrected for background were determined immediately after that of their respective standards, and no correction was made for physical decay. Equations for the least squares regression lines of the standards as percent of dose were calculated. The sample yields were determined by interpolation. The yields were expressed as percent of ingested dose; additionally the yield of the bone and intestinal samples was calculated as percent of absorbed dose.

Bone calcium (Study 3)

The National Bureau of Standards (NBS) 10 MW research reactor at Gaithersburg, MD was used as the neutron source. The detector signals from the Ge(Li) detector were fed into a computer-based Nuclear Data 4410 analyzer. Stability of the neutron flux of the reactor during the irradiation period was verified by determination of the calcium content of NBS Standard Reference Materials (Orchard Leaves 1571 and Bovine Liver 1577).

Excised, cleaned, and air-dried femurs were weighed and sealed in polyethylene tubing and identified by rat number and side. The samples and standards were sequentially placed in polyethylene capsules and irradiated for 15 sec in the reactor at a neutron flux of 6×10^{13} neutrons/cm^2/sec. After irradiation, the samples or standard were removed, resealed in plastic, allowed to decay for 1 min, and then analyzed. The femurs were placed on the detector in a reproducible position parallel to the detector window. Counting time was 5 min. The spectra obtained were entered directly into a computer at the facility. Results obtained as mg Ca per g air-dried bone were converted to mg Ca per g dried fat-free bone per g milk consumed.

Statistical analysis

Analysis of variance and regression were calculated using programs available from SAS Institute, (Cary, NC 27511) and using the facilities at the Food & Drug Administration's Parklawn Computer Center. When significant ($P < 0.05$) F tests were found, means were subjected to Duncan's multiple range test (Duncan, 1955).

RESULTS & DISCUSSION

THE CALCIUM CONTENT of the HTST and UHT milks fed in Study 1 was 116 and 113 mg per 100 mL and the ionized calcium was 13 and 12 mg per 100 mL, respectively.

Accumulated fecal activity at intervals from 100-200 hr after administration of the radionuclide-equilibrated milk was determined. The data for the low-calcium adapted groups are based on five readings during this time interval and for the high-calcium adapted groups, three readings (Fig. 1). The percentage of intestinal absorption at time 0 is the x-intercept of the regression line, while the percentage of ingested dose excreted per hour is the slope of that line. The slope was also expressed as a percentage of absorbed dose per hour using the determined absorption (Table 1). The average intestinal absorption of all low-calcium adapted animals fed HTST pasteurized milk was 100.26% and for animals fed UHT milk was 99.55%. The corresponding values for the high-calcium adapted animals consuming HTST milk was 87.51% and UHT milk 84.52%.

Two volume levels were fed in this study to ascertain if a saturable mechanism was involved. The volumes chosen were limited by the amount of milk that a fasted rat, unaccustomed to milk consumption, would freely consume within 1 hr. Since cows' milk is a fairly dilute source of calcium, saturation of the transport mechanism was not attained.

The intestinal absorption measured in this study was corrected for endogenous excretion of the mineral into the intestinal lumen. When the radionuclide was administered

orally, the fraction of the dose which was absorbed and then excreted into the lumen mixed with the unabsorbed radionuclide in the digesta and therefore could not be differentiated. After clearance of unabsorbed calcium from the intestinal tract, the gradual increase in activity of the accumulated feces represents the amount of the radionuclide moving into the lumen from the body pools. The time for an unabsorbed dose to clear the intestine is dependent on gut motility; however, 100 hr was sufficient for animals in this study. The rate of excretion of absorbed calcium is related to the physiological state of the animal. The mean percentage of the absorbed dose excreted per hour in the low-calcium adapted animals was 0.022, while in the high-calcium adapted animals it was 0.034. Since the calcium which is being measured is that which presumably did enter the body, its loss does not appear to be related to availability.

In Study 2, procedures for the equilibration of the radionuclide with the test milks and administration of the dose

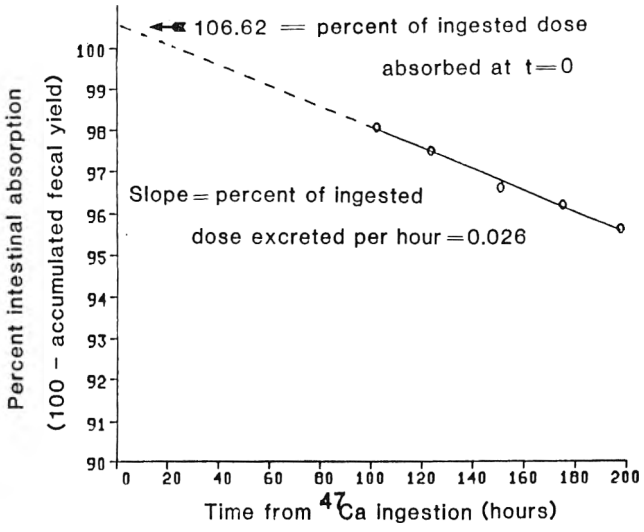


Fig. 1—Example of calculation of percent intestinal absorption corrected for endogenous fecal excretion.

were the same as for Study 1. The animals were those that had been used in Study 1 and they therefore were 42 days older. The time interval between these two studies was such that the radioactivity from the first test period had decayed to background levels. It was assumed that there were no latent effects of radioactivity on the animals that would alter the measured responses.

Inspection of the stored UHT milk, which had been held at 4°C for 58 days in clear glass bottles, showed no sediment or separation of constituents. The sensory attributes of the stored milk were equivalent to the other milks used in this study. The calcium content (mg per 100 mL) of the raw milk was 122; of HTST, 123; of UHT, 125, and of stored UHT, 124. Ionized calcium was not determined on the raw milk; ionized calcium (mg per 100 mL) of HTST, UHT, and stored UHT milks was 11, 10, and 9, respectively.

The percentage intestinal absorption was calculated on the basis of accumulated fecal excretion for 9 days after dosing. The true absorption was somewhat higher because the amount of dose absorbed before excretion into the intestinal lumen was not accounted for. The least squares means for those groups are shown in Table 2. The mean percentage for all animals fed the raw milk was 73.20; for HTST, 71.96; for UHT, 71.88; and for stored UHT, 71.12. Analysis of variance indicated no significant differences between these means.

The difference in the observed absorption between the high- and low-calcium adapted groups was highly significant ($P < 0.0001$). The mean intestinal absorption for all high-calcium adapted animals was 49.56% and for low-calcium adapted animals, 95.50%. The absorption intensity remained high during the intervening 42 days in the low-calcium group. The observed diminution of absorption in the high-calcium group is attributable to a diminished need for this mineral in calcium-sufficient animals as skeletal mineralization is attained. The response of all female animals was 73.38% and of males, 70.65%. These responses were not significant ($P > 0.0751$).

The least squares means of bone activity as percent of ingested dose measured postmortem in femurs excised from both sides of the animals and quantitated separately are shown in Table 2. This activity is an index of the amount of the calcium radionuclide absorbed by the intestine. The

Table 1—Absorption and rate of excretion of calcium radionuclide equilibrated with UHT or HTST milk and consumed by rats adapted to high- or low-calcium diet

Milk	Vol. milk ingested (mL)	Rat			% of ingested dose absorbed at t = 0	% of absorbed dose excreted/hr
		C.A.L.[a]	Sex	Weight (g)		
UHT	5	L	M	206 ± 8.7[b]	98.98 ± 1.86[b]	0.023 ± 0.005[b]
HTST	5	L	M	211 ± 12.2	100.03 ± 1.51	0.020 ± 0.005
UHT	10	L	M	196 ± 22.6	97.84 ± 2.79	0.019 ± 0.005
HTST	10	L	M	193 ± 19.3	100.83 ± 1.21	0.023 ± 0.005
UHT	5	L	F	158 ± 12.1	101.25 ± 1.66	0.028 ± 0.010
HTST	5	L	F	156 ± 8.1	100.82 ± 1.49	0.023 ± 0.007
UHT	10	L	F	156 ± 7.4	100.39 ± 0.95	0.021 ± 0.006
HTST	10	L	F	157 ± 7.5	99.34 ± 0.50	0.019 ± 0.003
UHT	5	H	M	212 ± 17.2	82.32 ± 9.76	0.038 ± 0.018
HTST	5	H	M	210 ± 19.0	92.01 ± 5.68	0.025 ± 0.009
UHT	10	H	M	214 ± 18.4	82.38 ± 5.39	0.036 ± 0.012
HTST	10	H	M	166 ± 5.7	88.84 ± 4.49	0.031 ± 0.018
UHT	5	H	F	162 ± 5.0	84.89 ± 4.51	0.032 ± 0.023
HTST	5	H	F	208 ± 14.7	83.75 ± 4.29	0.020 ± 0.005
UHT	10	H	F	157 ± 12.5	88.48 ± 3.27	0.027 ± 0.026
HTST	10	H	F	154 ± 12.3	85.44 ± 3.46	0.065 ± 0.028

[a] C.A.L. = calcium adaptation level. L = low; H = high.
[b] Mean of three observations ± one standard deviation.

Table 2—Least squares means for absorption and tissue yields of calcium radionuclide equilibrated with four milks and consumed by rats adapted to high- or low-calcium diet

				Tissue yield (%)					
				Ingested			Absorbed		
				Femur			Femur		
Milk	C.A.L.[a]	Sex	% Abs.[b]	Right	Left	Intestine	Right	Left	Intestine
Raw	H	M	49.13	2.04	1.97	0.57	4.09	4.05	1.18
		F	54.92	1.89	1.76	0.65	3.48	3.20	1.20
	L	M	95.88	3.79	3.89	0.19	3.95	4.04	0.20
		F	92.86	3.53	3.67	0.39	3.80	3.95	0.42
HTST	H	M	43.40	1.66	1.87	0.52	3.79	4.30	1.21
		F	53.59	1.80	1.81	0.66	3.36	3.38	1.22
	L	M	95.99	3.78	3.74	0.26	3.94	3.90	0.27
		F	94.88	3.84	3.88	0.31	4.05	4.09	0.33
UHT	H	M	52.20	1.77	1.82	0.41	3.57	3.69	0.89
		F	52.00	1.63	1.60	0.60	3.15	3.05	1.16
	L	M	95.69	3.90	3.92	0.16	4.08	4.10	0.17
		F	95.56	3.90	3.84	0.28	4.08	4.02	0.30
Stored UHT	H	M	46.05	2.12	2.08	0.58	4.61	4.53	1.26
		F	45.20	1.82	1.84	0.62	4.15	4.20	1.38
	L	M	95.23	3.76	3.73	0.23	3.95	3.92	0.24
		F	98.02	3.84	3.76	0.49	3.92	3.83	0.50

[a] C.A.L. = calcium adaptation level. H = high; L = low.
[b] % Abs. = % absorption.

Table 3—Response as mg Ca per g dry fat-free femur per g milk consumed by rats for 6 or 8 wk

Milk	Rat sex	Milk consumption period (wk)	Number of observations	Response mean ± 1 std. dev.
Raw	M	6	4	0.38 ± 0.22
	M	8	5	0.28 ± 0.03
	F	6	5	0.49 ± 0.06
	F	8	5	0.37 ± 0.06
HTST	M	6	5	0.39 ± 0.10
	M	8	5	0.30 ± 0.01
	F	6	5	0.45 ± 0.04
	F	8	4	0.32 ± 0.04
UHT	M	6	5	0.44 ± 0.07
	M	8	4	0.21 ± 0.12
	F	6	4	0.45 ± 0.15
	F	8	5	0.37 ± 0.04

least squares correlation (r) between femur activity and intestinal absorption was 0.957. As with data based on fecal excretion, the greatest difference in bone activity was observed between animals adapted to two calcium levels. The mean yield in the right femur for all low-calcium adapted animals was 3.79% of the ingested dose and for all high-calcium adapted animals 1.84% of the ingested dose. Corresponding values for the left femur were 3.80 and 1.85%. The differences between the two levels for both bones were highly significant ($P < 0.0001$).

The means of all animals (both adaptation levels) for both sides indicated that more of the ingested radionuclide present in the stored UHT equilibrated milk appeared in the bone. The activity yield in decreasing order was stored UHT, raw, HTST, and UHT for each side. These differences were not statistically significant.

Bone activity as a percentage of absorbed dose was calculated using the fecal excretion data (Table 2). When the data were adjusted for intestinal absorption, the difference between animals maintained on the two levels of dietary calcium was not statistically significant. Using the determined intestinal absorption to calculate the percentage of the absorbed radionuclide present in the bone, in a sense, factors out the intestinal influence. The predominant value of these data is the lack of significance between the low- and high-calcium adapted animals. This is consistent with the finding of Cohn et al. (1968) that the rate of calcium accretion into bone is not affected by dietary calcium level. Since the bone exchange mechanism is not different between groups, it appears that the femur yields as percent of ingested radionuclide are due to differences in intestinal absorption.

Some investigators add the residual activity in the intestinal tract to the feces in determining percentage of intestinal absorption (Carlsson, 1951). Activities of intestinal tracts removed from the animals used in this study were quantitated separately from the feces. The counts were low and therefore should be considered as estimates. The addition of this activity to that of the feces presupposes that it is derived from retained feces. In this study, the animals were killed 9 days after dose administration. It is assumed that all unabsorbed calcium radionuclide had cleared the intestinal tract and that which is present in the tissue is attributable to endogenous excretion. The mean percentage of ingested or absorbed doses in the high- and low-calcium adapted animals was significantly different ($P < 0.0001$). Mean percentage of ingested dose in this tissue in the high-calcium adapted animals was 0.577, while in the low-calcium adapted animals it was 0.294. Corresponding values for the mean percent absorbed dose were 1.188 and 0.307, respectively. This confirms the finding in Study 1 that the endogenous excretion of high-calcium adapted animals is higher than that of low-calcium adapted animals.

In Study 3, the rats were administered a weighed amount of raw and HTST or UHT milks based on the weight of diet consumed during the preceding 24 hr. This ratio was kept constant for all animals on a given day. Initially the animals in all groups scoured when milk was fed and therefore the amount of fluid was purposely kept low. Weight of the test milks was increased as the animals became more tolerant of the fluid.

The diet:milk ratio method of feeding was selected to avoid nutritional disproportionalities. Denis et al. (1973)

have shown that level of dietary protein determines the magnitude of physiological changes observed in growing animals fed calcium-deficient diets.

The results of the analyses of the femurs from these rats were expressed as mg Ca per g dry fat-free bone per g milk consumed (Table 3). Analysis of variance of these data showed no significant difference ($P > 0.2009$) between the three milks—raw, HTST, or UHT—at the 95% probability level. The effect of sex was significant ($P < 0.008$). The mean for all females was 0.410 and for males 0.357 mg Ca per g dry fat-free bone per g milk consumed. In Study 2 the percentage of absorbed radionuclide that appeared in the bones of male rats was significantly higher than that of female rats. In that instance, accretion was the measured parameter. In this study, resorption predominated over accretion as indicated by comparison of the 6- and 8-wk data. Rats maintained on the study for 6 wk had 0.450 mg Ca per g dry fat-free bone per g milk consumed while those on the study for 8 wk had 0.317 mg Ca per g dry fat-free bone per g milk consumed. This difference was significant ($P < 0.0001$). Bone remodeling was more active in male rats than in female rats at this age.

SUMMARY & CONCLUSIONS

STUDIES were performed to determine the availability of calcium in milk processed by a UHT system as compared with raw milk and that pasteurized by conventional HTST treatment. In addition, UHT milk stored for 58 days was evaluated. With the exception of the stored milk, all comparisons were made on milk derived from the same pool.

The experimental approach used was to add an extrinsic calcium radionuclide to the milk, administer the milk to young rats adapted to a low- or high-calcium diet, and quantitate the percent intestinal absorption (corrected for endogenous fecal contribution) and rate of endogenous loss. Using the same test animals, the experiment was repeated 42 days later. Percent intestinal absorption (uncorrected for endogenous fecal contribution), intestinal yield, and femoral yield were measured. The latter was measured separately on both femurs. The results obtained for intestinal and femoral activities were expressed both as percent of calcium radionuclide ingested and as percent absorbed.

The data for all parameters showed significant differences between the two calcium dietary levels consistent with the homeostatic mechanism. The low-calcium adapted animals showed significantly greater intestinal absorption in both trials. Likewise, the femurs of these low-calcium adapted animals had a higher yield of the ingested calcium radionuclide; however, when expressed as percent of absorbed dose the results did not differ significantly, indicating that dietary level does not affect the accretionary process in young rats. The intestinal yield of high-calcium adapted animals was greater than that of their low-calcium counterparts. Similarly the rate of endogenous excretion of calcium in high-calcium adapted animals was higher. These data indicate that either this excretory route functions in the homeostatic mechanism or more probably, the calcium excreted into the lumen is subject to the intensity of absorption in the intestine.

The intestinal absorption data indicated no significant differences between HTST and UHT milk in one trial or between raw, HTST, UHT, and stored UHT milks in another trial. The latter findings were confirmed by femoral yield, which is an index of gastrointestinal absorption.

Six- or 8-wk feeding studies, in which fluid milk was supplied to weanling rats as their sole source of calcium, resulted in no significant differences between the amount of Ca per g of dry fat-free bone per g milk consumed.

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A Rapid Immunoturbidimetric Method for Whey Proteins in Nonfat Dry Milk and Buttermilk

STEVEN P. GREINER, G. J. KELLEN, and DONALD E. CARPENTER

ABSTRACT

An immunoturbidimetric assay, using antibodies to whole bovine whey, was developed for the rapid screening of whey proteins in nonfat dry milk and buttermilk. Milk samples are heat-treated prior to analysis to denature the whey proteins for a more uniform response to antibodies. Of the whey proteins tested, the assay is most sensitive to bovine serum albumin and the least sensitive to β-lactoglobulin. Precision of the method is about 4% coefficient of variation with a minimum detectable level of 3% whey protein concentrate added to nonfat dry milk. Results are compared with electrophoretic analysis of whey proteins and high performance size exclusion chromatography of the glycomacropeptide from rennet whey.

INTRODUCTION

THE INCREASING USE of whey proteins in raw materials and finished products in the dairy industry has created a need for a rapid and sensitive method for the quantitative determination of whey proteins. The analysis of whey proteins has been the subject of numerous investigations using a variety of techniques. The whey protein nitrogen (WPN) method of Harland and Ashworth (1945) is commonly used to determine whey proteins in milk and dried milk. A method for analyzing whey protein adulteration by measuring the glycomacropeptide released into the whey by the action of rennet on κ-casein in milk was outlined by de Koning (1966) and Olieman and van den Beden (1983). Melachouris (1969) describes a discontinuous gel electrophoresis method for the analysis of whey proteins. Other techniques relying on phosphorous to nitrogen ratios (Douglas et al., 1982) and total half-cystine to protein ratios (de Koning and van Rooijen, 1971) have been successfully used for determining whey protein content in dairy products. Methods which rely on the antibody-antigen reaction such as immunodiffusion (Babajimopoulos and Mikolajcik, 1977; Larson and Hageman, 1963), immunoelectrophoresis (Dougherty, 1966; Kanno and Yamauchi, 1978; Lowenstein et al., 1975) and a radioimmunoassay (Beck and Tucker, 1977) are all very specific and sensitive, but rely on a nondenatured system to obtain a constant antibody response. All of these methods require expensive equipment or are too time consuming.

An immunoturbidimetric assay described by Lizana and Hellsing (1974) which uses polyethylene glycol to enhance the antibody-antigen reaction is a method which requires a minimum of equipment and less than an hour for analysis of several samples.

The purpose of this study was to develop a rapid and specific assay for the detection of whey protein adulteration of nonfat dry milk and buttermilk.

Authors Greiner, Kellen, and Carpenter are affiliated with Kraft, Inc., 801 Waukegan Rd., Glenview, IL 60025.

MATERIALS & METHODS

Reagents

A phosphate buffered saline solution (PBS 10X) is prepared by weighing 85g NaCl, 10.7g Na_2HPO_4, 3.4g $NaH_2PO_4 \cdot H_2O$ and 5g NaN_3 and diluting to 1L with distilled water. A phosphate buffered saline-5% polyethylene glycol solution (PBS-PEG) is prepared by adding 100 mL PBS 10X and 50g polyethylene glycol (obtained from Sigma Chemical Co. average M.W. 8000) to a final volume of 1L. The antibody solution (PBS-PEG-antibody) is prepared as a 1% solution of antibody (anti-whole bovine whey; Dakopatts Antibodies from Accurate Chemical and Scientific Corp.) in PBS-PEG buffer as required for each assay. A phosphate buffered saline solution (PBS) is prepared by adding 100 mL PBS 10X to a final volume of 1L. β-lactoglobulin, α-lactalbumin and bovine serum albumin (BSA) were obtained from Sigma Chemical Co.

Sample preparation

Samples (or standards) are prepared by dissolving the required amount of sample in PBS buffer. Under the conditions used, a 10 mg/mL solution of sample is preapred by dissolving 100 mg sample in 9.9 mL PBS. After thorough mixing and dissolution, the tubes are placed in a boiling water bath for 5 min.

Immunoturbidimetric assay

The samples and standards are added to the PBS-PEG buffer solution and to the PBS-PEG antibody buffer solution at a 10% (V/V) final ratio. In this study, 0.2 mL sample is placed in 1.8 mL of each buffer. These test solutions are incubated in a shaker at room temperature for 1 hr. The turbidity (absorbance) is measured in a spectrophotometer at 500 nm. The absorbance of the PBS-PEG test solution is subtracted from the absorbance of the PBS-PEG antibody test solution. This difference value represents the change in turbidity due to the formation of the antibody-whey protein complex. The polyethylene glycol enhances this reaction by increasing the excluded volume of the complex and by keeping the complex homogeneously suspended in solution.

A standard curve is prepared by combining solutions of a standard whey protein concentrate (WPC) and buttermilk or NFDM to produce a range of whey protein concentrations. The whey protein contents as a percentage of total sample of the buttermilk, NFDM, and WPC were derived from literature values and are taken to be 8.0, 8.0 and 34.0%, respectively. Whey protein content of representative samples of buttermilk, NFDM and WPC were determined electrophoretically and corroborate the literature values. These standards can be boiled as separate solutions or after they are combined.

Electrophoresis

Sodium dodecyl sulfate (SDS) electrophoresis was performed by the method of Laemmli (1970) with 10-20% polyacrylamide gradient gels. The gels were scanned with an LKB laser scanning densitometer interfaced to an Apple II plus computer and analyzed by the Gelscan curve resolving program. Whey protein content of buttermilk or NFDM was calculated from the ratio of β-lactoglobulin to the total of the protein bands in the electropherogram, assuming that β-lactoglobulin comprises 52% of the whey protein and basing the calculation on the total protein in the sample (Kjeldahl nitrogen x 6.38).

High performance size exclusion chromatography

High performance size exclusion chromatography (HPSEC) analysis of the glycomacropeptide from κ-casein was performed by the

method of Olieman and van den Beden (1983) with a DuPont Series 8800 gradient chromatographic system, a Varian Vari-chrom variable wavelength detector and two Bio Rad Biosil TSK 125 columns (300 mm x 7.5 mm) in series.

Double immunodiffusion

Pure preparations of β-lactoglobulin, α-lactalbumin, bovine serum albumin and whole whey were tested for reactivity against the whole whey antibodies with the double diffusion technique of Ouchterlony (1949). Antigen concentrations were 1%, while the antibody was used without prior dilution. Sixty μL were placed into each well.

RESULTS & DISCUSSION

THE ANALYSIS of whey proteins in NFDM and buttermilk is difficult due to the extent of denaturation caused by processing conditions, especially heat treatment. In the present method, all samples and standards were extensively heated to completely denature the whey proteins. The antibody response to the whey proteins was less when the whey proteins were denatured than when they were in their native conformation (Fig. 1). Although the response was considerably lower after heating, the standard curve for the standard samples at a concentration of 10 mg/ml was nearly linear in the range from about 8–21% whey protein.

Fig. 2 shows the time course for the antigen-antibody reaction and the effect of high whey protein levels on the response. At very high whey protein levels precipitation of the complex occurred resulting in lower absorbance values. For more accurate analysis, samples must be diluted appropriately and the incubation time chosen for complete reaction. Samples with turbidity values below the standard curve should be made more concentrated before analysis. For normal NFDM and buttermilk samples (not shown), the reaction was complete in 30 min at room temperature. Incubation at 37°C did not accelerate this assay.

In order to study the individual antibody-antigen reactions of α-lactalbumin, BSA and β-lactoglobulin, these major protein components of whey were subjected to double diffusion into antibodies to whole whey (Fig. 3). More precipitate was formed with BSA than for either β-lactoglobulin or α-lactalbumin. However, the precipitation line with β-lactoglobulin was very faint. This data was confirmed by observing the immunoturbidimetric response of the three proteins. The turbidity of the antibody-BSA complex was much greater than for the other whey proteins (Fig. 4). The β-lactoglobulin antibody complex

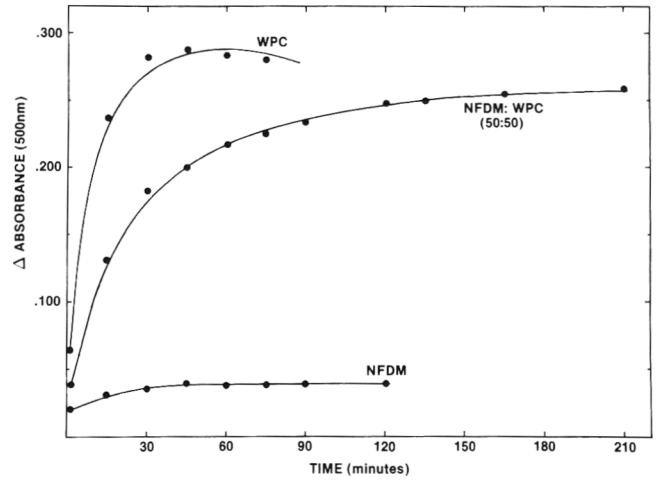


Fig. 2—Time course for antigen-antibody reaction.

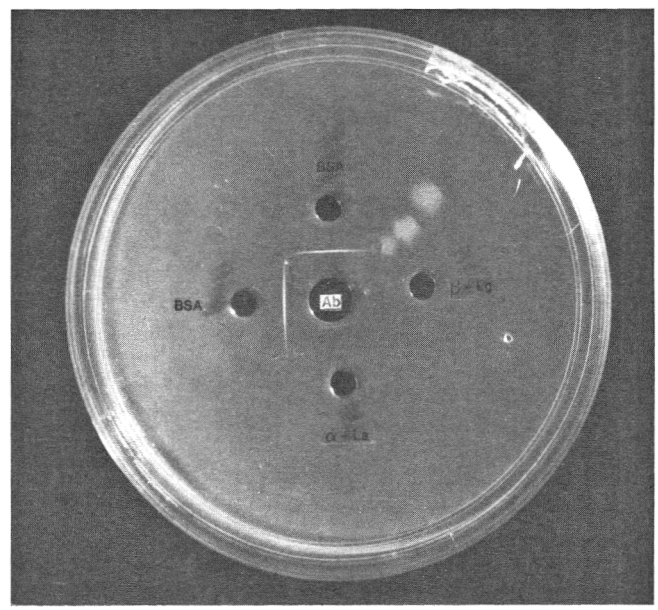


Fig. 3—Ouchterlony double diffusion plates of native β-lactoglobulin (β-Lg), α-lactalbumin (α-La) and bovine serum albumin (BSA) into whole whey antibodies (A).

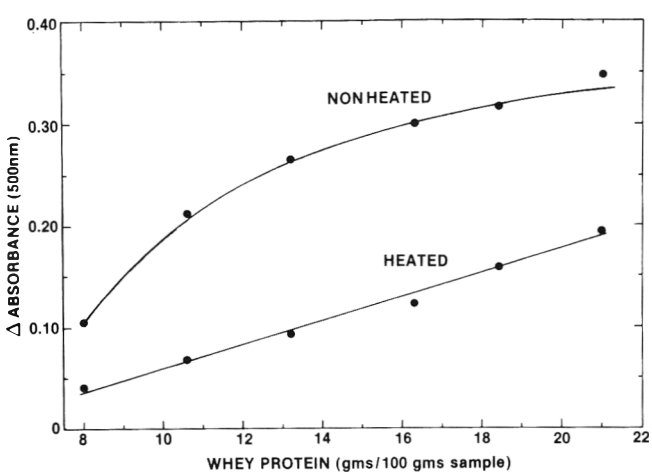


Fig. 1—Effect of sample heat treatment on the immunoturbidimetric response of buttermilk:WPC blends. Blends were heated for 5 min. at 100°C and absorbance measured after 1 h incubation at room temperature.

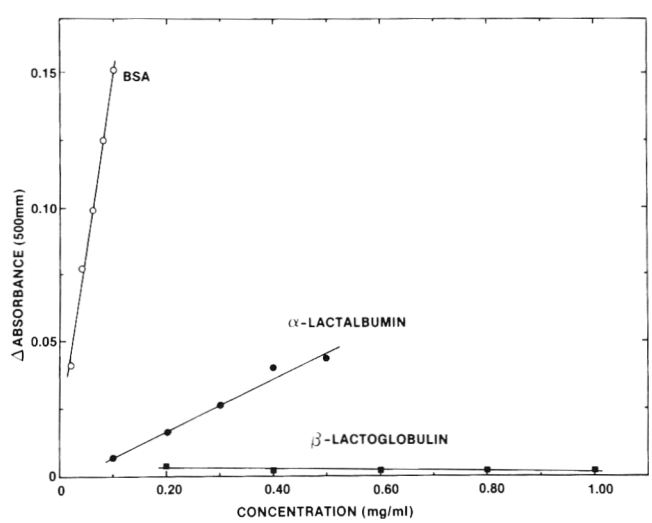


Fig. 4—Immunoturbidimetric response of individual native whey proteins. Protein concentrations approximate the concentrations used for the immuno test.

showed no turbidity at the concentrations used, indicating antigen may be in excess of the equivalence point. This data suggests that most of the turbidity was contributed by the BSA and to a lesser extent by the α-lactalbumin-antibody complexes or perhaps other antigens present in whey which were not tested. BSA content of 12 samples of NFDM as determined by electrophoresis varied from about 0.2–0.4% but there was no correlation of BSA with whey protein content determined by this immunoturbidimetric assay. This suggests that other antigens may be involved in the response. Due to lot to lot variation of antibody preparations, it is important to analyze antibody-antigen reactivity of each lot to ensure sufficient activity.

Samples of buttermilk and NFDM were obtained from different sources and analyzed for whey proteins by the Harland-Ashworth method, the SDS gel electrophoresis procedure of Laemmli, an HPSEC method described by Olieman and van den Beden (1983), which quantifies the glycomacropeptide, and the immunoturbidimetric assay. In comparing the four methods, the immunoturbidimetric method offers advantages in speed and ease of analysis.

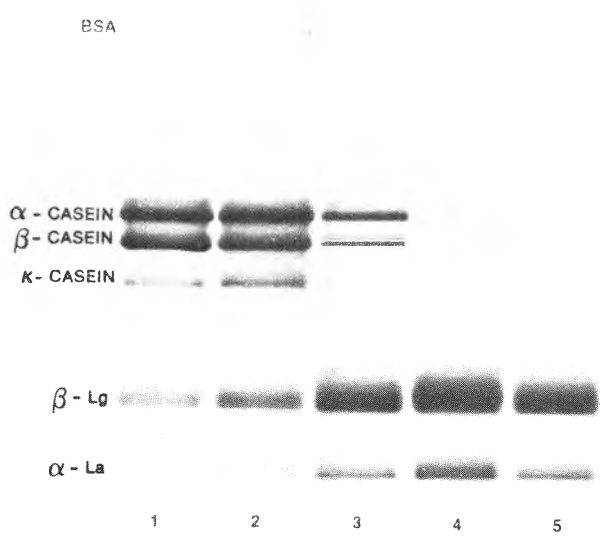


Fig. 5—SDS-polyacrylamide gel electrophoresis. About 50 μg sample were applied to each well as follows: Lane 1, NFDM; Lane 2, buttermilk; Lane 3, sample 500; Lane 4, sample 98; Lane 5, WPC.

Table 1—Precision and sensitivity of the immunoturbidimetric assay

Sample	Observations (#)	Δ Absorbance	C. V. (%)
Buttermilk			
100	5	0.167	3.6
500	5	0.313	2.6
NFDM	10	0.037	4.6
NFDM + 5% WPC	10	0.053	3.0

Probability $(0.014 \leq (\mu_1 - \mu_2) \leq 0.018) = 0.95$

The whey protein nitrogen value (WPN) obtained from the Harland-Ashworth method will vary greatly, depending on the degree of whey protein denaturation.

SDS gel electrophoresis overcomes the problem of varying degrees of whey protein denaturation, but is more complex to run, requires much longer analysis time, and is not precise. An electrophoretic separation of WPC, NFDM and several buttermilks is shown in Fig. 5. β-lactoglobulin was used to estimate total whey content since it is well resolved from other protein components on the gel.

Using the glycomacropeptide (GMP, produced by rennetting κ-casein during cheesemaking and found predominantly in the whey) as an indicator of rennet whey or WPC, HPSEC is sensitive to the addition of low levels of whey to NFDM. Sensitivity of this technique was less than 1% WPC added to NFDM.

The immunoturbidimetric assay is very specific for whey proteins. The precision and sensitivity of this method determined by the percent coefficient of variation (C.V.) from multiple samplings of buttermilk and NFDM are summarized in Table 1. The C.V. ranged from 2.6–4.6% with the samples containing higher concentrations of whey proteins having the lower percent C.V. values. Buttermilk sample 500 contained about 33% WPC as determined by electrophoretic analysis and has a much higher absorbance. A 95% confidence interval for the difference of means of a nonfat dry milk and the same sample with 5% additional WPC indicated that this level of adulteration with WPC can easily be detected.

The analyses of NFDM and buttermilk by these four methods are summarized in Table 2. Sample 446 with a low WPN value of 0.70 mg/g is a high heat NFDM with a high percentage of whey protein denaturation. Analysis for GMP indicated that no additional whey was added to these NFDM samples. Table 2 also shows the effect of high heat NFDM on WPN values. Even at 40% WPC, the WPN was

Table 2—Analysis of whey proteins in nonfat dry milk and buttermilk by four methods

Sample	Immunoturbidimetric	Electrophoresis	WPN (mg/g)	HPSEC[a] added whey solids
	Whey protein (g/100g sample)			
NFDM				
353	8.0	6.3	7.4	0
355	8.5	8.0	6.0	0
356	7.6	7.0	6.9	0
445	7.8	9.8	6.4	0
446	7.3	5.7	0.7	0
447	6.6	8.8	7.3	0
448	7.5	9.5	6.6	0
449	7.4	9.4	7.2	0
Low heat NFDM	8.0		6.6	
High heat NFDM	8.0		0.9	
High heat NFDM:WPC[b]	18.4		5.4	
Average of 99 NFDM samples: 7.9% (± 0.66)				
Buttermilk				
100	8.0	7.5	2.7	0
155	8.0	7.3	0.6	0
316	8.5	9.3	1.4	2
98	30.0	30	27	42
450	14.2	12	8.2	19
500	18.9	17	19	21

[a] The ratio of GMP to protein was determined for several samples of WPC and is the same for whey. Whey solids is expressed as the percent of GMP compared to that found in WPC. (GMP is found in ultrafiltered retentate and not in the permeate).
[b] The mixture of NFDM and WPC was 60:40.

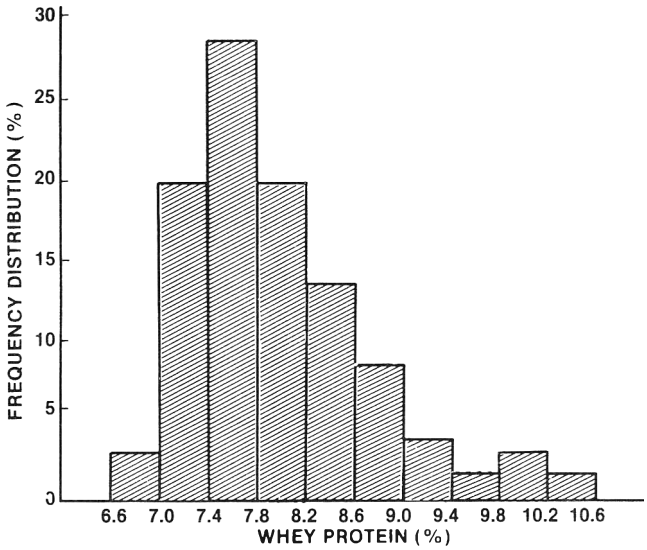


Fig. 6—Histogram for frequency distribution of whey protein in NFDM as determined by immunoturbidimetric assay (n = 99).

lower than for a low heat NFDM. Fig. 6 shows a frequency distribution of whey protein in nonfat milk as determined by the immunoturbidimetric assay (n = 99). Results which are higher than 10% whey protein should be further analyzed. The whey protein levels in buttermilk samples ranged from 7.0 to approximately 30%. Precision of the electrophoretic data from sample 500 was low because of band broadening of the β-lactoglobulin peak and subsequent poor integration of this peak due to uncertainties in the baseline. If buttermilk is processed from sweet cream or sweet cream:whey mixtures (75:25), the level of whey proteins should be in the range 6.5–10% of the sample. Several of the buttermilk samples contained whey proteins above the 10% level indicating the possible addition of WPC or buttermilk made from higher levels of whey cream. This was confirmed by the presence of high levels of GMP in these samples.

An immunoturbidimetric method was adapted for the analysis of whey proteins in NFDM and buttermilk solids. Although in the present study this method was used only for the analysis of whey proteins in nonfat dry milk and buttermilk it could be adapted to finished products such as processed cheese. Several references to the analysis of whey proteins in finished dairy products (Dougherty, 1966; Douglas et al., 1982; Hemmati and Keeney, 1979; Josephson and Holloway-Thomas, 1980) indicate that a rapid and reliable test such as the immunoturbidimetric method for whey proteins would be useful.

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A Statistical Approach to Error Analysis in Thermal Process Calculations

HUGO PATINO and JULIANNA R. HEIL

ABSTRACT

A method for the statistical analysis of thermal process calculations is presented. First, an analysis is made of some of the uncertainties involved in thermal death time and heat penetration experiments. That information is then used to evaluate the consequences on process lethality calculations. A method is presented for using the results for the assignment of safety factors. Finally, the limitations and implementation problems associated with this method are discussed, particularly the lack of fit of some sets of experimental data to the logarithmic bacterial death model.

INTRODUCTION

THE IMPORTANCE of thermal processing of hermetically sealed containers as a method of food preservation can be illustrated in several ways. One way is to look at the per capita consumption of vegetables in the U.S. Of the commercially produced vegetables, about 44% are processed and canned (Judge and Sons, Inc., 1983).

Relatively little information, however, has been published on the statistical analysis of the different sources of uncertainties. Hicks (1961) studied uncertainties due to physical measurements such as temperature and biological quantities such as spore counts. Lund (1978) investigated the statistical analysis of thermal process calculations, using the Monte Carlo procedures to generate distribution curves from which he estimated the effect of parameter variations on the lethality of the process. He concluded that, although the effect of variations had been shown, the application of that information to the assignment of safety factors still needed to be addressed. Similar simulations have been reported by Massaguer et al. (1983) who used a Poisson distribution to describe error populations, in contrast to the normal distribution used by Lund (1978).

Adequately planned recent experimental data for use as a basis for appropriate statistical analysis and quantification of the various sources of error in a given parameter are not available. Lack of recent data base constitutes a major weakness in recent publications. Lenz and Lund (1977) considered the uncertainty in destruction rates of microorganisms, but this was assessed in terms of the departure of the measured death rate constant (D value) from its fitted value, using data reported in 1920 by Bigelow and Esty. While their information is useful, it measures the combined effects of both experimental reproducibility and the lack of fit of the data to the model. The relative contribution or significance of either of the two components cannot be established from their procedure.

In this paper, a method is introduced for the statistical analysis of thermal process calculations. First, from experimentally generated data, some of the uncertainties involved in the determinations of thermal death and heat penetration experiments will be quantified, then the impact of uncertainties on the calculation of the process lethality will be evaluated. The possible use of this information in the assignment of safety factors, and an analysis of the limitations and implementation problems associated with this method, will be addressed.

Authors Patino and Heil are affiliated with the Dept. of Food Science & Technology, University of California-Davis, Davis, CA 95616.

MATERIALS & METHODS

SPORES of NCA 1518 were grown on 5% soil infusion agar containing 1.5% nutrient agar (DIFCO Laboratories, Detroit, MI), 0.5% agar, 0.05% glucose, 30 mg/L $MnSO_4$ and 50 mL/L of 5% soil infusion.

Spores of Putrefactive Anaerobe (PA) 3679 were grown according to the procedure of Goldoni et al. (1980) using the single stage inoculation and two stage incubation procedure in the formulation containing casein and meat particles.

Sampling-diluting-plating tests were done with spores of NCA 1518 suspended in 0.85% saline at concentrations ranging from 10^2 spores/mL for direct sampling-plating to 10^7 spores/mL for various levels of diluting-sampling-plating. Dextrose-tryptone agar (DIFCO) was used for plating and recovering NCA 1518, and fluid thioglycollate (DIFCO) with 1.5% agar in deep-agar-shake tubes was used for PA 3679.

Heat resistance experiments were done with spores of PA 3679, in thermal death time (TDT) retorts, using 10^7 spores/mL suspensions in pH 7 phosphate buffer scaled into 2 mL glass ampoules. The spores were heat shocked 15 min at 85°C in the ampoules. Destruction was evaluated by plating survivors diluted to give plate counts of 30–300 colonies/plate, i.e., spores/mL. Reproducibility within and among TDT retorts was tested by replicating actual time-temperature treatments for up to 10 ampoules per retort per test.

Heat penetration tests were done in a vertical still retort using 2.5 cm^3 diced potatoes packed in brine into 303x406 cans. Cans were filled with 300g potatoes and a brine was added leaving approximately 6 mm headspace. One coated 2.7 cm long copper-constantan thermocouple (O.F. Ecklund, Cape Coral, FL) was inserted into the brine at the geometric center of each 303x406 can. Time-temperature histories were recorded at 1 min intervals using a multi-channel data logger. Four cans were tested in each of three runs of 23 min at 250°F (121°C).

RESULTS & DISCUSSION

Error structure of the observations

Heat resistance experiments will determine the values of D and z (Stumbo, 1973). Several sources of variation which affect the results can be distinguished. One is the reproducibility and error distribution associated with the spore counts which results from the sampling, dilution, plating and counting procedures involved. This was tested using the traditional chi-squared goodness-of-fit test (Blank, 1980), as well as the normality test of Nelson (1981).

The random sampling of a spore suspension is considered to have a Poisson distribution (Finney, 1964) but when several other steps contribute to the final count, the distribution tends to normality. For a variable which is Poisson-distributed, the variance associated with the variable is proportional to the value itself: the larger the spore count to be measured the larger the variance. In such a case, it is usually easier to transform the variable, for example, use its logarithm, to have a distribution with a constant variance to which standard statistics can be applied. Table 1 presents the results of the sampling-diluting-plating experiments and their statistical analysis in terms of their logarithms. The

normality assumption could not be rejected at either 90 or 99% confidence levels, and the variance of the logarithms of the spore counts was constant as indicated by an F test with 99% confidence (Walpole and Myers, 1978).

One should be cautious, however, when interpreting the results. Breman (1973) recommended that the detection sensitivity of normality tests, such as the chi-square test, should be considered. He showed that to distinguish between two very different distributions, such as uniform and normal distributions, a very large sample size of 100 or more would be required.

From the previous results, the logarithm of the spore counts for the sampling-dilution-plating procedures from a stock suspension has an approximately normal distribution, with a standard deviation between 0.03 and 0.05. In the actual TDT experiments one has several other sources contributing to the lack of reproducibility of the results. Table 2 presents as an example, the results obtained with PA 3679 subjected to 255°F (124°C), for various lengths of time. There were two different runs, each had five samples (with duplicate counts for each sample). Results from the second run gave better reproducibility (0.033–0.182 standard deviations for the logarithm of the spore counts), compared to the first run (0.09–0.23 standard deviation values). A trend was observed that longer heating periods, where fewer viable cells are left, had larger standard deviations. The hypothesis of homogeneity of variances, however, could not be disproven with 99% confidence using Bartlett's test (Walpole and Myers, 1978).

Effect on D and z

The decimal reduction time D and the parameter z are determined from a survival curve and a phantom TDT curve as described by Stumbo (1973), using the logarithm of the spore counts. The effect of the uncertainty of those counts over the values of D and z was evaluated.

One way to study this effect is with a Monte Carlo simulation. First, a "perfect" set of data (log of counts vs time) was created for given values of D and z by setting the values of temperature and time and calculating the logarithm of the spore counts (Eq. 1). Then, random error with a structure similar to that found in the previous section for the logarithm of the spore counts was introduced to the "perfect" data, resulting in data simulating experimental data. From these data, the values of D and z are estimated in the conventional way (Stumbo, 1973). Two hundred simulations were done for each of several values of D ranging from 2 to 20, as well as for several z values between 15 and 18.

The results indicated that the current procedures of first finding the D and z values gave unbiased estimates of D and z, i.e., the average of the estimates of D and z corresponded to their "true" or fixed values. This is not always the case when least squares is used in a situation where some of its assumptions are violated. One of these assumptions is that the independent variable is measured with no error (Reilly and Patino, 1981). The standard deviation for the D values was between 3 to 6% of the D value, i.e., between 0.03D to 0.06D, compared to 0.10D used by Lenz and Lund (1977). The standard deviation of z was between 0.5 and 1.0°F compared to 2.0°F selected by Lenz and Lund (1977).

While one tends to think about D and z as separate parameters coming from the traditional "two-stage" procedure (of first estimating D values and from there estimating a z value), these two parameters are highly correlated. The model implied by the use of the traditional procedure can be shown to be

$$\log N = \log N_0 - \left[\frac{t}{D_{ref}}\right] 10^{\left[\frac{T - T_{ref}}{z}\right]} \qquad (1)$$

When the values of D and z are estimated simultaneously by using nonlinear least squares, they are highly correlated. The correlation between D and z was found to be approximately 0.92 to 0.96, which is typical for parameters involved in this type of nonlinear model. The information of the uncertainty of D and z will be used in a later section, where the effect on process lethality (F) will be assessed.

Uncertainty of heat penetration data

This aspect has received relatively more attention in the literature (Lund, 1978) and is typically expressed in terms of the standard deviation of f_h. The reported standard deviation of f_h ranged from 7% to 25% of its value (Herndon, 1971; Hicks, 1961). For comparison, Table 3 presents the results for diced potatoes in brine in 303 x 406 cans. As can be seen, the standard deviation of f_h varied from 15.90% to 25.56% of the average values, giving an overall standard deviation of 20.01%. These results were within the range of previously reported values. The impact of uncertainties in such typical heat penetration data on lethality calculations will be examined next.

Uncertainty in process lethaltiy, F_p

Ideally, one would like to know the true lethality F_p^* that a process has given:

Table 1—Results from the repeated sampling of a heat shocked spore stock suspension, after dilution, plating and counting. (Results expressed in terms of the logarithms of the spore counts) Passing criteria for the normality test was at 99% confidence

No. of dilutions	No. of replicates	Log_{10} (N) average	Std. dev.	Normality test
0	39	1.92	0.031	Passed
2	25	3.89	0.026	Passed
5	24	6.75	0.048	Passed
6	24	6.76	0.047	Passed

Table 2—Results from heat resistance experiments with spores of PA 3679 heated at 255°F for various lengths of time. (Values refer to the logarithm of the spore counts)

Corrected heating time (min)	Run No. 1		Run No. 2	
	Average	Std. dev.	Average	Std. dev.
0*	6.7490	0.1051	6.6729	0.0610
2	5.6588	0.1058	5.7053	0.0470
3	4.1596	0.0914	4.1244	0.0335
4	1.5331	0.1204	1.7100	0.0975
4.5	0.8948	0.1557	1.2082	0.1095
5	0.4003	0.2376	0.5918	0.1820

* Only heat shocked, 15 min at 185°F (85°C)

Table 3—Values of f_h (min) in replicated heat penetration experiments with potatoes in brine, packed in 303x406 cans

	Run 1	Run 2	Run 3
Can 1	31.95	36.01	42.51
Can 2	35.97	38.07	54.05
Can 3	49.80	50.27	43.17
Can 4	43.13	61.61	37.30
Average	40.21	46.49	44.25
Std. dev.	7.88	11.88	7.03
S.D./Avg.	19.62%	25.56%	15.90%

$$F_p^* = \int 10^{\left(\frac{T^*-T_{ref}}{z^*}\right)} dt^* \qquad (2)$$

However, the true values of the temperature (T*) and the true value of z (z*) are not known. Measured or calculated values are subject to some error or uncertainty that can be expressed as:

$$\begin{aligned} T &= T^* + \epsilon_T \\ z &= z^* + \epsilon_z \end{aligned} \qquad (3)$$

These errors, ϵ_T and ϵ_z, cause an error in the measurement or calculation of F_p^*:

$$F_p = F_p^* + \epsilon_{F_p} \qquad (4)$$

The properties of ϵ_{F_p} can be approximated from ϵ_T and ϵ_z by applying the "error propagation method" (Himmelblau, 1970):

$$\epsilon_{F_p} \approx \left(\frac{\partial F_p}{\partial T}\right)\epsilon_T + \left(\frac{\partial F_p}{\partial z}\right)\epsilon_z \qquad (5)$$

The distribution of ϵ_{F_p} can be found, for example, when the temperature is constant, such as during a holding period in a thermal process. Then, under normality assumptions for ϵ_T and ϵ_z, ϵ_{F_p} is normally distributed with the mean equal to zero and a standard deviation (σ_{F_p}) approximated by applying the standard formula for the variance of the sum of the two independent variables:

$$\sigma_{F_p} = 2.3025\, F_p \sqrt{\left(\frac{T-T_{ref}}{z^2}\right)^2 \sigma_z^2 + \left(\frac{1}{z}\right)^2 \sigma_T^2} \qquad (6)$$

This means that the uncertainty in F_p is proportional to its value. This generally agrees with previous simulations reported by Lund (1978), who showed that the dispersion of results increased with larger average lethality values. Similarly, the equation points out that the importance of an error in z, as quantified by its variance σ_z^2, is relative, depending on the difference between the temperature and the reference temperature, and the magintude of an error in temperature, as quantified by its variance σ_T^2, will depend on the value of z. For example, in terms of numerical values, with standard deviations of $\sigma_T = 1.0$, $\sigma_z = 0.5$, and $z = 18$, the value of σ_{F_p} would be $0.3776\, F_p$ for $T-T_{ref} = 10°F$, or $0.1557\, F_p$ for $T-T_{ref} = 5°F$, or $0.1279\, F_p$ for $T-T_{ref} = 0$. The relative importance of the two terms inside the square root will greatly depend upon the value of $T-T_{ref}$. Of course, the better the precision is for T and z, and the smaller their variances, the smaller will be the standard deviation for F_p.

For a thermal process, any time-temperature profile can be obtained and a general algebraic expression for σ_{F_p} cannot be found. However, from Eq. (5), it can be shown that

$$\sigma_{F_p} = \sqrt{\left(\frac{\partial F_p}{\partial T}\right)^2 \sigma_T^2 + \left(\frac{\partial F_p}{\partial z}\right)^2 \sigma_z^2}$$

where

$$\frac{\partial F_p}{\partial T} = \frac{\ln 10}{z} F_p$$

and

$$\frac{\partial F_p}{\partial z} = \frac{\ln 10}{z^2}\left[T_{ref}\, F_p - \int_{t_o}^{t_f} 10^{\left(\frac{T-T_{ref}}{z}\right)} T\, dt\right]$$

and to quantify σ_{F_p} for a given process, a numerical integration technique, similar to those used by the General Method, will be needed (Stumbo, 1973). What happens to the partial deviative of F_p with respect to temperature is clear. When F_p increases, the partial derivative also increases. The partial derivative of F_p with respect to z, however, does not seem to have an identifiable trend with respect to F_p. Table 4 illustrates this for several processes and products. Comparing the first two processes, a large difference in the value of the partial derivative for almost the same F_p value can be observed, but no clear trend can be found when all the seven processes are considered. Based on these observations, and on several simulated processes where only the come-up time or the holding time varied, it appeared that the value of the partial derivative depended not only on F_p and z but also on the particular time-temperature profile. This was expected considering the constant temperature example where σ_{F_p} was dependent on both F_p and $T-T_{ref}$.

The results of heat penetration studies in Table 3 are shown in Table 5. The values of the standard deviation ranged from 0.11 to 0.21 of the average F_p value, i.e., from 11% to 21% of its value. Using the values of the confidence intervals and the number of replicates reported by Lund (1978) in his simulations, the equivalent standard deviations would range from 7.8% to 32% of the average F_p value for purely convection-heating, and from 6% to 12% for purely conduction-heating.

Uncertainty in the required lethality, F_r

One would also like to know the true required lethality F_r^*. By definition,

$$F_r^* = D^* (\log a^* - \log b^*) \qquad (7)$$

but if D* is known only with some error or uncertainty ϵ_D:

$$D = D^* + \epsilon_D, \qquad (8)$$

then

$$F_r = F_r^* + \epsilon_{F_r} \qquad (9)$$

Table 4–Values of F_p and $(\partial F_p/\partial z)$ for various processes

Product	F_p	$(\partial F_p/\partial z)$
Potatoes in brine (Process 1)	19.78	0.24
Potatoes in brine (Process 2)	21.10	0.12
Potatoes in brine (Process 3)	14.86	0.41
Potatoes in brine (Process 4)	20.09	0.21
Green Peas in brine	12.79	0.53
Sweet Potatoes	36.26	0.81
Fish in Sauce	7.96	0.78

Table 5–Values of F_p from replicated heat penetration experiments with potatoes in brine

	Run 1	Run 2	Run 3
Can 1	12.89	12.64	15.29
Can 2	8.65	15.49	12.74
Can 3	11.67	12.24	17.52
Can 4	14.73	14.24	14.94
Average	11.98	13.65	15.12
Std. dev.	2.55	1.50	1.96
S.D./Avg.	0.21	0.11	0.13

where from Eq. (7)

$$\epsilon_{F_r} = (\log a^* - \log b^*)\,\epsilon_D \qquad (10)$$

The standard deviation of F_r can be calculated by using the properties of the variance of a random variable, and with Eq. (7), this can be shown to be

$$\sigma_{F_r} = \frac{F_r}{D}\,\sigma_D \qquad (11)$$

As in F_p, the standard deviation of F_r is also dependent on the value of F_r. From the results in the section on D and z, ϵ_D was between 0.03D and 0.06D, slightly lower than the 0.10D reported by Lund (1978). Assuming that σ_{F_r} would be between 0.03 F_r and 0.10 F_r, the results from this and the previous section can now be put to work.

Underprocessing distribution

Define $\delta^* = F_p^* - F_r^*$. Ideally, δ^* should be greater than or equal to zero to avoid underprocessing. It was shown in previous sections that F_p^* and F_r^* are not perfectly known. However, it is known that they are distributed about the central values F_p and F_r, with standard deviations σ_{F_p} and σ_{F_r}. That is,

$$\delta = \delta^* + \epsilon_\delta \qquad (12)$$

where

$$\epsilon_\delta = \epsilon_{F_p} - \epsilon_{F_r} \qquad (13)$$

In practice, $F_p = F_r = F_o$ is set. In this case, δ will have a mean value of zero and the standard deviation of δ can be found from Eq. (13), through Eq. (5) and (10). At this point the dependent nature of D and z makes a difference, because

$$\sigma_\delta = \sqrt{\sigma_{F_p}^2 + \sigma_{F_r}^2 - 2\,\mathrm{cov}\,(F_f, F_r)}$$

and the covariance between F_p and F_r is not zero, since F_p depends on z and F_r depends on D, where D and z have a correlation coefficient between 0.92 to 0.96.

For any given time-temperature profile, the value of $\sigma_{F_p}^2$ can be found using a numerical integration routine, and therefore the value of σ_δ can be calculated. It is instructive, however, to consider once more the constant temperature case. Here σ_δ can be found from the previous equation, and from Eq. (6) and (11),

$$\sigma_\delta = 2.3025\,F_o \sqrt{\left(\frac{T-T_r}{z^2}\right)^2 \sigma_z^2 + \left(\frac{1}{z}\right)^2 \sigma_T^2 + \left(\frac{1}{D}\right)^2 \sigma_D^2 + 1.9\left(\frac{T-T_{ref}}{z^2 D}\right)\sigma_D \sigma_z} \qquad (14)$$

assuming a correlation coefficient of 0.95 for D and z. Again, the contribution of the various sources of uncertainty to the total uncertainty in δ can be distinguished and the approximate probabilities for a designated amount of underprocessing can be calculated. For instance, one may ask for the probability of underprocessing by 10% or more the value of F_o. In this case

$$P(\delta < -0.10\,F_o) = P\left(Z < \frac{-0.10\,F_o}{\sigma_\delta}\right)$$

where Z represents a standard normal variable. The answer can be found by evaluating σ_δ for the appropriate values of D, z, $T-T_{ref}$, etc., and then using the standard normal tables.

A given safety factor can also be evaluated. For a calculated F_r one may determine a process to give a lethality F_p, representing a safety factor $\delta_o = F_p - F_r$. One can then calculate the probability that $\delta^* < 0$, that is, that we will underprocess the can from:

$$P(d < 0) = P\left(Z < \frac{0-\delta_o}{\sigma_\delta}\right)$$

Limitations and implementation problems

As Lund (1978) points out, methods currently used for the assignment of safety factors do work. A statistical method should therefore give results which are close to those currently found. Based on very limited examples, this has been found to be generally true for the method given in the previous section.

Example. As an example, let us consider the data presented by Stumbo (1973). This dealt with a product which had an average maximum spore concentration of 3 spores/gram, and each can had 570g of product. This contamination was to be reduced to the equivalent of having one spore per 100 cans. The values of D and z for the contaminant were 3 min and 10°C, respectively.

The conventional safety factor is applied when calculating the process for one can by considering the contamination in all 100 cans:

$$F_r = 3[\log(5 \times 570) - \log(.01)] = 16.3 \text{ min}$$

In the alternative statistical approach one would say that the required processing is such that it destroys the 5 x 570 spores in the can, and that it leaves alive one spore with a probability of 0.01. That is

$$F_r = 3[\log(5 \times 570) - \log 1] = 10.36 \text{ min}$$

One then finds the value of F_p such that the probability is of 0.01 of having an underprocessing (resulting in spores surviving). Suppose that the following apply at least approximately in this case: $\sigma_z = 1$ °F, $\sigma_D = 0.05D$, $\sigma_T = 1$. With these, and if $T - T_r = 0$ then $\sigma_\delta = 1.78$. From the tables of the Normal distribution, the expression $P(Z < Z_o) = 0.01$ corresponds to $Z_o = -2.33$. Then, the overprocessing δ_o (the mean of the distribution of δ) can be determined from:

$$0.01 = P(Z < -2.33) = P\left(\frac{0-\delta_o}{\sigma_\delta} < -2.33\right)$$

so that $\delta_o > 4.14$. This means that we should seek an $F_p >$ 10.36 min + 4.14 min, or $F_p > 14.4$ min. If we assume $T - T_r = 5$°F, then $F_p > 16.69$ min.

In practice, as previously expressed, one should look at the specific time-temperature profile to find out what the value of σ_δ is. One can note, however, that these results are in close agreement with that given by the traditional or conventional procedure.

There are several limitations for implementing this type of procedure. One will need to take a look at a large number of cases and compare the results. Also, since this is a statistical procedure, one should verify that the series of assumptions one makes apply in actual practice. One such assumption is the thermal death model assumed for the bacterial spores. While there is ample evidence which proves

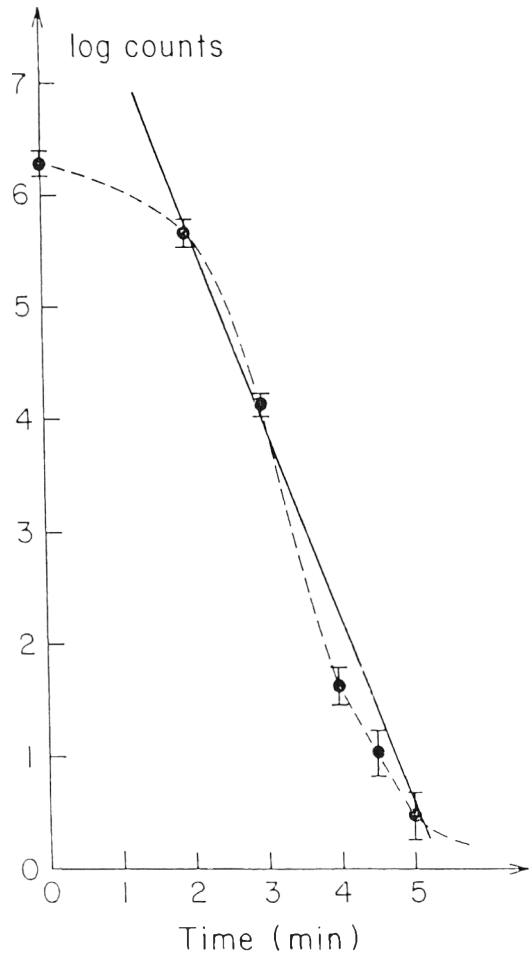


Fig. 1–Survival curve for PA 3697 spores in saline suspension at 255°F (124°C).

this is generally a good model, we have found for several sets of data that a significant lack of fit exists, for a 99% confidence level. An example of this is illustrated in Fig. 1. A possible solution to this limitation is the usage of other models that may take these deviations into account. This is still currently under research. Finally, when dealing with low probabilities of underprocessing one needs to work with the tails of the normal distribution. While the normality assumption has not been disproven, one needs to worry about deviations in the tail ends of the actual distributions. The results of this statistical approach, therefore, should be considered as approximations, and a larger set of data is needed to quantify the extent of these possible deviations.

SYMBOLS

a^* = true initial spore concentration, spores/g.
b^* = true final spore concentration, spores/g.
D = death rate constant or decimal reduction time. D^* is its true value.
D_{ref} = decimal reduction time at reference temperature.
F_p = lethality supplied by process. F_p^* is its true value.
F_r = lethality required from process. F_r^* is its true value.
f_h = inverse slope of heat penetration curve.
N = spore count (spores/mL) at any time.
N_o = initial spore count (spores/mL).
P = probability.
t = time, min.
T = temperature, same units as for z.
T_{ref} = reference temperature.
z = inverse of slope of phantom TDT curve. (z^* is its true value).
Z = standard normal variable.
δ = difference between F_p and F_r (δ^* is its true value).
ϵ = experimental error. The subscript denotes for which variable.
σ = standard deviation. The subscript denotes for which variable.
σ^2 = variance. The subscript denotes for which variable.

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Kinetics of the Degradation and Regeneration of Betanine

A. S. HUANG and J. H. VON ELBE

ABSTRACT

The degradation and regeneration kinetics of betanine in solution at pH 5.0 under nitrogen and oxygen were studied at 65, 75, 85 and 90°C. HPLC was used to monitor the concentration changes of betanine and its degradation products, betalamic acid and cyclodopa-5-0-glycoside. Betanine is partially regenerated. Rate constants for the degradation reaction were calculated and the activation energies for the forward and reverse reactions were determined to be 17.6 and 0.64 Kcal/mole, respectively. The enthalpy change ($\triangle H$) was calculated as 16.8 Kcal. The presence of betalamic acid and cyclodopa-5-0-glycoside favor regeneration of the pigment. A kinetic reaction model which accounts for the reversibility of the degradation reaction was adopted and can predict the amount of betanine remaining before and after regeneration of the pigment.

INTRODUCTION

BETANINE, the major pigment in beet, is a water soluble pigment which has been suggested as a colorant for many foods (von Elbe, 1977). Application of this pigment as food colorant is limited by its lack of thermostability in aqueous solution. Many studies have dealt with the thermostability of betanine (von Elbe et al., 1974; Saguy, 1979; Pasch and von Elbe, 1979; Sapers and Hornstein, 1979; Saguy et al., 1979). In the presence of oxygen, the degradation of betanine follows first-order reaction kinetics but deviates from first-order reaction kinetics in the absence of oxygen (Attoe and von Elbe, 1982). The degradation reaction of betanine in solutions has been shown to be partially reversible, and this regeneration of the pigment is pH dependent (von Elbe et al., 1981; Bilyk and Howard, 1982). The reported data suggest that to increase the thermostability of betanine, the level of oxygen present in the reaction mixture must be minimized and conditions for regeneration of the pigment maximized.

Betanine in solution upon heating hydrolyzes into betalamic acid (BA) and cyclodopa-5-0-glycoside (CDG) (Kimler, 1972; Schwartz and von Elbe, 1983). The regeneration of betanine, which is the reverse reaction of the hydrolysis process, involves a Schiff base condensation of the amine of CDG with the aldehyde of BA (von Elbe et al., 1981). Betanine rapidly forms when solutions of BA and CDG are combined (Schwartz and von Elbe, 1983). It is well known that Schiff base reactions are reversible and temperature and pH dependent (Capon, 1972). The objectives of this study were: (1) to apply a reaction model, utilizing kinetics that account for the reversibility of the degradation and (2) to examine if the model chosen will predict the degradation and regeneration of betanine under specified conditions, particularly in the absence of oxygen.

MATERIALS & METHODS

Preparation of betanine, betalamic acid and cyclodopa-5-0-glycoside

Betanine was purified from reconstituted beet powder using Sephadex G-25 column chromatography and preparatory high-performance liquid chromatography (HPLC) (Schwartz and von Elbe, 1980). BA and CDG were produced by heating pure betanine in solution (pH 5.0) at 90°C. The degradation products were separated and isolated by preparatory HPLC (Bondapak C_{18}/Porasil B column, Waters Assoc. Milford, MA) using CH_3CH + 5mM tetrabutylammonium phosphate (PICA, Waters Associates) 14: 86, v/v) as eluent (Schwartz and von Elbe, 1983). CDG was further purified by re-chromatographing on preparatory HPLC using CH_3OH + 5mM NaH_2PO_4 (12:88, v/v) as the eluent. Purified fractions were freeze-dried and stored at −15°C under nitrogen until used.

Authors Huang and von Elbe are affiliated with the Dept. of Food Science, Univ. of Wisconsin, Madison WI 53706.

Sample preparation and reaction conditions

Samples were prepared by dissolving appropriate amounts of pigment to achieve the desired conditions in a 0.1M citrate-phosphate buffer at pH 5.0. Two milliliter glass vials fitted with sleve-type rubber stoppers were used as reaction vessels. Approximately 1.5 mL of sample solution was introduced into each vessel. No correction for come-up time of the heating period was made, because of the short time involved compared to the overall heating period. At 90°C the come-up time was measured to be 10 sec while the minimum heating period was 2 min. Samples were flushed with nitrogen to reduce oxygen. Between 95-99% of the dissolved oxygen was removed within 2 min (Attoe and von Elbe, 1982). Air saturated solutions were used for comparison. Degradation of betanine and changes in concentration of BA and CDG were measured at 65, 75, 85 and 90°C. Degradation rates of BA and CDG were determined independently at 65 and 90°C. The samples were held at the desired temperature in a controlled water bath for the indicated time intervals. Six vials were removed for each time and temperature to allow for triplicate analysis immediately after heating and after holding for 20 hr at room temperature. This 20 hr holding time was to allow for regeneration to occur. Each vial was wrapped in aluminum foil during the heating and holding periods to present exposure to light (Attoe and von Elbe, 1981). Concentration of each compound was determined by HPLC using 12% CH_3CH in 5mM tetrabutylammonium phosphate as mobile phase on a μ-Bondapak C_{18} column (Schwartz and von Elbe, 1983). The compounds were monitored at 313, 436 and 546 nm using a Water's model 440 dual wavelength detector (Waters Associates, Milford, MA) and a Vari-Chrom liquid chromatography detector (Varian Associates, Inc., Palo Alto, CA). Betanine, BA and CDG were quantified applying reported molar absorptivity (ϵ) values (BA = 3.0×10^4, Kimler et al., 1971; Betanine = 6.5×10^4, Schwartz and von Elbe, 1980; CDG = 4.1×10^3, Wyler and Chiovini, 1968).

Data analysis

Replicated determinations for each reaction temperature and time were averaged to obtain one single datum point. Nonlinear regression analysis and nonlinear differential equations were solved numerically using a Sperry Univac 1100 computer system (Academic Computing Center, University of Wisconsin-Madison).

RESULTS & DISCUSSION

Reversibility of betanine degradation

Betanine in solution upon heating at high temperature, degrades to form BA and CDG at pH 5.0 (Schwartz and von Elbe, 1983). Fig. 1 shows a HPLC chromatogram of betanine in solution under nitrogen before (1) and after (2) heating at 90°C for 50 min. The chromatograms monitored at 313 and 436nm showed a decrease in betanine (B1; B2) with the accumulation of CDG and BA respectively, while the chromatogram monitored at 546nm only detected the

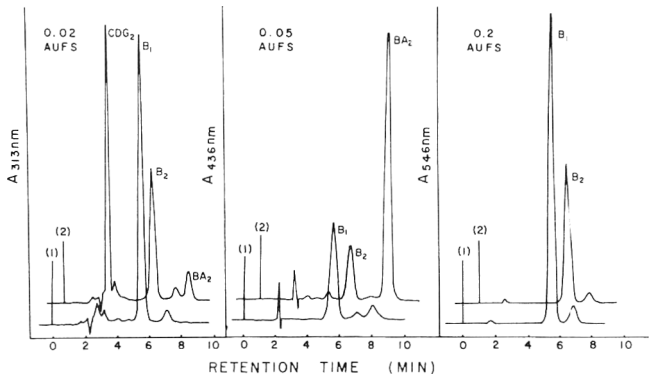


Fig. 1—HPLC chromatograms of betanine solutions monitored at 313, 436 and 546 nm before (1) and after heating (2) at 90°C under nitrogen for 50 min. Betanine (B), betalamic acid (BA) cyclodopa-5-0-glycoside (CDG).

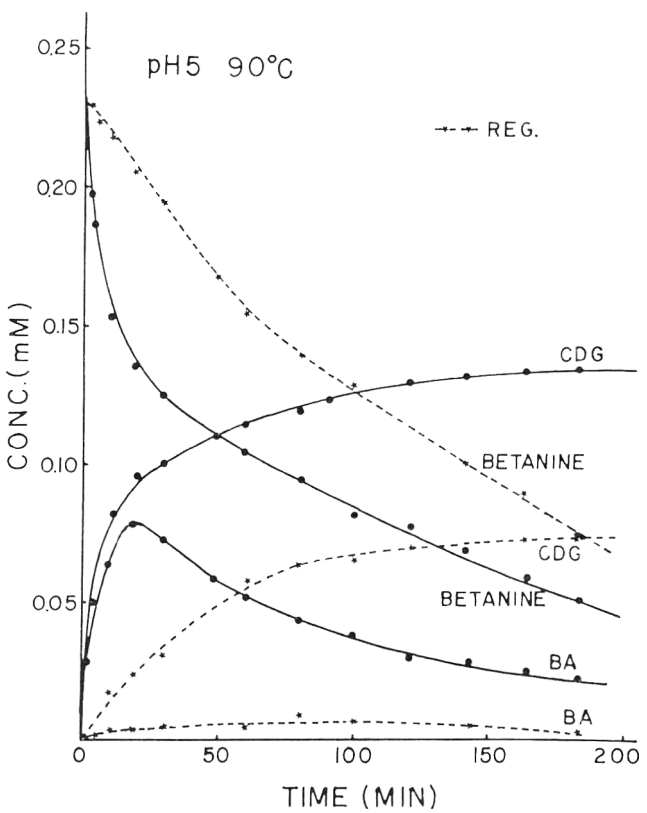


Fig. 2—Concentration changes of betanine, betalamic acid (BA) and cyclodopa-5-0-glycoside (CDG) at pH 5.0 after heating at 90°C before regeneration (solid line) and after regeneration (dashed line). Data points represent the average of duplicate determinations.

decrease in betanine. Typical concentration changes of betanine, BA and CDG before (solid line) and after (dashed line) regeneration, when 0.23mM solutions were heated at 90°C over time are shown in Fig. 2. In this study the small amount of isobetanine, formed during heating through the isomerization of betanine, was considered as betanine. In the first 20 min of heating, the concentration of betanine (solid line) decreased rapidly to 0.136 mM or 57.6%. During the same period, the concentrations of BA and CDG increased to more than 0.07 and 0.09 mM, respectively. After the first 20 min, the concentration of betanine decreased more slowly with a simultaneous decrease in BA, while the concentration of CDG increased slightly. This

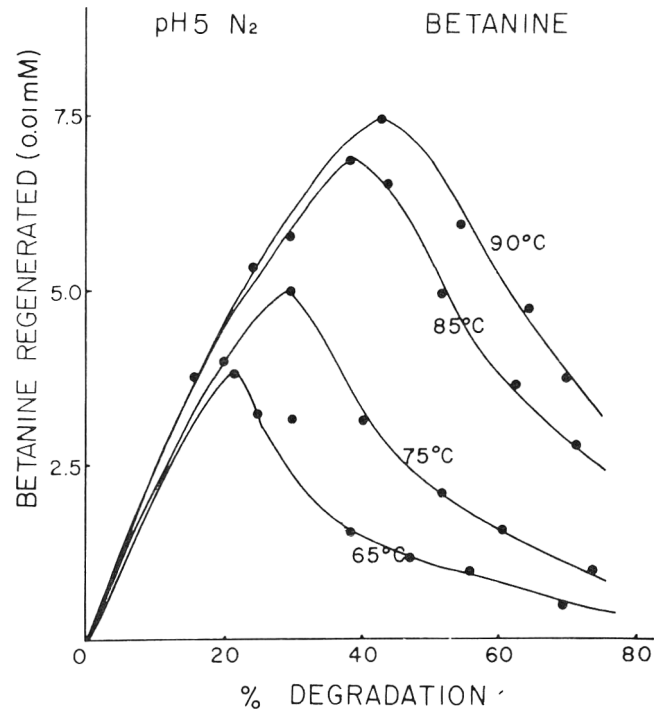


Fig. 3—Amount of betanine regenerated after heating at various temperatures. Initial betanine concentration was 0.25 mM.

change in degradation rate can not be described by either first or second-older reaction kinetics as noticed by Attoe and von Elbe (1982).

The increase in concentration of betanine after the 20 hr holding period was because of the regeneration of betanine from BA and CDG. This regeneration process was demonstrated by the lower concentrations of both BA and CDG when compared to the concentrations (solid line) before regeneration. This partial regeneration which was previously reported by von Elbe et al. (1981) was limited by the heat sensitivity of BA. In absence of oxygen BA will undergo an aldol condensation thereby making unavailable the aldehyde group required in the regeneration of betanine from BA and CDG. The data in Fig. 2 show that the BA concentration after regeneration is virtually zero and therefore complete regeneration of betanine is not possible.

Data represented in Fig. 3 show the difference in amount of pigment regenerated at different temperatures. The maximum amount regenerated depended upon the available amount of BA. At 90°C the maximum amount of BA available for regeneration was at a time when 40% of the betanine was degraded. Similar data obtained at 65°C showed that the maximum amount of BA in the reaction mixture was obtained after 20% of betanine was degraded. It should be noted though the percentage of pigment regenerated at 90°C was greater compared to that at 65°C, the total loss of betanine at 90°C was much greater than 65°C. The degree of the regeneration of betanine was therefore dependent on the temperature and the amount of BA present.

Rate equation

Any proposed reaction mechanism must yield a rate expression that is consistent with the kinetics observed experimentally (Hill, 1977). Since the regeneration of betanine involves a Schiff base condensation the reaction (Fig. 4) can be described by the following rate equations.

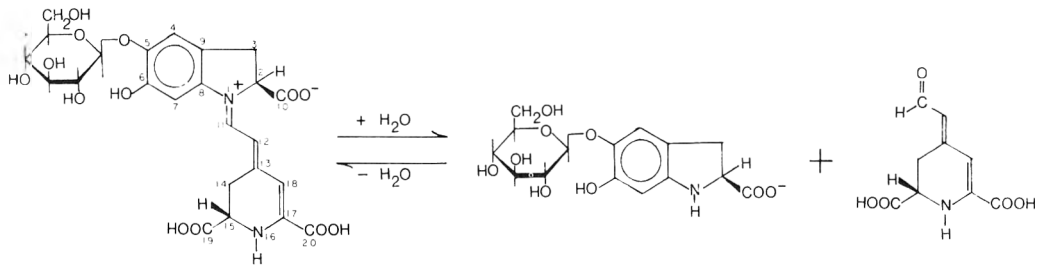


Fig. 4—Mechanism for the degradation of betanine (betanine ≒ cyclodopa-5-O-glycoside and betalamic acid) from von Elbe et al. (1981).

Table 1—Rate, equilibrium constants and activation energies for the degradation and regeneration reactions of betanine

Rate constant ($\times 10^2$)	Temperature (°C) 90	85	75	65	E_a (Kcal/mol)
k_1[a]	6.3 ± 0.3	4.5 ± 0.2	2.1 ± 0.1	1.01 ± 0.05	17.3 ± 0.8
k_{-1}[b]	86.7	85.6	83.3	81.0	0.64
k_2[a]	1.6 ± 0.2 (2.0)[c]	1.1 ± 0.1	0.53 ± 0.06	0.26 ± 0.02 (0.27)[c]	18.2 ± 2
k_3[a]	0.22 ± 0.03 (0.17)[c]	0.15 ± 0.02	0.048 ± 0.005	0.017 ± 0.002 (0.01)[c]	29 ± 3
Equilibrium Constant $K(\times 10^2)$	7.3 ± 0.3	5.3 ± 0.3	2.5 ± 0.1	1.25 ± 0.05	△H (Kcal/mol) 16.8 ± 2.5

[a] First order rate constant (min^{-1})
[b] Second order rate constant (min^{-1} mM^{-1})
[c] Values in parentheses were determined experimentally and derived by degrading pure BA and CDL in solution

$$dA/dt = -k_1A + k_{-1}BxC \quad (1)$$
$$dB/dt = k_1A - k_{-1}BxC - k_2B \quad (2)$$
$$dC/dt = k_1A - k_{-1}BxC - k_3C \quad (3)$$

where A, B, and C represent the concentrations of betanine, BA and CDG, k_1 and k_{-1} are the forward and reverse rate constants of betanine, and k_2 and k_3 are apparent degradation rate constants for BA and CDG respectively. First-order reaction kinetics were assumed for the degradation of BA and CDG.

These first order differential equations are not analytically solvable because of the existence of the nonlinear term (BxC). Verification of the model requires determination of the rate constants from experimental data and the application of computer to solve the nonlinear differential equations numerically.

Determination of rate constants

Both k_2 and k_3 are rate constants of a simple first-order reaction and are required to calculate k_1. Values for these two rate constants were determined by adding Eq. (1) and Eq. (2):

$$dB/dt + dA/dt = -k_2B \quad (4)$$

This equation states that the concentration change in betanine plus the concentration change in BA in a very short period of time (dt) equals the degradation rate of BA. Therefore the experimentally determined values of the changes of betanine and BA allow for the calculation of k_2.

Similarly the addition of Eq. (1) and Eq. (3) results in:

$$dC/dt + dA/dt = -k_3C \quad (5)$$

and the determination of changes in betanine and CDG allows for the calculation of k_3.

General differential method (Hill, 1977) was used to solve Eq. (4) and Eq. (5). Values of k_2 and k_3 were calculated for temperatures of 65, 75, 85 and 90°C. The Arrhenius equation was used to establish the temperature relationship among rate constants. Such use for k_2 results in the following equation:

$$(B_{t+\Delta t} - B_t) + (A_{t+\Delta t} - A_t) = \Delta t B_{t+\Delta t/2} k_2 \exp\left[\frac{-E_{a_2}}{R}\left(\frac{1}{T} - \frac{1}{T_r}\right)\right] \quad (6)$$

where B_t is concentration of BA at time t; A_t is concentration of betanine at time t; and T_r is the reference temperature (80°C).

A nonlinear regression analysis package (Nonlinear regression routine, Academic Computing Center, UW-Madison) was used to solve the equation. The reference temperature of 80°C was chosen, since it closely approached the mean value of temperatures used in this study.

Rate constants k_2 and k_3 were also evaluated by heating separately pure BA and CDG in solution under nitrogen at selected temperatures. Results showed little difference between numerically calculated rate constants and experimentally determined rate constants. The experimental values are shown in parenthesis in Table 1.

Plotting the ratio of B × C/A against percent degradation of betanine (Fig. 5) resulted in a plateau at each of the temperatures studied. The observed plateau suggested that a quasiequilibrium was established between betanine and its degradation products. However, because BA continued to degrade a true equilibrium was not reached. A reaction is in quasiequilibrium condition when the forward and reverse rates of the equilibrium step are much greater than the rates of other steps in the reaction sequence. The quasiequilibrium constant K_q can be used as the initial estimate of the true equilibrium constant K because the plateau suggests the equilibrium is closely approached.

The relationship between the equilibrium constant and the forward and reverse rate constants, k_1 and k_{-1} is as follows:

$$K = k_1/k_{-1} \quad (7)$$

Substituting Eq. (1) into Eq. (7) over the temperature range studied results in:

$$A_{t+\Delta t} - A_t = \Delta t\left(-A_{t+\Delta t/2} + \frac{1}{K}B_{t+\Delta t/2}k_{1r}\exp\left[\frac{-E_{a_1}}{R}\left(\frac{1}{T} - \frac{1}{T_r}\right)\right]\right) \quad (8)$$

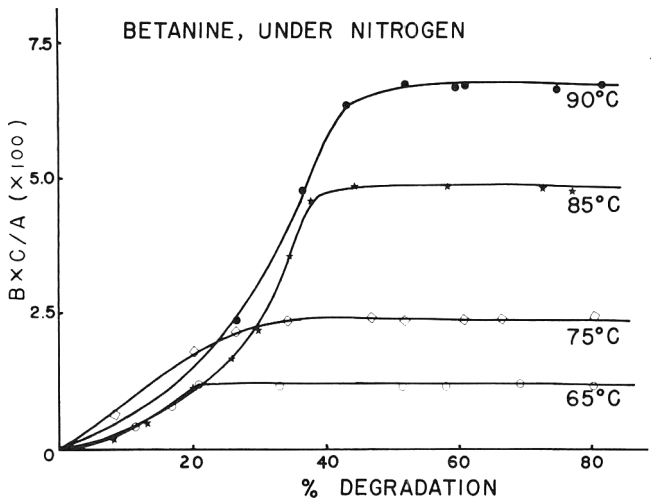


Fig. 5–Ratio of BxC/A vs % degradation at four temperatures. A,B,C = betanine, betalamic acid and cyclodopa-5-O-glycoside concentration, respectively.

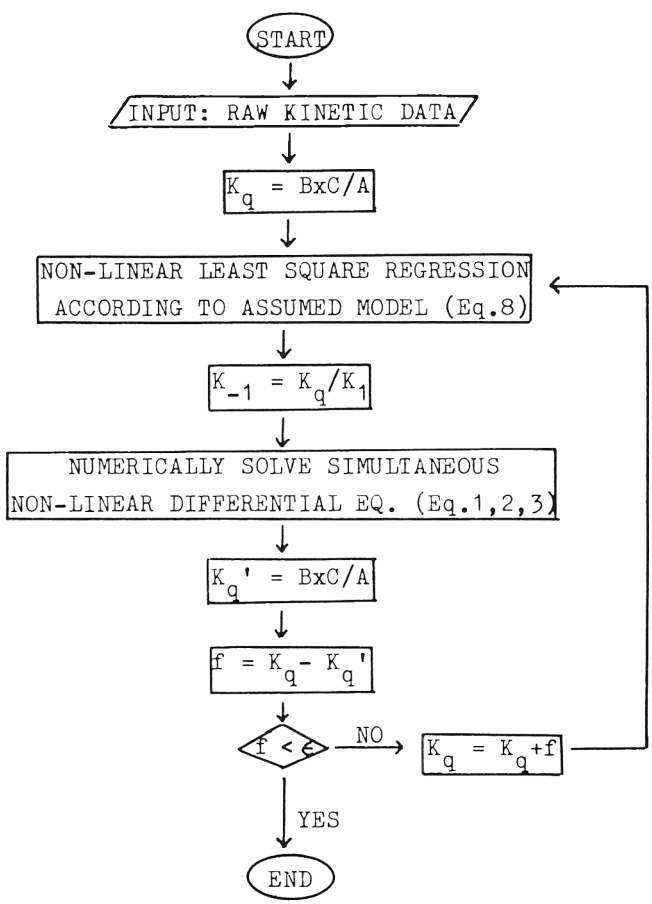


Fig. 6–Flowchart of the computer iteration process to calculate the ratio of the forward and reverse rate constants (k_1; k_{-1}) for the betanine degradation reaction.

The nonlinear regression analysis gives in k_1 and Ea_1. Values of k_{-1} were calculated from the relationship in Eq. (7). k_{-1} could not be determined experimentally because the time of the regeneration of betanine was too short.

The first-estimated values for k_1 and k_{-1} together with previously determined values for k_2 and k_3 allow for the evaluation of the simulatenous nonlinear differential equations [(Eq. (1), (2) and (3)] and a calculated value of B x C/A (K_q'). The difference between the experimentally determined K_q and mathematically determined K_q' depends on the magnitude of k_2 and k_3. If both the values of k_2 and k_3 are equal to zero, then $K_q' = K_q = K$. If k_2 and k_3, as in this study, are not equal to zero, the consecutive reaction mechanism applies and a correction factor (f) between the difference of K_q and K_q' must be applied.

Fig. 6 is a flow chart of the computer iteration process to establish the ratio of k_1/k_{-1}. Values for rate constant k_1, k_{-1}, k_2, k_3 in the absence of oxygen and the activation energies (E_a) are listed in Table 1.

The values for the rate constants k_1 and k_{-1} are at least four times the values of the constants k_2 and k_3. The concentration of BA during the degradation of betanine is approximately half of the concentration of betanine remaining at any one time during the heating period (Fig. 2). Therefore the degradation rate of betanine to BA and CDG (i.e., $k_1 \times A$) and the rate of the reverse reaction (i.e., $k_{-1} \times B \times C$) are approximately eight (4×2) times faster than the further degradation rates of BA and CDG. This result justifies the assumption of the existence of a quasi-equilibrium observed during the degradation of betanine in solution in the absence of oxygen.

The value of k_2 is approximately ten times larger than the value of k_3. Therefore, the degradation of BA was much faster compared to CDG and was the major cause of the loss of betanine under oxygen free condition, because it was no longer available for the regeneration of betanine.

The E_a of reverse reaction was determined as 0.64 Kcal or practically zero. According to the activated complex theory, the E_a of a reverse reaction equals the difference between the E_a of the forward reaction and the enthalpy change ($\triangle H$) of the equilibrium. The value of $\triangle H$ was obtained by regressing the equilibrium constant at each temperature according to the van't Hoff equation. The $\triangle H$ for the equilibrium of betanine and its degradative compounds was determined as 16.8 Kcal. According to the theory therefore the E_a of the reverse reaction can be calculated as 17.3-16.8 = 0.5 (Kcal). This calculated value was in close agreement with the value of the determined E_a. The small temperature dependence of the reverse reaction favors the preservation of betanine at low temperature but favors the accumulation of BA and CDG at high temperature.

Computer simulation of the change of betanine concentration

Computer simulation of the degradation and regeneration of betanine requires the capability to solve the rate equations [(Eq. (1), (2) and (3)]. The data needed are the values for rate constants k_1, k_{-1}, k_2, k_3 and the initial concentrations of betanine, BA and CDG. Concentration of betanine, BA and CDG over time is the numerical solution of the equations. The regeneration of betanine can be calculated from the shift in equilibrium constant when the temperature changes from temperature of reaction to room temperature (25°C). The value of K at room temperature was calculated according to the van't Hoff equation as 3.65×10^{-4}mM.

If the concentrations of betanine, BA and CDG before regeneration are A_i, B_i and C_i respectively, and x represents the amount of betanine regenerated, the equilibrium constant at the temperature of regeneration (i.e., room temperature) K_{rt} will be:

$$K_{rt} = (B_i - x)(C_i - x)/(A_i - x) \quad (9)$$

where x can be algebraically solved as:

$$x = 1/2[b - (b^2 - 4c)^{0.5}] \quad (10)$$

where $b = (B_i + C_i + K_{rt})$ and $c = (B_i C_i - A_i K_{rt})$.

The above calculation is based on the assumption that the excess BA and CDG resulted from lowering the temperature of reaction temperature to room temperature would condense to form betanine rather than degrading further. Based on the kinetics data for BA and CDG in Table 1, for the period of regeneration (less than 24 hr at 25°C) it can be assumed that only a small percentage of BA and CDG will degrade further. This assumption can also be justified by the experimental data (Fig. 2) which show that the amounts of BA and CDG decreasing during the regeneration period were approximately equal to the amount of betanine formed.

Computer derived concentration changes at 65 and 90°C of betanine, BA and CDG over time are shown in Fig. 7. The experimental data are represented by the data points while the computer simulated data are represented by the solid lines, before regeneration and broken lines, after regeneration. The computer simulation, as can be seen, agrees well with experimental data. The data illustrate again that the shape, as well as curve pattern, changes with temperature; and that the difference between the concentration of betanine before and after regeneration at 65°C is less compared with 90°C. A drastic change in degradation rate at 65°C occurred at a point where approximately 80% of betanine remained while at 90°C the change occurred after approximately 65% of betanine remained. These differences illustrate the importance of considering the regeneration process when the overall loss of betanine at elevated temperatures is to be determined.

In the study by Attoe and von Elbe (1982) the authors noted that the degradation of betanine in solution under nitrogen was dependent on the initial concentration and did not follow first order reaction kinetics. A computer simulation using the present model for this situation is illustrated in Fig. 8. The difference in degradation rates among different pigment concentrations can be explained considering the equilibrium constant. The equilibrium constant K_1 whether the initial concentration of betanine is high or low, is the same for all concentrations. Therefore the amount of betanine degrading in any specific time period to reach the equilibrium, expressed in percentage of betanine remaining, is much less when the initial concentration is high than when the initial concentration is low. For example, at 65°C K = 0.0125 mM. Under this condition an initial betanine concentration of 1.32 mM (725 ppm) only needs to degrade by approximately 9.2% to reach the quasiequilibrium while at an initial concentration of 0.025 mM (14 ppm) 49% of the betanine must degrade to reach the same K value.

von Elbe et al. (1974) reported that betanine in beet juice is more stable than pure betanine in solution. This difference in stability may be explained by the reversibility of the degradation reaction and the presence of some betalamic acid and CDG in beet juice. BA has been reported as a natural yellow pigment in beets (Kimler et al., 1971). Applying the computer simulation to the change in betanine concentration when CDG was present initially at concentration of 20 and 50% of the molar concentration of betanine, the half life values of betanine at 65°C, pH 5.0 increased from 28.6 hr to 55 hr and 76 hr, respectively. The presence of CDG at 20% of the molar concentration of betanine increased the stability of betanine by a value which closely approached the difference in betanine stabilities noted by von Elbe et al., (1974) between betanine in beet juice and betanine in pure solutions.

Betanine degradation under oxygen

Degradation of betanine in solution under an excess of oxygen has been described by a first-order reaction kinetics (Saguy et al., 1979; Attoe and von Elbe, 1982). This

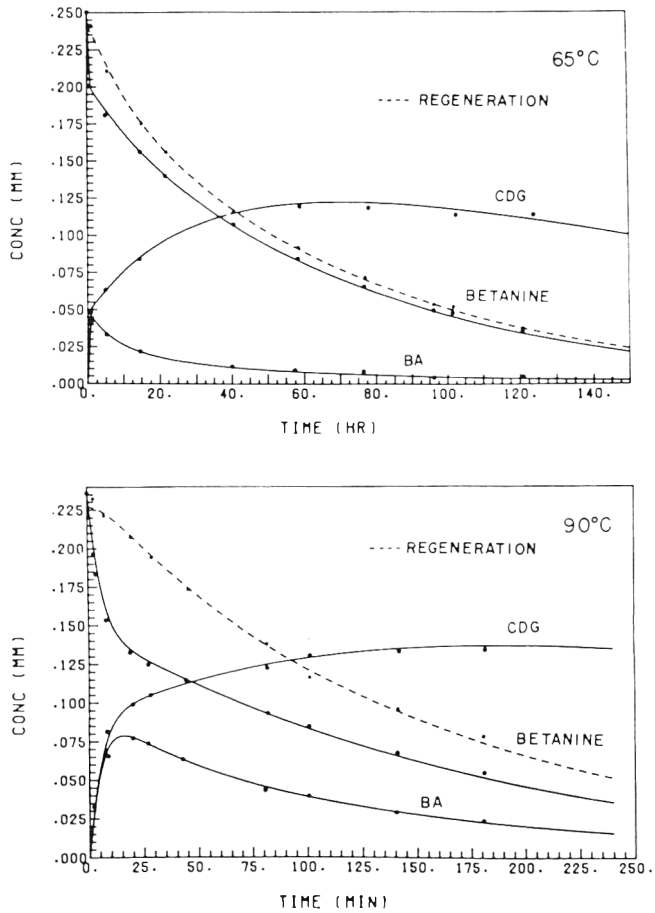


Fig. 7—Computer derived concentration changes at 65 and 90°C under nitrogen betanine, betalamic acid (BA), and cyclodopa-5-0-glycoside (CDG) over time.

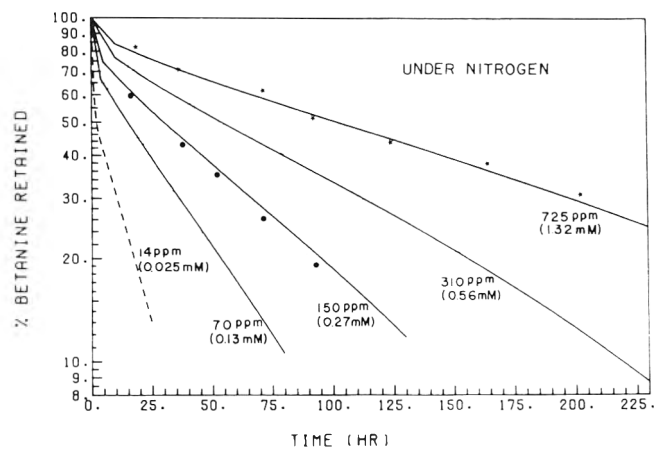


Fig. 8—A computer simulation of betanine in solution at various initial concentrations at 65°C, pH 5.0 [Adapted from Attoe and von Elbe (1982)]

phenomenon can also be described by the model under study. Under oxygen neither rate constants k_1 or k_{-1} is affected. Difference between the rate of degradation in absence or presence of oxygen is manifested in difference in the rate constants k_2 and k_3. The rate constant k_2 and k_3 at 90°C for BA and CDG were 0.045 and 0.074 mM^{-1} under oxygen and 0.016 and 0.00022 mM^{-1} under nitrogen.

Substituting Eq. (7) into Eq. (1) and doing some mathematical calculations result in:

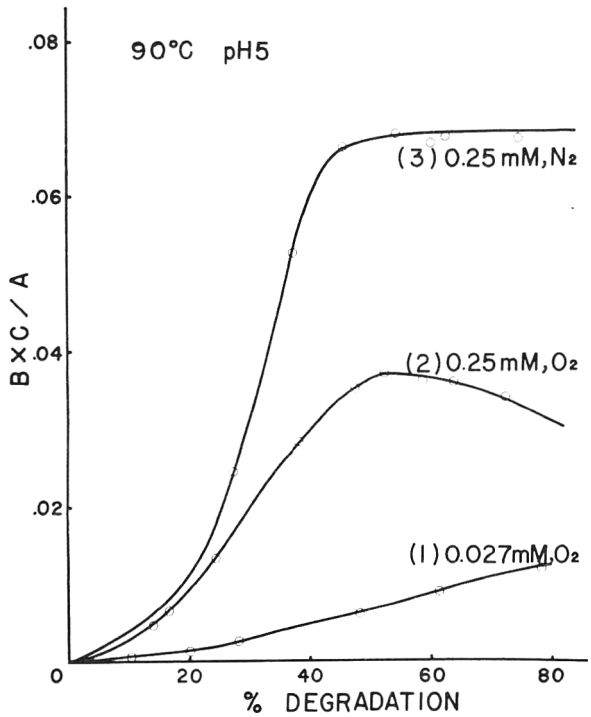


Fig. 9—Ratio of BxC/A vs % of degradation of betanine in solution in presence of no oxygen (line 3) and levels of oxygen equal (line 2) or greater (line 1) than the initial betanine concentration. A,B,C = betanine, betalamic acid and cyclodopa-5-0-glycoside concentrations, respectively.

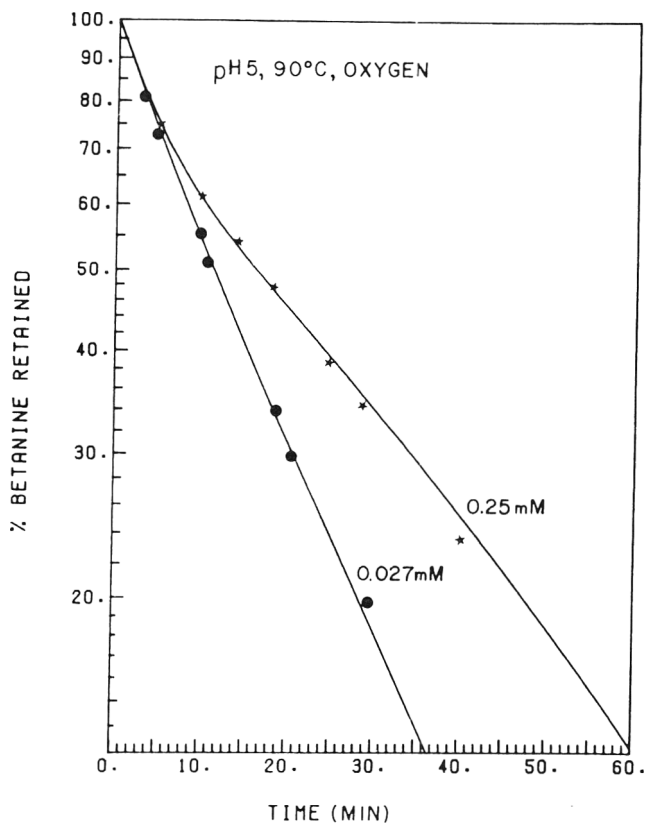


Fig. 10—A computer simulation of the betanine degradation at 90°C, pH 5.0 when the level of oxygen is greater than or equal to the initial betanine concentration.

$$dA/dt = [-1 + (B \times C/A)/K]k_1 \times A \qquad (11)$$

Under oxygen at low betanine concentration (i.e., excessive oxygen) the ratio of (B × C/A)/K is much smaller than 1, and the reaction kinetics appear to be first order. Data presented in Fig. 9 illustrate this condition. In a reaction where oxygen was in excess (line 1), when B × C/A was plotted against percentage of degradation, the ratio was approximately zero throughout the degradation time. However, when the betanine concentration equaled the oxygen concentration (line 2) the ratio B × C/A was approximately 50% of the ratio when oxygen was absent (line 3). Further increases in the betanine concentration would result in a ratio which approaches the ratio when O_2 was absent. This concentration dependency of the reactants explains the deviation from first order kinetics observed in the study by Attoe and von Elbe (1982). A computer simulation of the betanine degradation at 90°C, pH 5.0 using the proposed model when oxygen is in excess or equal to betanine concentration is shown in Fig. 10. The data verify first order kinetic mechanism when oxygen is in excess and deviation from first order kinetics when oxygen is limited.

CONCLUSION

THE DEGRADATION of betanine in solution resulted in the formation of betalamic acid and cyclodopa-5-0-glycoside. This reaction was partially reversible and was shown to depend upon the initial betanine concentration and temperature. A kinetic model was used which could account for the reversibility of the degradation reaction and therefore predict the amount of betanine remaining before and after regeneration of the pigment under various experimental conditions. The reaction rate constants and activation energies could be determined. The E_a of the forward reaction was calculated as 17.3 Kcal while that of the reverse reaction as 0.64 Kcal. The difference in these two values explained the greater percentage of regeneration of the pigment after heating at high temperature. The low temperature dependency of the reverse reaction caused the retention of betanine at low temperatures and explained why at temperatures slightly above room temperature (25°C) regeneration of the pigment was insignificant. Reversibility of the reaction was responsible for the deviation from first-order reaction kinetics observed in the degradation of betanine in the absence of oxygen (Attoe and von Elbe, 1982). The accumulation of BA and CDG during betanine degradation increased the rate of the reverse reaction and therefore decreased the amount of betanine that degradated.

This mechanism can also be applied to the degradation of betanine in solution in the presence of oxygen. The larger rate constant of BA and CDG degradation under oxygen account for the difference in the half-life value at the same temperatures when betanine was degraded under nitrogen or oxygen.

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—Continued on page 1129

Influence of Fruit Composition, Maturity and Mold Contamination on the Color and Appearance of Strawberry Wine

LETICIA S. PILANDO, RONALD E. WROLSTAD, and DAVID A. HEATHERBELL

ABSTRACT

Color composition of strawberry wines made from ripe, overripe, and mold-contaminated fruit (Benton and Totem varieties) was monitored over 6 wk storage. Overripe fruit with its higher anthocyanin and total phenolics gave wines with better color than fully ripe fruit. Totem fruit had higher anthocyanin, lower polyphenol oxidase activity and lower total phenolics than the Benton variety and produced wines with better color. Mold contamination increased juice viscosity, reduced fermentation rate, and accelerated color degradation. Anthocyanin content and Hunter L values of wines were highly correlated with panel color evaluations.

INTRODUCTION

A STRAWBERRY WINE of good quality has the appealing color of a premium rose wine. Unfortunately, the attractive color is often short-lived, changing to an undesirable brown color. Commercial producers of strawberry wine say that color degradation is highly variable, erratic and the principal cause for decreased consumer appeal; hence, optimization of strawberry wine color quality is important to the fruit wine industry.

Compositional differences due to maturity and variety influence color deterioration, mainly due to the instability of the anthocyanin pigment coupled with enzymic as well as nonenzymic browning. The polymerization of anthocyanins and reactive phenolics is a major cause of color deterioration in strawberry preserves (Abers and Wrolstad, 1979). Ascorbic acid accelerates destruction of anthocyanin pigments and contributes to browning (Poei-Langston and Wrolstad, 1981). Polyphenol oxidase (PPO) catalyzes browning reactions by acting on *d*-catechin present in strawberries (Wesche-Ebeling, 1980); β-glucosidase can also decolorize anthocyanins (Huang, 1955).

One could speculate that the higher level of anthocyanin pigments in overripe fruit would mask brown discoloration and give better color. Some wine makers believe, however, that the use of overripe fruit results in poorer color quality and stability. Pronounced browning has been reported in overripe frozen strawberries (Sistrunk and Moore, 1971), and this may account for poorer color in wine made from overripe fruit. Overripe fruit is highly susceptible to mold and rot, mainly by *Botrytis cinerea*. Mold contamination has been associated with quality deterioration in grape wines. *B. cinerea* affects the compositional balance of musts and produces "botryticine," an antibiotic that inhibits yeast development (Hildalgo, 1979). *Botrytis* infection is related to reduced acidity and increased pH, increased dry extract, reduced color intensity and reduced anthocyanins and tannins (Cassignard et al., 1977).

The purpose of this investigation was to determine the influence of maturity on strawberry composition and its relationship to wine color quality. Two varieties were studied, Benton and Totem, the former containing moderate levels of anthocyanin and the latter being high in pigment. The effect of mold contamination on strawberry wine quality was also investigated.

Authors Pilando, Wrolstad, and Heatherbell are affiliated with the Dept. of Food Science & Technology, Oregon State Univ., Corvallis, OR 97331.

MATERIALS & METHODS

Source and treatment of strawberry samples

Totem and Benton strawberries were hand-picked from field plots at the OSU Lewis-Brown farm throughout the 1981 season. The fruits were capped, washed, and categorized as fully ripe (FR), overripe (OR), or moldy (M) by color and texture. Fruits were individually quick frozen (IQF) at −40°C, placed in polyethylene bags, sealed in plastic containers, and stored at −10°C.

Compositional analysis

Extracts were prepared from 50g of liquid nitrogen powdered strawberries by extracting with 100 mL acetone and filtering. The filtrate was shaken in a separatory funnel with 240 mL chloroform and then allowed to partition. The chloroform-acetone phase was discarded and the aqueous phase was retained for analyses.

Total anthocyanin pigment was determined using a pH differential method (Wrolstad, 1976) and expressed as mg of pelargonidin-3-glucoside per g of fruit using a molar absorptivity of 22,400. Leucoanthocyanin was measured using the method of Swain and Hillis (1959) as absorbance units per g of fruit, and flavanol by the method of Swain and Hillis (1959) as mg catechin per g of fruit, using *d*-catechin (K & K Labs., Inc.) as the standard. Total phenolics were determined by the Singleton and Rossi (1965) procedure as gallic acid equivalents; total phenolics were also estimated from the absorbance at 280 nm. Nonflavonoid phenols were measured using the procedure of Watson (1975) and reported as absorbance units at 765 nm.

Soluble solids were determined on strawberry juice with an Abbe refractometer; total titratable acidity as meq citric acid per 100g fruit by titrating to pH 8.1 with 0.1N NaOH. Ascorbic acid was measured on liquid nitrogen powdered samples by the procedure by Loeffler and Ponting (1942) and expressed as mg ascorbic acid per 100g fruit. Measurement of pH was taken on slurries of the nitrogen powdered samples with a Corning pH meter model 125.

PPO extraction and activity assay were based on the procedure by Wesche-Ebeling (1980). Activity was measured using the Clark-type O_2 electrode, YSI model 53 (Yellow Springs Instrument Co., Inc., Yellow Springs, OH) with a Lauda K_2/R constant temperature circulator (30°C), and expresed as nmol of O_2 consumed per min per g of fruit.

β-Glucosidase extraction was based on the same procedure as that in the PPO extraction. The assay of Duerksen and Halvorson (1958) was used and a unit of activity reported as an absorbance change at 400 nm of 0.001/min/3 mL of reaction mixture.

Mold counts were done on the crushed samples and juice samples using the Howard Mold Count procedure described by Gould (1978) and reported as percent positive fields.

Wine-making

Wines were made following the procedure developed by Flores and Heatherbell (1984), using Benton fully ripe (B_{FR}), Benton overripe (B_{OR}), Benton fully ripe to which 15% by weight moldy Benton fruit had been added ($B_{FR/M}$), Totem fully ripe (T_{FR}), Totem overripe (T_{OR}), and Totem fully ripe to which 15% by weight moldy Totem fruit had been added ($T_{FR/M}$).

IQF strawberries (6.8 kg lots) were thawed at 20 - 23°C for 3 hr. (The samples with added moldy fruit were combined and mixed before thawing.) The strawberries were crushed with a hammermill (Model D Comminuting machine; W.J. Fitzpatrick Co., Chicago)

at 182 - 184 rpm. Two percent cellulose press aid (shredded filter paper) was added and the mixture was placed into a Willmes type bag press (Moffett Co., Inc., San Jose, CA). After pressing, 25 ppm SO_2 as a 1% sodium metabisulfite solution was added to the juice and allowed to stand for 2 hr. The juice was ameliorated to 22° Brix by adding sucrose (LSI liquid sugars/Northwest type "O"). One percent ammonium phosphate (J.T. Baker, Co., Phillipsburg, NJ) and 1% "active" yeast culture (Champagne strain, Scott Labs, Inc., San Rafael, CA) were added and mixed. The juice was transfered to 11.4L jars and allowed to ferment at 16°C until it reached 0.1 - 0.2% reducing sugars. The wines were racked and after adding 10 ppm SO_2 were bottled and stored in the dark at 25°C and at 3°C.

Color analyses

Color density (sum of the absorbance at 420 and 500 nm of strawberry wine), polymeric color (sum of the absorbance at 420 and 500 nm of bisulfite-treated wine), and % polymeric color (polymeric color/color density x 100) were determined from spectral analyses as described by Somers and Evans (1977) and Wrolstad (1976). Browning was measured as absorbance at 420 nm of the bisulfite-treated sample.

Hunter "L", "a", and "b" color parameters were measured in the transmission mode using a Hunter Model D 25 P-2 Color Difference Meter standardized against white tile (No. DC 122, L = +94.02, a = −0.9, b = +1.2). Samples were placed in 1.0 x 5.0 x 5.0 cm lucite cells. Diffuse transmittance measurements were made with the light source in the normal, aligned position (Arrangement I), excluding the specular component. Haze determinations were made by making measurements with the light source pivoted (Arrangement III); percent haze = $Y_{(\text{Arrangement I})}/Y_{(\text{Arrangement III})} \times 100$.

Table 1—Compositional analyses on Benton and Totem strawberries[a]

Composition	Benton		Totem	
	FR[b]	OR[c]	FR	OR
Total anthocyanin (mg/100g)	32.7	58.8	50.3	80.6
Flavanols (mg catechin/100g)	46.2	58.2	55.2	68.2
Leucoanthocyanins (abs units/g)	3.45	3.98	3.93	4.83
Total phenolics (mg gallic acid/g fruit)	1.50	2.27	1.31	2.06
(abs 280 nm)	0.83	1.12	0.81	1.11
Nonflavonoid phenolics (abs unit)	7.80	10.1	7.20	9.20
Ascorbic acid (mg/100g)	40.2	39.0	43.8	42.0
Soluble solids (°Brix)	7.8	10.4	7.6	8.7
Titratable acidity (mequiv/100g)	127	115	119	111
pH (23°C)	3.33	3.48	3.44	3.57
PPO (nmol/min/g)	465	400	302	201
β-Glucosidase	3	2	3	3

[a] All values reported are means of duplicate analyses
[b] Fully ripe
[c] Overripe

Sensory evaluation of color

Color quality of the wines was evaluated after 6 wk storage by a 12-member panel consisting of representatives from Oregon fruit wineries and OSU wine research personnel, all of whom were experienced in sensory evaluation of wines. A preliminary testing session was conducted where commercial strawberry wine samples were used to illustrate the attributes to be scored – color intensity, hue, clairy, browning, and over-all color quality and appearance. A desirability scale of 1 - 9 was used wherein 1 indicated "extremely undesirable," 5 for "neither desirable nor undesirable" and 9 for "extremely desirable."

Statistical analyses

A One-way Analysis of Variance (ANOVA) and simple correlation were done on the compositional analyses, color measurements of juice and wine, and the sensory evaluation data. Correlation coefficients of 0.85 and above were taken as meaningful.

RESULTS & DISCUSSION

COMPOSITIONAL ANALYSES of the fruit revealed that the Totem variety contained more anthocyanins, flavanols, leucoanthocyanins, and ascorbic acid whereas Benton was higher in PPO, total phenolics, nonflavonoid phenolics, and titratable acidity (Table 1). The overripe fruit was higher in anthocyanins, flavanols, leucoanthocyanins, total phenolics, pH, and soluble solids but lower in PPO, ascorbic acid, and titratable acidity than the fully ripe fruit. β-Glucosidase activity was extremely low for all the samples (Table 1). These results are consistent with reports that "processing-ripe" (dark red color, slightly soft) berries are higher in soluble solids, pH, and total anthocyanins but lower in titratable acidity than "firm-ripe" (well-developed red color, firm) berries (Spayd and Morris, 1981; Sistrunk and Moore, 1971). However, Reyes et al. (1982) reported lower soluble solids in overripe Benton than in fully ripe Benton.

Howard mold counts (Table 2) showed that the samples without added moldy berries were relatively free from mold contamination. The crushed strawberries with added moldy fruit had 30% and 50% positive fields for Benton and Totem, respectively. Processing into juice reduced the mold

Table 2—Howard mold counts and color analyses of strawberry juice[a]

	Benton			Totem		
	FR[b]	OR[c]	FR/M[d]	FR	OR	FR/M
Howard mold counts (% positive fields)						
Crushed strawberries	2.0	6.0	30.0	7.5	4.0	52.0
Strawberry juice	2.0	6.0	16.0	2.0	2.0	28.0
Color analyses						
Total anthocyanin, mg/L	176	398	193	258	445	309
Color density	3.78	6.18	3.98	4.32	5.58	5.36
Polymeric color	1.66	2.55	1.16	0.84	1.12	1.35
% Polymeric color	44.0	41.4	29.2	19.5	20.2	25.2
Browning	1.10	1.68	0.78	0.56	0.75	0.90
Hunter L	34.7	19.5	24.0	38.6	29.2	27.3
Hunter a	42.8	35.7	37.9	51.0	50.5	44.3
Hunter b	23.1	13.2	16.3	26.3	20.1	18.7

[a] All values reported are means of duplicate analyses
[b] Fully ripe
[c] Overripe
[d] Fully ripe with added moldy fruit

counts to 16% for Benton and 28% for Totem. Partial removal of the mycellia in the filtration unit operation could account for some of the mold count decrease. FDA regulations have set 45% as the maximum limit for acceptability of processing strawberries while only 3% is allowed for fresh strawberries (Harvey, 1982).

Color analyses of the juice samples are also shown in Table 2. Comparison of anthocyanin pigment of the juice with that originally present in the fruit (Table 1) revealed a loss in the order of 50%. The mold-contaminated juices contained more pigment than the fully ripe juices. Fungal enzymes may have facilitated the extraction of pigments through the breakdown of various polysaccharides. Benton was higher in polymeric color, % polymeric color, and browning index than the Totem. These observations could be attributed to the action of PPO and phenolics polymerization which would be greater in the Benton fruit with its higher PPO and total phenolics. The mold-contaminated juices were darker than the juice from fully ripe fruit as shown by the lower "L" values and higher color density. The development of browning was faster in the mold-contaminated Totem than in the fully ripe Totem but the opposite was observed for the Bentons. The mold-contaminated juices were more viscous and had slower fermentation rates than the uncontaminated juices.

Pigment analyses of the wines showed a tremendous decrease in anthocyanin after fermentation; only 3 - 9% of the anthocyanin originally present in the juice was retained (Table 3). Co-precipitation of anthocyanin pigments with yeast may have contributed to pigment loss. There was a progressive decrease through storage, with only 1 - 2% remaining after 6 wk. These measurements are for monomeric anthocyanin pigments whereas the color of the partially aged wine is chiefly polymeric. The Bentons showed higher pigment loss – 3.0, 4.4, and 4.1% remaining for B_{FR}, B_{OR}, and $B_{FR/M}$, respectively, compared to the 8.6, 7.0, and 5.7% for the T_{FR}, T_{OR}, and $T_{FR/M}$, respectively.

Polymeric color increased by 30 - 50% after fermentation and steadily increased during wine storage (Fig. 1). The Bentons were higher in % polymeric color. Polymerization of catechins, catalyzed by PPO, produce repeated quinone units having a high absorbance in the 400 - 500 nm range. Wines from the overripe strawberries, which were higher in total phenolics but lower in PPO, were higher in % polymeric color than the wines from fully ripe fruit. $T_{FR/M}$ and $B_{FR/M}$ were higher in % polymeric color than the T_{FR} and B_{FR}. Possibly, laccase from the mold contributed to this increase. After 6 wk, 80 - 90% of the color was due to the more stable polymeric pigments.

Browning index gradually increased in the aging wines (Fig. 2). B_{OR} and T_{OR} having the most browning. $B_{FR/M}$ and $T_{FR/M}$ had more browning than the B_{FR} and the T_{FR}. The browning reaction is the sum of the oxidative browning of phenolics, PPO-catalyzed browning reaction, Maillard browning, and ascorbic acid browning.

Hunter measurements were taken to measure the lightness, "L," redness, "a," and the yellowness, "b," of the

Table 3–Change in anthocyanin pigment content (mg/L) of wines through storage at 25°C[a]

	Benton			Totem		
	FR[b]	OR[c]	FR/M[d]	FR	OR	FR/M
Fruit	327	588	ND[e]	503	806	ND
Juice	176	398	193	258	445	309
Wine, 0 wk	5.3	17.7	7.95	22.5	31.2	17.5
Wine, 1st wk	4.5	15.2	7.9	20.4	28.3	16.5
Wine, 2nd wk	4.3	12.4	6.3	15.4	24.9	13.3
Wine, 3rd wk	3.1	10.2	5.6	9.5	16.9	8.7
Wine, 4th wk	1.8	8.9	3.8	9.3	14.4	8.0
Wine, 5th wk	1.6	7.6	4.0	6.7	11.6	7.8
Wine, 6th wk	1.4	5.7	2.8	5.9	9.2	5.5

[a] All values are means of duplicate readings
[b] Fully ripe
[c] Overripe
[d] Fully ripe with added moldy fruit
[e] Not determined

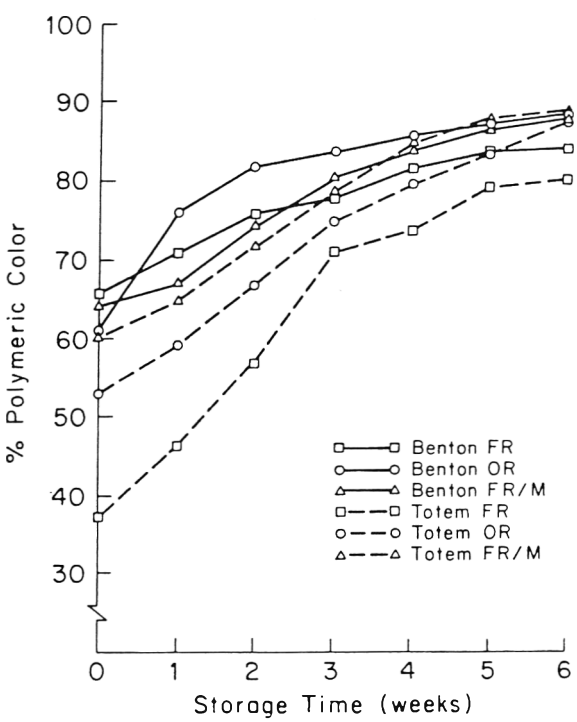


Fig. 1–Effect of storage on the % polymeric color of wines.

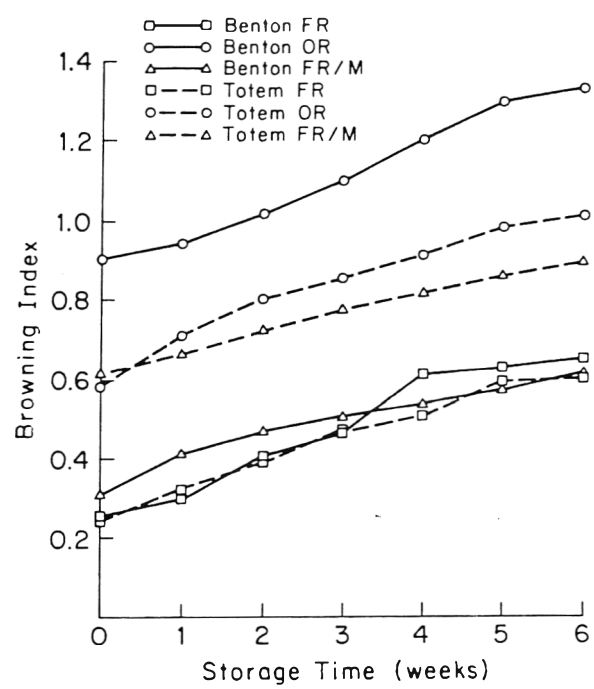


Fig. 2–Effect of storage on the browning index (abosrbance at 420 nm) of wines.

wines during storage. The "L" values generally increased during storage which showed the bleaching and lightening of the wines (Fig. 3). The B_{FR}, $B_{FR/M}$ and $T_{FR/M}$ showed a drastic increase after 2 wk storage. Varying patterns in the "a" values demonstrated the different color changes occurring during storage (Fig. 4). $T_{FR/M}$, $B_{FR/M}$, and B_{OR} showed increasing "a" values whereas T_{FR} and T_{OR} decreased while B_{FR} was relatively constant. The "b" values generally increased through storage (Fig. 5).

In the sensory evaluation, T_{OR} was rated superior, T_{FR}, $T_{FR/M}$, and B_{OR} were acceptable while the $B_{FR/M}$ and B_{FR} were unacceptable, based on the over-all color and appearance (Table 4). One-way ANOVA of the sensory data indicated statistically significant differences between the Totem and Benton variety, between the overripe and fully ripe, and between fully ripe and mold-contaminated wines (Table 5). There was no significant difference in the wines stored at refrigerated (3°C) and room (25°C) temperatures after 6 wk.

Simple correlation ($|r| \geq 0.85$) pointed out the interrelationships between composition, color measurements and sensory scores of the wines (Table 6). Total phenolics, nonflavonoid phenolics, and soluble solids of the fruit were highly correlated with color density, polymeric color, browning index, and Hunter "a" values of the wines. Likewise, anthocyanin content, titratable acidity, pH, and PPO of the fruit were highly correlated to anthocyanin content of the wine. PPO was highly correlated to over-all color and appearance, color intensity, and browning. Over-all color and appearance, color intensity, and hue measured

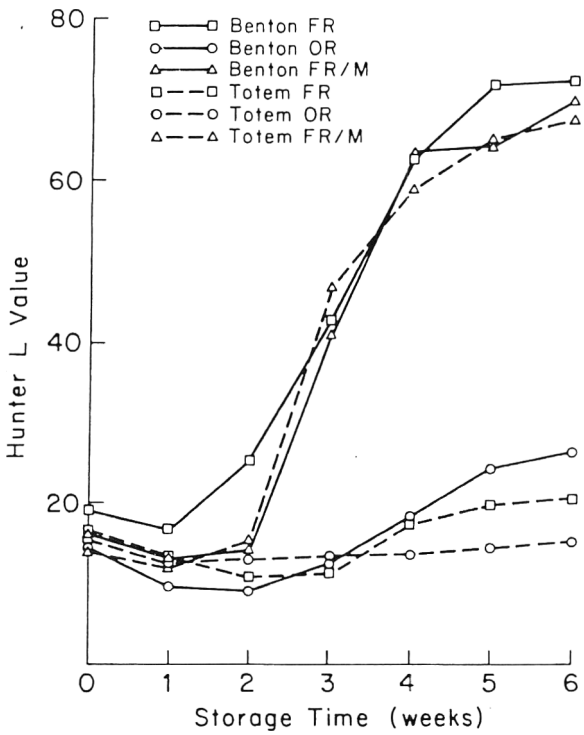


Fig. 3—Effect of storage on the Hunter "L" values of wines.

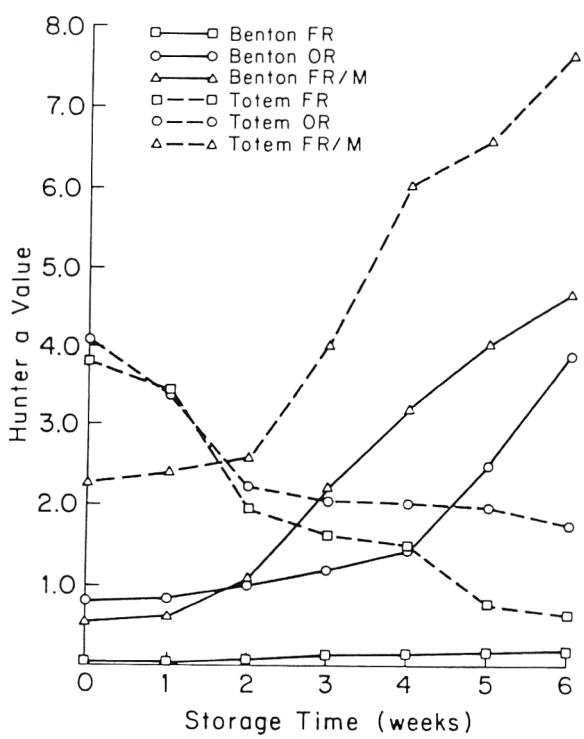


Fig. 4—Effect of storage on Hunter "a" values of wines.

Table 4—Sensory evaluation of strawberry wines stored for 6 wk at 25°C[a]

	Color intensity	Hue	Browning	Clarity	OCA[b]
Benton FR[c]	2.5	2.4	4.1	3.0	2.5
Benton OR[d]	5.5	6.0	4.7	5.8	5.0
Benton FR/M[e]	3.7	3.7	4.7	5.2	3.8
Totem FR	6.3	6.2	6.0	7.0	6.0
Totem OR	7.0	7.1	6.4	7.6	7.1
Totem FR/M	5.1	5.2	5.2	3.8	4.5

[a] All reported values are means of 12 panel scores
[b] Color and appearance
[c] Fully ripe
[d] Overripe
[e] Fully ripe with added moldy fruit

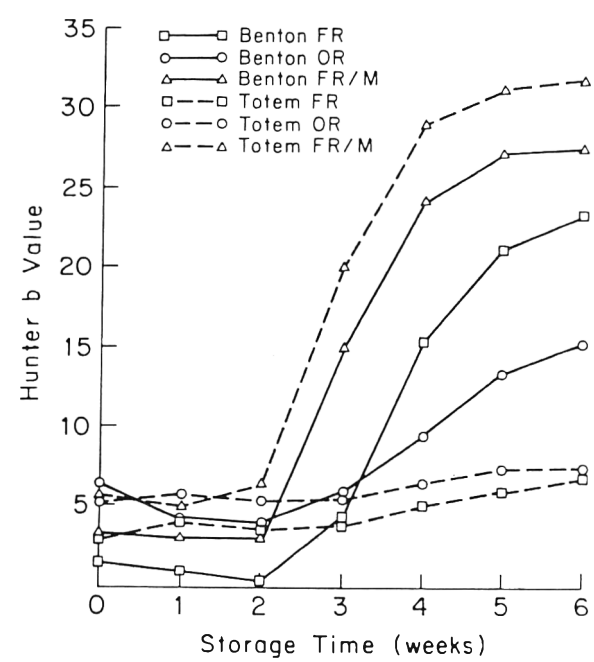


Fig. 5—Effect of storage on Hunter "b" values of wines.

Table 5—One way ANOVA on the influence of variety, maturity and mold contamination on the sensory analysis of strawberry wines[a]

Index	Benton		Totem				
	FR[b] vs OR[c]	FR vs FR/M[d]	FR vs OR	FR vs FR/M	Benton FR vs Totem FR	Benton OR vs Totem OR	Benton FR/M vs Totem FR/M
Color intensity	1%	5%	NSD	5%	1%	1%	1%
Hue	1%	1%	NSD	5%	1%	5%	1%
Browning	NSD[e]	NSD	NSD	NSD	1%	1%	NSD
Clarity	1%	1%	NSD	NSD	1%	1%	1%
Over-all color and appearance	1%	5%	5%	1%	1%	1%	NSD

[a] All values are in % significance level from 12 scores
[b] Fully ripe
[c] Overripe
[d] Fully ripe with added moldy fruit
[e] No significant difference

by sensory analysis were highly correlated to anthocyanin content, flavanols, leucoanthocyanins, titratable acidity, and pH. The anthocyanin content of the wine and the Hunter "L" were found to be important predictors of color since they were both highly correlated to practically all of the sensory indices for color.

These findings indicated that Totem was a better variety than Benton for wine-making purposes because of the higher anthocyanin pigment content and lower PPO and total phenolics. The overripe fruit, which contained more pigments, produced wines of better color. Mold-contamination caused color degradation problems due to the presence of pigment destabilizing enzymes like laccase and β-glucosidase and other compounds that inhibit fermentation.

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Table 6—Simple correlation analysis on compositional analyses, wine color parameters, and sensory evaluation indices[a]

Variables		Correlation Coefficient, r	Variables		Correlation Coefficient, r
ACN	WACN	0.97	WHA	WCD	0.93
TPH	WHA	0.90	ACN	CINT	0.86
TPH	WCD	0.99	FLV	CINT	0.88
TPH	WPC	0.99	pH	CINT	0.88
TPH	WBRI	0.99	PPO	CINT	−0.89
NFP	WHA	0.93	PPO	BRN	−0.98
NFP	WCD	0.98	PPO	CLY	−0.91
NFP	WPC	0.99	PPO	OCA	−0.95
NFP	WBRI	0.99	CINT	WACN	0.99
SOS	WHA	0.99	OCA	WACN	0.99
SOS	WCD	0.94	HUE	WACN	0.99
SOS	WPC	0.94	HUE	WHL	0.97
SOS	BRI	0.94	OCA	WHL	0.96
TA	pH	−0.99	CINT	WHL	−0.93
TA	WHL	−0.91	CLY	WHL	−0.91
TA	WACN	−0.96	CLY	WHZ	−0.89
pH	WHL	−0.89	BRN	WACN	0.88
pH	WHZ	−0.86	WHL	WACN	−0.86
pH	WACN	0.98	OCA	WHZ	−0.85
WHL	WACN	−0.93			

[a] Codes used for the multiple correlation analysis: ACN — Anthocyanin pigment content; FLV — Flavanols; LEU — Leucoanthocyanins; TPH — Total phenolics; NFP — Nonflavonoid phenolics; ASC — Ascorbic acid; TA — Titratable acid; pH — pH; SOS — Soluble solids; PPO — Polyphenol oxidase; CINT — Color intensity; HUE — Hue; BRN — Browning; CLY — Clarity; OCA — Overall color and appearance; ACNJ — Anthocyanin content, juice; CDJ — Color density, juice; PCJ — Polymeric color, juice; PPCJ — Percent polymeric color, juice; BRIJ — Browning index, juice; HLJ — Hunter L, juice; HAJ — Hunter a, juice; HBJ — Hunter b, juice; WCD — Color density, wine; WPC — Polymeric color, wine; WPPC — Percent polymeric color, wine; WBRI — Browning index, wine; WHL — Hunter L, wine; WHA — Hunter a, wine; WHB — Hunter b, wine; WHZ — Haze, wine; WACn - Anthocyanin content, wine.

Characterization of Tannase Activity on Tea Extracts

R. L. THOMAS and K. MURTAGH

ABSTRACT

Assay of tannase activity was performed directly, using extracts of black tea leaf, by a pH stat method at the pH optimum of the enzyme (6.0). Reaction rates were calculated from the initial rates of the volume of titrant vs. time plots, and activity was expressed as μmoles/min. The enzyme was unstable to pH levels above 6.0 and temperatures above 40°C. Optimum temperature was 35°C. Using saturating amounts of enzyme, the pH stat was also used to determine total hydrolyzable gallate for kinetic studies and to determine the effect of gallated polyphenols on loss of solids in instant tea processing.

INTRODUCTION

TANNASE (Tannin acyl hydrolase EC. 3.1.1.20) is an inducible enzyme produced by various species of *Aspergillus* and *Penicillium* when grown in the presence of tannic acid (Knudson, 1913; Weetall and Detar, 1974). This enzyme hydrolyzes esters of phenolic acids, including the gallated polyphenols found in tea (Halsam and Tanner, 1970; Katwa et al., 1981; Coggon et al., 1979).

The use of tannase in processing instant tea and the production of leaf products of increased extractability have been reported (Coggon et al., 1975, 1979; Sanderson and Coggon, 1977; Takino, 1976; Pintauro, 1977). The enzyme improves the cold water solubility characteristics of tea by hydrolyzing tea cream, the cold water insoluble precipitate that forms from the complexing of gallated polyphenols and caffeine (Pintauro, 1977). However, the high cost of the enzyme has precluded its use (Ross, 1984). Also, there is very little information available concerning the characteristics of the enzyme with tea substrates.

Several methods for assaying tannase utilizing many different substrates have been reported. Spectrophotometric assays have been most commonly used. Parmentier (1970) described an assay based on the differences in ultraviolet absorption of gallic acid and methyl gallate. Iibuchi et al. (1967) utilized a similar approach with tannic acid as the substrate. Halsam and Tanner (1970) were very critical of using a spectrophotometric method based on small differences in ultraviolet absorption of gallic acid and its methyl ester or tannic acid and thus developed a spectrophotometric assay utilizing p-nitrophenyl esters of gallic acid as substrate. These authors reported conflicting results concerning the pH optimum of tannase. Parmentier (1970) reported a pH optimum of 6.0 with methyl gallate as substrate, and Iibuchi et al. (1967) reported 5.5 as the optimum with tannic acid as substrate. However, Halsam and Tanner (1970) reported the optimum to be much lower. They showed two optima at 4.2 and 4.8 with p-nitrophenyl gallate as substrate. It is not uncommon for enzymes to demonstrate different pH optimum with different substrates (Thomas and Jen, 1980), and these previous studies do not offer dependable information for using tannase in tea processing.

Authors Thomas and Murtagh are affiliated with the Dept. of Food Science, Clemson Univ., Clemson, SC 29631.

Assays based on the titration of liberated gallic acid have been reported, including the use of a pH stat (Halsam and Stangroom, 1966; Iibuchi et al., 1972). The disadvantage of using the pH stat is that the pH of the assay must be sufficiently close to the pH optimum of the enzyme. The endpoint of the titration of gallic acid has been determined to be 6.8 by Iibuchi et al. (1972). Therefore, the reported low pH optimum of tannase by some researchers has discouraged the use of the pH stat in assays with other substrates, such as gallated polyphenols of tea.

The purpose of this study was to measure and characterize tannase activity using tea compounds as substrate for optimizing the use of this enzyme in tea processing.

MATERIALS & METHODS

Tannase

Tannase (ENZECO Tannase R), lot numbers K13779 and K16069, was a gift from Enzyme Development Corporation, New York. The enzyme was supplied as a tan colored powder and was stored at 4°C in a dessicator until used. The enzyme was dissolved in distilled water or in 10 mM K_2HPO_4 buffer (pH 6.0) and held on ice for assays.

Tea extracts

Extracts of commercial black and green teas were prepared by adding 50g tea leaf to 500 mL distilled water and boiling for 5 min. The extracts were filtered first through glass wool and then paper toweling to remove the leaf. Tea solids were determined by drying samples at 90°C overnight in a convention oven. Total solids of these extracts typically were 4.5–5.5%.

pH stat

A Radiometer titrator consisting of a PHM 84 research pH meter, TTT 80 titrator, ABU 80 autoburette, TTA 80 titration assembly and a REC 80 servograph was used in performing the enzyme assays. Instrumental parameters were endpoint 6.0 (pH stat), 14 min/full scale pen speed, proportional band 0.2, and burette speed 60 (indicating maximum titrant delivery to be 6 mL/min).

pH stat assay

Substrate was prepared by diluting the above tea extracts 1:50, and 15 mL aliquots of the dilution were used in each titration. Temperature and pH were adjusted according to experimental parameters as described below. The substrate was stirred continuously and 0.4 mg tannase, in a volume of 50 μL, was added with a micropipette. This amount of enzyme was found to be the minimal amount to achieve maximum initial rates (Fig. 3). Autotitration with 0.5 mM NaOH using the pH stat was begun immediately after enzyme addition and was allowed to continue for 10 min. Except for the pH studies the endpoint setting of the pH stat was 6.0. Reaction rates were calculated from the initial slopes of the pH stat curves (volume of titrant vs. time) and expressed as μmoles/min.

pH studies

The pH optimum was determined using timed assays at different pH values held constant with the pH stat, followed by titration to an endpoint of 6.8 for lower pH values. This was to ensure complete ionization of gallic acid, which has an endpoint of 6.8 (Iibuchi et al., 1972). Any additional titrant utilized was added to the pH stat volume to calculate activity. At pH's above 6.8 this step was not necessary.

To determine pH stability of tannase, enzyme solutions of 8 mg/ml were made in 10 mM sodium acetate buffers for pH 3.5–5.5 and in 10 mM KH_2PO_4 buffers for pH 6.0–7.5. The enzyme solutions were held for 2 hr at 25°C, the pH was adjusted to 6.0, and activity remaining was determined at pH 6.0 using the pH stat.

Temperature studies

Temperature optimum was determined by assaying enzyme activity with 1:50 dilutions of tea extracts by the pH stat with an endpoint of 6.0 at temperatures varying from 15–50°C in increments of 5°C. The tea extracts were first adjusted to pH 6.0 and were held in a water-jacketed vessel during the assay.

To determine temperature stability, enzyme solutions of 8 mg/mL in 10 mM KH_2PO_4 buffer (pH 6.0) were prepared and held for 20 min at temperatures ranging from 15–50°C in increments of 5°C. Residual enzyme activity was determined by the pH stat at pH 6.0 and 25°C.

Hydrolyzable gallate

The pH stat was used to determine the total amount of hydrolyzable gallate in the tea extracts by increasing the amount of enzyme added until no further increase in titrant volume was observed. At this point it was assumed that complete hydrolysis of gallate had occurred. From the volume and normality of the titrant used, the milliequivalents of hydrolyzed gallate in the reaction vessel and the molarity of hydrolyzable gallate present in the tea solutions was determined.

Kinetics

Based on the concentrations of hydrolyzable gallate in the tea solutions, appropriate dilutions were made to obtain solutions of varying concentrations of hydrolyzable gallate. These solutions were assayed at optimum pH and temperature to obtain kinetic characteristics of the enzyme.

Recovery of tea solids

Tea extracts were prepared as described above, adjusted to pH 6.0, and placed in a shaking water bath at 35°C. Tannase was added to individual samples at an equivalent rate of 1, 5, 10, and 20 g tannase per pound of tea solids. The solutions were held for 90 min, and the pH was maintained at 6.0. Afterward, the solutions were heated to 90°C for 10 min to inactivate the enzyme, cooled to 4°C, and held overnight at 4°C. The samples were centrifuged at 12,000 × *g* for 20 min at 4°C to separate the precipitated tea cream. Cream loss was determined by the difference in % solids before and after centrifugation.

Replications

Data from the pH, temperature, and kinetic studies are means of triplicate analyses. Determinations of hydrolyzable gallate and tea cream loss are means of duplicate analyses.

RESULTS & DISCUSSION

USING TIMED ASSAYS followed by titration of the liberated gallic acid to a 6.8 endpoint, a pH optimum of 6.0 was observed with a tea solution as substrate (Fig. 1). Based on the pH optimum curve, it was concluded that the optimum was sufficiently close to pH 6.8 to utilize the pH stat as a continuous method to assay tannase with tea as the substrate for further characterizations. The pH stability of tannase (Fig. 1) indicates the enzyme was not stable to pH's above the optimum but was very stable below the optimum.

The temperature optimum was found to be 35°C, and the enzyme was not stable at temperatures above 40°C (Fig. 2). This is in contrast to the current technical information provided by the supplier of the enzyme, who reports an optimum of 45°C and range of use between 3°C and 60°C (Anonymous, 1983).

Another application of the pH stat was to quantitate the amount of substrate as hydrolyzable gallate present in the tea extracts (Fig. 3). Increasing amounts of enzyme were added until complete hydrolysis was assured. High levels of enzyme were not inhibitory. The amount of NaOH utilized

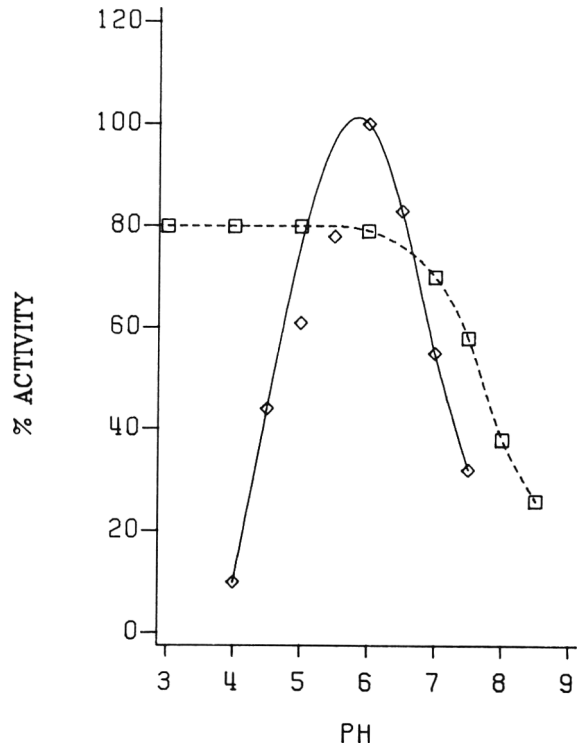


Fig. 1—pH optimum (——) and pH stability (·····) of tannase with tea extract as substrate.

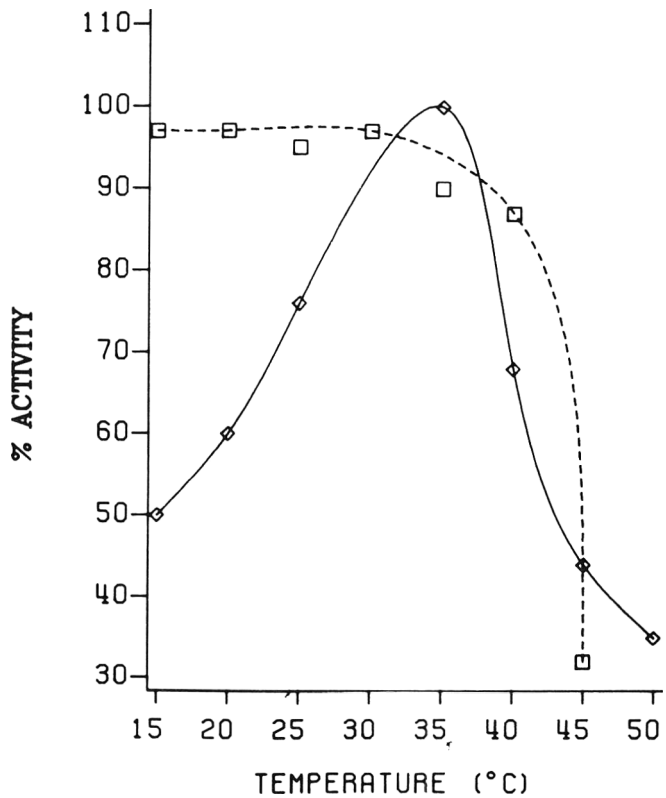


Fig. 2—Temperature optimum (——) and temperature stability (·····) of tannase with tea extract as substrate.

at complete hydrolysis was used to determine the equivalents of gallic acid released by enzyme action. The commercial brands of tea used showed a large variation in the amount of hydrolyzable gallate. Values from 0.06–0.36

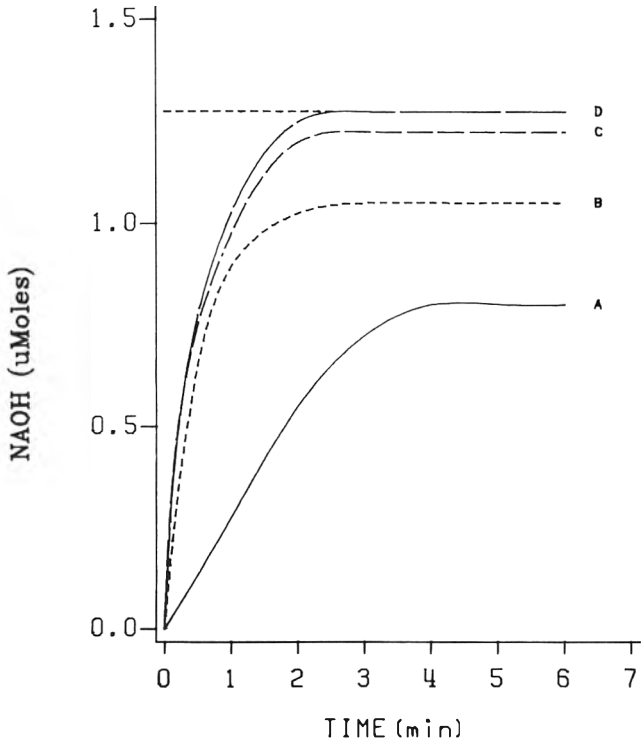


Fig. 3—Effect of enzyme amount on reaction rate. Curves A, B, C, and D represent 0.08, 0.20, 0.40, and 2.0 mg of tannase, respectively. The amount of titrant utilized at complete hydrolysis (------) was used to determine total hydrolyzable gallate.

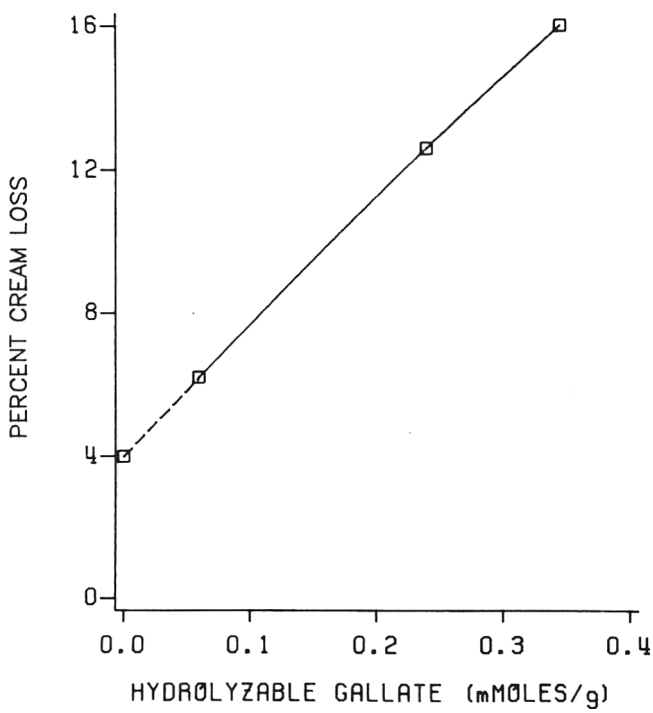


Fig. 4—Relationship of concentration of hydrolyzable gallate in tea leaf to the degree of cream loss in processing. Each point except the extrapolated value was determined from a different tea blend. All extracts were adjusted to 4% solids and held overnight at 4°C to induce cream formation.

mmoles per gram tea leaf were observed. The degree of cream loss in processing these teas (4% solids extracts) to a soluble instant product was directly related to the concentration of hydrolyzable gallate (Fig. 4). However, it was apparent that a certain amount of tea cream could be found in the absence of any hydrolyzable gallate. By extrapolation, it was estimated that under these experimental conditions 4-5% of tea cream is formed by interactions among tea polyphenols, caffeine, and possibly other constituents that do not involve the gallic acid moiety of the gallated polyphenols. This was confirmed by the observation that even with large amounts of tannase added to similar tea extracts, there was still a 3.5-5% tea cream loss (Fig. 5). It might be expected that at solids levels higher than 4%, which would be typical in commercial manufacturing, the cream loss not attributable to gallate would be higher.

Since substrate could be quantitated, kinetic studies were performed. Fig. 6 shows the effect of substrate concentration, expressed as hydrolyzable gallate, on reaction rates with black tea and green tea as substrates. The kinetics of the enzyme with green tea substrates was very similar to those with black tea substrates. Maximum velocity was observed at concentrations of approximately 1 mM

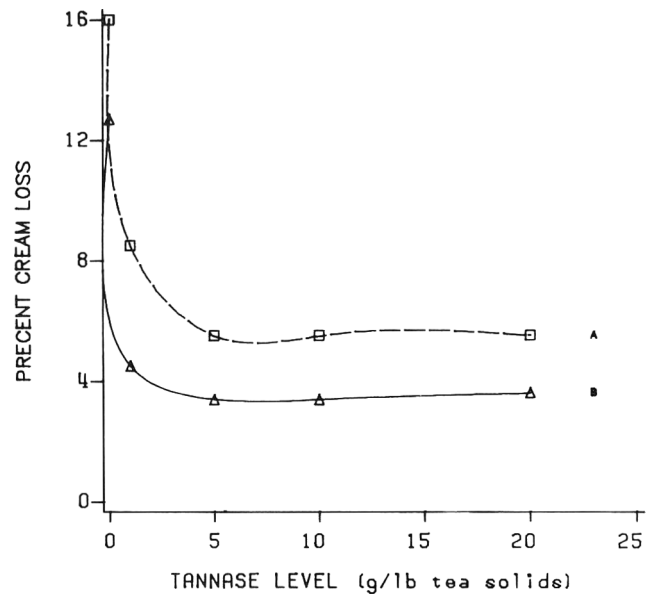


Fig. 5—Effect of tannase level on tea cream loss. A and B represent two different black tea blends. Extracts from both were adjusted to 4% solids before tannase treatment.

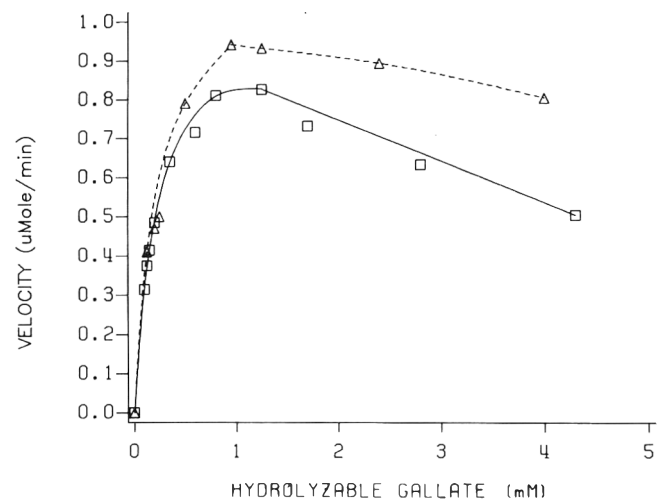


Fig. 6—Effect of substrate concentration on reaction rates with black tea (-------) and green tea (——) as substrates.

Table 1—Kinetic characteristics of tannase with gallated tea polyphenols as substrate

Tea extract[a]	Km[b] (mM)	V_{max} (μmoles/min)	Substrate conc at V_{max}
A_1	0.20	0.95	1.0
A_2	0.14	0.70	0.9
A_3	0.13	0.87	1.0
B	0.17	0.43	1.1
C	0.18	0.83	1.2

[a] Extracts A_1, A_2, A_3 were prepared from different lots of the same commercial black tea blend. Extract B was a different black tea blend, and Extract C was a green tea.
[b] Based on concentrations of hydrolyzable gallate. Values obtained from Lineweaver-Burke plots.

hydrolyzable gallate for both types of tea substrates. Substrate inhibition was only slight with black tea but was more pronounced with green tea indicating that in instant tea processes that involve conversion of green teas to black teas, it would be more desirable to use tannase for cream solubilization after conversion is completed. Also, it appears that black tea extracts relatively high in solids can be effectively treated with tannase without concerns of substrate inhibition.

Lineweaver-Burke plots with different teas as substrate showed that all had nearly the same K_m values, but V_{max} varied among the teas and among different lots of the same tea blend (Table 1). The kinetics indicated that noncompetitive inhibitors may be present. The substrates of green tea are the various gallated forms of catechin and epicatechin, and the substrates of black tea are the oxidized, polymerized products of oxidation of the green tea substrates, the theaflavins and thearubigins (Sanderson, 1972). Therefore, tea extracts represent several closely related substrates, and this probably accounts for much of the observed variability in Table 1. Nevertheless, this type of kinetic data can be valuable to tea processors who wish to establish an assay for tannase based on standard tea blends primarily utilized in their process.

It is always desirable to characterize enzymes with the actual substrates in question, although this is often impossible. The pH stat method can be used to assay a variety of enzymes which liberate acids or bases as products and actual substrates can be utilized. However, a requirement to be met in the pH stat assay is that the pH optimum of the enzyme be near the endpoint pH of the released product being titrated. Since the pH optimum of tannase with tea compounds as substrate was near the titration endpoint of gallic acid, the pH stat could be used to assay and characterize tannase with tea substrates. The direct assay of tannase activity on tea provided more dependable information concerning the use of the enzyme in tea processing.

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A Model System of Natural Orange Juice Cloud: Effect of Calcium on Hesperidin-Pectin Particles

N. BEN-SHALOM, RIVKA PINTO, J. KANNER, and MONIKA BERMAN

ABSTRACT

Hesperidin forms with pectin a particle which could be isolated by gradient sucrose centrifugation. Using this technique we were able to separate soluble stabilized pectin which was bound to hesperidin and a soluble pectin which was hesperidin-free. Pectin particles in which the degree of esterification (D.E.) was 25%, flocculated in the presence of 100 ppm calcium, while those with a D.E. of 30% required 200 ppm calcium. A D.E. of 36% was found to be the critical level at which precipitation by calcium ions occurred. A 23% D.E. of the flocculated cloud peel juice was found to fit well with a model system of a ratio of 1:4.4 of calcium and pectin with 25% D.E. and 100 ppm calcium.

INTRODUCTION

CLOUD from orange juice contains mainly pectins, proteins and lipids (Baker and Bruemmer, 1969). Hesperidin, which seems to be located in a soluble form in the vacuole of the intact cell, crystallizes out of the cell during membrane damage (Bennett and Albach, 1981). Thus, crystallized hesperidin was found in the orange cloud (Mizrahi and Berk, 1970).

Pectin esterase is the main enzyme which determines orange cloud flocculation. Precipitation of the cloud components by enzymatic deesterification of the pectin polymer followed by interaction with calcium ions is a well-established mechanism (Krop and Pilnik, 1974). Addition of ammonium oxalate, which removes the bivalent cation, prevents flocculation of the cloud components (Krop and Pilnik, 1974). Treatment of the cloud particles with polygalacturonase before or in conjunction with pectin esterase prevents cloud flocculation due to the formation of short chains which cannot precipitate by formation of calcium bridges (Baker and Bruemmer, 1972).

Cloud formation between pectin and hesperidin in our model system seems to be a result of a specific interaction between the polysaccharide polymer and the insoluble flavonoid (Ben-Shalom et al., 1984). The stability of this cloud seems to be a function of the ratio between the amount of flavonoid and the molecular weight of the polymer (Ben-Shalom et al, 1984).

The aim of the present study was to use the model system of hesperidin pectin particles to determine the critical degree of esterification of pectin in the juice which caused flocculation by calcium ions.

MATERIAL & METHODS

PECTIN (polygalacturonic acid and methyl ester) from citrus fruit [73% D.E. m.w. 84,300 daltons, determined according to Christensen (1954)], was purchased from Sigma Chemical Co. (St. Louis, MO). Citric acid, tri-potassium-citrate, sucrose and sodium benzoate obtained from British Drug Houses Ltd. (Poole, England). Hesperidin was obtained from the Jaffora Factory (Rehovot, Israel), and purified in our laboratory with formamide as described by Pritchell and Merchant (1946). Concentrate Valencia juice (60° Bx) and concentrate Valencia peel juice (50° Bx) were received from Jaffora Factory (Rehovot, Israel).

The authors are with the Dept. of Food Technology, Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel 50 250.

Pectinesterase from citrus fruit was purified on cross-linked pectate according to Rombouts et al. (1979). The cloud model system contains a solution of citric acid (1%), tri-potassium citrate (0.67%), benzoate (0.1%) and pectin (0.2%) at pH 3.8 as described previously (Kanner et al., 1982). In some cases the buffer was changed, as mentioned. Hesperidin was dissolved in 0.2N NaOH, added to the pectin solution, and then stirred vigorously. A water extracted (1:1 = peel:water) peel juice, 5° Bx, was prepared by slicing the peel to 1 mm (with a food processor) and stirring for 45 min at 45°C. The extracted juice was separated from the pulp by cheesecloth, heated for 5 min at 85°C and centrifuged for 10 min at 350^{x} *g*. Separation of hesperidin-pectin particles on sucrose gradient was done with 60% and 70% sucrose. The hespiridin-pectin particles were dissolved in 40% sucrose and put over 60% sucrose. Separation of the particles was done by Beckman ultracentrifuge at 15,000 rpm for 30 min. The effect of calcium on the flocculation of hesperidin-pectin with various degrees of esterification, was investigated using citrus pectin with an initial D.E. of 73%. The pectin was deesterified with purified orange pectinesterase to various D.E.'s and formed stabilized colloidal particles with the various pectic acids by addition of hesperidin to the solution. The particles were separated on sucrose gradient and mixed with various concentrations of calcium (10–1,000 ppm), Stability was determined by measuring turbidity at 660 nm.

Hesperidin was separated from the insoluble components of the juice by 66% alcohol and determined by Spectra Physics HPLC model SP 8000 with UV detector and reverse phase C-18 column. The mobile phase included solvent A (1.5% phosphoric acid) and solvent B (1.5% phosphoric acid, 20% acetic acid, 25% acetonitrile and 43.5% water). The gradient was 50–100% solvent B over 10 min linear, held 3 min. The flow rate was 2 mL/min and the hesperidin was detected at 285 nm.

Calcium was determined by atomic absorption. Pectin was determined in the alcohol-insoluble solids of the juice, by the m-phenyl phenol reagent according to Blumenkrantz and Asboe-Hansen (1973).

Methanol was converted to methyl nitrite and determined by gas chromatography according to Litchman and Upton (1972) and its modification by Versteeg (1979).

RESULTS & DISCUSSION

THE FORMATION of stabilized colloidal particles between hesperidin and pectin is shown in Fig. 1. Addition of hesperidin (previously solubilized in NaOH) to 0.1N sodium acetate buffer, pH 4.0, caused the flavonoid to precipitate. Addition of hesperidin (0.15%) to a pectin solution (0.2%) initiated the formation of stabilized colloidal particles in the solution.

To check whether the pectin serves as a nucleation site for crystal formation, the concentration of hesperidin was kept constant and the concentration of pectin was increased (Fig. 2). The initial rate of the cloud formation was dependent on pectin concentration, but after 4 hr the three concentrations of pectin reached almost the same cloud intensity. According to Weimann's law (Adamson, 1976), the more rapid the nucleation, the greater the number of nuclei formed before relief of the supersaturation occurs; hence, we assumed that the hesperidin "recognizes" the pectin molecule and use it as a nucleation site. In the presence of 0.3% and 0.4% pectin, two different processes were observed: a rapid one, connected with nucleation of hesperidin with the pectin molecule; and a slow one,

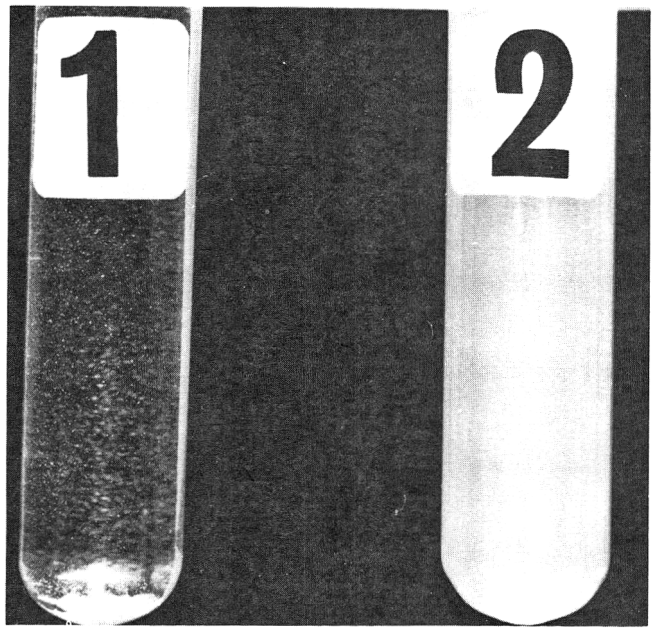


Fig. 1—Formation of stabilized cloud between pectin and hesperidin. (1) Addition of hesperidin to a 0.1N sodium acetate buffer, pH 4.0(2) Addition of hesperidin (0.015%) to a pectin solution (0.2%) in acetate buffer, pH 4.0.

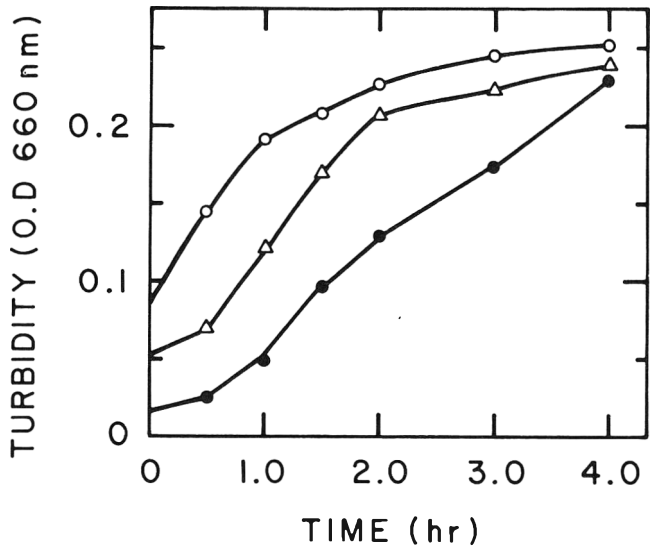


Fig. 2—Effect of various concentrations of pectin on the cloud formation with hesperidin (0.015%) •—0.2% pectin added; △—0.3% pectin added; ○—0.4% pectin added.

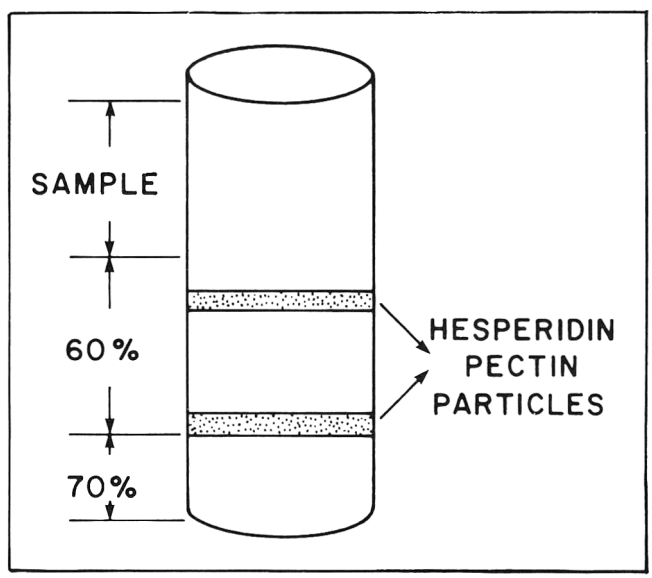


Fig. 3—Separation of hesperidin-pectin particles on sucrose gradient.

connected with the continuous growth of the hesperidin crystals.

To examine the possibility that hesperidin and pectin form real particles, the cloud components were separated on a sucrose gradient. Two bands were found in 60% sucrose (Fig. 3). Resuspension of the particles in 10% sucrose led to a stable cloud. This indicated that hesperidin is tightly connected with pectin and could not be separated by force of gravity.

The ratio of hesperidin to pectin in the separated colloidal particles was found to be 1:2. Consequently, it appears that in our model system, which included 15 mg hesperidin and 200 mg pectin, only 15% of the total pectin was responsible for the formation of the stabilized cloud. Eighty-five percent of the pectin did not enter the 60% sucrose layer and remained on top of the gradient. Increasing the ratio between hesperidin and pectin in the solution led to flocculation (Ben-Shalom et al., 1984). Thus, resuspended particles after sucrose gradient centrifugation could be stabilized in the solution only due to the binding to soluble pectin. This seems to explain part of the mechanism of stabilization of the orange cloud particles. Soluble pectin appears to be the main stabilizer of the juice cloud components, and one must differentiate between a soluble pectin which is bound to the juice particles and stabilizes them, and a soluble pectin which is not bound to the particles and is not important for their stability, as was also demonstrated by Baker and Bruemmer (1969).

Part of the explanation for the limited amount of hesperidin (15 mg) which could be stabilized by 200 mg pectin lies in the observation that hesperidin stabilization is molecular weight-dependent (Ben-Shalom et al., 1984). Thus, only pectin with molecular weight between 84,000 and 76,000 daltons can stabilize hesperidin without any flocculation. The pectin used in this study was a commercial product with an average molecular weight of 84,000 daltons. This material contained molecules which covered a whole range of molecular weights, some which could not stabilize hesperidin in the solution.

The effect of calcium of hesperidin-pectin particles was studied in reaction mixtures which contained particles of hesperidin-pectin with different degrees of esterification (D.E.) and different calcium concentrations (Fig. 4). Pectin (D.E. 73%, M.W. = 84,000 daltons) deesterified with purified orange pectin esterase to various D.E., was used to make stabilized clouds by the addition of hesperidin to the solution. The particles were separated from the soluble pectin by sucrose gradient and then reconstituted in 10% sucrose to form a stabilized cloud. Each fraction of cloud material with a different degree of esterification was mixed with various concentrations of calcium and its stability was determined by measuring the turbidity. Hesperidin-pectin particles with a D.E. of at least 40% did not flocculate with

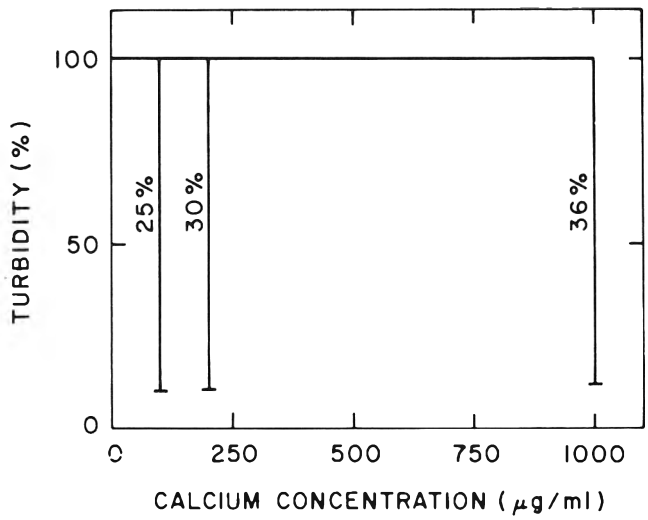


Fig. 4—Effect of calcium on the flocculation of hesperidin-pectin with various degrees of esterification.

Table 1—Comparison among the model system of hesperidin-pectin particles in whole orange juice and peel juice during calcium-initiated flocculation of the particles

Components	Model system (mg/reaction mixture)	Peel juice (mg/g)	Juice (mg/g)
Hesperidin	1.10	1.25	0.78
Calcium	0.50	0.80	0.10
Pectin	2.20	4.50	0.50

as much as 1,000 ppm calcium. (Fig. 4). Flocculation of the cloud components began to be observed only with 36% D.E. At that D.E., only 1,000 ppm calcium initiated a loss of turbidity. Particles of hesperidin-pectin with 30% D.E. flocculated at 200 ppm and 100 ppm calcium, respectively. In all cases, as soon as the flocculation process began, most of the turbidity was lost.

To test the assumption that the hesperidin-pectin particles could be used as a good bio-assay to study the flocculation behavior of the whole juice cloud particles, 5° Bx peel juice was used because it is a rich source of hesperidin. The juice was incubated with two different concentrations of orange pectin esterase, at 35°C and pH 4.8 (Fig. 5). The reaction was stopped when juice flocculation was observed. The concentration of the pectinesterase in the reaction mixture was found to influence the rate of methanol release, at both concentrations of pectinesterase used.

To obtain the D.E. of the flocculated juice, alcohol-insoluble solids fraction was prepared from the untreated juice. The D.E. of the alcohol-insoluble solids was calculated from the uronic acid content (45%) and the methanol content (12.1%). From these results it was found that the juice pectin was 73% (Fig. 6). The D.E. of the juice decreased as a function of pectin esterase activity. The flocculation of the juice was observed in both concentrations of pectin esterase, at a D.E. of 23%.

Flocculation initiated by calcium ions was studied in the model system of hesperidin pectin particles, whole orange juice and peel juice. Hesperidin pectin particles with D.E. of 25% were separated on sucrose gradient and then reconstituted in 10% sucrose and mixed with 100 ppm calcium in a 5 mL reaction mixture. Peel juice and whole juice were

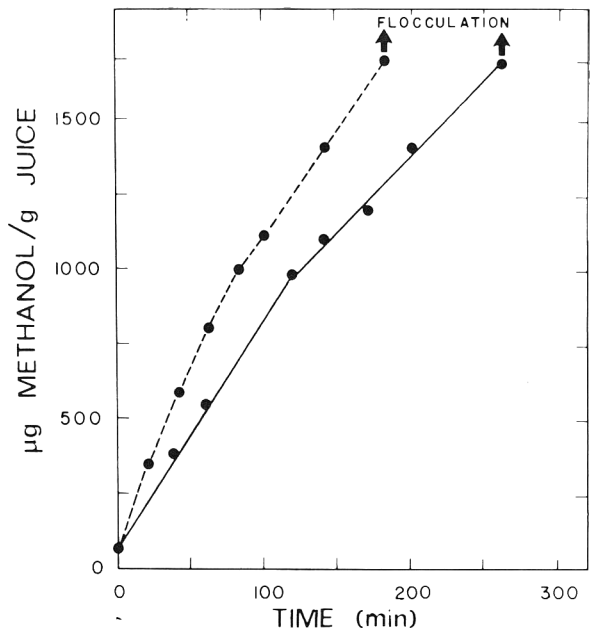


Fig. 5—Effect of pectinesterase activity on the D.E. of peel juice and its flocculation. 3 mg (- - - -) and 5 mg (———) pectinesterase incubated with peel juice at pH 4.8 and 35°C. The decrease in their D.E. was followed until flocculation was observed. At zero time the D.E. of the juice was found to be 73%.

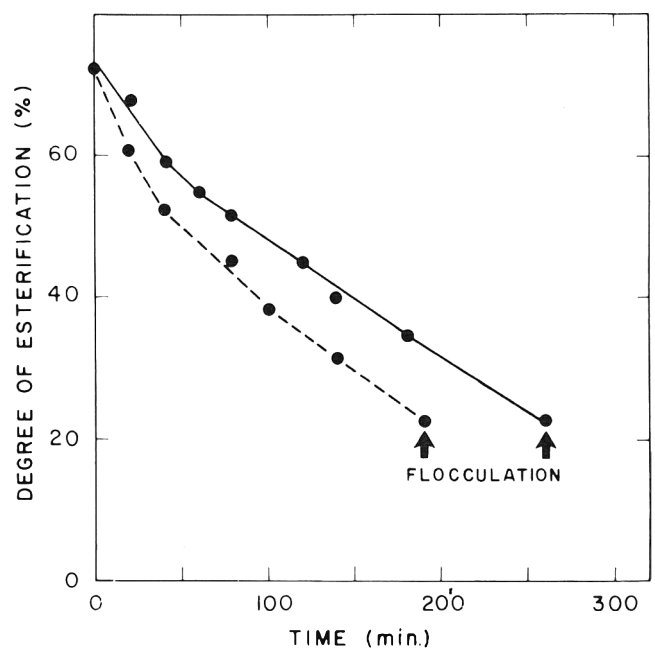


Fig. 6—Effect of pectin esterase on the release of methanol from peel juice. Peel juice was incubated at pH 4.8 and 35° C with 3 mg (- - - -) and 5 mg (———) orange pectinesterase. The reaction was stopped when flocculation of the juice was observed.

analyzed for hesperidin, calcium and pectin amount in 10° Bx reconstituted juice (Table 1).

The peel juice contained 4.5 mg/g juice pectin, 1.25 mg/g hesperidin and 0.8 mg/g juice calcium (Table 1). Based on the ratio between the calcium and the pectin (1:5.6), these results fit well the data obtained from the juice with a calcium-to-pectin ratio of 1:5, and with our model system

—Continued on page 1142

Compatibility of Gelatin with Broad Bean Globulin in an Aqueous Medium

O. ANDERSSON, H. SCHMANDKE, V. I. POLYAKOV, V. Ya. GRINBERG, T. M. BIKBOV, A. P. DANILENKO, A. L. LEONTJEV, and V. B. TOLSTOGUZOV

ABSTRACT

The phase state of mixture of gelatin and Broad Bean globulins in an aqueous medium has been studied. Phase diagrams were determined which demonstrated the compatibility of gelatin with total globulin and of gelation with 11S globulin of Broad Beans. Results indicate that compatibility of gelatin with total globulin differs only slightly from the compatibility with 11S globulin. The increase in compatibility of gelatin with 11S globulin with increasing ionic strength is particularly interesting. As a consequence it appears possible to extend the region of the one-phase mixed compositions suitable for the production of mixed gels.

INTRODUCTION

THE SOURCES of raw materials for the production of traditional gel forming substances are limited, as is information related to their functional properties. Consequently the search for new raw material of this class is of particularly interest (Tolstoguzov, 1978). Present studies have reported numerous plant proteins with good gelling properties. It is important to study these plant proteins not only in the pure state but also in mixtures with other gelling substances. The use of mixtures makes it possible to control the functional properties since the properties of the mixed gel are determined to a high degree by the initial composition of the mixture and of its phase state. The purpose of the present work was the investigation of thermodynamic compatibility of protein gel-formers (gelatin and Broad Bean Globulin). In the present paper equilibrium in the systems water-gelatin-broad bean globulin and water-gelatin-11S Broad Bean globulin was studied.

Authors Andersson and Schmandke are affiliated with the Central Institute of Nutrition, Academy of Sciences of the GDR, 1505 Bergholz-Rehbrücke, German Democratic Republic. Authors Polyakov, Grinberg, Bikbov, Danieleko, Leontjev and Tolstoguzov are affiliated with the Institute of Organoelement Compounds, USSR Academy of Sciences, Vavilov St. 28, Moscow V-312, USSR.

MATERIALS & METHODS

ALKALINE PRECURSOR GELATIN ("Biochimreaktiv" — U.R.S.S.) was purified according to Loeb (1932). The average molar mass of this gelatin preparation was calculated from diffusion and sedimentation velocity data in phosphate buffer (pH 5,9; ionic strength 0.1) to be about 160 kg/mol. The isoelectric point lies at pH 4.9.

Broad bean flour was prepared by dehulling broad beans (*Vicia faba* var, minor, cultivar "Fribo", harvested in 1981) and subsequent milling.

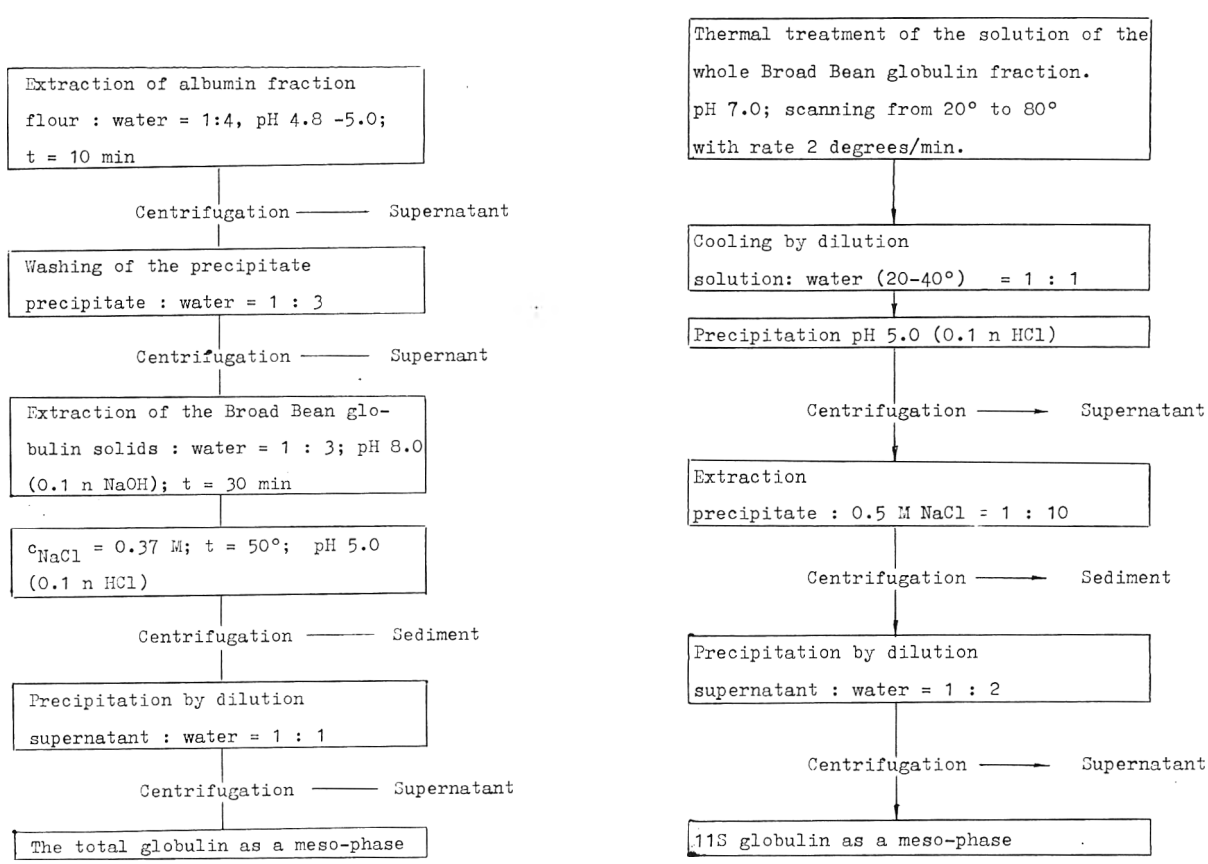


Fig. 1—Scheme for fractionation of total globulin from Broad bean flour (left) and of 11S globulin from total globulin (right).

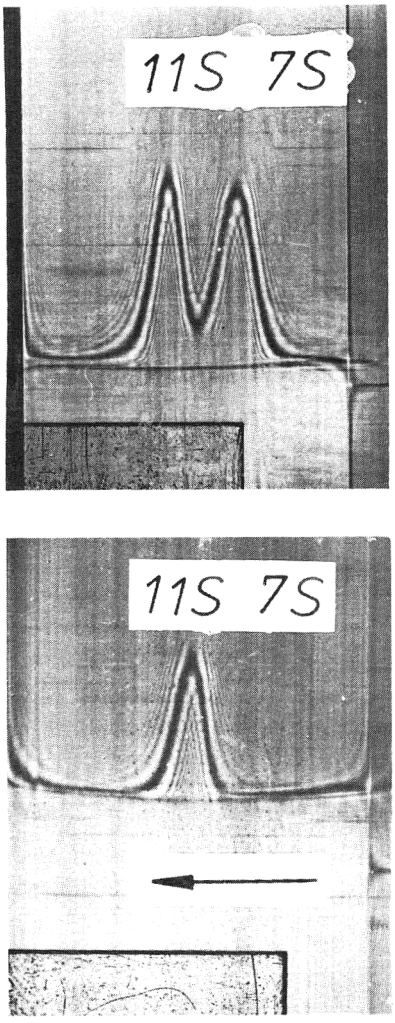


Fig. 2—Sedimentogram of the globulin preparations, the arrows indicate the direction of sedimentation (top) two peaks of equal height: 7S and 11S globulins in total globulin; (bottom) 95% 11S globulin and about 5% 7S globulin.

The flour contained 4.7% nitrogen, 1.7% total lipids, and 6.0g lysine per 16g nitrogen.

The total globulin and the 11S globulin were fractionated from the Broad Bean flour according to the scheme shown in Fig. 1. The yields were about 8% and 1 - 2%, respectively, of the flour. This fractionation process was not optimized to give a maximum yield. In Fig. 2 the sedimentograms are given for the total globulin and the 11S globulin. They were obtained by ultracentrifugation (MOM 3170 B, Hungary) at 50,000 rpm and 25°C and the protein concentration in phosphate buffer (pH 7.6; ionic strength 0.5) was 0.7%. As can be seen from ratio of peak squares on sedimentograms (Svedberg and Pedersen, 1940), the total globulin contains about 50% 7S globulin and 50% 11S globulin. The 11S globulin contains about 95% 11S globulin as the main component and about 5% 7S globulin.

Stock protein solutions were prepared at previously fixed concentrations, pH values, and ionic strengths. These solutions were purified by centrifuging at 0.95 x 10^5g on an ultracentrifuge UZP-60 ("Biofispribor" – U.R.S.S.). Mixed solutions were prepared with different percentages of the protein components and stirred for 2 hr at 40°C. For the construction of the phase diagram the phase-volume-ratio method (Koningsveld and Staverman, 1967, 1968; Polyakov et al., 1980, 1985a) was used. The separation to yield the pure phases took place when standing for 2 hr at 40°C. After this time the separation was complete.

RESULTS

FIG. 3 shows the phase diagram for the system water-gelatin-Broad Bean globulin at pH 7.0 and 40°C. The given curve, a binodal, marks off area B from region A. Mixtures in the region B separated to yield two phases, the compositions of which are points of the binodal. In region A the mixtures were one-phased. In the two-phase region B gelatin was preponderant in one phase nd total globulin the other phase. The phase diagram is asymmetrical since the threshold value of separation (symbol ◓) lies near the axis of the gelatin concentration. The threshold value of separation is that point on the binodal indicating the composition of the mixture with the lowest sum of the content in gelatin and globulin. In relation to this the critical point (symbol ●) (Koningsveld and Staverman 1967, 1968; Albertson 1971) is shifted on the binodal towards the axis of the globulin concentration. The coordinates for the threshold value of separation are $c^{◓}_{gelat}$ = 6.4%; $c^{◓}_{glob}$ = 2.6% and for the critical point $c^{●}_{gelat}$ = 2.4%; $c^{●}_{glob}$ = 11.6%.

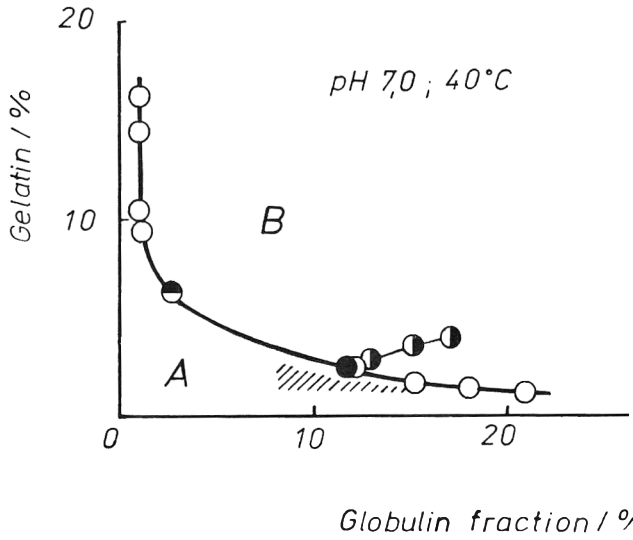


Fig. 3—Phase diagram of the system water-gelatin-Broad Bean globulin fraction at pH 7.0 and 40°C; —○— points of the binodal; —◓— threshold value of separation; —●— critical point; —◑— points of the rectilinear diameter indicating the separation to yield equal volumes of phases; A — region of the one-phase state; B — region of two-phase state.

Fig. 4 shows the phase diagram of the water-gelatin-11S globulin system at pH 7.0 and 40°C. The asymmetry of the phase diagram is somewhat more pronounced than in the preceding case. The critical point is nearly identical to the preceding case. The coordinates for the threshold value of separation are $c^{◓}_{gelat}$ = 6.2%; $c^{◓}_{11S}$ = 1.4% and for the critical point $c^{●}_{gelat}$ = 2.0%; $c^{●}_{11S}$ = 12.0%.

Fig. 5 shows the phase diagram for gelatin with 11S globulin in 0.5M NaCl at pH 6.0 and 40°C. It is interesting that the compatibility of the components was considerably increased by the presence of sodium chloride. The coordinates for the threshold value of separation are $c^{◓}_{gelat}$ = 7.2%; $c^{◓}_{11S}$ = 5.5%. The critical point lies near $c^{●}_{gelat}$ = 3.4%; $c^{●}_{11S}$ = 15.4%.

DISCUSSION

THE RESULTS indicate a small increase in the compatibility of the proteins in the systems studied when passing from 11S globulin to the total globulin fraction. This increase is probably associated with the content of the 7S globulin in the total globulin, the molecular mass of the former being smaller than that of the 11S globulin. According to Bailey and Boulter (1970, 1972) the molar mass of the 11S globulin (= legumin) is 350 kg/mol and of the 7S

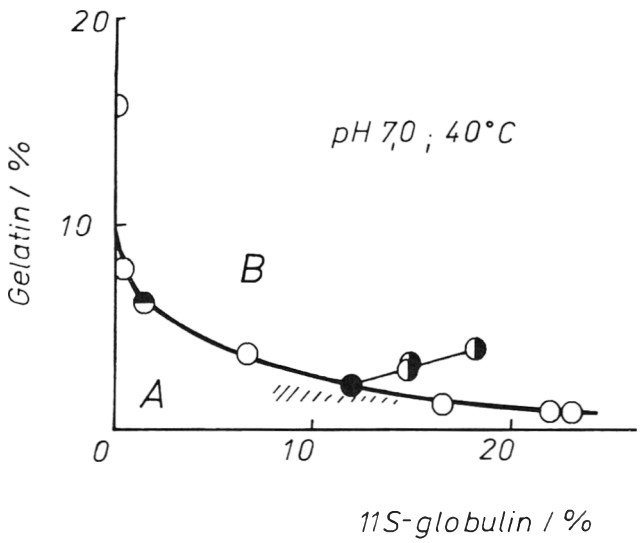


Fig. 4—Phase diagram of the system water-gelatin-11S Broad Bean globulin at pH 7.0 and 40°C. Designations cf. Fig. 3.

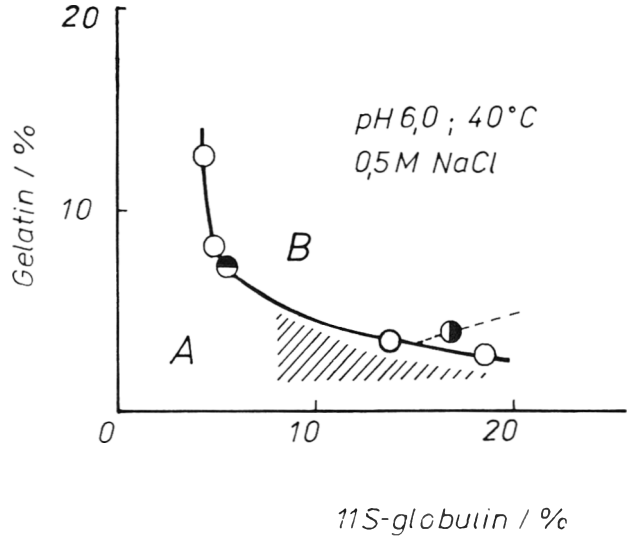


Fig. 5—Phase diagram of the system water-gelatin-11S Broad Bean globulin at pH 6.0 and 40°C; c_{NaCl} = 0.5 mol/L; designations cf. Fig. 3.

globulin (= vicilin) 220 kg/mol. From these the average molar mass of the present total globulin is calculated to be 285 kg/mol. The influence of NaCl on the compatibility of the proteins in the system water-gelatin-11S globulin is analogous to that of casein with soybean globulin (Polyakov, 1985b). This dependence apparently could be general for protein systems. On the one hand, the increase of the concentration of neutral salts leads to an increase of self-association of the proteins and thus favors a decrease in compatibility. On the other hand, the decrease of the Coulomb repulsion in the presence of neutral salts obviously eases the formation of mixed clusters of gelatin-globulin, thus promoting compatibility. It appears most likely that the latter situation prevails. The position of the critical point relative to the threshold value of separation indicates that under these test conditions gelatin is more hydrophilic than 11S globulin and total globulin fraction (Hsu and Prausnitz, 1974). Therefore 11S globulin and total globulin formed more concentrated phases after the phase separation.

For gelation two points are of importance. First, it is necessary that the mixture was one phase and, second, the quality of the gel will be better when the concentrations of both components are higher than their critical concentration for gel formation. As is well known, this concentration of gelatin is 1.0 - 1.4% and for the Broad Bean globulins 7.0 - 8.0%. In Fig. 3, 4 and 5 these regions are indicated by cross-hatching.

Therefore, gelatin-11S globulin and gelatin-total globulin mixtures in aqueous medium were separated into two phases at higher protein concentrations. These two-phase mixtures are susceptible to syneresis and form two-phase gels of low quality. Gels of higher quality can be prepared from homogeneous mixtures in regions limited by the binodal and concentrations according to the gel-points of each of the proteins. According to the phase equilibria data the one-phase region can be extended by addition of sodium chloride. Consequently, the region of mixtures for production gels of high quality can be extended.

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Thermal Degradation of Texture in Apples

R. C. ANANTHESWARAN, M. R. McLELLAN, and M. C. BOURNE

ABSTRACT

Thermal degradation of texture in Cortland and Spigold varieties of apples was studied using instrumental Texture Profile Analysis. The loss of hardness was modeled as a first order reaction and the activation energy was found to be 26.5 kcal/mole and 15.6 kcal/mole for Cortland and Spigold, respectively. The z value for the loss of hardness was found to be 24.8°C and 42.2°C, respectively, for Cortland and Spigold. The temperature of processing was found to significantly change hardness, cohesiveness, and chewiness in both the varieties. In the case of Spigold, time of processing was found to significantly affect hardness and cohesiveness. And in the case of Cortland, time of processing was found to significantly change hardness, springiness and chewiness.

INTRODUCTION

APPLES are very widely processed in the United States. In 1982, nearly 200 million pounds of apple slices were frozen in the United States (Anon, 1984). Of some concern to the apple industry is the growing demand for a premium frozen product with no chemical additives. An efficient thermal process could be used to inactivate enzymes without the use of enzyme inhibitors such as SO_2. Control of thermal softening in the apple tissue is vital to producing a quality product. The study of thermal degradation of apple tissue may lead to a better understanding of controllable mechanisms leading to methods for producing superior thermally treated products.

The effect of heating on fruits have been reviewed by Holdsworth (1979). A major change caused by thermal processing is loss in texture due to the breakdown of the cellular material. Such changes are product specific. They also vary with the maturity and condition of the fruit. Fletcher (1971) found that the variation in the mechanical properties of apple slices were higher in processed samples than the raw fruit. The effect of temperature on these mechanical properties were marginal over small ranges in temperature.

The inherent variability and nonhomogeneity in biological systems increases the variance associated with physical measurements in these systems especially when the sample represents a large population. Among fruits, apples exhibit a very complex phenomenon with respect to their change in firmness with ripening and processing (Mohsenin 1970, Van Woensel and De Baerdemaeker, 1983). Factors such as the size of the fruit, location of measurement, cultural practices are all equally responsible for the variation in their firmness (Worthington and Yeatman, 1968).

Huang and Bourne (1983) used two apparent first-order kinetic mechanisms to describe thermal softening of vegetables. The softening curve, consisting of a log extrusion force versus process time, had an initial steep negative slope that was almost linear. This was followed by a second straight line with a lower slope value at longer process time. Equating this entire curve to that obtained for the sum of two independent simultaneous first-order processes occuring at different rates, they evaluated two rate constants: one contributing to the initial high rate of softening (mechanism I) and the other responsible for the softening at prolonged heating times (mechanism II). They also evaluated the Arrhenius activation energy values for these rate constants, and found them to be in the range of 15-35 kcal/mole for mechanism I and 0.5-24 kcal/mole for mechanism II. Carrots had the lowest value of the activation energy and green peas had the highest value. Mechanism I was attributed to pectic changes in the interlamellar layer.

It is intuitively known that hardness will decrease with heating, but the effect on other textural parameters like cohesiveness, springiness and chewiness is not readily apparent although these textural parameters do play an important role in the quality of cooked apple slices. The objective of this study was to determine the effect of time and temperature of thermal processing on the textural attributes of apples.

MATERIALS & METHODS

Processing

Rome and Spigold cultivars were hand harvested according to optimum harvest dates as determined over a 10-yr period (LaBelle, 1973). The apples for each cultivar were randomly picked from different trees at different locations in the orchard. They were held in refrigerated storage at 0°C and 90% RH for a few months until ready for processing. Only fully mature fruit of high quality was used. Pressure readings were taken during storage and at the time of processing using a Effegi fruit tester. The average Effegi readings for Cortland and Spigold at the time of processing were 9.1 lbf and 10.2 lbf, respectively.

Apples were mechanically peeled and cored, and then diced into 1.25 cm cubes in a dicer (Model #G878 Urschell Lab., Inc., IN). The dices were then passed through a set of two vibrating screens under a spray of water to remove oversized and undersized pieces. Two hundred grams of the dices were filled into a 303 size can followed by 225 g water at a temperature equal to that of processing or 100°C. They were then promptly sealed and placed in a preheated still retort. Up to five cans per cultivar were used at each time-temperature combination. A range of temperature from 87.7-121°C, and a range of processing time from 5-45 min was used. At the end of the designated processing, the cans were quickly cooled under cold running water and then stored at 0°C for about a month, allowing the dices and water mixture to equilibrate.

Texture measurement

Texture profile analysis (TPA), as described by Bourne (1968, 1974) for Instron Universal Testing Machine, was used to characterize the textural attributes of the apple dices. TPA involves analyzing the force-time curve for a product to evaluate various textural parameters such as: hardness, fracturability, cohesiveness, chewiness, and springiness. Hardness-1 is the firmness measured during the initial compression. Hardness-2 measures the residual amount of hardness left at the end of the first compression. Cohesiveness is defined as the ratio of the force area during the second compression to that during the first compression. Chewiness is defined as the product of hardness, cohesiveness, and springiness. Springiness is

Author Anantheswaran, formerly with Cornell University, is now with the Food Processing Center, Dept. of Food Science & Technology, Univ. of Nebraska, Lincoln, NE 68583. Authors McLellan and Bourne are affiliated with the Dept. of Food Science & Technology, NYS Agric. Expt. Station, Cornell Univ., Geneva, NY 14456.

defined as the height that the food recovers between the end of first compression and the start of the second compression. These parameters have shown excellent correlations with sensory attributes (Szczesniak et al., 1963). Abbot et al. (1984) found that a combination of several Instron Texture Profile parameters greatly improved prediction of sensory attributes.

Before the test, the can was brought back to approximately 24°C by immersing it in warm water. A minimum of 20 regular cubes were picked randomly from the can for texture profile analysis. The dice (1.25 cm cube) was placed on a metal platform under the crosshead containing the load cell. It was then compressed twice to 92% of its original height in an Instron Universal Testing machine, using a crosshead speed of 5 cm/min.

The generated force distance curves were digitized using a Summagraphics Digitizer (Summagraphics Corp., CT). The data were then analyzed by a program written in Fortran for a Prime 400 computer (Bourne et al., 1978), and the various texture profile parameters were evaluated. An Analysis of Variance was carried out to determine the effect of time and temperature of processing on the texture profile parameters.

RESULTS & DISCUSSION

A SIMILAR PHENOMENON to that reported by Huang and Bourne (1983) was observed in apples in the present investigation; however, mechanism I ceased to be dominant in apples at a much shorter time period in comparison to vegetables. This might be attributed to differences in the carbohydrate and pectin contents in apples and vegetables. Based on preliminary experiments with processing times as short as 2.5 min, it was found that a large drop in hardness did occur during 2–3 min of thermal processing. During heating periods after the initial 2–3 min, the rate of softening was found to be much slower. It is postulated that mechanism I, as described by Huang and Bourne (1983), in apples generally ends in less than 5 min. All discussions, henceforth, will pertain to mechanism II as studied with processing times of 5 min and longer.

Hardness

There was a high positive correlation (r = 0.995) between hardness-1 and hardness-2 for the both varieties. This is to be expected since hardness-2 measures the residual amount of hardness left at the end of the first compression. The extent of tissue destruction and failure during the first compression was independent of the time and temperature of processing, resulting in the same ratio of original hardness being lost at the end of first compression.

Fig. 1a and 1b illustrate the effect of heating on hardness-1 for Spigold at the three lower temperatures and the four higher temperatures, respectively. Similar results were found with Cortland apples.

It is known that most physical changes in foods can be modeled as a first order process and it is the widely accepted model for softening. Hardness was modeled as a first order reaction. Hence the slopes of the predicted lines for the raw data presented in Fig. 1a and 1b represent the apparent rate of the reaction, the reaction constant (k) being equal to 2.3 times the negative slope as derived from the classical first order rate equation (Barrow, 1974). The samples processed at high temperature for a fairly long period of time were too soft to be handled for TPA, resulting in no data being obtained.

As expected, the rate of softening (Loss in hardness) increased with temperature (Fig. 2). This trend applied to both cultivars, although specific differences did occur. The most notable break from this overall trend occurred in the 121°C process. For Cortland apples the rate dropped significantly from the 115°C process. The Spigold apples increased slightly in rate. Although we have no apparent explanation, it is presummed to be related to differences in flesh structure from one cultivar to another.

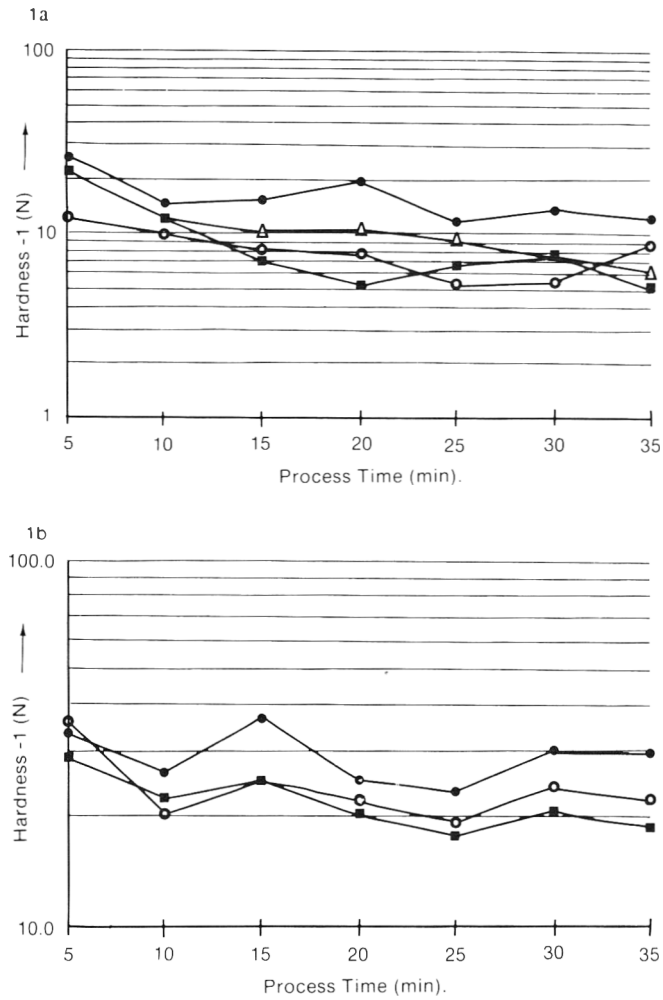


Fig. 1—Hardness-1 vs processing time for Spigold apples. 1a illustrates the response at the four higher temperatures (● 104.4°C, △ 110.0°C, ■ 115.6°C, and ○ 121.1°C); Ib illustrates the response at the three lower temperatures (● 87.8°C, ○ 93.3°C, and ■ 100.0°C). Separate graphs are for clarity of presentation only.

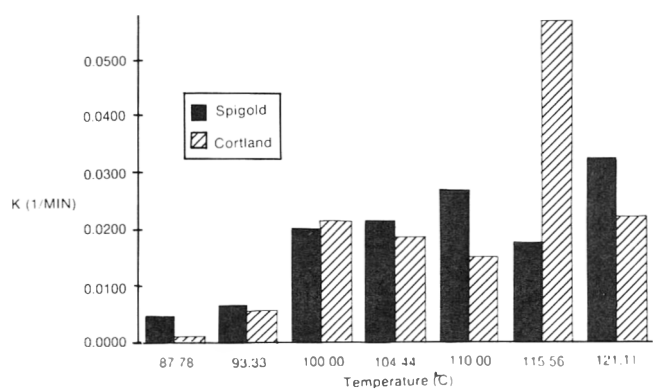


Fig. 2—Apparent rate constants for loss of hardness.

From the slope of the Arrhenius plot of ln(k) versus the reciprocal of the absolute temperature, the apparent activation energy was calculated as 26.5 ± 7.9 kcal/mole (r^2 = 68.7%) and 15.6 ± 3.8 kcal/mole (r^2 = 76.4%) for Cortland and Spigold, respectively. This is within the range reported for fruits and vegetables (Lund 1982; Huang and Bourne, 1983).

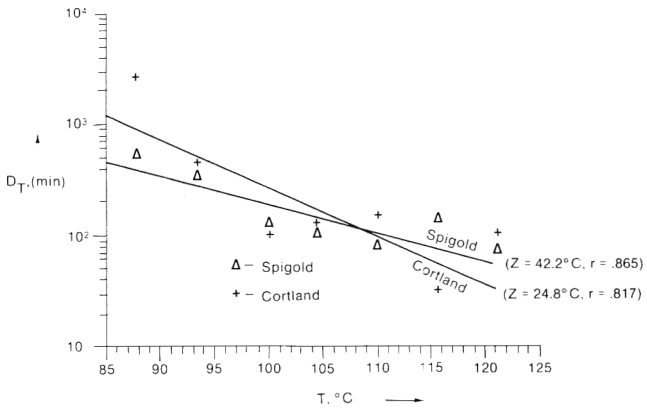


Fig. 3—Z value for loss of hardness.

Table 1—F-statistics from an Analysis of Variance on the texture profile parameters for two factors: process time and process temperature

	df	Hardness	Cohesiveness	Springiness	Chewiness
Spigold					
Time	11	13.09**	3.13**	1.57^{NS}	2.31^{NS}
Temp	6	115.72**	45.30**	0.68^{NS}	8.11**
Residual	df = 58				
Cortland					
Time	13	2.35**	1.72^{NS}	2.62**	3.51*
Temp	6	11.90**	31.81**	2.05^{NS}	3.14**
Residual	df = 56				

** — Significant at the 0.01 level
NS — Not significant
df — Degrees of freedom

The D value (D_T) for the loss of hardness was calculated as the negative reciprocal of the slope of the temperature-time curves for both cultivars. From the plot log(D_T) versus the process temperature, the Z_T value for the softening was calculated as 24.8°C and 42.2°C, respectively, for Cortland and Spigold cultivars (Fig. 3). This identifies the varietal differences in apples as a dominant factor in determining their behavior on thermal processing.

Other textural parameters

The other textural parameters determined were cohesiveness, chewiness and springiness. An analysis of variance was carried out to test for the significance of time and temperature of processing on these parameters (Table 1).

Cohesiveness increased during processing. For both the varieties, it increased significantly with temperature of processing. Time of processing had no significant effect on cohesiveness for Cortland samples, whereas Spigold did increase in this characteristic with time of processing.

Chewiness was found to decrease significantly with temperature of processing. In the case of Cortland chewiness decreased with the time of processing; however, no significant changes occurred in Spigold.

Springiness is a measure of the elasticity of the sample. In the case of Spigold there were no significant changes in springiness with increased temperature or time of processing. Whereas, in Cortland significant increases occurred with increased time of processing.

CONCLUSIONS

THE PRESENT STUDY shows that textural changes in apples during thermal processing are very complex. The varietal effect is still the dominant factor that determines the effect of thermal processing. During the first few minutes of thermal processing the hardness decreased considerably. The loss of hardness beyond 5 min of thermal processing was modeled as a first order reaction and the activation energy was found to be 26.5 kcal/mol and 15.6 kcal/mol for Cortland and Spigold varieties of apples, respectively. The z value for loss of hardness was determined to be 24.8°C and 42.2°C, respectively, for Cortland and Spigold.

The temperature of processing was found to change significantly cohesiveness and chewiness in both cultivars. In the case of Spigold, time of processing was found to change significantly cohesiveness, and gumminess. And in the case of Cortland, time of processing was found to change significantly springiness and chewiness. Detailed studies of textural changes in apples during the first few minutes of thermal processing are being planned.

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Solubility and Foaming Properties of Phytate-Reduced Soy Protein Isolate

B. H-Y. CHEN and C. V. MORR

ABSTRACT

A pilot scale ion exchange process was developed to produce a 75 - 77% phytate-reduced soy protein isolate. The solubility and foaming properties of this isolate were compared to those of control and commercial soy protein isolates as a function of protein concentration (5 and 10%, w/v), pH (3, 6 and 9) and preheat temperature (25, 60 and 80°C). Phytate-reduced soy protein extract exhibited minimum solubility at pH 4.8 - 5.0, compared to 4.2 - 4.5 for control soy extract. Phytate-reduced soy protein isolate was most soluble and functional at pH values below its isoelectric point (pH 3), whereas control and commercial soy isolates were generally most soluble and functional at pH values above their isoelectric point (pH 6 and 9).

INTRODUCTION

UTILIZATION of soy protein isolates in human food product manufacture is increasing at a faster rate than for any other soy protein product and is projected to continue at an annual rate of about 7% through the year 2000 (Sharpe, 1984). However, there is also a generally recognized need to improve the solubility and functionality of soy protein isolates to make them more acceptable to the food industry.

Soy protein isolates commonly contain up to 2% phytate, the hexaphosphate form of myo-inositol (Brooks and Morr, 1982). This polyvalent anionic compound is highly reactive and capable of forming complexes with negatively charged protein molecules at alkaline pH values through calcium and magnesium bonding mechanisms and with positively charged protein molecules at pH values below their isoelectric point by charge neutralization (Cheryan, 1980; Maga, 1982). Although phytate removal reportedly alters the isoelectric point, solubility and functionality of soy proteins (O'Dell and deBoland, 1976; Hartman, 1979), there is a noticeable lack of documented information on this matter (Morr, 1984).

The major purpose of this study was to produce sufficient phytate-reduced soy protein isolate to compare its solubility and foaming properties with those of control and commercial soy protein isolates as a function of several important pretreatments. These latter experiments were conducted with soy isolate dispersions at two protein concentrations (5 and 10%, w/v), three pH values (3, 6 and 9) and after preheating at one of three temperatures (25, 60 and 80°C) for 30 min.

MATERIALS & METHODS

Protein isolates

Control and phytate-reduced soy protein isolates were prepared from commercial, low temperature processed, defatted soy flakes (Ralston Purina Company, St. Louis, MO). Alkaline soy extract was adjusted to pH 4.5 with 0.1N HCl and centrifuged at 1,000 x *g* to recover the proteins, which were freeze dried as control soy protein isolate (Brooks and Morr, 1982). The laboratory ion exchange process of Brooks and Morr (1982) was modified to produce enough phytate-reduced soy protein isolate for this study. Alkaline soy extract was passed through a 3L bedvolume column of Rexyn 101 cation exchanger (Fisher Scientific, Atlanta, GA) to remove Ca and Mg ions and then through a 12L bedvolume column of Rexyn 202 anion exchanger (Fisher Scientific, Atlanta, GA) to remove phytate ions (Chen, 1983; Rodriguez et al., 1985). Extract flow rates were maintained at about 50 and 100 mL min^{-1} for the cation and anion exchange treatments, respectively, to assure complete reaction. The ion exchange treated extract was then adjusted to pH 4.8 - 5.0 and centrifuged at 1,000 x *g* to recover the proteins, which were freeze-dried as phytate-reduced soy protein isolate. The yield of phytate-reduced soy protein isolate was about 30 - 32% of the defatted flakes used, which was similar to that for control isolate. Commercial soy protein isolate was obtained from Ralston Purina Company (St. Louis, MO) and was designated SUPRO-620.

Isoelectric point determination

Aliquots of control and phytate-reduced soy extracts were adjusted to pH values ranging from 3.4 to 6 with 0.1N HCl or NaOH and centrifuged at 11,000 x *g* for 20 min. The concentration of soluble proteins remaining in the supernatant fractions was determined by dye-binding (Bradford, 1976).

Isolate protein solubility

The method of Morr et al. (1985) was used to determine protein solubility of freeze-dried soy isolates. About 500 mg of each isolate was accurately weighed into a 150 mL beaker and carefully dispersed into about 40 mL of 0.1M NaCl solution. The homogeneous dispersions were immediately adjusted to pH 3, 6, or 9 with 0.1N HCl or NaOH and stirred at slow speed for 1 hr. The protein dispersions were made to 50 mL with additional 0.1M NaCl solution and centrifuged at 20,000 x *g*. The soluble protein content of the resulting supernatant fractions was determined by Biuret (Gornall et al., 1949).

Foaming experiments

Soy protein isolates were dispersed in distilled, deionized water at approximately 5 and 10% protein concentration (w/v). Each dispersion was divided into 3 aliquots which were adjusted to pH 3, 6, or 9 with 0.1N HCl or NaOH. Each aliquot was then made to 5 or 10% protein concentration and further subdivided into 3 aliquots which were held at 25°C or heated at 60° or 80°C for 30 min with constant agitation in a shaker bath and then cooled to 25°C.

The above pretreated soy isolate dispersions were examined for protein solubility and foaming properties within a period of 60 min after their pretreatment was completed (Chen, 1983). An aliquot of each isolate dispersion was centrifuged at 15,000 x *g* and it supernatant fraction was examined for soluble protein content by Biuret (Gornall et al., 1949).

Foaming properties of the isolate dispersions were determined at 25 ± 1°C with a Kitchen Aid model K5-A mixer equipped with a stainless steel bowl and wire whip beater. A 100 mL aliquot of each dispersion was weighed into the bowl and whipped at a speed setting of 6 to produce maximum foam expansion. The whipping process was interupted after each 3 min time interval to determine the degree of foam expansion achieved. Foam expansion was determined by weighing a 100 mL aliquot of foam in a plastic weighing boat to ±0.01g and using the expression:

$$\text{Percent foam expansion} = \frac{\text{Wt. of 100 mL unwhipped dispersion} - \text{Wt. of 100 mL foam}}{\text{Wt. of 100 mL foam}} \times 100$$

Authors Chen and Morr are affiliated with the Dept. of Food Science, Clemson Univ., Clemson, SC 29631.

The foam was returned to the bowl and whipping was resumed for an additional 3 min period at which time the procedure was repeated. The whipping procedure was discontinued at the point where maximum foam expansion was achieved and the entire 100 mL of foam was transferred from the weighing boat onto the top of a pyrex funnel (7.5 cm inner top diameter, 0.4 cm inner stem diameter and 7.0 cm stem length). A thin layer of glass wool was positioned over the stem opening to retain the foam in the funnel. The length of time (min) required for the first drop of liquid to drain from the funnel stem and the percent of liquid (w/w) drained from the funnel during the first 30 min period were taken as indicators of foam stability.

Analytical

Freeze-dried laboratory isolates and spray-dried commercial soy protein isolate were analyzed for moisture by drying in a 100°C atmospheric oven for 2 hr (AOAC, 1980), for ash by ignition in a 550°C electric muffle furnace for 3 hr ((AOAC, 1980) and for protein content by micro-Kjeldahl (AOAC, 1980) using a nitrogen conversion factor of 5.71 (Morr, 1982). Phytate was determined by the procedure of Brooks and Morr (1982).

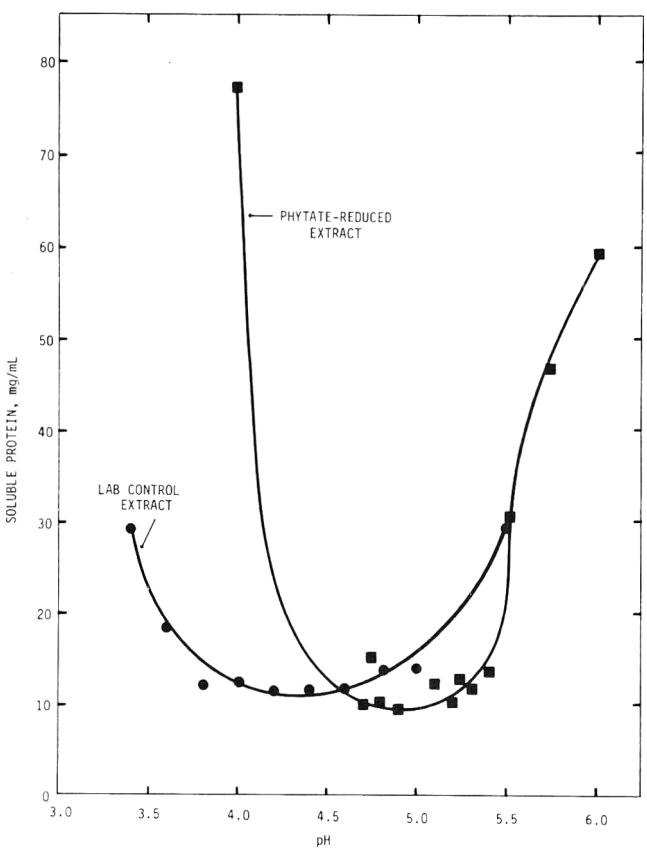


Fig. 1—pH-soluble protein concentration curves for control and phytate-reduced soy extracts prior to drying. Extracts were adjusted to pH values indicated with HCl or NaOH, centrifuged at 11,000 x g and assayed for soluble protein by dye-binding (Bradford, 1976).

RESULTS & DISCUSSION

Isoelectric point of soy extracts

Control soy extract exhibited minimum protein solubility at pH 4.2 - 4.5 compared to values of 4.8 - 5.0 for phytate-reduced extract prior to freeze drying (Fig. 1). The most obvious difference in solubility of these two protein systems is the substantially greater solubility of phytate-reduced soy proteins below pH 4 - 4.5. This improvement in protein solubility of phytate-reduced soy proteins at acid pH is undoubtedly due to a greater positive molecular charge in the absence of polyanionic phytate ions. The lower solubility of phytate-reduced soy proteins at pH between 4.5 and 5.5 (Fig. 1) is merely typical of their isoelectric point range.

Soy isolate composition

Mean compositional data for soy protein isolates are in Table 1. The isolates contained about 4 - 5% moisture and 1.6 - 3.7% ash. The lower ash content of phytate-reduced soy isolate is consistent with previous reports that the ion exchange process removes Ca, Mg and phytate ions (Brooks and Morr, 1982). The isolates contained about 75 - 82% protein. The phytate-reduced isolate contained 0.43% phytate compared to 1.9% for control isolate. Thus, the pilot scale process used in this study removed only 75 - 77% of the phytate from soy isolate compared to a $\geqslant$ 95% removal by the laboratory ion exchange process (Brooks and Morr, 1982). The problem of incomplete phytate removal by the pilot scale process is probably due to use of improperly designed ion exchange columns that failed to provide adequate contact of soy extracts with the ion exchanger media.

Isolate protein solubility

Protein solubility data for freeze-dried laboratory isolates and spray-dried commercial isolate are in Table 2. Phytate-reduced isolate was more soluble than control isolate at pH 3 but less soluble than control isolate at pH 6 and of equivalent solubility at pH 9. These latter solubility data are in good agreement with those provided for liquid extracts prior to drying (Fig. 1). Commercial isolate was less soluble than either of the two laboratory isolates at all pH values examined.

Table 2—Solubility of soy protein isolates as a function of pH, %[a]

Protein isolate	pH 3		pH 6		pH 9	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
Control	61.3	±0.26	22.3	±0.28	70.2	±4.27
Phytate-reduced	83.7	±2.25	11.6	±0.80	70.3	±9.25
Commercial	8.9	±0.96	12.1	±0.45	37.9	±4.45

[a] Mean values from three replicate determinations. Procedure of Morr et al. (1985).

Table 1—Composition of soy protein isolates, %[a]

Protein isolate	Moisture		Ash		Protein		Phytate	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Control	4.57	±0.06	3.62	±0.02	74.7	±0.58	1.90	±0.08
Phytate-reduced	4.22	±0.18	1.65	±0.04	82.2	±1.80	0.43	±0.01
Commercial	5.05	±0.03	3.74	±0.00	77.7	±1.28	1.00	±0.05

[a] Mean values from three replicate determinations.

Isolate foaming properties

Detailed data for protein solubility and foam expansion and stability as a function of protein concentration, pH, and preheating conditions are given in Table 3 for each of the isolates. Values from 3 to 7 of the experimental pretreatment conditions that resulted in greatest solubility and foam expansion values were pooled in Tables 4 and 5. Mean optimum protein solubility values ranged from 93 - 99% (Table 4). These solubility values were generally independent of protein concentration and preheating temperature but were strongly dependent upon pH. Phytate-reduced isolate was, as expected, most soluble at pH 3, but the other isolates were most soluble at pH 9.

Phytate-reduced soy protein isolates exhibited the largest mean optimum foam expansion value of 1336% (Table 4). Mean optimum foam expansion values for the other two isolates were 320 (commercial) and 984% (control). Mean optimum foam expansion values were generally independent of protein concentration for these protein isolates, which disagrees with reported findings of Eldridge et al. (1963). Mean optimum foam expansion performance of phytate-reduced soy isolate was generally independent of preheating temperature, whereas, the performance of the other two isolates was somewhat improved by preheating their dispersions at 60 or 80°C, which agrees with Eldridge et al. (1963). Foam expansion performance of these different isolates as a function of protein concentration, pH and preheating conditions was quite variable. For example, control isolate performed best at pH 6, whereas, phytate-reduced isolate performed best at pH 3.

Mean optimum foam stability data revealed that stable foams were provided by isolate dispersions when examined under optimum experimental conditions. Greatest foam stabilities were consistently found at 10% protein concentration conditions and at pH 3 and 9 for both laboratory isolate dispersions, whereas commercial isolate exhibited foam stability values that were independent of pH.

Although protein solubility and foaming performance of these soy isolate dispersions were generally related to pretreatment conditions employed, numerous exceptions were evidenced that failed to fit a pattern. For example, phytate-reduced isolate exhibited greatest mean solubility and foam expansion at pH 3 and at both 5 and 10% protein concentration. Although the optimum foam expansion value of this latter isolate was provided by the 25 and 60°C pretreatments, its protein solubility was independent of preheating conditions. It therefore appears necessary to experimentally determine the pretreatment conditions needed to provide optimum foaming performance of each of these soy isolates. These findings tend to confirm the complex nature of the foaming phenomenon and the inadequate state of knowledge concerning the physicochemical properties and reactions necessary to allow protein molecules to promote and stabilize aqueous foam systems (Cherry and McWatters, 1981).

Table 3—Foaming properties of soy protein isolate[a]

Protein conc (%, w/v)	Preheat temp (°C)	pH	Protein sol.[b] (%)	Maximum foam expansion (%)	Foam stability: Time of 1st drop (min)	Foam stability: Drainage (%)
			(Control)			
5	25	3	70.8	753	6.2	47
5	25	6	34.8	645	1.1	60
5	25	9	100	863	9.4	39
5	60	3	79.2	726	9.1	37
5	60	6	24.0	948	1.6	63
5	60	9	100	833	3.7	64
5	80	3	75.5	755	10.4	33
5	80	6	8.8	897	2.4	55
5	80	9	84.0	802	4.4	63
10	25	3	65.0	763	18.5	8.6
10	25	6	51.1	666	35.5	0
10	25	9	100	749	22.8	3.5
10	60	3	c	475	≥60	0
10	60	6	33.7	1108	6.8	41
10	60	9	100	645	14.8	16
10	80	3	c	497	≥60	0
10	80	6	10.8	790	6.1	32
10	80	9	96.1	469	≥60	0
			(Phytate-reduced)			
5	25	3	100	1539	22.6	14
5	25	6	21.0	511	25.0	0
5	25	9	95.1	735	14.7	16
5	60	3	100	1304	23.6	11
5	60	6	4.5	541	0.7	70
5	60	9	89.5	676	3.4	63
5	80	3	93.1	741	9.8	43
5	80	6	13.6	673	2.6	60
5	80	9	64.4	611	3.5	56
10	25	3	100	1164	32.9	0
10	25	6	8.2	676	4.0	33
10	25	9	89.0	564	58.9	0
10	60	3	c	442	≥60	0
10	60	6	16.4	643	4.5	34
10	60	9	96.8	541	22.1	2.2
10	80	3	c	285	≥60	0
10	80	6	3.2	859	6.3	34
10	80	9	56.8	465	≥60	0
			(Commercial)			
5	25	3	55.9	277	13.8	6.5
5	25	6	25.2	62	<0.5	91
5	25	9	91.3	74	<0.5	90
5	60	3	42.7	141	7.6	1.3
5	60	6	34.2	57	<0.5	91
5	60	9	87.2	332	<0.5	91
5	80	3	33.4	250	2.0	47
5	80	6	38.1	35	1.8	32
5	80	9	95.4	320	<0.5	94
10	25	3	11.6	36	≥60	0
10	25	6	25.3	136	≥60	0
10	25	9	92.4	283	≥60	0
10	60	3	36.5	36	≥60	0
10	60	6	30.9	44	27.2	0
10	60	9	75.5	315	≥60	0
10	80	3	39.4	102	≥60	0
10	80	6	27.4	31	8.5	84
10	80	9	63.3	315	≥60	0

[a] Mean values from three replicate determinations.
[b] Dispersions were centrifuged at 15,000 x g and soluble protein assayed by Biuret.
[c] Gel formed.

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Table 4—Solubility and foaming properties of soy protein isolates examined at optimum experimental conditions[a]

Protein isolate	Solubility, %[b]			Maximum foam expansion, %			Foam stability: Time of first drop, min			Foam stability: Drainage, %		
	n	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD
Control	5	99.2	±1.8	3	984	±110	3	60	±0	4	0	±0
Phytate-reduced	5	98.0	±3.1	3	1336	±189	4	59.7	±0.6	6	0	±0
Commercial	3	93.0	±2.1	4	320	± 8	7	60	±0	7	0	±0

[a] Data-selected from Table 3 for experimental conditions that provided highest solubility and foaming performance.
[b] Dispersions were centrifuged at 15,000 x g and soluble protein assayed by Biuret.

Table 5—Selected experimental conditions that provide optimum solubility and foaming properties to soy protein isolates

	Solubility[a]	Foam expansion	Foam stability
Control	5 and 10% protein conc pH 9 25, 60 and 80°C preheat	5 and 10% protein conc pH 6 60 and 80°C preheat	10% protein conc pH 3 and 9 60 and 80°C preheat
Phytate-reduced	5 and 10% protein conc pH 3 25, 60 and 80°C preheat	5 and 10% protein conc pH 3 25 and 60°C preheat	10% protein conc pH 3 and 9 25, 60 and 80°C preheat
Commercial	5 and 10% protein conc pH 9 25 and 80°C preheat	5 and 10% protein conc pH 9 60 and 80°C preheat	10% protein conc pH 3, 6, and 9 25, 60 and 80°C preheat

[a] Pretreated dispersions were centrifuged at 15,000 x g and soluble protein assayed by Biuret.

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of hesperidin-pectin particles with 25% D.E. and 100 ppm calcium with a ratio of 1:4.4. These figures are slightly lower than those of Rouse (1949), who estimated that the D.E. necessary for cloud precipitation is 38% for 25% cloud loss, and higher than those of Baker (1979), who showed that low ester pectin treated with pectinesterase precipitated the cloud at a D.E. of 14% or less.

This seems to be an important observation that can help us to understand better the factors that initiate spontaneous flocculation of the juice during storage. If the D.E. of the pectin is above 36%, pectinesterase or calcium ions cannot be the reasons for flocculation and therefore other factors should be looked for.

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Functional Properties of Dry Milled Fractions From Wild Oats (*Avena fatua* L.)

PETER R. CHANG and FRANK W. SOSULSKI

ABSTRACT

When processed into groats, wild oats provided meal, bran and flour fractions which contained 22, 28 and 18% protein, respectively. While nitrogen solubilities were low due to the steam treatments used to inactivate lipase, water hydration capacities of the wild oat products were high. Oil absorption characteristics of the bran were more than twice those of the flour but oil emulsions formed with wild oat products were poor. Starch contents of the meals and flours were 55 and 70%, respectively, and their Visco/Amylograph curves were characterized by low initial pasting temperatures, high peak viscosities at 95°C and setback values that were intermediate to those of corn flour.

INTRODUCTION

WILD OATS (*Avena fatua* L.) are the most important weed pest in the grain industry, and annual crop losses attributed to this weed are estimated at a billion dollars in Canada and a half billion in the United States (Miller and Mitich, 1976). In major commercial seed cleaning plant associated with grain terminals and flour mills, wild oats are separated primarily as Mixed Feed Oats for disposition as livestock feeds. Mechanical dehulling of these feed grades yield 50 - 60% of wild oats groats (Sosulski and Sosulski, 1985). Wild groats appear to have an exceptionally high nutritive value, containing over 20% protein (N x 6.25), 9% lipid and 55% starch. Previously, Tkachuk and Mellish (1977) reported that wild groats contained 19.6% protein (N x 5.7) and 1.6% oil. In feeding trials with weanling rats, the protein efficiency ratios (PER) obtained when wild oat flakes were the sole protein source in the diets was 2.0, about 90% of the PER value for the casein control (Sosulski et al., 1985). In these experiments, the apparent digestibility of protein and energy utilization were 87 and 94%, respectively.

Due to the ample supply of raw material, wild groats has the potential of becoming a major new grain commodity. Because of its unique composition, wild oat flakes, meal and flour could find markets as high protein and energy supplements in breakfast cereals, infant foods and specialty diets. Before wild oat products are utilized widely by the food industry, their functional properties alone and in blends with other ingredients will need to be determined. The functionality of protein concentrates from oats has been determined (Ma, 1983), but the main applications are as flakes and meals in breakfast cereals and infant foods (Youngs et al., 1982). Oat flours have not competed effectively against other cereal flours, and the markets as a functional ingredient in foods are limited to breakfast cereal blends.

The objectives of the present investigation were to compare the functional properties of wild oat meal, bran and flour with those of domestic groats (*Avena sativa* L.) as well as commercial flours prepared from wheat, corn, rice and potato. Because wild groats contained appreciably more lipid material than occur in these commercial flours, or even the domestic groat products, the functionality of meal, bran and flour from defatted wild groats were also determined.

Author Sosulski is affiliated with the Dept. of Crop Science & Plant Ecology, Univ. of Saskatchewan, Saskatoon, Sask., Canada S7N 0W0. Dr. Chang is now with the Ontario Centre for Farm Machinery & Food Processing Technology, Chatham, Ontario, Canada N7M 5J5.

MATERIALS & METHODS

Plant materials

Domestic groats and wild groats were processed and supplied by Robin Hood Multifoods Inc., Saskatoon. Mixed Feed Oat samples were purified to contain 97% wild oats and less than 2.0% other grains (mainly wheat and barley) and 0.5% of foreign material (mainly chaff and hulls). For both wild and domestic oats, the hulls were cracked on impact hullers and separated from the groat (kernel) by air aspiration. The groats were steamed to inactivate the lipase enzyme in the bran layers of the groat and kiln-dried before storage. This heat treatment was necessary to stabilize the lipids and prevent the rapid onset of rancidity after the groat is ground.

Defatted wild groats were prepared by extracting the lipids with hexane for 16 hr on a Goldfisch extractor followed by removal of solvent at 40°C in a vacuum oven. Oat meals were prepared by grinding the full-fat and defatted groats in a Krups coffee mill and sifting on a 20W screen. Groats were also milled into flour and bran fractions with a Brabender Quadrumat Junior flour mill. Bran was sifted on 60-mesh sieve and the throughs were added to the flour fraction.

To serve as comparative controls for the oat samples, commercial flours of wheat, corn, rice and potato were obtained from a local wholesaler.

Analytical methods

Proximate analyses and neutral detergent fiber (NDF) (or insoluble dietary fiber) were determined by AACC (1983) procedures and starch content by the dual enzyme method of Banks et al. (1970). The pH values were measured on 10% dispersions (w/v) of products in distilled water. Nitrogen solubility tests were done with the 2-hr extraction method (Method 46-23) of the AACC (1983) at pH 2,4,6,8 and 10 in distilled water and 0.25M NaCl.

Amino acids were determined by hydrolysis of proteins with distilled 6N HCl for 24 hr at 110°C under nitrogen. Hydrolysates were evaporated to dryness *in vacuo*, dissolved in buffer, and analyzed on a Beckman Model 119 BL analyzer with separate hydrolyses for sulfur-containing amino acid and tryptophan determinations (Olson et al., 1978).

Functional tests

Functional property tests were determined on samples of products which were adjusted to an equal dry weight basis. Water hydration capacity was measured by the AACC (1983) method 88-04, and is expressed as the amount of water bound by a 100-g sample on a dry moisture basis. Oil absorption was measured by the centrifuge procedure of Lin et al. (1974) using a centrifugal force of 2,000 x *g* for 25 min. The results are expressed as grams corn oil bound by 100g sample, using 0.918 g/mL as the density of corn oil.

Emulsificaton capacity was based onthe oil titration method of Beuchat (1977) where the breakpoint was defined subjectively as the point at which the emulsion coalescence broke, resulting in liquid separation with substantial loss of consistency. Emulsion stability was measured as the volume of water separated at time intervals up to 24 hr. Effects of concentration on emulsion capacity were evaluated at sample concentrations of 1, 2, 3 and 4% (w/v). The 2% slurry was used to determine the effects of pH on emulsion

capacity using 1N HCl or 1N NaOH for pH adjustment. Salt (NaCl) concentrations of 0.0, 0.2, 0.4, 0.6 and 0.8 molarity in 2% (w/v) slurries were employed to study the effects of salt on emulsion capacity.

Foaming capacity (whippability) and foaming stability of samples were measured by the Lin et al. (1974) procedure using low speed mixing for 30 sec and high speed whipping for 6 min.

Pasting curves were determined on a Brabender Visco/Amylograph equipped with a 700 cmg cartridge. Slurry concentration was 11% (dry basis) in 450 mL of water. The slurries were heated from 30 - 95°C and held at that temperature for 15 min before cooling at 1.5°C per min for 30 min to determine the setback on cooling.

Apparent (cold) viscosity was measured on 10% slurries at room temperature using the NV sensory system of a Haake Rotovisco Model RV-3 (Haake, Karlsruhe, West Germany) at a rotor speed of 90.5 rpm. The SV sensory of the Rotovisco at the same rotor speed was used to determine the firmness of gels prepared by cooking 10% slurries for 30 min in a boiling water bath and then refrigerating the pastes for 18 hr at 5°C.

Color measurements on the dry flours were performed on a Hunterlab D25D2M Digital Color Difference Meter equipped with the M optical head. A white tile (No. C2-5470; L = 94.7, a = −0.9, b = 0.5) was used to standardize the instrument where L = 100 (white), L = 0 (black); +a = red, −a = green; +b = yellow, −b = blue.

Data reported in the tables and figures are means of duplicate analyses.

Table 1—Proximate and chemical composition of domestic and wild oat meals and milled products, and the control flours, dry basis

Crop and milled fraction	Protein[a] %	Fat %	Fiber %	Ash %	Starch %	NDF[b] %
Domestic groats						
Meal	15.3	8.7	2.1	2.1	62.3	7.4
Bran	20.9	11.7	4.4	4.1	38.9	21.5
Flour	12.7	7.9	0.7	1.0	76.1	1.1
Wild groats						
Meal	21.9	10.0	2.8	2.5	55.1	8.2
Bran	28.0	12.6	4.9	4.3	35.6	18.5
Flour	17.9	9.1	0.8	1.3	70.2	2.5
Defatted wild groats						
Meal	22.9	2.2	2.4	2.7	61.2	8.1
Bran	33.5	5.7	5.1	5.7	27.4	22.6
Flour	18.2	1.2	0.6	1.3	72.7	1.2
Wheat flour	17.3	1.3	0.4	0.5	74.3	1.3
Corn flour	7.0	1.4	0.7	0.3	87.8	0.9
Rice flour	9.3	0.7	0.4	0.6	81.0	1.2
Potato flour	9.6	0.1	1.9	3.6	74.3	2.5
Standard deviation	0.6	0.3	0.3	0.2	2.9	0.5

[a] N x 6.25.
[b] Neutral detergent fiber.

RESULTS & DISCUSSION

Composition

Wild groats or meal contained 43% more protein than domestic oat meal, the protein level of 22% being higher than values for any of the commercial cereal products (Table 1). Wild oat meal was also high in crude fat, 10%, and contained more crude fiber, ash and NDF than domestic oat meal. However, the starch level of 55% was substantially less than the 62% in domestic groats. Defatting the wild groat increased the concentrations of most nonlipid components except fiber in the processed products.

Milling the groats gave flour yields of 66 and 68%, respectively, for wild and domestic groats, and 68% in the case of defatted wild groats. The bran fraction of wild groats contained 28% crude protein and over 12% crude fat (Table 1). The NDF content in the bran was 18.5% but the starch level was reduced to about 35.5%. While depleted in protein and lipid, relative to the original groat, the wild oat flour was still a rich source of protein, lipid and starch. The milling of the defatted wild groat gave a more efficient fractionation of protein, fat, starch and NDF between bran and flour than was possible for the full-fat groat.

Among the commercial controls, only wheat flour was similar in composition to the defatted wild oat flour (Table

Table 2—Composition of amino acids (AA) in the proteins of domestic and wild groat products, and the NAS (1980) pattern of human requirements (g amino acid/100g protein)

Amino acid	Domestic groats			Wild groats			Standard deviation	Human requirements
	Meal	Bran	Flour	Meal	Bran	Flour		
Essential for human nutrition								
Histidine	2.2	2.2	2.0	2.3	2.3	2.2	0.02	1.7
Isoleucine	3.6	3.6	3.4	3.7	3.7	3.8	0.09	4.2
Leucine	7.4	7.4	7.1	7.4	7.6	7.4	0.04	7.0
Lysine	4.1	4.1	3.6	3.7	3.9	3.6	0.04	5.1
Methionine	1.7	1.8	1.8	1.4	1.4	1.3	0.10	2.6
Phenylalanine	5.0	4.9	4.6	5.5	5.6	5.3	0.20	7.3
Threonine	2.7	2.7	2.5	2.5	2.5	2.4	0.05	3.5
Tryptophan	1.6	1.8	1.6	1.7	1.7	1.6	0.07	1.1
Valine	5.3	5.2	5.2	5.3	5.7	5.3	0.27	4.8
Nonessential for human nutrition								
Alanine	4.6	4.7	4.2	4.4	4.7	4.4	0.07	—
Arginine	6.7	6.9	6.3	6.9	7.2	6.7	0.19	—
Aspartic acid	8.7	8.5	8.3	8.4	8.7	8.6	0.12	—
Half-cystine	2.9	3.1	2.8	2.7	2.8	2.5	0.05	—
Glutamic acid	20.0	20.2	19.7	19.6	19.5	19.8	0.14	—
Glycine	5.0	5.1	4.5	4.6	4.9	4.4	0.02	—
Proline	5.3	5.3	4.8	5.1	5.2	5.1	0.08	—
Serine	4.7	4.8	4.4	4.6	4.8	4.5	0.03	—
Tyrosine	3.4	3.6	3.2	3.7	4.0	3.4	0.40	—
Total	97.2	98.3	92.3	96.0	98.7	94.8	—	37.3
Ammonia	2.5	2.3	2.4	2.4	2.5	2.5	0.05	—
AA score	77	77	71	71	71	69	—	100
Limiting AA	Thr	Thr	Lys	Thr	Thr	Thr	—	—

1). Corn and rice flours were characteristically low in protein, fat, fiber and ash, but contained particularly high contents of starch. Potato flour had somewhat greater quantities of fiber and ash, but starch content was similar to levels in oat and wheat flours.

The high protein contents of the wild groat products were particularly significant because there was 3.6% lysine in flour proteins and 3.9% lysine in bran proteins (Table 2). The domestic groat products contained slightly higher levels of lysine, which perhaps reflected the negative correlation between lysine and total protein in oats (Youngs et al., 1982). Ma (1983) reported lysine levels of 3.6% in the domestic groat but a purified bran fraction obtained during wet processing had 5.5% lysine.

In an earlier study (Sosulski et al., 1985), lysine was reported to be the first limiting amino acid relative to human requirements (NAS, 1980). The present data (Table 2) show that threonine was marginally more deficient than lysine in most of the present wild groat samples. Total sulfur-containing amino acid contents exceeded the human requirement level of 2.6 g/100g protein, and methionine contents were sufficient to satisfy at least one-half of this requirement. Thus, the amino acid scores of 69 - 72% for wild groat products were relatively high compared to the corresponding values of 45 and 55%, respectively, for wheat or corn (Sosulski, 1977). Rice and potato have similar amino acid scores of over 70%, but their protein contents were very low (Table 1).

The high lipid levels of 8.7 and 10.0%, respectively, in domestic and wild oat meals (Table 1) would make a substantial contribution to the nutritive value of oat-based foods since the utilization of energy was 94% (Sosulski et al., 1985). However, oats contain an active lipase in the bran layers, and green groats cannot be ground without initiating the development of soapy flavors and rancidity (Youngs et al., 1982). Steam treatment and kilning are commonly employed by the food industry to stabilize the groats, flakes and flours. The present domestic and wild groats were treated similarly during manufacture so that the roller-milled brans and flours did not develop off-flavors during the course of the study.

The compositions of 7.4 and 8.2% of NDF in the domestic and wild oat meals, resp., or 21.5 and 18.5% in the respective brans (Table 1), were relatively low compared to the levels in commercial bran products used in high fiber foods (Sosulski and Cadden, 1982). Yet Chen and Anderson (1979) found that the addition of oat bran to a rat diet containing cholesterol would lower the accumulation of cholesterol and triglycerides in both plasma and liver. Gould et al. (1980) confirmed that a high fiber diet based on oatmeal would effectively reduce the serum cholesterol in adults with hypercholesterolemia. Because of the high yields of bran during milling, the wild groat would be an

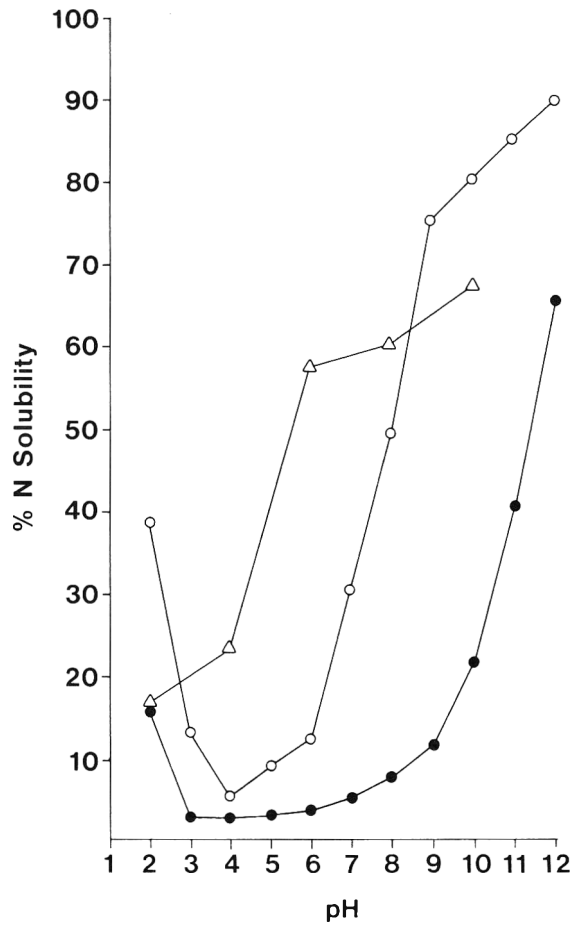


Fig. 1—Nitrogen solubility curves for wild groats before (○ – ○) and after (● – ●) steam treatment and kiln drying and, for the unheated groat, in 0.25M NaCl (△ – △).

Table 3—Nitrogen solubilities, pH of dispersion and absorption properties of domestic and wild oat meals, brans and flours, and control flours, dry basis

Crop and milled fraction	Nitrogen solubility, %					pH of dispersion	Water hydration capacity (%)	Oil absorption (%)
	pH 2	pH 4	pH 6	pH 8	pH 10			
Domestic groats								
Meal	11.9	2.5	2.6	8.5	24.4	6.4	118	75
Bran	3.5	1.8	2.4	14.3	22.3	6.5	217	137
Flour	45.7	3.5	4.8	16.2	23.4	6.2	110	62
Wild groats								
Meal	14.8	0.2	0.7	4.6	25.6	6.3	130	73
Bran	1.1	0.7	1.9	10.0	25.6	6.4	191	120
Flour	54.7	2.0	1.6	3.9	29.5	6.1	120	56
Defatted wild groats								
Meal	15.3	0.2	1.3	5.7	25.5	6.3	132	88
Bran	3.8	1.4	1.2	8.5	25.1	6.4	229	178
Flour	52.0	2.4	2.1	6.4	23.1	6.2	124	80
Wheat flour	82.5	78.3	15.4	31.5	96.0	5.6	93	101
Corn flour	12.5	6.3	6.8	11.4	20.2	6.0	92	73
Rice flour	49.5	3.0	4.8	12.3	18.6	6.2	86	75
Potato flour	62.7	62.5	63.7	66.4	66.6	6.0	523	129

Table 4—Oil emulsion and foaming capacity and stability of processed products of domestic and wild groats, and control flours

Crop and milled fractions	Emulsion capacity, g/g	Volume of water separated, mL				Volume inc. on whipping %	Volume of foam after whipping, mL		
		10 min	1 min	3 min	24 min		10 min	1 hr	2 hr
Domestic groats									
Meal	29.0	31	41	43	44	6	103	101	100
Bran	35.3	14	32	38	42	10	104	102	101
Flour	28.5	32	40	43	45	4	102	102	100
Wild groats									
Meal	29.1	31	37	39	41	3	102	101	100
Bran	33.7	26	37	40	42	8	104	101	100
Flour	27.3	3	8	18	27	10	106	102	100
Defatted wild groats									
Meal	30.9	8	36	40	45	20	106	102	102
Bran	34.8	15	35	38	41	10	104	100	100
Flour	29.4	22	31	36	42	26	110	102	100
Wheat flour	29.4	0	0	0	0	90	176	166	156
Corn flour	15.6	42	43	44	44	2	102	102	102
Rice flour	13.8	38	40	41	41	10	105	103	102
Potato flour	26.6	0	0	40	42	8	107	106	104

Table 5—Effects of concentration of defatted wild oat bran, pH and salt concentrations on oil emulsification capacity

Bran conc (w/v) %	Emulsion capacity g/g	pH[a]	Emulsion capacity g/g	Salt[a] conc M	Emulsion capacity g/g
1	63.0	2	39.2	0.0	34.7
2	34.7	4	35.8	0.2	36.4
3	29.5	6	34.5	0.4	45.1
4	29.2	8	35.6	0.6	43.2
		10	42.5	0.8	41.2

[a] Conducted at a bran concentration of 2% (w/v).

excellent commercial source of bran for fiber-supplemented foods and diets. For this reason, the wild oat bran was investigated as a functional component in food systems.

Protein solubility

The solubilities of the proteins (nitrogen) in the domestic and wild groat products were very low within the pH range 4 - 8 (Table 3). The proteins in the flours showed somewhat higher solublity at pH 2, about 50%, but the solubility at pH 10 was low in the flour and other groat products. In corn flour, the solubility of proteins was low over the pH range 2 - 10. Rice flour proteins exhibited a solubility pattern much like that of oat flours. In contrast, wheat proteins showed high solubility at pH 2 - 4 and pH 10. Potato flour proteins had an unusual pattern of high solubuility across the pH range tested.

Steam treatment of the groats, necessary to stabilize the lipids before grinding the groats, was expected to denature the proteins and decrease nitrogen solubility. Therefore, nitrogen solubility curves were determined for the groats before and after the steam treatment. While the unheated wild groat sample showed higher nitrogen solubilities than the treated sample, the wild oat proteins still had a broad range of insolubility over the pH range 3 - 6 (Fig. 1). Ma (1983) showed much higher solubility for oat protein concentrate which represented only 65 - 70% of the original groat proteins. The present results demonstrate that the proteins in commercial meals or flours which have been stabilized by heat would be insoluble over the range of pH commonly used in foods.

The solubility of the unheated wild oat proteins was determined at pH 7 using a range of salt (NaCl) concentrations from 0.01 - 0.75M. The solubility of wild oat proteins increase from 11% at 0.01 NaCl to 60% in 0.25M NaCl, above which the increases were small. Using 0.25M NaCl, the solubility pattern over the pH range 2 - 10 showed high values between pH 6 - 10 (Fig. 1). Thus, in certain food applications, such as in ground meat emulsions, the functionality of oat proteins may be high. Lipase activity would need to be controlled early in the meat processing system by heat treatment or use of low pH.

Ten percent dispersions of the oat products in distilled water exhibited a narrow pH range of 6.1 - 6.5 (Table 3). Wheat flour had a more acid pH (5.6) than corn, rice or potato flours. These pH values were within the range of least solubility for each protein source. Most of the functional tests were conducted at or near these natural pH levels.

Water hydration and oil absorption

The water hydration capacities of the domestic and wild oat meals and flours were similar at 118 - 130% of the sample weights but the brans exhibited water absorptions of about 200% (Table 3). Defatting the wild groats did not alter the water hydration characteristics of the processed products significantly. Wheat, corn and rice flours exhibited lower water absorptions than oat flours but potato flour showed its characteristic high water hydration capacity.

The oil absorption properties of the domestic and wild oat meals were intermediate between the corresponding bran and flour fractions, the values for the brans being over twice those of the flours (Table 3). The domestic oat bran and flour had similar oil absorption properties to those of the wild oat fractions, and the defatted wild oat bran and flour gave the highest values. Corn and rice flours were similar in oil absorption to the oat flours while wheat and potato flours gave high values in the range of the full-fat oat brans.

Emulsion capacity and stability

Emulsification capacities of oat bran fractions were slightly higher than those of the meals or flours but all values were relatively high (Table 4). Wheat and potato flours gave comparable emulsion capacities to the processed groat products but corn and rice flours exhibited about one-half the emulsification capacities of other flours.

Table 6—Viscosities of domestic and wild oat meal, bran, flour, and control flour slurries, and the color measurements on the dry products

Crop and milled fraction	Brabender Visco/Amylograph curve					Apparent viscosity cps	Gel firmness cps	Hunterlab color difference		
	Initial pasting temp[a] °C	Peak viscosity BU	Peak temp °C	Viscosity after 15 min[b] BU	Setback on cooling[c] BU			L	a	b
Domestic groat										
Meal	55.5	810	95	560	400	7.8	3376	83.0	−0.1	11.1
Bran	40.5	750	95	510	550	42.0	3107	74.1	0.3	14.1
Flour	57.0	850	95	630	370	6.8	3604	86.5	−0.8	8.9
Wild groat										
Meal	54.0	840	95	530	430	8.8	1906	76.9	−0.2	11.1
Bran	48.0	660	95	370	540	29.8	1719	73.0	0.4	13.5
Flour	63.0	990	95	700	260	6.0	2340	82.6	−0.6	10.2
Defatted wild groat										
Meal	57.0	1000	95	610	310	9.3	2527	87.4	−0.4	5.7
Bran	36.0	780	95	460	590	50.8	2071	76.8	0.3	10.7
Flour	52.5	1190	95	750	240	7.8	2796	89.0	−0.7	4.7
Wheat flour	61.5	530	95	400	410	2.1	2817	88.0	−1.0	8.5
Corn flour	66.0	850	87	590	980	2.9	4838	86.5	−2.6	23.4
Rice flour	72.0	880	95	530	520	2.3	4486	88.5	−1.2	5.2
Potato flour	61.0	910	43	270	200	140.0	2403	82.8	−2.1	14.1

[a] Temperature when curve reached 10 Brabender units (BU).
[b] Viscosity after holding temperature at 95°C for 15 min.
[c] Difference between 15-min height and viscosity on cooling.

The stability studies showed that wheat flour emulsions were fully stable over the 24-hr test period (Table 4). Potato flour maintained an emulsion for only 1 hr whereas most of the other brans and flours performed poorly as emulsifying agents. Only wild oat flour appeared to be partially effective over the 24-hr period.

Additional studies were conducted on conditions for the emulsification test using the defatted wild oat bran which had shown high oil absorption (Table 3) and emulsification capacity (Table 4). Low bran concentration of 1% gave a much improved emulsification capacity (Table 5) but the emulsion was not stable. High pH and salt concentration also favored emulsion formation but these were not stable beyond 30 - 60 min.

Foaming properties

Like corn, rice and wheat flour, the groat samples were ineffective in developing stable foams on whipping (Table 4). The lipids in the domestic and wild groat products were not a major factor in depressing foam stability since the defatted products were only slightly improved in foaming capacity and stability. Only the wheat flour sample exhibited strong foaming properties which were stable over the 2-hr test period.

Viscosity characteristics

The amylograph curves for the oat products were compared with the characteristic curve for corn flour (Table 6). On this basis, the oat meals and flours had low initial pasting temperatures, high peak viscosities after 95°C was reached, high viscosities during holding at 95°C, but setback values were only intermediate. Compared to the oat flours, the oat brans had even lower initial pasting temperatures, lower peak viscosities but setbacks were higher. Wild oat products were lower in viscosities than domestic oat products, but the highest viscosities were attained with the defatted wild oat products.

Compared to the oat flours, wheat flour had a low peak viscosity, rice flour exhibited a low initial pasting temperature and potato flour had a low peak temperature (Table 6).

Apparent or cold viscosity was also determined on 10% slurries with the Haake Rotovisco Instrument to demonstrate the very high apparent viscosities of the oat brans (Table 6). Wild oat bran was lower in slurry viscosity than the domestic oat bran but the defatted bran gave the highest viscosity value, next to that of potato flour. Even the slurries of oat meals and flours were three to four times more viscose than wheat, corn and rice flours.

The firmness of gels formed by refrigeration of the cooked pastes were very high for corn and rice flours (Table 6). The values for the domestic oat products were intermediate at 3107 - 2340 cps as compared to the wild oat products (1719 - 2340 cps), although defatting the products resulted in somewhat higher gel firmness values. Oat brans developed lower viscosity gels than the flours in both oat species, probably because this property was associated with starch contents.

Product colors

The wild oat products were slightly darker in appearance than domestic oat products, and defatting resulted in higher L values (Table 6). The oat brans were darker than the flours, having an attractive tan appearance. Corn and potato flours were more yellow-brown than oat brans which contrasted with the bright white appearance of wheat and rice flours.

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Relation Between Oxygen-17 NMR and Rheological Characteristics of Wheat Flour Suspensions

S. J. RICHARDSON, I. C. BAIANU, and M. P. STEINBERG

ABSTRACT

Water binding has a significant influence on the rheological properties of wheat flour suspensions. Wheat flour-water suspensions at concentrations from 60 - 95% wet basis were subjected to rheological and NMR analyses in order to ascertain the relation between the two methods. The rheological data obtained by the Haake RV-3 Rotoviscometer followed the Power Law, thus comparisons were based on consistency coefficient (a) and flow behavior (b) values. The NMR spectrometer measured oxygen-17 relaxation rates (34 MHz in water and in deuterium oxide) as an indication of water mobility. The data showed a general inverse relationship between consistency coefficient and water mobility. However, the data indicated three distinct regions, each showing a linear relation with water mobility decreasing as apparent viscosity increased.

INTRODUCTION

THE PHENOMENA of water binding by wheat flour and its components have been investigated by several researchers (Vail and Bailey, 1940; Bushuk and Winkler, 1957; Webb et al., 1970; Steinberg and Leung, 1975; Bushuk and Mehrotra, 1977; Leung et al., 1979, 1983). However, the specific nature of water binding and water mobility in wheat flour suspensions and doughs has still not been fully elucidated. The importance of investigating the water binding in the wheat flour-water system lies in its influence on the rheological properties of the doughs and their baking performance (Finney and Shogren, 1972).

It is the gluten protein components of the wheat flour that are primarily responsible for the unique viscoelastic characteristics of the dough (Wall and Beckwith, 1969; Redman, 1971). Gluten is composed of two fractions, glutenins and gliadins. Previous rheological work with these fractions (Wall and Beckwith, 1969) has shown that the shear stress is a function of protein concentration; gliadin suspensions behaved like Newtonian fluids whereas glutenin suspensions showed non-Newtonian characteristics.

Rheological studies on wheat doughs are extensive (Wall and Beckwith, 1969; Smith et al., 1970; Bloksma, 1971; Ewart, 1972; Launay and Bure, 1973; Hoseney, 1979; Navickis et al., 1982; Haddad, 1984). However, none could be found that cover the batter and wet dough range, which is the major emphasis of this work; Mewis (1980) gave a general review of suspension rheology, and Urbanski (1981) worked on soybean components.

Rheological methods are used to probe the macroscopic functional property of the wheat flour-water system. On the other end of the spectrum, a technique that has been used to probe the microscopic or molecular mobility of water in a variety of systems is Nuclear Magnetic Resonance (NMR) Spectroscopy (Fuller and Brey, 1968; Steinberg and Leung, 1975; Berendsen, 1975; Leung et al., 1976; Woessner, 1977; Edzes and Samulski, 1978; Tricot and Niederberger, 1979; Renou et al., 1983; Lang and Steinberg, 1983; DiNola and Brosio, 1983). A report by Barker et al. (1974) suggested the possible correlation between NMR linewidths and rheological properties in viscoelastic liquids. Baianu et al. (1982) proposed NMR measurements for wheat variety identification and correlation with rheological testing as a practical application of NMR technology.

The specific objective of this study was to test the hypothesis that the rheological properties of a wheat flour-water system are related to the mobility of its water component, using Oxygen-17 NMR to avoid problems of cross-relaxation and chemical exchange encountered with proton NMR.

Authors Richardson, Baianu, and Steinberg are affiliated with the Dept. of Food Science, Univ. of Illinois at Urbana, 1304 W. Pennsylvania Ave., Urbana, IL 61801.

MATERIALS & METHODS

THE WHEAT FLOUR, purchased in a local store, was a commercial grade, white, all-purpose flour. For the period of the tests, the wheat flour was stored at 5 ± 2°C. Moisture content of the dry flour was determined in triplicate to be 11.0% by a modification of the air-oven method (AOAC, 1980); the modification employed a time/temperature of 130°C for 6 hr. Protein content (N x 5.7) was determined in duplicate to be 10.6% by the microkjeldahl method (AOAC, 1980).

The wheat flour-water suspensions and doughs were prepared by hand mixing at room temperature the appropriate amount of distilled water or 99.8% deuterium oxide (D_2O) for 2.5 min to obtain the selected moisture contents (60 - 95% wet basis). At these high moisture contents the flour hydrated readily so mixing time was not critical. Replacement of D_2O for H_2O was done on a molar basis.

The mixing time of 2.5 min corresponded to the optimum mixing time according to the farinograph plots (AACC, 1969) for 47.5% w/w moisture content. Physical dough tests (farinograph and extensograph) on the wheat flour showed it to possess normal functional dough characteristics and standard mixing behavior (arrival time 1.5 min and peak time 2.5 min).

Rheological measurements

The Haake RV-3 Rotoviscometer (Haake, Inc., Saddlebrook, NJ) equipped with a DMK 500/50 dual measuring head was employed for the rheological measurements. For wheat flour-water (or D_2O) systems of lower viscosity, the NV sensory system was used (sample size 10 mL); higher viscosities required the SV-II sensor system (sample size 8 mL). A constant temperature circulator was used to equilibrate the samples in the sensor system to 25 ± 1°C prior to measurements.

Measurements were made using the Haake linear shear rate program 50, operating 0 - 150 sec^{-1}. Flow curves were plotted as rpm versus torque with a Haake Model 3533 X-Y recorder. These values were then converted to shear rate and shear stress, respectively, by the use of calibration constants. Samples were analyzed in triplicate. Mineral oils of precisely determined viscosities (50 cp; 1000 cp; and 20,000 cp from Brookfield, Co). were employed as standards.

NMR measurements

A laboratory-built NSF-250 Multinuclear Spectrometer (Oldfield and Meadows, 1978) operating at 34 MHz resonance frequency was used for the oxygen-17 (^{17}O) NMR measurements. Both H_2O and D_2O dispersion systems were probed. Single pulse experiments were done in duplicate at 20°C. A pulse width of 51 microseconds and a recycling time of 0.22 sec was used. The number of scans required for adequate signal-to-noise ratio depended on the concentration of the sample and ranged from 625 for the 95% sample to 4000 for the 60% sample. The spectra were retained in an 8K point array which provided adequate resolution.

The ^{17}O NMR spin-spin (T_2) relaxation parameter (Deslauriers and Smith, 1980) was determined by measuring the line widths at half-height for each spectrum at each concentration. To correct for any residual magnetic field inhomogeneity, the net line broadening $\Delta\nu_B$ was calculated by subtracting the line width of liquid water or of 0.2% HDO in D_2O (ν_{free}) from that of the sample (ν_{obs}). The net or differential relaxation rate (ΔR_2, sec^{-1}), which is the inverse of ΔT_2, was calculated from the line widths by: ΔR_2 (sec^{-1}) = $\pi\Delta\nu_B$ (sec^{-1}).

RESULTS & DISCUSSION

Nuclear magnetic resonance data

The dependence of the ^{17}O NMR relaxation rates on flour concentration in both H_2O and D_2O (Fig. 1) indicated a transition in water mobility occurring at approximately 85% moisture for both the H_2O and D_2O curves. Such a transition from one mobility to another was predicted by Derbyshire (1982). Based on theory, he ascribed the observed change in gradient to aggregation of polymers at high concentration and stated that it usually occurred at 20% by weight, while for our wheat flour it occurred between 15 and 20%. In a study by Halle et al. (1981), this change in gradient was observed between 5 and 10% lysozyme and human plasma albumin solutions. Oakes (1976) showed a marked increase in the relaxation rate at 10% native bovine serum albumin, and attributed this change in gradient to a cooperative effect of adjacent protein molecules on surrounding water.

The D_2O curve (Fig. 1) shows a lower relaxation rate at each concentration than the H_2O curve because the D_2O minimizes the proton exchange line broadening that occurs with water (Mantsch et al., 1977).

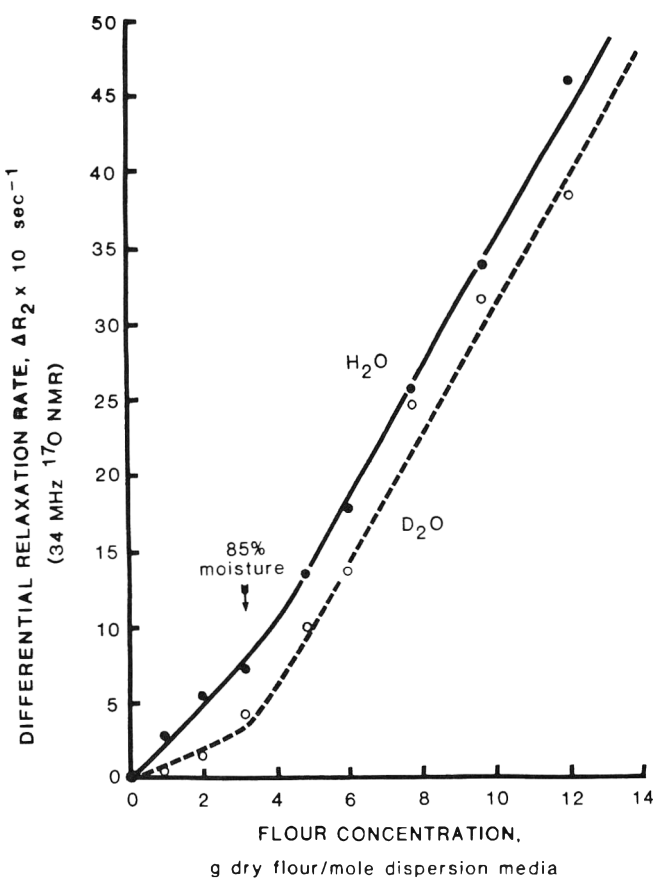


Fig. 1—Oxygen-17 NMR (34 MHz) differential relaxation rates for dispersions of wheat flour in water (H_2O) and in deuterium oxide (D_2O) at moisture contents of 60 - 95%.

Rheological data

Shear stress-shear rate values calculated from Rotoviscometer data were fitted to the Power Law equation:

$$\text{stress} = a\,(\text{rate})^b \qquad (1)$$

where a is the consistency coefficient and b is the flow behavior index. Since

$$\log(\text{stress}) = \log a + b \log(\text{rate}) \qquad (2)$$

a plot of log (stress) vs log (rate) should give a straight line with intercept of log a and slope of b.

The correlation coefficient of fit for the Power Law at each concentration for the wheat flour suspension in water plotted according to Eq. (2) (Fig. 2) ranged between 0.999 and 0.995 for the entire range of flour concentrations in both H_2O and D_2O. The Power Law plots for D_2O were similar to those for H_2O (data not shown). As shown in Table 1, there was no statistically significant difference (Steel and Torrie, 1980) in Power Law parameters between H_2O and D_2O.

Previous studies investigating substitution of D_2O for H_2O in wheat flour doughs have reported a significant difference in rheological behavior between D_2O and H_2O systems (Leung et al., 1983; Tkachuk and Hlynka, 1968; Kretovich and Vakar, 1964; Hoseney, 1979). However, these workers studied dough at about 45% moisture content, whereas the present study was done with suspensions at higher moisture contents, 60 - 95%.

Flour concentration regions of different rheological behavior can be observed (Fig. 3) by following changes in magnitude and direction of both a and b; b was equal to one until the concentration reached 3.0g dry flour/mole water (85.7% moisture), indicating the system remained Newtonian up to this point. With further increase in concentration, the b value decreased, signaling a transi-

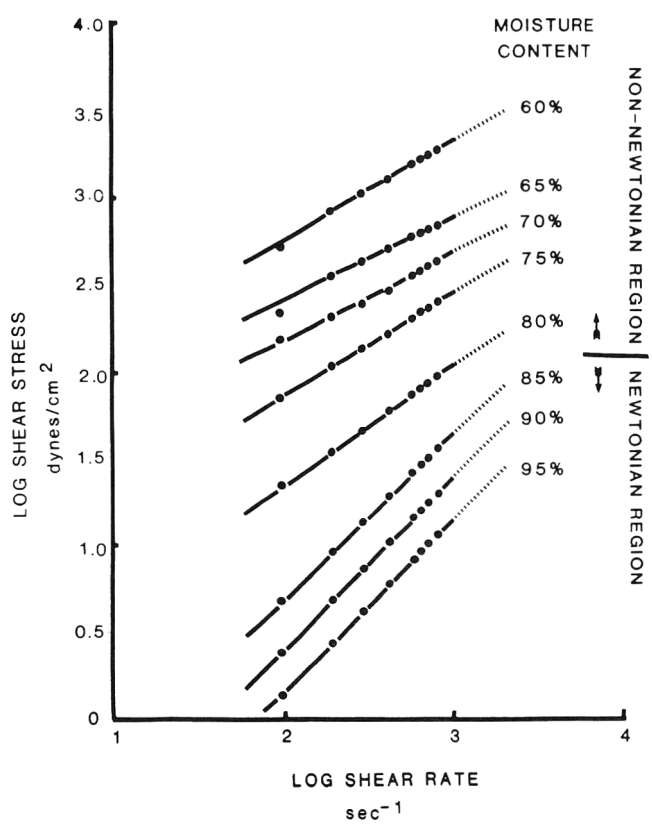


Fig. 2—Power Law plots of rheological data for dispersions of wheat flour in water at moisture contents of 60 - 95%.

tion to pseudoplastic behavior at this point. It decreased to about 0.48 at a concentration of 6.5 (73.5% moisture), indicating an increase in pseudoplasticity with increasing concentration in this region. With a further increase in concentration beyond this point, the b value increased. This point represented a second transition in rheological behavior to a decreased pseudoplasticity with increasing concentration. The b value increased linearly to 12.0 (60% moisture), the highest concentration studied.

This second transition showing a change from negative to positive slope for b value was not consistent with a report by Launay and Bure (1973) who found that b value decreased linearly with flour concentration over the range 9.7 to 30. This inconsistency may be ascribed to a difference in instrumentation; they used the cone-plate sensory system, and we used the rotating cylinder. Wheat flour doughs possess both elastic and viscous rheological properties (Launay and Bure, 1973). The onset of gluten development introduces the elastic component into the system. We hypothesize that our readings were affected by this elastic component. Evidence for this hypothesis is given by the coincidence of two observations. First, the elastic component becomes tactually apparent when the flour concentration reaches about 6. Second, the b value in Fig. 3 stopped descending and started to rise at a concentration of 6.5. Thus, the increase in gluten development with increasing concentration overshadowed the increase in pseudoplasticity to give the ascending line for b observed in Fig. 3. Since the term viscoelasticity describes the combination of pseudoplasticity and elasticity, this is the label given to the third region.

Fig. 3 shows three lines demarcating three rheological regions based on flour concentration. Of signifiance is the finding that the a plot shows the transition points at about the same concentrations as the b plot, thus lending validity to the demarcation of three rheological regions.

Relation of NMR to rheology

Since the rheological behavior was found to be the same in D_2O as in H_2O (Table 1), the water data can be used for rheological characteristics. In the case of NMR, the D_2O data were taken instead of H_2O data because the former minimizes proton exchange line broadening. Fig. 4 shows the relationship of a and b values to ^{17}O NMR relaxation rate. The b value curve has the same shape as when plotted against concentration in Fig. 3. This was expected because the relaxation rate was directly related to concentration (Fig. 1) at concentrations above 3. The a value curve (Fig. 4) showed three linear portions; the slopes were successively 1.2×10^{-3}, 0.10, and 0.64. These a lines are labeled Regions I, II, and III to indicate three viscosity relationships. Thus, the NMR response was directly related to apparent viscosity only within each region, and there was no overall relation.

Evidently, the two instrumental methods are measuring different parameters; the Rotoviscometer is measuring the macroscopic or bulk flow properties of the entire system, while the ^{17}O NMR spectrometer detects the average (free and bound) molecular mobility of the water component only. From Fig. 1 and 3, we can observe a similar transition in both the relaxation rate and the rheological behavior at approximately 85% moisture. Thus, in the dilute flour concentration range where the free water predominates, both instruments are similarly affected. As the moisture content decreases below 85%, Region II, aggregation of flour particles caused a decrease in the average mobility of the water and a shift from Newtonian to pseudoplastic flow. However, due to the large number of experimental variables that affect rheology (Bloksma, 1971; Launay and Bure, 1973; Rao et al., 1975) it is unlikely that aggregation is the only phenomenon being measured.

With increased flour concentration, the Rotoviscometer measurements are increasingly influenced by flour particle interactions, whereas the NMR spectrometer still measures only water mobility. This difference causes a transition

Table 1–Power Law parameters expressing rheological behavior of wheat flour suspensions in water and in deuterium oxide at various concentrations[a]

Moisture content (%)	Slope b		Intercept log a	
	H_2O	D_2O	H_2O	D_2O
95	1.000	0.995	–1.852	–1.835
90	0.999	0.978	–1.600	–1.546
85	0.961	0.977	–1.232	–1.274
80	0.677	0.704	0.001	–0.070
75	0.583	0.590	0.694	0.682
70	0.491	0.499	1.196	1.178
65	0.529	0.527	1.727	1.730
60	0.594	0.593	2.011	2.012

[a] Statistics: Homogeneity of regression of the H_2O and D_2O power law coefficients was tested (Steel and Torrie, 1980) under supervision of R.D. Seif, and no difference in either log a or b was found between H_2O and D_2O at the 0.05 level of significance for each concentration.

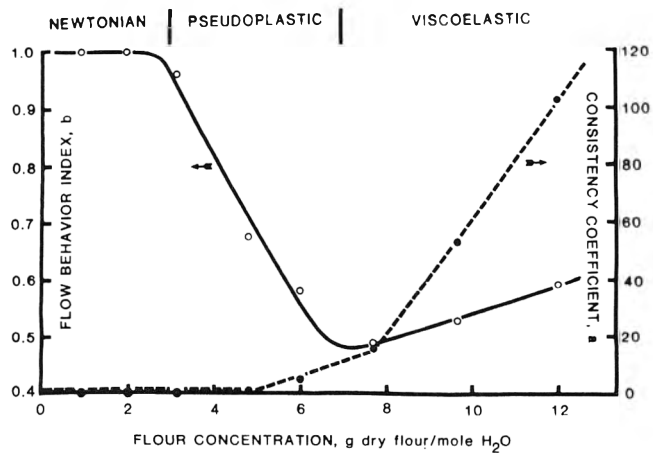


Fig. 3–Newtonian, pseudoplastic, and viscoelastic flour concentration regions as defined by the dependence of Power Law parameters on concentration.

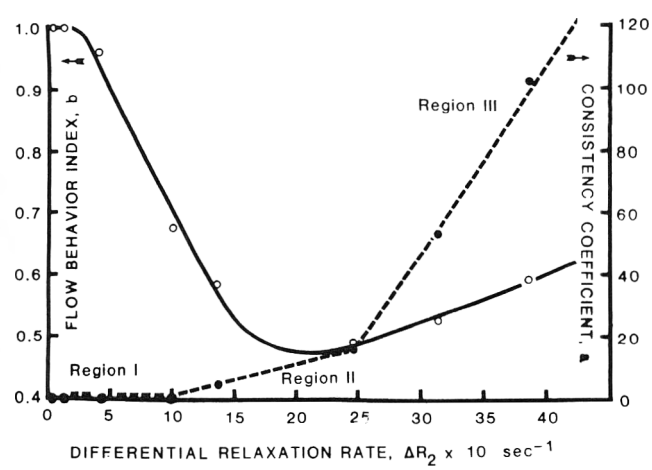


Fig. 4–Relation of Power Law parameters to oxygen-17 (34 MHz in D_2O) differential relaxation rate.

(Fig. 4) at about 70% moisture from Region II to III. It is hypothesized that this transition to Region III is caused by the onset of gluten formation, which is apparent by sight and feel. With further increase in concentration, the NMR spectrometer continues to show a linear decrease in water mobility and is not affected by gluten formation (Fig. 1). However, the Rotoviscometer is greatly affected by gluten formation (Fig. 3) which results in the large slope for Region III (Fig. 4).

Leung et al. (1983) stated that the "water mobility (in a flour-water system) as determined by proton and deuteron relaxation does not reflect the different rheological properties of soft and hard wheat flour doughs." They reported that addition of up to 15% vital (wheat) gluten and 20% wheat starch to flour doughs changed the mixing properties but had little or no effect on NMR readings. Their interpretation of the deuterium NMR data is consistent with the current view that the mobility and conformation of the macromolecules determine the different properties of doughs containing different types of wheat flours. This work in the high moisture range also supports the current view.

CONCLUSIONS

OXYGEN-17 NMR monitored water mobility throughout the 60-95% moisture content range. Variation of the Power Law parameters (Rotoviscometer) with flour concentration showed three distinct rheological regions: Newtonian, pseudoplastic and viscoelastic. The two instruments measured different parameters; the Rotoviscometer measured flow properties while the NMR detected average molecular mobility of the water. The consistency coefficient and the flow behavior index showed three distinct regions with differential relaxation rates. Thus, the NMR response was directly related to apparent viscosity only within each region, so there was no overall relation. Further work is needed to develop NMR methodology which will differentiate the water states responsible for the rheological properties observed.

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Essential Elements and Cadmium and Lead in Fresh, Canned, and Frozen Green Beans (*Phaseolus vulgaris L.*)

ANTHONY LOPEZ and H. L. WILLIAMS

ABSTRACT

Sixteen essential elements, cadmium and lead were determined in fresh, canned, and frozen green beans (*Phaseolus vulgaris* L.). Samples were taken during processing to determine where changes in element content occurred. Canned green beans contained lower concentrations of calcium, copper, iron, magnesium, manganese, phosphorus, potassium, and zinc, but had higher amounts of chloride, nickel, and sodium than fresh beans. No change in silicon was observed. Iron, phosphorus and potassium were lower in frozen than in fresh green beans, but calcium, sodium and zinc were higher. There was no change in chloride, copper, magnesium, manganese and silicon due to freezing. Element retention ranged from 51 - 100% for canned and from 73 - 171% for frozen green beans.

INTRODUCTION

MOST PREVIOUS WORK done on the element content of green beans included only macro-elements, i.e., calcium, iron, phosphorus, potassium, and sodium (Watt and Merril, 1963; Anonymous, 1962; Ensminger et al., 1983; Bowes and Church, 1963). Although some work has been done on the other elements, little has been published to show the effects of canning and freezing on all the elements.

Objectives of the research were (1) to determine and compare the concentration of 18 elements, including cadmium and lead, in fresh, canned, and frozen green beans, and (2) to determine the effects of unit operations in canning and freezing on the concentration of these elements in the finished product.

MATERIALS & METHODS

Canned green beans

Samples of fresh, blanched, and canned green beans (*Phaseolus vulgaris L.*) were obtained from Hanover Brands, Inc., Hanover, PA. They were harvested in Snowhill, MD, in the summer of 1981, commercially canned within 8 hr of harvesting and were of the Blue Lake GV-2 variety. Truckloads of green beans were received at the processing plant. The beans were conveyed to the processing line (fresh sample taken), where they were put through shakers to remove dirt and debris. After cutting ends off, washing and inspecting, the beans were cut into pieces and washed again. After another inspection (before blanch sample taken), they were blanched 3 min at 77 - 88°C (170 - 190°F) (after blanch sample taken) and then 624g (22 oz) of beans were placed in 40 oz (401 x 602) epoxy enameled cans. Approximately 482g (16.30 fl oz) of 1.7% brine were added to each can before sealing. Cans were then placed in Hydro-Cooker retorts, processed for 30 min at 116°C (240°F) and cooled in water for 21 min (finished product sample taken) before storing.

Frozen green beans

Samples of fresh, blanched, and frozen green beans were obtained from Consumers Packing, Inc. in Lancaster, PA. The beans were of the Blue Lake variety, small sieve size, and were harvested in the Hanover, PA area in the summer of 1981. They were brought to the plant by truck, placed on the processing line and treated as the canned green beans were. After blanching the beans were chilled prior to conveying to the Flo-Freezer at -29°C to -32°C (-20°F to -25°F), allowed to flow through for 10 min and packed in 283g (10 oz) packages (finished product sample taken). Frozen samples were stored at -18°C to -21°C (0° to -6°F).

Sampling

Nine samples were taken at each step of the canning and freezing processes over a 2-day period. Sampling from the line was coordinated so that fresh, in-process, and finished product samples were of the same batch. Two samples of processing water were taken at each plant during the sampling period.

Statistical design and analysis

Fresh, before blanch, and after blanch samples weighed 0.5 - 1.0 kg each. Six cans of the canned green beans and six packages of the frozen green beans were used for each of the nine finished product samples. The canned green bean samples were stored at 21°C (70°F) for 20 wk before being opened, drained and mixed. The frozen green bean samples were stored at -8°C (18°F) for 7 wk before being opened and mixed. Nine samples were chosen in order to keep the variation around 15%. This was determined by running preliminary analyses on three canned green bean samples and determining the number of samples needed to give 15% variation for a paired "t" test. The data were analyzed using Duncan's test. Analyses of variance were performed at the 1% and 5% levels to test for significant differences.

Reagents

Nitric acid, A.C.S. grade 70%; Whatman #41 ashless filter paper; atomic absorption standards; and hydrochloric acid, reagent A.C.S. grade 37.5% were obtained from Fisher Scientific Company, Fair Lawn, NJ. Lanthanum oxide, 98% pure, was obtained from J.T. Baker Chemical Company, Phillipsburg, NJ (Baker); it was dissolved in water and hydrochloric acid for a 5% (w/v) solution. Certified cesium chloride (Fisher) was dissolved in water for a 0.5% (w/v) solution.

For phosphorus and silicon determinations the following A.C.S. certified Fisher reagents were used: Potassium dihydrogen phosphate was used to prepare a 500 ppm standard; ammonium molybdate to prepare a 10% solution; 53.5g ammonium chloride was dissolved in water, 70 mL of 30% ammonium hydroxide was added and the solution was diluted to 1L; and 1-pentanol and diethyl ether.

For the chloride determination sodium carbonate for a 5% (w/v) solution, silver nitrate to prepare a 1000 ppm standard, and nitric acid, 70% were used. All three reagents were Fisher, A.C.S. certified.

Sample preparation

Samples were prepared for analyses by mixing and pureeing with a Cuisinart food processor, freezing at -8°C (18°F) and freeze drying on a Virtis freeze dryer for 48 hr.

Table 1—Moisture content of green bean samples

Green bean sample	Canned (%)	Frozen (%)
Fresh	91.69 ± 0.16[a]	89.00 ± 0.93
Before Blanch	92.26 ± 0.28	89.44 ± 0.68
After Blanch	91.98 ± 0.47	89.46 ± 0.80
Finished Product	93.19 ± 0.10	89.91 ± 0.58

[a] Standard deviation.

Authors Lopez and Williams are affiliated with the Dept. of Food Science & Technology, Virginia Polytechnic Institute & State Univ., Blacksburg, VA 24061.

The freeze-dried samples were finely ground with the food processor. Duplicates were weighed into 250 mL Erlenmeyer flasks and boiled with 25 mL of 5N nitric acid until dissolved. Freeze-dried samples represented 5g of sample on a wet basis. After the wet ashed samples were filtered and made up to 50 mL, the solutions were ready for analysis by atomic absorption spectrophotometry (AAS).

Canned green bean samples were prepared for chloride determination following AOAC Methods of Analysis 3.071 (AOAC, 1980). Duplicate 0.40 ± 0.12g freeze-dried samples were moistened with 20 mL 5% Na_2CO_3 solution, dried at 80°C for 4 hr and ignited at 450°C overnight. After washing and filtering the ash with hot water, the filter papers were ashed at 450°C for 3 hr. The ash was dissolved in 4 mL of HNO_3 (1 + 4), filtered, and diluted to 100 mL. Samples of plant processing waters, fresh brines and processed brines were diluted 1/2 and contained 4% conc HNO_3.

Analytical methods

Elemental analyses were performed with a Perkin-Elmer Model 403 atomic absorption spectrophotometer. A 4-inch burner head and standard air-acetylene flame were used for elemental analyses except for molybdenum and tin for which a nitrous oxide-acetylene burner and flame were used. Single element hollow cathode lamps were used for all elements. The instrument settings and other experimental conditions were in accordance with the manufacturer's specifications.

Appropriate dilutions were used to determine calcium, magnesium, potassium, and sodium in all samples. Samples diluted for calcium and magnesium analyses contained 1% (w/v) lanthanum to overcome potential anionic interferences. Cesium chloride at a final concentration of 2000 ppm cesium was used for sodium and potassium analyses.

Duplicate aliquots of the wet-ashed solutions were used for the indirect determination of phosphorus and silicon. Aliquots of all the green bean samples, plus the water and brine samples, were analyzed by the procedure described by Parker (1972).

Chloride was also determined indirectly (Parker, 1972). Except for the canned green bean samples, duplicate 0.20 ± 0.07g of the freeze-dried green bean samples were analyzed. Two milliliters of the canned green bean solutions prepared by the AOAC method as well as aliquots of the processing waters and brines were analyzed by the chloride procedure.

Table 2—Concentration of elements in canned green beans

Element	Fresh (mg/100g)	Before blanch (mg/100g)	After blanch (mg/100g)	Finished product (mg/100g)
Cadmium	<0.001[a]	<0 001[a]	<0.001[a]	<0.001[a]
Calcium	56.1 ± 2.6[b]	53.1 ± 4.0	70.8 ± 5.4	47.0 ± 1.7
Chloride	33.2 ± 2.1	30.3 ± 1.6	21.2 ± 4.2	491 ± 23
Chromium	<0.01[a]	<0.01[a]	<0.01[a]	<0.01[a]
Cobalt	<0.02[a]	<0.01[a]	<0.01[a]	<0.02[a]
Copper	0.05 ± 0.01	0.04 ± 0.00	0.03 ± 0.01	0.03 ± 0.00
Iron	0.56 ± 0.28	0.35 ± 0.04	0.45 ± 0.06	0.36 ± 0.04
Lead	<0.04[a]	<0.04[a]	<0.04[a]	<0.04[a]
Magnesium	23.3 ± 0.6	20.3 ± 0.5	23.5 ± 3.3	12.8 ± 0.6
Manganese	0.25 ± 0.03	0.23 ± 0.02	0.37 ± 0.03	0.15 ± 0.01
Molybdenum	<0.2[a]	<0.2[a]	<0.2[a]	<0.2[a]
Nickel	<0.02[a]	<0.02[a]	<0.02[a]	0.30 ± 0.02
Phosphorus	22.8 ± 2.2	20.6 ± 1.5	22.2 ± 1.8	15.5 ± 1.0
Potassium	254 ± 9	227 ± 6	188 ± 10	129 ± 4
Silicon	2.16 ± 0.54	1.45 ± 0.24	2.15 ± 0.49	2.15 ± 0.33
Sodium	0.78 ± 0.11	0.85 ± 0.14	1.47 ± 0.53	319 ± 14
Tin	<7[a]	<7[a]	<7[a]	<7[a]
Zinc	0.18 ± 0.02	0.15 ± 0.01	0.16 ± 0.01	0.11 ± 0.00

[a] Element below the detection limit of the AAS instrument.
[b] Standard deviation.

Table 3—Statistical analyses of concentration of elements in canned green beans[a]

Element	Fresh vs before blanch	Fresh vs after blanch	Fresh vs finished product	Before blanch vs after blanch	Before blanch vs finished product	After blanch vs finished product
Cadmium	b	b	b	b	b	b
Calcium	*	**	**	**	**	**
Chloride	ns	ns	**	ns	**	**
Chromium	b	b	b	b	b	b
Cobalt	b	b	b	b	b	b
Copper	ns	**	**	**	**	*
Iron	**	*	**	ns	ns	ns
Lead	b	b	b	b	b	b
Magnesium	**	ns	**	**	**	**
Manganese	**	**	**	**	**	**
Molybdenum	b	b	b	b	b	b
Nickel	b	b	**	b	**	**
Phosphorus	**	ns	**	*	**	**
Potassium	**	**	**	**	**	**
Silicon	**	ns	ns	**	**	ns
Sodium	ns	ns	**	ns	**	**
Tin	b	b	b	b	b	b
Zinc	**	*	**	ns	**	**

[a] Statistical differences as analyzed by Duncan's Test: ns = no significant difference at 1% and 5% levels; * = significant difference at 5% level; ** = significant difference at 1% level.
[b] Element below the detection limit of the AAS instrument.

Accuracy of analytical procedures

Analyses were done on five replicates of 0.5g of National Bureau of Standards Citrus Leaves (Standard Reference Material #1572). These samples were analyzed in parallel following the analytical procedures used in this work. Data on the 14 elements analyzed showed variations of 5.2% or less for calcium, magnesium, manganese, potassium, sodium, and zinc; and 7.8%, 9.1%, 15.0%, and 23.1% for iron, copper, lead, and phosphorus, respectively; cadmium, chromium, molybdenum, and nickel were below the detection limit of the instrument. The 23.1% variation between the phosphorus results was probably due to different indirect methods being used for determining phosphorus. However, when standard deviations were considered, there was no difference between the NBS certified values and the values obtained following the procedures used in this work.

RESULTS & DISCUSSION

ALL DATA are presented on a wet weight basis. Moisture was determined in duplicate on all nine samples (Table 1). The concentration and statistical analyses of 16 essential elements and cadmium and lead are given for canned green beans in Tables 2 and 3 and for frozen green beans in Tables 4 and 5. Table 6 shows the concentration of the same elements in fresh brine, processed brine, and plant water. Retention of the elements is given in Table 7 and percentages of recommended dietary allowances (RDA) and estimated safe and adequate daily dietary intake values per 100g of fresh, canned, and frozen green beans are shown in Table 8. The reported calculated percentages of established RDAs and estimated safe and adequate daily dietary intake values assumed 100% availability of the element. This was done to provide some measure of the contribution of elements in processed foods to the diet. Availability of iron and zinc to the human body varies with valence, solubility, and chemical species of the element present in the food.

Cadmium in all samples was below the detection limit of the AAS instrument (<0.001 mg/100g). Bielig and Treptow (1978) reported cadmium values of 0.0029 mg/100g for raw green beans; 0.002 and 0.0014 mg/100g for before blanch green beans; and 0.0016 and 0.0011 mg/100g for

Table 4—Concentration of elements in frozen green beans

Element	Fresh (mg/100g)	Before blanch (mg/100g)	After blanch (mg/100g)	Finished product (mg/100g)
Cadmium	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]
Calcium	84.5 ± 7.1[b]	88.1 ± 5.8	89.9 ± 8.4	92.5 ± 7.4
Chloride	27.3 ± 5.8	25.8 ± 3.6	25.5 ± 4.7	23.9 ± 2.4
Chromium	<0.01[a]	<0.01[a]	<0.01[a]	<0.01[a]
Cobalt	<0.02[a]	<0.02[a]	<0.02[a]	<0.02[a]
Copper	0.08 ± 0.02	0.08 ± 0.01	0.07 ± 0.01	0.07 ± 0.01
Iron	0.82 ± 0.21	0.73 ± 0.25	0.55 ± 0.14	0.60 ± 0.16
Lead	<0.04[a]	<0.04[a]	<0.04[a]	<0.04[a]
Magnesium	25.2 ± 1.9	24.5 ± 2.1	24.5 ± 2.3	24.2 ± 1.3
Manganese	0.52 ± 0.17	0.48 ± 0.16	0.46 ± 0.16	0.46 ± 0.13
Molybdenum	<0.2[a]	<0.2[a]	<0.02[a]	<0.2[a]
Nickel	<0.02[a]	<0.02[a]	<0.02[a]	<0.02[a]
Phosphorus	30.5 ± 2.8	28.5 ± 4.1	25.0 ± 2.8	27.0 ± 1.6
Potassium	241 ± 21	230 ± 21	219 ± 21	208 ± 8.5
Silicon	2.77 ± 0.61	2.95 ± 0.77	2.20 ± 0.47	2.37 ± 0.57
Sodium	0.56 ± 0.14	1.01 ± 0.19	1.19 ± 0.31	1.11 ± 0.28
Tin	<7[a]	<7[a]	<7[a]	<7[a]
Zinc	0.24 ± 0.03	0.30 ± 0.10	0.24 ± 0.05	0.41 ± 0.17

[a] Element below the detection limit of the AAS instrument.
[b] Standard deviation.

Table 5—Statistical analyses of concentration of elements in frozen green beans[a]

Element	Fresh vs before blanch	Fresh vs after blanch	Fresh vs finished product	Before blanch vs after blanch	Before blanch vs finished product	After blanch vs finished product
Cadmium	b	b	b	b	b	b
Calcium	ns	ns	*	ns	ns	ns
Chloride	ns	ns	ns	ns	ns	ns
Chromium	b	b	b	b	b	b
Cobalt	b	b	b	b	b	b
Copper	ns	ns	ns	ns	ns	ns
Iron	ns	**	*	ns	ns	ns
Lead	b	b	b	b	b	b
Magnesium	ns	ns	ns	ns	ns	ns
Manganese	ns	ns	ns	ns	ns	ns
Molybdenum	b	b	b	b	b	b
Nickel	b	b	b	b	b	b
Phosphorus	ns	**	*	*	ns	ns
Potassium	ns	*	**	ns	*	ns
Silicon	ns	ns	ns	*	ns	ns
Sodium	*	**	**	ns	ns	ns
Tin	b	b	b	b	b	b
Zinc	ns	ns	**	ns	*	**

[a] Statistical differences as analyzed by Duncan's Test: ns = no significant difference at 1% and 5% levels; * = significant difference at 5% level; ** = significant difference at 1% level.
[b] Element below the detection limit of the AAS instrument.

after blanch green beans. Schroeder et al. (1967) found cadmium values of 0.001 mg/100g for fresh green beans and 0.003 mg/100g for canned green beans. Mills and Jones (1979) reported a calculated cadmium value of 0.006 mg/100g for frozen green beans.

Calcium decreased significantly from 56.1 mg/100g in fresh beans to 47.0 mg/100g in canned beans. This decrease was due to calcium leaching from the beans into the brine which contained 13.2 mg/100mL calcium after processing. These values are comparable to those reported by Watt and Merrill (1963) with 56 mg/100g for raw green beans, 45 mg/100g for canned drained solids and 15 mg/100g for canned drained liquid. Calcium of frozen green beans (92.5 mg/100g) was significantly higher than that found in the fresh samples (84.5 mg/100g). This increase in calcium probably came from Ca contained in plant water (7.05 mg/mL) used to wash and blanch the green beans. Mills and Jones (1979) reported a calculated calcium value of 81 mg/100g for frozen green beans. Canned and frozen green beans, respectively, supply 5.9% and 11.6% of the established RDA for calcium in a 100g serving.

The chloride of canned green beans (491 mg/100g) reflected a significant increase over the amount found in fresh green beans (33.2 mg/100g) due to the addition of brine (1,116 mg/100mL). There was no significant difference in the concentration of chloride present in fresh (27.3 mg/100g) and frozen (23.9 mg/100g) green beans. Bridges (1941) reported a chloride value of 24 mg/100g for fresh green beans. A 100g serving of fresh or frozen green beans supplies less than 1% of the estimated safe and adequate daily dietary intake of chloride, while canned green beans supply 14.4% in a 100g serving.

The chromium value of <0.01 mg/100g was below the detection limit of the AAS instrument. Toepfer et al. (1973) found a chromium value of 0.04 mg/100g in fresh green beans. Thomas et al. (1974) reported 0.022 mg/100g chromium in frozen green beans. Cobalt was also below the detection limit of the AAS instrument with <0.01 and <0.02 mg/100g. Thomas et al. (1974) reported a cobalt value of 0.002 mg/100g for frozen green beans. Mills and Jones (1979) also calculated a value of 0.0011 mg/100g for frozen green beans.

With a retention value of 60%, copper significantly decreased from 0.05 mg/100g in fresh green beans to 0.03 mg/100g in canned green beans. Processed brine which contained 0.02 mg/100mL suggested the decrease was due to leaching of copper. There was no significant change in copper due to freezing, with values of 0.08 mg/100g and 0.07 mg/100g for fresh and frozen green beans, respectively. These values are comparable to those reported by Elkins (1979), Furr et al. (1976), Gormican (1970), and Koivistoinen et al. (1974). Fresh, canned and frozen green beans are poor sources of copper, with 100g supplying less than 4% of the safe and adequate daily dietary intake average value.

Iron in canned green beans (0.36 mg/100g) was significantly lower than that found in fresh green beans (0.56 mg/100g) with a retention of 64%. This decrease was probably due to leaching of iron from the product into the brine, with processed brine containing 0.34 mg/100mL. Iron in frozen green beans (0.60 mg/100g) was also significantly lower than that found in fresh green beans (0.82 mg/100g), with a retention of 73%. Canned and frozen green beans at 100g servings supply 3.6% and 6.0% of the established RDA for iron, respectively.

Lead of <0.04 mg/100g in fresh, canned, and frozen green beans was below the detection limit of the AAS instrument. Zuber (1972) calculated lead values of 0.03 - 0.10 mg/100g and stated that 20 - 50% of the lead was removed by washing. Mills and Jones (1979) reported a lead value of 0.126 mg/100g, assuming 90% moisture.

Magnesium in canned green beans (12.8 mg/100g) was significantly lower than that found in fresh green beans (23.3 mg/100g). The retention value was 55%. Processed brine contained 11.4 mg/100mL of magnesium suggesting that the element was leached out due to processing. There was no significant difference in the concentration of magnesium found in fresh (25.2 mg/100g) and frozen (24.2 mg/100g) green beans. These results are comparable to values reported by Watt and Merrill (1963), Ensminger et al. (1983), Furr et al. (1976), Bowes and Church (1963), Elkins (1979), and Gormican (1970). Fresh and frozen green beans supply 6.7 - 7.2% of the RDA for magnesium, while canned green bean solids supply 3.7% in 100g servings.

In a 100g serving, fresh and frozen green beans supply 6.7% to 13.9% of the estimated safe and adequate daily dietary intake average value for manganese, while canned green bean solids supply only 4.0%. There was a significant decrease in manganese from the fresh (0.25 mg/100g) to canned (0.15 mg/100g) samples due to processing and the leaching of the element into the brine. There was no significant difference between the fresh (0.52 mg/100g)

Table 6—Concentration of elements in freshly prepared brine, processed brine, and plant water used in processing canned and frozen green beans

Element	Fresh brine (mg/100 mL)	Processed brine (mg/100 mL)	Plant water (Canned) (mg/100 mL)	Plant water (Frozen) (mg/100 mL)
Cadmium	<0.0003[a]	<0.0003[a]	<0.0003[a]	<0.0003[a]
Calcium	<0.85[a]	13.2 ± 0.7[b]	<0.85[a]	7.05 ± 0.67
Chloride	1116 ± 25	540 ± 19	1.28 ± 0.007	2.38 ± 0.32
Chromium	<0.002[a]	<0.002[a]	<0.002[a]	<0.002[a]
Cobalt	0.03 ± 0.00	0.02 ± 0.00	<0.003[a]	<0.003[a]
Copper	<0.002[a]	0.02 ± 0.00	<0.002[a]	<0.002[a]
Iron	0.001 ± 0.00	0.34 ± 0.02	0.04 ± 0.01	<0.01[a]
Lead	0.04 ± 0.00	0.03 ± 0.01	<0.008[a]	<0.008[a]
Magnesium	0.64 ± 0.05	11.4 ± 0.6	0.55 ± 0.06	1.08 ± 0.06
Manganese	0.006 ± 0.000	0.12 ± 0.00	<0.004[a]	<0.004[a]
Molybdenum	<0.03[a]	<0.03[a]	<0.03[a]	<0.03[a]
Nickel	0.22 ± 0.01	0.16 ± 0.01	<0.005[a]	<0.005[a]
Phosphorus	<0.0005[a]	7.45 ± 0.78	<0.001[a]	<0.001[a]
Potassium	3.00 ± 0.13	135 ± 4	2.04 ± 0.00	2.26 ± 0.42
Silicon	0.19 ± 0.02	2.07 ± 0.50	<0.003[a]	<0.003[a]
Sodium	626 ± 18	307 ± 12	1.53 ± 0.11	1.02 ± 0.09
Tin	<1.50[a]	<1.50[a]	<1.50[a]	<1.50[a]
Zinc	<0.0002[a]	0.09 ± 0.00	0.21 ± 0.06	0.06 ± 0.02

[a] Element below the detection limit of the AAS instrument.
[b] Standard deviation.

and frozen (0.46 mg/100g) samples. These results can be compared to values reported by Bridges (1941), Mills and Jones (1979), Peterson et al. (1946), Elkins (1979), and Koivistoinen (1974).

Molybdenum in canned and frozen green beans was below the detection limit of the AAS instrument (<0.2 mg/100g). Furr et al. (1976) reported molybdenum at 0.01 and 0.02 mg/100g in fresh green beans. Schroeder et al. (1970) found molybdenum values of 0.066 mg/100g in fresh green beans and 0.069 mg/100g in canned green beans.

Nickel values of <0.02 mg/100g were below the detection limit of the AAS instrument for all samples except canned green beans. Canned green beans contained 0.30 mg/100g of nickel or 1,500% of the amount in fresh product, probably caused by a pick up of Ni from processing equipment. Furr et al. (1976) found nickel in fresh green beans to be 0.039 and 0.044 mg/100g. Nickel values of 0.0076 mg/100g and 0.035 mg/100g in frozen green beans have been reported by Mills and Jones (1979) and Thomas et al. (1974), respectively.

Fresh samples contained 22.8 and 30.5 mg/100g phosphorus, while canned and frozen samples contained 15.5 and 27.0 mg/100g, respectively. Both canning and freezing processes significantly lowered phosphorus, with retention values of 68% and 89%, respectively. The loss of phosphorus during canning was due to the leaching of the element into the brine. The loss of phosphorus during the freezing process was probably due to washing and blanching in water. Fresh, canned, and frozen green beans are rather poor sources of phosphorus, supplying less than 4% of the established RDA in a 100g serving. Watt and Merrill (1963) reported phosphorus values of 44 mg/100g in fresh green beans, 25 mg/100g in canned green beans, and 33 mg/100g in frozen green beans.

Fresh, canned, and frozen green beans were fairly good sources of potassium with a 100g serving supplying 3.4 – 6.8% of the estimated safe and adequate daily dietary intake average value. Processing significantly decreased the concentration of potassium in green beans, as reflected by retention of 51% for the canned product and 86% for the frozen product. Fresh green bean potassium values of 254 and 241 mg/100g are consistent with results reported by Watt and Merrill (1963), Ensminger et al. (1983), and Furr et al. (1976). The canned green bean value of 129 mg/100g is similar to literature values (Anonymous, 1962; Elkins, 1979). The frozen green bean value of 208 mg/100g can be compared to reported data (Anonymous, 1962; Mills and Jones, 1979).

Silicon concentrations of 2.15 to 2.77 mg/100g in fresh and processed samples indicated no significant differences due to either processing method. Mills and Jones (1979) found silicon at 5.75 mg/100g in frozen green beans.

The high sodium of canned green beans (319 mg/100g) was due to the addition of brine to the processed product, as evident by an increase of 40,897% in the amount in the fresh product. The sodium concentration of fresh and frozen green beans ranged from 0.56 – 1.11 mg/100g, with a significant increase due to processing. Fresh and frozen green beans are very poor (<0.1%) sources of sodium; however, a 100g serving of canned green beans supplies 14.5% of the estimated safe and adequate daily dietary intake. Fresh green bean sodium values in this work are comparable to those reported (Anonymous, 1962; Furr et al., 1976). A canned green bean sodium value of 236 mg/100g was reported by Watt and Merrill (1963). Sodium in frozen green beans, comparable to the value reported here, was reported by Watt and Merrill (1963), Bowes and Church (1963), Anonymous (1962), Mills and Jones (1979), and Gormican (1970).

Tin concentration of <7 mg/100g was below the detection limit of the AAS instrument. Tin analyses of green beans packed in plain and lacquered cans have been reported by Cameron and Brittain (1971).

Zinc in canned green beans decreased significantly due to processing, while zinc in frozen green beans increased significantly over fresh green beans. All samples were poor sources of zinc with less than 3% of the established RDA in a 100g serving. Fresh and canned green bean values of 0.11 – 0.24 mg/100g were comparable to results reported by Sandstead (1973), Elkins (1979), and Schroeder et al.

Table 7—Retention of elements in canned and frozen green beans, on the basis of element concentration in fresh green beans being 100%

Element	Fresh vs canned green beans retention (%)[a]	Fresh vs frozen green beans retention (%)[a]
Cadmium	b	b
Calcium	84	109
Chloride	1,479	88
Chromium	b	b
Cobalt	b	b
Copper	60	88
Iron	64	73
Lead	b	b
Magnesium	55	96
Manganese	60	88
Molybdenum	b	b
Nickel	1,500[c]	b
Phosphorus	68	89
Potassium	51	86
Silicon	100	86
Sodium	40,897	198
Tin	b	b
Zinc	61	171

[a] Retention = canned (or frozen)/fresh x 100.
[b] Element below the detection limit of the AAS instrument.
[c] Concentration of element in fresh green beans was below detection limit of the AAS instrument.

Table 8—Percentages of recommended dietary allowances and estimated safe and adequate daily dietary intakes per 100g of fresh, canned and frozen green beans

Element	Fresh green beans	Canned green beans	Fresh green beans	Frozen green beans
Cadmium	a	a	a	a
Calcium	7.0[b]	5.9	10.6	11.6
Chloride	0.98[c]	14.4	0.80	0.70
Chromium	a	a	a	a
Cobalt	a	a	a	a
Copper	2.0[c]	1.2	3.2	2.8
Iron	5.6[b]	3.6	8.2	6.0
Lead	a	a	a	a
Magnesium	6.7[b]	3.7	7.2	6.9
Manganese	6.7[c]	4.0	13.9	12.3
Molybdenum	a	a	a	a
Nickel	a	c	a	a
Phosphorus	2.8[b]	1.9	3.8	3.4
Potassium	6.8[c]	3.4	6.4	5.5
Silicon	d	d	d	d
Sodium	0.04[c]	14.5	0.03	0.05
Tin	a	a	a	a
Zinc	1.2[b]	0.7	1.6	2.7

[a] Element below the detection limit of AAS instrument.
[b] Recommended Dietary Allowance as set by the Food & Nutrition Board, National Research Council (Anon., 1980) % RDA in 100g serving for adult male.
[c] Estimated safe and adequate daily dietary intake (%) in 100g serving for adults (Anon., 1980). Percentages are calculated on the average values for the ranges of recommended intakes.
[d] No estimated safe and adequate daily intake has been recommended.

(1967). The frozen green bean value of 0.41 mg/100g can be compared to concentrations of 0.31 and 0.35 mg/100g reported by Gormican (1970) and Mills and Jones (1979).

SUMMARY

OF ALL THE ELEMENTS which were above the detection limit of the instrument, calcium, copper, iron, magnesium, manganese, phosphorus, potassium and zinc decreased significantly in canned green beans, due to processing. In most instances this decrease was due to the element being leached into the brine. Chloride, nickel and sodium increased significantly in the canned product, due to the addition of brine. There was no change in silicon due to processing.

In the freezing process there were fewer changes in element concentration. Of the elements which were above the detection limit, iron, phosphorus and potassium decreased significantly due to freezing but calcium, sodium and zinc increased significantly. There was no change in chloride, copper, magnesium, manganese, and silicon due to freezing.

Fresh, canned or frozen green beans supplied less than 13% of each of the established RDAs or estimated safe and adequate daily dietary intake values, except for chloride and sodium. Frozen green beans supplied more of the mineral RDAs than canned green beans.

The concentration of elements in canned green beans ranged from 51% to 100% of the amount present in the fresh product, while in frozen green beans it ranged from 73% to 171%, excluding sodium, chloride, and nickel.

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Thermodynamic Analysis of the Freezing and Thawing of Foods: Enthalpy and Apparent Specific Heat

C. S. CHEN

ABSTRACT

On the basis of the principles of freezing point depression, equations for the temperature rate of ice formation, apparent specific heat, and enthalpy were derived and expressed in terms of solids content and temperature. A unique characteristic constant for each food system can be determined experimentally from the calorimetric measurements. The new equations differ only slightly with similar equations previously developed by Bartlett in 1944 and by Schwartzberg in 1976. Reliability and accuracy of the methods are demonstrated with literature data for meat, fish and fruit juices. Enthalpy and apparent specific heat values for a range of temperature between 20° and −40°C and solids content between 10 and 50% are presented.

INTRODUCTION

FOODS UNDERGOING freezing and thawing involve gradual changes in ice-water phase, temperature, and enthalpy. These changes are interrelated. Enthalpy vs temperature change can be measured by calorimetric methods.

Calorimetric investigations on freezing of meat, fish, fruits and vegetables were reported by Short and Bartlett (1944), Riedel (1951, 1956, 1957), Fleming (1969), and others. An extensive compilation of experimental enthalpy and ice content data has been summarized in ASHRAE (1981). Enthalpy data are useful for calculating refrigeration loads.

Apparent specific heat along with thermal conductivity and density comprises the thermal diffusivity. Apparent specific heat values change drastically at freezing and thawing over a wide range of temperatures (Short and Bartlett, 1944). Its accuracy will in turn affect the accuracy of the calculated thermal diffusivity which characterizes the Fourier's equations for freezing and thawing calculations.

Several theoretical methods were presented to correlate the experimental data (Short and Bartlett, 1944; Heldman, 1982; Heldman and Singh, 1981; Schwartzberg, 1976; Schwartzberg et al., 1977). Empirical equations were also proposed (Chang and Tao, 1981; Succar and Hayakawa, 1983). These methods greatly simplify the calculation procedures and are useful to convert the scarce experimental data for generalized heat transfer calculations (e.g., Schwartzberg et al., 1977).

The thermodynamic methods of Bartlett (1944) and Schwartzberg (1976) are especially useful for understanding the principles of freezing and thawing processes.

This work was to investigate the applicability and limitations of thermodynamic methods for analyzing the food freezing processes.

ASSUMPTIONS AND THERMODYNAMIC METHODS

CONSIDER a food containing W_{wo} kg of total water, W_{so} kg of soluble substances (e.g., acids, salts, sugars in solution) and W_u kg of insoluble substances (e.g. cellulose, fats and starch) being cooled below its initial freezing temperature. The system now contains W_I kg of ice, and W_w kg of unfrozen water. For any temperature change below the freezing point there is a corresponding change in the amounts of W_I and W_w due to ice crystallization. The ice-water relation can be expressed as:

$$W_I = W_{wo} - W_w \tag{1}$$

For the purpose of analysis, the following five assumptions were made (Bartlett, 1944): (1) The food is a heterogeneous system in a state of thermodynamic equilibrium at constant pressure; (2) The system is at all times above the eutectic temperature; (3) Raoult's law is applicable; (4) The thermal capacity of the solution phase is a linear function of concentration and is independent of temperature; (5) The thermal capacities of solid phases are constant. Since Raoult's law is valid only for an ideal solution, its applicability and limitations for foods need be established experimentally.

On the basis of the above assumptions, the freezing point depression equation has been derived and expressed either in a differential form (Bartlett, 1944) as:

$$\frac{d \ln a_w}{dT} = \frac{M_w L}{RT^2} \tag{2}$$

or in an integral form as:

$$\ln a_w = \frac{M_w L}{R}\left(\frac{1}{T_o} - \frac{1}{T}\right) \tag{3}$$

where a_w is water activity; L, latent heat of fusion of water (Kcal/kg); R, ideal gas constant (= 1.987 Kcal/kg-mole · °K); T_o, freezing point of water (= 273.1°K); T, freezing point of food (°K); M_w, molecular weight of water (kg/kg-mole).

The freezing point depression equation has provided the thermodynamic basis for many modern studies on food freezing processes (Heldman, 1974, 1982; Heldman and Singh, 1981; Schwartzberg, 1976).

The unfrozen water is expressed in mole fraction and equated to water activity by Raoult's law:

$$a_w = \frac{W_w/M_w}{W_w/M_w + W_{so}/M_s} = \frac{W_w}{W_w + EW_{so}} \tag{4}$$

in which $E = M_w/M_s$ and M_s is effective molecular weight of dissolved solids.

By solving Eq. (1) and (4), the relation between water activity and the amount of ice is:

$$W_I = W_{wo} - EW_{so}\frac{a_w}{1 - a_w} \tag{5}$$

It is convenient to transform the ice-water relation to mass fraction by dividing Eq. (5) by total weight of food:

$$X_I = X_{wo} - EX_{so}\frac{a_w}{1 - a_w} \tag{6}$$

in which X_I is ice fraction (kg/kg food); X_{wo}, initial moisture fraction (kg/kg food); X_{so}, soluble solids fraction (kg/kg food).

For a food containing a given amount of bound water, Eq. (4) was modified by Schwartzberg (1976):

$$a'_w = \frac{W_w - W_B}{W_w - W_B + EW_{so}} \tag{7}$$

in which W_B is bound water (kg); a'_w, modified definition of water activity. By a similar transformation, the ice-water relation becomes:

Author Chen is with the Florida Department of Citrus, 700 Experiment Station Road, Lake Alfred, FL 33850.

$$X_I = X_{WO} - EX_{SO}\frac{a'_W}{1 - a'_W} \quad (8)$$

The equations (6) and (8) are in a similar form, therefore, the following developments are applicable for both cases.

Temperature rate of ice formation

Define "s" as kilogram mass of ice formed per degree fall in temperature per kilogram of food and express "s" as:

$$s = -\frac{\partial X_I}{\partial T} \quad (9)$$

Assuming M_s constant and differentiating Eq. (6) with respect to a_w, one can obtain:

$$\frac{\partial X_I}{\partial a_w} = -EX_{SO}\frac{1}{(1 - a_w)^2} \quad (10)$$

From Eq. (2), one can obtain:

$$\frac{\partial a_w}{\partial T} = \frac{a_w M_w L}{RT^2} \quad (11)$$

By using chain rule and combining Eq. (9), (10) and (11), the "s" function becomes:

$$s = -\frac{\partial X_I}{\partial T} = -\left(\frac{\partial X_I}{\partial a_w}\right)\left(\frac{\partial a_w}{\partial T}\right) = \frac{EX_{SO}\cdot a_w \cdot M_w \cdot L}{(1 - a_w)^2 \cdot RT^2}$$

$$= \frac{X_{SO}}{M_s}\cdot\frac{a_w}{(1 - a_w)^2}\cdot\frac{M_w^2 L}{RT^2} \quad (12)$$

Using $\ln a_w \simeq a_w - 1$, from Eq. (3), it can be shown:

$$1 - a_w \simeq \frac{LM_w}{RTT_o}(T_o - T) \quad (13)$$

Substituting Eq. (13) and $t = T - T_o$, Eq. (12) can be expressed as:

$$s = \frac{X_{SO}}{M_s}\cdot\frac{RT_o^2}{L}\left[\frac{1}{t^2} + \frac{1}{t(T_o + t)}\cdot\frac{LM_w}{RT_o}\right] \quad (14)$$

The second term in the bracket can be dropped since its contribution is less than 2% as compared to the first term at temperatures in the freezing zone and Eq. (14) is simplified to:

$$s = \frac{X_{SO}}{M_s}\cdot\frac{RT_o^2}{L}\cdot\frac{1}{t^2} \quad (15)$$

Apparent specific heat equation

It is known that specific heat is dependent on both chemical composition and temperature (Staph and Woolrich, 1951). The temperatures can be divided into two regions: above and below freezing point.

At temperatures above freezing point, the specific heat can be estimated by the following equation if the chemical composition is not known:

$$C = 1 - 0.55\,X_{SO} - 0.15\,X_{SO}^3 \quad (16)$$

where C is the specific heat above freezing (kcal/kg °C).

At temperatures below freezing point, modified Siebel's equation was used (ASHRAE, 1981):

$$C_f = C_o + 0.3\,X_{SO} \quad (17)$$

where C_f is the specified heat of material below freezing (kcal/kg °C) and C_o, a constant varying between 0.2 to 0.5 depending on food substance. An averaged value of 0.37 was assumed for C_o for foods containing less than 60% solids. Eq. (17) assumes the specific heat of frozen material is a linear function of concentration and does not include any effects of freezing/melting enthalpy.

Define apparent specific heat as including both sensible and latent heat changes, then:

$$C_e = C_f + s\cdot L = 0.37 + 0.3X_{SO} + \frac{X_{SO}}{M_s}\cdot\frac{RT_o^2}{t^2} \quad (18)$$

where C_e is the apparent specific heat below freezing (kcal/kg °C). Eq. (18) is limited for foods with moisture greater than 40% and it differs only slightly with equations derived by Bartlett (1944) and by Schwartzberg (1976).

Enthalpy equation

By thermodynamic definition, the apparent specific heat can be written as:

$$C_e = \left(\frac{\partial H_f}{\partial T}\right)_p \quad (19)$$

where H_f is the enthalpy below freezing (kcal/kg); the subscript p denotes constant pressure. Enthalpy is measured with a reference temperature, t_r, which is often chosen at −40°C for practical purposes. By substituting Eq. (18) for C_e and integrating Eq. (19) between a reference temperature, t_r, and t, the following equation for enthalpy is obtained for temperatures below freezing point:

$$H_f = (t - t_r)\left(0.37 + 0.3X_{SO} + \frac{X_{SO}}{M_s}\cdot\frac{RT_o^2}{t\,t_r}\right) \quad (20)$$

For temperatures above freezing, Eq. (20) becomes:

$$H = H_I + (t - t_I)(1 - 0.55X_{SO} - 0.15X_{SO}^3) \quad (21)$$

where H_I is the enthalpy corresponding to initial freezing point (kcal/kg); t_I initial freezing point, (°C).

Determination of effective molecular weight

Effective molecular weight is required for calculating enthalpy from Eq. (20). A method for determining M_s from calorimetric measurements can be developed by rearranging Eq. (20):

$$M_s = \frac{X_{SO}\cdot RT_o^2}{t\cdot t_r\left(\frac{H_f}{t - t_r} - 0.37 - 0.3X_{SO}\right)} \quad (22)$$

Experimental values of enthalpy in the range within 10°C below freezing point were employed for the calculations of M_s.

The calculated values for M_s varied with solids content which indicated the derivation from the ideal solution law. A characteristic constant (N) dependent on the substance and invariant to change in solids content was sought. For lean beef and cod muscle, the following equation was deduced on the basis of experimental data:

$$M_s = \frac{N}{1 - X_{SO}} \quad (23)$$

where N = 535.4 for lean beef and 404.9 for cod muscle.

For fruit juices:

$$M_s = \frac{N}{1 + 0.25X_{SO}} \quad (24)$$

where N = 200 for both orange juice and apple juice. The value of N has a unit of kg/kg mole.

Determination of initial freezing point

The highest temperature (initial freezing point) at which freezing started is difficult to determine precisely. The following method was developed on the basis of the enthalpy Eq. (20). An approximate initial freezing point was obtained by determining the inflection point of the enthalpy-temperature plot of calorimetric data. The enthalpy value corresponding to the approximate initial freezing point was then substituted into the following equation to calculate the corresponding initial freezing point, t_I:

$$H_I = (t_I - t_r)\left(0.37 + 0.3X_{SO} + \frac{X_{SO}}{M_s}\cdot\frac{RT_o^2}{t_I\cdot t_r}\right) \quad (25)$$

Evaluation of accuracy

Standard errors of estimate or the root mean square error (or RMS) were calculated to express the accuracy of the calculated vs. experimental data (Mickley et al., 1957).

Table 1—Calculated vs experimental enthalpy values for lean beef meat with 74% moisture content (reference temperature −40°C)

	Enthalpy (Kcal/kg)					
				Calculated		
Temp °C	Experimental Diagram[a]	Diagram[b]	Avg	Schwartz-berg[c]	Succar & Haya-kawa[d]	Proposed[e]
−40	0	–	0.0	0.0	0.0	0.0
−30	4.5	–	4.5	5.37	4.96	4.92
−25	6.8	–	6.8	8.19	7.59	7.52
−20	9.7	–	9.7	11.18	10.40	10.29
−18	10.9	–	10.9	12.46	11.61	11.48
−16	12.4	–	12.4	13.81	12.90	12.75
−14	13.9	–	13.9	15.27	14.30	14.12
−12	15.5	15.5	15.5	16.89	15.86	15.65
−10	17.5	17.5	17.5	18.76	17.68	17.43
− 9	18.7	18.5	18.6	19.85	18.75	18.47
− 8	19.8	19.5	19.7	21.08	19.96	19.65
− 7	21.1	21.5	21.3	22.56	21.39	21.05
− 6	22.8	23.0	22.9	24.28	23.15	22.77
− 5	25.0	25.0	25.0	26.55	25.41	24.99
− 4	27.8	28.0	27.9	29.70	28.57	28.09
− 3	32.6	33.0	32.8	34.62	33.49	32.97
− 2	42.2	42.0	42.1	43.98	42.79	42.29
− 1.5	–	–	–	53.09	–	–
− 1.0	70.36	70.0	70.2	71.07	–	69.33
− 0.99	–	–	–	71.89	69.90	–
0.0	–	71.5	71.5	–	–	71.08
+10.0	–	79.5	79.5	–	–	79.57
RMS error				1.43	0.50	0.38

[a] Mollier diagram of Riedel (1957) quoted by Succar and Hayakawa (1983)
[b] Mollier diagram of Riedel (1957) quoted by Rolfe (1968)
[c] Schwartzberg (1976)
[d] Succar and Hayakawa (1983)
[e] Eq. (20) and (21)

Table 2—A comparison of various calculated apparent specific heat values for lean beef with 74% moisture

	Apparent specific heat (Kcal/kg°C)					
Temp °C	Rolfe[a] Diag.[e]	Schwartzberg[b] Diag.[e]	Calc.	Succar & Hayakawa[c] Diag.[e]	Calc.	Proposed[d] Calc.
−12	1.0			0.90	0.84	0.82
−11		1.0	0.96			0.89
−10	1.0			1.10	1.00	0.98
− 9.5		1.0	1.12			1.04
− 9.0	1.0			1.15	1.13	1.11
− 8.5		1.0	1.28			1.19
− 8.0	2.0			1.20	1.31	1.28
− 7.5		2.0	1.50			1.39
− 7.0	1.5			1.50	1.57	1.53
− 6.5		1.5	1.84			1.71
− 6.0	2.0			1.95	1.97	1.93
− 5.5		2.0	2.37			2.21
− 5.0	3.0			2.50	2.62	2.58
− 4.5		3.0	3.29			3.08
− 4.0	5.0			3.80	3.82	3.77
− 3.5		5.0	5.14			4.79
− 3.0	9.0			9.40	6.39	6.36
− 2.5		9.0	9.61			8.96
− 2.0	28.0			18.60	13.64	13.74
− 1.5		28.0	25.82			24.10
− 1.0	1.5					53.63
− 0.99				55.90	53.08	–
0.0			–	0.85		

[a] Rolfe (1968)
[b] Schwartzberg (1976)
[c] Succar and Hayakawa (1983)
[d] Eq. (18)
[e] Experimental data obtained through the same Mollier diagram of Riedel (1957)

Table 3—Calculated vs. experimental enthalpy values for cod muscle with 82% moisture content (reference temperature −40°C)

	Enthalpy (Kcal/kg)				
	Experimental			Calculated	
Temp °C	Riedel[a]	Diagram[b]	Diagram[c]	Schwartzberg[d]	Proposed[e]
−40	0.0	0	0	0.00	0.00
−30	4.60	4.5	4.54	5.58	4.69
−25	–	–	–	8.50	7.17
−20	10.07	9.5	10.03	11.60	9.83
−18	11.35	–	11.23	12.93	10.98
−16	12.71	12.0	12.66	14.33	12.20
−14	14.17	–	14.09	15.84	13.53
−12	15.78	–	15.76	17.51	15.02
−10	17.60	17.0	17.68	19.44	16.77
− 9	18.65	–	18.87	20.56	17.79
− 8	19.82	19.5	20.06	21.83	18.96
− 7	21.17	–	21.26	23.31	20.35
− 6	22.83	22.0	22.93	25.11	22.06
− 5	24.94	–	25.08	27.43	24.28
− 4	27.90	27.0	28.19	30.66	27.40
− 3	32.60	31.5	32.72	35.69	32.32
− 2	42.18	40.5	42.28	45.24	41.74
− 1.5	–	–	–	–	54.54
− 1.0	72.27	65.0	71.18	72.88	69.14
0.0	78.91	79.0	77.15	–	77.99
+10.0	87.70	88.0	–	–	86.95
+20.0	96.52	–	–	–	95.92

[a] Riedel (1956)
[b] Mollier diagram of Riedel (1960) quoted by Rolfe (1968)
[c] Mollier diagram of Riedel (1960) quoted by ASHRAE (1981)
[d] Schwartzberg (1976)
[e] Eq. (20) and (21)

RESULTS & DISCUSSION

Lean beef

Mollier diagram for lean beef (Riedel, 1957) was used by Rolfe (1968) and Succar and Hayakawa (1983) to obtain the experimental data of enthalpy. Using the averaged data for 74% moisture, the calculated M_s was 723.5 which was used to compute the enthalpy data. The computed results compared with experimental data and other methods are shown in Table. 1. Heldman's method could not be included since it requires more information on the product properties than is available and it has been shown to be the least accurate among the existing methods (Succar and Hayakawa, 1983). The proposed method appears to be as accurate as the method of Schwartzberg.

Since the enthalpy equation is accurate, its thermodynamically related equation of apparent specific heat (Eq. 18) should also be accurate. A comparison of calculated apparent specific heat values from Eq. (18) along with literature results is shown in Table 2. Considerable discrepancies among the literature values are due to errors associated with the conventional method of the graphical differentiation. The proposed method is an alternative to the conventional method.

Fish

Mollier diagram for cod muscle (Riedel, 1960) was used by Rolfe (1968) and ASHRAE (1981) for obtaining numerical data. Using data of Riedel (1956), the calculated M_s for cod was 493.8. The calculated results of enthalpy compared with experimental data are shown in Table 3. The proposed method appears to be in good agreement with experimental data. The calculated apparent specific heat values are shown in Table 4.

Both the results in Tables 2 and 4 indicate that the discrepancies among the data are greater or greatest at or near the initial freezing temperature.

Table 4—A comparison of various calculated apparent specific heat values for cod muscle with 82% moisture content

Temp °C	Apparent specific heat (Kcal/kg°C) Riedel[a] Expt.	Rolfe[b] Diag.[e]	Schwartzberg[c] Diag.[e]	Schwartzberg[c] Calc.	Proposed[d] Calc.
−40	0.44	0.45		0.54	0.46
−30	0.50	0.5		0.57	0.48
−25	—	—		0.59	0.51
−20	0.62	0.6		0.64	0.56
−18	0.66	—	0.6	0.67	0.59
−16	0.71	0.8		0.71	0.63
−14	0.76	—		0.77	0.70
−12	0.85	—		0.87	0.80
−10	0.99	1.3		1.02	0.96
− 9	1.11	—	1.3	1.15	1.09
− 8	1.26	1.8		1.31	1.27
− 7	1.49	—	1.8	1.56	1.53
− 6	1.85	2.5		1.94	1.92
− 5	2.47	—	2.5	2.57	2.58
− 4	3.66	4.5		3.72	3.80
− 3	6.41	9.0		6.23	6.42
− 2	16.10	24.5		13.37	13.91
− 1.5	—	—	24.5	23.37	24.4
− 1	25.95	6.0		51.95	54.37
0	0.97	0.9		0.9	0.9
+10.0	0.89	0.9		0.9	0.9
+20.0	0.89	0.9		0.9	0.9

[a] Riedel (1956); Averaged values of cod and haddock
[b] Rolfe (1968)
[c] Schwartzberg (1976)
[d] Eq. (18)
[e] Experimental data obtained through the same Mollier diagram of Riedel (1960)

It should be noted that the apparent specific heat in the freezing zone includes the latent heat effects. Woolrich (1966) discussed the inherent difficulties in obtaining accurate apparent specific heat experimentally. The experimental procedure is to take the heat inputs required to raise the temperature of the material and divide it by the temperature rise. This actually results in an average specific heat over the temperature interval. Since at or near the initial freezing point, large heat inputs are required for small temperature rises, the average values so obtained in this region are of doubtful validity. The mathematical equations are also subject to errors for temperatures near zero.

Orange juice

Calorimetric data of orange juice with 89% moisture content were reported by Riedel (1951). The calculated M_s was 194.6. The calculated vs experimental enthalpy values with reference to 20°C are shown in Table 5. The good agreement between the calculated and experimental results allows the use of Eq. (18) and (20) to predict the apparent specific heat and enthalpy for other temperatures using any other reference temperatures.

Effects of solids content

The values of effective molecular weight were found to be dependent on the solids content for all three different foods studied. The concept of effective molecular weight is based on the use of the freezing point depression equation from which all the literature methods (including the proposed method) have been derived (Short and Bartlett, 1944; Bartlett, 1944; Heldman, 1974; Heldman and Singh 1981; Schwartzberg, 1976). Such variability has also been noted by Schwartzberg (1976).

In an analysis of Mollier diagram for beef (Riedel, 1957) and cod (Riedel, 1960), respectively, Eq. (22) was deduced and the calculated values of N were 535.4 and 404.9 for

Table 5—Calculated vs experimental enthalpy values for orange juice with 89% moisture content (reference temperature 20°C)

Temp °C	Enthalpy (Kcal/kg) Experimental[a]	Calculated
−29.92	101.5	100.3
−19.65	95.0	94.7
−14.50	91.2	91.1
− 9.34	85.7	85.9
− 4.17	73.0	72.7
− 0.05	18.7	18.8
20	0	0

[a] Riedel (1951); Enthalpy = 0 at 20°C.

Table 6—Calculated vs experimental enthalpy values for partially dried lean beef and cod muscle with 50% moisture content (reference temperature −40°C)

Temp °C	Enthalpy (Kcal/kg) Lean beef Diag.[a]	Lean beef Calc.[c]	Cod muscle Diag.[b]	Cod muscle Calc.[c]
−40	0.0	0.0	0.0	0.0
−30	5.5	5.78	5.5	5.96
−20	10.0	12.13	11.0	12.68
−18	12.0	13.55	13.0	14.23
−16	14.0	15.07	15.0	15.91
−14	16.0	16.73	17.0	17.76
−12	17.5	18.59	18.5	19.89
−10	20.0	20.78	21.5	22.45
− 9	21.5	22.07	23.5	23.99
− 8	23.5	23.55	25.5	25.78
− 7	25.5	25.30	27.5	27.93
− 6	28.0	27.47	30.0	30.63
− 5	31.5	30.29	34.0	39.28
− 4	37.5	34.27	39.0	39.28
− 3	42.5	40.55	43.0	42.94
− 2	43.5	44.30	43.5	43.63
− 1	44.0	44.99	44.5	44.33
0	45.5	45.69	45.0	45.03
10	52.5	52.65	52.0	51.99
20	59.5	59.61	58.5	58.95

[a] Mollier diagram of Riedel (1957)
[b] Mollier diagram of Riedel (1960)
[c] See text.

Table 7—Calculated vs experimental enthalpy values for various fishes

Temp °C	Haddock 83.6% moisture Expt.[a]	Haddock 83.6% moisture Calc	Cod 80.3% moisture Expt.[a]	Cod 80.3% moisture Calc.	Perch 79.1% moisture Expt.[a]	Perch 79.1% moisture Calc.
−40	0	0.00	0	0.00	0	0.00
−30	4.63	4.61	4.56	4.77	4.54	4.83
−20	10.11	9.64	10.03	10.03	9.88	10.16
−18	11.38	10.75	11.31	11.21	11.11	11.37
−16	12.72	11.94	12.69	12.47	12.45	12.65
−14	14.15	13.23	14.18	13.84	13.87	14.06
−12	15.71	14.66	15.84	15.39	15.44	15.64
−10	17.47	16.33	17.73	17.21	17.21	17.51
− 9	18.49	17.31	18.80	18.28	18.21	18.62
− 8	19.64	18.43	19.99	19.51	19.31	19.89
− 7	20.99	19.74	21.35	20.98	20.59	21.40
− 6	22.64	21.35	23.01	22.78	22.13	23.27
− 5	24.76	23.44	25.12	25.14	24.06	25.72
− 4	27.75	26.37	28.05	28.46	26.71	29.17
− 3	32.49	30.96	32.70	33.71	30.86	34.64
− 2	42.19	39.73	42.16	43.77	39.29	45.15
− 1	73.37	65.21	71.16	73.11	67.69	75.51
0	80.66	78.02	77.16	77.26	75.92	76.39
10	89.51	87.08	85.89	86.12	84.52	85.19
20	98.38	96.14	94.65	94.99	93.12	93.98

[a] Riedel (1956)

Table 8—Calculated vs. experimental enthalpy values for apple juice (reference temperature 20° C)

Temp °C	Enthalpy (Kcal/kg) Juice 87.2% moisture Expt.[a]	Calc.	Concentrate 71.9% moisture Expt.[a]	Calc.	Concentrate 49.8% moisture Expt.[a]	Calc.
−22.92	99.3	98.8	80.7	81.2	55.3	54.9
−19.65	92.6	92.9	71.6	72.7	42.9	42.3
−14.50	88.3	89.0	65.0	66.3	33.0	32.0
− 9.34	82.0	83.2	53.8	55.5	21.4	20.9
− 4.17	66.7	68.9	24.9	23.6	–	17.3
− 0.05	18.7	18.6	17.0	16.8	14.8	14.3
+20.0	0	0	0	0	0	0

[a] Riedel (1951); Enthalpy = 0 at 20°C.

beef and cod, respectively. The calculated vs experimental enthalpy values for partially dired lean beef and cod muscle with 50% moisture content are shown in Table 6. The results indicate good agreement for a wide range of temperatures for both lean beef and cod muscle. It should be noted that accuracy of the experimental data is limited by the interpolation of values from the original Mollier diagrams. The proposed methods are, therefore, valid for both fresh and partially dried meat and fish. The calculated vs experimental enthalpy values for various fishes are shown in Table 7. Conversely, the fruits and vegetable juices behave quite differently. Based on the Mollier diagram for fruit and vegetable juices (Riedel, 1951), Eq. (23) was deduced and the calculated value of N was 200 for both orange and apple juices. The calculated vs experimental enthalpy values for apple juice and apple juice concentrate are shown in Table 8. The good agreement between the calculated vs experimental data for a wide range of temperatures and moisture content indicates the validity of the methods for fruit and vegetable products.

The values of M_s increase as solids content increases for both lean beef and fish while the values decrease as solids content increases for fruit and vegetable juices. The values of N are higher for foods containing a higher amount of protein and lower for foods containing mostly carbohydrate (sugars and orange juice).

Limitations of Raoult's law

Bartlett's method (misnamed as Heldman's method) applies Raoult's law of water activity and the freezing point depression equation to calculate effective molecular weight. This method is simple and requires only a single initial freezing point datum. The fact that the calculated results of enthalpy deviated with experimental data as shown by Succar and Hayakawa (1983) may reflect the limitations of Raoult's law for calculating the effective molecular weight. Schwartzberg (1976) suggested a method for modifying Raoult's law by using Eq. (7). He used the modified equation for calculating water activity. The proposed method employs Raoult's law in the derivation of "s" function which defines the ice formation as a function of temperature. The characteristic constants (N) for various foods are derived from the calorimetric data and are invariant to change in solids content.

The good agreement between the calculated results and experimental data of enthalpy for both methods of Schwartzberg and the proposed is logical since the calorimetric measurements include the effects of bound water. There is no contradication between these two methods. In fact, the bound water can be measured by calorimetric methods (Duckworth, 1971).

In the following part of this series further comparison of various methods will be examined on the basis of ice content data of Mollier diagrams.

SUMMARY & CONCLUSION

ON THE BASIS of the principles of freezing point depression, a mathematical expression was developed for the temperature rate of ice formation. Applying the derived thermodynamic relations, equations were developed for apparent specific heat and enthalpy. By rearranging the derived enthalpy equation, the required parameters can be determined from the calorimetric measurements. The experimentally determined parameters can then be used to calculate each of three quantities: (1) temperature rate of ice formation; (2) apparent specific heat; (3) enthalpy.

The derived equations are slight variations of equations previously derived based on the same principles (Bartlett, 1944; Schwartzberg, 1976). However, the proposed use of calorimetric data for the characterization of different food systems is more reliable than the use of initial freezing point data first suggested by Bartlett (1944) and applied in contemporary literature (Heldman, 1974; Schwartzberg, 1976; Heldman and Singh, 1981).

The proposed mathematical expressions and methods are broadly applicable for different food systems and are reliable for moisture contents in the range greater than 40%.

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Thermodynamic Analysis of the Freezing and Thawing of Foods: Ice Content and Mollier Diagram

C. S. CHEN

ABSTRACT

On the basis of the freezing point depression equation, an ice content equation was derived. The required parameters are similar to equations for enthalpy and apparent specific heat previously developed. Validity and accuracy of the equation are demonstrated with experimental data for meat, fish and fruit juices. Cohesive data for enthalpy, apparent specific heat and ice content are calculated for a wide range of temperatures between 20 and −40°C. The calculated results are consistent with values obtainable through enthalpy-moisture content-temperature (Mollier) diagrams developed by Riedel.

INTRODUCTION

ONE OF THE physical phenomena in the freezing preservation of foods is the formation of ice. The amount of ice is a function of chemical composition and temperature, and is largely independent of the freezing rate (Bartlett, 1944; Short and Bartlett, 1944). Knowledge of the amount of ice in food is useful in predicting frozen food properties (Heldman, 1982), and is useful in minimizing undesirable effects of ice on frozen foods.

This work was to investigate the applicability and limitations of applying the freezing point depression for predicting ice formation during freezing and to evaluate the thermodynamic methods for describing the Mollier diagrams.

MATERIALS & METHODS

Derivation of an ice content equation

On the basis of the freezing point depression equation, Chen (1985) developed an equation for predicting ice crystallization as a function of freezing temperature (see Part 1),

$$s = -\frac{\partial X_I}{\partial t} = \frac{X_{so}}{M_s} \cdot \frac{RT_o^2}{L} \cdot \frac{1}{t^2} \tag{1}$$

where s is mass fraction of ice formed per degree fall in temperature (kg/kg food °C); L, latent heat of fusion of water (= kcal/kg); X_I, mass fraction of ice (kg/kg food); X_{so}, initial solids content (kg/kg food); M_s, effective molecular weight of soluble solids (kg/kg-mole); R, ideal gas constant (= 1.987 Kcal/kg-mole °K); T_o, freezing point of water (= 273.1 °K); $t = T - T_o = (T - 273.1)$°K and is therefore numerically equal to freezing temperature in °C ($-40°C \leq t < 0°C$); T, freezing temperature in °K. By rearranging and integrating between a temperature t and the initial freezing temperature t_1, Eq. (1) becomes:

$$\int_{X_I}^{o} -dX_I = \int_{t}^{t_1} \frac{X_{so}}{M_s} \cdot \frac{RT^2}{L} \cdot \frac{1}{t^2} \cdot dt \tag{2}$$

The latent heat of fusion of water is known to be dependent on temperature (Randall, 1930) and can be approximated by

$$L = 79.8 + 0.506\,t \tag{3}$$

Author Chen is with the Florida Department of Citrus, 700 Experiment Station Road, Lake Alfred, FL 33850

By denoting $L_o = 79.8$; $L_1 = 0.506$ and substituting Eq. (3) for L in Eq. (2) then performing the integration, Eq. (2) yields an equation for ice content:

$$X_I = \frac{X_{so}}{M_s} \cdot \frac{RT_o^2}{L_o} \left[\frac{(t_1 - t)}{t\ t_1} + \frac{L_1}{L_o} \ln \frac{(L_o + L_1 \cdot t_1)\,t}{(L_o + L_1 \cdot t)\,t_1} \right] \tag{4}$$

The second term in the bracket can be dropped since its contribution is less than 2% as compared to the first term at temperatures in the freezing zone and, therefore, Eq. (4) is simplified to

$$X_I = \frac{X_{so}}{M_s} \cdot \frac{RT_o^2}{L_o} \cdot \frac{(t_1 - t)}{t\ t_1} \tag{5}$$

Eq. (5) justifies the use of latent heat of fusion of water at 0°C for pracitcal purposes.

The ice content (I), is expressed as a percent of the total initial moisture content:

$$I = \frac{100 X_I}{X_{wo}} \tag{6}$$

where X_{wo} is initial moisture content (kg/kg food).

Determination of effective molecular weight

Effective molecular weight required for calculating ice content from Eq. (5) was determined by the method previously described (Chen, 1985). For lean beef and cod muscle, the following relationship was used.

$$M_s = \frac{N}{1 - X_{so}} \tag{7}$$

where N = 535.4 for lean beef and 404.9 for cod muscle.

For fruit juices

$$M_s = \frac{N}{1 + 0.25 X_{so}} \tag{8}$$

where N = 200 for both orange juice and apple juice. The value of N has a unit of kg/kg mole.

Determination of initial freezing point

The highest temperature (initial freezing point) at which freezing started was determined on the basis of an enthalpy equation previously developed (Chen, 1985):

$$H_1 = (t_1 - t_r)\left(0.37 + 0.3\,X_{so} + \frac{X_{so}}{M_s} \cdot \frac{RT_o^2}{t_1 \cdot t_r}\right) \tag{9}$$

where H_1 is enthalpy corresponding to initial freezing point (Kcal/kg); $t_r = T_r - T_o$, a reference temperature (°C); T_r, a reference temperature (°K).

Comparison with literature methods

On the basis of the same freezing point depression equation, Bartlett (1944) developed an ice content equation as (it should be noted that Bartlett defined $t = T_o - T$ instead of $t = T - T_o$ in this paper):

$$X_I = X_{wo} - \frac{EX_{so}}{e^{(-at + bt^2)} - 1} \quad (10)$$

and

$$EX_{so} = X_{wo}\,[e^{(-at_1 + bt_1^2)} - 1] \quad (11)$$

where $a = LM_w/RT_o^2 = 9.703 \times 10^{-3}\ (°C)^{-1}$; $b = 4.794 \times 10^{-6}\ (°C)^{-2}$; $E = M_w/M_s$; M_w is molecular weight of water (= 18 kg/kg-mole). The parameters M_s and E are determined from Eq. (11) using the initial freezing point.

Heldman (1974) applied the same freezing point depression equation directly to characterize the effective molecular weight by using the initial freezing point. The effective molecular weight so determined is then used to calculate the ice content for temperatures below freezing. His procedures can be shown to be similar to those of Bartlett (1944) and can be expressed as:

$$X_I = X_{wo} - \frac{EX_{so}}{e^{-a_1 t} - 1} \quad (12)$$

and

$$EX_{so} = X_{wo}\,(e^{-a_1 t_1} - 1) \quad (13)$$

where $a_1 = \frac{\lambda}{RT_oT} = \frac{LM_w}{RT_oT}$; λ is molar heat of fusion of water (Kcal/kg-mole); $T_oT \simeq T_o^2$ for small values of $(T - T_o)$ (see Heldman and Singh, 1981, p. 161). Therefore, "a_1" ≃ "a" and Heldman's method is an approximation of Bartlett's method. Since the terms e^{bt^2} is negligibly small, the two methods are essentially equivalent.

Evaluation of accuracy

The values of root mean square error (RMS) or standard error of the estimate were calculated to assess the accuracy of predicted vs experimental data (Mickley et al., 1957).

RESULTS & DISCUSSION

EXPERIMENTAL CALORIMETRIC DATA for meat, fish, fruits and vegetables (Riedel, 1951; 1956; 1957) were used for testing the accuracy and reliability of enthalpy and apparent specific heat equations by several researchers (Schwartzberg, 1976; Succar and Hayakawa, 1983; Chen, 1985). Riedel's data were used to test ice content predictions by Heldman (1974). The parameters for Riedel's data for lean beef, cod muscle, and orange juice for fresh and partially dried (or concentrated) products were calculated and are shown in Table 1.

The effective molecular weight for the proposed method was calculated using either Eq. (7) or Eq. (8) depending upon the products. Experimental enthalpy H_1's corresponding to t_1's were taken from Riedel's diagrams. The initial freezing points were calculated from Eq. (9) for each value of H_1. The calculated t_1's were used in Eq. (11) and (13) to calculate M_s for Bartlett's and Heldman's methods, respectively. $X_{wo} = 1 - X_{so}$ was used for all the calculations (fat content was assumed to be negligible).

It should be noted that the values of effective molecular weight for both Bartlett's and Heldman's methods are almost identical indicating the similarity of both methods. However, the values of the calculated effective molecular weight vary greatly dependent on the values of the initial freezing point used.

Ice content

The parameters shown in Table 1 were used to calculate the ice contents for various freezing temperatures. The calculated vs. experimental results for lean beef, cod muscle, and organe juice are shown in Tables 2, 3, and 4, respectively. Solids content at 50% represents partially dried or concentrated foods.

The method of Bartlett (1944) using the initial freezing point to derive the effective molecular weight for predicting ice content of frozen foods appears to be applicable to fruit juices. It is not as accurate for meat and fish and greater discrepancies are evident when these products are partially dried. Nevertheless, Bartlett's developments have provided the fundamental basis for modern studies on food freezing (Heldman, 1974; Heldman and Singh, 1981; Schwartzberg, 1976).

The proposed method using the effective molecular weight derived from the enthalpy data appears to be applicable for both fresh and partially dried or concentrated foods in the range tested. Some discrepancies between the calculated vs experimental results at or near the initial freezing point are observed. The reasons for the discrepancies were discussed previously (Chen, 1985).

Bound water (unfreezable water)

The existence of bound water has been observed experimentally (Duckworth, 1971). Schwartzberg (1976) used the knowledge of bound water to modify Raoult's law and showed better results for predicting apparent specific heat than that predicted by Heldman's method (Succar and Hayakawa, 1983).

The calculated bound water content at −40°C is shown in Table 5. The calculations for Schwartzberg's method are:

Table 1—Experimental and calculated characteristics of various foods (Enthalpy = 0 at −40°C)

	Moisture content $100\,X_{wo}$ (%)	Enthalpy H_1 (kcal/kg) Diagram[a]	Initial freezing point t_1 (°C) Diagram[a]	Initial freezing point t_1 (°C) Calc.[b]	Calculated effective molecular weight M_s (kg/kg mole) Schwartzberg[c]	Heldman[d]	Bartlett[e]	Proposed[f]
Lean beef	74	70	−1.0	−0.98	677	661.9	661.6	723.5
	50	44	−2.8	−2.61	677	701.9	700.9	1070.8
Cod muscle	82	77	−0.9	−0.87	519	466.1	465.8	493.8
	50	42	−3.5	−3.58	519	509.2	508.2	809.8
Orange juice	89	85	−1.2	−1.17	–	194.8	194.7	194.6
	50	41.5	−11.5	−11.25	–	156.1	155.2	177.8

[a] Mollier diagram of Riedel (1957, 1960, 19851, 1956)
[b] Eq. (7)
[c] Schwartzberg (1976)
[d] Heldman (1974)
[e] Bartlett (1944)
[f] See text Eq. (7) and (8).

Table 2—Calculated vs. experimental ice content for lean beef at different solids content

Temp °C	Ice content, %					
	Solids content, 26%			Solids content, 50%		
	Expt.[a]	Bartlett[b]	Proposed[c]	Expt.[a]	Bartlett[b]	Proposed[c]
−40	88	98.0	89.2	64	94.6	61.1
−20	87	95.5	87.0	63	88.0	56.8
−12	84	92.3	84.0	55	79.2	51.0
−10	82	90.6	82.5	51	74.8	48.2
−9	81	89.5	81.5	47	71.9	46.2
−8	79	88.2	80.2	44	68.2	43.8
−7	78.5	86.4	78.6	40	63.5	40.7
−6	76.5	84.1	76.5	34	57.2	36.6
−5	73.0	80.8	73.5	26	48.4	30.9
−4	70	75.9	69.0	13	35.2	22.2
−3	63	67.7	61.5	2.0	13.2	7.80
−2	47	51.2	46.5	0	0	0
−1	1.5	2.0	1.5			
0	0	0.0	0.0			
RMS errors		7.5	0.7		23.6	4.7

[a] Riedel (1957); Rolfe (1968)
[b] Bartlett (1944)
[c] Eq. (5)

Table 3—Calculated vs. experimental ice content for cod muscle at different solids content

Temp °C	Ice content, %					
	Solids content, 18%			Solids content, 50%		
	Expt.[a]	Bartlett[b]	Proposed[c]	Expt.[a]	Bartlett[b]	Proposed[c]
−40	92	98.2	92.5	61	92.5	59.1
−20	90	96.0	90.4	57	83.5	53.4
−12	87	93.1	87.7	49	71.4	45.7
−10	86	91.7	86.3	44	65.3	41.9
−9	85	90.7	85.4	40	61.3	39.4
−8	84	89.5	84.3	36	56.2	36.2
−7	83	87.9	82.8	31	49.7	32.1
−6	81	85.9	80.8	25	41.0	26.7
−5	78	82.9	78.1	16	28.9	19.1
−4	76	78.6	74.0	5	10.7	7.6
−3	70	71.3	67.1	0	0.0	0.0
−2	57	56.7	53.4			
−1	20	13.1	12.2			
0	0	0.0	0.0			
RMS errors		5.1	2.7		20.8	2.6

[a] Riedel (1960); Rolfe (1968)
[b] Bartlett (1944)
[c] Eq. (5)

$$X_B = BX_{so} \tag{14}$$

where X_B is mass fraction of bound water (kg/kg food); B = 0.25 for meat and fish; 0.30 for sucrose solution; 0.15–0.20 for glucose solution (Schwartzberg, 1976).

Bound water content can also be expressed as:

$$J_B = \frac{100\ X_B}{X_{wo}} \tag{15}$$

where J_B is bound water content as a percent of the total initial moisture content.

The calculations for the proposed method are, at −40°C:

$$X_I = \frac{X_{so}}{M_s} \cdot \frac{RT_o^2}{L_o} \cdot \frac{(t_1 + 40)}{(-40)t_1} \tag{16}$$

Table 4—Calculated vs. experimental ice content for orange juice at different solids content

Temp °C	Ice content, %					
	Solids content, 11%			Solids content, 50%		
	Expt.[a]	Bartlett[b]	Proposed[c]	Expt.[a]	Bartlett[b]	Proposed[c]
−40	–	97.6	97.9	–	75.7	75.1
−20	95	94.7	94.9	45	46.1	44.0
−12	88	90.8	91.0	6	6.6	6.1
−10	87	88.8	89.1	0	0	0
−9	86	87.5	87.7			
−8	85	85.9	86.1			
−7	83	83.8	84.0			
−6	80	81.0	81.2			
−5	76	77.0	77.3			
−4	70	71.2	71.4			
−3	61	61.3	61.6			
−2	41	41.7	42.0			
−1	0	0.0	0.0			
0	0	0.0	0.0			
RMS errors		1.3	1.4		0.4	0.4

[a] Riedel (1951)
[b] Bartlett (1944)
[c] Eq. (5)

Table 5—Calculated vs experimental bound water content at −40°C

Foods	Solids content (%)	Water content (%)	Bound water content at −40°C (%)			
			Expt.[a]	Schwartzberg[b]	Bartlett[c]	Proposed[d]
Lean beef	26	74	12	8.8	2.0	10.8
	50	50	36	25	5.4	38.9
Cod fish	18	82	8	5.5	1.8	7.5
	50	50	39	25	7.5	41.9
Orange juice	11	89	2	1.9-3.7	2.4	2.1
	50	50	17	15-30	24.3	24.9

[a] Riedel (1951, 1956, 1957, 1960)
[b] Schwartzberg (1976)
[c] Tables 2, 3, 4
[d] Tables 2, 3, 4

and

$$J_B = 100\ (1 - X_I/X_{wo}) \tag{17}$$

The calculated amount of bound water closely agrees with the experimental data as shown in Table 5. Although this agreement is greatly dependent upon the accuracy of the initial freezing point. Thus Eq. (16) provides only an estimate of bound water and its predicted results should be verified with more reliable experimental methods (Duckworth, 1971).

Comparison with Mollier diagrams

Variations with temperature of enthalpy, apparent specific heat and ice content for a number of foods have been established by the well-known enthalpy-moisture content-temperature (Mollier) diagrams by Riedel (1951, 1956, 1956, 1960). The Mollier diagrams and their tabulated values for lean beef, cod muscle and fruit and vegetable juices are available in many sources (e.g., Charm, 1971; Rolfe, 1968; ASHARE, 1981).

Using the methods developed in this series (Chen, 1985) the computed values of enthalpy, apparent specific heat, and ice content for lean beef, cod muscle and orange juice are shown in Table 6, 7 and 8, respectively.

Table 6—Calculated results of enthalpy, apparent specific heat and ice content of lean beef[a]

Temp °C	Enthalpy Kcal/kg	Apparent specific heat Kcal/kg°C	% Ice
−40	0.0	0.48	89.2
−30	4.9	0.51	88.5
−20	10.3	0.58	87.0
−18	11.5	0.61	86.5
−16	12.8	0.66	85.9
−14	14.1	0.72	85.1
−12	15.7	0.82	84.0
−10	17.4	0.98	82.5
−9	18.5	1.10	81.5
−8	19.7	1.28	80.2
−7	21.1	1.53	78.6
−6	22.8	1.93	76.5
−5	25.0	2.58	73.5
−4	28.1	3.77	69.0
−3	33.0	6.36	61.5
−2	42.3	13.74	46.5
−1	69.3	53.63	1.5
0	71.1	0.85	0.0
10	79.6	0.85	0.0
20	88.1	0.85	0.0

[a] Moisture content = 74%; Characteristic constant = 535.4; Effective molecular weight = 723.5; Initial freezing point = −0.98°C.

Table 7—Calculated results of enthalpy, apparent specific heat and ice content of cod muscle[a]

Temp °C	Enthalpy Kcal/kg	Apparent specific heat Kcal/kg°C	% Ice
−40	0.0	0.46	92.5
−30	4.7	0.48	91.8
−20	9.8	0.56	90.4
−18	11.0	0.59	90.0
−16	12.2	0.63	89.4
−14	13.5	0.70	88.7
−12	15.0	0.80	87.7
−10	16.8	0.96	86.3
−9	17.8	1.09	85.4
−8	19.0	1.27	84.3
−7	20.4	1.53	82.8
−6	22.1	1.92	80.8
−5	24.3	2.58	78.1
−4	27.4	3.80	74.0
−3	32.3	6.42	67.1
−2	41.7	13.91	53.4
−1	69.1	54.37	12.2
0	78.0	0.90	0.0
10	87.0	0.90	0.0
20	95.9	0.90	0.0

[a] Moisture content = 82%; Characteristic constant = 404.9; Effective molecular weight = 493.8; Initial freezing point = −0.87°C.

The calculated values are as accurate as those obtainable from their corresponding Mollier diagrams (e.g., Rolfe, 1968; ASHARE, 1981).

CONCLUSIONS

ON THE BASIS of three broadly different food systems being investigated the following conclusions are reached: (1) the method of using the initial freezing point to calculate the ice content from the freezing point depression equation (Bartlett, 1944; Heldman, 1974; Heldman and Singh, 1981) is found to be accurate for fruit juice. It is not as accurate for fresh meat and fish and is highly inaccurate for partially dried products; (2) the proposed method using

Table 8—Calculated results of enthalpy, apparent specific heat and ice content of orange juice[a]

Temp °C	Enthalpy Kcal/kg	Apparent specific heat Kcal/kg°C	% Ice
−40	0.0	0.46	97.9
−30	4.7	0.50	96.9
−20	10.2	0.61	94.9
−18	11.4	0.66	94.3
−16	12.8	0.73	93.5
−14	14.4	0.83	92.4
−12	16.2	0.98	91.0
−10	18.4	1.24	89.1
−9	19.7	1.44	87.7
−8	21.3	1.71	86.1
−7	23.2	2.11	84.0
−6	25.6	2.73	81.2
−5	28.7	3.75	77.3
−4	33.3	5.63	71.4
−3	40.7	9.70	61.6
−2	55.1	21.31	42.0
−1	85.4	0.94	0.0
0	86.4	0.94	0.0
10	95.7	0.94	0.0
20	105.1	0.94	0.0

[a] Moisture content = 89%; Characteristic constant = 200; Effective molecular weight = 194.6; Initial freezing point = −1.17°C.

the parameters derived from calorimetric measurements (Mollier diagram) is found broadly applicable for meat, fish, and fruit juice for both fresh and partially dried (or concentrated) products; (3) the proposed ice equation and the equations previously derived for enthalpy and apparent specific heat are capable of predicting the data described by the enthalpy-moisture content-temperature (Mollier) diagrams for meat, fish, and fruit juices developed by Riedel (1951, 1956, 1957, 1960).

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Rheological Characterization of Grain Legume Pastes and Effect of Hydration Time and Water Level on Apparent Viscosity

M. S. CHHINNAN, K. H. McWATTERS, and V. N. M. RAO

ABSTRACT

An extrusion capillary viscometer was employed to obtain shear stress-shear rate data of two types of cowpea pastes, traditional and experimental. The power law model was found to describe adequately flow behavior of the pastes with consistency coefficients of 77.45 and 59.02, and flow behavior indices of 0.456 and 0.458 for experimental and traditional pastes, respectively. Effect of hydration time (0 - 180 min) and water level (54 - 62%) on the apparent viscosity of experimental paste was determined by extrusion capillary and Brookfield viscometers with correlation coefficient of 0.953. Water level had a significantly greater effect than hydration time on apparent viscosity of cowpea paste.

INTRODUCTION

FOODS derived from grain legumes are an important part of the human diet, especially in the developing countries. Some of the popular dishes prepared from cowpeas in West Africa and tropical areas of the world are made by deep-fat frying or steaming paste prepared from dry peas (Dovlo et al., 1976; Seigel and Fawcett, 1976). The paste is prepared by either of two methods. The traditional method involves soaking the seeds in water for a specific amount of time, manually decorticating the seeds, grinding into paste, and also sometimes whipping the paste to incorporate air (Seigel and Fawcett, 1976); the second method involves the use of flour (prepared separately elsewhere by mechanical decortication and milling) which is hydrated by mixing with water, and whipped (if necessary) to obtain the paste of desired consistency (Seigel and Fawcett, 1976; Williams, 1980). In the preparation of akara, a traditional Nigerian deep-fat fried hush-puppy-like product made from cowpeas, the incorporation of air during the whipping step is an important factor affecting the textural quality of the finished product.

Extensive studies have been reported on the rheological characteristics of fluid foods including paste-like food materials (Campbell et al., 1979; Holdsworth, 1971). It is an established fact that most fluid food products do not follow the simple Newtonian rheological model in which a linear relationship exists between shear stress, τ, and shear rate, $\dot{\gamma}$,

$$\tau = \eta\dot{\gamma} \quad (1)$$

Instead of being able to characterize the flow behavior with a simple viscosity constant, η, at least two parameters and more complex models, non-Newtonian, are needed.

A very widely used nontime dependent rheological model is

$$\tau = k\dot{\gamma}^n \quad (2)$$

The apparent viscosity, η_a, is calculated from the following expression:

$$\eta_a = \frac{\tau}{\dot{\gamma}} = k\dot{\gamma}^{n-1} \quad (3)$$

Parameters k and n are all that is required to characterize the fluid. 'k' is known as the consistency index and increases with increasing solids content. The flow behavior index 'n' is a measure of the departure from Newtonian flow. For n = 1 flow is Newtonian and $\eta_a = \eta = k$.

One of the most generalized mathematical models for nontime dependent fluid having a yield stress term is the Herschel-Bulkley model:

$$\tau = \tau_o + k\dot{\gamma}^n \quad (4)$$

The apparent viscosity, η_a, is again a function of shear rate and is determined by the ratio of shear stress to shear rate.

Most fundamental studies of rheological behavior involve determining all the parameters of a mathematical model such as those given in Eq. (1), (2) or (4); whereas other studies involve determining a parameter representing the consistency or apparent viscosity of the material. Holdsworth (1971), Bourne (1982) and Charm (1978) have described various instruments, cylindrical, cone and plate, and tube viscometer, which can be used to generate shear stress-shear rate data for absolute characterization of fluids.

Rao and Otoyapalomino (1974) used gravity flow tube viscometer for determining flow properties of tropical fruit purees. Charm (1978) determined parameters k, n and τ_o of Eq. (4) for orange juice concentrate by employing a single cylinder rotational viscometer. Steffe et al. (1983) compiled data from the literature with respect to parameters in Eq. (2) and (4) for fluid foods, but no information on legume pastes and slurries is included.

The Brookfield Viscometer has been used to determine fundamental flow properties of fluid foods such as fruit juices and purees (Holdsworth, 1971) but its most wide application has been for obtaining empirical flow properties such as apparent viscosity or some other index of viscosity (Bourne, 1982).

dePadua and Maroun (1984) studied rheological behavior of Venezuelan arepa dough prepared from precooked corn flour (40% precooked corn flour, 59.4% water and 0.6% salt). They measured apparent viscosity of 25% aqueous dispersion of arepa dough with a Brookfield Viscometer Model RVT equipped with a cylindrical spindle rotating at 5 rpm and found an increase in apparent viscosity with increased fraction of fine particles. A Brookfield Viscometer Model RVT and Model C Helipath Stand equipped with a T-C spindle turning at 2.5 rpm was employed by McWatters and Brantley (1982) to compare apparent viscosities of pastes prepared from whole soaked cowpeas and hydrated cowpea meal. Mayfield et al. (1978) employed an extension capillary viscometer to obtain apparent viscosity values of mechanically deboned poultry meat; however, it can be employed to obtain a wide range of shear stress – shear rate data for thick materials such as meat batters.

The purpose of this investigation was to characterize the rheological behavior of cowpea pastes and study the effect of hydration time and water level on apparent viscosity. Although cowpeas were used in this study, the

Authors Chhinnan and McWatters are with the Dept. of Food Science, Univ. of Georgia Agricultural Experiment Station, Experiment, GA 30212. Author Rao is with the Dept. of Food Science, Univ. of Georgia, Athens, GA 30602.

scope of this investigation is not limited to this particular legume. Paste made from whole seeds by the traditional method and that made from flour is subsequently referred to as traditional and experimental paste, respectively. Specific objectives of this study were to: (1) Obtain shear stress-shear rate data with an extrusion capillary viscometer for traditional and experimental cowpea pastes and use a power law model to describe the flow characteristics; (2) Determine the effect of hydration time and water level on apparent viscosity of experimental pastes using the extrusion capillary and Brookfield viscometers; and (3) Establish a possible correlation between apparent viscosities obtained from two different types of viscometers.

MATERIALS & METHODS

Cowpeas

Cowpeas (*Vigna unguiculata*), California blackeye cultivar, were used in the study. These were obtained from Dr. A.E. Hall (Univ. of California, Riverside) and stored at 1°C and 65% relative humidity until the time of use. They were removed from storage at least 4 hr prior to use to bring them to room temperature.

Flour and paste preparation

California blackeye peas (4.5 kg) were decorticated by abrasive action in a mini-PRL rollover dehuller (Nutana Machine Company Ltd., Saskatoon, Canada) equipped with resinoid disks (Hudda, 1983) and operated at 1,000 rpm for six min. Seeds were passed through an Alamco seed cleaner (Ames, Iowa) equipped with 3.2 mm screen and ground into flour in a Retsch microjet ultracentrifugal mill. Model ZM1 (Haan, West Germany), equipped with a 1.0 mm screen and operated at 10,000 rpm. Experimental paste was prepared by adding a known amount of water to 200g cowpea flour. Cowpea flour to water ratio of 1:1 was used for obtaining shear stress – shear rate profile in reference to objective #1 (conditions used by McWatters and Brantley, 1982). For all other tests, flour was hydrated by adjusting the moisture level (wet basis) to 54, 56, 58, 60 or 62%. The water/flour mixture was stirred gently with a spoon or spatula for 2 min, covered and held undisturbed for 0, 30, 60 or 180 min.

For preparing traditional paste, 200g dry cowpeas were soaked in 400 mL tap water at room temperature for 30 min. Loosened seed coats were removed manually, and cotyledons were washed and blotted dry. Two hundred thirty-two grams rehydrated decorticated peas were blended with 167 mL tap water for 5 min at speed 3 in an Oster Kitchen Center blender (Model 965-04F) to form a smooth paste.

All test samples were replicated twice.

Viscosity measurements

Extrusion capillary viscometer. The extrusion capillary cell designed and constructed by the Instrument Shop of the University of Georgia was used with the Instron Universal Testing Machine, Model 1122 or 1130 (Canton, MA). The cell consisted of a cylinder (jacketed for water circulation) and piston arrangement to which an orifice or capillary attachment was connected. Details of the device are given by Mayfield et al. (1978).

Water from a temperature-controlled water bath was circulated through the cylinder jacket in order to maintain a constant temperature of 23 ± 1°C in the test material and the cell throughout the experiment. For all viscosity measurements, paste (traditional or experimental) was whipped for 1.5 min in a N-50 Hobart mixer at speed 3 (McWatters and Brantley, 1982). The cylinder was filled completely with the whipped paste; extra care was exercised to avoid air-pockets. Force required to extrude the paste at a steady rate was meaasured at a constant cross-head speed with the capillary and the orifice attachment. Instron Model 1122 (located in the Department of Poultry Science, University of Georgia, Athens) was used in determining the shear stress-shear rate profile of the experimental (flour:water = 1:1, and zero hydration time) and traditional pastes at six cross-head speeds of 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 mm/min. Instron Model 1122 was chosen due to the ease of selecting cross-head speeds via push-button switches. This model could not be used for all of the extrusion capillary tests because of its unavailability for the entire period of the study. All other tests were conducted with Model 1130 (located in the Agricultural Engineering Department at this location); the tests involved only the experimental pastes (water levels of 54, 56, 58, 60 and 62%, and hydration time of 0, 30, 60 and 180 min). Both Instron models were equipped with 50 ± 0.05 kg capacity load cells. The Instron Model 1130 was operated at the minimum cross-head speed of 30 mm/min.

Brookfield viscometer. Paste viscosity was measured at room temperature, 23 ± 1°C, with a Brookfield Viscometer Model RVT and Model C Helipath Stand equipped with a T-B spindle rotating at 5 rpm. Measurements were made on whipped experimental pastes corresponding to five water levels and three hydration times as described earlier. At least four readings were taken per test sample.

Analysis of data

Extrusion Capillary Viscometer. The generalized power law mathematical model, Herschel-Bulkley model, for non-Newtonian fluids was assumed [Eq. (4)]. Estimation of parameters, τ_o, k and n is discussed below.

The shear stress, τ, was calculated as,

$$\tau = \frac{\Delta p R}{2L} \tag{5}$$

where, ΔP is the pressure drop across the capillary which was calculated from

$$\Delta P = \frac{F_c - F_o}{A} \tag{6}$$

The shear rate $\dot{\gamma}$ was calculated from Rabinowitz's equation (Charm, 1978)

$$\dot{\gamma} = \frac{V}{R} 3 + \frac{d(\ln \bar{V}/2R)}{d[\ln(\tau - \tau_o)]} \tag{7}$$

where,

$$\bar{V} = Q/4\pi R^2 \tag{8}$$

Eq. (7) can be rewritten as

$$\dot{\gamma} = Q\,(3 + b)/\pi R^3 \tag{9}$$

where,

$$b = \frac{d\,(\ln \bar{V}/2R)}{d\,[\ln(\tau - \tau_o)]} = \frac{d\,(\ln Q/2R^3)}{d\,[\ln(\tau - \tau_o)]} \tag{10}$$

The volumetric flow rate, Q, was calculated from the following expression

$$Q = s \times A \tag{11}$$

The parameter b in Eq. (9) is also referred to as a correction factor; its value equals unity for Newtonian fluids. As seen from Eq. (10), b is a derivative of $\ln(Q/2R^3)$ with respect to $\ln(\tau - \tau_o)$; thus, it was estimated as the slope of linear regression of $\ln(Q/2R^3)$ on $\ln(\tau - \tau_o)$. Prior to performing the regression analysis, the presence of yield stress, τ_o, was examined by plotting τ versus $4Q/\pi R^3$; if no yield stress existed, τ_o was taken equal to zero. $4Q/\pi R^3$ is also known as uncorrected shear rate. It may be noted that for Newtonian fluids, b = 1, which yields $\dot{\gamma} = 4Q/\pi R^3$ in Eq. (9).

Logarithmic transformation of Eq. (4) yields

$$\ln(\tau - \tau_o) = \ln(k) + n \ln(\dot{\gamma}) \tag{12}$$

After obtaining the shear stress and shear rates associated with a test, linear regression of $\ln(\tau - \tau_o)$ and $\ln(\dot{\gamma})$ was performed. The intercept and slope values thus obtained estimated ln(k) and n, respectively.

Apparently viscosity at a given extrusion rate was calcualted by the following formula:

$$c\eta_a = \frac{\tau}{\dot{\gamma}} \tag{13}$$

$\dot{\gamma}$ was calculated from Eq. (9) and τ was either τ_{obs} or τ_{pred}. τ_{obs} refers to an observed shear stress value as computed from Eq. (5) and τ_{pred} refers to a predicted value as obtained from power law model [Eq. (2) or (4)].

Brookfield Viscometer. The dial readings (range 0 – 100) of the viscometer were converted to apparent viscosity, $B\eta_a$, values in Pa ∘ s by multiplying by a factor of 0.8. The conversion factor

was determined from information supplied by the manufacturer for a T-B spindle connected to RVT model rotating at 5 rpm.

Statistical analysis. Appropriate linear regression analyses of the extrusion capillary and Brookfield Viscometer data were performed using GLM procedures of SAS, Statistical Analysis System (Helwig and Council, 1979). Correlation co-efficient of extrusion capillary apparent viscosity, ${}^{c}\eta_a$, and Brookfield apparent viscosity, ${}^{B}\eta_a$, was also determined by a SAS procedure. Significant differences between mean apparent viscosities, ${}^{c}\eta_a$ and ${}^{B}\eta_a$, for various water level and hydration time treatments were determined by Student-ized Range, Q, method as outlined by Snedecor and Cochran (1967). Significance level of 5% was used for all tests.

RESULTS & DISCUSSION

Shear stress-shear rate profile

The first step in characterizing the flow properties of experimental and traditional pastes using the capillary extrusion device was to examine the presence of yield stress, τ_o. Shear stress, τ_{obs}, and uncorrected shear rate, $4Q/\pi R^3$, plots did not provide a definite indication of presence or absence of yield stress, τ_o (Fig. 1). It was decided to analyze the data both for $\tau_o = 0$ and $\tau_o = 0$; for $\tau_o = 0$, τ_o was taken as 118.9 and 69.9 Pa for the experimental and the traditional paste, respectively (these correspond to the minimum shear stress values in Fig. 1). The estimated values of parameters, k (fluid consistency index) and n (flow behavior index), for the experimental and traditional paste are given in Table 1. Root mean square errors, RMSE, were computed for two models [Eq. (2) and (4)] and two types of pastes using the following formula (Table 1).

$$RMSE = \left[\frac{1}{m}\Sigma(\tau_{obs} - \tau_{pred})^2\right]^{1/2} \quad (14)$$

RMSE provides an indication of goodness of fit of a model; a smaller value means a better fit. RMSE values for models assuming no yield stress were lower than the models with finite yield stress, thus, indicating a preference for the two parameter model [Eq. (2)] over the three parameter model [Eq. (4)] for both types of pastes. Plots of observed and predicted shear stress against shear rate, for both the traditional and experimental pastes, illustrate a satisfactory fit provided by the power law model, $\tau = k\dot{\gamma}^n$ (Fig. 2).

Examination of parameters k_1 and n_1 in Table 1 indicates that the two types of pastes exhibit similarities in flow characteristics. In fact, flow behavior index, n_1, values were found to be almost equal for both types of pastes (0.456 and 0.458 for experimental and traditional, respectively). Consistency of the experimental paste was greater than the traditional paste as indicated by k_1 (fluid consistency index) values in Table 1. Moisture content of the experimental and the traditional paste was 55% and 59%, respectively. It is believed that changing the solid-water ratio of the experimental paste will not significantly alter the n_1 value but will affect the k_1 value (Holdsworth, 1971). Thus, it should be possible to lower the k_1 value of experimental paste by increasing its moisture content resulting in equal consistency index, k_1, values for experimental and traditional pastes.

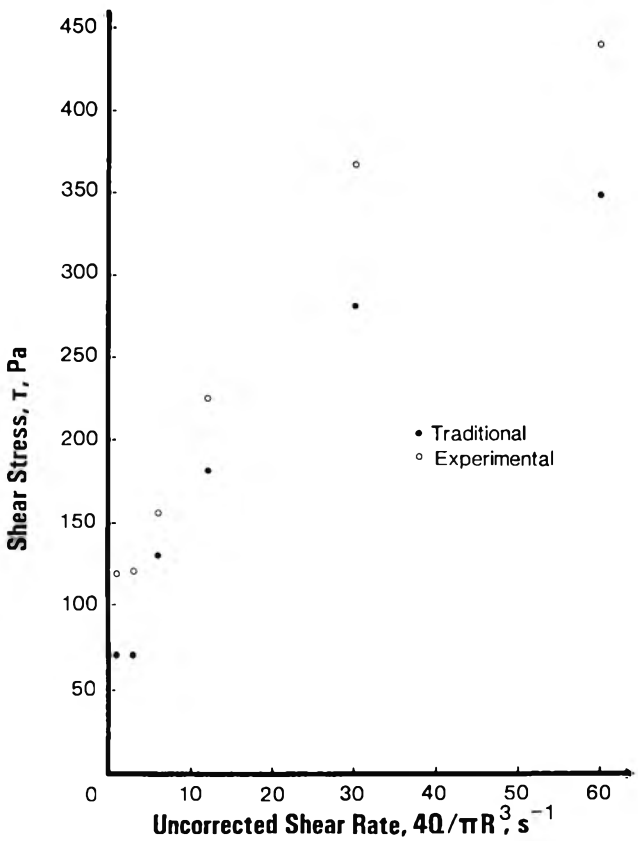


Fig. 1—Shear stress and uncorrected shear rate data for traditional and experimental cowpea pastes pertinent to extrusion capillary viscometer.

Apparent viscosity of experimental paste

Apparent viscosities of experimental paste for various hydration times and water levels as obtained by the extrusion capillary and Brookfield methods are presented in Tables 2 and 3. Water level was observed to have a considerably greater affect on ${}^{c}\eta_a$ (apparent viscosity determined by the extrusion capillary method) and ${}^{B}\eta_a$ (apparent viscosity determined by the Brookfield method) than hydration time.

The apparent viscosity values, ${}^{c}\eta_a$, presented in Table 2 were computed from Eq. (13) employing τ_{obs} values. The shear rate value, $\dot{\gamma} = 152.16\ s^{-1}$, was calculated from Eq. (9) using a cross-head speed, s, of 30 mm/min. The correction factor, b, was taken as 0.376 from Table 1.

The Brookfield Viscometer readings associated with the 54% water level and 0, 30, 60 min hydration times were off-scale; as a result, corresponding ${}^{B}\eta_a$ values are not included in Table 3. Selecting a different T-spindle and/or

Table 1—Rheological parameters of the experimental and traditional cowpea pastes

Model	Paste type	Model parameters				RMSE[a]
		τ_o	b	k	n	
$\tau = k_1\dot{\gamma}^{n_1}$	Experimental	–	0.376	77.45	0.456	22.9
[Based on Eq. (2)]	Traditional	–	0.458	59.02	0.458	14.4
$\tau = \tau_o + k_2\dot{\gamma}^{n_2}$	Experimental	118.9	0.949	8.11	0.949	25.4
[Based on Eq. (4)]	Traditional	69.9	0.669	20.80	0.669	23.8

[a] Root mean square error computed from Eq. (14).

rotation speed allowed measurements to be taken on paste at the 54% water level, but it was imperative to have the same spindle and rpm combination throughout the study (Cooley et al., 1953). The T-B spindle rotating at 5 rpm was found to be the best suitable combination.

The main reasons various hydration times were considered in this study was that commercial flour producers, particularly in Nigeria, have no general consensus on hydration time requirements for preparing paste from flour. From our study we found that if apparent viscosity of the paste is considered as an indicator of its performance in the preparation of the final product, then the hydration time should have relatively little affect. In fact, for water levels ranging between 56 - 62%, the hydration time had no significant affect (5% level) on ${}^{c}\eta_a$ (Table 2). According to the Brookfield measurements, only one hydration time (180 min) significantly affected the apparent viscosity ${}^{B}\eta_a$.

The parameters in Table 1 for the experimental paste are based on 55% water level and zero hydration time. The experimental paste shear stress value estimated as τ_{pred} = 766.2 Pa from Eq. (2) in Table 1 and $\dot{\gamma}$ = 152.16 s^{-1} (computation of which is described earlier) yielded ${}^{c}\eta_a$ = 5.03 Pa.s. Table 2 indicates that the water level of 55% should yield apparent viscosity much greater than 5.03 Pa.s. This discrepancy can be attributed to the fact that parameters given in Table 1 correspond to experimental data for cross-head speeds between 0.2 - 10 mm/min. Values predicted for cross-head speeds outside the test range can deviate considerably from true values.

McWatters and Brantley (1982) used an RVT Brookfield viscometer to measure viscosity of experimental pastes prepared from Dixiecream cowpea meal using meal to water ratios of 1:1 (55% moisture content) and 10:11 (59% moisture content). The observations were presented in terms of dial readings of the instrument. Those dial readings were converted by the present authors into apparent viscosity values at 284 and 206 Pa.s corresponding to paste moisture content of 55% and 59%, respectively. Reduction in apparent viscosity with increase of paste moisture content is noted; however, the magnitude of the apparent viscosity values from McWatters and Brantley's data cannot be directly compared with those in Table 3 because of the different T-spindles and rpm used by the authors of the two studies.

Correlation coefficient, r, of apparent viscosities obtained from the capillary extrusion and Brookfield viscometers for experimental pastes corresponding to various hydration times and water levels (Tables 2 and 3) was 0.953 (significant at 1% level). Apparent viscosity determined by the capillary method has a fundamental basis (Rao and Otoyapalomino, 1974) whereas that determined by the Brookfield method using the T-spindle is of empirical nature. It is faster and easier to measure ${}^{B}\eta_a$ than ${}^{c}\eta_a$; therefore, a good correlation between ${}^{B}\eta_a$ and ${}^{c}\eta_a$ suggests that ${}^{B}\eta_a$ values are equally meaningful as ${}^{c}\eta_a$.

Table 2—Apparent viscosity (Pa.s), c_{η_a}, of the experimental paste determined by the extrusion capillary method at various hydration times and water levels of cowpea meal[d]

Hydration time, min	Water level, %				
	54	56	58	60	62
0	12.78^{ab}	8.10^{a}	5.33^{a}	4.38^{a}	2.58^{a}
15	11.92^{bc}	8.12^{a}	5.46^{a}	4.71^{a}	3.02^{a}
30	13.94^{a}	8.06^{a}	6.26^{a}	4.35^{a}	3.11^{a}
60	11.90^{bc}	7.65^{a}	5.37^{a}	4.53^{a}	2.31^{a}
180	10.83^{c}	7.57^{a}	5.57^{a}	3.95^{a}	3.49^{a}

[a-c] Values having different letters within a column are significantly different at $P \leq 0.05$.
[d] Estimated shear rate = 152.16 s^{-1}

SUMMARY & CONCLUSIONS

COWPEAS were chosen as a model grain legume in this investigation. Two types of cowpea pastes, traditional (prepared from whole, soaked peas) and experimental (prepared from hydrated cowpea flour) were used in obtaining shear stress-shear rate data with an extrusion capillary viscometer. The data fitted to a two parameter (flow behavior index and fluid consistency index) power

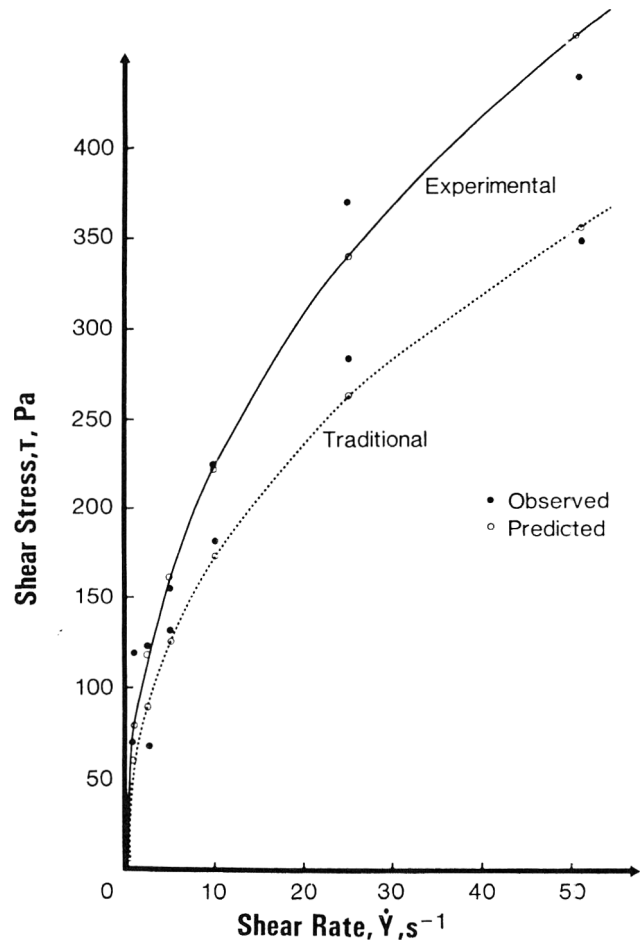


Fig. 2—Plots of observed and predicted shear stress and shear rate data for traditional and experimental cowpea pastes pertinent to extrusion capillary viscometer.

Table 3—Apparent viscosity (Pa.s), B_{η_a}, of the experimental paste determined by the Brookfield method at various hydration times and water levels of cowpea meal[c]

Hydration time, min	Water level, %				
	54	56	58	60	62
0	—[d]	61.00^{a}	46.64^{a}	32.72^{a}	19.68^{a}
15	76.16^{a}	56.64^{ab}	44.16^{ab}	33.04^{a}	20.92^{a}
30	—	56.88^{ab}	42.08^{ab}	28.72^{ab}	18.16^{a}
60		56.32^{ab}	44.40^{ab}	30.24^{ab}	18.20^{a}
180	74.20^{a}	52.32^{b}	39.63^{b}	27.56^{b}	16.00^{a}

[a-b] Values having different letters within a column are significantly different at $P \leq 0.05$.
[c] Values obtained with T-B spindle connected to RVT model of Brookfield viscometer rotating at 5 rpm.
[d] Instrument readings off-scale.

law model adequately characterized the flow behavior within the tested shear rate range. Consistency index values were different for the two pastes; however, the flow behavior index values were almost equal indicating similar flow characteristics.

Effects of hydration time and water level on the apparent viscosities of experimental pastes were determined. Apparent viscosities were determined by a Brookfield Viscometer, a commonly used instrument both in quality control and research applications, and the extrusion capillary viscometer. It was found that water level had a much greater affect on apparent viscosity than hydration time. An excellent correlation between the Brookfield and capillary extrusion viscometer data indicated that the Brookfield viscometer can be used equally effectively as the extrusion capillary viscometer in determining apparent viscosity of legume pastes.

NOMENCLATURE

General

A = cross-section area of piston, m^2
b = correction factor, Eq. (10)
F = force required to extrude material through the capillary or orifice, N
k = fluid consistency index
L = length of capillary, m
m = number of observations, Eq. (14)
n = flow behavior index
Q = volumetric flow rate, m^3/s
R = capillary or orifice radius, m
s = cross-head speed, m/s
V = average flow rate, m/s

Δp = pressure drop across capillary, Pa
$\dot{\gamma}$ = shear rate, s^{-1}
η = viscosity, Pa.s
τ = shear stress, Pa
τ_o = yield stress, Pa

Subscripts and superscripts

1 = parameters in Eq. (2), see Table 1
2 = parameters in Eq. (4), see Table 1
a = apparent
B = Brookfield
c = capillary or extrusion capillary
o = orifice
obs = observed
pred= predicted

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A Research Note

Effect of Carbon Dioxide Modified Atmosphere on the Compressibility of Stored Baked Goods

DIETRICH KNORR and RICHARD I. TOMLINS

ABSTRACT

Whole wheat flour breads, white breads and biscuits were stored in flexible packages flushed with either air, 100% nitrogen or 100% carbon dioxide. Compressibility tests were performed during a storage time of 13 - 15 days for bread and 7 - 15 days for biscuits. Significantly lower regression coefficients ($P < 0.01$) were found for white and whole wheat breads and biscuits stored in carbon dioxide modified atmosphere than for the air stored samples. No significant differences were found between regression coefficients of air and nitrogen stored bread samples. Mold growth was inhibited on breads and biscuits by carbon dioxide atmosphere during 13 - 15 days of storage, but occured between the 5th and 7th day of storage on the air packaged samples.

INTRODUCTION

CONTROLLED and modified atmosphere storage using gases such as carbon dioxide, nitrogen, carbon monoxide, ozone, ethylene oxide, sulphur dioxide and propylene oxide or combinations thereof have been successfully used to extend the shelf life of foods (ICMSF, 1980; Jay, 1978), such as fruits and vegetables (Banwart, 1981; Kader, 1980); meat, poultry and fish (Finne, 1982, Gray et al., 1984; Banks et al., 1980; Christopher et al., 1980a, b; Hanna et al., 1981; Bailey et al., 1979) and for bakery items (Cerny, 1979; Brummer et al., 1980; Ooraikul, 1982; Seiler, 1983).

Most of the work carried out with baked goods dealt with the use of carbon dioxide and nitrogen, where carbon dioxide was shown to be a more effective inhibitor of mold growth at higher residual concentrations than nitrogen (Cerny, 1979). Several studies have been conducted to compare the effect of different packaging materials on the shelf life extension of baked goods stored in carbon dioxide modified atmospheres (Seiler, 1983; Brummer et al., 1980, Feldheim and Metschulat, 1979). Seiler (1984) stated recently that "it is now generally accepted that carbon dioxide is an excellent preservative for bakery products in that it can give long increases in the shelf life of all parts without affecting flavour or appearance." Although there have been several attempts to explain the antimicrobial action of carbon dioxide (King and Nagel, 1975; Enfors and Molin, 1981), its action is still not completely understood. The use of carbon dioxide-modified atmosphere has been shown (Bogadtke, 1979; Cerny, 1979; Brummer et al., 1980; Ooraikul, 1982; Seiler, 1983) to inhibit mold growth on baked goods beyond a shelf life at which excessive staling is expected.

Since the storage of breads in elevated CO_2 concentrations substantially improves the microbiological shelf life, it was of interest to determine whether the firming of the bread crumb would in any way be affected. This is of relevance since breads stored for extended periods in CO_2 atmospheres may be rejected by the consumer on the basis of the physicochemical changes to the crumb (Kulp and Ponte, 1981). The measurement of crumb firmness is now used as an acceptable indicator of the rate of staling of bread (Pomeranz and Shellenberger, 1971; Dahle and Montgomery, 1978; Roewe et al., 1982). Consequently this work attempted to examine the effect of CO_2-modified atmospheres on the compressibility of stored soft baked goods.

Author Knorr is with the Dept. of Food Science & Human Nutrition, Univ. of Delaware, Newark, DE, 19716. Author Tomlins, formerly with the Food Science Unit. Oxford, Polytechnic, Oxford OX30BP, is now with BOC Ltd., Deb Park Road, London SW19 3UF, England.

MATERIALS & METHODS

Bread

Whole wheat and white bread was made by the "no time" procedure at a commercial bakery. The baking formula was: 1 kg flour (70% Canadian hard wheat; 30% English soft wheat); 48g yeast; 12g salt; 16g fat and 640g water. No improvers or preservatives were added. Prepared doughs were fermented for 45 min at 27°C. Molded dough (500g) was placed in loaf tins and fermented for 30 min at 27°C. The loaves were baked at 230°C for 35 min, allowed to cool under ambient conditions and then sliced into 9 mm slices.

Biscuits

Forty grams "quick drop" biscuits were prepared by using: 1 kg flour (unbleached); 12g salt; 18g baking powder (full strength, double acting; Davis, RJR Foods Inc., Winston-Salem, NC); 12g shortening (Crisco all vegetable shortening; Procter & Gamble, Cincinnati, OH); and 580g milk. The biscuits were baked in muffin tins at 230°C for 16 min.

Packaging

Bread slices of 9 mm thickness were packaged in 20 x 30 cm nylon pouches (coated with PVDC/polyethylene laminate; Transparent Paper p.l.c., Lancashire, England) and biscuits were packaged in 20 x 30 cm Nylon/Surlyn pouches (International Paper Co., New York, NY). The pouches were evacuated and gas flushed with either air, 100% nitrogen or 100% carbon dioxide and then heat sealed (Multivac 300/22 Model, Wolfertschwenden, Germany; Statovac Vacuum Packer Model ST-1, Woodward Research Co., Inc., Rochelle Park, NJ). Samples were stored at 19°C ± 1°C.

Microbiological analysis

Bread samples that showed visible signs of mold when sampled for compressibility were analyzed as follows: A portion of the bread showing mold growth was excised aseptically, placed on Malt Extract Agar (Oxoid Ltd., Basingstoke, England) and incubated at 25°C for 7 days. Slide cultures of the molds were prepared and the genera present were identified by their spore structures. Biscuit samples were analyzed for visible mold growth.

Compressibility tests

Three to four samples of each variable were tested in 24- or 48-hr intervals immediately after removing from the pouches. Compressibility tests were carried out using a Universal Testing Machine (Model 1140, Instron Corp, Canton, MA) at a crosshead speed of 50 mm · mm^{-1}. Bread slices were compressed to 45% of initial thickness using a compression anvil of 36 mm diameter. The top of each biscuit was cut off and the biscuits were compressed between two uniaxial plates to 50% of initial thickness (35 mm).

Statistical analyses

Regression analyses were carried out using the SPSS package and comparison of regression coefficients was obtained after Sachs (1974).

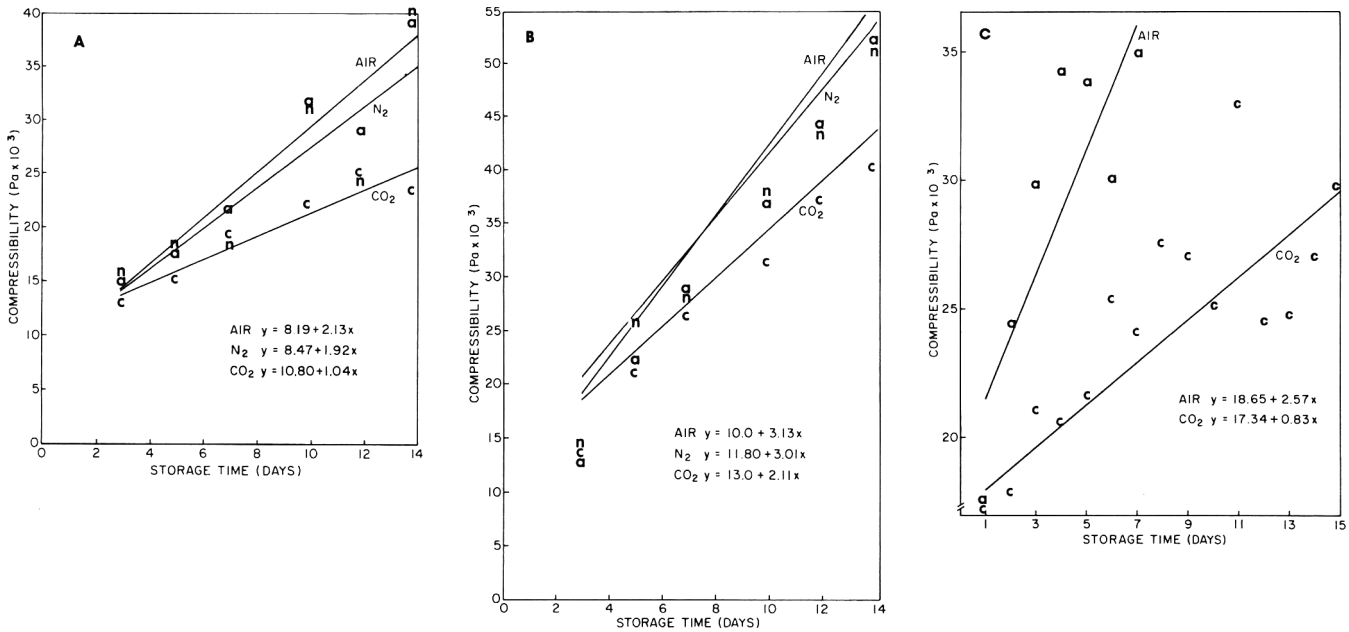


Fig. 1—Mean values, regression lines and regression equations of compressibility of white bread (A), whole wheat bread (B), and biscuits (C) stored in air (a), nitrogen (b), or carbon dioxide (c) atmosphere for 7 - 15 days at 20 ± 20°C.

Table 1—Comparison of regression coefficients of compressibility data of baked goods stored in air, nitrogen or carbon dioxide atmosphere

Baked goods	Comparison	Storage time (days)	d.f.	t-value
White Bread	Air vs N_2	14	32	0.13 ns
	Air vs CO_2	14	34	13.75*
	N_2 vs CO_2	14	36	11.36*
Whole Wheat Bread	Air vs N_2	13	34	3.81*
	Air vs CO_2	13	37	13.85*
	N_2 vs CO_2	13	41	10.31*
Biscuit	Air vs CO_2	7	38	10.63*

ns Nonsignificant difference ($P > 0.01$)
* Significant difference ($P < 0.001$)

RESULTS & DISCUSSION

COMPRESSIBILITY DATA for white bread and whole wheat bread slices as well as for biscuits stored in air, nitrogen and carbon dioxide for 5 to 15 days are given in Fig. 1. Comparison of regression coefficients for white bread (Fig. 1A) revealed nonsignificant differences ($P > 0.01$) in compressibility between air and nitrogen modified atmosphere samples. The regression coefficient for the samples stored in carbon dioxide modified atmospheres was found to be significantly lower ($P < 0.01$) than for the air stored samples. Mold growth occurred on the air samples on the fifth day of storage resulting in a slight surface discoloration while no mold growth was observed on the nitrogen and carbon dioxide samples during the 13 days of experiments.

Regression coefficients for whole wheat bread (Fig. 1B) were larger than the ones obtained for white bread indicating a product dependence of the modified atmosphere on compressibility of bread. The same effect of carbon dioxide-controlled atmosphere on the compressibility of whole wheat bread could be found as with white bread samples resulting in a significantly ($P < 0.01$) lower regression coefficient as compared to air or nitrogen samples (Table 1). Nonsignificant differences ($P > 0.01$) were found between regression coefficients of air and nitrogen samples. Growth of *Penicillium* and *Cladosporium* species was observed at the sixth day of storage on whole wheat bread slices stored in air as well as in nitrogen. No mold growth was found on the samples stored in carbon dioxide-modified atmospheres until termination of the experiments. A significantly lower ($P < 0.001$) regression coefficient was identified for biscuit samples stored for 7 days in carbon dioxide-modified atmosphere than the ones for air stored samples (Fig. 1C, Table 1). Visible mold growth occurred on the air packaged samples on the seventh day of storage, but remained absent on the carbon dioxide packaged samples throughout the 15 days of storage.

The microbiological data imply occurrence of a critical oxygen concentration for the aerobic molds on the whole wheat breads at an earlier stage of storage than on the white breads and/or a reduced inhibitory effect of nitrogen per se in the case of whole wheat bread. A critical oxygen concentration of about 6% has been reported for various bread types (Anon 1979). Ooraikul (1982) showed a decrease in nitrogen atmosphere from 100% to 31.5% during 7 days of storage of crumpets packaged in polyethylene coated nylon film and a decrease in carbon dioxide atmosphere from 100% to only 87% during 14 days of storage.

Our data confirm that carbon dioxide is an effective preservative for bakery products (Cerny, 1979; Seiler, 1983) and indicate that it also delays the firming of bread and biscuits. The manner by which carbon dioxide exerts an effect on the firming of stored baked goods is still unknown. But with evidence accumulating which indicates that compounds such as carbonic acid and ethanol also alter the rate of firming (Russell, 1983) it is expected that increased attention will be given to the study of aging of soft baked goods.

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A Research Note

B Vitamins in Selected Variety Breads Commercially Produced in Major U.S. Cities

GUR RANHOTRA, JANETTE GELROTH, FRANCES NOVAK, and RUTH MATTHEWS

ABSTRACT

Three enrichment vitamins (thiamin, riboflavin, niacin) and three other (folic acid, pantothenic acid, vitamin B_6) B vitamins were determined in 10 types, up to four brands of each type, of variety breads (including tortillas) purchased from four major U.S. cities. In general, bread types traditionally enriched and not containing nonflour components as significant ingredients met the vitamin enrichment standards. In many products, the contents of all six vitamins varied appreciably from brand to brand. In contrast to wholewheat bread and some enriched breads, corn tortillas were consistently low in all vitamins except vitamin B_6.

INTRODUCTION

THE CONSUMPTION of variety (specialty) breads in the U.S. has been increasing steadily in recent years (Ranhotra and Winterringer, 1981). Eating quality and nutritional considerations/perceptions appear to have influenced this trend. Studies were initiated in the fall of 1982 to widen the base on which nutritional perceptions of variety breads can be substantiated. This encompassed developing detailed compositional information on 10 types of popular variety breads (up to four brands of each type) which were obtained from four major U.S. cities. Earlier we reported on the content of proximate components (Ranhotra et al., 1984) and minerals (Ranhotra et al., 1985) in these products. This paper summarizes information on the content of six B vitamins in the same products.

MATERIALS & METHODS

Products and processing

All relevant information on the 129 bread products, including wholewheat (100%) bread and corn tortillas, was detailed earlier (Ranhotra et al., 1985). For cities other than Chicago, all brands of a product were composited for analysis; for Chicago, the various brands were analyzed individually.

Analytical

Product samples were analyzed in triplicate for each of the vitamins reported. Thiamin and riboflavin were determined fluorometrically and niacin by the cyanogen bromide method using the standard AACC methods (1977). The other three vitamins were determined microbiologically using *Lactobacillus casei* (folic acid), *Lactobacillus plantarum* (pantothenic acid), and *Saccharomyces carlsbergenis* (vitamin B_6) as the test organisms. Various published methods were tested and modified to provide reproducible results. Folic acid was determined by autoclaving samples in phosphate buffer (pH, 6.1) followed by conjugase (chick pancreas conjugase) treatment. Assay and standard series were prepared, inoculated, incubated (18 hr) and the turbidity read spectrophotometrically at 545 nm. Samples for pantothenic acid determinations were autoclaved with Tris buffer (pH, 8.3), centrifuged, and aliquots treated with alkaline phosphatase and pigeon liver conjugase (Sigma Chem. Co., St. Louis, MO). The solutions were diluted, filtered, and an assay series made. After inoculation and incubation (18 hr), transmittance was read at 620 nm. Samples for vitamin B_6 (total) were autoclaved in 0.44N HCl (1 hr), centrifuged, and an aliquot diluted to volume. The pH was adjusted to 6.5 and an assay series prepared, steamed, inoculated, incubated (40 hr), and transmittance read at 620 nm. Culture media for all determinations and chick pancreas conjugase were obtained from Difco Labs. (Detroit, MI).

Authors Ranhotra, Gelroth, and Novak are affiliated with the Nutrition Research Group, American Institute of Baking, Manhattan, KS 66502. Author Matthews is with CND/HNIS/USDA, Hyattsville, MD 20782.

RESULTS & DISCUSSION

THE CONTENT of three enrichment and three additional B vitamins is listed in Tables 1 and 2, respectively. As identified earlier (Ranhotra et al., 1985), only some of the products from Chicago were enriched products (brands analyzed individually). In contrast, all brands of a traditionally enriched product (this excludes wholewheat bread and tortillas) from the other three cities were, in fact, enriched.

Enrichment vitamins

According to the standards of identity (CFR, 1980), white and related variety breads should contain, on a 100-g product basis, 0.40, 0.24, and 3.30 mg thiamin, riboflavin, and niacin, respectively. Average vitamin values (Table 1) for oatmeal, cracked wheat, Italian, French, and pita breads approached or matched these standards. Exceptions were noted for the two Italian breads from Washington. Exceptions were also noted for certain products from Chicago primarily because not all products purchased from Chicago were enriched products. In most cases, the average content of the enrichment vitamins in pumpernickel, raisin, and mixed grain breads failed to meet enrichment standards primarily because the enriched flour used was obviously diluted when ingredients such as rye meal, raisins, and nonwheat grains were used. The content of thiamin and riboflavin in wholewheat bread was low compared to other products except tortillas; in tortillas, the content was quite low because of losses during processing (Ranhotra and Bock, in press). Brand-to-brand differences in the content of thiamin and riboflavin (also niacin) in the two aforementioned products – wholewheat bread and tortillas – were minimal. This contrasts with such differences which were observed in the other eight bread types.

Other vitamins

B vitamins naturally occurring in cereal grains tend to be high in the wholemeal and in the nonendospermic fractions of these grains. Thus, wholewheat and other less-refined bread products (mixed grain and cracked wheat bread, for example) tended to be a better source of these other three vitamins (not enrichment vitamins) than the refined products (Table 2). Apparently because of losses during processing (high pH, for example), tortillas were quite low in two (folic acid and pantothenic acid) of these three vitamins; the third vitamin, namely vitamin B_6, averaged the highest in tortillas, however. Some brand-to-brand and city-to-city differences are seen in the content of these vitamins in products analyzed, but, in general, such differences tend to be of a lesser magnitude than is the case for the enrichment vitamins. One exception to this

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Table 1—Enrichment vitamins in bread (product as-purchased basis)

Vitamin	Bread	City				
		Los Angeles	Dallas	Washington	Chicago[a]	All cities[b]
Thiamin (mg/100g)	Pumpernickel	0.42	0.28	0.29	0.17 ± 0.05	0.29 ± 0.10
	Raisin	0.23	0.33	0.32	0.29 ± 0.08	0.29 ± 0.05
	Oatmeal	0.38	0.35	0.39	0.26 ± 0.11	0.35 ± 0.06
	Wholewheat	0.23	0.17	0.22	0.26 ± 0.13	0.22 ± 0.04
	Cracked Wheat	0.54	0.32	0.34	0.30 ± 0.15	0.38 ± 0.11
	Mixed grain	0.28	0.35	0.26	0.19 ± 0.03	0.27 ± 0.07
	Italian	0.39	0.51	0.25	0.21 ± 0.13	0.34 ± 0.14
	French	0.44	0.41	0.36	0.35 ± 0.23	0.39 ± 0.04
	Pita	0.57	0.48	0.53	0.28 ± 0.04	0.47 ± 0.13
	Tortillas	0.08	0.06	0.10	0.09 ± 0.06	0.08 ± 0.02
Riboflavin (mg/100g)	Pumpernickel	0.53	0.22	0.30	0.26 ± 0.18	0.33 ± 0.14
	Raisin	0.19	0.24	0.23	0.24 ± 0.04	0.23 ± 0.02
	Oatmeal	0.25	0.22	0.23	0.23 ± 0.02	0.23 ± 0.01
	Wholewheat	0.17	0.12	0.13	0.18 ± 0.11	0.15 ± 0.03
	Cracked Wheat	0.28	0.22	0.19	0.22 ± 0.10	0.23 ± 0.04
	Mixed grain	0.28	0.25	0.25	0.16 ± 0.04	0.24 ± 0.05
	Italian	0.28	0.29	0.18	0.15 ± 0.09	0.23 ± 0.07
	French	0.32	0.25	0.24	0.19 ± 0.13	0.24 ± 0.08
	Pita	0.36	0.25	0.29	0.10 ± 0.02	0.25 ± 0.11
	Tortillas	0.05	0.04	0.04	0.05 ± 0.01	0.05 ± 0.01
Niacin (mg/100g)	Pumpernickel	4.70	3.03	4.02	2.60 ± 0.28	3.59 ± 0.95
	Raisin	2.32	2.67	2.47	2.28 ± 0.36	2.44 ± 0.18
	Oatmeal	3.54	2.59	3.08	3.19 ± 0.24	3.10 ± 0.39
	Wholewheat	4.27	4.40	4.18	4.66 ± 0.98	4.38 ± 0.21
	Cracked Wheat	4.53	3.59	4.10	4.63 ± 1.77	4.21 ± 0.47
	Mixed grain	4.33	4.73	4.70	3.60 ± 0.29	4.34 ± 0.53
	Italian	4.82	5.98	3.05	2.89 ± 1.47	4.19 ± 1.48
	French	4.94	4.64	4.63	3.38 ± 1.81	4.40 ± 0.69
	Pita	6.58	3.57	4.09	2.27 ± 0.16	4.13 ± 1.81
	Tortillas	2.21	2.34	1.97	1.92 ± 0.30	2.11 ± 0.20

[a] Values are average of up to four brands ± SD
[b] Values are grand average of four-city averages ± SD

Table 2—Other B vitamins in bread (product as-purchased basis)

Vitamin	Bread	City				
		Los Angeles	Dallas	Washington	Chicago[a]	All cities[b]
Folic acid (μg/100g)	Pumpernickel	36	37	30	40 ± 2	36 ± 4
	Raisin	35	37	36	35 ± 1	36 ± 1
	Oatmeal	28	36	30	31 ± 2	31 ± 3
	Wholewheat	53	55	59	48 ± 7	54 ± 5
	Cracked Wheat	37	47	39	50 ± 5	43 ± 6
	Mixed grain	47	59	40	60 ± 8	52 ± 10
	Italian	36	41	21	32 ± 3	33 ± 9
	French	35	38	15	35 ± 2	31 ± 11
	Pita	12	30	20	17 ± 1	20 ± 8
	Tortillas	6	17	20	18 ± 1	15 ± 6
Pantothentic acid (mg/100g)	Pumpernickel	0.341	0.326	0.369	0.456 ± 0.049	0.373 ± 0.058
	Raisin	0.358	0.331	0.310	0.351 ± 0.015	0.338 ± 0.022
	Oatmeal	0.396	0.306	0.377	0.367 ± 0.024	0.362 ± 0.039
	Wholewheat	0.670	0.613	0.658	0.648 ± 0.042	0.647 ± 0.025
	Cracked Wheat	0.532	0.476	0.604	0.606 ± 0.030	0.555 ± 0.063
	Mixed grain	0.448	0.435	0.616	0.603 ± 0.022	0.526 ± 0.097
	Italian	0.370	0.392	0.384	0.418 ± 0.034	0.391 ± 0.020
	French	0.437	0.396	0.360	0.465 ± 0.048	0.415 ± 0.046
	Pita	0.463	0.445	0.357	0.439 ± 0.052	0.426 ± 0.047
	Tortillas	0.189	0.116	0.186	0.161 ± 0.022	0.163 ± 0.034
Vitamin B_6 (mg/100g)	Pumpernickel	0.185	0.188	0.220	0.108 ± 0.024	0.175 ± 0.048
	Raisin	0.050	0.045	0.045	0.074 ± 0.011	0.054 ± 0.014
	Oatmeal	0.051	0.038	0.040	0.052 ± 0.021	0.045 ± 0.007
	Wholewheat	0.193	0.181	0.163	0.178 ± 0.011	0.179 ± 0.012
	Cracked Wheat	0.096	0.097	0.095	0.102 ± 0.002	0.098 ± 0.003
	Mixed grain	0.108	0.134	0.125	0.170 ± 0.056	0.134 ± 0.026
	Italian	0.050	0.044	0.039	0.049 ± 0.007	0.046 ± 0.005
	French	0.064	0.037	0.044	0.045 ± 0.009	0.048 ± 0.012
	Pita	0.032	0.015	0.015	0.028 ± 0.009	0.023 ± 0.009
	Tortillas	0.290	0.231	0.271	0.286 ± 0.028	0.270 ± 0.027

[a] Values are average of up to four brands ± SD
[b] Values are grand average of four-city averages ± SD

appears to be the content of vitamin B_6 in pumpernickel bread; Chicago products averaged considerably lower in vitamin B_6 than products from the other three cities.

Nutrition information

Bread consumption in the U.S. averages about 100g (equal to four slices) per day (Gustafson, 1983). Four slices of French and Italian breads would provide a maximum of 26% of thiamin towards our need expressed as U.S. Recommended Daily Allowance (U.S.-RDA) (NCC, 1975). Maximum amounts of riboflavin (19%), niacin (22%), folic acid (14%), and pantothenic acid (6%) would be provided by four slices of pumpernickel bread, wholewheat and French breads, wholewheat bread and wholewheat bread, respectively. Tortillas provided the maximum amount – 14% of the US-RDA/100g – of vitamin B_6. Tortillas also provided an appreciable amount of niacin but their contribution of the other four vitamins was the lowest among products tested.

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A Research Note

Utilization of Defatted Sesame Flour in Fermented Salami

O. A. CRUZ and H. B. HEDRICK

ABSTRACT

Fermented salami was prepared containing 0, 9, 18 and 27% defatted sesame flour (DSF). Chemical and sensory analyses were performed on the products. Results indicated that DSF could be used at either the 9 or 18% level without detrimental effect upon sensory attributes but salami containing 27% DSF will have less desirable flavor.

INTRODUCTION

COMMINUTED MEAT PRODUCTS are widely consumed throughout the world, but unfortunately their cost is often high and availability of meat is scarce in some countries. To reduce costs of these products and to utilize available plant proteins, there is increasing interest in the use of plant proteins as functional ingredients in meat products. Soybean protein has received most of the attention because of its nutritional value (Young et al., 1979) and functional properties in food processing (Kinsella, 1979). The growing industry based on the functional properties of soy protein has stimulated interest in the potential use of other oilseeds (Dench et al., 1981) in meat products. Although studies have been reported on the properties of defatted sesame flour and meal (Dench et al., 1981), their use in fortifying some foods (Bressani et al., 1963; Attschul, 1969; Pomeranz et al., 1969; Rooney et al., 1972), and in formulating high protein beverages (Parpia, 1966; Tasker et al., 1966; Jaffe and Chavez, 1971), very little work has been done on the use of defatted sesame flour in meat products.

In view of the apparent functionality of defatted sesame flour, the present work was carried out to evaluate the use of defatted sesame flour as a meat substitute in fermented salami.

MATERIALS & METHODS

Materials

Utility grade cow beef and pork trimmings, containing approximately 27 and 29% fat, respectively, were used. Both beef and pork had been frozen for approximately 30 days and was defrosted at 3°C prior to use. Defatted sesame flour (DSF) was obtained from the "Fundacion Centro de Investigaciones del Estado Para la Produccion Experimental Agroindustrial (CIEPE), San Fellipe, Edo Yaracuy, Venezuela.

Author Hedrick is with the Dept. of Food Science & Nutrition, Univ. of Missouri, Columbia, MO 65211. Author Cruz is with the Fundacion Cieppe, APPO 100, Zona Industrial San Felipe, Edo Yaracuy, Venezuela.

Formulation and processing

Four salami formulations were prepared and evaluated (Table 1). The all beef and pork salami represented the control. Three formulations were prepared containing 9, 18 and 27% DSF. DSF was hydrated 1:5, sesame flour to water, and allowed to stand for 30 min prior to mixing with the meat and other ingredients. The pH of the DSF was 6.7. The trimmings were ground separately, first through a 6.4 mm grinder plate and then through a 3.2 mm plate. Six 3.4 kg batches of ingredients were prepared per treatment. Each batch of ingredients was mixed for 6 min in a ribbon mixer (Leland Model 100DA). The salami mixtures were then stuffed into size 5 (maximum diameter 88 mm) fibrous casings (Union Carbide). After stuffing, the sausages were placed in a smokehouse at 35°C and 95% relative humidity for 24 hr. Then the smokehouse was increased to 68°C and 65% relative humidity for 4 hr; afterwards the temperature was increased to 74°C for an additional 1.5 hr. The sausages were then removed from the smokehouse, chilled at 3°C for 18 hr and then vacuum packaged. The packaged sausages were stored at 3°C for either 15 or 30 days. At the time of packaging, samples were obtained for chemical analyses and color evaluation from each replication of the four treatments.

Analytical methods

Six samples of sausage from each treatment were analyzed in duplicate for moisture and nitrogen (AOAC, 1980) and fat (modified Babcock according to Koniecko, 1979). pH and total acidity determinations were performed as described by Keller et al. (1974).

Color values of "L" (measure of total light reflected were determined on 3 mm slices of salami with a color/difference meter (Hunter Model D25D2). The meter was standardized against Hunter Tile No. C23395 (L = 67.6, a = 22.7, b = 9.8, Y = 45.7, X = 53.4 and Z = 42.8).

Sensory evaluation

Sensory evaluations were performed by a six member experienced panel. Panelists were selected on the basis of their ability to repeat their ratings and to detect differences between control samples consistently. The panelists evaluated 3 mm thick slices cut at cross section to the long axis of the sausages. Samples were identified by random three digit numbers and were presented to the panelists cold (approximately 5°C) in two sessions on each of three consecutive days. There was a total of 36 observations per treatment in a partially balanced design with two associate classes (Design S6 in Bose et al., 1954). The panelists evaluated samples after 0, 15 and

Table 1—Formulations for fermented salami

Treatment number	Level of DSF, %[a]	Beef trimmings %	Pork trimmings %	Starter culture[b] %	Salt %	Dextrose %	Spices[c] %	Sodium nitrite ppm
1	0	60.0	40.0	0.02	3.0	1.0	1.0	156
2	9	54.4	36.6	0.02	3.0	1.0	1.0	156
3	18	43.8	33.2	0.02	3.0	1.0	1.0	156
4	27	43.2	29.8	0.02	3.0	1.0	1.0	156

[a] The level of DSF is expressed on a 1:5 rehydrated basis with cold water.
[b] Lactacel 75, Microlife Technics, Sarasota, FL.
[c] Ingredients in spice formulation: spice extractives, dextrose, spices, paprika, garlic powder, onion powder, natural smoke flavor and tricalcium phosphate. Griffith Laboratory, Union City, CA.

Table 2—Composition, pH, total acidity, cooking losses, color and sensory panel scores of fermented salami

Variable	Treatment			
	Control all meat	Meat +9% defatted sesame flour	Meat +18% defatted sesame flour	Meat +27% defatted sesame flour
Moisture, %	56.29[a]	58.23[b]	60.32[c]	61.50[d]
Fat, %	23.50[a]	22.02[b]	19.86[c]	18.43[d]
Protein, %	16.67[a]	16.42[a]	15.83[b]	15.67[b]
pH	4.98[a]	4.72[b]	4.63[bc]	4.54[c]
Total acidity, %	0.70[a]	0.73[b]	0.75[b]	0.84[c]
Cooking loss, %	7.68[a]	8.09[b]	9.39[c]	10.08[c]
Hunder Color Difference Meter "L" Value	49.70[a]	50.48[a]	51.73[b]	53.32[c]
Tenderness score				
0 days storage	6.25[a]	6.25[a]	6.36[a]	6.25[a]
15 days storage	5.78[a]	6.25[a]	6.53[a]	6.31[a]
30 days storage	6.17[a]	6.39[a]	6.17[a]	5.97[a]
Juciness score				
0 days storage	6.25[a]	6.36[a]	6.36[a]	6.36[a]
15 days storage	6.28[a]	6.06[a]	6.39[a]	6.22[a]
30 days storage	6.08[a]	6.06[a]	5.86[a]	6.06[a]
Flavor score				
0 days storage	5.45[a]	5.95[b]	5.09[a]	4.64[c]
15 days storage	6.06[a]	5.61[ab]	5.31[b]	4.53[c]
30 days storage	5.86[a]	5.47[b]	4.64[c]	4.31[c]
Acid score				
0 days storage	5.45[ab]	5.78[a]	5.14[b]	4.53[c]
15 days storage	5.70[a]	5.50[ab]	5.25[b]	4.50[c]
30 days storage	5.45[a]	5.47[a]	4.53[b]	4.44[b]

[a,b,c,d] Means bearing different superscripts on the same line are different (P<0.05).

30 days storage for tenderness, juciness, flavor and acidity desirability. The scoring system used for each characteristic was 1 = very undesirable, 2 = undesirable, 3 = moderately undesirable, 4 = slightly undesirable, 5 = slightly desirable, 6 = moderately desirable, 7 = desirable, 8 = very desirable. Panelists were instructed that a score of 1 should indicate tenderness as being very tough; juciness – very dry, flavor – very intense, and acid – imperceptible; and a score of 8 should indicate tenderness as being very tender, juciness-very juicy, flavor-very pronounced, and acid-very pronounced.

Statistical analysis

The statistical model contained the main effect of treatment. Computations were done using the GLM procedure given in Statistical Analysis Systems User's Guide (SAS, 1979). Least squares means were separated by least significant difference (Snedecor and Cochran, 1979).

RESULTS & DISCUSSION

Salami prepared with DSF contained more moisture and less fat (P<0.05) than the all-meat salami (Table 2). Likewise, salami prepared with 18 and 27% DSF contained less (P<0.05) protein than the all meat salami or that containing 9% DSF. These differences can be attributed to water added for hydration of the DSF and to the amounts of hydrated DSF in the initial sausage formulations. The pH of salami containing DSF was lower (P<0.05) than the all-meat salami and in general decreased as the level of DSF was increased. The lower pH level of the salami containing DSF compared to the all-meat salami was probably related to the carbohydrate present in the DSF facilitating acid production by bacteria which were added during processing (Boloorforooshan and Markakis, 1979). Total acidity was lower (P<0.05) for the DSF salami and in general increased as the level of DSF was increased. The color values (lighter color) of salami containing 18 and 27% DSF were higher (P<0.05) than those of the all meat and 8% DSF salami. These observations are in agreement with the report of Berry et al. (1979) that dry fermented salami containing structured soy protein fiber (SSPF) was lighter in color than all meat salami.

Sensory panel data show that tenderness and juciness desirability scores were similar for all treatments during the three storage periods (Table 2). Some differences were noted for flavor and acid desirability scores. There was a trend for the all-meat and 8% DSF salami to be scored more desirable for flavor and acid than the 18 and 27% DSF salami. The lower flavor and acid desirability scores for salami containing 27% DSF are in agreement with the report of Berry et al. (1979) for dry fermented salami containing 30% SSPF.

Results from this study indicate that DSF can be used in the production of fermented salami at 9 or 18% levels without detrimental effect upon sensory attributes. DSF at the 27% level, except for tenderness and juciness, may result in unacceptable flavor.

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A Research Note

"Coagulation Test" for Cooked Meat Temperature: Effect of Sample Preparation Methods

W. E. TOWNSEND, J. E. THOMSON, and J. R. HUTCHINS

ABSTRACT

The "Coagulation Test," to determine the temperature to which meat had been cooked, was studied relative to the effect of variations in sample preparation methods on the coagulation temperature, biuret protein content and spectrophotometric absorbance value of 0.9% saline or water filtrates obtained from extracts of samples of rare, medium and well done portions of roast beef. Sample preparation methods were: (1) 5-mm cubes, (2) ground once, (3) ground three times, or (4) homogenized at 12,000 rpm for 30 sec. Sample preparation method had no significant effect on the coagulation temperature, biuret protein content or absorbance values of filtrates from extracts of rare, medium and well done portions of roast beef. No significant differences were found between 0.9% saline and distilled water filtrates.

INTRODUCTION

THE ANIMAL and Plant Health Inspection Service/Veterinary Services (APHIS/VS) and the Food Safety and Inspection Service (FSIS) of the USDA both require that certain meat and poultry products be heat processed to specific internal temperatures; e.g., cooked beef and roast beef to a minimum internal temperature of 62.8°C (145°F), imported canned hams, picnics and luncheon meat to 69°C (156°F) (USDA-APHIS, 1982; USDA-FSIS, 1982). These requirements by APHIS and FSIS were instituted to ensure the destruction of viruses and other organisms that cause diseases in animals and man.

Presently, FSIS is using a protein "Coagulation Test" for monitoring the internal temperature of both beef and pork products cooked to temperatures lower than 65°C (150°F) (USDA-FSIS, 1981). This test is based on the measureable loss in protein solubility as the temperature of the product is increased; however, because visual observation is involved, the test is considered empirical and subjective.

FSIS technical personnel (McNeal, 1980; Soderberg, 1980; Woods, 1980) and several investigators (Coretti, 1957; Visacki et al., 1966; Popescu and Din, 1982) have suggested a need to evaluate possible variability in the steps in the present FSIS "Coagulation Test" (sample preparation, extraction, filtration, etc.) that may affect the temperature at which the proteins in the filtrate coagulate.

The major objective of this study was to evaluate the effect of variations in sample preparation methods on the coagulation temperature of 0.9% saline and distilled water filtrates obtained from extracts of samples of rare, medium and well done portions of roast beef. In addition, biuret protein and spectrophotometric characteristics were evaluated to determine their relationship to coagulation temperature.

MATERIALS & METHODS

Preparation of filtrate samples

Two commercially prepared cooked beef roasts were obtained locally, trimmed of excessive fat, cut into portions determined to be rare, medium and well done by visual examination by an experienced investigator. Subportions were then prepared by one of three methods: (1) 5-mm cubes, (2) ground once, or (3) ground three times with a Toledo (Model 5126) grinder, through a grinder plate with 3mm holes. Fifty gram samples from each subportion were wrapped in aluminum foil and stored at -35°C until analyzed.

Samples were thawed overnight at 3°C and the soluble proteins extracted, with occasional stirring, from each 50g sample (cubes, ground once, or ground three times) for 20 min with 100 mL of either 0.9% saline or distilled water per sample. Homogenized samples were prepared by adding 100 mL of either 0.9% saline or distilled water to the 50g meat sample (ground once through a grinder plate with 3mm holes in a Sorvall Omni-mixer operated for 30 sec at 12,000 rpm. The meat slurry from each sample preparation procedure was strained to remove muscle tissue debris, and this primary extract filtered in accordance with the standard Food Safety and Inspection Service "Coagulation Test" procedure (USDA-FSIS, 1981). All experiments were replicated twice.

Coagulation temperature

The coagulation temperature of the filtrates was determined by the "Coagulation Test" (USDA-FSIS, 1981).

Protein concentration

One mL of each filtrate was analyzed for protein concentration by the biuret procedure of Layne (1975). Crystalline bovine serum albumin (Sigma Chemical Company) was used for preparation of the standard curve for each protein determination.

Heme pigment absorbance values

Heme pigment absorption spectra were obtained by scanning 3.5 mL of each filtrate in 1cm cuvet cells from 350 to 750 nm in a Model 35 Beckman spectrophotometer. Filtrates were scanned at 50 nm/min, with a chart speed at 2 in/min, and chart span at 2.0 A. Absorbance values at 490-500, 537-542, 575-578 and 630 nm were obtained from the spectra. The wavelengths of 537-542 and 575-578 nm; and 490-500 and 630 nm represent the maxima for oxymyoglobin and metmyoglobin, respectively. (Bernofsky et al., 1959; Fox, 1966).

Statistical analysis

Data were analyzed by the general linear model and Duncan's multiple range test procedures of the SAS program (SAS, 1979).

RESULTS & DISCUSSION

STATISTICAL ANALYSIS of the data showed that sample preparation method had no significant effect on the coagulation temperature, biuret protein content and absorbance values of filtrates from extracts of rare, medium and well done portions of roast beef. However, several observations of interest were noted.

Coagulation temperatures for filtrates obtained from extracts of rare, medium and well done portions of roast beef were 2°, 3°, and 3-6°C higher, respectively, for filtrates obtained from samples prepared as cubes or ground three times than those samples ground only once or homogenized (Table 1). Coagulation temperatures of filtrates extracted by distilled water did not significantly differ from those extracted by saline. The exact reason for the slightly higher coagulation temperature and lower protein content of filtrates from samples prepared as cubes or ground three times than those ground once or homogenized is not readily

The authors are affiliated with the USDA-ARS, Poultry Meat Quality and Safety Research Unit, Richard B. Russell Agricultural Research Center, P.O. Box 5677, Athens, GA 30613.

Table 1—Coagulation temperature of filtrates from extracts of roast beef.

Preparation method	Roast beef doneness:	Rare		Medium		Done	
	Extracted by:	0.9% Saline	Dist Water	0.9% Saline	Dist Water	0.9% Saline	Dist Water
Cut into 5-mm cubes		50.8[a]	51.9	53.8	51.7	54.4	56.1
Ground once		48.9	49.9	51.1	52.5	53.8	53.8
Ground three times		51.7	51.9	52.2	53.0	54.8	53.6
Homogenized (30 sec at 12,000 rpm)		49.1	50.8	49.1	51.1	48.0	50.8

[a] Each value represents mean (°C) of two replications.

Table 2—Effect of sample preparation methods on absorbance values (490-500, 537-542, 575-578 and 630 nm) of saline and distilled water filtrates from rare, medium and done portions of roast beef

Preparation method	0.9% Saline				Distilled water			
	Absorbance values at:[a]				Absorbance values at:			
	490-500	537-542	575-578	630	490-500	537-542	575-578	630
Rare portion								
Cut into 5-mm cubes	0.44[b]	0.50	0.39	0.14	0.37	0.58	0.50	0.08
Ground once	0.42	0.54	0.44	0.14	0.44	0.53	0.43	0.13
Ground three times	0.43	0.49	0.39	0.13	0.38	0.48	0.40	0.11
Homogenized (30 sec at 12,000 rpm)	0.68	0.65	0.47	0.24	0.60	0.71	0.56	0.19
Medium portion								
Cut into 5-mm cubes	0.29	0.24	0.17	0.10	0.27	0.26	0.19	0.08
Ground once	0.35	0.31	0.23	0.13	0.28	0.31	0.23	0.10
Ground three times	0.21	0.23	0.18	0.07	0.32	0.30	0.44	0.12
Homogenized (30 sec at 12,000 rpm)	0.49	0.39	0.26	0.19	0.44	0.40	0.28	0.16
Done portion								
Cut into 5-mm cubes	0.08	0.07	0.04	0.03	0.08	0.09	0.07	0.03
Ground once	0.22	0.19	0.13	0.08	0.22	0.20	0.14	0.08
Ground three times	0.19	0.16	0.11	0.07	0.21	0.18	0.14	0.08
Homogenized (30 sec at 12,000 rpm)	0.32	0.24	0.28	0.13	0.27	0.28	0.22	0.09

[a] Absorbance values determined from heme pigment spectra at 490-500, 537-542, 575-578 and 630 nm, respectively.
[b] Values represent mean absorbance values of two replications.

apparent at this time. A possible explanation for the slightly higher coagulation temperatures and lower protein content might be that the frictional shear forces between the cutter blades and grinder plate during grinding were sufficient to cause some denaturation of the tissue proteins. However, this was not the case in our study, and requires further basic protein investigations. In contrast to the coagulation temperatures, more soluble proteins were extracted from samples ground once or homogenized than those cubed or ground three times. In general, when the protein content of the filtrate was lower, the coagulation temperature of the filtrate was higher. Similar observations have been reported by Davis and Anderson (1983), Popescu and Din (1982) and Townsend et al. (1984). In our study, there was a significant ($P<0.05$) correlation (-0.720) between degree of doneness and soluble protein content. Although sample preparation methods had no significant effect on the absorbance values of filtrates, there was a decrease in magnitude (Table 2) of the absorbance values as degree of doneness increased. Similar decreases of magnitude of absorbance values have been reported by Helmke and Froning (1971) for turkey rolls prepared from thighs and drumsticks, and by Townsend and Thomson (1984) for beef top round, pork loin and turkey breast. These observations suggest the potential of using a spectrophotometric technique for monitoring the adequacy of doneness of meat and poultry products. More research needs to be conducted to relate absorbance values to actual end-temperatures of the products.

As reported by Townsend et al. (1984), the fact that the sample preparation methods, as used in our study, had no significant effect on the coagulation temperatures of the filtrates may be a reflection of the empirical and subjective nature of the "Coagulation Test" procedure itself.

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A Research Note

Thiobarbituric Acid-Reactive Substances in Chicken Fat and Unsaturated Fatty Acids

HIROKO KOSUGI and KIYOMI KIKUGAWA

ABSTRACT

When autoxidized chicken fat and unsaturated fatty acids were subjected to thiobarbituric acid (TBA) test in trichloracetic acid, all samples revealed two absorption maxima at 532 and 455 nm. Rate of increase in the TBA-reactive substances (TBARS) at 532 nm was different from that in the TBARS at 455 nm. It was suggested that there were several kinds of TBARS in the autoxidized samples: the TBARS at 532 nm which were unstable in the autoxidation process and slowly liberated malonaldehyde; the TBARS at 532 nm which liberated malonaldehyde rapidly; and the TBARS at 455 nm which liberated other aldehydes unstable after reaction with TBA.

INTRODUCTION

IT HAS BEEN KNOWN that meat with fat containing unsaturated fatty acids undergoes deterioration during storage owing to autoxidation of the unsaturated fatty acids (Turner et al., 1954; Younathan and Watts, 1960; Chipault and Hawkins, 1971), and there are a number of observations with lipid oxidation of muscle foods (Melton, 1983; Dawson and Gartner, 1983). The thiobarbituric acid (TBA) test has been commonly used to measure the oxidative deterioration of fat samples (Gray, 1978; Sinnhuber and Yu, 1977). The TBA-reactive substances (TBARS) at 532 nm have been assumed to liberate mostly malonaldehyde (Yu and Sinnhuber, 1957) and the TBARS at 455 nm to liberate alkanals and 2-alkenals (Jacobson et al., 1964; Marcuse and Johansson, 1973), but they are not well characterized. The objectives of this research were to investigate the development of TBARS of fats during autoxidation under controlled condition and attempt to characterize the TBARS.

MATERIALS & METHODS

METHYL LINOLEATE (95%), methyl oleate, malonaldehyde bis(dimethylacetal) and TBA were obtained from Tokyo Kasei Kogyo Company, Ltd., Tokyo, Japan. Purified chicken fat was prepared by warming and pressing of the subcutaneous fat obtained commercially and washed with hot distilled water several times. Fatty acid composition of the chicken fat was determined by gas liquid chromatography. Methyl esters of the unsaturated fatty acids and the chicken fat were autoxidized by the active oxygen method (AOM) (King et al., 1933). Peroxide number was determined according to the method of Wheeler (1932).

The TBA test was performed according to the methods of Ottolenghi (1959) and Fong et al. (1973). Each sample (25 mg) was heated in a mixture of 1.0 mL of 0.5% (w/v) trichloracetic acid (TCA) at 60°C for 90 min. After cooling, the mixture was extracted with 3.0 mL of chloroform to give a clear aqueous phase. Absorption spectrum of the aqueous phase was recorded with a Shimadzu UV-240 UV-visible recording spectrophotometer. The TBA test of the TCA extracts was also performed (Vyncke, 1975). The autoxidized sample (25 mg) was extracted with 2.9 mL of 0.5% (w/v) TCA at 60°C for 40 or 90 min, and the mixture was shaken with 3.0 mL of chloroform to remove the oil. To the aqueous extract was added 1.0 mL of 0.5% (w/v) TBA and the mixture was heated at 60°C during the indicated period to measure absorbance.

Author Kosugi affiliated with Ferris Women's College, 37 Yamatecho, Nakaku, Yokohama 231, Japan. Author Kikugawa is affiliated with Tokyo College of Pharmacy, 1432-1 Horinouchi, Hachioji, Tokyo 192-03, Japan.

RESULTS & DISCUSSION

THE PROPERTIES ot the TBARS of the autoxidized chicken fat, methyl linoleate and methyl oleate were investigated (Fig. 1). The test was carried out on the AOM-autoxidized samples of the early stage (before reaching maximum peroxide level) and advanced stage (after the maximum peroxide level). The TBARS at 532 nm of the early stage (Fig. 1A, C, E) progressively increased with TBA-reaction time. Unoxidized samples showed little increase in absorbance under the same conditions, indicating that the samples were not oxidized during the test periods. The progressive increase in absorbance of the early stage samples indicated the presence of a large amount of the TBARS at 532 nm which slowly liberated malonaldehyde in TCA medium. On the other hand, the TBARS of the advanced stage (Fig. 1B, D, F) increased to reach maximum level within 90 min, as well as those of the standard precursor of malonaldehyde, malonaldehyde bis(dimethylacetal) (Fig. 1G). The fat samples of the advanced stage may contain only the TBARS at 532 nm which rapidly provided malonaldehyde. The TBARS levels at 455 nm increased to reach maximum at around 20–40 min, and subsequently decreased (Fig. 1A-F), indicating that the aldehydes were ustable in the TBA reaction. However, the maximum TBARS levels at 455 nm of the advanced stage were higher than those of the early stage.

The chicken fats of the early and the advanced stages were extracted with TCA, and the extracts were subsequently reacted with TBA for various periods (Fig. 2A, B). Absorbance at 532 and 455 nm was always higher in the 90 min extracts than in the 40 min extracts. Malonaldehyde and other aldehydes were released from TBARS and remained stable in the TCA extracts. While absorbance at 532 nm increased to reach maximum within 1 hr, absorbance at 455 nm leveled off after 1 hr of TBA reaction, indicating that aldehydes other than malonaldehyde were unstable and degraded after reaction with TBA. The TCA extracts of the autoxidized methyl linoleate (Fig. 2C, D) showed similar results.

The TBARS at 532 nm in the early stages of autoxidation and those in the advanced stages were different in nature in each of the autoxidized samples. The TBARS in the early stages were gradually destroyed in autoxidation as Tarladgis and Watts (1960) reported. While the TBARS at 532 nm in the advanced stages rapidly released malonaldehyde, those in the early stages slowly liberated the aldehyde. While several discussions have been made with respect to the TBARS at 532 nm (Dahle et al., 1962; Pryor et al., 1976; Ohkawa et al., 1978), the present results indicate that the TBARS at 532 nm of the fat samples were composed of various kinds of precursors.

Every autoxidized sample produced the TBARS at 455 nm. The precursors gradually increased with time of autoxidation, and they may liberate alkanals and 2-alkenals; these aldehydes produced unstable yellow pigment (Jacobson et al., 1964; Marcuse and Johansson, 1973). Several conflicting reports have appeared on the formation and the properties of yellow pigment (Tarladgis et al., 1960; de Koning and Silk, 1963; Porter et al., 1976), demonstrating

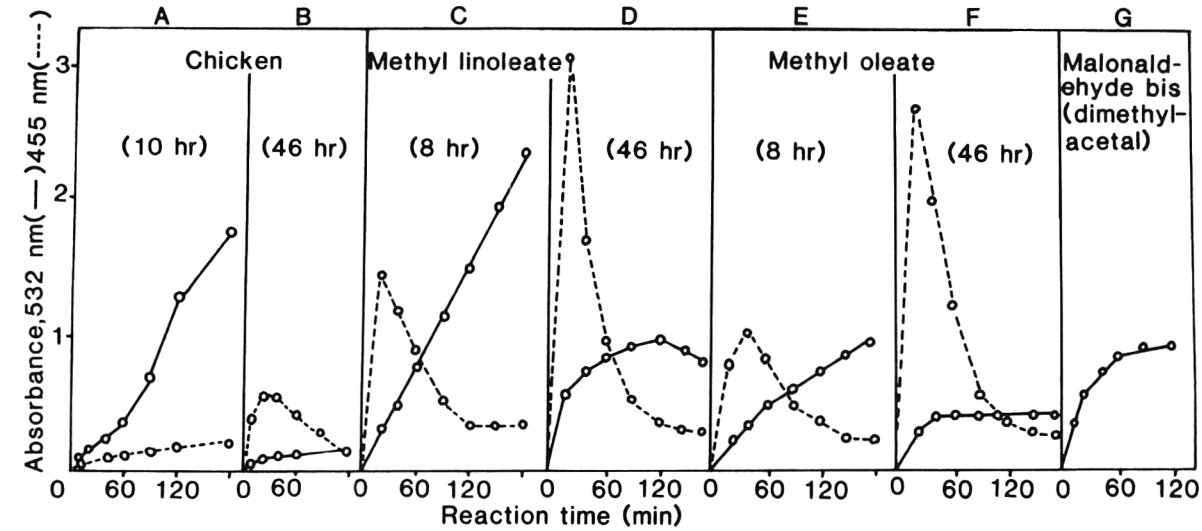


Fig. 1—Time course of TBA reaction of the AOM-autoxidized samples. Time in parenthesis indicates time of AOM-autoxidation. A standard solution of 0.80 mM malonaldehyde bis(dimethylacetal) was similarly treated. Unoxidized chicken fat contained 29.6% saturated fatty acids and 64.5% unsaturated fatty acids (42.2% oleic, 13.9% linoleic, 7.2% palmitoleic and 1.2% linolenic). Peroxide numbers (meq/kg) of chicken fat were 280 (10 hr), 1000 (23 hr) and 750 (46 hr); methyl linoleate, 1080 (8 hr), 1100 (21 hr) and 700 (46 hr); and methyl oleate, 600 (8 hr), 1650 (23 hr) and 600 (46 hr).

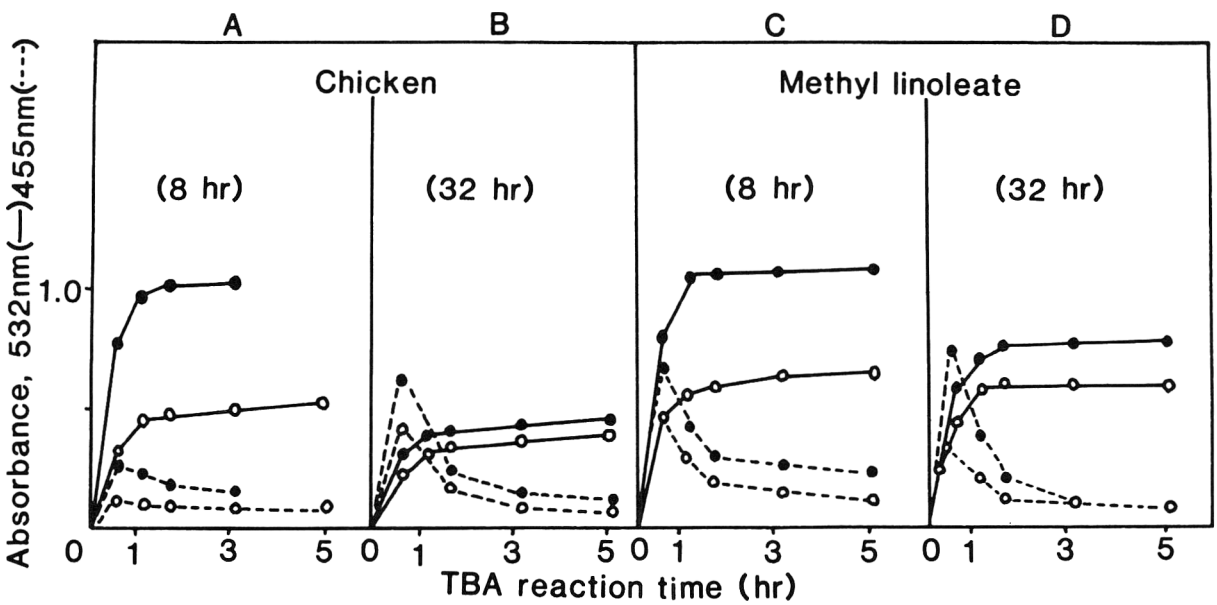


Fig. 2—Time course of TBA reaction of the TCA-extract of the AOM-autoxidized samples. The autoxidized sample was extracted with TCA during 40 min (○) and 90 min (●), and the extract was reacted with TBA. Time in parenthesis indicates time of AOM-autoxidation.

that red TBA-malonaldehyde adduct was changed into yellow pigment or a yellow intermediate was changed into red pigment. From the present results, it was impossible to rationalize the relation between the TBARS at 455 nm and those at 532 nm.

In conclusion, it was suggested that there were several kinds of TBARS in the autoxidized samples: the TBARS at 532 nm which were unstable in the autoxidation process and slowly liberated malonaldehyde; and the TBARS at 455 nm which liberated other aldehydes unstable after reaction with TBA.

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A Research Note

Postmortem Loss of Carnitine from Skeletal Muscle of Pork and Beef

JOANNE M. NELSON, DENNIS R. CAMPION, and JAMES O. REAGAN

ABSTRACT

The carnitine content of beef muscle postmortem was more than twofold higher than the content of pork muscle. For both pork and beef, carnitine was distributed among the fractions, free carnitine >> short-chain acylcarnitine > long-chain acylcarnitine concentration. Based on total carnitine content, the only loss of carnitine from pork muscle occurred as a result of cooking. Cooking also reduced total carnitine content of beef muscle. In addition, total carnitine content of the beef steaks was lower after thawing. Time in the retail case did not influence carnitine content.

INTRODUCTION

HUMAN CARNITINE (β-hydroxy-α-trimethyl-amino butyric acid) deficiencies have recently been reported and are characterized by overall muscle weakness and lipid accumulation (Engel, 1980; Di Mauro et al., 1980). Deficiencies may be caused by many factors such as (1) the body not synthesizing sufficient amounts of carnitine, (2) excessive release of carnitine from the cells, (3) impaired transport of carnitine into cells, (4) excessive catabolism, and (5) excessive loss from body gluids (Engel, 1980). Normally sufficient quantities of carnitine are synthesized within the body. But several disease states involving carnitine deficiency have been treated successfully by addition of carnitine to the diet.

The carnitine content of skeletal muscle is greater than that of plants (Snoswell and Koundakjian, 1971; Panter and Mudd, 1969). Thus, muscle represents a potentially valuable and natural dietary source of carnitine. Since carnitine is water soluble, loss of water and associated leaching of nutrient during aging, thawing, cooking, etc., may influence carnitine content of meat (Mitchell, 1978). The present study was designed to establish whether postmortem handling and cooking influenced carnitine content.

MATERIALS & METHODS

EIGHT MARKET WEIGHT HOGS (104.5 ± 38 kg) were obtained from the University of Georgia swine farm and slaughtered at the University Food Science abattoir. Six steer carcasses (230.6 ± 10.5 kg) were randomly selected at a commercial packing plant. Longissimus muscle pH and temperature were recorded 15 - 30 min and 24 hr postmortem. Upon completion of these measurements at 24 hr, the short loins from the beef carcasses were removed, put into cardboard boxes and transported to the Food Science Department, University of Georgia.

Four days postmortem the pork loins were removed, cut into 1.7 cm thick chops and vacuum packaged in pairs. The beef short loins were cut into 2.3 cm thick steaks and wrapped in pairs and placed and stored in freezer paper. Chops and steaks were placed in a freezer (-24°C) for 2 wk. Chops and steaks were thawed overnight at 20°C. The thawed meat was wrapped in polyvinyl chloride film and placed on trays in a display case for up to 3 days.

Duplicate samples weighing approximately 2g were taken from each pork loin and beef short loin at time of death (day 0) and 24 hr (day 1) and 4 days postmortem. These samples were kept in liquid nitrogen until analyzed for carnitine. In a preliminary study it was determined that freezing and storing in liquid nitrogen did not influence the carnitine content (data not shown). Samples from the chops and steaks that had been stored in the freezer 2 wk were taken after thawing (day 19) and after one (day 20) and three days (day 22) in the display case.

Additional chops and steaks were taken at days 19, 20 and 22 and cooked on Faberware Open Hearth electric broilers. The chops were turned at 48.9°C as determined by thermocouples and removed at 70°C. Steaks were turned at 54.4°C and removed at 72.8°C. Cook loss was determined on beef steaks by taking weights before and after cooking. Samples of cooked meat from each of the time periods were taken and stored in liquid nitrogen.

Part of each sample frozen in liquid nitrogen was freeze dried to constant weight and dry matter determined. Another part was homogenized in ice-cold 3% perchloric acid at half speed with a Polytron homogenizer for 60 sec intervals (pork, 1g wet weight per 2 mL; beef, 1g wet weight per 10 mL). The homogenate was centrifuged at 300 x *g* for 10 min. The supernatant was assayed for free carnitine (fraction 1) and short chain acylcarnitine (fraction 2). The pellet was assayed for acid-insoluble, long-chain acylcarnitine (fraction 3). The three fractions were derived according to Brass and Hoppel (1978). Total carnitine is the sum of fractions one, two and three.

Twenty-five microliter aliquots from the three fractions were transferred to tubes (12 x 75 mm). Then 225 μL of a solution containing 0.2M Trishydroxymethyl-2 amino ethane sulphonic acid, 6 mM 2 N-ethyl maleimide, and 1.2 x 10^5 dpm [1-^{14}C] acetyl coenzyme A, pH 7.2 (Carter and Stratman, 1980) was added. The ^{14}C-labeled isotope was obtained from Amersham (Arlington Heights, IL) and had a specific activity of 55 μCi/mmole. Ten microliters (5 mg protein) of carnitine acetyl transferase (EC 2.3.1.7) were added, the tubes were vortexed, and incubated in a 37°C water bath for 30 min. The reaction was terminated by the addition of 0.6 mL of a continuously stirred slurry of Dowex 1 x 8 anion exchange resin containing 0.44 mL H_2O. The resin was treated prior to use with 1N HCl, filtered under vacuum, and washed with water until the filtrate had a neutral pH (McGarry and Foster, 1976). The tubes were vortexed, placed on ice for 10 min, vortexed again, and placed on ice for an additional 10 min. The tubes were then centrifuged at 3,000 x *g* for 5 min. Two-tenths milliliters of supernate were transferred to scintillation vials and 5 mL of scintillation fluid were added. Counts were obtained by the use of a liquid scintillation counter (Searle Isocap/300, TM Analytic, Inc., Elk Grove, IL).

A stock standard (10 X solution, 267 μM) of DL-carnitine (Sigma Chemical Co., St. Louis, MO) was prepared. The L-carnitine content of this solution was determined spectrophotometrically (Pearson and Tubbs, 1967).

Analysis of variance was conducted according to Steel and Torrie (1960). Means were separated either by the method of Duncan (1955) or by the student's t-test, as appropraite.

RESULTS & DISCUSSION

TEMPERATURE and pH values obtained in this study on day 0 and day 1 were typical of values reported by many others. In pork, the pH and temperature on day 0 were pH 6.43 and 38.4°C, and pH 5.66 and 5.4°C on day 1. In beef, the pH and temperature on day 0 were pH 6.29 and 37.8°C, and pH 5.66 and 6.2°C on day 2.

In the uncooked state, the various carnitine fractions remained remarkably constant in value over the entire time course of the initial 4-day holding period, 2-wk freezing

Authors Nelson, Campion, and Reagan are affiliated with the Dept. of Food Science, Univ. of Gerogia, Athens, GA 30602. Author Campion is also affiliated with the USDA-SE-ARS, Animal Physiology Unit, Richard B. Russell Agricultural Research Center, Athens, GA 30613.

Table 1—Mean values for carnitine content in pork and beef skeletal muscle[a]

Fraction	Days Postmortem					
	0	1	4	19	20	22
		(Unfrozen)			(Frozen[b])	
			Pork			
Total carnitine[c]	837 ± 67	622 ± 51	736 ± 92	674 ± 52	660 ± 37	748 ± 74
Fraction one	493 ± 53	437 ± 31	533 ± 57	561 ± 45	574 ± 40	582 ± 32
Fraction two	278 ± 40[d]	129 ± 29[e]	150 ± 72[d,e]	64 ± 29[b]	28 ± 22[b]	127 ± 79[e]
Fraction three	66 ± 7	56 ± 7	53 ± 10	49 ± 8	58 ± 16	39 ± 3
			Beef			
Total carnitine[c]	2994 ± 120[d]	2664 ± 94[d,e]	2512 ± 156[e]	1950 ± 182[f]	1890 ± 173[f]	1593 ± 142[f]
Fraction one	1861 ± 87[d,e]	2019 ± 80[d]	1603 ± 199[e,f]	1487 ± 58[f]	1556 ± 82[f]	1321 ± 125[f]
Fraction two	1072 ± 66[d]	584 ± 13[d,e,f]	846 ± 302[d,e]	424 ± 147[e,f]	299 ± 189[f]	232 ± 112[f]
Fraction three	61 ± 9[d]	61 ± 7[d]	63 ± 9[d]	39 ± 8[d]	35 ± 5[e]	40 ± 5[e]

[a] Means (± standard error) expressed in nmol/g dry weight.
[b] Samples frozen on day 4, thawed on day 18, then placed in retail case.
[c] Total carnitine is the sum of fractions one, two and three.
[d,e,f] Means within the same row and species with different superscripts were significantly different at $P < 0.05$.

Table 2—Mean values (± standard error) for carnitine content of uncooked and cooked pork and beef muscle

Fraction	Uncooked[a] (nmole/g dry wt)	Cooked[b] (nmole/g dry wt)
	Pork	
Total carnitine[c]	694 ± 32[d]	576 ± 17[e]
Fraction one	572 ± 19[d]	500 ± 19[e]
Fraction two	73 ± 29	32 ± 12
Fraction three	49 ± 4	44 ± 2
	Beef	
Total carnitine	1811 ± 98[d]	1473 ± 81[e]
Fraction one	1455 ± 56[d]	1178 ± 51[e]
Fraction two	318 ± 85	269 ± 55
Fraction three	38 ± 3[d]	26 ± 3[e]

[a] Chops and steaks sampled at day 19, 20 and 23. Values were pooled across days as the effect of day and the interaction cooking method x day were not significant ($P > 0.05$).
[b] Chops and steaks cooked at day 19, 20 and 23. Values were pooled across days as the effect of day and the interaction cooking method x day were not significant ($P > 0.05$).
[c] Total carnitine is the sume of fractions one, two and three.
[d,e] Means within same row and species with different superscripts were significantly different at $P < 0.05$.

period, and during the 3 days of display in the retail case (Table 1). Relatively more carnitine was present as free carnitine (fraction one) while the least amount was found in the acid insoluble fraction (fraction three).

The relative distribution of the carnitine content among the three fractions for beef muscle was similar to that observed for pork muscle (Table 1). However, the carnitine content of the uncooked beef muscle was influenced by postmortem handling procedure. Generally, the carnitine content of the various fractions was reduced after thawing. Thaw loss in these steaks was 5.1 ± 0.2%. Thus, at least part of the loss in carnitine, which is water soluble, was probably due to moisture loss attendant upon thawing. This assumption is supported by the work of Harris and Von Loesecke (1960). They found that after freezing, 10% of the water soluble vitamins could be lost in the drip. But Jul (1978) observed that freezer storage of meat had no effect on vitamin content except for the water soluble vitamin, riboflavin. The carnitine content of the fractions was stable during the three days in the display case. In this respect, the beef and pork muscle responded similarly.

During the initial holding period, the total carnitine content of the beef muscle decreased from day 0 to day 4. Values for fractions one and two were quite variable, however. Only fraction three, the acid insoluble carnitine, remained particularly stable over this time period.

Values for total carnitine for both pork and beef were significantly reduced by cooking, 17% in pork and 18% in beef. This decrease particularly reflected the loss in free carnitine content (fraction one). Cook loss for the beef steaks was 27.2%. The significant moisture loss from beef (and pork) muscle as a result of cooking can partially explain the loss of free carnitine that resulted from cooking. The loss of other water soluble vitamins as a result of cooking was observed previously (Mitchell, 1978).

Statistical comparisons for carnitine content between pork chops and beef steaks were not performed. However, it was obvious that beef muscle contained more total carnitine due to a greater content of carnitine in fractions one and two. Beef muscle has a higher myoglobin content and muscles with a higher myoglobin content have a greater capacity for aerobic metabolism (Lawrie, 1952). Furthermore, Borum (1980) observed that red meat contained significantly more carnitine than white meat.

Pork and beef muscle were similar to other species, e.g., rat (Brass and Hoppel, 1977) and sheep (Snoswell and Koundakjian, 1972) in that the free carnitine fraction was higher than the other two fractions. Others (Snoswell and Linzell, 1975) noted that the carnitine content of muscle can change in animals with varying seasons and pasture conditions. From the present study, it was determined that, with the exception of retail display, postmortem handling of meat can influence the carnitine content of both pork and beef muscle.

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A Research Note

Flavor and Nutrient Composition of a Sausage Ball Snack-type Food Made From Pork or Catfish

L. C. MEDEIROS and C. D. DUDLEY

ABSTRACT

Ground catfish flavored with pork sausage seasoning was used in a sausage ball snack-type food and the product was compared to pork sausage balls for sensory and nutrient differences. Triangle tests were not significant ($P > 0.05$). Protein ($p \leq 0.01$), fat ($p \leq 0.01$), and nitrogen free extract ($p < 0.05$) were significantly different between the two products. Moisture, crude fiber, ash, sodium, and potassium content were not significantly different ($P > 0.05$). In the proportions used in this study, catfish sausage is useful as a substitute for pork sausage.

INTRODUCTION

POND-RAISED FISH have a mild flavor which would allow the flesh to be used as a meat analog. Recent research (Arocha and Toledo, 1982) has shown that various species of fish can be successfully used in the production of frankfurter-type sausages. The objective of this study was to determine whether there was a sensory difference between pork sausage balls and catfish sausage balls and to determine their nutrient composition.

MATERIALS & METHODS

Preparation of samples

Farm-raised catfish fillets were ground using a hand grinder with a 5 mm plate. Commercial pork sausage seasoning was mixed by hand into the ground fish. Pork sausage was prepared with approximately 80% lean (Boston butt) and 20% fat. Cheddar cheese was grated using the coarse side of a hand grater. All ingredients were weighed on a top loading balance according to the proportions listed in Table 1. Ingredients were hand mixed, formed into 2-4 cm balls, and cooked approximately 12 min at 205°C in a conventional oven. Because of possible variation in flavor and nutrient composition of the fish and other ingredients, the experiment was repeated using ingredients purchased on separate occasions from different vendors.

Sensory evaluation

Twenty-four (trial 1) and 14 (trial 2) experienced taste panelists were chosen from the food science faculty and student body. To mask color differences between the two products, panelist were blindfolded during all sensory tests. Randomly arranged triangle tests were used to determine if a detectable difference existed between sausage balls made from catfish or pork. Blindfolded panelists were also asked to identify the sample or samples made with catfish and to comment on the products. Taste panel trials were conducted on two days to avoid taste fatigue.

Nutrient analysis

Pork and catfish products were analyzed for nutrients, sodium, and potassium as described by Mustafa and Medeiros (1985).

Author Dudley is affiliated with the Dept. of Home Economics, Mississippi State Univ. Mississippi State, MS 39762. Author Medeiros (to whom inquiries should be directed) is now with the Dept. of Home Economics, The Univ. of Wyoming, University Station, Box 3354, Laramie, WY 82071.

Statistical analysis

Statistical analysis of sensory data followed the method of Roessler et al. (1948) for two and three sample testing. Nutrient data were analyzed for statistical difference using the student t-test for samples with an even number of observations (Ferguson, 1976).

RESULTS

TASTE PANELISTS were unable to detect a sensory difference ($P > 0.05$) between the catfish sausage balls and pork sausage balls. In trial 1, 10 of the 24 panelists, and in trial 2, eight out of 14 panelists were able to make the correct choice in triangle tests ($P > 0.05$). Panelists commented that texture differences or seasoning intensity were the only distinguishing characteristics rather than quality of flavor. A "fishy" flavor was not detected in these products. When the blindfolded panelists were asked to identify from texture or flavor only which sample or samples were made from catfish, only four of the eight panelists who correctly identified the odd sample in the second triangle test could distinguish between samples ($P > 0.05$). In trial 1, six of the 10 panelists made correct identifications ($P > 0.05$). Differences which existed were evidently inconsistent and would have limited use as a basis for correct selection of the odd sample.

There was a significant difference between cooked catfish sausage balls and pork sausage balls for protein ($P \leq 0.01$), fat ($P \leq 0.01$), and nitrogen-free extract ($P \leq 0.05$) (Table 2). Pork samples contained more protein and fat and less nitrogen-free extract than catfish samples indicating that calculated physiological caloric values for the pork

Table 1—Formulation of catfish and pork sausage balls

Ingredient	Pork (g/100g)	Catfish (g/100g)
Meat[a]	50.0	50.0
Pork sausage seasoning	1.3	1.3
Cheese	34.0	34.0
Binder[b]	14.6	14.6

[a] Pork sausage (80% lean/20% added fat) or ground catfish
[b] Preformulated biscuit mix

Table 2—A comparison of proximate analysis, sodium and potassium content of catfish or pork sausage balls

	N	Pork[a] (g/100g)	Catfish[a] (g/100g)
Moisture	3	42.30 ± 0.61	46.53 ± 1.41
Protein	7	22.37 ± 0.06	19.95 ± 0.30**
Fat	7	19.10 ± 0.10	14.78 ± 0.53**
Fiber	7	0.99 ± 0.14	0.77 ± 0.07
Ash	7	3.56 ± 0.24	3.54 ± 0.24
Nitrogen free extract	3	12.25 ± 0.07	13.61 ± 0.40*
Sodium	18	0.69 ± 0.43	0.83 ± 0.53
Potassium	18	0.22 ± 0.11	0.22 ± 0.20

[a] Mean ± std. error;
* $p < 0.05$
** $p < 0.01$

samples (310.38 kcal/100g) would also be higher than the catfish sausage balls (267.26 kcal/100g). Moisture, fiber and ash content did not differ ($P > 0.05$) between samples. Differences in sodium and potassium content were not significant ($P > 0.05$).

DISCUSSION

THE OBJECTIONABLE "fishy" flavor of some varieties of fish has hindered the use of fish as meat analogs. The farm-raised catfish has a very mild flavor which could be modified by various meat flavorings and seasonings. Pork sausage seasoning dominates the flavor of pork sausage. In the study, catfish sausage substituted for pork sausage produced a comparable product with the only apparent difference being texture or seasoning strength.

Fish subjected to processing such as deboning, mincing or grinding have been reported to change from a flaky structure to a mushy, random structure (Arocha and Toledo, 1982; Knorr and Regenstein, 1983; Rockower et al, 1983). Catfish fillets used in this study could only be ground once without complete destruction of the flaky structure. Even with the inclusion of other ingredients, the crispy outer crust of the pork sample versus the softer texture of the catfish sample was noticeable to some panelist, but not to the extent that it could be used as a consistent basis of selection in the triangle test.

Panelists who commented that their choices during the triangle test were based on flavor, made their selection on the intensity or strength of flavors. However, the proportion of spice in both products was constant. Differences in the perception of flavor by some panelists can be explained by the random nature of the pork sausage seasoning. Large pieces of pepper may have been dominant in one bite, but completely absent in another.

Since the differences between the two product were not consistent, substitution of catfish sausage for pork sausage as an ingredient in a snack food product is possible. The advantage of this substitution would be to persons who choose to limit fat and calories in their diets. Catfish sausage balls are comparable to pork sausage balls with only minor sensory differences between the two products. Any changes in the formulation designed to further reduce sodium or fat content would be limited because of the textural problems associated with minced or ground fish in processed food products.

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A Research Note

Partial Characterization of a Collagenolytic Enzyme Fraction From the Hepatopancreas of the Freshwater Prawn, *Macrobrachium rosenbergii*

W.K. NIP, C.Y. LAN, and J.H. MOY

ABSTRACT

Textural changes of freshwater prawn during ice-chilled/refrigerated storage are believed in part to be due to an autolytic process. A collagenolytic enzyme fraction, isolated from the hepatopancreas of the freshwater prawn, was most active at 37°C and pH 6.5–7.5 and was also active at 0°C. The addition of this enzyme fraction to prawn tissue increased the hydrolysis of prawn collagen at 0°C for 96 hr indicating that this enzyme fraction might be responsible for the textural change in prawn during ice-chilled storage.

INTRODUCTION

PRAWNS *(Macrobrachium rosenbergii)* produced by aquaculture have a shelf-life of only 2–3 days under ice-chilled conditions (Lee, 1979; Hanson and Goodwin, 1977). Quality deterioration starts with a breakdown of tissue resulting in "mushy texture" after cooking (Nip et al., 1985). This phenomenon is most pronounced in the first section of the abdomen (tail). The cause of this mushiness, which may be due to autolytic enzymes, microorganisms or a combination of both, has not been reported.

Studies on the storage stability of ice-chilled/refrigerated *M. rosenbergii* have been reported by Angel et al. (1981) and Waters and Hale (1981). However, neither of these research groups addressed the mushiness problem in *M. rosenbergii.* Rowland et al. (1982) reported some observations on the trypsin-induced morphological changes in the tails of iced *M. rosenbergii.*

Percent soluble collagen and protein in *M. rosenbergii* increased significantly during ice-chilled storage (Nip et al., 1985). Baranowski et al. (1984) showed the presence of a collagenolytic enzyme in the prawn hepatopancreas. The objective of this study was to isolate and partially characterize this collagenolytic enzyme.

MATERIALS & METHODS

Extraction and chromatographic separation of the collagenolytic enzyme

Aquacultured prawns (11 to 18 counts/kg) obtained live were purged overnight in running tap water at 22°C. They were immobilized in an ice-slurry and the hepatopancreas were removed from the prawns (ea. 30g per kg). The hepatopancreas were homogenized with three volumes of 0.05M tris-HCl buffer at pH 7.4 for 5 min at 4°C. The mixture was centrifuged at 4°C at 42,000 x *g* for 30 min. The supernatant was collected, and the extraction of the residue was repeated. The supernatants were pooled, centrifuged at 4°C for 60 min at 12,000 x *g* and then filtered through a 0.05 μm sterile Nalgene filter under aseptic condition.

The chromatographic separation of the collagenolytic enzyme followed the procedure developed by Eisen et al. (1973). Protein concentration was determined according to the Bio-rad Protein Assay procedure (Bio-rad Inc., 1979). Collagenolytic activity of column effluent was based on the amount of insoluble bovine collagen hydrolyzed in a specific time at 37°C. A reaction mixture containing 1 mL enzyme solution, 1 or 2 mL pH 7.0 0.05M tris-HCl-0.005mM $CaCl_2$ buffer and 8 mg insoluble bovine collagen was incubated at 37°C for 4 or 8 hr. The filtrate from the reaction mixture was hydrolyzed with 6N HCl for 3 hr at 130°C and analyzed for hydroxyproline by the method of Woessner (1961).

Characterization of isolated collagenolytic enzyme fraction

To study the effects of pH, temperature and/or time on the isolated collagenolytic enzyme fraction, insoluble bovine collagen was used as the substrate. The reaction mixture containing 40 μL of enzyme solution and 1 mL of substrate was incubated at 0°C and 37°C for various intervals. The reaction was stopped by adding 2 mL of 95% alcohol. The filtrate was hydrolyzed with 6N HCl and assayed for hydroxyproline (Woessner, 1961).

Tests on prawn tail tissue were conducted to determine the effect of the prawn collagenolytic enzyme fraction on raw prawn tail tissue at ice-chilled condition. The tissue was prepared from the first abdominal sections of raw prawn tails. After deveining, lyophilization and blending, the 40–60 mesh fraction was sterilized with propylene oxide. Three hundred sixty mg prawn tail tissue were suspended aseptically in 100 mL of pre-sterilized appropriate pH buffers. A 1 mL aliquot was transferred aseptically into sterile 10 mL plastic test tubes. Tests at 0°C for 96 hr at pH 6, 7, and 8 were conducted. After incubation, the samples were extracted at 95°C for 1 hr with occasional agitation. After centrifugation at 11,000 x *g* for 30 min and removal of supernatant, the residue was washed with distilled water and re-centrifuged. The supernatants were pooled, hydrolyzed with 6N HCl and analyzed for hydroxyproline (Woessner, 1961). A conversion factor of 2 mg hydroxy-

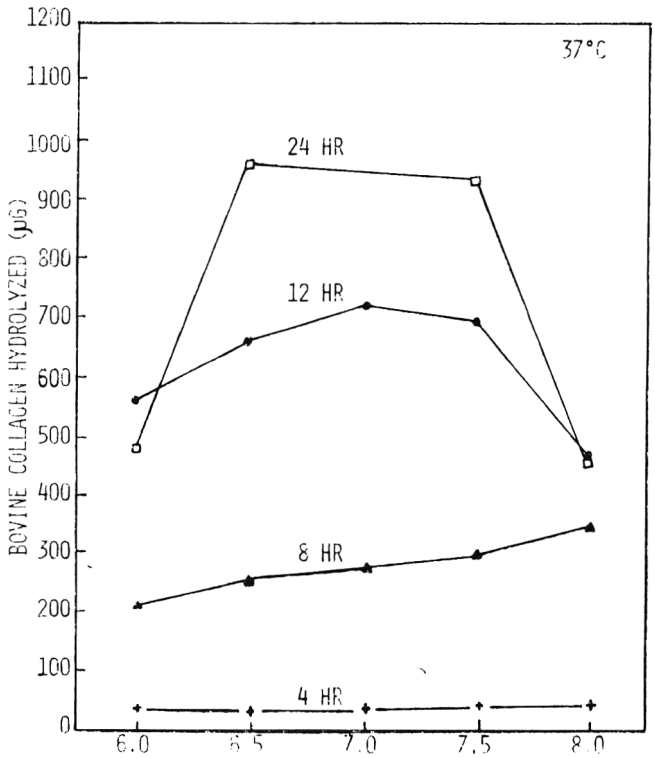


Fig. 1—Effect of pH and incubation time on hydrolysis of insoluble bovine collagen 37° C.

Authors Nip, Lan, and Moy are affiliated with the Dept. of Food Science & Human Nutrition, Univ. of Hawaii at Manoa, 1920 Edmondson Road, Honolulu, HI 96822.

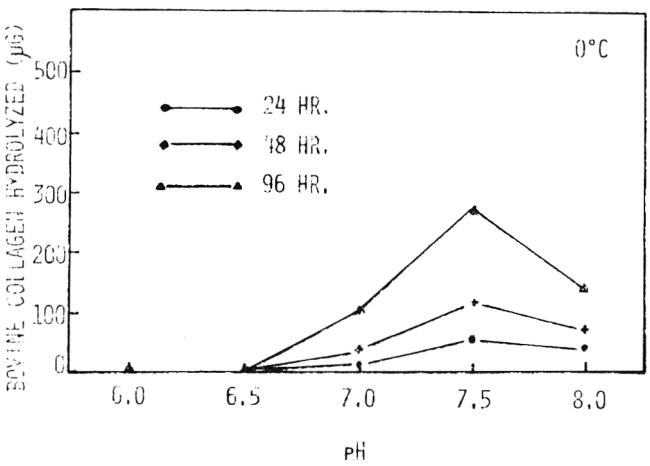


Fig. 2—Effect of pH and incubation time on hydrolysis of insoluble bovine collagen at 0° C.

proline to 100 mg insoluble prawn collagen was used (Nip et al., 1981).

RESULTS & DISCUSSION

Isolation of prawn collagenolytic enzyme

The ammonium sulfate precipitation and chromatographic purification increased the specific activity of the crude enzyme extract from 0.03 μg bovine collagen hydrolyzed/min/mg protein to 23.2 μg bovine collagen hydrolyzed/min/mg protein for the collagenolytic enzyme fraction. The presence of a collagenolytic enzyme in the prawn hepatopancreas confirmed our earlier findings (Baranowski et al., 1984).

Collagenolytic activity

It is clear from Fig. 1 and 2 that this prawn hepatopancreas collagenolytic enzyme was active at a pH range 6 5–7.5. Freshwater prawn showed a similar pH range after immobilization and during ice storage (Nip et al., 1985). At 37°C collagenolytic activity was demonstrated clearly after 4 hr incubation (Fig. 1). Collagenolytic activity was demonstrated at 0°C after 24 hr of incubation or longer at pH 6.5–8.0 (Fig. 2). Because of the difference in optimum pH at 37°C and 0°C, the collagenolytic enzyme might exist in different forms or not be in a pure state.

Effect of prawn collagenolytic enzyme on prawn tissues

Incubation of isolated collagenolytic enzyme with presterilized raw prawn tissue was conducted. The prawn tissue itself hydrolyzed 12.4 μg of prawn collagen at pH 6–8 at 0°C after 96 hr. However, with the addition of the prawn hepatopancreas collagenolytic enzyme the amounts of prawn collagen hydrolyzed were 49.6, 37.2, and 24.8 μg at pH 6.7, and 8, respectively. This confirmed our earlier findings (Baranowski et al., 1984) that collagenolytic enzyme was present in the prawn hepatopancreas and was active in the solubilization of collagen in prawn tissues. This suggested that as the ice-chilled period was prolonged with an increase in pH in the prawn tissues (Nip et al., 1985) collagenolytic enzyme began to act on the prawn collagen and induce mushiness in the tissue.

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A Research Note

HPLC Determination of Sugars in Raw and Baked Sweet Potatoes

DAVID H. PICHA

ABSTRACT

Isocratic HPLC techniques were developed for the quantitative analysis of sugars in raw and baked sweet potato roots. The major sugars in raw roots, sucrose, glucose, and fructose, were quantitated with a cation exchange resin column (Aminex HPX-87C) heated to 75°C using HPLC-grade H_2O as the mobile phase and refractive index detection. The major sugars in baked roots (convection oven or microwaved) were maltose, sucrose, glucose, and fructose. They were quantitated with an aminopropyl bonded phase column (Bio-Sil Amino 5S) at 21°C using a 70% CH_3CN:30% H_2O mobile phase and refractive index detection. Over 95% recoveries of all sugars added to sweet potato roots were obtained.

INTRODUCTION

THE PREVIOUS LITERATURE on sweet potato carbohydrate components is mostly based on total sugar or total reducing sugar measurements with limited information on the individual sugars. Colorimetric procedures for sugar determination (Somogyi, 1952; Ting, 1956) are not specific enough to quantify all the individual sugars. Immobilized enzymes used for sugar analyses require a different enzyme for each sugar assayed (Huntington, 1978). Gas-liquid chromatography (GLC) has been used successfully in quantifying sugars in fruit and vegetable products (Kline et al., 1970) but has the disadvantages of a lengthy derivatization procedure and formation of α- and β- anomers of certain sugars. Paper and thin-layer chromatography (TLC) can separate sugars, but the results are only semi-quantitative.

Advances in high performance liquid chromatography (HPLC) systems have made this technique the method of choice for many researchers interested in sugar determinations (Conrad and Palmer, 1976; Binder, 1980; Jupille et al., 1981; Wilson et al., 1981).

The objective of this study was to develop HPLC procedures for the quantitative analysis of sugars in raw and baked sweet potatoes.

MATERIALS & METHODS

Samples

Six different sweet potato cultivars were evaluated for sugars in the raw and baked conditions. Baked roots consisted of heating in a convection oven for 75 min at 170°C or microwaving at 1500 watts with a frequency of 2450 megahertz for 15 min.

Preparation of root extracts

Raw or baked unpeeled roots were longitudinally cut in half and uniformly grated over the entire surface to a depth of about 3 mm. Grated tissue from six different roots was combined together for each determination. A total of eight determinations per cultivar was made. Exactly 10.00g of randomly selected tissue were homogenized in 80% ethanol for 1 min at high speed using a Virtis 45 homogenizer. The resulting slurry was immediately boiled for 15 min, cooled and filtered through Whatman #4 paper; the residue and original container were washed with additional 80% ethanol and made to a final volume of 100mL. About 5 mL were filtered through a 0.45μ filter membrane before injecting in the HPLC.

Preparation of sugar standards

Sugar standard preparation followed the same procedure as root extract preparation. For a 1% standard of any sugar, 1.000g of analytical grade sugar (Sigma Chemical Co., St. Louis, MO) was homogenized in 80% ethanol, boiled, filtered, made up to 100 mL with 80% ethanol and filtered before injecting into the HPLC.

HPLC analyses of raw roots

A Beckman series 340 liquid chromatograph equipped with a model 112 pump, model 210 injector fitted with a 20 μL sample loop and a model 156 refractive index detector was used. The detector signal was electronically integrated by a Varian 401 integrator in the external standard mode using an attenuation of 16 and a chart speed of 0.5 cm/min. Sugars were separated with a 300 mm x 7.8 mm i.d. column packed with Aminex HPX-87C resin (Bio-Rad Labs, Richmond, CA) heated to 75°C. Plumbed between the injector and the analytical column were respectively: a 2μ Rheodyne 7302 column inlet filter; a 40 x 4.6 mm ion exclusion guard cartridge packed with Aminex HPX-85H resin; and a 40 x 4.6 mm anion/OH guard cartridge packed with Aminex A-25 resin (both from Bio-Rad Labs). The mobile phase was degassed HPLC grade H_2O at a flow rate of 1.2 mL/min.

HPLC analyses of baked roots

A 5 μL sample loop attached to an identical liquid chromatograph, and integrator system as described above was used. Sugars were separated with a 250 mm x 4 mm i.d. column packed with Bio-Sil Amino 5S resin (Bio-Rad Labs) maintained at room temperature (21°C). Plumbed between the injector and the analytical column were respectively: a 2μ column inlet filter as described above and a 40 x 4.6 mm amino bonded phase guard cartridge packed with Bio-Sil NH_2 resin (Bio-Rad Labs). The mobile phase was degassed HPLC-grade 70% CH_3CN:30% H_2O at a flow rate of 1.0 mL/min.

Recovery studies and sugar purity confirmation

The percent recovery of each sugar from both raw and baked roots was determined by weighing out two equal 10.00g portions from the same root tissue; one portion was spiked with a known amount of sugar (1.00g fructose, 1.00g glucose, 2.00g sucrose, 5.00g maltose; or exactly one-tenth this amount) and the other was not. The samples were prepared as described above. Percent recoveries were based on the difference between the total amount in the spiked versus unspiked samples.

Identification and purity of the sugars in both raw and baked sweet potato samples were confirmed by TLC separation on silica gel (Lato et al., 1969).

RESULTS & DISCUSSION

IDENTIFICATION of each sugar was based on HPLC retention times and confirmed by TLC. Detector response to all sugars was linear between 0 to 15% concentration. Less than 3% variability in individual sugar concentration was found between triplicate injections of the same sample. Comparisons between extracting solvents showed 80% ethanol to be superior to CH_3OH/H_2O, CH_3CN/H_2O or

Author Picha is affiliated with the Dept. of Horticulture, Louisiana Agricultural Experiment Station, Louisiana State Univ. Agricultural Center, Baton Rouge, LA 70803.

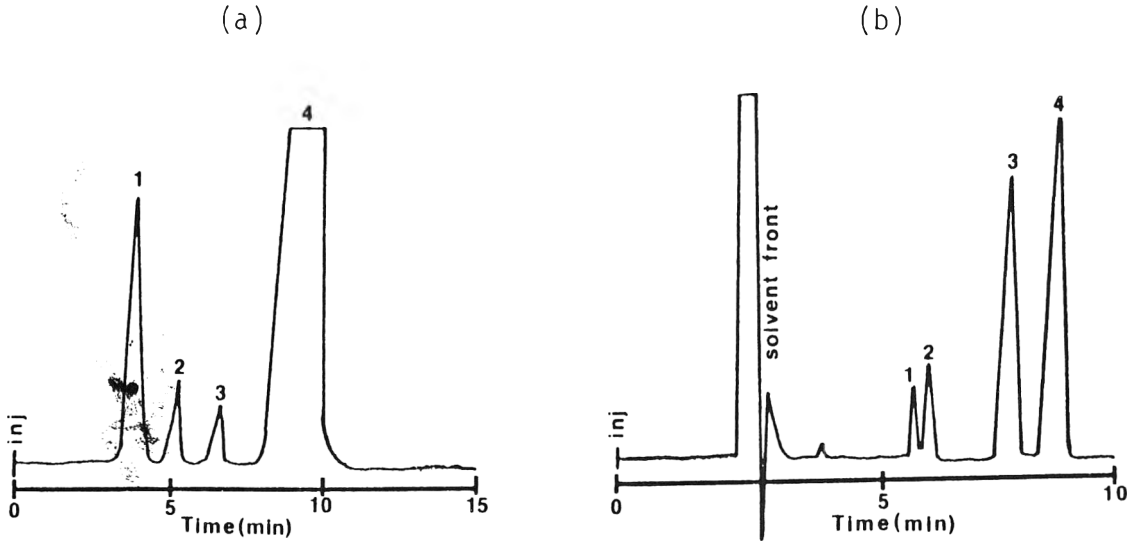


Fig. 1—(a) Typical chromatogram of sugar separation in raw sweet potatoes on an Aminex HPX-87C column at 75°C using a H_2O mobile phase at 1.2 mL/min, refractive index detection and 20 µL injected. Peaks: (1) sucrose; (2) glucose; (3) fructose; (4) ethanol. (b) Typical chromatogram of sugar separation in baked sweet potatoes on a Bio-Sil Amino 5S column at 21°C using a 70% CH_3CN:30% H_2O mobile phase at 1.0 mL/min, refractive index detection and 5 µL injected. Peaks: (1) fructose; (2) glucose; (3) sucrose; (4) maltose.

Table 1—Sugar concentrations in raw and baked roots of six different sweet potato cultivars held at 15.6°C for 3 months

	% Sugar (Fresh weight basis)[a]							
	Sucrose		Glucose		Fructose		Maltose	
Cultivar	Raw	Baked	Raw	Baked	Raw	Baked	Raw	Baked
Centennial	4.10	5.17	0.24	0.27	0.30	0.43	0	9.33
Jasper	3.63	5.14	0.44	0.42	0.43	0.41	0	7.75
Travis	2.87	3.26	1.50	2.73	1.15	1.99	0	4.02
Jewel	2.78	3.98	1.22	1.29	1.01	1.20	0	7.55
Whitestar	2.50	3.35	0.40	0.39	0.39	0.40	0	14.12
Rojo Blanco	1.30	1.59	0.95	1.22	0.65	0.97	0	10.77

[a] Each value represents a total of 48 roots (8 different determinations x 6 roots per determination).

H_2O alone in extracting sugars from sweet potato tissue. The recovery of each sugar added to sweet potato tissue at two different concentrations was: fructose at 0.10% and 1.00%, 95% and 96% recovery; glucose at 0.10% and 1.00%, 96% and 99% recovery; sucrose at 0.20% and 2.00%, 98% and 98% recovery; maltose at 0.50% and 5.00%, 97% and 96% recovery.

Fig. 1a illustrates a typical chromatogram of sugar separation in raw sweet potato roots using an Aminex HPX-87C column. The major sugar, sucrose, elutes first followed by the monosaccharides glucose and fructose. Baseline separation of all sugars occurs within 8 min. After elution of the very large ethanol peak, no other compounds were found. The system was capable of detecting as low as 0.01% of each sugar. Both the disaccharides sucrose and maltose elute at 4.4 min. However, no maltose was found in any raw root sample (determined on the Bio-Sil Amino 5S column described below) so the disaccharide peak for raw sweet potato roots is composed entirely of sucrose.

Fig. 1b illustrates a typical chromatogram of sugar separation in baked roots using a Bio-Sil Amino 5S column. A large ethanol-water solvent peak elutes before the monosaccharides, fructose and glucose, followed by the disaccharides, sucrose and maltose, respectively. Baseline separation existed between sucrose and maltose and about 80% of baseline separation existed between fructose and glucose. Total analysis time for all four sugars was 10 min. The system was capable of detecting as low as 0.01% sucrose and maltose.

Sugar concentration in raw and convection oven baked roots of six different sweet potato cultivars stored for 3 months at 15.6°C is listed in Table 1. Large differences in sugars exist within the sweet potato germplasm. Sucrose was the most abundant sugar in raw roots of all cultivars. Glucose was the second most abundant sugar in three out of six cultivars, with the other three cultivars having about the same concentration of glucose and fructose. Travis, Jewel and Rojo Blanco had a better balance of the three sugars while Centennial, Jasper and Whitestar roots contained very little glucose and fructose. Maltose was the most abundant sugar in baked roots of all cultivars followed by sucrose and then the monosaccharides. Sucrose was higher in baked roots compared to raw roots of all cultivars while the monosaccharides increased in the baked state in only several cultivars. Sugar concentrations in microwaved roots were similar to those reported in Table 1 for the convection oven treatment.

Quantitation of maltose and sucrose in baked roots can only be made using the Bio-Sil Amino 5S column since these disaccharides coelute on the Aminex HPX-87C. Sugars in raw roots can be quantitated using either column since maltose was not found. Run times were shorter on the Bio-Sil column and no column heater was necessary, but the mobile phase required CH_3CN which is considerably more expensive than H_2O. With each system, the amount of sample preparation was limited and excellent sugar resolution resulted without the presence of interfering compounds.

—Continued on page 1210

A Research Note

Effects of Blanching, Soaking, and Cooking on the HCN Yields, Nitrogen, Ash, and Minerals of Lima Beans (*Phaseolus lunatus*)

M. A. AKPAPUNAM

ABSTRACT

Blanching, soaking, and cooking two cultivars of lima bean reduced the HCN concentrations to a safe level. Beans soaked for 12 hr had lower HCN levels than those soaked for 6 hr. Traditional processing was also effective in reducing the HCN concentration in the beans. The treatment of the beans also resulted in a reduction in ash, and the concentration of some minerals.

INTRODUCTION

LIMA BEAN (*Phaseolus lunatus*) is one of the pulses used as human food in Nigeria. The dried shelled beans are commonly used in the same way as cowpeas (Aykroyed and Doughty, 1964; Akpapunam and Markakis, 1981). Cultivation and utilization of the beans in Nigeria are, however, on the decline. One major reason for this is the high amount of cyanogentic glucoside in the beans (Oyenuga, 1968). Under damp conditions, or when the tissues are broken down by chewing or grinding, an enzyme present in the seeds causes the liberation of hydrogen cyanide (Purseglove, 1977), making raw or improperly cooked beans unsafe for consumption.

Preparation of the beans for human consumption is usually a long and tedious task. The seeds are cooked in three stages during which the water from the first and second cookings are normally discarded. This method is known to reduce the HCN concentration to innocuous levels (Oyenuga, 1968). Apart from being tedious, such cooking method could lead to great losses in nutrients.

Lima bean cultivars from various parts of the world vary widely in HCN-producing capacity. Montgomery (1969) reported HCN values of 312 mg/100g, 300 mg/100g, and 210 mg/100g for lima beans from Java, Puerto Rico, and Burma, respectively, while values as low as 17 mg/100g and 10 mg/100g were reported for some American varieties.

Since current research efforts in Nigeria have not produced lima bean varieties with low levels of HCN, it is necessary to find ways to reduce the toxic components in the beans. The present study was therefore undertaken to find simpler and more efficient ways of processing the beans to reduce their HCN concentrations. The study also evaluated the efficacy of the traditional processing of lima beans for which there are no records.

MATERIALS & METHODS

ONE-HUNDRED FIFTY grams lima bean cultivars (broad and small limas) obtained from a local market in Bendel State, Nigeria, were blanched at 100°C for 5 min then soaked in 28°C distilled water for 6 hr or 12 hr and drained. One hundred grams of the presoaked beans were dried in an oven at 100°C for 6 hr while the remaining 50g were cooked to tenderness (approximately 1½ hr) in a glass beaker. The cooked beans were drained and oven dried at 100°C for 6 hr. All samples treated as above were ground to 60 mesh size and stored at 5°C for analysis.

Author Akpapunam is affiliated with the Dept. of Food Science & Technology, Rivers State Univ. of Science & Technology, P.M.B. 5080, Port Harcourt, Nigeria.

Traditional cooking

Fifty grams of each of the two lima bean cultivars were cooked to tenderness in a covered glass beaker. The cooking was done in three stages of 30 min intervals; the water from the first and second stages were discarded before the final cooking. After cooking, the beans were drained, oven dried at 100°C for 6 hr and ground to 60 mesh size.

Chemical analysis

Hydrogen cyanide (HCN) was determined using the iodimetric method of Knowles and Watkins (1950), while nitrogen and ash were determined by the AOAC (1975) methods.

The minerals, P, Fe, and Ca were analyzed according to the AOAC (1975) standard methods; P and Fe colorimetrically, and Ca titrimetrically following precipitation as calcium oxalate. Na and K were analyzed by a flame photometric method (Pearson, 1976).

RESULTS & DISCUSSION

TABLE 1 shows the hydrogen cyanide yields and nitrogen and ash contents of the raw and treated lima bean cultivars. Both the traditional processing and the treatments described above were effective in reducing the HCN concentrations in the beans to levels below the 20 mg/100g recommended safe limits (Montgomery, 1969). When the broad and small blanched bean cultivars were soaked for 6 hr, a reduction of 47.3% and 49.6% in HCN were observed, while soaking for 12 hr resulted in 69.3% and 71.9% reduction, respectively. Cooking the soaked beans resulted in a further 40.5% average reduction in HCN for beans soaked 6 hr, while a reduction of 20.8% and 19.4% were observed, respectively, for broad and small beans soaked 12 hr.

Table 1—Hydrogen cyanide yields, nitrogen and ash contents of raw and treated lima beans (dry weight basis)[a]

Treatment	Hydrogen Cyanide Yield (mg/100g)	Total Nitrogen (%)	Ash %
Raw			
Broad lima	113.2 ± 8.8	3.6 ± 0.1	3.8 ± 0.2
Small lima	143.0 ± 5.1	3.7 ± 0.1	3.9 ± 0.1
Soaked 6 hr			
Broad lima	65.9 ± 1.3	3.6 ± 0.2	3.7 ± 0.1
Small lima	72.0 ± 3.7	3.5 ± 0.1	3.8 ± 0.3
Soaked 12 hr			
Broad lima	34.7 ± 2.9	3.7 ± 0.1	3.4 ± 0.2
Small lima	40.1 ± 4.9	3.8 ± 0.3	3.2 ± 0.3
Soaked 6 hr-cooked			
Broad lima	13.8 ± 1.2	3.5 ± 0.2	3.4 ± 0.1
Small lima	14.1 ± 3.8	3.6 ± 0.3	3.5 ± 0.1
Soaked 12 hr-cooked			
Broad lima	11.2 ± 1.2	3.5 ± 0.2	2.8 ± 0.4
Small lima	12.5 ± 2.5	3.4 ± 0.3	2.5 ± 0.1
Traditionally cooked			
Broad lima	14.7 ± 4.9	3.4 ± 0.1	2.1 ± 0.2
Small lima	15.1 ± 3.2	3.5 ± 0.2	2.3 ± 0.1

[a] Mean of four determinations ± S.D.

Table 2—Mineral content of raw and treated lime beans (mg/100g dry weight)[a]

Treatment	Calcium	Phosphorus	Iron	Sodium	Potassium
Raw					
Broad lima	89.5 ± 1.2	451.0 ± 3.6	6.7 ± 0.1	11.6 ± 1.2	1501.3 ± 13.6
Small lima	108.7 ± 1.9	489.3 ± 4.1	7.2 ± 0.2	10.9 ± 0.6	1609.7 ± 15.0
Soaked 6 hr					
Broad lima	89.4 ± 2.1	449.3 ± 2.4	6.4 ± 0.4	11.7 ± 0.4	1499.6 ± 21.0
Small lima	109.1 ± 1.4	489.1 ± 5.7	7.3 ± 0.3	10.2 ± 0.3	1589.9 ± 30.1
Soaked 12 hr					
Broad lima	89.1 ± 1.0	446.4 ± 3.2	6.5 ± 0.1	11.5 ± 0.8	1467.9 ± 9.7
Small lima	107.9 ± 0.8	487.8 ± 5.3	7.1 ± 0.1	10.6 ± 1.0	1561.1 ± 5.5
Soaked 6 hr-cooked					
Broad lima	88.5 ± 0.5	443.6 ± 4.0	6.1 ± 0.3	9.3 ± 0.9	873.8 ± 4.2
Small lima	107.1 ± 0.4	475.3 ± 1.3	7.1 ± 0.4	9.7 ± 0.2	953.6 ± 11.2
Soaked 12 hr-cooked					
Broad lima	88.3 ± 0.6	440.8 ± 6.7	5.8 ± 0.1	8.9 ± 0.6	781.7 ± 15.8
Small lima	107.1 ± 1.3	470.7 ± 5.8	6.6 ± 0.2	7.8 ± 1.2	891.3 ± 5.6
Traditionally cooked					
Broad lima	87.5 ± 1.7	431.8 ± 7.2	6.1 ± 0.4	6.3 ± 0.1	661.5 ± 11.7
Small lima	101.4 ± 0.6	461.3 ± 6.6	6.4 ± 0.3	5.8 ± 0.4	709.3 ± 18.0

[a] Mean of four determinations ± S.D.

Traditionally, cooked beans lost 87.0% and 89.4% of their HCN concentrations, respectively.

There were slight changes in total nitrogen of the bean cultivars following treatment. The ash content, however, decreased considerably. The decreases were more pronounced in the traditionally cooked beans and the beans soaked for 12 hr before cooking. The loss of ash content may be due to leaching of some of the mineral elements into the soaking and cooking water.

There were noticeable reductions in the mineral level of the beans following various treatments (Table 2). The most seriously affected were K and Na. The losses were highest for traditionally cooked beans followed by the beans cooked after 12 hr and 6 hr soaking, respectively. The mineral losses may be due to the resultant leaching losses observed for the ash contents of the samples.

The results of this study show that it may be possible to prepare lima bean seeds with low levels of HCN.

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A Research Note

Changes in Flavor Components of Garlic Resulting from Gamma Irradiation

JOONG HO KWON and HYUNG SIK YOON

ABSTRACT

Formation of diallyl disulfide, identified as a main flavor component of garlic, was gradually decreased by irradiation of 0.05-0.5 kGy, but increased slightly at higher doses of 1.0-3.0 kGy. In garlic stored for 5 months, a dose of 0.1 kGy had little influence on the amount of diallyl disulfide, but higher dose irradiation caused an apparent reduction in the amount of the component.

INTRODUCTION

IRRADIATION with ionizing radiation has been applied in the fields of sprout inhibition, disinfestation of insects, sterilization, delay of fruit ripening and improvement of sensory properties in foods. Recently, the storage of garlic utilizing gamma irradiation was investigated by many researchers (Khan and Wahid, 1978; El-Oksh et al., 1971; Kwon et al., 1984), and it has been made clear that irradiation doses of 50-150 Gy effectively inhibited sprouting and prevented spoilage and weight loss during storage. Matsushima et al. (1974) reported that the sprout inhibition of onions with irradiation appeared to suppress the development of di-n-propyl disulfide, a main flavor component of onions. In Korea, garlic is widely used as a condiment and as a food. Since Semmler (1892) first described the importance of diallyl disulfide and diallyl trisulfide in the flavor of garlic distillates, Saghir et al. (1964) and Brodnitz et al. (1971) have attempted the identification of its flavor components obtained from headspace vapor and solvent extract, respectively, and the alliinase in garlic was partially purified (Mazelis and Crews, 1968). In relation to garlic preservation, however, the chemical or biochemical aspects of irradiation effects on its flavor have not yet been investigated except for partial studies on enzyme activity (Wadanabe and Tozaki, 1967). This study concerns investigations on the effect of gamma irradiation at several doses on the flavor component of garlic and the changes in the amount of this component during storage.

MATERIALS & METHODS

Materials

The garlic bulbs (*Allium sativum* L.) used were obtained from a local farm at the time of harvest (July, 1982). They were cured in circulating air at 22-25°C and a relative humidity (RH) of 60-65% for 4 wk after harvest.

Irradiation and storage

The cured garlic bulbs were irradiated by a 10 kilo curie Co-60 gamma irradiator (U.K., 25 Gy/hr) with doses of 0, 0.05, 0.1, 0.5, 1.0 and 3.0 kGy, and stored at 3±1°C and a R.H. of 75-85% for 5 months.

Author Kwon is affiliated with the Korea Advanced Energy Research Institute, Seoul, 130-02, Korea. Author Yoon is with the Dept. of Food Technology, Kyungpook National Univ., Daegoo, 635, Korea.

Determination of main flavor component

The flavor components were extracted by soaking five unpeeled garlic cloves in five times their volume of Spectro-Grade hexane (Oaks et al., 1964). Garlic extract was fractionated with a Varian 3700 gas chromatograph equipped with flame photometry detector, and peak areas were calculated with a Varian CDS-III data system. Identification of diallyl disulfide, a main component, was carried out by comparison of the retention time, and gas chromatography-mass spectrometry (GC-MS, Varian MAT 212 system) was also used.

RESULTS & DISCUSSION

PRELIMINARY STUDIES showed the optimum extraction time of flavor components with hexane was 3 hr at 25°C. A gas chromatogram of the hexane extract in garlic represented a relatively simple pattern, and a main peak, which was more than 70% of the total peak area, was identified as diallyl disulfide by comparison to the retention time of a reference compound, and by GC-MS analysis. This result

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Table 1—Effect of gamma irradiation dose on the formation of diallyl disulfide in hexane extract of garlic

Irradiation dose (kGy)	Relative amount (%)
0	100
0.05	95 ± 1.24[a]
0.1	94 ± 1.02[a]
0.5	84 ± 2.16[a]
1.0	89 ± 1.03[a]
3.0	90 ± 1.54[a]

[a] Mean ± standard deviation of triplicate experiments

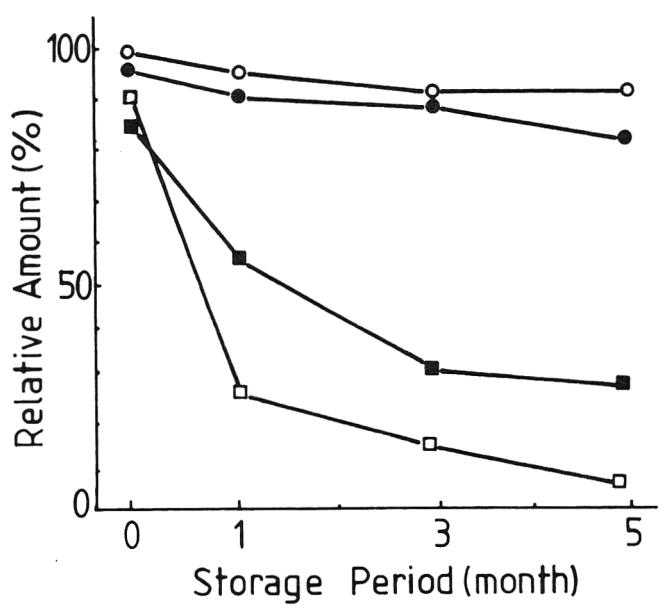


Fig. 1—Effect of gamma irradiation on diallyl disulfide content of garlic during storage at 3±1°C. Each value is the mean of triplicate experiments; ○, 0 kGy; ●, 0.1 kGy; ■, 0.5 kGy; □, 3.0 kGy.

A Research Note

Enzymatic Browning in Five Olive Varieties

V. SCIANCALEPORE

ABSTRACT

Enzymatic activities (polyphenoloxidase and peroxidase) and o-diphenols content were estimated in fruits of five olive varieties differing in browning rate. Significant differences were observed among the varieties. The browning rate correlated with o-diphenols content and polyphenol oxidase activity, but not with peroxidase activity.

INTRODUCTION

IN A PREVIOUS PAPER it was reported that the large differences in the enzymatic browning of five olive varieties were directly related to the polyphenol oxidase (PPO) (E.C. 1.14.18.1) activities of the tissue (Sciancalepore and Longone, 1984). In the course of this study, it was noted that many tissues with high peroxidase (POD) (E.C. 1.11.1.7) activities were characterized by low browning rate, relative to tissues with low POD activities. Therefore, it was of interest to determine whether there was an inverse relationship between browning rate and POD content in green olives.

The relationship between enzymatic browning, o-diphenols content, activities of PPO and POD in the fruits of five olive varieties is examined here.

MATERIALS & METHODS

GREEN OLIVES of the following varieties were used: *Ascolana Tenera, Bella di Spagna, Pasola, San Agostino,* and *San Francesco.* The fruits were hand-picked in September 1984 from trees grown at the horticultural orchard of Bari University (Italy). Three samples were harvested for each variety and analyzed immediately.

Sample preparation

Fresh fruit pulp (100g) of each olive sample was partially defatted by homogenizing twice with 200 mL chilled, glass-distilled petroleum ether in an Ultra-Turrax homogenizer for 5 min each time, and excess solvent was removed through Whatman No. 1 filter paper on a Buchner funnel. The resulting material was dried overnight at room temperature (25°C) to remove residual solvent. This olive sample was used for extraction of enzymatic solution.

Preparation of the crude enzymatic extract

Partially defatted olive sample was homogenized for 2 min with 100 mL 0.05M sodium phosphate buffer (pH 6.2 and 5g Polyclar AT to bind phenolic compounds occurring naturally in the fruit. The suspension was squeezed through eight layers of cheesecloth, and the filtrate centrifuged at 18,000 x *g* for 20 min. The supernatant (crude extract) was dialyzed overnight against extraction buffer. After dialysis, enzymatic activity of crude extract was determined.

PPO activity was assayed in a reaction mixture that included, in a total volume of 6 mL, 3 mL 0.1M sodium citrate buffer (pH 5), 2 mL 0.02M 4-methyl catechol (substrate) in the same buffer, and various amounts of the crude extract. Absorbance at 410 nm was recorded with a Varian 950 spectrophotometer. Activity was linear with time for the first 60 sec of the reaction.

POD activity was assayed in a reaction mixture that included, in a total volume of 3 mL, 2.2 mL 0.05M citrate-phosphate buffer (pH 5.5), 0.2 mL of the same buffer containing 0.5% guaicol and 0.15% hydrogen peroxide, and various amounts of the crude extract. Absorbance at 470 nm was recorded with a Varian 950 spectrophotometer. Activity was linear with time for the first 60 sec of the reaction.

Protein concentrations were measured by the method of Lowry et al. (1951) using bovine serum albumin as the standard protein.

o-Diphenols of fresh olive pulp were determined by the method of Vazquez et al. (1971) and expressed as mg pyrocatechol per 1g fresh fruit pulp, using a calibration curve of pyrocatechol treated with the same method.

Browning rate of fruit homogenate was determined as described in a previous paper (Sciancalepore and Longone, 1984).

Statistical analysis

Analysis of variance (Snedecor and Cochran, 1967) was applied to all data. Duncan's (1955) new multiple range test was used to locate differences among means.

RESULTS & DISCUSSION

THE MEAN VALUES of the characteristics studied in the mesocarps of five olive varieties are arranged according to browning rate, with *Ascolana Tenera* having the highest browning rate (Table 1). This variety also had the highest PPO activity, which was significantly correlated (r = 0.992) with the browning rate (Fig. 1). The varieties showed sig-

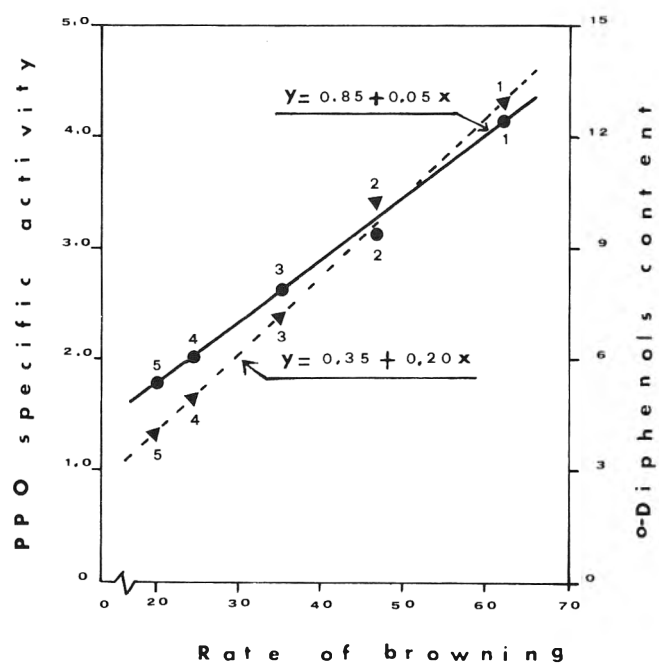


Fig. 1—Relationship between rate of browning and PPO specific activity (—●—●—) and o-diphenols content (—▲—▲—) in five olive varieties. Varieties no. 1 - 5 are reported in Table 1. Units used for PPO specific activity, o-diphenols content and browning rate are referred in subscripts to Table 1.

Author Sciancalepore is affiliated with Istituto di Industrie Agrarie, Universita degli Studi di Bari, via Amendola 165/a, 70126 Bari, Italy.

Table 1—Means[a] for browning rate, PPO, and POD activities, and o-diphenols content in five olive varieties

Variety	Browning rate[b]	Specific activity[c] (OD/min/mg protein) PPO	POD	o-Diphenols[d]
1. *Ascolana Tenera*	62.8 A	4.05 A	2.05 B	12.62 A
2. *San Agostino*	47.1 B	3.14 B	1.84 B	10.33 B
3. *Bella di Spagna*	33.8 C	2.73 BC	3.92 A	7.32 C
4. *San Francesco*	24.8 D	2.05 CD	1.82 B	4.80 D
5. *Pasola*	19.9 D	1.84 D	1.62 B	4.31 D

[a] Where letters following means within a column differ, means differ significantly ($P < 0.01$).
[b] Browning rate is expressed as absorbance at 410 nm per gram weight of fruit pulp after 5-min incubation.
[c] Enzymatic activity and protein content were determined in the dialyzed crude extract (18,000 x g supernatant) of olive mesocarp; PPO and POD activities were assayed respectively at 410 and 470 nm.
[d] o-Diphenols are expressed as mg of pyrocatechol per 1g fresh fruit pulp.

nificant differences in o-diphenol contents; the values correlated positively (r = 0.994) with the browning rate (Fig. 1). The highest POD activity was observed in *Bella di Spagna* variety which had a medium browning rate compared with those of the other varieties tested. No correlation was found between POD activity and browning rate of the crude homogenates of olives, in agreement with data of Sharon-Raber and Kahn (1983) on avocado mesocarp of six cultivars. This latter finding, therefore, does not support our assumption of the existence of an inverse relationship between browning rate and POD activity in olives.

This study provides more information on the browning of green olives and may be useful in selecting varieties of fruit with low rate of browning.

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FLAVORS IN GAMMA IRRADIATED GARLIC . . . From page 1193

confirmed prior reports on garlic flavor components (Freeman and Mossadeghi, 1971; Oaks et al., 1964; Bordnitz et al., 1971).

Table 1 shows the effect of gamma irradiation dose on the formation of flavor component in a hexane extract of garlic. The amount of diallyl disulfide was gradually decreased by irradiation with doses from 0.05-0.5 kGy as compared with a nonirradiated sample, while at higher doses the reduction was not as great. The results obtained in this study correlated in some degree with the reports on gamma-irradiated onions (Kawakishi et al., 1971). During storage, the amount of diallyl disulfide decreased depending upon irradiation dose increase (Fig. 1). After 5 months of storage the amount of diallyl disulfide was reduced by 0.5 and 3.0 kGy irradiation, whereas an irradiation dose of 0.1 kGy, which appeared to be optimum for sprout inhibition in garlic (Kwon et al., 1984), only brought about a slight decrease in the amount of diallyl disulfide, as compared with the nonirradiated garlic.

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A Research Note

Effect of Fertilization, Postharvest Handling and Blanching Temperature on the Drained Weight of Canned Summer Squash

W. C. HURST, V. N. M. RAO, D. GRANBERRY, G. SOCHA, and P. E. KOEHLER

ABSTRACT

There was no apparent effect on drained weight or firmness when three rates of calcium fertilization and two rates of potassium fertilization were applied to a test planting from which two harvests of raw summer squash were canned. However, high Ca (L_2) decreased dry matter and increased structural carbohydrates and tissue Ca in the canned product. Precooling increased drained weight and dry matter, but drastically reduced firmness of the canned product. Noncooled canned squash had lower drained weights and pH, but greater firmness and higher water soluble pectin and cellulose content. Blanching at 99°C increased drained weight; blanching at 88°C decreased drained weight, but increased calgon-soluble pectin content.

INTRODUCTION

CANNED SUMMER SQUASH is an important commodity marketed to institutional food service businesses – cafeterias, restaurants, and public school systems – for meal preparation. In 1982, 636,000 cases of #10 cans of processed squash and pumpkin were sold to this clientele, while an additional 1.4 million cases of #303 cans were distributed to retail grocers for sale (Almanac, 1984). A major quality control problem of squash canners is the textural breakdown during heat processing, resulting in low cut-out squash drained weights. Consequently, most packers overfill raw product into cans before processing in order to satisfy minimum USDA drained weight standards after processing. This causes both economic and legal problems for the processor.

It has been shown that cultural practices, such as low potassium (K) fertilization rates (Van Buren and Peck, 1981) and preharvest calcium (Ca) sprays (Morris et al., 1980; Eaves et al., 1972), increased the firmness of canned green beans, canned raspberries and canned blackberries. Longer postharvest holding time and higher temperatures have caused firmer canned peas (Weckel et al., 1964) and higher drained weights in canned snap beans (Sistrunk, 1965). Whittenburger (1952) has shown that hydrocooling cherries after picking promoted firming and increased the drained weight of the heat processed product. Several authors (Van Buren et al., 1960; Sistrunk, 1969) suggested low temperature blanching to promote firming of canned vegetables, but Tressler and Evers (1957) recommended that squash be blanched 3 min in boiling water.

Since earlier studies by Hurst et al. (1981) have shown that Ca addition during processing did not improve canned squash drained weights, this study was initiated to determine the influence of K and Ca fertilization, postharvest cooling and blanching temperature on the drained weight of canned squash.

Authors Hurst, Rao, Socha, and Koehler are with the Food Science Division, The Univ. of Georgia, Athens, GA 30602. Author Granberry is affiliated with the Horticulture Dept. and is located at the Extension Research Center, P.O. Box 189, Attapulgus, GA 31715.

MATERIALS & METHODS

HYRIFIC (straight neck) variety squash were cultivated in a sandy loam soil whose fertility and pH (6.4) had been previously checked to insure plant nutrient availability. Two levels of potassium fertilizer (K_2O/KCl) supplied at a rate of 163 kg/ha (P_1) and 258 kg/ha (P_2), and three levels of calcium fertilizer ($CaCO_3$) supplied at a rate of 855 kg/ha (L_0), 1136 kg/ha (L_1) and 1500 kg/ha (L_2) were applied by rototilling into the soil of test plots. Following squash seed planting, appropriate irrigation, disease, insect, and weed control measures were taken to insure plant viability. The field experiment consisted of six treatments and three replications in a randomized block design. Immature squash were hand harvested when their diameter reached approximately 3.5 cm and placed in mesh bags. Bags were segregated by fertilizer treatment and either precooled (PC) to 7°C, or held (NC) 18 hr at ambient conditions (34°C) before processing. Squash were taken from two harvests of three replications of each treatment and bags were transported 322 km to a commercial canning plant. On arrival, squash were cleaned and cut into uniform 3.5 cm cross-sectional slices. Two blanching temperatures, 99°C (HB) and 88°c (LB), were employed before filling the squash by hand into 303 x 406 plain enameled cans. Fill-in-weights were recorded, and processing followed the procedure outlined by Hurst et al. (1981) using an approved scheduled process.

Analytical procedures

Triplicate squash cans of each treatment were analyzed for % drained weight, sloughing, dry matter, pH, shear values, structural carbohydrates and K and Ca content. Percentage drained weight was determined according to standard methods (USDA, 1959). Sloughing or the amount of insoluble solids precipitated in the bottom of the can (as sediment), dry matter and pH were determined by the procedure of Hurst et al. (1981). Shear force was determined using a Kramer standard shear cell on an Instron Universal Testing Machine, Model 1130. A load cell calibrated to a full scale of 22.7 kg was used to shear 50g drained canned squash. A crosshead speed of 12.7 cm/min was used. Shear energy value were calculated by integrating the areas under the shear force curves using a planimeter. Structural carbohydrates, including water soluble pectin (WSP), calgon-soluble pectin (CSP), protopectin (SP), hemicellulose (Hemi), and cellulose (CEL) were determined on each 0.25g squash (dry weight) sample according to the procedures described by Smittle et al. (1980). K and Ca contents were determined by Emission Plasma ICP. Sample preparation followed the procedure outlined by Hurst et al. (1981). All data were analyzed statistically on the IBM 370 SAS package. Duncan's multiple range test was applied to the means of main effects when F values were significant.

RESULTS & DISCUSSION

POSTHARVEST COOLING and blanching temperature had the most pronounced effect on canned squash drained weight while K and Ca fertilization apparently had no effect (Table 1). A significant interaction of K x Ca (not graphed) showed that the lowest drained weight (71.34%) was exhibited by squash of the first harvest grown in P_1 x L_2 test plots, while squash from the second harvest grown in the same fertilization regime exhibited the highest drained weight (76.02%). This indicates that the role of Ca in fruit development may change as the growing plant ages. Drained weights were highest (75.30%, 76.00%) in both harvests when P_1 x L_0 fertilization regimes were utilized. There was no effect on any of the variables analyzed due to the level of K applied, (Table 1 and 2).

Table 1—Effects of K and Ca fertilization, postharvest handling and blanching temperature on the drained weight and firmness of canned squash[a]

Main effects	Drained weight[b] (%)	Dry matter[c] (%)	Sloughing (ml)	Shear force[d] (N/50g)	Shear energy[d] (J/50g)	Squash pH (tissue)	K[d] (ppm)	Ca[d] (ppm)
K^+ Fertilization								
P_2	74.64a	6.08a	4.5a	46.60a	635.94a	5.40a	62.33a	7.29a
P_1	74.63a	5.99a	4.1a	49.92a	672.94a	5.39a	63.71a	7.03a
Ca^{++} Fertilization								
L_2	74.53a	5.53b	5.2a	46.40a	601.86a	5.38a	61.21a	7.76a
L_1	74.24a	6.27a	3.6a	49.80a	674.58a	5.39a	61.28a	7.70a
L_0	75.15a	6.28a	4.2a	48.62a	688.40a	5.41a	66.33a	6.19b
Postharvest Trt.								
PC	75.24a	6.34a	4.8a	39.59b	559.48b	5.43a	60.82a	7.29a
NC	73.98b	5.68b	3.8a	57.57a	756.42a	5.36b	65.32a	7.03a
Blanch Trt.								
HB (99°C)	75.52a	6.18a	5.5a	47.00a	607.99a	5.39a	61.18a	7.40a
LB (88°C)	73.79b	5.86b	3.2a	49.44a	698.60a	5.39a	64.87a	6.92a

[a] Mean separation within columns and main effects by Duncan's multiple range test, 5% levels
[b] % drained weight = drained weight/fill-in-weight x 100% (wet basis)
[c] Calculated as % dry matter
[d] Wet weight measurement

Table 2—Effects of K and Ca fertilization, postharvest handling and blanching temperature on the % structural carbohydrates of canned squash[a]

Main effects	Pectic substances[b] %WSP	%CSP	%SP	Hemicellulose[b] (%)	Cellulose[b] (%)
K^+ Fertilization					
P_2	147.83a	44.04a	16.46a	35.42a	29.53a
P_1	140.06a	44.84a	18.64a	32.60a	27.81a
Ca^{++} Fertilization					
L_2	152.91a	50.65a	22.18a	37.79a	31.91a
L_1	144.50ab	43.94ab	14.50b	29.06b	27.88b
L_0	133.63b	38.38b	15.69b	34.84ab	27.56b
Postharvest Trt.					
PC	138.22b	41.43a	17.55a	36.63a	27.60b
NC	149.51a	47.47a	17.59a	31.33a	30.63
Blanch Trt.					
HB (99°C)	141.92a	38.83b	16.33a	35.63a	30.00a
LB (88°C)	145.74a	49.84a	18.76a	32.40a	28.23a

[a] Mean separation within columns and main effects by Duncan's multiple range test, 5% level
[b] % represents 1 x 10^3 actual value (wet basis)

However, high Ca (L_2) decreased dry matter and increased the % structural carbohydrates (Table 2) and tissue Ca in canned squash (Table 1). Increases in the pectic substances (WPS, CPS, SP) may be attributed to the higher concentration of Ca^{++} ions in the L_2 tissue which would lead to greater pectin solubility during heating (Doesburg, 1965; Van Buren, 1979). However, there is no ready explanation why the Ca concentration in L_1 squash did not affect the structural carbohydrates similarly. There was no change in firmness, drained weight, sloughing or dry matter content among the Ca fertilization treatments, except in L_2 where dry matter decreased. The fact that L_2 drained weight was not affected by lower dry matter may be explained by the higher % Hemi and % Cel which would be available for hydration (Sterling and Shimazu, 1961) upon heating. Thus, a greater % of the drained weight may have been water to replace the dry matter loss. Precooling increased canned squash drained weight and dry matter at the apparent expense of decreasing firmness (Table 1). Precooling apparently reduced enzymatic tissue activity yielding a lower % Cel (Table 2) which resulted in a softer product. In contrast, non-cooled squash were significantly firmer and exhibited higher shear values. These may have been caused by the holding conditions (34°C; 18 hr) and the presence of ca^{++} ions which activated cellular enzymes to initiate the pectin demethylation process (Bartolome and Hoff, 1972) leading to a surface-toughening effect on the squash tissue (Whittenberger and Nutting, 1950). Increases in % WSP and % Cel (Table 2) further substantiate enzymatic activity and the likelihood of increased firmness in non-cooled squash. Although statistically different, changes in pH between pre-cooled and noncooled squash are not thought to be of practical importance (Table 1). Blanch temperature had no effect on firmness or dry matter of canned squash, but high blanching (99°C) increased drained weight (Table 1). Low blanching (88°C) may have been insufficient to inactivate, but instead activated, tissue degrading enzymes leading to increased % CSP (Table 2). This fact becomes more plausible when considering filled cans were held 2 hr before sealing and processing.

In summary, the commercial squash packer could benefit economically (increased drained weight) and legally (avoidance of over-filling) by the precooling and high temperature blanching of squash during processing. However, in light of its decreased firmness, a sensory evaluation of precooled canned squash should follow to determine its consumer acceptance.

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A Research Note

Stability of 3,6-Dimethyl-2,3,3a,4,5,7a-hexahydrobenzofuran in Aqueous Buffer Solutions

R. HUOPALAHTI, and E. HÄNNINEN

ABSTRACT

3,6-Dimethyl-2,3,3a,4,5,7a-hexahydrobenzofuran, one of the major aroma compounds in the dill herb, was isolated from the flowering dill and its stability in aqueous buffer solutions in the pH range 2 - 8 at different temperatures between 5 and 100°C was tested. The decomposition followed first-order kinetics and was dependent on the pH of the solution. The studied compound had maximal stability at pH 4. The half-life of the benzofuranoid at 20°C and pH 4 was 122 days.

INTRODUCTION

3,6-Dimethyl-2,3,3a, 4,5,7a-hexahydrobenzofuran has been identified in the dill herb by many investigators (Belafi-Rethy et al., 1974; Embong et al., 1977; Chou and Iwamura, 1978; Göckeritz et al., 1979; Lawrence, 1980; Schreier et al., 1981; Huopalahti et al., 1981; Porter et al., 1983), but its amount has been reported to vary widely depending on different factors such as the harvesting time, i.e. the state of maturity (Huopalahti and Linko, 1983; Porter et al., 1983) and the isolation procedure of aroma compounds (Huopalahti et al., 1981). This compound has not been identified in any other spice or herb.

In recent studies 3,6-dimethyl-2,3,3a,4,5,7a-hexahydrobenzofuran has been identified as one of the major aroma components in the dill herb, exhibiting a pleasant odor similar to fresh dill (Schreier et al., 1981; Huopalahti and Linko, 1983).

Although 3,6-dimethyl-2,3,3a,4,5,7a-hexahydrobenzofuran can be synthetized (Ohloff et al., 1966), its use in the flavor industry as a pure synthetic compound is not reported. One reason for this can be the lack of detailed information on the stability of this benzofuranoid. Therefore, in the present work the decomposition of this compound in aqueous buffer solutions in the pH range 2 - 8 at temperatures between 5 and 100°C was studied.

MATERIALS & METHODS

Isolation and purification of 3,6-dimethyl-2,3,3a,4,5,7a-hexahydrobenzofuran

Aroma compounds were isolated by extracting 200g of crushed flowering dill for 6 hr with 450 mL of a mixture of redistilled n-pentane and diethyl ether (1;2, v/v) by using a modified Soxhlet-technique as described by Huopalahti et al. (1981). Three concentrated dill extracts (50 mL) were combined and cleaned up by vacuum distillation (41 - 47°C at 4 mmHg) to remove lipids, waxy materials and plant pigments. The further purification of this fraction was performed by preparative gas chromatography (Perkin Elmer F 21) and by distillation under vacuum (41 - 42°C at 4 mmHg). The purity of 3,6-dimethyl-2,3,3a,4,5,7a-hexahydrobenzofuran was confirmed by GC. The purified sample was stored in a sealed vial at −25°C.

Preparation of the test solutions

Stock solutions (10^{-3} or 10^{-4} mole/L) of 3,6-dimethyl-2,3,3a,4,5,7a-hexahydrobenzofuran were made in 500 mL of the appropriate buffers. For stability tests 10 mL of the appropriate stock solution was pipetted into vials, which were sealed tightly under nitrogen. In the stability tests at 5°C and 20°C, when the storage times were several weeks, a small drop of mercury was added to the vials to prevent microbial growth. In the pH tests the vials were stored at 20°C and the temperature tests were carried out at pH 4. Samples in all tests were taken at intervals. The vials were opened and the solutions were extracted with redistilled diethyl ether (3 x 10 mL). The extracts were combined, 0.1 mg of linalool (internal standard) was added and the solutions were diluted to 50 mL with diethyl ether.

Gas chromatography (GC)

The quantitative gas chromatographic determinations were performed on a Varian 3700 gas chromatograph equipped with a flame ionization detector connected to a Hewlett Packard 3388A integrator. The fused silica capillary columns (0.32 mm i.d. x 25 m; film thickness 0.20 μm) coated with OV-351 were used inthe stability tests. For the purity tests of 3,6-dimethyl-2,3,3a,4,5,7a-hexahydrobenzofuran the fused silica columns (0.31 mm i.d. x 25 m; film thickness 0.23 μm) coated with Chirasil-Val (Alltech Associates) were applied. The oven temperature was programmed from 30°C to 150°C at 5°C/min after an isothermal period of 2 min. At the same time the temperature of Varian on-column injector (with the coolant of liquid CO_2) was programmed from 30°C to 240°C at 120°C/min. The temperature of the detector was 250°C and the flow rate of the carrier gas (helium) was 1.5 mL/min. The quantitative estimations of the benzofuranoid (at the beginning: 3.0 μg/mL or 30 μg/mL) were performed by using linalool as the internal standard (2.0 μg/mL). The injected volume was 1.0 μL. The corrected values were calculated by assuming the calibration factor of linalool as 1.

Gas chromatography-Mass spectrometry (GC-MS)

The identification of isolated and purified 3,6-dimethyl-2,3,3a,4,5,7a-hexahydrobenzofuran was carried out on a VG Analytical 7070E mass spectrometer by using the same capillary columns as in the GC-analysis. An energy of 70 eV was used. The data system employed was VG-11-250.

Nuclear magnetic resonance (NMR) and infra-red (IR)-spectroscopy

The 400 MHz (^{1}HNMR) and 100 MHz (^{13}CNMR) spectra were obtained on an JEOL JNM-GX400 instrument. IR-analyses were carried out on a Perkin Elmer 180 equipment.

RESULTS & DISCUSSION

THE FLOWERING DILL was used in the isolation procedure, because at that growth stage dill has reached its maximal concentration of 3,6-dimethyl-2,3,3a,4,5,7a-hexahydrobenzofuran (Huopalahti and Linko, 1983; Porter et al., 1983). The yield of the benzofuranoid in this procedure was 0.1% from the fresh dill with purity over 99%. The optically active stationary phase was used in the purity tests to ascertain that the isolated fraction contained only one isomer, (+)-(3S, 3aS, 7aR)-3,6-dimethyl-2,3,3a,4,5,7a-hexahydrobenzofuran. The stereochemical structure was conformed by NMR- and IR- spectroscopy.

The rate constants of the decomposition of 3,6-dimethyl-2,3,3a,4,5,7a-hexahydrobenzofuran at two different concentrations in the pH range of 2-8 are shown in Table 1.

Authors Huopalahti and Hänninen are affiliated with the Dept. of Chemistry & Biochemistry, Laboratory of Food Chemistry, Univ. of Turku, SF-20500 Turku, Finland.

Table 1—First-order rate constants of decomposition and half-life of 3,6-dimethyl-2,3,3a,4,5,7a-hexahydrobenzofuran in pH range 2 - 8 at two different concentrations at 20°C.

pH	Concentration mole/L	Rate constant s^{-1}	Half-life d
2	10^{-3}	0.79×10^{-7}	101
2	10^{-4}	0.79×10^{-7}	101
3	10^{-4}	0.75×10^{-7}	107
4	10^{-4}	0.66×10^{-7}	122
5	10^{-4}	0.77×10^{-7}	104
6	10^{-4}	0.82×10^{-7}	98
7	10^{-4}	1.14×10^{-7}	70
8	10^{-4}	1.78×10^{-7}	45
8	10^{-3}	2.00×10^{-7}	39

The decomposition of the benzofuranoid followed first-order kinetics in the pH range used. The differences between the rate constants at different concentrations were insignificant, because the differences were within the limits of accuracy of the gas chromatographic method. The benzofuranoid turned out to be a rather stable compound, more stable at pH 2 than at pH 8 with maximal stability at pH 4.

The decomposition of 3,6-dimethyl-2,3,3a,4,5,7a-hexahydrobenzofuran at different temperatures is presented in Table 2. The decomposition increased with temperature and at 100°C was about 60 fold compared to that at 20°C. However, at 100°C the half-life was 2 days, which must be regarded as very good.

Dill is mainly used for flavoring various foods such as salads, sauces, soups, sea foods and pickled vegetables, which are stored at room temperature or in the refrigerator and whose pH is about 4. If the synthetic compound of 3,6-dimethyl-2,3,3a,4,5,7a-hexahydrobenzofuran was available, it could be suitable for flavoring most foodstuffs mentioned above, because the data of Table 1 and 2 show that the shelf-life of the benzofuranoid at room temperature and pH 4 is a few months, at 5°C almost a year. On the other hand, it must be kept in mind that the benzofuranoid is rather sensitive to oxidation and is very easily lost during processing by vaporization.

Table 2—First-order rate constants of decomposition and half-life of 3,6-dimethyl-2,3,3a,4,5,7a-hexahydrobenzofuran in the temperature range 5 - 100°C at pH 4; concentration was 10^{-4} mole/L

Temperature °C	Rate constant s^{-1}	Half-life d
5	0.27×10^{-7}	297
20	0.66×10^{-7}	122
50	1.46×10^{-7}	55
100	40.0×10^{-7}	2

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A Research Note

Effect of Germination on Phytic Acid, Protein and Fat Content of Rapeseed

LILIAN U. THOMPSON and MARIA R. SERRAINO

ABSTRACT

Rapeseed was germinated over a 7-day period and the changes in phytic acid, protein and fat contents were monitored. Two-day germination period reduced the phytic acid by 13% and slightly increased the fat and protein by 6 and 4%, respectively. A 65% decrease in phytic acid, 10% increase in protein and 46% decrease in fat were observed after 7 days of germination. The free fatty acid values increased from 1 to 120. While reduction in phytic acid and increase in protein are desirable, the reduction in fat and its increased hydrolysis to free fatty acids after 7 days may be considered detrimental if rapeseed oil is to be extracted for commercial purposes.

INTRODUCTION

RAPESEED is a major oilseed crop which is also a good source of protein. Its use for human consumption, however, is limited by, among others, high phytic acid concentration (Ohlson and Anjou, 1979). Phytic acid can form insoluble complexes with minerals and/or protein, resulting in their reduced bioavailability (Cheryan, 1980; Erdman, 1979).

Germination has been shown to reduce the phytic acid concentration of mature seeds and this has been attributed to increased phytase activity (Eskin and Wiebe, 1983; Chang, 1967; Mayer, 1958). Phytase is an enzyme which can hydrolyze phytic acid to inositol and free orthophosphate. Similarly, germination may reduce the phytic acid concentration in rapeseed but this has not been studied. There has been uncertainty as to whether rapeseed contains any phytase. Thus the objective of this study was to determine the changes in phytic acid content of rapeseed during germination. Since rapeseed is considered a good source of oil and protein, changes in these macronutrients were also observed.

MATERIALS & METHODS

RAPESEEDS (*Brassica Napus* var Tower) were soaked in water (2 mL/g) for 24 hr and then germinated at 20-22°C in the dark while single layered in containers lined with several layers of wet cheesecloth. The seeds were rinsed once a day with water to prevent any bacterial growth. At the end of 1, 2, 3, 5, and 7 days of germination, samples were freeze-dired. The ungerminated and freeze-dried germinated samples were ground to pass thru a 60-mesh sieve and analyzed for moisture, protein and fat using the AOAC (1980) methods and phytic acid by the method of Latta and Eskin (1981). The extracted fat was analyzed for free fatty acid value according to Triebold and Aurand (1963). The reported data represent means of two experiments with samples per experiment analyzed at least in duplicate.

RESULTS & DISCUSSION

THE EFFECT of germination on the composition of rapeseed is shown in Fig. 1. After 2 days of germination, phytic acid decreased by 13% while fat and protein increased by 6% and 4%, respectively. Continuing the germination up to 7 days resulted in 65% loss of phytic acid, 46% loss of fat and 10% increase in protein. Free fatty acid values rose continuously from 1 in the ungerminated seed to 120 in the seed germinated for 7 days.

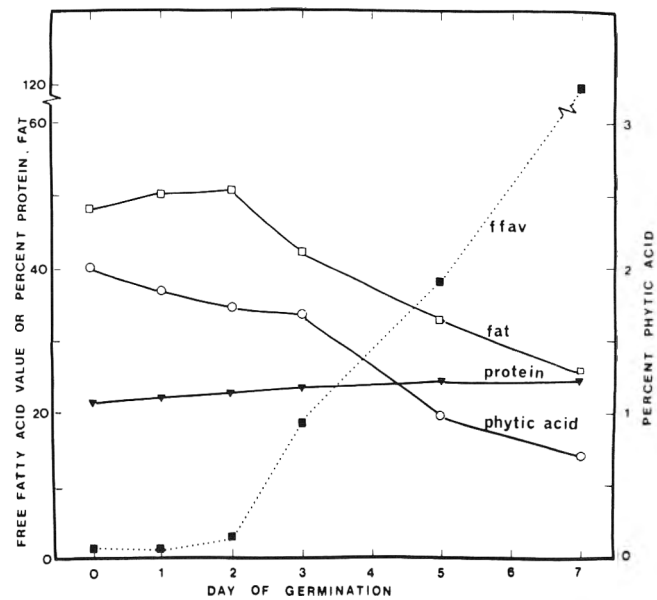


Fig. 1—Changes in phytic acid, protein and fat content (moisture-free basis) during germination of rapeseed.

This study suggests that germination may be used to reduce the phytic acid concentration is rapeseed. This also suggests that phytase is present in rapeseed and its activity is increased by germination. However, if germination is to be used for reducing phytic acid in rapeseed which has to be processed for oil, it should not be done beyond the second day. The further increase in protein and loss in phytic acid beyond the second day are desirable but the fat reduction coupled with increased free fatty acids is objectionable. Hydrolysis of fats to free fatty acids by lipases, which obviously increased in concentration during germination, gives the oil a rancid flavor.

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Authors Thompson and Serraino are affiliated with the Dept. of Nutritional Sciences, Univ. of Toronto, Toronto, Ontario, Canada M5S 1A8.

A Research Note

Interfacial Tensions of Commercial Vegetable Oils with Water

L.R. FISHER, E.E. MITCHELL, and N.S. PARKER

ABSTRACT

The pendant drop method, the current method of choice for interfacial tension measurement, was used to obtain the interfacial tensions of a number of vegetable oils with water. Contrary to the reports of earlier workers, all triglycerides examined had similar interfacial tensions with water, all values being in the range 23–26 mNm^{-1}. Long chain fatty acids, principal contaminants of commercial oils, had little effect on the interfacial tension. When the oils were exposed to alkaline conditions, the mono- and diglycerides formed by hydrolysis caused the interfacial tension to drop rapidly, in agreement with previously reported results for the effect of mono- and diglycerides on interfacial tensions.

INTRODUCTION

LORD RALEIGH (1890) was the first to measure the interfacial tension between a triglyceride (castor oil) and water. The value of ca. 21 mNm^{-1} which he obtained is regarded as sufficiently reliable to have been quoted in even the most recent literature (Dickinson and Stainsby, 1982). There have been few subsequent measurements of triglyceride/water interfacial tensions, despite their obvious importance to food emulsion technology and to the understanding of such biological processes as lipid absorption (Linthorst et al., 1977), and many of those which have been published are of very dubious value.

The presence of surface active contaminants could have affected some measurements (Benerito et al., 1954), but a more serious criticism of published work to date is that the experimental methods chosen (invariably the Du Nouy ring or some variant of capillary rise) have been quite inappropriate to the task in hand (Padday, 1969), the results being strongly dependent on contact angle, which is an unmeasured and often unmeasurable variable. The wide scatter in most results so far published suggests that the use of such techniques has led to substantial errors.

The method of choice for the measurement of liquid/liquid interfacial tensions is the pendant drop technique, where the interfacial tension is calculated from the dimensions of a drop of one of the liquids hanging from a capillary tube and immersed in the other liquid (Padday, 1969; Ambwani and Fort, 1979). The method is an equilibrium method (unlike the Du Nouy ring) and the results are independent of the contact angle of the liquid/liquid interface with the supporting capillary tube. The purpose of this study was the application of this technique to the measurement of interfacial tensions between some commonly available vegetable triglycerides and water.

MATERIALS & METHODS

SUNFLOWER OIL (Vegetable Oils P/L, Mascot, N.S.W., Australia), olive oil (Abel and Lemon, Concord West, N.S.W., Australia) and peanut oil (Southern Packers (Aust) P/L, Ingles Street, Port Melbourne, Victoria, Australia) were commercial products purchased at a local supermarket. Coconut oil and MCT810 ("medium chain triglyceride"; glycerol esterified with a mixture of caproic and capric acids, mean molecular weight 480; Nihon Oils and Fats Co., Ltd.) were gifts of Mr. A.C. Fogerty of the CSIRO Division of Food Research. These oils were either used as received or deacidified by a standard aqueous extraction technique (Walker, 1983). The water was laboratory distilled water which had been further treated by passing through a charcoal bed followed by distillation in a quiescent quartz still. All glass, stainless steel and fluorocarbon components (the only materials to come in contact with the liquids) were cleaned in chromic acid, washed thoroughly with double-distilled water and oven-dried.

The pendant drop apparatus has been described in detail by Ambwani and Fort (1979), the only difference between our apparatus and that described by them being our use of a helium/neon laser, beam-expanded to give a parallel beam ca. 5 mm in diameter, as a light source. The sample temperature was 25.0±0.1°C for all measurements reported here. Measurements were taken on at least six different drops for each of the systems studied. Densities were determined using an Anton Paar precision density meter fitted with an ultrathermostat.

RESULTS & DISCUSSION

Equilibrium interfacial tensions

Equilibrium interfacial tensions are listed in Table 1. All deacidified oils studied have interfacial tensions with water in the range 24–26 mNm^{-1}. Since the only difference between the oils is in the structure of the hydrocarbon chains, these results are in agreement with the analogous case of the hydrocarbon oils, where the details of the hydrocarbon chain structure make only small differences to the hydrocarbon/water interfacial tension (Jasper et al., 1970). The agreement is in marked contrast to the scattered results reported by earlier workers (Pound, 1923, 1926; Halpern, 1949; Kuhrt et al., 1950; Benerito et al., 1954; Singleton and Benerito, 1955; Feuge et al., 1972; Linthorst et al., 1977). For example, Singleton and Benerito (1955) quoted

Table 1—Interfacial tensions of commercial and deacidified triglycerides with water at pH 6.4 and 25.0°C[a,b]

Triglyceride	Interfacial tension (mNm^{-1})[c]	
	Commercial	Deacidified
Peanut oil	22.8 ± 0.8[d]	24.45 ± 0.13
Sunflower oil	23.91 ± 0.06	25.35 ± 0.05
Coconut oil	23.60 ± 0.10[e]	
MCT810	24.77 ± 0.14[e]	
Olive oil	23.56 ± 0.08	24.17 ± 0.10
Deacidified olive oil + 1% w/w oleic acid		23.17 ± 0.04
Deacidified olive oil + 5% w/w oleic acid		22.5 ± 0.2

[a] All values are averages for six droplets except that for commercial peanut oil (eight readings).
[b] Interfacial tension values were calculated using the following density values (g/mL): peanut oil 0.9128; sunflower oil 0.9168; coconut oil 0.9204; MCT810 0.9475; olive oil 0.9071.
[c] Errors quoted are standard errors.
[d] The large error is probably due to particulate matter ("haze") in the oil.
[e] Refined sample not needing deacidification.

Authors Fisher, Mitchell, and Parker are affiliated with the CSIRO Division of Food Research, P.O. Box 52, North Ryde, N.S.W. 2113, Australia.

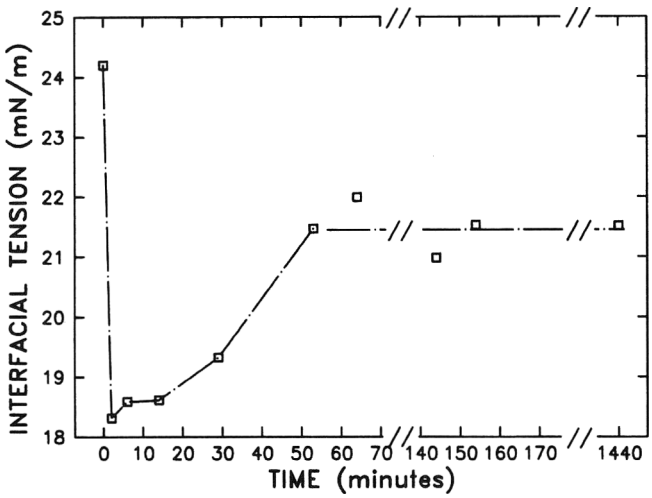


Fig. 1—Interfacial tension of deacidified olive oil in contact with aqueous sodium hydroxide solution, initial pH 9.5.

interfacial tensions for purified triglycerides against water ranging from 12.8 mNm^{-1} for coconut oil through 17.6 mNm^{-1} for olive oil to 22.9 mNm^{-1} for peanut oil. It is difficult to reconcile such a wide range of values with the known similar structures of the three triglycerides.

Effect of fatty acids

The relatively minor differences between "as received" and deacidified oils are rather surprising and were investigated further by measuring the effect of adding back oleic acid, a possible contaminating acid, to deacidified olive oil. The results are given in Table 1 and show that there is only a small lowering of the interfacial tension even when as much as 5% w/w oleic acid is present. The pK of oleic acid is ca. 4.9 (Albert and Sergeant, 1962), so that about 3% of the acid is in the unionized form. Clearly, neither the ionized or unionized form of the acid is particularly surface-active in this system compared to the case for the hydrocarbon/water interface, where a similar concentration of the unionized acid alone would be sufficient to reduce the interfacial tension from 50 mNm^{-1} to below 30 mNm^{-1} (Ward and Tordai, 1952).

Effect of alkaline pH

Alkaline pH may be expected to lower the oil/water interfacial tension by hydrolysis of the oil to produce free acid and strongly surface active (Kuhrt et al., 1950) mono- and diglycerides. As shown in Fig. 1, the interfacial tension drops rapidly over a period of a minute or so after the aqueous solution at pH 9.5 has been brought into contact with olive oil. Actual interfacial tensions are difficult to measure during this period, but the change in drop shape is clearly observable. The finite amount of OH^- ions in the aqueous hanging drop (ca. 10^{-10} moles) is gradually depleted, as shown by the change in color of phenolphthalein indicator added to the droplet (a blank experiment using decane instead of olive oil showed that the loss of color was not due to dissolution of the indicator in the oil phase). With the loss of alkali, the interfacial tension gradually rises again, presumably owing to the dissolution of the mono- and di-glycerides formed at the interface into the bulk oil.

In conclusion, the results presented here show that, contrary to earlier reports, there are only minor differences between the interfacial tensions of various vegetable triglycerides and water. The presence of relatively large amounts of fatty acid does not greatly affect the interfacial tension. Under alkaline conditions, hydrolysis at the interface produces mono- and diglycerides which lower the interfacial tension.

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A Research Note

System Design and Calibration of a Continuous Flow Apparatus for Kinetic Studies

K.R. SWARTZEL and V.A. JONES

ABSTRACT

A heat exchange apparatus is described to determine reaction kinetics of fluid food constituents during continuous flow. During operation fluids are exposed to well defined thermal treatments. The thermal evaluation method associated with the apparatus allows a variety of effects to be examined rapidly (i.e. pH, supporting media, level of concentration, temperature range etc.). In addition, kinetic data generation at processing conditions simulating actual conditions (velocities, Reynolds numbers, Nusselt numbers, shear stress at the wall, etc.) is possible. Description of the design and calibration procedure is given. Design flexibility allows for constituent examination at any time-temperature range useful to the food processor.

INTRODUCTION

THE PRACTICE of using continuous thermal processing and aseptic packaging for preserving fluid foods is a growing trend. Compared to in-can processing, continuous thermal processing offers reduced energy and packaging costs, and favors product quality retention. Thermal treatments simultaneously produce beneficial and undesirable changes in products. Selection of time and temperature treatment depends upon destruction of spoilage and disease causing microbial spores and on physical, chemical and biological transformations that occur within the product. Reliable kinetic data are required to assess both desirable and undesirable changes resulting from thermal processes. Traditional batch methods for generating reaction kinetic data become less reliable at the high temperatures of continuous flow thermal processing where exposure times are very short.

Well established methods for obtaining thermal kinetic data for food constituents include thermal death time (TDT) tube, TDT can, capillary tubes and thermoresistometer (Stumbo, 1973). More recently differential scanning calorimetry (DSC) has found many uses for kinetic data generation (Lund, 1983; Grieme and Barbano, 1983). However, these are all batch methods requiring relatively small representative samples and, therefore, may not be indicative of kinetics for continuous processes. Small batch sizes also limit the ability to make comparisons between objective measurements and subjective evaluations. Another limitation of batch techniques is that extrapolation of kinetic data to the high temperatures and short times normally associated with continuous flow thermal sterilization processes is often required. A recently developed method utilizing a nonisothermal reactor may eliminate these problems (Swartzel, 1984). This new method of generating kinetic data was based on a previously developed thermal evaluation technique (Swartzel, 1982). The thermal process can be described in terms of an equivalent single time (t_E) and temperature (T_E) independent of activation energy.

Authors Swartzel and Jones are affiliated with the Depts. of Food Science and Biological & Agricultural Engineering, North Carolina State Univ., Raleigh, NC 27695-7624.

The objective of this work was to develop a system for thermal kinetic studies of fluid foods during continuous processing and to calibrate the system in terms of t_E and T_E values as a function of processing parameters.

MATERIALS & METHODS

Apparatus

Design criteria for the heat exchanger system include: (1) the ability to establish well-defined thermal histories, (2) the flexibility to operate over a broad process and product range, (3) the ability to aseptically sample, (4) the ability to process enough product so that subjective studies are possible without requiring such quantities that would result in prohibitive run cost, (5) ease of operation and calibration, and (6) the ability to be cleaned and sterilized.

An existing flow system shown in Fig. 1 (Model XLV No-Bac Unitherm, manufactured by Cherry-Burrell Inc., Cedar Rapids, IA) as described by Biziak et al. (1982) was modified for the kinetic studies by installing three additional thermocouples in each of the two tubular heaters. All tube outside diameters were 1.58×10^{-2} m with a wall thickness of 2.10×10^{-3} m. The holding tube length was 2.07m.

System calibration

To establish the system calibration, T_E and t_E values were determined as described by Swartzel (1984) for constant initial heater inlet temperature (T_o), product flow rates (V), and process or holding tube temperatures (T_p). Values for V were 605.2, 757.0, 946.3, 1185.5 and 1703.3 L/hr; T_p, 410.8, 416.3, and 421.9°K; and T_o was held constant at 385.2°K.

RESULTS

THE CALIBRATION is shown graphically in Fig. 2. Empirical expressions were derived as:

$$Tp = -1221.824 + 7.054\,(T_E) - 0.000455\,(T_E)\,(t_E) + 0.00799\,(t_E)^2 - 0.00747\,(T_E)^2 \quad (5)$$

with

$$r^2 = 0.99 \text{ and c.v.} = 0.055$$

and

$$V = 2176.458 + 679.897\,(t_E) - 1.983\,(t_E)\,(T_E) + 2.765\,(t_E)^2 \quad (6)$$

with

$$r^2 = 0.91 \text{ and c.v.} = 11.22$$

To extend or reduce the limits of the reactor, the piping may be altered (i.e. change the holding tube length or diameter, increase or decrease the flow rate, or alter the incoming product temperature).

Empirical determination of rate constants

If the thermal treatment chosen were 418°K for 4 sec the flow rate required (Eq. 2) would be 1703.3 L/h at process temperature (Eq. 1) of 420.2°K; 418°K for 6.3 sec, V = 1135.5 L/h and T_p = 420.0°K; 418°K for 12 sec, V = 757.0 L/h and T_p = 419.8 °K. With the input of the initial (c) and final constituent concentrations (c-x) for each run, where x denotes the concentration of product formed or

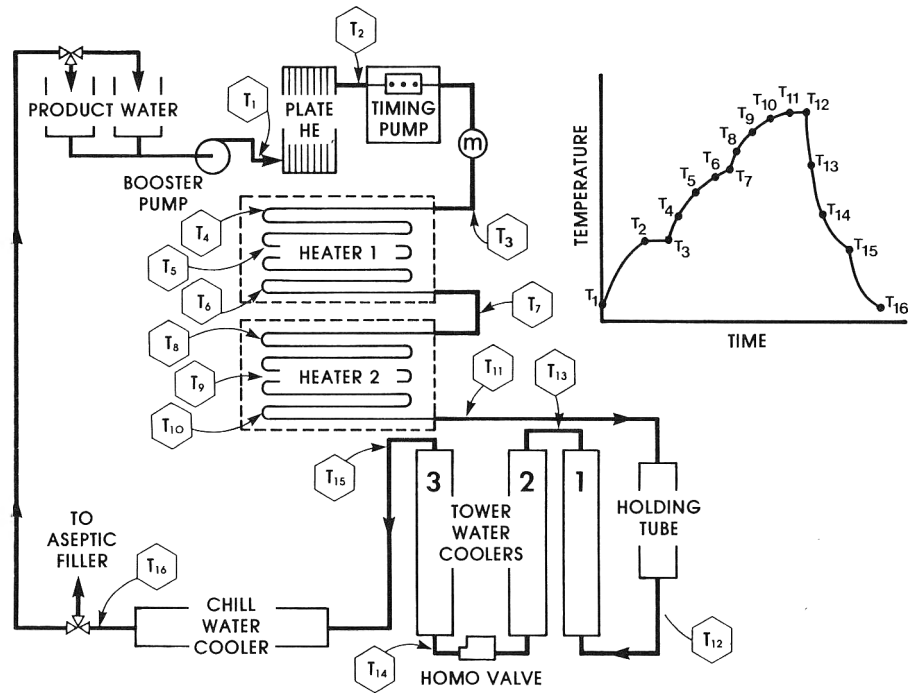


Fig. 1—Flowchart and temperature history pattern for continuous flow reactor with thermocouple locations.

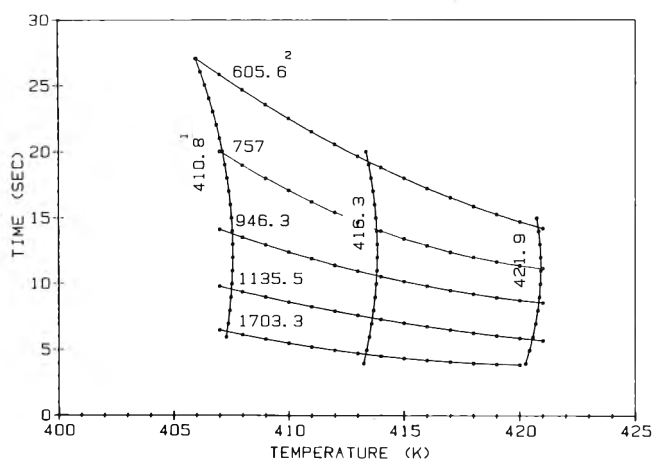


Fig. 2—Empirical design criteria for continuous flow reactor. [1] Holding tube temperature (°K); [2] Product flow rate (L/hr).

reactant consumed, the order of the reaction can be easily determined from the use of established rate laws (Moore and Pearson, 1981). The experimental data are received in the form of a series of values of x at different time t, including the initial value when t=zero. Values of t_E, c and x are substituted into each of the rate law equations in turn until constant values for the rate, k, or a straight line plot is obtained when the concentration expression is plotted against time. Thus, if it is suspected that a reaction is of the first order, a graph of log (c-x) versus time is plotted. If the result is a straight line the reaction is in fact first order. If the result does not, however, fit the first-order law, they must be substituted into the other rate equations until one is found that yields a constant value of k.

With the development of rate constants, additional kinetic parameters (D values, z values, Q_{10}, E_a, etc.) can then be determined by established equations (Lund, 1975).

DISCUSSION

THIS METHOD for determining reaction kinetics of fluid food constituents during continuous flow is useful for any continuous flow heat exchanger as long as true mass mean temperature profiles can be obtained. Once the reactor is calibrated, a specific reaction's order and corresponding kinetic parameters can be determined as long as the initial and final concentration (or some indirect measurement) can be obtained.

Presently several constituents are being tested with the continuous flow reactor. Simultaneously kinetic data are being obtained by traditional batch methods for comparison. Reaction mechanisms at ultra high temperature are also being examined. Phase Two of this current study involves construction of a laboratory-sized reactor requiring less product. With the smaller unit extensive tests may be performed since less product and time would be required for each run. It is hoped that the second unit will be useful in determining kinetics of constituents which may be used as calibration materials for thermal system evaluation. If such materials could be developed, evaluations of thermal systems, which are difficult to define, mathematically, would be much easier and more reliable.

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A Research Note

Reduction of Bitterness in Grapefruit Juice with β-Cyclodextrin Polymer in a Continuous-Flow Process

PHILIP E. SHAW and CHARLES W. WILSON, III

ABSTRACT

Levels of the bitter components naringin and limonin were reduced an average of 50% or more in clarified grapefruit juice by treatment of about 16 bed volumes of juice with β-cyclodextrin polymer in a continuous flow process. Dilute alkaline solution regenerated the column effectively, ethanol was only about half as effective as dilute alkali. Cloudy grapefruit juice was also debittered in the same column using reversed flow to prevent column plugging. This process was less efficient because liquid flow was inadequate to maintain a fluid bed. A preliminary batch process showed ability of the polymer to be regenerated at least 19 times with no loss in ability to adsorb bitter components.

INTRODUCTION

EXCESSIVE BITTERNESS is a sufficient problem in early-season grapefruit juice so that maximum levels of the two major bitter components, naringin and limonin, are regulated in juice processed in Florida from August 1 to December 1 (Florida Department of Citrus, 1975). Grapefruit juice could be an attractive base for blended fruit drink beverages because of its relatively low cost if the bitterness could be reduced considerably. At present, there is no acceptable commercial method for reducing levels of bitter components in citrus juices. Thus, processed grapefruit juice that is excessively bitter is blended with less bitter juice to produce an acceptable product, and the potential market for use of grapefruit juice in blended juice drinks is restricted.

Several experimental procedures are available for reduction of naringin and/or limonin in grapefruit juice. Some enzymes convert naringin (Kefford and Chandler, 1970) or limonin (Maier et al., 1977) to nonbitter compounds and one of these, naringinase, was used in immobilized form to reduce naringin levels in citrus juices (Ono et al., 1978; Gray and Olson, 1981). Immobilized bacteria have been used to reduce limonin levels in citrus juices as well (Hasegawa et al., 1983). Certain porous polymers and ion exchange resins reduce naringin and limonin in grapefruit juices (Johnson and Chandler, 1982; Puri, 1984), and some of these resins significantly reduce the acidity. Cyclodextrin monomer can be dissolved in grapefruit juice to reduce the bitter flavor by forming inclusion compounds with naringin and limonin (Konno et al., 1981, 1982; Misaki et al., 1981), although the bitter components are not removed from the juice by this treatment (Shaw and Wilson, 1983). In earlier studies, β-cyclodextrin polymer reduced the levels of naringin and limonin in clarified (Shaw and Wilson, 1983) and in cloudy (Shaw et al., 1984) grapefruit juices using either batch or fluid-bed processes. In those studies, relatively small amounts (1 - 6g) of polymer were used, and the effects of multiple regeneration of the polymer were not studied. The current study reports larger scale debittering of clarified and cloudy grapefruit juices with β-cyclodextrin polymer using continuous flow columns. Effects of repetitive regeneration on effectiveness of the column are also reported.

Authors Shaw and Wilson are affiliated with the USDA-ARS Southern Region, South Atlantic Area, U.S. Citrus & Subtropical Products Lab., Post Office Box 1909, Winter Haven, FL 33883.

MATERIALS & METHODS

Materials

Clarified grapefruit juice was prepared by centrifugation of cloudy single-strength grapefruit juice at 2 gal/hr (Westphalia Model SB-7 centrifuge) followed by ultrafiltration at 8 gal/hr (Romocon) with a 100 PM cellulose acetate membrane. The resulting clarified juice was used in the gravity flow experiments described below. Three 20-l batches of this juice were used, each with slightly different levels of naringin and limonin. Thus, there was a slight difference in naringin and limonin content for various starting juices in Tables 1 and 2.

Cloudy grapefruit juice to which no flavor fractions had been added was prepared by reconstitution of 1350 mL of 55°Brix frozen concentrated grapefruit juice to 10.0°Brix by addition of 7650 mL of deionized water. The reconstituted juice was filtered through a 60-mesh screen (Millipore 316 stainless screen for 47 mm analytical filter holder) to remove traces of large pulp that might plug the screen used in the fluid bed apparatus. The pulp content of 6% was unchanged by filtration through the 60-mesh screen.

β-Cyclodextrin polymer was prepared as described earlier (Shaw et al., 1984). The dried polymer was ground in a mortar and screened to give 20-40 mesh beads used in this study.

Debittering methods

Preliminary batch process. A 125 mL sample of cloudy grapefruit juice was stirred mechanically with 5.0g of β-cyclodextrin polymer at room temperature for 60 min. The polymer was removed by filtration of the mixture through a 60-mesh screen and regenerated by stirring it twice for 30 min each time with 100 mL portions of 2% NaOH solution followed by filtration through a 60-mesh screen. The regenerated polymer was then washed with 8 x 25 mL of water and treated as above with 125 mL of cloudy juice. The process was repeated a total of 20 times. Every third regeneration 0.4g of fresh polymer was added (to replace polymer lost by transfer of material during washing and drying) before proceeding with subsequent debittering cycles.

Fluid bed column. A slurry of 69g of β-cyclodextrin polymer (20-40 mesh) in 40% ethanol was packed into a 3.2 cm i.d. x 30 cm glass column (200 mL bed volume) with gravity flow at 15 mL/min. A 48-mesh nylon screen was used to prevent polymer from flowing out the 8 mm i.d. exit tube. Flow was then reversed by pumping solvent with a Masterflux tubing pump at 15 mL/min for 30 min to help settle the polymer bed. Gravity flow with water was begun at 15 mL/min with a screw clamp on the exit tube to control flow rate. Clarified juice was pumped onto the top of the column to maintain a 6 cm level above the fluid bed during debittering, washing and regeneration with either dilute NaOH solution or ethanol. Cloudy grapefruit juice was pumped into the bottom of the column at 15 mL/min.

Analytical methods

Naringin values were determined using the Davis test which measures nonbitter flavanone glycosides as well as naringin. Total acid was determined from titratable acidity (Praschan, 1975). Limonin was determined by an HPLC method reported earlier (Shaw and Wilson, 1984).

RESULTS & DISCUSSION

β-CYCLODEXTRIN POLYMER was used in a continuous flow process to reduce the levels of the bitter components

Table 1—Levels of naringin (ppm) in grapefruit juice treated with β-cyclodextrin polymer[a]

Column fraction	Bed volumes[b]	Naringin[c] in juice from regeneration number (flow rate)[d]							Reverse[e] flow
		S[f] (15)	1 (15)	2 (7.5)	3 (7.5)	4 (7.5)	5 (15)	6 (20)	7 (15)
Control	–	980	960	850	900	860	1040	1000	940
1	2.25	115	200	130	200	170	500	190	360
2	4.5	280	325	240	310	280	630	400	680
3	6.75	425	450	350	410	350	675	550	545
4	9.0	480	560	450	480	480	780	625	500
5	11.25	360[g]	640	490	530	550	860	680	650
6	13.5	500	670	580	580	600	900	700	800
7	15.75	640	700	600	630	640	880	820	840
8	18	720	540[g]	580	650	740	900	880	850
9	20.25	800	630	640	680	580[g]	890	880	900
10	22.5	855	780	720	750	740	940	900	850
Wash fraction:		2% NaOH	2% NaOH	1 - 4% NaOH[h]	4% NaOH	EtOH	2% NaOH	2% NaOH	2% NaOH
1	1.5	2540	2900	1800	2000	1760	3000	2320	70
2	3.0	1825	1920	1020	1260	240	820	760	480
3	4.5	545	720	510	580	110	475	600	480
4	6.0	320	430	380	340	80	390	450	310
5	7.5	180	–	480	250	85	285	170	210
6	9.0	160	–	360	190	80	230	140	145
7	10.5	–	–	250	200	80	195	110	105
8	12.0	–	–	185	155	80	–	–	85
Total % naringin recovered in washes[i]		76	97	109	86	51	148	90	52

[a] β-Cyclodextrin polymer (69g) in 200 mL bed volume.
[b] Juice fractions 450 mL and wash fractions 300 mL each.
[c] Determined by Davis test (ppm).
[d] Flow rate in mL/min.
[e] Cloudy grapefruit juice used.
[f] S = starting fresh polymer.
[g] Column stood overnight at 10°C with some of the juice fraction in contact with polymer.
[h] Wash fractions 1 - 4 with 1% NaOH, fractions 5 - 8 with 4% NaOH.
[i] Based on total mg naringin removed in 10 x 450 mL of juice.

Table 2—Levels of limonin (ppm) in grapefruit juice treated with β-cyclodextrin polymer[a]

Column fraction	Bed volumes	Limonin[b] in juice from regeneration number (flow rate)							Reverse flow
		S (15)	1 (15)	2 (7.5)	3 (7.5)	4 (7.5)	5 (15)	6 (20)	7 (15)
Control	–	4.1	4.1	3.5	3.5	3.8	4.0	4.0	8.0
1	2.25	T[c]	T	N[d]	N	T	T	T	2.3
4	9.0	T	T	N	T	1.4	2.4	2.2	3.0
7	15.75	1.5	T	T	T	1.9	3.0	2.4	7.0
10	22.5	1.5	1.0	T	T	2.9	3.2	2.8	7.7

[a] Same as conditions used in Table 1.
[b] Limonin value determined by HPLC (Shaw and Wilson, 1984).
[c] T = trace (<0.5 ppm).
[d] N = not detected (<0.1 ppm).

naringin and limonin in clarified and cloudy grapefruit juice samples. A preliminary smaller scale batch process was used to determine the capacity of the polymer for repetitive regeneration. Thus, twenty 125-mL portions of cloudy grapefruit juice were tested in succession with the same 5g sample of β-cyclodextrin polymer after regeneration each time with 2% sodium hydroxide solution. The polymer maintained its ability to lower naringin to an average of 53% and limonin to an average of 42% of the original values throughout the 19 regenerations.

Clarified grapefruit juice was debittered in a larger scale continuous process using gravity flow to study effects of changes in flow rate and regeneration conditions on column performance (Tables 1 and 2). With fresh and regenerated polymer, a gradual loss in ability to remove naringin and limonin was noted as grapefruit juice up to 22.5 bed volumes was passed through the column. Naringin was reduced an average of about 50% in the first 13.5 - 18 bed volumes collected when clarified juice was debittered with polymer that had been regenerated with dilute alkali. Although the later fractions (9 - 18 bed volumes) had less than 50% naringin removed, they could be blended with early fractions to achieve an average of 50% reduction in naringin. Flow rates of 7.5, 15 and 20 mL/min were studied, representing contact time for the juice of 26, 13 and 10 min, respectively. In preliminary batch studies 10 - 15 min was determined as the minimum contact time needed for the adsorption process to occur (Shaw and Wilson, 1983). In this study, a flow rate of 7.5 or 15 mL/min appeared more effective than one of 20 mL/min in reducing both naringin and limonin contents. In a few cases where juice fractions stood overnight in contact with the polymer, lower levels of naringin than normal were found (footnote g, Table 1).

For column regeneration, 2% sodium hydroxide was as effective as a stronger (4%) sodium hydroxide solution. Use of a 1% sodium hydroxide solution (6 bed volumes) followed by 4% solution indicated a 1% solution to be less effective than either of the stronger alkaline solutions in removing naringin. When ethanol was used to regenerate the

polymer (data in column 4 of Table 1), the column was about half as effective in debittering juice as when dilute alkali was used. In addition, the naringin not removed by the ethanol wash apparently was removed in the subsequent alkaline wash (last value, column 5). From six to nine bed volumes of 2 - 4% sodium hydroxide solution were sufficient to regenerate the column.

The column seemed to lose some ability to remove limonin in later regeneration cycles (Table 2), although this trend was not seen in the batch study described above. Limonin recovery in regeneration washes was not quantitative (Shaw et al., 1984). The column was less effective in removing naringin or limonin from cloudy juice. In that case, it was operated in the reverse flow direction to prevent column plugging from pulp in the juice. Reduction in limonin (Table 2) paralleled that of naringin with virtually no removal occurring by the seventh fraction. Loss of effectiveness in the reversed flow study was undoubtedly due to the inability of liquid flow alone to maintain an adequate fluid bed for intimate mixing of polymer and juice.

In the regeneration step with alkaline solution, a dark reddish-yellow color developed on the polymer that could be used as an indication of flow characteristics of the column. With reversed flow, uneven color development occurred, indicating inadequate mixing. Naringin recovery values indicated only about half the adsorbed naringin was removed during the regeneration step, also indicating inadequate mixing of polymer and liquid. Use of magnetic stirring or nitrogen gas as reported in an earlier study (Shaw et al., 1984) causes mechanical breakdown of some polymer beads to afford particles small enough to pass through the screen and end up in the debittered juice. Thus, the continuous flow gravity flow column was more effective in lowering the levels of the naringin and limonin than was the reverse flow column. In reverse flow, the column was less effective because liquid flow was inadequate to cause complete mixing of juice and polymer.

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A Research Note

Changes in Volatile Components of Passion Fruit Juice as Affected by Centrifugation and Pasteurization

MAY-CHIEN KUO, SU-LIN CHEN, CHUNG-MAY WU, and CHU-CHIN CHEN

ABSTRACT

Volatile components of fresh passion fruit (hybrid variety, cultivar Tai-Non No. 1) juice (A), de-starched juice (B), pasteurized and de-starched juice (C) and headspace volatiles that were recovered during centrifugation of fresh juice (D) were compared. Charcoal adsorption was used to recover the headspace compounds. The yields of volatiles obtained from the different treatments were 31.69 mg/kg juice for A, 18.97 mg for B, 12.27 mg for C, and 7.93 mg for D, respectively. The pasteurization process (1 min at 85°C) caused a 35% loss in volatile components.

INTRODUCTION

PASSION FRUIT is a kind of tropical fruit now grown in most of the tropical and sub-tropical countries of the world. In Taiwan, the hybrid passion fruit (cultivar Tai-Non No. 1), which originated from the cross-breeding of purple passion fruit (*Passiflora edulis* Sims.) with yellow passion fruit (*Passiflora edulis* f *flavicarpa*), has been cultivated commercially for its stronger flavor intensity (Chen et al., 1982). In hybrid passion fruit, the presence of about 1 - 2% starch will make heat processing such as pasteurization and concentration impossible or impractical, if the starch is not removed before processing. The methods adopted to remove starch include centrifugal separation (Casimir et al., 1981) and enzymic digestion of gelatinized starch by microbial α-amylase (Kwok et al., 1974). However, microbial α-amylase seemed ineffective in reducing the viscosity of gelatinized juice (Kwok et al., 1974).

The purpose of this study was to investigate the changes of volatile components of passion fruit juice as affected by the centrifugal separation of starch and the subsequent pasteurization.

MATERIALS & MEHTODS

Sample preparation

Ripe, fully colored passion fruit (cultivar: Tai-Non No. 1) were purchased from Taitung, Taiwan, immediately after harvest. The fruit was washed, and the juice was extracted with a Passy-Press extractor (S.A. Bertuzzi., Italy) equipped with a brush finisher (60/80 mesh). Most of the starch (>97%) in fresh juice (sample A) was removed by a centrifugal separator (Escher Wyss, H-400, Switzerland) operated at 2,800 rpm. The headspace components (sample D) generated during centrifugation were adsorbed on a charcoal filter (10 cm x 5 cm) which was installed vertically over the juice collecting tank. The de-starched juice (sample B) was pasteurized (sample C) through a plate heat-exchanger (Alfa-Laval, P-20-BV, Sweden) at 85°C for 1 min and cooled quickly to room temperature.

Authors Kuo, Wu, and C-C. Chen are affiliated with the Food Industry Research & Development Institute, P.O. Box 246, Hsinchu, 300, Taiwan, Republic of China. Author S-L. Chen is affiliated with the Institute of Food Science & Technology, National Taiwan Univ., Taipei, Taiwan, Republic of China. Author C.-C. Chen is presently with the Dept. of Food Science, Cook College, Rutgers, The State Univ. of New Jersey, New Brunswick, NJ 08903.

Vacuum distillation and adsorption

The isolation apparatus used was similar to that originally proposed by Gross (1963). Juice A, B or C (1 kg) was placed in a 2L flask immersed in a 40°C water bath. A glass tube (120 mm x 15 mm i.d.), containing 10g of granular charcoal (2.5 mm, E. Merck) and maintained at 40°C by a heating tape, was installed horizontally between the sample flask and the condensers. The whole operation was carried out under vacuum (5 mm-Hg) for 1 hr and repeated twice.

Treatments of charcoal

Granular charcoal was washed for 8 hr with glass distilled pentane and ether (1:1) and then activated at 260°C overnight under nitrogen flow of 100 mL/min. Volatiles adsorbed on charcoal were extracted with 120 mL glass distilled pentane and ether (1:1) in a Soxhlet extractor for 24 hr. Ethyl cinnamate (10.01 mg, 99%, Wako, Japan) dissolved in pentane and ether (1:1) was used as standard. The extracts were dried over anhydrous sodium sulfate, concentrated to ca. 0.2 mL by a Vigreaux column and the remaining solvent purged by nitrogen.

GC and GC-MS analyses

Sample were analyzed using a Hewlett-Packard model 5985B gas-chromatography-mass spectrometry system equipped with a flame ionization detector. A 25m x 0.19 mm fused silica capillary column coated with Carbowax-20M (WCOT, Applied Science, USA) was used. GC conditions were as follows: 30°C, 1 min; 60°C, 1 min; rate, 2°C/min; final temperature, 200°C; helium flow, 2 mL/min. The samples were injected in the spitless mode. The linear retention indices were calculated according to the modified Kovat's method by Majlát et al. (1974), using normal pariffins (C_8 – C_{22}, Alltech Associates, USA) as references. The MS conditions were as follows: ionization voltage, 70 eV; electron multiplier voltage, 2200V; ion source temperature, 200°C.

RESULTS & DISCUSSION

CHARCOAL has been used in the enrichment, isolation and determination of volatile compounds from foods (Jennings and Nurstens, 1967; Clark and Cronin, 1975; Schaefer, 1981). The break-through volume of many volatiles is far greater on charcoal than on porous polymers (Schaefer, 1981). Charcoal was used to serve both for preparative and analytical purposes.

Fig. 1 shows the gas chromatogram of volatiles of fresh passion fruit juice. Sixty-two of eighty compounds that were detected by GC-MS were identified.

Table 1 shows the quantitative changes of volatile components of passion fruit juice as affected by centrifugation and pasteurization. The identifications were accomplished by comparing retention indices with previous reports and published mass spectra (Murray et al., 1972; MSDC, 1974; Jennings and Shibamoto, 1980; TNO, 1981; Chen et al., 1982; Engel and Tressl, 1983). The amounts of volatiles yielded by different treatments as estimated from Table 1 were about (A) 31.69 mg, (B) 18.97 mg, (C) 12.27 mg, and (D) 7.93 mg, respectively, based on 1 kg samples.

Column D of Table 1 lists the volatile components assorbed on the charcoal filter during centrifugation. Components with high volatility such as ethyl butyrate (peak 3), ethyl hexanoate (peak 13), hexyl acetate (peak 16) and cis-3-hexenyl acetate (peak 19) were easily vaporized by the

centrifugal force. Those of medium to low volatility such as hexyl butyrate (peak 27), hexyl hexanoate (peak 47) and cis-3-hexenyl hexanoate (peak 50) were less affected by the centrifugation and remained in the juice. The effect of centrifugation in this study can be seen very clearly by comparing the quantitative differences between column A and B in Table 1. Some volatiles were entrapped by the starchy materials, which may explain partly the differences between column A and the total columns B and D in Table 1.

In Table 1, compounds in column C were in most cases lower than those in column B except for linalool (peak 40) and α-terpineol (peak 52). The quantitative loss of volatiles due to pasteurization was about 35%. Recently, Engel and Tressl (1983) found that monoterpene alcohols (such as linalool, α-terpineol, nerol and geraniol) of passion fruit may be generated from the corresponding nonvolatile glycosides by thermal degradation under acidic conditions.

Table 1—Volatile components of passion fruit juice at different treatments

Peak[a] No.	Components	I_k[b] CW-20M	x 10^{-6} g/kg juice[c] A[d]	B[d]	C[d]	D[d]
1.	ethyl acetate	868	55	66	13	10
2.	unknown	898	46	+[e]	25	40
3.	ethyl butyrate	1025	2762	+	28	1756
4.	butyl acetate	1044	45	+	+	25
5.	methyl pentanoate	1050	38	–[f]	–	+
6.	unknown	1084	14	–	–	16
7.	unknown	1095	11	–	–	11
8.	unknown	1130	343	53	40	148
9.	methyl hexanoate	1141	+	28	19	16
10.	myrcene	1169	167	41	27	34
11.	limonene	1177	17	+	–	43
12.	butyl butyrate	1191	77	+	+	53
13.	ethyl hexanoate	1218	1406	179	113	745
14.	p-cymene	1229	+	22	21	9
15.	2-heptyl acetate	1245	28	+	+	10
16.	hexyl acetate	1252	691	91	52	340
17.	ethyl 3-hexenoate	1278	76	14	+	36
18.	terpinolene	1285	32	12	+	6
19.	cis-3-hexenyl acetate	1297	1100	117	67	480
20.	ethyl heptanoate	1303	+	+	3	4
21.	1-hexanol	1326	1203	1526	924	80
22.	unknown (p-cymene-like)	1333	11	23	33	7
23.	cis-3-hexen-1-ol	1353	687	695	361	45
24.	2-nonanone	1360	25	13	10	13
25.	acetic acid	1365	5	+	+	5
26.	2-heptyl butyrate	1394	23	70	44	+
27.	hexyl butyrate	1406	6487	2982	2003	1493
28.	unknown	1415	106	64	45	40
29.	ethyl octanoate	1430	62	21	19	15
30.	cis-3-hexenyl butyrate	1440	2272	892	567	589
31.	trans-3-hexenyl butyrate	1450	218	113	68	60
32.	2-nonyl acetate	1453	+	+	+	5
33.	octyl acetate	1460	13	11	+	4
34.	ethyl cis-4-octenoate	1466	51	33	23	16
35.	unknown (138/194)	1473	188	159	46	36
36.	hexyl pentanoate	1482	64	51	28	20
37.	cyclohexyl butyrate	1486	85	61	42	21
38.	2-cyclohexyl ethyl acetate	1492	59	33	24	12
39.	unknown (68/138)	1499	75	52	35	18
40.	linalool	1508	99	96	182	30
41.	unknown	1512	6	+	11	9
42.	1-octanol	1520	147	140	90	15
43.	ethyl 4,7-octadienoate	1543	78	29	18	10
44.	4-terpineol	1559	56	35	25	6
45.	2-heptyl hexanoate	1578	159	143	92	56
46.	unknown (acid)	1584	60	95	55	5
47.	hexyl hexanoate	1604	6503	5092	3529	682
48.	6-but-2-enylidene-1,5,5-trimethylcyclo-hex-1-ene	1606	374	295	153	41
49.	ethyl benzoate	1625	272	254	130	94
50.	cis-3-hexenyl hexanoate	1639	2489	1983	1413	269
51.	trans-2-hexenyl hexanoate	1649	267	222	151	25
52.	α-terpineol	1654	177	182	189	13
53.	ethyl3-hydroxyhexan-oate	1666	54	55	23	7
54.	unknown (acid)	1683	136	129	64	33
55.	cyclohexyl hexanoate	1689	81	77	81	10
56.	benzyl acetate	1692	44	26	+	5
57.	citronellyl propionate	1700	40	19	14	5
58.	geranyl acetate	1723	57	40	14	5
59.	unknown	1763	43	52	37	5
60.	hexyl octanoate	1791	267	99	+	6
61.	caproic acid	1797	550	966	540	164
62.	unknown (thymol-like)	1804	48	59	38	11
63.	ethyl laurate	1820	46	82	25	5
64.	benzyl butyrate	1821	47	84	20	5
65.	trans-3-octenyl hexanoate	1831	194	149	38	10
66.	cis-3-hexenyl octanoate	1842	113	165	50	4
67.	unknown	1850	23	19	30	+
68.	gamma-heptalactone	1857	24	20	39	+
69.	unknown	1868	122	118	69	5
70.	geranyl isobutyrate	1874	180	180	151	12
71.	cis-3-hexenyl nonanoate	1890	147	154	74	7
72.	β-ionone	1900	68	102	52	14
73.	unkonwn (acid)	1911	20	40	17	4
74.	phenyl-3-propyl acetate	1926	32	43	14	4
75.	unknown	1935	19	74	13	4
76.	unknown	1943	26	+	32	+
77.	hexyl benzoate	2020	5	+	+	+
78.	ethyl myristate	2027	40	142	71	14
79.	gamma-decalactone	2101	31	44	24	+
80.	ethyl hexadecanoate	2106	42	48	24	15

[a] Numbers refer to Fig. 1
[b] Calculated Kovats' indices
[c] Average of two experiments
[d] A, fresh juice; B, de-starched juice; C, de-starched and pasteurized juice; D, headspace components from centrifuged fresh juice trapped by charcoal
[e] Trace component
[f] Nondetected

These reactions are good examples of the results of thermal treatments on passion fruit juice. In this study, linalool and α-terpineol in column B increased after pasteurization, with linalool increasing about twofold (182 μg vs 96 μg) but the increase in α-terpineol was negligible (189 μg vs 182 μg) (columns B and C). This is a good indication about the treatment of the juice during pasteurization.

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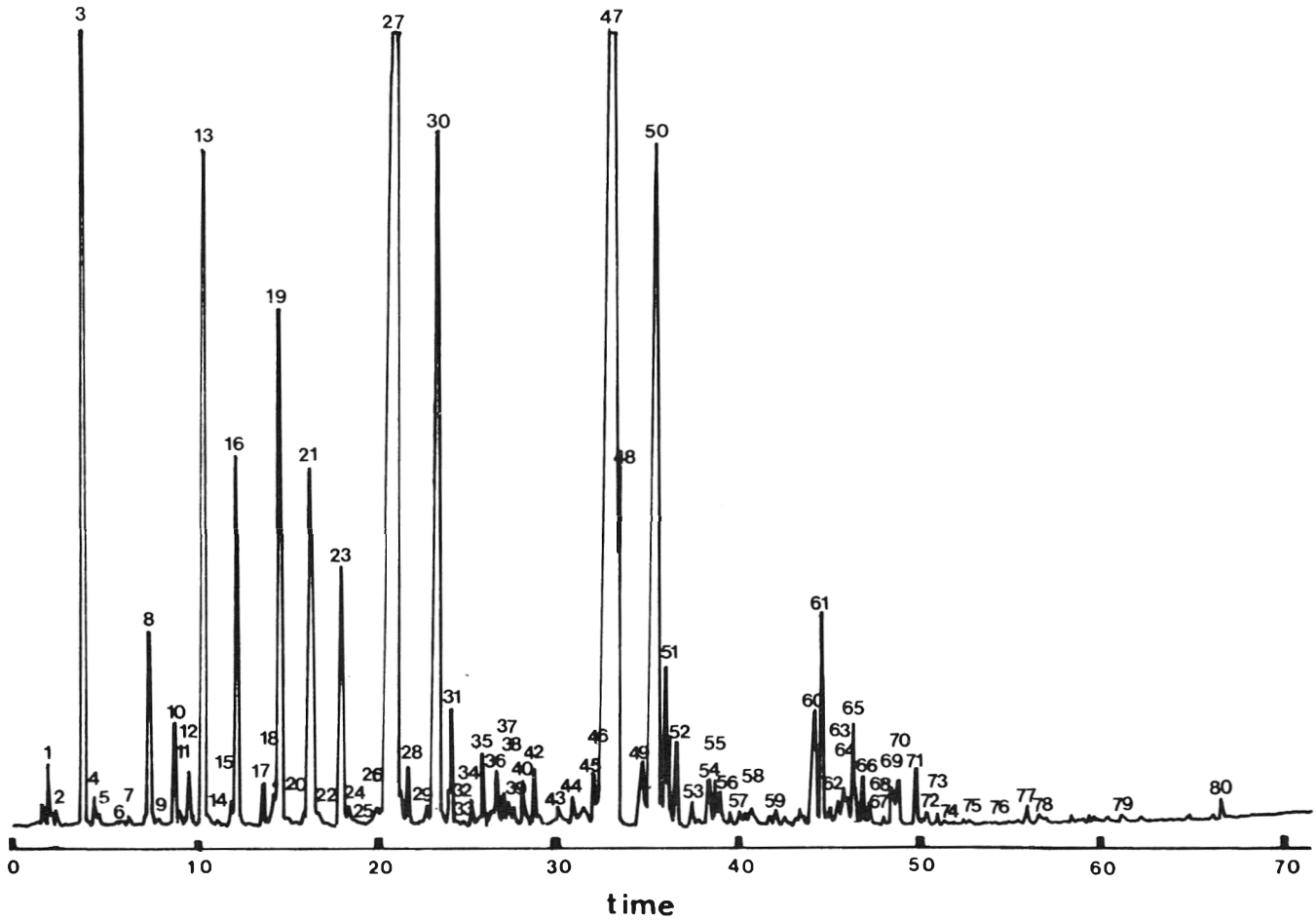


Fig. 1—Capillary gas chromatogram of volatiles of fresh passion fruit juice. The peak numbers correspond to those in Table 1.

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A Research Note

A Comparison of Enzymatic and Lime Treatments for Extraction of Alcohol Soluble Solids from Citrus Peel

M. R. MARSHALL, T. R. GRAUMLICH, R. J. BRADDOCK, and M. MESSERSMITH

ABSTRACT

Digestion of citrus wastes by pectolytic and cellulolytic enzymes was compared to the currently employed lime method of solubilizing solids from citrus peel. Maximal soluble solids removed from the pressed peel occurred after 6 hr incubation at 45°C with a pectinase concentration of 1.0 PGu/g peel mixture. Decreasing the particle size of the peel caused a 4 - 10% increase in solids removal after enzyme treatment. Addition of cellulase enzyme (20 CEu/g peel mixture) to pectinase enzyme (1.0 PGu/g peel mixture) removed an additional 15% soluble solids from peel. Glucose, fructose, arabinose, and xylose were the major sugars extracted from enzyme-treated peel, while sucrose was the major sugar in lime-treated peel samples. Organic acids (citric, malic, and quinic) were present at higher concentrations in lime-treated peel extracts than in enzyme-treated peel extracts.

INTRODUCTION

APPROXIMATELY 50% by weight of the citrus fruit in the form of peel, seeds, and pulp is discarded or dried and sold as cattle feed (Kesterson and Braddock, 1976). In this paper the combination of peel, pulp and seeds from citrus will be referred to as citrus peel. Drying citrus peel removes water from an initial moisture content of 80% to a final value of 10 - 20%. Before drying in direct fired or steam tube dryers, citrus peel is mixed with lime ($Ca(OH)_2$) and then pressed which reduces the moisture content of peel from 80 to 70%. The liquid formed is commonly called press liquor, which is around 10°Brix. Press liquor may be concentrated by evaporators to produce citrus molasses which can be added back to citrus peel prior to drying or sold as a separate by-product.

Water removal by waste heat evaporators is considerably more energy efficient than direct fired or steam tube dryers (Braddock et al., 1977). Liquefaction of the peel could potentially increase efficiencies in drying operations as well as increase the availability of citrus peel solids for utilization as fermentation substrates. The purpose of this investigation was to determine the feasibility of utilizing enzymatic treatment to increase the extraction of orange peel solids during dewatering operations.

MATERIALS & METHODS

COMBINED CITRUS PEEL from 'Valencia', 'Hamlin', and 'Pineapple' oranges was collected after juice extraction from commercial extractors (Model 391, FMC Corp., Lakeland, FL). Peel samples were comminuted with a Norwalk Model 201 triturator (Norwalk Foundary and Machine Co., St. George, UT) to achieve a fairly uniform coarse particle size of 0.8 - 1.0 cm^2 (coarse peel). After comminution the peel was mixed and stored at −18°C prior to use.

Commercial pectinase (polygalacturonase) and cellulase enzymes were obtained from Biocon (Biocon, Inc., Lexington, KY). Polygalacturonase and cellulase activity were determined by measuring the release of reducing groups from sodium polypectate (Miller, 1959) and glucose from microcrystalline cellulose (Bergmeyer and Bernt, 1974), respectively. One polygalacturonase unit was defined as the release of 1 μmole galacturonic acid per min at 40°C, pH 4.5. One cellulase unit was defined as the release of 0.010 mg glucose per hr at 37°C, pH 5.0.

Citrus peel-enzyme mixtures were compared to the currently employed method of lime treatment for dewatering citrus peel (Kesterson and Braddock, 1976). Lime treatment was accomplished by mixing 0.15 - 0.25% $Ca(OH_2)$ (based on peel wet weight) with citrus peel for 15 min. Peel-enzyme mixtures were incubated at 45°C. After incubation both lime and enzyme-treated peel samples were individually pressed using a Norwalk 201 hydraulic press (Norwalk Foundary and Machine Co., St. George, UT). Total and dry weights and total hexose sugars (anthrone method; Herbert et al., 1971) were determined for peel extracts (press liquor). Significant microbial populations (less than 100/g peel mixture) were not detected by plate count on orange serum agar. Specific sugars and organic acids were determined by gas-liquid chromatographic analysis of their trimethylsilyl ether derivatives (Kline et al. 1970; Fernandez-Flores et al., 1970).

Statistical analysis was performed using the Tektronix System with Tektronix Plot 50 Statistics package (Tektronix Inc., Beaverton, OR).

RESULTS & DISCUSSION

SOLUBLE SOLIDS in extracts of pressed peel increased as enzyme concentration and incubation time increased (Table 1). Analysis of Variance (ANOVA) demonstrated that a significant ($p < 0.001$) removal of solids from the peel occurred at various enzyme concentrations and incubation times. Zero hour controls and incubated controls (no enzyme) were not significantly different ($p > 0.05$) (Table 1). Maximum solids removal with polygalacturonase was at 6 hr incubation, with no significant ($p > 0.05$) increase in solids removal occurring at the later incubation period (12 hr). Increasing the pectinase concentration from 0.2 to 2.0 PGu/g peel mixture caused a 15% increase in solids removal from peel at 6 hr incubation. Lime treatment of citrus peel removed 25% soluble solids from citrus peel (Table 1). This was similar to the reported 30% soluble solids removed from citrus peel for commercial liming operations (Kesterson and Braddock, 1976). Enzyme concentrations greater than 0.5 PGu/g peel for 1 hr incubation period and all enzyme concentrations at the longer incubation periods significantly removed more soluble solids from citrus peel than lime treatment. Nishio and Nagai (1979) reported that 83% of the total solids were released during incubation of mandarin peel with a commercial and laboratory produced pectinase. However, their digestion conditions were 24 hr at 40°C with a total solids concentration of 0.8% compared to our conditions of 6 hr incubation at 45°C with a total solids concentration of 20%.

Particle size also affected the extractibility of solids from citrus peel. Peel was comminuted as described from a coarse particle size of 0.8 - 1.0 cm^2 to a fine particle size of 0.1 - 0.3 cm^2 (fine peel). Solids removal after enzyme treatment (1.0 PGu/g peel) of finely ground peel was 32, 41, 51, and 58% for 1, 2, 4, and 6 hr incubation, respectively. In comparison to coarse peel (Table 1; 1.0 PGu/g peel), reducing the particle size demonstrated a significant

Authors Graumlich and Braddock are with the Univ. of Florida, IFAS, Citrus Research & Education Center, 700 Experiment Station Road, Lake Alfred, FL 33850. Author Marshall's present address is Dept. of Food Science & Human Nutrition, Univ. of Florida, IFAS, Gainesville, FL 32611. Author Messersmith's present address is Biocon, Inc., 2348 Palumbo Drive, Lexington, KY 40509.

Table 1—Comparison of lime treatment to pectinolytic and cellulolytic treatment of citrus peel. All peel was adjusted to 80% moisture and incubated at 45°C

Peel treatment		% Solids removed (dry weight)[a] Reaction time (hr) 0.25	1	2	4	6	12
Incubated controls		19 ± 2.6[b]	20 ± 2.2	19 ± 1.9	19 ± 2.0	21 ± 3.0	20 ± 3.0
Lime treatment		25 ± 1.9	–	–	–	–	–
Pectinase (PGu/g peel)	0.2	–	23 ± 1.7	30 ± 1.9	33 ± 2.0	36 ± 1.9	38 ± 1.7
	0.5	–	27 ± 1.9	34 ± 2.0	39 ± 1.9	43 ± 1.9	46 ± 1.6
	1.0	–	31 ± 1.7	37 ± 1.9	44 ± 1.9	48 ± 1.7	50 ± 1.7
	2.0	–	34 ± 1.7	43 ± 1.9	49 ± 2.0	51 ± 1.9	52 ± 3.0
Pectinase + Cellulase (PGu/g peel + CEu/g peel)	0.2 + 4	–	–	–	–	49 ± 1.9	–
	1.0 + 20	–	–	–	–	62 ± 1.6	–

[a] Means ± the 95% confidence interval (C.I.)
[b] Zero incubated control and not peel incubated at 15 min

Table 2—Sugar and organic acid content of experimental peel extracts adjusted to 20°Brix after enzyme treatment (2.0 PGu/g peel and 20 CEu/g peel) for 6 hr at 45°C and lime treatment[a]

Sugar/Organic acid	Enzyme (mg/g dry wt press liquor)	Lime (mg/g dry wt press liquor)
Arabinose	59.7	<0.02
Xylose	9.2	<0.10
Fructose	149.4	49.8
Glucose	287.4	89.0
Galactose	29.1	31.2
Sucrose	<0.02	326.4
Malonic	0.26	<0.02
Malic	0.92	11.52
Citric	1.63	18.22
Quinic	1.33	4.98
Galacturonic	16.40	15.70

[a] All peel samples were adjusted to 80% moisture and incubated at 45°C.

($p < 0.05$) increase of 4 - 10% solids removal from citrus peel.

Nishio et al. (1979) and Hara et al. (1982) reported that cellulase and xylanase were necessary for maceration of mandarin orange peel. A combination of pectinase (polygalacturonase) and cellulase was found to be more effective than treatment with pectinase alone (Table 1). Treatment of coarse peel with 0.2 PGu/g and 4 CEu/g was equivalent to treatment with 1.0 PGu/g. Cellulase activity associated with crude pectinase preparations was approximately 2 CEu/g at levels of 1.0 PG/g. Treatment with pectinase (1.0 PGu/g peel) and cellulase (20 CEu/g peel) removed significantly ($p < 0.05$) more solids than pectinase treatment (1.0 PGu/g) alone. Cellulase activity greater than that associated with commercial pectinase was apparently necessary for increased solubilization of citrus peel solids.

Sugar and organic acid analyses performed on the extracts of enzyme and lime-treated pressed coarse peel are presented in Table 2. Arabinose, xylose, fructose, and glucose were higher in extracts from enzyme-treated peel than lime-treated peel extracts (Table 2). Galactose was slightly lower in enzyme-treated samples than lime-treated samples (Table 2). Sucrose was higher in the lime-treated peel extracts (326.4 mg/g dry weight) than enzyme-treated extracts (<0.02 mg/g dry weight). Glucose, fructose, and sucrose were the main sugars present in citrus peel (Ting and Deszyck, 1961), and this was consistent with our findings in the press liquor from enzyme and lime-treated peel.

Malic, citric, and quinic acids were lower in enzyme-treated peel extracts than lime-treated peel extracts (Table 2). Malonic acid was higher in enzyme-treated peel extracts than in lime-treated extracts. Galacturonic acid concentrations were similar for both the enzyme-treated and lime-treated peel extracts. Oxalic and succinic acids were not determined. Citric, malic, malonic, oxalic, quinic, and succinic acids were reported to be the major organic acids in orange peel (Ting and Deszyck, 1959; Clements, 1964; Vandercook, 1977).

In conclusion, soluble solids in extracts of pressed peel were influenced by incubation time, enzyme concentration, and a combination of pectinase and cellulase. Percent solids removed from citrus peel was higher following enzyme treatment in comparison to conventional lime treatment. Decreasing the particle size of the peel caused a 4 - 10% increase in the removal of soluble solids. Glucose, fructose, arabinose, and xylose were higher in extracts from enzyme-treated peel than lime-treated peel, while the organic acids were generally lower in extracts of enzyme-treated peel than that of lime-treated peel.

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A Research Note

Measurements of Monochromatic Visible Light Changes within Food Products using Laser and Fiber Optics

P. -G. FYHN and E. SLINDE

ABSTRACT

Light irradiance within dry sausages was studied. Monochromatic light provided by lasers was transmitted into and out of the sample through optical fibers. The light irradiance was detected as a function of the distance between the excitation point at the end of the source fiber and the end of the detector fiber. The mean penetration depths within dry sausages decreased at 457 nm from 1.7 to 0.6 mm, and at 625 nm from 2.8 to 2.1 mm from production day to finished product (28 days later). Use of fiber optics in combination with lasers represents a possible method for continuous spectral measurements within food products.

INTRODUCTION

IN MANY PROCESSES where visible light or other spectral information are important quality parameters, measurements within the products are often difficult to make. Use of optical fibers to make monochromatic visible light changes during processing detectable, may represent a possibility for continuous measurements within food products. Using a laser it is possible to transmit light of sufficient energy into the sample through one fiber, and to receive the transmitted or reflected light from the sample through another fiber. Such equipment has, for example, been used to measure optical properties of the human brain (Svaasand and Ellingsen, 1983).

In the present study lasers in combination with optical fiber cables were used to test the possibility for measuring monochromatic visible light changes within ripening dry sausages.

MATERIALS & METHODS

FROM INDUSTRIALLY PRODUCED dry sausages (salami), samples were taken on the first day and at the end of the fermentation period, on the 28th day. The samples were immediately frozen and stored. Prior to analyses, the samples were thawed and analyzed within 3 hr. The final product consisted of 20% protein, 39% fat and 36% water, plus salts and spices.

In the present study few wavelengths were available and the apparatus (Fig. 1) was rather primitive. The light beam was generated by two sources: An argon-ion laser, giving light at 457, 488 and 514 nm (Argon-Ion Laser, Spectra Physics 165 (5w)), and a tunable dye laser at 625 and 650 nm (Argon-Ion Laser. Spectra Physics 365, color DCM). The light was transmitted through approximately 1m of silicone-clad optical fiber with fused silica core, diameter 0.2 mm, and measured with a silicone PIN detector. The laser beams were chopped and the measured signals were analysed with a standard lock-in amplifier. The optical fibers with a diameter of 0.2 mm, were mounted in hypodermic needles with an outer diameter of 1.2 mm. The needles were both oriented towards the center of the sample, at a fixed angle (β = 30°) to each other. The source fiber was fixed near the center of the sample (r = 0; Fig. 1B). The dry sausage samples were cylindrical with a volume of approximately 300 cm^3.

RESULTS & DISCUSSION

LASERS provide monochromatic, visible light of sufficient energy to make measurements within samples possible (Fig. 1). The light is transmitted through an optical fiber into the sample and a detector fiber transmits reflected light to the detector (Fig. 1A). The decrease in light irradiance is detected as a function of the distance from the excitation point at the end of the source fiber to the point where the detector fiber is located (Fig. 1B). According to Svaasand and Ellingsen (1983) the penetration depth, δ, is given by the following equation;

$$\delta = (r_2 - r_1)/\ln(x_1/x_2 \cdot r_1/r_2) \quad (1)$$

where r_1 and r_2 are the distances from the end-surface of the source fiber to the detector fiber giving irradiances of

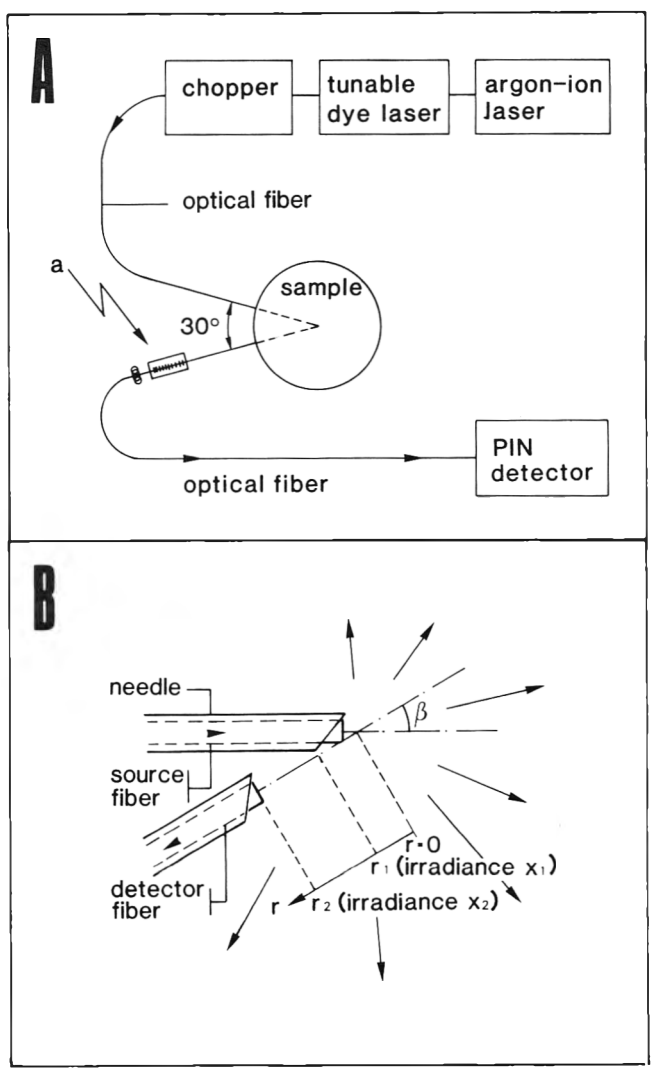


Fig. 1(A) Principles of apparatus for color measurement by optical fibers. When dry sausages were measured a sample of 300 cm^3 was used. a = screw to set distance between fiber ends. (B) Detail of measuring region, arrows indicate source flux in all directions; β is the angle between source and detector fiber.

Authors Slinde and Fyhn are affiliated with the Norwegian Food Research Institute, P.O. Box 50, N-1432 Aas-NLH, Norway.

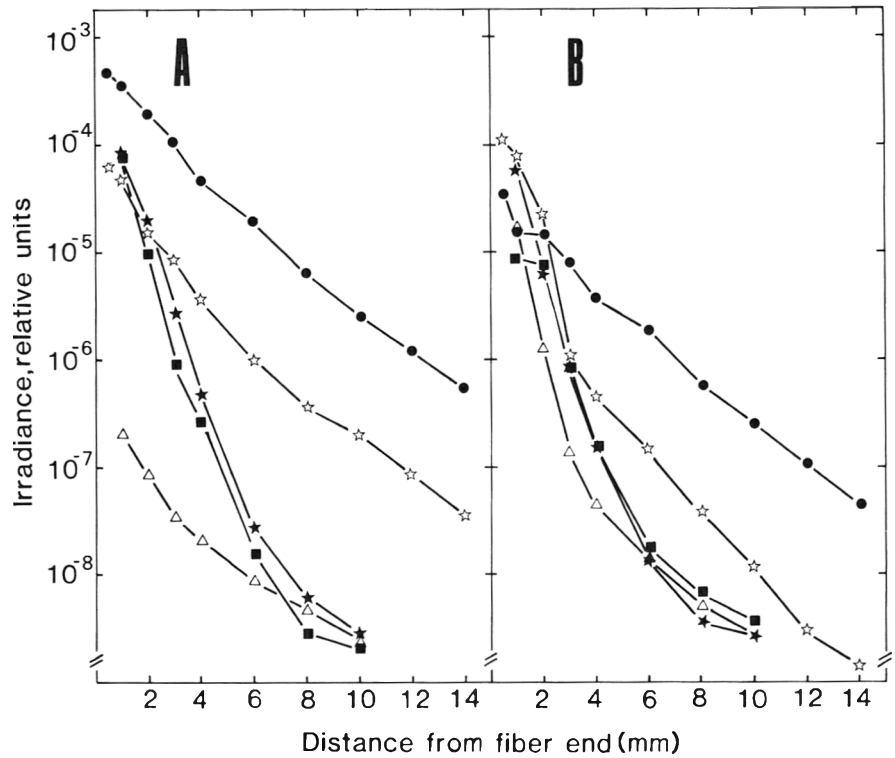


Fig. 2—Irradiance in dry sausages versus distance from end of source fiber to detector fiber at 457 (△), 488 (■), 514 (★), 625 (☆) and 650 nm (●) on day of the production (day 1, A) and at the end of fermentation (day 28, B). Deviation from linearity when the distance increases, is due to low irradiance which cannot be properly detected. The penetration depth (mm) was calculated for day 1 and day 28 to be: δ (457 nm) = 1.7 and 0.6; δ (488 nm) = 0.7 and 0.9; δ (514 nm) = 0.8 and 0.7; δ (625 nm) = 2.8 and 2.1; δ (650 nm) = 2.9 and 2.9.

x_1 and x_2, respectively (Fig. 1B). In brain tissue Svaasand and Ellingsen (1983) found that the intensity of the transmitted light showed an exponential decrease at distances larger than δ. The sensitivity of the method was independent of the orintation of the fibers. i.e. the irradiance was identical in all directions. The equation for the penetration depth is valid in the region used for light transmittance measurements (Birth, 1982).

Fig. 2 shows the detected irradiance for dry sausages as a function of the distance from the end-surface of the source fiber at five wavelengths on the day of production (Fig. 2A) and at the end of fermentation (Fig. 2B). The penetration depth is calculated from the slope of the curves in the linear region, i.e. at a distance greater than the penetration depth (Svaasand and Ellingsen, 1983).

When the color component, nitrosoheme, is formed in dry sausage there is a change of color (Slinde and Nordal, 1978). Among the wavelengths used in the present study the most pronounced changes in penetration depths were expected at wavelengths 457 nm (near the Soret region) and at 625 nm, as were indeed found (Fig. 2). Thus the method may be used to follow color development during dry sausage fermentation. From equation (1) it is seen that, ideally, measurements at only two distances is sufficient for determination of the penetration depth. Due to inhomogeneties, such as pieces of fat in dry sausages, the penetration depth has to be calculated from the slope of the curves, otherwise the errors become large.

The possibility of measuring spectral changes by optical fibers inserted into samples has advantages compared to surface reflectance measurements, such as where the surface pigmentation is different from internal. Continuous line measurements within samples during processing are made possible, with the advantage of keeping the sample in the processing atmosphere. The method is nondestructive if the pin holes from fiber insertions are disregarded.

A further example substantiates the potential of the method: In the case of strawberry jam it has been found that the absorbances at the wavelengths 425 nm and 505 nm strongly correlate to sensory analysis (Spayd and Morris, 1981). For a series of industrially produced jams of acceptable color we found that the penetration depth was 1.1 ± 0.1 mm at 509 nm. Thus the method may be used for quality control.

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A Research Note

Comparison of Media and Influence of Petri Plate Position (Inverted or Upright) for Determining Fungal Populations in Bulk-Stored, Dry, Seed-Based Foods

L. R. BEUCHAT and R. Y.-Y. CHIOU

ABSTRACT

Total populations of fungi and the presence of aflatoxigenic aspergilli were determined in 109 bulk-stored foods predominantly from seed origin offered for sale by retail grocers. Six media were evaluated for their suitability to support development of fungal colonies. The influence of the position of the Petri dish (inverted or upright) on colony formation during incubation was determined. Overall, dichloran-rose bengal-chloramphenicol agar, oxytetracycline-glucose-yeast extract agar, antibiotic-supplemented plate count agar and rose bengal-chlortetracycline agar were superior to acidified potato dextrose agar for recovering yeasts and molds. Considering any given medium, the position of the plate did not significantly ($P \leq 0.05$) influence colony development. Aflatoxigenic strains of *Aspergillus flavus* or *A. parasiticus* were detected in 3.7% of the samples.

INTRODUCTION

THE PRESENCE of fungal propagules on cereal grains and legumes as well as products produced from them is to be expected but does not represent a public health hazard unless moisture, temperature and atmospheric gas conditions are such that growth and subsequent mycotoxin production might occur. However, there has been some concern among public health officials regarding the lack of sanitation and handling procedures inherently associated with marketing practices in which bulk-stored dry foods and so-called health foods are sometimes offered for sale directly to the consumer (Mislivec et al., 1979; Mossel and Shennan, 1976). Food mycologists generally agree that spread plate techniques are superior to pour plate methods for enumerating yeasts and molds in foods (Flannigan, 1974; Jarvis et al., 1983). However, there is often disagreement as to whether Petri dishes in which diluted food samples have been deposited should be incubated in an inverted or upright position. The study reported here was designed to determine fungal populations and presence of aflatoxigenic aspergilli in bulk-stored cereal- and legume-based foods offered for sale by retail grocers. Six media and the position of the plate during incubation were evaluated.

MATERIALS & METHODS

A TOTAL of 109 bulk-stored, dry food samples was purchased from eight retail grocers in the Atlanta, GA area. Products evaluated consisted of one sample each of popcorn and pumpkin seed, three samples of barley, coconut and sesame seed, six samples of lentils (green and red), oats (rolled, split and pearled) and rice (brown short and long), seven samples of sunflower seed (raw and roasted), eight samples of granola, eleven samples of peas (blackeye, chick, split green and yellow) and nuts (almond, cashew, mixed, peanut, pecan and walnut), twelve samples of wheat (bran, bulghar, germ and pearled), fourteen samples of flours (buckwheat, corn oat, pumpernickel, rye, soy and wheat) and sixteen samples of beans (adzuki, black, kidney, mung, navy, pinto, red and soy). Duplicate samples (50g) were combined with 450 mL 0.1M potassium phosphate buffer (pH 7.0) containing 0.01% Tween 80 and vigorously shaken for 2 min. Serial dilutions (0.1 mL) of samples were surface plated in quadruplicate on plates of test media which had been poured 18-24 hr earlier and "dried" at 22°C.

Five media were evaluated for their suitability to support fungal colony formation: dichloran-rose bengal-chlortetracycline (DRBC, pH 5.6) agar as reported by King et al. (1979) and modified by Hocking (1981) and Pitt (1983) to contain chloramphenicol in place of chlortetracycline; oxytetracycline-glucose-yeast extract (OGY, pH 6.5) agar (Mossel et al., 1970); plate count agar (PCA, pH 7.0) containing 100 μg/mL each of chloramphenicol and chlortetracycline-HCl; potato-dextrose agar (PDA) acidified to pH 3.5 with tartaric acid; and rose bengal-chlortetracycline (RBC, pH 7.2) agar (Jarvis, 1973). A sixth medium, *Aspergillus flavus/parasiticus* agar (AFPA, pH 6.7) (Pitt et al., 1983), was employed to detect and enumerate this group of molds.

Duplicate plates of all dilutions on DRBC, OGY, PCA, PDA and RBC were incubated in inverted and upright positions for 5 days at 25°C before yeast and mold colonies were enumerated; AFPA plates were incubated for 2 days at 37°C.

The methods of Hesseltine et al. (1966) and Chiou et al. (1984) for detecting aflatoxin production by isolates of *A. flavus* and *A. parasiticus* was followed, with some modifications. Isolates from the 109 samples were picked from AFPA plates, purified and cultured on PDA slants for 14 days at 30°C. Sterile distilled water (3 mL) was deposited on the surface of the cultures and conidia were dislodged by gently rubbing with a sterile inoculating loop. Duplicate inocula of 0.5 mL heavy conidial suspension were transferred to 30 mL sterile potato dextrose broth (pH 6.7) in 125-mL Erlenmeyer flasks. Five milliliters of culture were removed on the fourth and seventh day of incubation at 30°C, combined with 5 mL chloroform and vigorously shaken. Water was removed from the samples and the chloroform was evaporated to about half its original volume. Presumptive and confirmed tests for aflatoxin were done by determining the presence of fluorescent blue, green or blue-green spots on thin layer chromatography plates (K5 Silica gel, 250μ, 20 × 20 cm, Whatman chemical Separation Division, Clifton, NJ) spotted with 25 and 10 μL sample extract and developed in chloroform:acetone (9:1, vol:vol). The presence of specific aflatoxins (B_1, B_2, G_1 and G_2) was confirmed by comparing R_f values with those from commercial aflatoxin standards as well as from extracts of control samples originally inoculated with a known aflatoxin producer (*A. parasiticus* NRRL-2999).

Mean values of populations of fungi enumerated in 1308 tests (109 samples, six media, two plate positions) were calculated and significant differences between means for media as well as for inverted versus upright plates were determined. Statistically significant ($P \leq 0.05$) differences between mean values were determined by subjecting data to analysis of variance and Duncan's multiple range test (SAS, 1979).

RESULTS & DISCUSSION

SINCE ONE of the objectives of this study was to determine if one or more media were superior to others for enumerating fungi in a wide range of dry seed-based foods, it was desirable to determine significant differences between means of composites of values of all 109 food samples for each test medium (Table 1). Significantly higher populations were detected in 11 of 545 tests (five enumeration media, 109 samples) (2.0%) when plates were incubated in an inverted position; conversely, significantly higher populations were detected in 17 (3.1%) tests when plates were incubated in an upright position.

Authors Beauchat and Chiou are affiliated with the Dept. of Food Science, Univ. of Georgia, Agricultural Experiment Station, Experiment, GA 30212.

Table 1—Comparison of inverted and upright position of plates for enumerating colony-forming units (CFU) in a composite of 109 dry foods

Plate position	Recovery medium					
	Total CFU/g[a]					*A. flavus/ parasiticus* (CFU/g)[b]
	DRBC	OGY	PCA	PDA	RBC	AFPA
Inverted	4020ab	4264ab	3670b	1573c	5541a	21
Upright	4522ab	4192ab	4047ab	1410c	4390ab	24
Composite	4271a	4228a	3863a	1491b	4965a	23

[a] Mean values for total yeasts and molds enumerated on inverted and upright plates which are not followed by the same letter are significantly different ($P \leq 0.05$). Likewise, mean values of composite populations not followed by the same letter are significantly different ($P \leq 0.05$).
[b] No significant differences ($P \leq 0.05$) were detected between mean values for *A. flavus/parasiticus* population detected on inverted and upright plates.

Inverted RBC plates were superior to inverted PCA plates, and PDA was significantly inferior to DRBC, OGY, PCA and RBC, regardless of plate position during incubation. There were no significant differences between DRBC, OGY, PCA and RBC, regardless of plate position. A composite of mean values for populations enumerated on inverted and upright plates for each test medium further confirms observations that, overall, DRBC, OGY, PCA and RBC perform equally well and are superior to PDA for recovering fungi from dry foods predominantly consisting of or derived from cereals and legumes. However, some consideration should be given to selection of a medium based on predilection of the predominant type of fungus in the sample to be analyzed. When spreading molds are present, DRBC would be the medium of choice, since colony development is restricted, thus facilitating enumeration. Fewer bacterial colonies developed on antibiotic-supplemented PCA, and yeast colonies appeared to develop best on OGY for foods examined here.

When the data were collapsed even further, i.e., mean values for all inverted and all upright plates of DRBC, OGY, PCA, PDA and RBC media were independently determined, significant differences in fungal populations were not observed when comparing the position of the plates during incubation. The composite mean values were 3814 CFU/g on inverted plates and 3712 CFU/g on upright plates. Likewise, the number of *A. flavus* and *A. parasiticus* colonies detected on AFPA was not influenced by plate position (Table 1).

Colonies developing on AFPA which were judged to belong to the *A. flavus/parasiticus* group were detected in only 16 of the 109 samples. In presumptive tests of aflatoxin, 10 of 34 isolates from these samples were positive on the fourth day of incubation; one additional isolate was positive for aflatoxin production on the eleventh day. Among these eleven isolates, five were confirmed to be aflatoxin producers. These isolates originated for four food samples (3.7% of total analyzed): rice, brown rice and two samples of walnuts. In light of the demonstrated ability of *A. flavus* to survive in dry foods for extended periods of time (Beuchat 1979, 1984), these samples could represent a public health hazard if they were subjected to environmental conditions which would enable the mold to grow and produce aflatoxin.

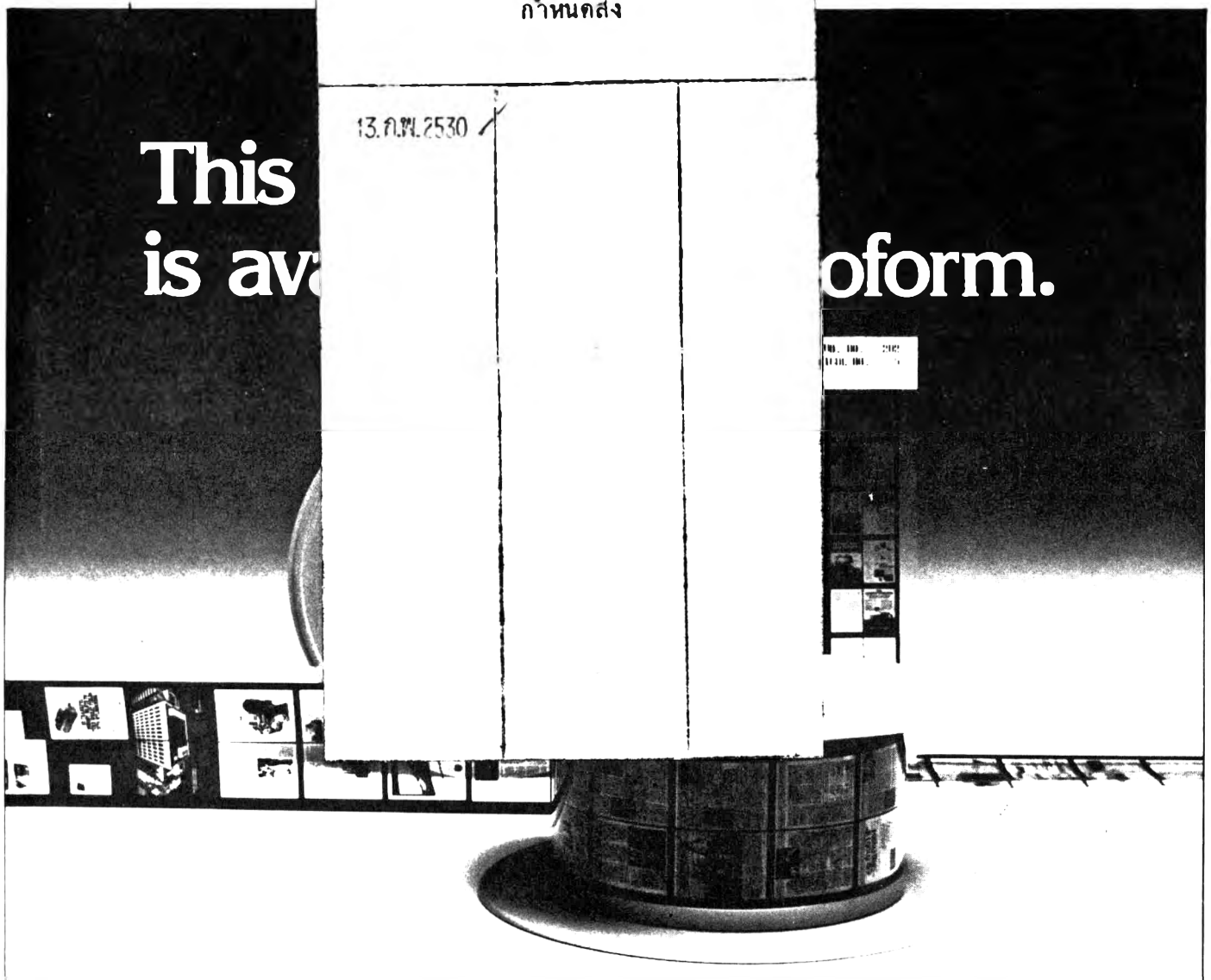
It was of interest to compare composite data for total fungal populations by their source, i.e., the store from which they were purchased. Populations detected in foods from four stores were considered low (320–720 CFU/g), from three stores intermediate (1200–3100 CFU/g) and from the eighth store as high (14000 CFU/g). It is interesting to note that store number 8 was the only store included in the study which is strictly a health food outlet.

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