1 January-February 1987
Volume 52, No. 1

Coden: JFDAZ 52(1):1-244 (1987)
ISSN: 0022-1947

JOURNAL OF FOOD SCIENCE

Research, Applied Science and Engineering

CATION OF
ITUTE OF FOOD TECHNOLOGISTS

Research Notes

AUTHOR INDEX
Volume 52: Number 1

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January-February 1987
Volume 52, No. 1

JOURNAL OF FOOD SCIENCE

ISSN:0022-1147

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Enzymatic Determination of Trimethylamine and Its Relationship to Fish Quality

K. WONG and T.A. Gill

ABSTRACT

An enzymatic procedure was developed to determine trimethylamine (TMA) in fish muscle extracts. The method utilized TMA dehydrogenase extracted and purified from *Hyphomicrobium X* and gave excellent correlations with the picric acid and HPLC methods. The minimum detectable TMA level was 0.05 μmoles. The entire enzymatic procedure required 15–20 min to complete. The method may be used in the laboratory as a spectrophotometric analysis or may be used semi-quantitatively as a visual color comparison test outside the laboratory. The method greatly simplifies the procedures for TMA determination and enables the estimation of microbiological quality of fish with very little laboratory equipment.

INTRODUCTION

BEATTY AND GIBBONS (1936) reported the separation of a fraction of the volatile nitrogenous bases (TVB) from decomposing cod muscle which was found to develop exclusively from bacterial decomposition. Beatty (1938) later showed that approximately 99% of the TVB fraction isolated from press juice in spoiling cod consisted of trimethylamine (TMA). Hoogland (1958) demonstrated the potential use of TMA as an objective quality indicator. In a detailed study of cod and haddock spoilage, he showed a high positive correlation between subjective grading scores of experienced fish graders and TMA index where TMA index = log (1 + TMA value). Although the production of TMA in spoiling fish was observed to be exponential with time for any set of spoilage conditions, TMA index was observed to increase in direct proportion to postmortem age for any set of spoilage conditions. Laycock and Regier (1971) demonstrated that all *Altermonas putrefaciens* isolates from spoiling haddock were TMA producers and concluded in the same study that this organism was largely if not wholly responsible for observed TMA production in haddock. Dyer and Dyer (1949, 1950) concluded that for most fish, the usual sensory borderline of acceptable quality for cod and similar fish was 15 mg TMA-N per 100g tissue.

The Dyer (1945) method as modified by Tozawa et al. (1971) has been used for many years for the determination of TMA in fish. Other methods of analysis have been proposed including gas chromatography (Ruiter, 1973; Miller et al., 1972; Gruger, 1972; Kuwata et al., 1980; Tokunaga et al., 1977); ion specific electrode (Chang et al., 1976); high performance liquid chromatography (Gill and Thompson, 1984) and enzymic assay (Large and McDougall, 1975) to name but a few. Methods of TMA analysis for fish proposed to date share one or more of the following disadvantages: lack of specificity, cannot be performed outside the laboratory environment, expensive and time consuming. None of the methods proposed to date are applicable to routine testing large numbers of fish and few can be readily performed by personnel with little or no laboratory experience.

The objective of the present study was to develop a rapid visual color test for the estimation of TMA in fish extracts. This method would eliminate most of the disadvantages inherent in previously reported methods.

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MATERIALS & METHODS

Enzyme preparation

Hyphomicrobium X was grown in 25L fermentors with 0.25% TMA-HCl as the sole carbon source under anaerobic conditions as described by Meiberg and Harder (1978). Bacterial cells were suspended in 0.05M phosphate buffer pH 7.5 in a ratio of 10g per 50 mL buffer. TMA dehydrogenase was prepared from a cell-free extract as de-

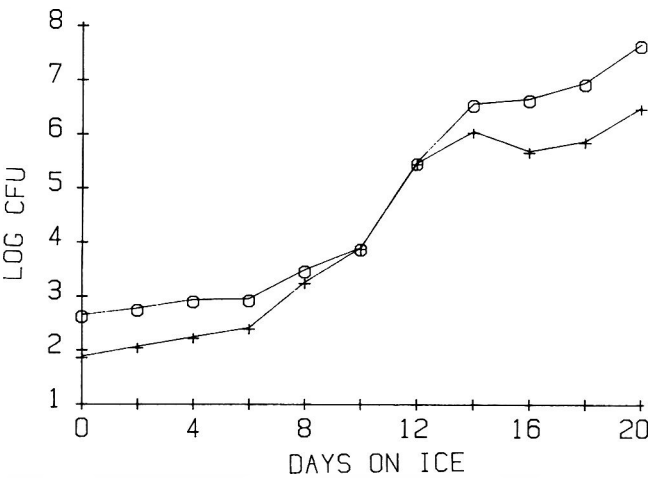

Fig. 1—Growth of bacteria on iced gutted cod. Plate counts were perfomed on plates incubated at 21°C (□) and 5°C (+).

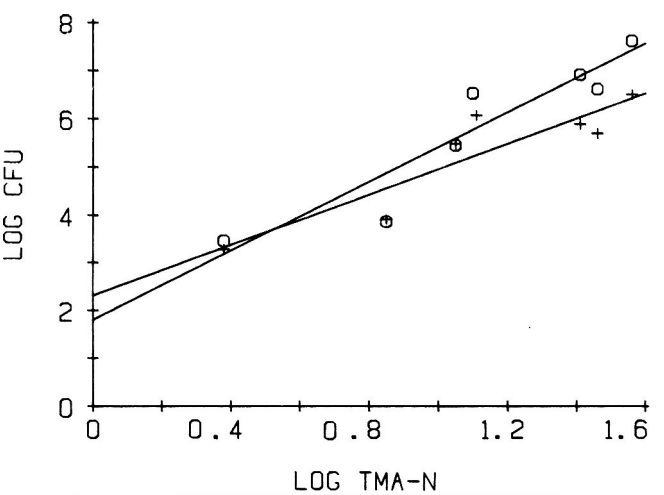

Fig. 2—Relationships between log colony forming units and log TMA-N in iced cod tissue. Data point represent an average of three determinations. The lines are derived by least squares best fit. Bacterial counts were performed on plates incubated at 21°C (□) and 5°C (+).

Table 1—Correlation matrix for cod spoilage data

	TMA-N	Log TMA-N	Bact. 21°C	Log bact. 21°C	Bact. 5°C	Log. bact. 5°C	Overall grade	Log overall grade	Odor score	Log odor score
TMA-N	1.00	0.909	0.835	0.896	0.626*	0.804	0.722	0.729	0.890	0.936
Log TMA-N		1.00	0.771	0.937	0.626*	0.909	0.773	0.858	0.748	0.790
Bact. 21°C			1.00	0.633	0.835	0.559*	0.864	0.804	0.665	0.577
Log bact. 21°C				1.00	0.791	0.980	0.909	0.918	0.766	0.771
Bact. 5°C					1.00	0.749	0.810	0.615	0.760	0.648
Log. bact. 5°C						1.00	0.732	0.865	0.704	0.735
Overall grade							1.00	0.986	0.789	0.786
Log overall grade								1.00	0.797	0.828
Odor score									1.00	0.955
Log odor score										1.00

* Not significant at the 95% level.

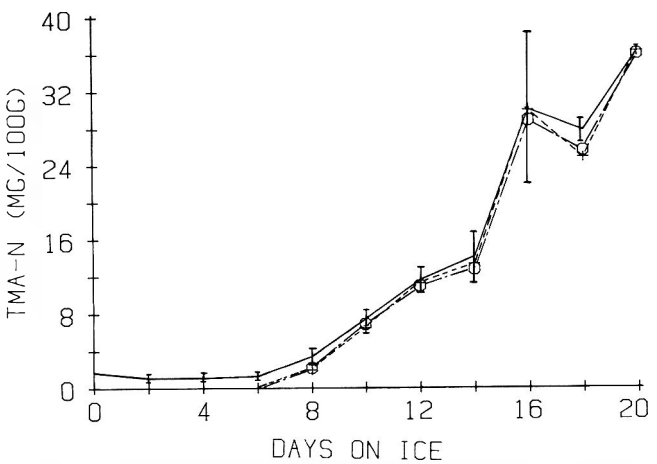

Fig. 3—Production of TMA-N in iced gutted cod. Determinations were performed by the picric acid method (——), the HPLC procedure (+ – – – – – +) and the new spectrophotometric enzyme assay (□ – – – □). Error bars indicate standard deviations for determinations performed on three individual fish by the picric acid method. The average standard deviation for values determined by the picric acid, HPLC and enzymic methods were 1.77, 3.44 and 2.89, respectively.

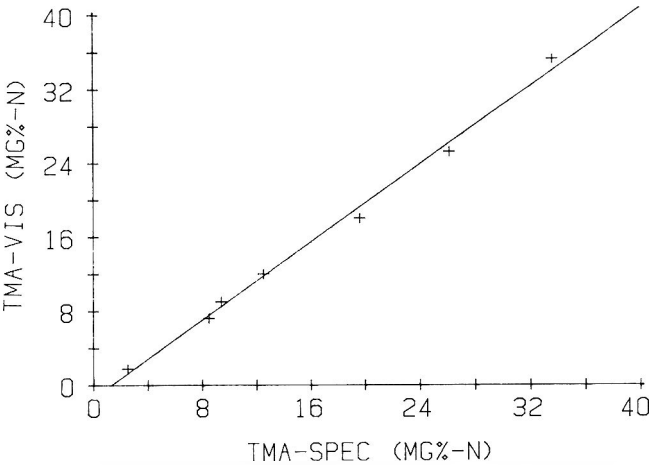

Fig. 4—Performance of the visual color test as compared to spectrophotometric procedure for the determination of TMA in fish extracts. Both tests were performed with TMA dehydrogenase prepared from Hyphomicrobium X.

Table 2—Linear regression parameters for three methods of TMA analysis[a]

	Enzyme vs HPLC	HPLC vs Picric	Enzyme vs Picric
Slope	0.983	1.014	0.999
Intercept	0.269	−1.014	−0.779
r^2	0.998	0.994	0.997

[a] n = 7

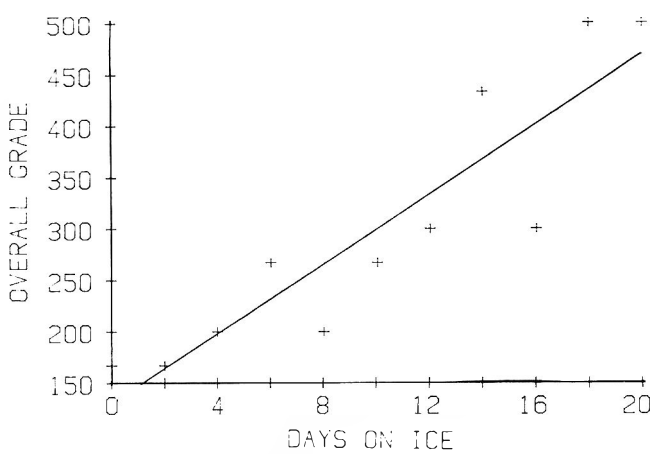

Fig. 5—Relationship between overall subjective grade and time of spoilage for iced gutted cod.

scribed by Meiberg and Harder (1978). Purification of the enzyme was accomplished by ultrasonic cell disruption, 15 min heat treatment at 60°C, ammonium sulfate precipitation and ion exchange chromatography. The activity of the fraction precipitating between 50 and 85% ammonium sulfate saturation was typically 70 units per mg protein. DEAE cellulose, DEAE Sephadex (Pharmacia) or Accell QMA (Waters) ion exchange chromatography yielded specific activities of 425, 390 and 310 units per mg protein, respectively. Recoveries ranged from 70 to 80%.

Enzyme assay

The method described by Colby and Zatman (1973) was used to follow purification of TMA dehydrogenase. A typical assay mixture contained 100 μmoles K_2HPO_4, 0.05 mg dichlorophenolindophenol (DCPIP), 16 μmoles phenazine methosulfate (PMS), 0.1 μmoles TMA and enzyme, all in a total volume of 3.0 mL, final pH of 7.4. The reference curvette contained all of the components except TMA, which was replaced with distilled water. The reaction at 30°C was monitored spectrophotometrically at 600 nm. One unit of activity was defined as the amount of enzyme required to catalyze the reduction of 1 nmole DCPIP per min.

Enzymatic determination of TMA in fish extracts

The method was based on the oxidation of TMA with PMS in the presence of TMA dehydrogenase. The reduced $PMSH_2$ formed converts *p*-iodonitrotetrazolium violet (INT) to red formazan. The reaction mixture contained 200 μmoles N-tris [hydroxmethyl]methyl-2-aminoethanesulfonic acid (TES) buffer, 0.5 mL 10% (v/v) Triton X-100 solution, 0.1 mL 0.2% (w/v) INT in ethanol, 8 μmoles PMS, 92 units of TMA dehydrogenase, and neutralized perchloric acid fish extract containing ≤ 0.3 μmoles TMA. The final pH of the reaction mixture was 7.4 (adjusted with KOH) and final volume of 2.5 mL. Dilutions ranging from 1:1 to 1:20 of most neutralized fish extracts were typically used for the assay procedure.

A blank reaction mixture for the spectrophotometric determinations contained all of the components of sample mixtures except for fish extract which was replaced with water. After 20 min at 30°C, 0.5 mL 10% H_3PO_4 was added to terminate the reaction and the absorbance at 500 nm was recorded immediately. Due to the reactivity of PMS and INT with light, all incubations were carried out in the dark. The

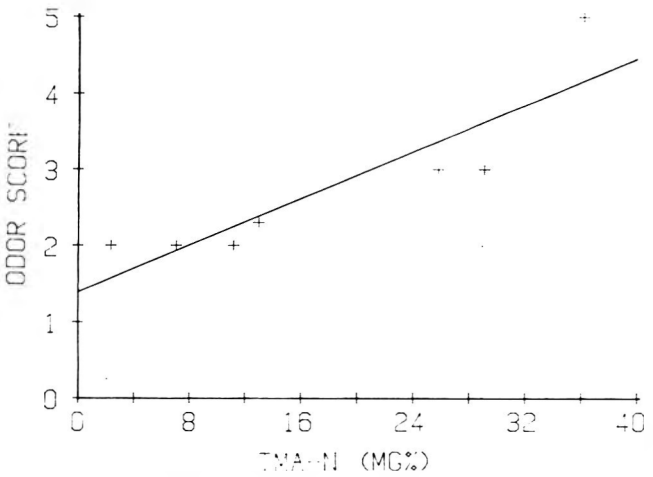

Fig. 6—Relationship between odor score and enzyme test data (spectrophotometric procedure) for TMA-N. All data points are averages for three individual iced cod. The line was determined by linear regression analysis.

levels of TMA were calculated from a set of standards prepared from TMA-HCl.

Alternatively, the TMA was estimated semi-quantitatively by five inexperienced judges. Five color standards containing 0, 0.05, 0.1, 0.2, and 0.3 μoles TMA were used. Judges were asked to compare colors of unknown samples with standards, thus estimating the TMA level to the nearest 0.05 μmoles.

Alternative methods of TMA analysis in fish

Trimethylamine was determined in fish extracts by the picric acid procedures of Dyer (1945) as modified by Tozawa et al. (1971). TMA was also determined by high performance liquid chromatography (HPLC) using the method of Gill and Thompson (1984).

Storage of fish and perparation of extracts

Gutted whole cod (*Gadus morhua*) still in rigor were purchased from a local fish market. Two layers of fish were packed in ice and held in a 6°C cold room. Three fish were taken at random every 2 days for physical grading according to Woyewoda et al. (1986) by an experienced grader. Essentially, the fish were graded according to the following criteria: (1) extent of gutting and washing; (2) texture of the fish; (3) odor at the neck; (4) general appearance of eyes and body; (5) odor and color of gills; and (6) presence or absence of blood clot, texture and odor of the fillets.

The overall grades obtained were further analyzed by the method of Woyewoda et al. (1985). Essentially, grades A,B,C, and R (reject) obtained were arbitrarily assigned numbers of 1,2,3, and 5, respectively. Multiplying these numbers by the percentage of fish in each category and summing the derived scores would give an overall grade for the three fish. For example, all three fish graded as A would have a score of 100, ie. 100×1; three fish with 67% grade A and 33% grade B would have a score of $(67 \times 1) + (33 \times 2) = 133$. From this scheme, the lower the score, the fresher the fish.

Fillet odor scores were analyzed separately by replacing the grades A,B,C, and R with 1,2,3, and 5, respectively. Again, the lower the scores, the fresher the fillets.

Microbiological enumerations were performed by the standard methods (Sharf, 1966). Mesophilic counts were carried out at 21°C and psychrophilic counts performed at 5°C.

Perchloric acid extracts were carried out as follows. Fillets were removed and minced in a Cuisinart food processor, and 100g mince were further blended in 200 mL 6% perchloric acid for 2 min in a Waring Blendor at high speed. The homogenate was filtered through Whatman #1 filter paper and 50 mL aliquots frozen for further analysis. Analyses for TMA were then performed on extracts which were thawed and neutralized to pH 6.8 to 7.0 with 30% KOH.

RESULTS & DISCUSSION

FIGURE 1 shows the proliferation of spoilage bacteria on iced cod. It has been known for some time that *Altermonas (Pseudomonas)* is the bacterial genus of primary importance in the spoilage of N. Atlantic fish species (Laycock and Regier, 1971; Shewan, 1971). It has also been shown that plate counts carried out at mesophilic temperatures may not necessarily reflect the degree of fish spoilage (Liston, 1979). Figure 1 illustrates that for most samples, the bacteria counts performed at 5°C were generally lower than those at 21°C although this is not always necessarily true.

Figure 2 and Table 1 illustrate that the relationship between log bacterial counts and log TMA level is essentially linear. The correlation coefficients (r) relating log bacterial numbers at 21°C and 5°C to log TMA were 0.937, 0.909, respectively. Both values were significant at the $P \leq 0.01$ level.

Figure 3 depicts the production of TMA in iced cod with time. Measurements were mean values for three individual fish as analyzed by the enzymic (spectrophotometric), picric acid and HPLC procedures. Excellent agreement among the three methods was observed. Linear regression parameters for the relationships among the three procedures are given in Table 2. All three coefficients of determination (r^2) were significant at the $P \leq 0.01$ level. The minimum detectable TMA level in a fish muscle extract was approximately 0.05 μmoles for the spectrophotometric enzymic assay. The average coefficient of variation on replicate samples with concentration near the detection limit for the assay was 4.3%. These data compare favorably with the other two methods of TMA analysis.

Figure 4 illustrates the performance of the visual color test compared to the spectrophotometric TMA assay. The slope, intercept and coefficient of determination (r^2) for the best fitting linear regression equation were 1.05, −1.38 and 0.99, respectively. The regression equation was significant at the $P \leq 0.01$ level. The visual color test was more rapid than any of the previously described techniques and yielded essentially the same results. The equipment required for the color test included only a blendor and balance for sample preparation as well as a 30°C thermostatically controlled incubator or heating block. TMA dehydrogenase was stable for at least 3 months when held at −25°C and did not react with trimethylamine oxide, dimethylamine or momomethylamine. Choline was found to be a competitive inhibitor. However, the volume of extract used did not inhibit enzyme activity. Color development was rapid and after the addition of H_3PO_4, the color was stable for at least 1 hr in the dark or at least 30 min in a well-illuminated room.

The subjective assessement of iced cod quality included categories for texture, odor at neck and gills, general appearance, color of gills, appearance of eyes and color of tissue. The overall grade was converted to a numerical score as described by Woyewoda et al. (1985). The relationship between overall grade and time of spoilage is illustrated in Fig. 5 where the data points are depicted as averages of the numerical scores for three individual fish. Although the correlation coefficients for the relationships between overall grade vs TMA-N, overall grade vs log TMA-N, log overall grade vs TMA-N are significant at the $P \leq 0.05$ level, the relationship between odor score and TMA-N was slightly better (Fig. 6). It would perhaps be expected that TMA level should best reflect the development of off-odors in seafood products. It should however be noted that the application of parametric statistical analysis of non-parametric data such as subjective grades, is dangerous (Siegel, 1956). The use of TMA-dehydrogenase greatly simplifies the procedure and enables the estimation of microbiological quality with very little laboratory equipment. Further work will involve acceleration and immobilization of the TMA color reaction.

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—Continued on page 6

Impact of Infrared Broiling on the Thiamin and Riboflavin Retention and Sensory Quality of Salmon Steaks for Foodservice Use

Y. TAKAHASHI and M.A. KHAN

ABSTRACT

Salmon steaks were broiled using infrared radiation and compared to convection oven baking. Total percent cooking losses of moisture and fat content were not significantly different. Samples broiled by infrared oven retained 87.2% and 92.6% of thiamin and riboflavin content, respectively. There were no significant differences for vitamin retention by both methods. Appearance and color of salmon steaks baked in the convection oven were rated significantly higher than infrared broiled samples. Tenderness and juiciness scores for infrared broiled steaks were significantly higher than those for convection oven baked steaks. There were no significant differences in panel scores for odor, flakiness, flavor and overall acceptability of steaks prepared by both methods.

INTRODUCTION

INFRARED RADIATION has considerable potential for use in the preparation of meats, particularly in foodservice operations. However, there are limited published reports on the impact of infrared radiation on nutritional and sensory quality of meats. Since there are practically no reported studies on the impact of infrared radiation on the nutritional and sensory qualities of salmon, this review is based on published data on pertinent studies with other food items. Ang et al. (1975, 1978) studied thiamin and riboflavin retention in frozen prepared food products reheated by different methods, including the use of infrared and convection ovens. They found that the reheating time varied from product to product, but the infrared method was always faster than the convective heating.

VanderMey (1985) did not find a significant difference in thiamin content of beef steaks either on as cooked or on a dry, fat-free basis, although the gas broiled steaks retained slightly more thiamin than those which were broiled in infrared broiler. There were no significant differences among panelist's sensory evaluations for appearance, flavor, juiciness, tenderness and overall acceptability of steaks prepared by the two methods. Charley and Goertz (1958) studied the effect of different oven temperatures on palatability of salmon. The taste panel recognized the differences in palatability among various parts of the fish but not the differences in quality, due to different oven temperatures.

The conveyorized tube broiler (CTX Model #80, CTX-Pet Industries, Fenton, MO) produces a greater number of servings per unit of time when compared to the conventional method (Khan and VanderMey, 1985). It was of interest to determine whether it could be used in foodservice operations with the production of equivalent or better quality products than conventional methods. The purpose of this study was to compare salmon steaks cooked by the infrared tube broiler with those cooked by convection oven with respect to moisture, fat, thiamin and riboflavin content, in addition to sensory attributes.

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MATERIALS & METHODS

TWO WHOLE FRESH SALMON (without head) were purchased from a local retail store on four consecutive weeks. Each fish (A and B) was cut into eight steaks (Fig. 1), about 2.5 cm thick. Each steak was weighed to the nearest tenth of a gram, labeled as per the position and stored at 4°C until cooked. The fifth steak from each fish was used for the determination of moisture, fat, thiamin, and riboflavin contents of the raw sample. The first, third, sixth, and eight steaks from fish A and the second, fourth, and seventh steaks from fish B were used, in this order, for broiling in a conveyorized infrared tube broiler. The rest of the steaks, i.e., the second, fourth, and the seventh steaks from fish A and the first, third, sixth, and eighth steaks from fish B, were used for roasting in a convection oven (Market Forge, Everett, MA, Model M-2600). The same order was followed for all replications in this study. A diagram for the infrared tube broiler (CTX Model #80, CTX-Pet Industries, Fenton, MO) is shown in Fig. 2. The unit uses 208 V, single phase, 60 Hz, 23 amp and 4.7 kw of electricity. The broiler has upper and lower temperature controls. The product to be broiled is put on the entrance conveyor and it is carried through the chamber. The degree of searing as well as the speed of the conveyor can be controlled. The temperature and the cooking time needed to yield a final internal temperature between 60° and 65°C were determined in preliminary trials. The convection oven-cooked salmon steaks were placed directly on a 38 × 56 cm ungreased pan and roasted on the center rack in the oven at 163°C for 18 to 20 min until the internal temperature was between 60° and 65°C as determined by thermocouples placed at the center of the steaks. For both cooking methods no seasonings were added. Seven steaks were cooked by each method for each replication. Immediately after cooking, weight was recorded, from which cooking losses were calculated. Similar portions of the steaks were used for chemical (moisture, fat and vitamin) analyses and for sensory evaluations for all treatments in this study.

Thiamin determinations were conducted as described by Lee et al. (1981) using a modification of the thiochrome method outlined by the Association of Vitamin Chemists (1966). Activated Bio-Rex 70 resin (Bio-Rad Laboratories, Richmond, CA) and takadiastase enzyme (Ac-

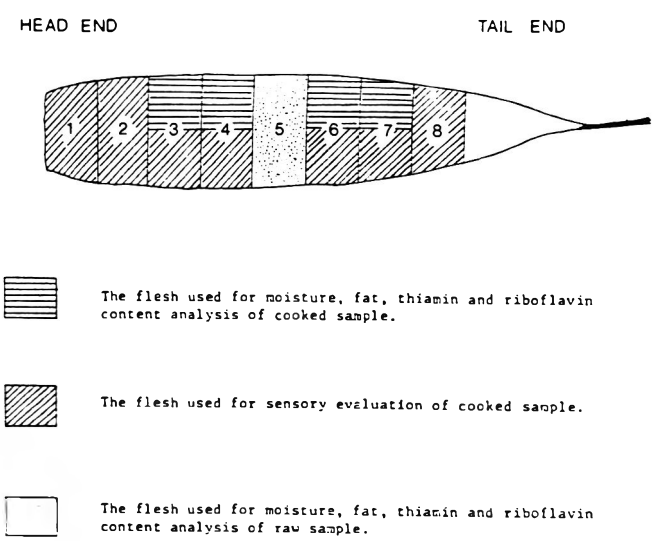

Fig. 1—Top view of salmon showing steaks and analysis performed on each steak.

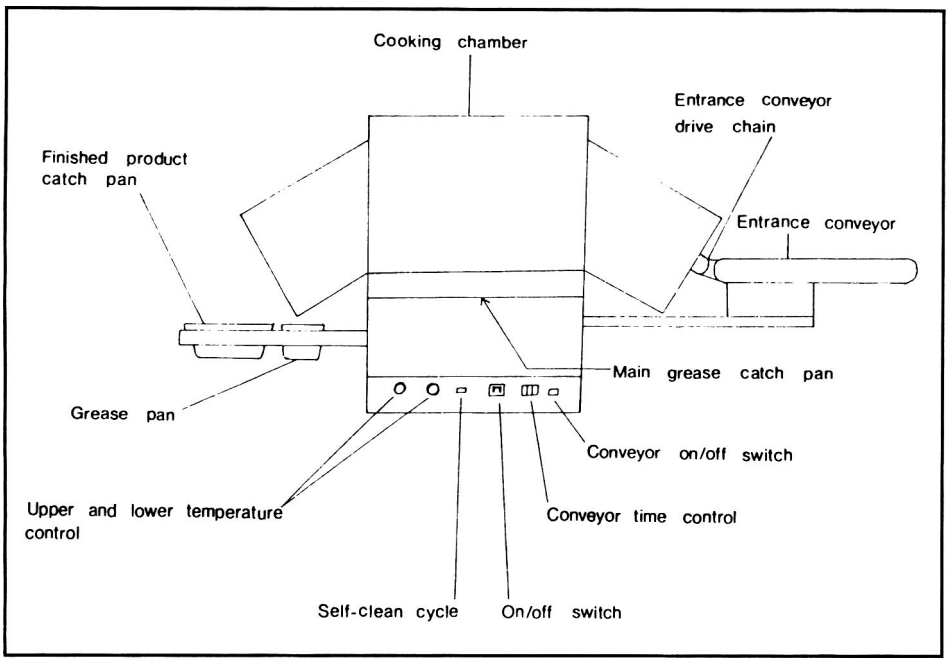

Fig. 2—Conveyorized infrared tube broiler.

curate Chemical and Scientific Corp., Westburg, NJ) were used in the analysis. Moisture and fat determinations were conducted according to the AOAC (1975) procedures. Riboflavin determinations were done using a fluorometric method (AOAC, 1980). Duplicate samples for each replication were used.

Sensory evaluations were conducted by an 8- to 10-member trained taste panel under incandescent light at room temperature between 2:00 and 3:00 p.m. Ten samples from the upper part of the dorsal half of the muscle above the lateral line were taken from steaks cooked by each method. The samples were cut uniformly and served within approximately 2 hr of preparation at room temperature on a polystyrene plate with a plastic fork, two pieces of unsalted crackers and a glass of water. Each week the panelists received the randomly selected samples. Sensory attributes were evaluated on a 0 (the lowest end of the scale) to 15 metric scale, measurements being recorded to the nearest tenth of a centimeter. Attributes evaluated, and their rankings, were appearance (very undesirable to very desirable); color (very undesirable to very desirable); odor (very undesirable to very desirable); tenderness (very tough to very tender); flakiness (difficult to flake to easy to flake); juiciness (very dry to very juicy); flavor (unacceptable to excellent); and overall acceptability (unacceptable to excellent).

A two-way analysis of variance was conducted to determine the statistical significance. When observations were significantly different, a test for Fisher's least significance differences (Snedecor and Cochran, 1980) was conducted. Interrelationships among the percent total cooking losses, percent moisture and fat contents and percent thiamin and riboflavin retentions were analyzed by correlation methods outlined by Snedecor and Cochran (1980).

RESULTS & DISCUSSION

THE TOTAL PERCENT COOKING LOSSES when the convection oven was used (13.2%) were higher than those for the infrared broiler (11.8%); however, this difference was not found to be significantly different. The percent moisture (Table 1) content of the infrared cooked sample was slightly higher than that of the convection oven cooked samples. The mean percent fat content of the raw sample (5.9%) was close to 5.3% and 5.7% reported by Stansby (1976) and Karrick and Thurston (1964), respectively. Between the infrared broiled and convection oven baked samples, the former had lower percent fat content, although this difference was not significant. Also, Stansby (1976) reported that moisture content in salmon is inversely related to the fat content. Simple correlations between fat and moisture content of raw and cooked salmon steaks in this study were found to be significant at the 1% level.

Thiamin and riboflavin retention

Thiamin (Table 1) content of the cooked salmon sample was found to be 1.797 and 1.795 mcg per gram for the infrared and the convection oven method, respectively. Thiamin content of the raw cooked samples did not differ significantly on original weight basis. There was a significant difference in thiamin content on a dry, fat-free basis between the raw and cooked samples at the 5% level. There was no significant difference in thiamin content among the samples broiled by the infrared and the convection oven methods. This difference was not significant between treatments and among replicates. In this study, probably the longer cooking time at lower temperature and the shorter cooking time at higher temperature compensated for each other. Ang et al. (1975, 1978) reported that thiamin retention after convection oven and infrared oven heatings was similar.

There were no significant differences in the riboflavin contents between the samples on an as cooked basis or on a dry, fat-free basis between the two methods. The percent riboflavin retention in salmon steaks did not differ significantly among the methods studied, although the percentage riboflavin retention was much higher than the percent thiamin retention. Correlation coefficients between total cooking losses and percent moisture content versus thiamin and riboflavin retention were assessed with the assumption that as the cooking losses increased, the retentions of the vitamins decreased. There was no significant correlation between these variables. Also there was no significant correlation between the cooking losses and the percent fat and moisture content.

Sensory evaluation

The scores of the various attributes are presented in Table 2. Appearance of the convection oven baked salmon was rated significantly higher ($p < 0.01$) than the infrared broiled salmon. The color of the convection oven baked salmon was significantly different ($p < 0.05$) than the infrared broiled steaks. The color of the convection oven baked salmon was lighter than those baked by the infrared broiler. There were no significant differences among the scores for odor. Infrared broiled salmon was scored significantly higher ($p < 0.05$) for tenderness than the convection oven baked salmon. There was no significant difference in the scores for the flakiness. Also, there was no significant difference among the flavor scores for salmon

Table 1—Moisture, fat, thiamin, and riboflavin content of raw, infrared broiled and convection oven baked salmon steaks[a,b]

	Raw steaks	Infrared broiler	Convection oven
Percent moisture	70.7 ± 4.4[a**]	69.4 ± 3.6[ab**]	67.8 ± 3.6[b**]
Percent fat	5.9 ± 4.5 NS	5.2 ± 3.2 NS	5.6 ± 3.9 NS
Thiamin content mcg/g (as cooked)	1.825 ± 0.048 NS	1.797 ± 0.099 NS	1.795 ± 0.203 NS
Thiamin content mcg/g (dry, fat-free basis)	7.801 ± 0.162*[a]	7.071 ± 0.3306[b*]	6.752 ± 0.747[b*]
Percent thiamin retention (as cooked)	100	87.2 ± 4.60 NS	85.5 ± 9.9 NS
Riboflavin content mcg/g (as cooked)	1.690 ± 0.216 NS	1.758 ± 0.134 NS	1.787 ± 0.968 NS
Riboflavin content mcg/g (dry, fat-free basis)	7.233 ± 0.990 NS	6.924 ± 0.586 NS	6.721 ± 0.334 NS
Percent riboflavin retention (as cooked)	100	92.6 ± 6.5 NS	92.7 ± 9.4 NS

[a] Values are the mean ± standard deviation of at least two samples per replicate.
[b] Where letters differ horizontally, the values are significantly different (* at 5% level and ** at 1% level).
[NS] No significant difference.

Table 2—Sensory evaluation of infrared broiled and convection oven baked salmon steaks[a,b,c]

Attribute	Infrared broiler	Convection oven
Appearance	6.79 ± 0.65[a**]	9.16 ± 0.67[b**]
Color	6.88 ± 0.77[a*]	9.62 ± 0.83[b*]
Odor	8.43 ± 1.00 NS	8.99 ± 0.83 NS
Tenderness	10.86 ± 0.62[a*]	8.07 ± 1.06[b*]
Flakiness	9.22 ± 0.71 NS	8.26 ± 1.65 NS
Juciness	11.24 ± 0.85[a**]	7.45 ± 1.37[b**]
Flavor	8.97 ± 0.93 NS	9.56 ± 0.68 NS
Overall acceptability	8.10 ± 0.91 NS	9.31 ± 0.99 NS

[a] Values are the mean ± standard deviation.
[b] Scores are based on a 15-point scale with 0 as the lowest possible score and 15 as the highest possible score.
[c] Where letters differ horizontally, the values are significantly different (* at 5% level and ** at 1% level).
[NS] No significant difference.

prepared by both methods. However, there was a significant difference ($p < 0.01$) among the scores for juiciness. The infrared broiled salmon was rated higher than the convection oven baked samples. This may be attributed to the relatively short cooking time of the infrared method. The overall acceptability of the salmon steaks cooked by the two methods did not differ significantly.

Since the important aspect of this study was to evaluate the effects of infrared broiling and convectional baking on quality characteristics of salmon steaks prepared for foodservice use, it may be concluded that infrared broiling did not have any adverse effect on the thiamin and riboflavin retention. Also, except for the appearance, the sensory quality was not significantly different from the convection oven baked salmon steaks.

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Ms received 4/7/86; revised 7/28/86; accepted 8/5/86.

This paper is partly based on a thesis submitted by the senior author in partial fulfillment of an M.S. degree at the University of Illinois at Urbana-Champaign.

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Ms received 3/28/86; revised 9/8/86; accepted 9/12/86.

The present study was funded through a Natural Sciences and Engineering Research Council of Canada Strategic Grant.

Novel Products from Underutilized Fish Using Combined Processing Technology

ENDEL KARMAS and ELLEN LAUBER

ABSTRACT

Mincing or surimi-processing, followed by fermentation, extrusion and intermediate moisture food (IMF) processing were combined to investigate new ways to create fish protein based snack foods. A dough of fermented fish (minced or surimi), wheat flour, corn starch, and water was extruded and subsequently dried resulting in an IMF product at pH 5.2, water activity 0.90 and moisture about 30%. The products had a chewy texture, were shelf-stable and could be processed into flavored, high-protein snack foods.

INTRODUCTION

THERE ARE MANY SPECIES of fish that could provide high quality protein but are underutilized for various reasons — including small fish size, dark meat, high fat content, strong flavor, high bone content, unacceptable textural properties and other factors (Crawford et al., 1979; Hiltz et al., 1976; Jauregui and Baker, 1980; Kelleher et al., 1982; Knorr and Regenstein, 1983; Tseo et al., 1983). In spite of these shortcomings, it would be desirable to utilize such fish, because fish protein is well-balanced in essential amino acid composition and easily digestible.

With the advent of mechanical deboning machines, underutilized fish, such as red hake and whiting, can be processed rapidly by removing flesh from the bones, thus producing minced fish. Minced fish is the raw material for surimi production which can subsequently be used in the formulation of products due to its unique texture-forming properties (Lee, 1984). Recently, there has been a growing demand in the United States for restructured seafood of various kinds. The underutilized fish protein resources can be tapped and high quality protein snack foods can be developed whereby the original identity and functional shortcomings of these fish can be masked.

The purpose of this study was to utilize underutilized fish in new product development; to use surimi to improve the textural and flavor characteristics of fish; to combine fermentation, intermediate moisture processing, extrusion and flavoring technology to create novel flavorful and texturized as well as shelf-stable snack food products; and to test and compare the various final products for water activity, texture firmness, palatability and shelf-stability.

MATERIALS & METHODS

FIGURE 1 summarizes the overall fish preparation and treatments. Whiting (*Merluccius bilinearis*) fillets were ground twice through a 4-mm plate and divided into four portions. Two minced portions were prepared into surimi, after which one surimi portion was formed into a dough, while the other was pre-cooked and fermented prior to being formed into a dough. The third minced portion was precooked, fermented and formulated into a dough, whereas the fourth minced portion was formed into a dough without prior treatment. All doughs were subsampled and tested for pH, water activity and moisture prior to being extruded. The extrudates were subdivided and dried at 93°C for 0, 10, and 30 min. All extrudates were tested for pH, water activity and moisture. Instron shear-compression cell measurements were performed on extrudates dried at 93°C for 30 min.

To produce the surimi, minced fish was placed in a fine-meshed bag and washed for 1 min in cold tap water (1 part fish to 5 parts water) twice to remove the water-soluble constituents. After the second washing, the surimi was dewatered by applying pressure to the bag until the flesh was paste-like in consistency. As was mentioned above, the minced fish and surimi were pre-cooked in a double-boiler, with stirring, for 10 min and subsequently cooled to about 37°C prior to the fermentation.

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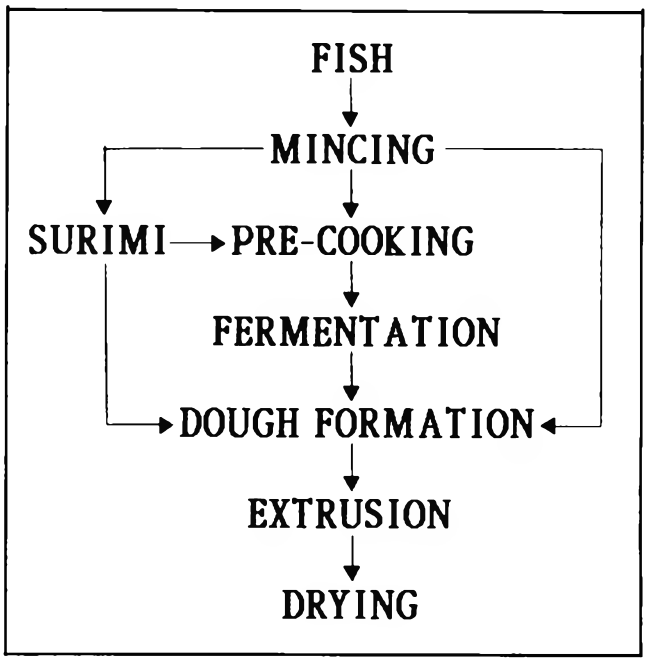

Fig. 1—Flow diagram of fish preparation and treatments.

Table 1—pH values of initial fish and the final extrudates dried 30 minutes at 93°C

Samples	Fish	Extrudates
Minced fish	6.8	6.6
Surimi	6.9	6.5
Minced fish, fermented	4.9	4.8
Surimi, fermented	5.1	5.2

Table 2—Storage categories of meat products[a]

Category	Criteria	Temperature of storage
Storable	$a_w \leq 0.95$ & pH ≤ 5.2 or $a_w \leq 0.91$ or pH ≤ 5.0	No refrigeration required
Perishable	$a_w = 0.95$–0.91 or pH = 5.2–5.0	$\leq +10$°C
Easily perishable	$a_w > 0.95$ or pH > 5.2	$\leq +5$°C

[a]Leistner et al. (1981)

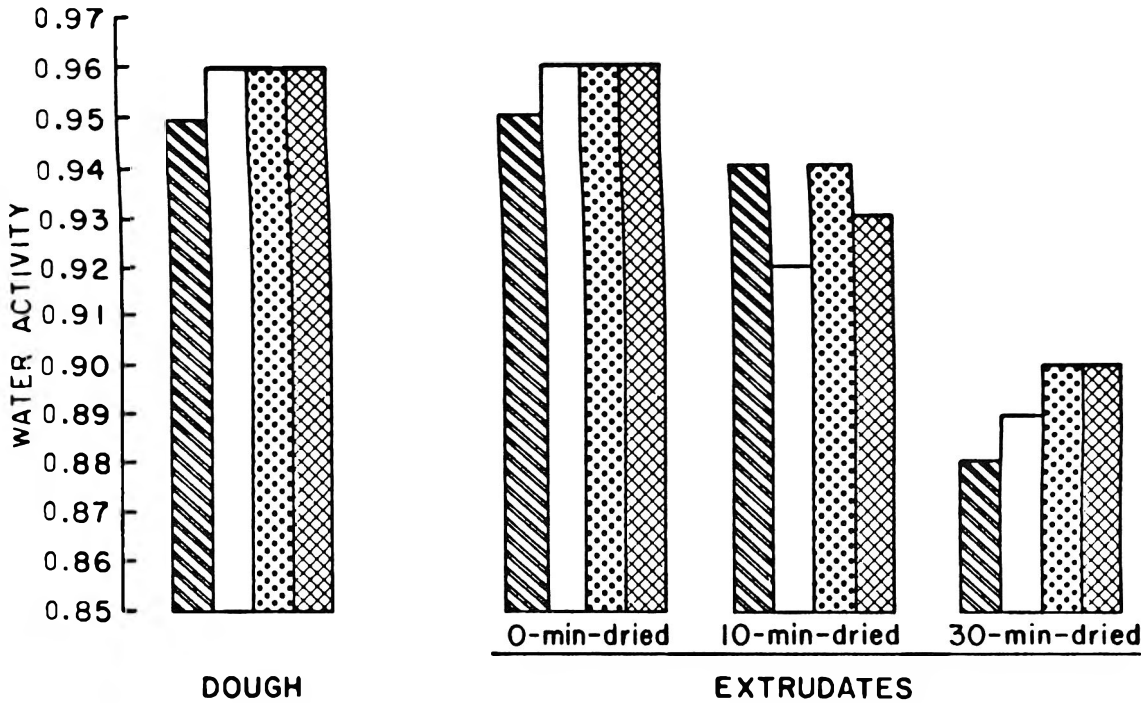

Fig. 2—Water activity of dough and 0-, 10-, and 30-min-dried extrudates: ▨ minced fish; ▭ minced fish, fermented; ⊠ surimi; ▨ surimi, fermented.

For fermentation, Lactacel 75, a highly concentrated, frozen lactic acid starter culture of *Pediococcus cerevisiae* (Microlife Technics, Sarasota, FL) was used. The fish portion was mixed with 0.02% *Pediococcus* culture and 0.63% glucose and incubated at 37°C for 12 hr until a pH of about 5.0 was reached.

After numerous trials, a favorable composition of the fish dough was found to be: fish or surimi 35.7%, wheat flour 35.7%, corn starch 4.8% and water 23.8%. The doughs were extruded with a C.W. Brabender (Hackensack, NJ), Model 2001, 1.9-cm laboratory single-screw extruder. The following processing parameters were used: Zone 1–80°C, Zone 2–180°C, Zone 3–200°C; screw speed–200 rpm.

The pH was measured with a Corning Research pH-Meter, Model 12. The water activity was determined with a Lufft (Stuttgart, West Germany) Water Activity Meter. Moisture was determined by heating 2-g samples in a drying oven for 16 hr at 105°C (Karmas, 1980).

For texture testing, the extruded samples, with a 0.9-cm diameter, were cut into pieces about 4 cm long weighing about 2g each. The sample sticks were placed in the base of an Instron multiblade shear-compression cell, perpendicular to the blades. The Instron Universal Test Machine (Canton, MA) was calibrated in pounds of force. The force-deformation curves were produced at a crosshead speed of 25.4 cm/min. Each curve was analyzed for the maximum force and total curve area (the total work done on the sample). All tests were performed in at least five replicates allowing statistical analyses.

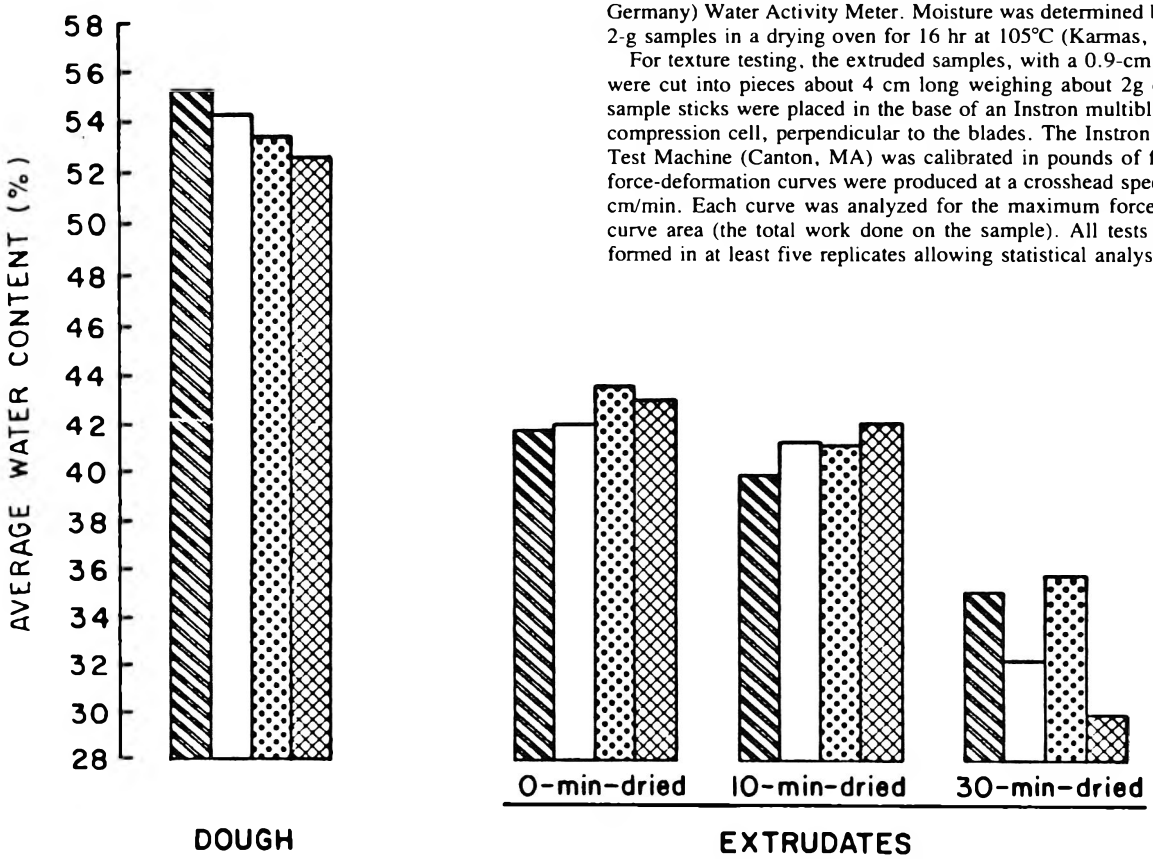

Fig. 3—Average water content of dough and 0-, 10-, and 30-min-dried extrudates: ▨ minced fish; ▭ minced fish, fermented; ⊠ surimi; ▨ surimi, fermented.

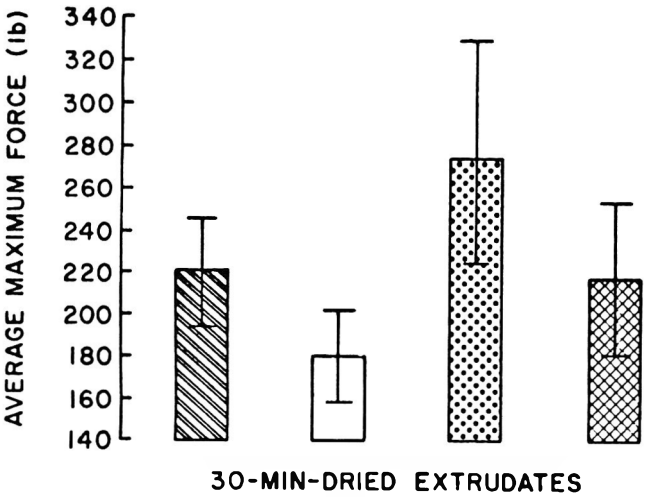

Fig. 4—Average maximum force of 30-min-dried extrudates. The vertical lines in the bar graphs indicate the standard deviation: ▨ minced fish; ▭ minced fish, fermented; ▩ surimi; ▩ surimi, fermented.

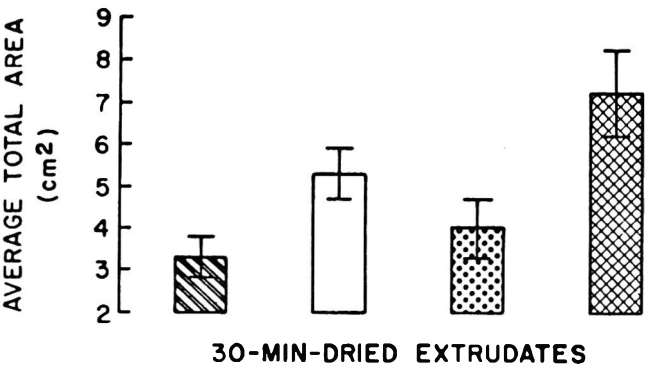

Fig. 5—Average total area of 30-min-dried extrudates. The vertical lines in the bar graphs indicate the standard deviation: ▨ minced fish; ▭ minced fish, fermented; ▩ surimi; ▩ surimi, fermented.

RESULTS & DISCUSSION

THE FERMENTED FISH SAMPLES and the final (30-min-dried) extrudates containing fermented fish had lower pH values than the non-fermented samples as is evident from Table 1. Lactic acid which reduced the pH was produced by the *Pediococcus* culture. The *Pediococcus* organisms have the unique ability to produce lactic acid rapidly over the temperature range 24–43°C. Before fermentation, the minced fish as well as surimi were pre-cooked to remove any competing microorganisms and thus promote a faster generation of lactic acid. A low pH was desirable, because most bacteria, inhibited by high acid, gave the product an extended shelflife.

Figures 2 and 3, respectively, illustrate the water activity and average water content of the dough and extrudates. All 30-min-dried extrudates had a water activity less than 0.90 and an intermediate moisture of 30–35%. According to Hilmarsdottir and Karmas (1984), no growth of *Staphylococcus* aureus occurred when the a_w was 0.90 and pH was less than 5.3. According to Table 2 (Leistner et al., 1981), meat products are storable without refrigeration when a_w is less than 0.95 and, at the same time, pH is less than 5.2; or, independently, a_w is less than 0.91 or pH is less than 5.0. In the present study, the pH-values and a_w were below these values thus making the product shelf-stable if properly packaged. By definition, these final extrudates are intermediate moisture foods (Karel, 1973; Robson, 1976). Intermediate moisture foods behave like dried foods in their microbial stability (Labuza et al., 1972). In such foods type E *Clostridium botulinum* is no danger, because its minimum water activity requirement is about 0.94.

The maximum shear-deformation force is defined as "firmness under tension," whereas the total area under the shear force-deformation curve signifies the cohesiveness of the test material or the total work needed to rupture the sample. Figures 4 and 5 indicate that the fermented samples, which required the least amount of force to cut through the sample, required that more total work be done on the sample. This means that the fermented samples were chewier, whereas the non-fermented samples were less cohesive.

This novel product was flavored with barbecue, taco, nacho cheese, chocolate, mint and other flavors. No fish taste was obvious, particularly in the surimi-based product. The surimi-processing in connection with lactic acid fermentation may be responsible for the elimination of fishy flavor. Final protein of the product was between 20–25%. With high-temperature short-time extrusion cooking combined with extrusion forming, the product can be given any desired shape.

In summary, mincing and surimi-processing changed the nature of the underutilized fish flesh to an appropriate starting raw material. The novel fish products made of fermented minced fish or fermented surimi had the following desirable characteristics: (1) Low pH, less than 5.2; reduced water activity of 0.90 or less; and intermediate moisture of 30–32%. These properties gave the product an extended shelflife. (2) The product had a high, complete protein content in the range of about 20–25%. (3) The extrusion-formed texture was highly palatable and chewy. (4) Furthermore, the product could be flavored in many different ways, such as chocolate, barbecue, mint, nacho cheese, for consumption as a snack food; and/or used in different forms, such as a dry soup ingredient.

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Ms received 7/21/86; received 9/12/86; accepted 10/3/86.

This is a New Jersey Agricultural Experiment Station Publication No. D-10104-1-87 supported by State Funds as a part of a M.S. thesis written by Ellen Lauber.

Influence of Processing on the Volatile Compounds Characterizing the Flavor of Pickled Fish

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ABSTRACT

Fresh Lake Michigan smelt (*Osmerus mordax*) were processed using a traditional sodium chloride brine (48 hr), vinegar (48 hr), and sugar-vinegar (final solution) pickling process. Substantial extractions of fresh fish volatile carbonyls and alcohols into brines occurred during processing. Only traces of carbonyls and modest concentrations of alcohols could be detected in finished pickled fish. Model aqueous systems containing selected amino acids and carbonyls in 10% NaCl solutions showed 50–99% reductions of carbonyls after reaction times of only 2.25 hr at 21°C. Model systems containing aqueous 5% acetic acid and selected long-chain alcohols did not yield measurable (1 ppb) concentrations of corresponding acetate esters when stored at 4°C for up to 2 wk.

INTRODUCTION

IN THE UNITED STATES, pickled fish refers to uncooked fish products that have been preserved by first salt-brining and finally by acidifying in vinegar which usually contains a variety of spices (Jarvis, 1950). Although pickled fish is popular in Europe and Asia, in these countries the term, pickle curing, often is used synonomously with salt curing, the technology of preserving fresh fish in brines formed by salt dissolving in tissue fluids (Burgess et al., 1967). Domestically, pickled fish have been traditionally consumed by certain ethnic groups, but these products have been increasingly purchased by nonethnic consumers as specialty deli items that are often marketed in condiments, such as sour cream or wine sauce.

High quality pickled fish is generally considered to possess very mild but distinct fresh fish-like flavors that are reminiscent of fresh, green-plant-like flavors. The flavor of pickled fish is less pronounced in fishiness character than either unprocessed fresh fish or abused, oxidized fishery products. Aromas of freshly harvested fish are characterized by six-, eight-, and in some cases nine-carbon volatile carbonyls and alcohols that are derived from polyunsaturated lipids by the action of endogenous enzymes (Josephson et al., 1983a, b; 1984a, b). Autoxidation of polyunsaturated lipids in fresh fish leads to the development of oily and stale fish aromas that are caused at least in part by the presence of the 2,4-heptadienals and the 2,4-decadienals (Meijboom and Stroink, 1972; Swoboda and Peers, 1977; McGill et al., 1974) along with other oxidation products.

Porkorny (1980) observed that when oxidized fish oil was reacted with amino acids or proteins at room temperature, the mild flavor of raw or pickled fish appeared. This seems to indicate that the flavor of pickled fish depends to a significant extent upon the presence of the fresh fish alcohols rather than the harsher-flavored carbonyls. The mild nature of pickled fish flavors compared to fresh fish aromas also suggests that the processing steps involving salt brining, washing in fresh water, and acidifying with vinegar also provide influential effects on final flavors.

Therefore, the purpose of this research was to quantify the fresh fish volatile aroma compounds at each stage of the processing of smelt to pickled fish, and to assess the contributions of the compounds that were measured to the characteristic flavor of finished pickled fish. Additionally, studies were included to evaluate the contribution of certain equilibrium reactions involving carbonyls and alcohols that could influence amounts of these compounds in pickled fish.

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MATERIALS & METHODS

Source of fish

Fresh Lake Michigan smelt (*Osmerus mordax*) were chosen for study because they are a modestly fatty fish, the type considered most suitable for pickling purposes (Burgess et al., 1967). Smelt were obtained freshly-harvested from a commercial source (Two Rivers, WI), and were eviscerated, washed, and drained prior to processing by a traditional pickling procedure (Jarvis, 1950). Some were then vacuum packaged in barrier pouches (Freshtuff; 2 cc/254 sq cm/24 hr at 23°C; American Can Co., Neenah, WI), and held at − 25°C for 30 days before use.

Processing of pickled fish

Fresh smelt lots (2.25 kgs each) were processed in rigid polyethylene containers (20 L capacity) by first placing each lot in 4 L of a 10% NaCl brine (wt. basis; 4°C), and holding for 48 hrs. After holding, the lots of fish were washed twice each with 3L distilled water, and this washwater was discarded. Brined and freshened smelt were next acidified by placing each lot in 2L vinegar (grain distilled; 5% acidity; 4°C) and holding for an additional 48 hr. Each lot was then transferred to 2L final pickling brine which was prepared by dissolving sucrose (49%, wt basis) in vinegar with mild heating (60°C), and then cooling to 4°C before use. Smelt were held at 4°C in the final pickling solution, and were evaluated periodically up to 10 wk. Spices, chopped onions, and other condiments traditionally employed were omitted from the final pickling brine to facilitate quantification of fresh fish volatile alcohols and carbonyls.

Isolation of volatile compounds

Volatile compounds were isolated from both fish and processing solutions throughout the preparation sequence.

Fresh unprocessed smelt. Approximately 100g fresh, unwashed, gutted and deheaded smelt were blended in 500 mL saturated NaCl with an internal standard (3.7 μg ethyl heptanoate in 50 uL ethyl ether). These samples were purged under a stream of nitrogen (2 hr; 120 mL/min; 21°C) for collection onto Tenax GC (60-80 mesh, ENKA N.V., Holland) as described by Olafsdottir et al. (1985). Volatiles were eluted from each Tenax trap with 1 mL ethyl ether (Mallinckrodt Inc., Paris, KY), and concentrated to approximately 10 μL under a stream of nitrogen.

Following initial salt-brining. Volatile aroma compounds from 800 mL portions of the 10% salt brine were purge-collected after adding an internal standard, and then concentrated. Approximately 100 gm lots of smelt also were blended in saturated NaCl solution with an internal standard, and volatile aroma compounds were collected and concentrated.

Following acidification in vinegar. Lots of 800 mL of the vinegar-based acidification solution were neutralized to pH 7.5 with 5N NaOH to suppress collections of acetic acid prior to adding the internal standard, and then were purge-collected and concentrated. In addition, approximately 100 gm lots of smelt were blended in saturated NaCl, and each was also adjusted to pH 7.5 prior to adding the internal standard. Each was then purge-collected and concentrated.

Finished pickled fish and sweetened vinegar solution. Volatile aroma compounds from 800 mL portions of the sucrose-vinegar solution or smelt lots of approximately 100g were isolated in a manner similar to that described for samples obtained from the initial acidification processing step.

GC-MS of volatile aroma compounds

Ethyl ether extracts were separated with a Varian 1740 gas chromatogram (Varian Associates, Palo Alto, CA) equipped with an effluent splitter (10:1 in favor of the exit port) and a flame ionization detector (FID). The effluent splitter allowed simultaneous FID and odor evaluations of eluting compounds. Separations were carried out with a 3 m × 2 mm i.d. silane deactivated glass column packed with 7% Carbowax 20 M on 80-100 mesh Chromosorb W AW/DMCS, programmed from 50° to 200°C at 2°C/min. Nitrogen carrier gas, hydrogen, and air flow rates were 24, 24, and 240 mL/min, respectively, and injection port and detector temperatures were 250° and 235°C, respectively.

Fused silica capillary GC analysis (Carbowax 20 M; 60 m × 0.25 mm i.d.; J & W Scientific, Inc., Rancho Cordova, CA) of volatiles was carried out in conjunction with mass spectrometric analysis (GC-MS) using a Finnigan 4021 GC-MS and an INCOS 2300 data system (Finnigan Instruments, Sunnyvale, CA). Compounds were identified by computer matching of full or partial mass spectra of compounds published in "EPA/NIH Mass Spectral Data Base" (Heller and Milne, 1975, 1980) and manual matching with published spectral data. Coincidence of retention indices (I_E; Van den Dool and Kratz, 1963) for unknown and authentic compounds, and where possible agreement of aromas of eluting unknown compounds, was also employed in assigning identities.

Analysis of commercial pickled herring and vinegar

Commercial pickled herring products and commercial vinegars were obtained from a local retail market. The herring samples were all prepared in wine sauce, and included Vita Herring Party Snacks, (Vita Food Products, Inc., Chicago, IL), Noon Hour Imported Fillet of Herring (Noon Hour Food Products, Inc., Chicago, IL), and Ma-Baensch's Herring Tidbits (Baensch's Food Products Co. Inc., Milwaukee, WI). Volatile aroma compounds from the tissue of these samples were purge-collected onto Tenax GC. Vinegar samples (5% acidity) included two white distilled grain vinegars (Kohl's Corp., Milwaukee, WI and H.J. Heinz, Co., Pittsburgh, PA), and an apple-flavored, distilled grain vinegar (H.J. Heinz, Co., Pittsburgh, PA). Each of these samples was analyzed for volatile compounds after neutralizing 100g portions to pH 7.5 with 5 N NaOH to suppress collection of acetic acid. Additionally, the general flavor characteristics of smelt pickled in each vinegar type was noted by the authors.

Model systems

Carbonyls and amino acids. A combination of hexanal, 2,4-heptadienal, and 1-octen-3-one (1 ppm final concentration each), and a mixture of lysine, taurine, cysteine and glycine (either 10, 50 or 100 ppm final concentration each) were added to 1L quantities of 10% NaCl solution or 1L quantities of vinegar (Kohl's, 5% acidity), and then were held at 4°C. Combined mixtures in NaCl brine were stirred for 15 min (4°C) prior to initiating purge-collection (2 hr; 120 mL/min; 21°C) of volatiles onto Tenax GC from 100 mL portions of reaction solutions. Combined mixtures in vinegar solutions were stirred for 2.25 hr (4°C), and then either neutralized to pH 7.0 with 5N NaOH prior to purge-collecting the volatiles, or were purged directly (24 min; 600 mL/min; 21°C) onto Tenax GC. Solutions of alcohols and carbonyls prepared as above without amino acids were also analyzed to serve as a basis for determining the percent reduction of each carbonyl. 1-Penten-3-ol and 1-octen-3-ol were added (1 ppm each) as internal standards for the quantitative estimation of carbonyl compounds in model systems.

Alcohols and acetic acid. A mixture of 1-penten-3-ol, 1-octen-3-ol, 6-nonen-1-ol, 2,6-nonadien-1-ol, and 1,11-dodecadiene (internal standard) at a final concentration of 6 ppm each was added to 1L of a 5% acetic acid solution (4°C) that had been prepared from glacial acetic acid (J.T. Baker Chemicals, Phillipsburg, NJ). After neutralization to pH 8.0 with 5 N NaOH to suppress acetic acid collection, volatiles were purge-collected (2 hr; 120 mL/min; 21°C) from 100 mL portions of the reaction mixture after holding at 4°C for 15 min, 1, 2, 7, and 14 days, respectively, onto Tenax GC as previously described. Additionally, a mixture of 5% acetic acid, the selected alcohols, and 1% H_2SO_4 was analyzed after 15 min, 1, 6, and 10 days to assess the extent of the esterification reactions when catalyzed by a mineral acid.

RESULTS & DISCUSSION

SMELT were chosen as the model for this study because they are a small, moderately fatty fish, and unlike many other freshwater fish which contain only the six- and eight-carbon fresh fish volatile aroma compounds, they also possess the nine-carbon volatiles (Josephson et al., 1984a). Concentrations of the fresh fish volatile aroma compounds found in smelt through the pickling process are shown in Table 1. As has been noted earlier for a variety of fish (Josephson et al., 1983a, 1984a), the freshly harvested smelt contained enzymically formed fresh fish alcohols in greater abundance than the carbonyls (Table 1). However, the lower thresholds exhibited by the volatile carbonyls compared to the alcohols result in dominating contributions of carbonyls to the intensity and overall odor quality of freshly harvested fish (Olafsdottir, 1985). For example, the recognition threshold for 1,5-octadien-3-one (0.001 ppb; Swoboda and Peers, 1977) is five orders of magnitude lower than that for 1,5-octadien-3-ol (10 ppb; Whitfield et al., 1982). As a result, much of the intensity and odor quality of fresh fish is contributed by carbonyls.

Notable reductions in concentrations of both carbonyls and alcohols can be seen for the smelt following each the NaCl brining and vinegar acidification processing stages (Table 1). After 2 days in the vinegar solution, only trace quantities of eight- and nine-carbon volatile carbonyls were detected in the smelt tissue (neutralized to pH 7.5), but volatile alcohols were still present in fairly high above threshold concentrations (Table 1). Both 1-octen-3-ol and 1,5-octadien-3-ol were found near or above threshold concentrations in the finished pickled fish, and remained as such through 10 wk at 4°C in the final pickling solution.

The fate of the carbonyls and alcohols which disappeared from the smelt was investigated through analysis of tissue samples and brine solutions obtained at each processing stage (Table 2). These data indicate that substantial amounts of fresh fish alcohols and carbonyls were extracted from the fish by the preserving solutions, and therefore, extraction into the aqueous solutions constitutes a notable means for depletion of volatiles during processing of pickled fish. Between the salt brining and vinegar acidification processing stages, smelt were also washed or freshened with two successive distilled water rinses of 3L each, and these rinses undoubtedly also participated in the depletion of volatile compounds although analytical data were not obtained for them.

After 2 days in 10% NaCl brine, hexanal increased substantially in concentration from that found in freshly harvested smelt (Table 1). Since hexanal is an abundant product of autoxidation, this may reflect carry-over of oxidizing polyunsaturated lipids which accumulated as surface films in brining vessels. Measurements for hexanal in brine solutions showed variability over time (Tables 1 and 3), and these data appear to support such an interpretation. Low concentrations (0.1–1 μg/L) of autoxidatively derived conjugated carbonyls (i.e., the 7-carbon-, and 10-carbon-dienals) also were noted sporadically in the volatiles isolated from the brines. However, the oxidatively-formed conjugated carbonyls were never detected in samples of smelt tissue at any stage of pickling, and the lower limit of detection for these compounds was about 0.5 ppb.

The increase in concentration of 1-penten-3-ol noted at 5 wks of brining in the sugar-vinegar solution (Table 1) paralled the increase observed for hexanal, and this probably reflects a common n-6 positioned fatty acid hydroperoxide precursor for these two compounds. A direct cleavage of the n-6 sited hydroperoxide of linoleic or arachidonic acid would yield hexanal while a rearrangement and cleavage between the n-5 and n-6 sites would yield 1-penten-3-ol. These two mechanisms have been demonstrated to be involved in the formation of 3,6-

Table 1—Quantification of volatile carbonyls and alcohols in smelt after each processing stage during the preparation of pickled fish

Compound	Control (Initial)	Concentration at stage of processing or holding: 2 Day NaCl	2 Day Vinegar	2 Day Vinegar/ Sugar	1 wk Vinegar/ Sugar	2 wk Vinegar/ Sugar	5 wk Vinegar/ Sugar	10 wk Vinegar/ Sugar	Odor potency for compound: Recognition threshold (ppb)	Ref.[a]
	µg/kg (ppb)									
Carbonyls										
Hexanal	57	162	139	79	60.1	65.3	51	130	4.5	1
1-Octen-3-one	1.0	0.5	tr[b]	tr	tr	tr	tr	tr	0.005	3
1,5-Octadien-3-one	1.0	0.7	tr	tr	tr	tr	tr	tr	0.001	2
(E)-2-Nonenal	2.9	1.8	—[c]	—	tr	tr	tr	tr	0.08	3
(E,Z)-2,6-Nonadienal	5.7	3.3	—	—	tr	tr	tr	tr	0.01	3
Alcohols										
1-Penten-3-ol	12.4	57.7	40.4	35.4	14.4	13.1	24.4	84	400	1
1-Octen-3-ol	100.5	71.2	18.3	10.2	15.8	15	15.1	21	10	4
1,5-Octadien-3-ol	49.8	34	8.6	5.0	9.9	10.3	8.5	11.7	10	5
2-Octen-1-ol	12.0	10.3	2.4	1.9	1.4	1.4	3.3	0.8	40	4
2,5-Octadien-1-ol, 6-Nonen-1-ol[d]	36.3	33.0	7.3	5.1	4.6	6.4	2.0	4.3	—[e]	—
3,6-Nonadien-1-ol	30.1	29.5	5.9	4.2	3.4	4.1	3.8	2.7	10	3

[a] Threshold in water: (1) Frazzalari (1978); (2) Swoboda and Peers (1977); (3) Buttery (1981); (4) Pyysalo and Suihko (1976); (5) Whitfield et al., 1982).
[b] Trace (Based on odor assessment from effluent from a gas chromatographic peak region; < 0.1 ppb).
[c] Not detected.
[d] 2,5-octadien-1-ol and 6-nonen-1-ol co-elute on a 7% packed Carbowax 20M.
[e] Not available.

Table 2—Quantification of volatile carbonyls and alcohols in brining solutions after each processing stage during the preparation of pickled fish

Compound	Concentration at stage of processing or holding: 2 Day NaCl	2 Day Vinegar	1 Wk Vinegar/ Sugar	2 Wk Vinegar/ Sugar
	µg/kg (ppb)			
Carbonyls				
Hexanal	5.8	28.6	16.0	7.9
1-Octen-3-one	0.09	*[a]	*	0.05
1,5-Octadien-3-one	0.05	tr[b]	tr	tr
(E)-2-Nonenal	1.4	0.11	0.13	0.03
(E,Z)-2,6-Nonadienal	1.9	0.1	0.13	0.04
Alcohols				
1-Penten-3-ol	10.5	1.0	0.5	0.38
1-Octen-3-ol	2.5	1.2	1.0	1.1
1,5-Octadien-3-ol	2.0	0.6	0.5	0.84
2-Octen-1-ol	0.56	0.16	0.13	0.13
2,5-Octadien-1-ol, 6-Nonen-1-ol[c]	2.3	0.56	0.56	0.88
3,6-Nonadien-1-ol	2.5	0.5	0.44	0.55

[a] Quantification precluded by chromatogram aberration.
[b] Trace (Based on odor assessment from effluent from a gas chromatographic peak region; < 0.1 ppb).
[c] 2,5-Octadien-1-ol and 6-nonen-1-ol co-elute on a 7% packed Carbowax 20M column.

nonadienal (Frankel, 1980) and 1,5-octadien-3-ol (Wurzenburger and Grosch, 1986) from a 12-hydroperoxy-8, 12, 15-octadecatrienoic acid precursor.

Acetic acid co-elutes with 1-octen-3-ol and 1,5-octadien-3-ol when aroma extracts from acidified samples are analyzed directly on Carbowax 20M gas chhromatography columns. Neutralization of acetic acid in solutions to pH 7.5 with 5N NaOH virtually eliminated the acetic acid peak, but carbonyl-amino reactions (Montgomery and Day, 1965; Rizzi, 1976) occurring during collection of volatiles from the neutralized samples appeared to reduce recoveries of some carbonyls in these samples. The magnitude of this effect was investigated using vacuum packaged frozen smelt which were processed into pickled fish (Table 3), and samples of tissue and pickling solution at pH 3.6 and 7.5 were each analyzed for volatiles. These data show that at pH 7.5 carbonyl-amino type reactions were occurring during purge-collections, and the reactions caused measurable reductions (0–50%) in concentrations, especially for 1-octen-3-one. Thus, these data were used to develop correction factors for each carbonyl measured, and other data (Table 1 and 2) were adjusted accordingly to compensate for changes in concentrations that were attributable to the pH.

Greater losses of carbonyls as compared to alcohols during the processing steps do not seem to reflect their relative solubilities in aqueous systems. Alcohols seemingly could be more soluble than corresponding carbonyls in water, and thus would be more easily extracted into brining solutions. On the other hand, free amino acids and peptides from fish tissue also leach into the brining solutions, and their participation in initial carbonyl-amino reactions with fresh fish volatile carbonyls might be postulated even though brown pigments do not develop. To test the potential extent of the influences of carbonyl-amino acid reactions participating in flavor modification of pickled fish per se, model systems of selected amino acids and carbonyls were allowed to react (2.25 hr) in either a 10% NaCl or 5% acetic acid solution before analysis (Table 4).

Since the carbonyl-amino acid reaction would have proceeded as long as favorble conditions existed and reactants were present, any losses in volatile carbonyls reflect the total extent of the reaction occurring during the course of analysis of samples which were held at both 4°C and 21°C for collection of volatiles. The three different structural-types of carbonyls represented in the tests show that 1-octen-3-one was the most rapidly-reacting carbonyl included. Since 1-octen-3-one and 1,5-octadien-3-one (fresh fish ketones) exhibit very low aroma thresholds, the loss of their aroma contributions through reactions with free amino acids could account for a substantial portion of the diminished aroma intensities observed for salt-brined fish as compared to freshly harvested fish. Similarly, reaction of carbonyls with amino acids in refrigerated fresh fish could contribute to the suppression of fresh fish aromas which are noted before the onset of stale and putrid spoilage aromas (Olafsdottir, 1985).

Increasing concentrations of amino acids enhanced the reduction of only hexanal and 2,4-heptadienal which indicates that the reaction of 1-octen-3-one with amino acids was greatly favored over the other carbonyls, and as such it effectively competed for reaction substrate at the lower concentrations of amino acids.

Imposition of a low pH (2.55) effectively inhibited the reaction of carbonyls with amino acids (Table 4), but when these solutions were neutralized to pH 7.0 and then purged for only 24 min at an accelerated flow rate of 600 mL/min at 21°C, substantial reductions in carbonyls were also seen (Table 4). These data emphasize the extremely rapid rate of reaction be-

Table 3—Quantification of volatile carbonyls during vinegar processing stages from samples which were either purged directly (pH = 3.6) or neutralized to pH 7.5 before initiating purge-collecting

Carbonyl	Concentration at stage of processing or holding							
	Vinegar/Sugar							
	Vinegar 2 day		2 day		1 wk		5 wk	
	pH 3.6[a]	7.5[b]	3.6	7.5	3.6	7.5	3.6	7.5
	µg/kg (ppb)							
Tissue Sample								
Hexanal	76	35	143	167	230	213	392	285
1-Octen-3-one	4.6	5.0	8.2	8.0	18	12	36	17
1,5-Octadien-3-one	tr[c]	tr	tr	tr	tr	tr	tr	tr
(E)-2-Nonenal	1.1	0.5	1.4	0.7	1.0	0.8	3.4	1.9
(E,Z)-2,6-Nonadienal	2.7	1.9	3.8	2.7	3.7	2.4	2.3	1.1

	Vinegar/Sugar					
	Vinegar 2 day		1 week		5 week	
	pH 3.6	7.5	3.6	7.5	3.6	7.5
	µg/kg (ppb)					
Pickling solution						
Hexanal	26	30	6.8	6.4	46	51
1-Octen-3-one	5.2	2.7	0.6	0.3	4.7	3.0
1,5-Octadien-3-one	tr	tr	tr	tr	tr	tr
(E)-2-Nonenal	0.3	0.5	0.1	0.1	0.6	0.7
(E,Z)-2,6-Nonadienal	0.7	0.9	0.2	0.2	0.6	0.7

[a] Quantified from samples purged at pH 3.6.
[b] Quantified from samples which were first neutralized to pH 7.5 before initiating purge-collection.
[c] Trace (Based on odor assessment from effluent from a gas chromatographic peak region; <0.1 ppb).

Table 4—Percent reduction of selected fresh fish carbonyl compounds during residence for 2.25 hr in aqueous model systems containing added amino acids

Amino acid[a] concentration (ppm)	Percent reduction in carbonyl added		
	1-Octen-3-one[b]	Hexanal[b]	2,4-Heptadienal[b]
In pH 6.1, NaCl (19%) solution			
40	99	63	53
200	99	85	54
400	99	99	76
In pH 2.55, acetic acid (5%) solution			
40	0	0	0
200	0	0	0
400	0	0	0
In pH 7.0, neutralized solution with rapid purge[c]			
40	67	22	32
200	98	50	35
400	98	72	43

[a] Model systems contained a mixture of glycine, taurine, lysine and cysteine each added either at 10, 50, or 100 ppm.
[b] Added at 1 ppm to appropriate model system.
[c] Reaction system held for 2.5 hr at pH 2.55, then neutralized to pH 7.0, and purged for 24 min at 600 mL/min (Olafsdottir et al., 1985).

Table 5—Concentrations of short-chain fermentation-derived alcohols and acetate esters in commercial distilled vinegars

Volatile compounds	Concentrations in vinegar samples		
	1[a] (white grain)	2[a] (white grain)	3[a] (apple-flavored grain)
	(ppb)		
Esters			
Butyl acetate	0	25	41
Isobutyl acetate	0	144	247
Isoamyl acetate	0	539	737
Alcohols			
2-Methyl-1-propanol	0	23	26
3-Methyl-1-butanol	0	294	269

[a] 1 = Kohl's, Milwaukee, WI; 2 = Heinz, Pittsburgh, PA; 3 = Heinz, Pittsburgh, PA.

tween carbonyls and amino acids at neutral pH values. Overall, the data seem to indicate that reductions of carbonyls in pickled fish compared to fresh fish largely reflected their extractions into neutral processing solutions. However, since an initial low pH prevented the carbonyl-amino reaction (Table 4), it was theorized that the imposition of a low pH to pickled fish upon acidification would result in a release of carbonyls from amino acid complexes through reversible reaction mechanisms. Yet, when similar model systems (pH 7.4; 10% NaCl) containing free carbonyls and amino acids first were allowed to form carbonyl-amino acid complexes, and then were acidified to a 5% acetic acid level, little evidence for the regeneration of carbonyls from carbonyl-amino acid complexes was obtained. A lack of regeneration of carbonyls in the acidic medium at 21°C was noted throughout a 36 hr holding period. This result was not anticipated, and it suggests that either the initial stages of the Maillard reaction are not readily reversible under these conditions or that another reaction mechanism involving carbonyl depletion also occurred simultanously in the aqueous systems.

Experiments were designed to determine whether fresh fish alcohols could be depleted through reactions with acetic acid to form acetate esters during vinegar acidification, and these were based on a model system composed of 1-penten-3-ol, 1-octen-3-ol, 6-nonen-1-ol and 2,6-nonadien-1-ol (6 ppm each) in an aqueous solution of 5% acetic acid held at 4°C. Samples were analyzed at 0, 1, 4, 7, and 14 days after mixing. Acetate esters of these alcohols could not be found in any of the samples indicating that this mechanism does not contribute noticeably to alterations of flavors during pickling of fish. Similar results were obtained when 1% H_2SO_4 was included in the model system as a catalyst for the esterification.

Although acetate esters of fresh fish alcohols could not be detected, all samples of experimental and retail pickled fish analyzed with the exception of fish processed with one vinegar (Kohl's, Table 5) contained relatively large concentrations of short-chain acetate esters. Since these esters would be expected to affect the final flavor of pickled fish, their occurrence in commercial distilled grain vinegars was investigated. The identification of numerous volatile esters and alcohols in vinegar has been previously reported (Kahn et al., 1972; Mecca and Vecchio, 1977). In Table 5 it can be noted that the acetate esters of n-butanol, 2-methyl-1-propanol, and 3-methyl-1-butanol as well as the 4- and 5-carbon methyl-branched alcohols

were found in 2 of 3 samples of vinegars analyzed. Free n-butanol was not detected in the vinegars.

These volatile esters would be expected to have their origin in the initial yeast fermentations carried-out principally for the formation of ethanol. In such fermentations, the 4- and 5-carbon alcohols are produced from amino acid precursors (Sentheshanmuganathan, 1960). The secondary fermentation of ethanol to acetic acid by *Acetobacter* (Casida, 1968) is not recognized as a major source of neutral volatiles, but it is possible that the esterification of alcohols with acetic acid might be enhanced during the process. Fermented apple cider which is sometimes employed in vinegar production could also directly contribute short-chain, branched-alcohols and esters because these compounds occur abundantly in apples where they strongly influence the flavor (Buttery, 1981).

The absence of short chain acetate esters and branched-chain alcohols in one retail vinegar suggests that either pure ethanol was employed as a substrate for the vinegar fermentation or that a highly efficient distillation system was utilized in preparing this product. When each of the retail vinegar samples was used to prepare pickled smelt, it was observed that the short-chain esters and alcohols in the vinegars greatly affected the resulting pickled fish flavors. The presence of these fermentation products provided smoothness to finished pickled smelt flavors, and ameliorated the harsh flavor of acetic acid. Therefore, selection of vinegar for pickled fish appears to be an important factor along with selection of spices for providing finished pickled fish flavors.

Analysis of samples of retail pickled herring in wine sauce revealed flavor-active concentrations of 1-penten-3-ol, 1-octen-3-ol, 1,5-octadien-3-ol as well as hexanal, 1-octen-3-one, 1,5-octadien-3-one and (E,Z)-2,6-nonadienal in all three samples (Table 6), and these compounds would contribute to the mild fish flavors in these products.

In summary, evidence has been provided to show that extractions of volatile fresh fish alcohols and carbonyls into processing solutions accounts for some of the reductions in the intensity of pickled fish flavors compared to that of freshly harvested fish. The remaining fresh fish alcohols and carbonyls contribute to the mild but distinct flavor of pickled fish. Although only trace quantities of the eight- and nine-carbon volatile carbonyls were found in finished pickled fish, these compounds still appear to be sufficiently abundant to contribute flavor notes because they exhibit exceedingly low recognition threshold values. Analysis of model systems containing selected amino acids and carbonyls demonstrated that carbonyl-amino acid reactions occur rapidly and result in large reductions in concentrations of selected carbonyls at neutral pH, but not at low pH conditions. Lowering of the pH of model systems containing carbonyl-amino complexes did not regenerate significant amounts of the free carbonyls that were provided to the systems initially.

Table 6—Concentrations of influential carbonyls and alcohols in retail samples of pickled herring packed in wine sauce

	Concentrations in pickled herring samples		
	1[a]	2[a]	3[a]
	(ppb)		
Alcohols[b]			
1-Penten-3-ol	75.8	79.4	61.0
1-Octen-3-ol	8.2	33.3	24.1
1,5-Octadien-3-ol	15.5	15.4	8.3
2,5-Octadien-1-ol	2.3	—[c]	—
Carbonyls[d]			
Hexanal	69	50	209
1-Octen-3-one	9.3	tr	18
1,5-Octadien-3-one	tr	tr	tr
(E,Z)-2,6-Nonadienal	3.0	1.5	2.1

[a] 1 = Vita; 2 = MaBaensch's; 3 = High Noon brands.
[b] Alcohols were quantified from samples neutralized to pH 7.5.
[c] Not detected.
[d] Carbonyls were quantified from samples witout neutralization (pH = 3.55).

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Ms received 5/2/86; revised 7/21/86; accepted 8/8/86.

Research supported by the College of Agriculture & Life Sciences and the Sea Grant Program, Univ. of Wisconsin-Madison.

Some Characteristics of the Enzymic Lipid Peroxidation System in the Microsomal Fraction of Flounder Skeletal Muscle

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ABSTRACT

The microsomal lipid peroxidation system of flounder muscle was characterized as to response to temperature, cofactor requirements, inhibitors, and activators. The enzyme-catalyzed reaction had a rate-limiting step prior to the formation of lipid hydroperoxides. The K_M for NADH of the enzymic system in the presence of 0.1 mM ADP was approximately 1 μM. In the presence of Fe^{+3} both cysteine and ascorbate also catalyzed lipid peroxidation. While neither NADH nor ADP had an effect in the presence of ascorbate-Fe^{+3}, NADH stimulated the cysteine-Fe^{+3} oxidation and ADP completely inhibited it. Inhibitor studies indicated that it was a free radical reaction, an -SH group was involved and O_2 reduction products and singlet oxygen participated.

INTRODUCTION

LIPID OXIDATION is well recognized as a major form of deterioration in stored muscle foods. Although lipid oxidation in muscle tissue has been thought to be primarily nonenzymic in nature, the possible importance of enzymic oxidations is becoming more apparent as discussed by German and Kinsella (1985). It had been observed in the 1960s that the microsomal fraction from liver had the capacity to catalyze the oxidation of its lipid component in the presence of NADPH and iron and that this reaction was enhanced in the presence of ADP (Hochstein and Ernster, 1963). The presence of an enzyme-catalyzed lipid peroxidation system in the microsomal fraction of muscle tissue was demonstrated in 1976 (Lin and Hultin, 1976). This system was considered enzymic because it was unstable to heat, showed a preference for NADPH compared to NADH (if nonenzymic, these two reducing agents should have been comparable), and the reaction was inhibited by disruption of the membrane with detergents.

In some earlier work, a similar lipid peroxidative system in the microsomal fraction of fish muscle which had some interesting features was reported (McDonald et al., 1979; Hultin et al., 1982). Unlike the avian and mammalian muscle systems (Lin and Hultin, 1976; Rhee et al., 1984), the microsomes of fish muscle functioned very much better in the presence of NADH as compared to NADPH. This is significant because NADH is the form of the cofactor found in muscle tissue. In addition, the fish muscle microsomal fraction was very active at relatively low temperatures and even had significant activity in the frozen state (Apgar and Hultin, 1982).

Our objective was to study in more detail the kinetic characteristics of the microsomal fraction of winter flounder muscle. Winter flounder was chosen because this was a convenient fish to catch and maintain alive allowing control of postmortem events and standardization of procedures.

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MATERIALS & METHODS

Materials

Winter flounder (*Pseudopleuronectes americanus*) were caught either by net or by rod and reel in Ipswich Bay, MA and kept alive in circulating salt water tanks until processed.

Glutathione, p-hydroxymercuribenzoate, diphenylfuran, superoxide dismutase, catalase, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Company (St. Louis, MO). The disodium salts of reduced nictoinamide adenine dinucleotide (NADH) and adenosine 5'-diphosphate (ADP) were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). All other chemicals were reagent grade or the purest available.

Methods

All experiments were performed at least in duplicate, and results presented are the averages. Generally sufficient fish were used to provide microsomal samples for 4 days of assays; this was usually one to three fish per preparation, depending on the number of assays to be done.

Preparation of microsomal fraction. The flounder were sacrificed by decapitation immediately after being removed live from the tanks. The fillets were removed, skinned and immediately minced in a Rival meat grinder. The rest of the procedure has been described (Apgar and Hultin, 1982). The microsomal fraction was that which sedimented between 21,400g for 30 min and 104,000g for 60 min with a treatment with 0.6M KCl to solubilize actomyosin. The microsomes were suspended in 0.12M KCl and 5 mM histidine, pH 7.3 at a protein concentration of from 5 to 10 mg per mL.

Determination of protein. Protein of the microsomal preparation was determined by the method of Lowry et al. (1951) as modified for membranes by Markwell et al. (1978).

Measurement of lipid oxidation in the microsomal fraction. Incubations were performed in a shaking water bath under an atmosphere of air in 25 mL Erlenmeyer flasks. The exact concentrations of the various components used and the specific conditions are given in the text. Initiation of the reaction was generally done by addition of the microsomes. Thiobarbituric acid (TBA) reactive-substances were determined by a modification of the procedure of Buege and Aust (1978). On the day of use, a trichloroacetic acid (TCA/TBA) stock solution was prepared consisting of 15% w/v TCA and 0.375% w/v TBA in 0.25M HCl. After mild heating and agitation to dissolve the components, 3 mL 2% butylated hydroxytoluene (BHT) in absolute ethanol was added per 100 mL of the TCA/TBA stock solution. At appropriate time intervals, 1.0 mL aliquot of the sample medium was added to the TCA/TBA stock solution in a test tube and immediately mixed thoroughly with a Vortex mixer. The sample was then heated in a boiling water bath for 15 min and cooled to room temperature before centrifugation in an IEC clinical centrifuge at the maximal speed setting (approximately 1600 × *g*) for 10 min. The absorbance of the supernate was measured at 532 nm and the results reported as nmol malonaldehyde (MDA) per mg of protein using a molar extinction coefficient of $1.56 \times 10^5 M^{-1} cm^{-1}$.

Lipid hydroperoxides (LOOH) were determined by the procedure of Buege and Aust (1978). At appropriate time intervals, a 1 mL sample of the assay medium was added to 5 mL of chloroform/methanol (2:1), mixed thoroughly and centrifuged in an IEC clinical centrifuge at a speed setting of 4 (approximately 1000 × *g*) to separate the phases. The upper layer was removed by suction and 3.0 mL of the lower chloroform layer was placed in a test tube and taken to dryness in a 45°C water bath under a stream of nitrogen. While still under this stream of nitrogen, 1.0 mL of acetic acid/chloroform (3:2) was added, followed by 0.05 mL of a saturated solution of KI. The

test tube was rapidly stoppered and mixed and the reaction was allowed to take place exactly 5 min in the dark. The reaction was terminated by adding 3.0 mL of 0.5% cadmium acetate. After mixing, the solution was centrifuged at a speed setting of 4 in the IEC centrifuge and the absorbance of the top layer was determined at 353 nm. The results are reported as nmol LOOH per mg of protein using a molar extinction coefficient of $1.73 \times 10^4 M^{-1}cm^{-1}$.

Total carbonyl was determined using 2,4-dinithrophenylhydrazine. After a period of incubation sufficient for adequate reaction, 2.0 mL of the microsomal assay mixture was added to 2.0 mL of 20% TCA which contained 0.03% propyl gallate. The sample was centrifuged in an IEC clinical centrifuge at maximal speed for 15 min. Two milliliters supernate were then added to 3.0 mL of an aqueous saturated solution of 2,4-dinitrophenylhydrazine that had been purified by extraction four times with reagent grade hexane. Three milliliters of hexane were added and the mixture was vortexed exactly 3.0 min to extract the hydrozones. After centrifugation in an IEC clinical centrifuge at 600 × g for 5 min, the absorbance of the upper hexane layer was read at 340 nm. Results are reported as nmols of carbonyls per mg of protein using a molar extinction coefficient of $1.28 \times 10^4 M^{-1}cm^{-1}$, obtained by constructing a standard curve using acetone.

NADH regeneration system. It had been demonstrated that there was a potent system in fish muscle microsomes that destroyed NADH (McDonald et al., 1979). In some of the experiments described in this paper where the reaction period was long or where the level of NADH used was low, the lipid oxidation reaction stopped after a relatively short period of time. In these cases, it was necessary to use an NADH regeneration system. This was accomplished by the addition of glucose-6-phosphate dehydrogenase to the lipid oxidation assay medium at a concentration of 1 unit of enzyme per milliliter along with 0.5 mM glucose-6-phosphate. This system will be referred to in this paper as the NADH-regeneration system.

RESULTS

THE PROCESS of lipid oxidation is complex involving a set of initiation reactions to generate free radicals which are responsible for the chain reaction which generally ends by mutual destruction of paired radicals. The methods of analysis used for lipid oxidation measure different stages or properties of the process. We compared the production of lipid hydroperoxides which occurred early in the process of lipid oxidation with two measurements of final breakdown products. One of these, the TBA-reactive substances, was thought to be relatively specific for malonaldehyde and a few related compounds and the other was a measure of total carbonyls (Fig. 1). The general features of the progress curves for these three measurements were very similar. On a molar basis, there was somewhat greater production of carbonyls than TBA-reactive substances (calculated as malonaldehyde), and there was approximately a six-fold greater production of LOOH than either total carbonyls or TBA-reactive substances. All curves showed a typical lag phase.

The effect of pH on lipid peroxidation in the presence of NADH, ADP, and $FeCl_3$ was similar to what had been observed for red hake (McDonald et al., 1979) microsomal lipid peroxidation. The maximum rate was found at a pH around 6.7, and there was a fairly broad range over which high activity was observed. Likewise, increasing ionic strength, whether obtained by NaCl or KCl caused a decrease in the rate of lipid peroxidation, with approximately a ⅓ reduction at an ionic strength of 0.2 and greater than 50% reduction at an ionic strength of 0.4. As had been previously observed with flounder muscle microsomes in a frozen system (Apgar and Hultin, 1982), a decrease in specific activity with an increase in protein concentration in the assay medium (Table 1) was found.

The effect of temperature on both the formation of TBA-reactive substances (MDA) and LOOH is shown in Fig. 2. Similar patterns were again seen between these two measurements of lipid peroxidation with the exception that at the highest temperature (37°C), the LOOH decreased after 30 min. This was undoubtedly due to the instability of the lipid hydroperoxides at this high temperature. As the temperature increased, the lag period decreased and the rate of oxidation during the linear portion of the reaction increased.

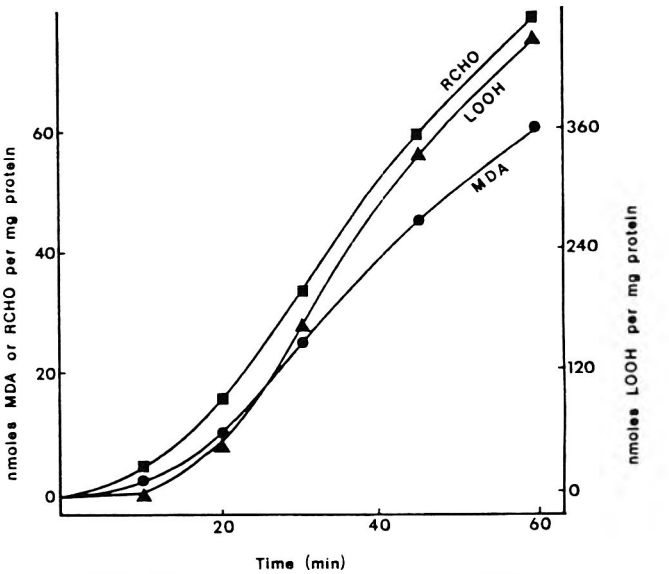

Fig. 1—Flounder muscle microsomal lipid peroxidation measured by three techniques. The reaction medium contained 0.5 mg protein per mL, 0.2 mM NADH, 0.2 mM ADP, 0.05 mM $FeCl_3$, 0.12 M KCl and 5 mM histidine, pH 7.1. The reaction was carried out at 6°C. MDA:TBA-reactive substances; RCHO:total carbonyls; LOOH:lipid hydroperoxides.

Table 1—Effect of microsomal protein concentration on lipid peroxidation specific activity

Protein conc (mg/mL)	Activity, nmol MDA[a] per mg protein-hr
0.25	16
0.5	11
0.75	8
1.0	4

[a] TBA-reactive substances, calculated as malonaldehyde. The assay medium contained 0.5 mM NADH, 0.1 mM ADP, and 0.015 mM $FeCl_3$ in 0.12 KCl, 5 mM histidine, pH 7.1.

In determining some kinetic properties of the microsomal lipid peroxidation system, it was observed that linearity of the reaction was dependent upon the NADH concentration used. At the normal concentration of 0.1 mM NADH, linearity was reasonably good over the range of 30 to 40 min. However, this was not the case with lower concentrations. Due to our previous observations that fish muscle microsomes contained a system which removed NADH, we though that this lack of linearity might be due to an NADH oxidase. In this case, a recycling system to reduce the oxidized NAD should maintain the NADH at a constant concentration and provide linearity. As described in the Materials & Methods section, glucose-6-phosphate dehydrogenase and glucose-6-phosphate were used for this. Activities at low NADH concentrations in the presence of this regeneration system are shown in Fig. 3. Linearity was very good for up to 30 min at NADH concentrations as low as 5 μM. Thus, the regeneration system was used in all experiments in which the concentration of NADH was low, i.e., less than 0.1 mM or when excessive loss of NADH could be a factor.

The dependence of the rate of flounder microsomal lipid peroxidation on NADH concentration is presented under two sets of conditions in Fig. 4a and 4b. In the former, there was no ADP added and the $FeCl_3$ concentration was high (0.15 mM). The data in Fig. 4b were obtained in the presence of 0.1 mM ADP as well as the lower concentration of 0.015 mM $FeCl_3$. Both of these sets of experiments used the NADH regeneration system. Double reciprocal plots of the data in Fig.

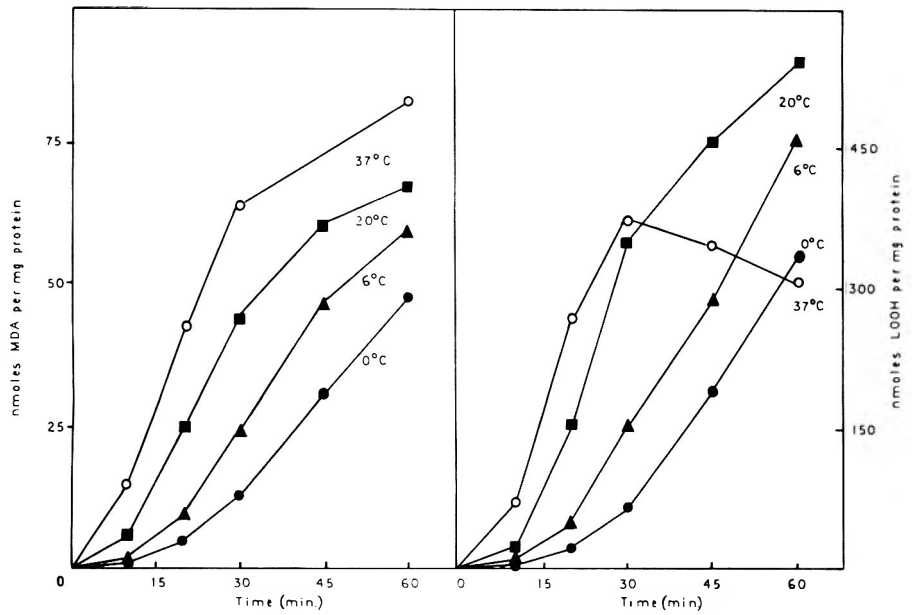

Fig. 2—Production of TBA-reactive substances (MDA) and LOOH by flounder microsomes as a function of temperature. The reaction conditions were similar to those described in Fig. 1 except the concentrations of ADP and NADH were 0.1 mM and the temperature was varied as indicated.

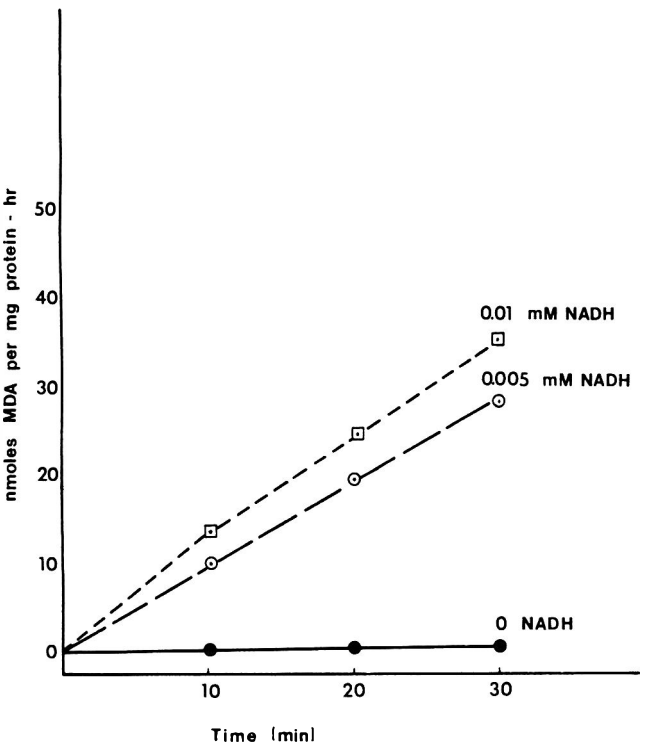

Fig. 3—Effect of an NADH-regeneration system on flounder muscle microsomal lipid peroxidation at low concentrations of NADH. Reaction conditions were similar to those described in Fig. 1 except the concentration of ADP was 0.1 mM, $FeCl_3$ was 0.015 mM, and NADH was varied as indicated. In addition, an NADH regeneration system was added which consisted of 0.5 mM glucose 6-phosphate and 1 unit per mL of glucose 6-phosphate dehydrogenase. MDA:TBA-reactive substances.

4a and Fig. 4b indicated that the maximal velocity of the reaction in the absence of ADP and with the high $FeCl_3$ concentration was 149 nmol MDA per mg of protein-hr and the K_M for NADH was approximately 10 μM. The system in the presence of 0.1 mM ADP and the lower $FeCl_3$ concentration had a maximal velocity of 178 nmol per mg of protein-hr and a K_M for NADH of approximately 1 μM. These data illustrated the importance of ADP to reactions since its presence not only lowered the K_M, but increased the maximal velocity even though the iron concentration had been lowered by an order of magnitude. In the presence of ADP there was gerater susceptibility of the lipid peroxidation system to inhibition by NADH than in the absence of ADP.

The effect of increasing concentrations of ADP on lipid peroxidation at two levels of $FeCl_3$ (0.5 mM and 0.015 mM) and in the presence of the NADH regeneration system is shown in Fig. 5. High concentrations of ADP were inhibitory at both levels of iron while the optimal level of ADP for the system appeared to be independent of whether a low or high concentration of Fe^{+3} was used. This optimal concentration was approximately 0.1 mM.

It had been shown that when phosphate was used as buffer in place of histidine, there was significant inhibition of the lipid oxidation reaction in red hake microsomes (Hultin et al., 1982). The effect of increasing phosphate concentrations at two levels of iron on flounder muscle microsomes is illustrated in Table 2. Phosphate had roughly the same inhibitory effect whether 0.015 mM or 0.15 mM $FeCl_3$ was used. Table 3 shows the decreasing inhibitory effect of phosphate as the concentration of ADP in the reaction medium increases. A concentration of 0.5 mM phosphate showed a decreased inhibitory effect at concentrations of ADP greater than 0.1 mM while with 1.0 mM phosphate, the loss of the phosphate inhibitory effect occured at slightly lower ADP concentrations. The control for each of the samples in Table 3 was the corresponding sample without the addition of phosphate. Since the activities of the controls varied with ADP concentration, the percentages given in Table 3 are based on different absolute activities. The effect of 0.5 mM and 1.0 mM phosphate at low NADH concentrations is shown in Fig. 6. Each of the phosphate concentrations used produced a roughly constant percentage of inhibition.

Cysteine in the presence of Fe^{+3} is capable of catalyzing microsomal lipid oxidation (Fig. 7). No detectable lipid oxidation was observed when Fe was not in the reaction mixture. When NADH was added to the reaction medium in addition to the cysteine and Fe^{+3}, oxidation was stimulated while the addition of ADP completely inhibited the cysteine-Fe-catalyzed lipid oxidation. An interesting observation was the fact that in the presence of the normal lipid peroxidation system (NADH, Fe^{+3}, ADP), the addition of cysteine caused an initial

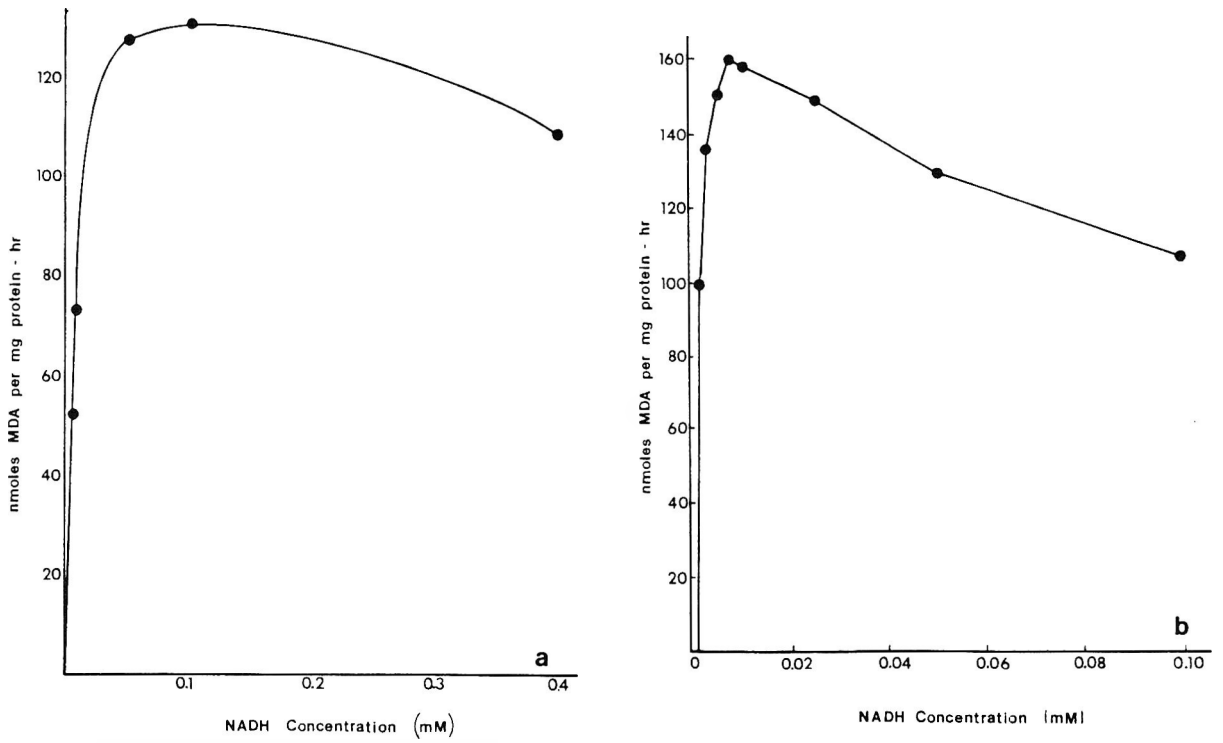

Fig. 4—The effect of NADH concentration on the rate of flounder muscle microsomal lipid oxidation. Reaction conditions were as described in Fig. 1 except that the concentration of $FeCl_3$ was 0.15 mM and the NADH regeneration system was used. The concentration of NADH was as indicated in the graphs: (a) in the absence of ADP; (b) in the presence of 0.1 mM ADP. MDA:TBA-reactive substances.

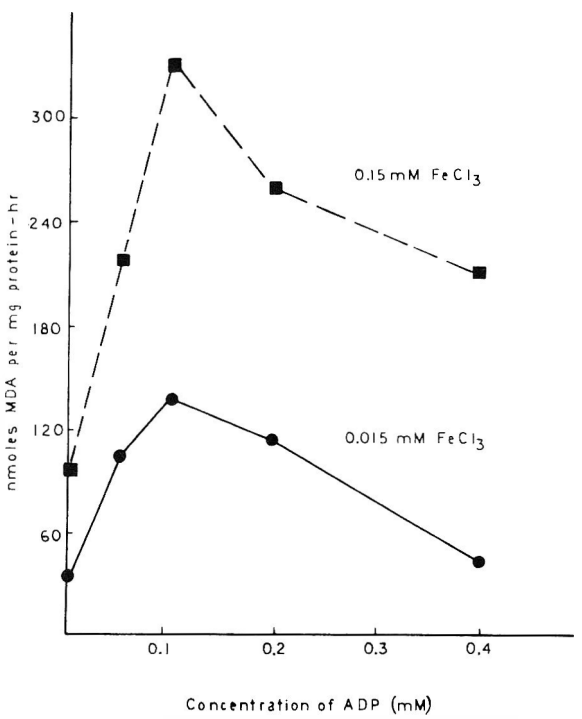

Fig. 5—Effect of ADP concentration on flounder muscle microsomal lipid peroxidation at two concentrations of $FeCl_3$. Reaction conditions were similar to those described in Fig. 1 except the concentration of NADH was 0.01 mM in the presence of the NADH-regeneration system and the $FeCl_3$ concentration was as indicated. MDA:TBA-reactive substances.

Table 2—Inhibitory effect of phosphate on lipid peroxidation at two levels of $FeCl_3$[a]

Phosphate (mM)	% Inhibition	
	0.015 mM $FeCl_3$	0.15 mM $FeCl_3$
0.5	62.5	66.4
1.0	81.7	84.2
2.5	91.7	87.9

[a] In addition to the indicated sodium phosphate and $FeCl_3$ concentrations, the reaction media contained 0.5 mg progein per mL in 0.12M KCl, 5 mM histidine at pH 7.1. The temperature of the reaction was 6°C.

Table 3—Inhibitory effect of phosphate on lipid peroxidation as a function of ADP concentration[a]

ADP (mM)	% Inhibition	
	0.5 mM PO_4^{-3}	1.0 mM PO_4^{-3}
0	70	90
0.05	76	93
0.075	70	86
0.10	66	79
0.15	48	64

[a] In addition to the indicated concentrations of ADP and Na phosphate, the reaction media contained 0.5 mg protein per mL, 0.01 mM NADH, 0.015 mM $FeCl_3$, 0.12 M KCl, 5 mM histidine at pH 7.1 and the NADH regenerating system described in Fig. 4. The temperature of reaction was 6°C.

rapid buildup of lipid hydroperoxides which was followed by a rapid breakdown such that there were almost no detectable hydroperoxides after about 40 min (Fig. 8).

The microsomal fraction of flounder muscle catalyzed the oxidation of the membrane lipid (approximately 45 nmoles of MDA per mg protein in 30 min) in the presence of 0.2 mM ascorbate and 0.015 mM $FeCl_3$. Unlike the situation with cysteine, however, the addition of ADP or NADH to the system had relatively little effect.

Cupric ion catalyzed lipid peroxidation of the microsomes (Table 4). The reaction was somewhat less than that of the corresponding Fe^{+3} salt. The Cu^{+2}-catalyzed reaction did not show the selectivity for NADH vs NADPH that iron did.

In Table 5, a series of additives is evaluated as to their effect on three modes of lipid peroxidation in the flounder microsomal fraction. These reaction modes were those catalyzed by NADH, by NADPH (both in the presence of ADP and $FeCl_3$),

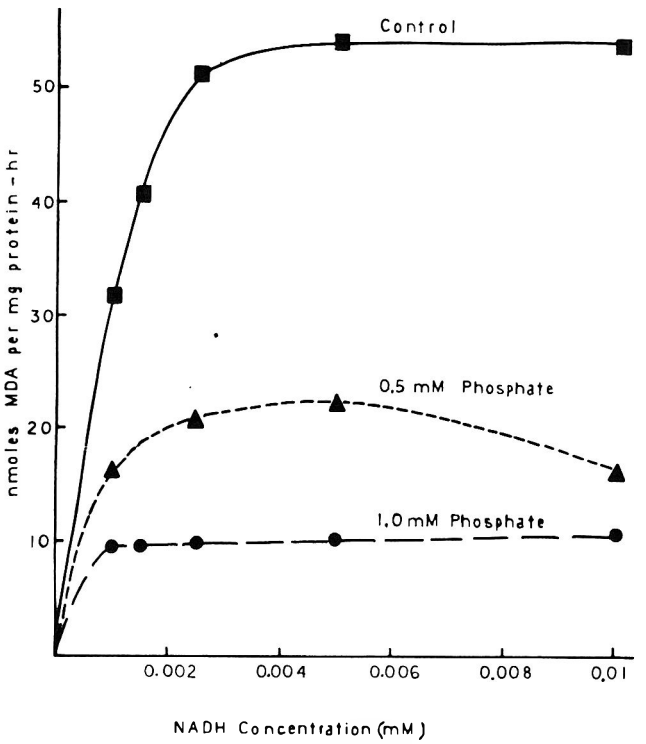

Fig. 6—Effect of inorganic phosphate on flounder muscle microsomal lipid peroxidation at low levels of NADH. The reaction medium contained the NADH concentrations indicated along with the NADH-regeneration system, 0.1 mM ADP, 0.015 mM $FeCl_3$, and sodium phosphate as indicated (pH 7.1). Other conditions were the same as described in Fig. 1. MDA:TBA-reactive substances.

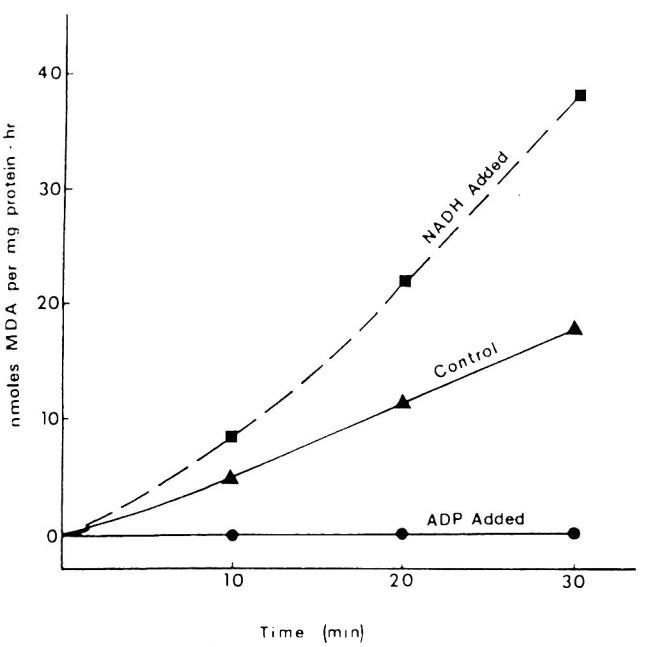

Fig. 7—Cysteine-Fe^{+3} oxidation of lipids in flounder muscle microsomes. The reaction medium of the control curve (▲——▲) contained 0.2 mM cysteine and 0.015 mM $FeCl_3$. Other conditions were as described in Fig. 1. The upper curve (■– – –■) contained 0.1 mM NADH in addition to the cysteine-FE^{+3}. The curve at the bottom (●——●) contained 0.1 mM ADP in addition to the cysteine-Fe^{+3}. MDA:TBA-reactive substances.

and by Fe^{+3} (the nonenzymic system). The NADPH-system contained a higher concentration of the reductant as well as the $FeCl_3$ to boost its activity to levels sufficient to detect

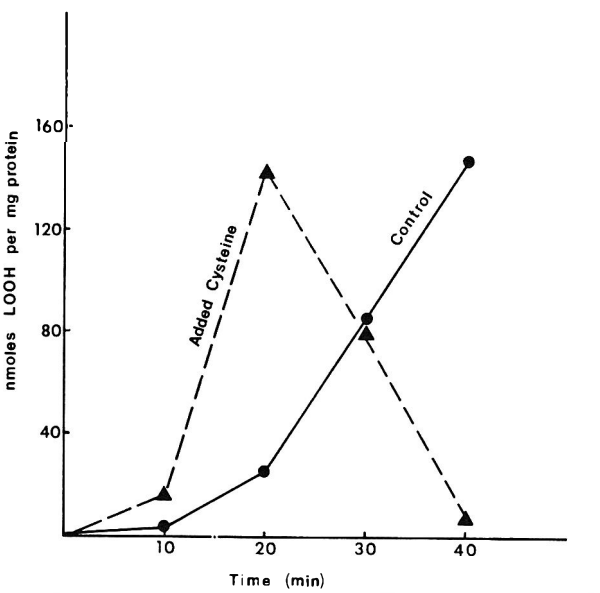

Fig. 8—Effect of cysteine on the production of lipid hydroperoxides during oxidation of flounder muscle microsomes. The control sample was as described in Fig. 1. The system with the added cysteine contained in addition 2 mM of this compound.

Table 4—Cu^{-2}-catalyzed lipid oxidation in flounder muscle microsomes[a]

Added component(s)	nmol MDA[b] per mg protein-hr
Cu^{-2}	0.4
Cu^{-2} + ADP	0.9
Cu^{-2} + NADH	3.5
Cu^{+2} + NADH + ADP	5.0
Cu^{+2} + NADPH	4.0
Cu^{+2} + NADPH + ADP	5.3

[a] The reaction media contained 0.5 mg protein per mL, 0.12M KCl, and 5 mM histidine, pH 7.1. Copper was added as 0.015 mM $CuCl_2$ and, when present, the concentration of ADP or NAD(P)H was 0.4 mM. Incubation temperature was 6°C.
[b] TBA-reactive substances calculated as malonaldehyde.

Table 5—Effect of some additives on various modes of lipid peroxidation of flounder microsomes[a]

Additive	% of MDA[b] produced compared to control		
	NADH-catalyzed[c]	NADPH-catalyzed[d]	Nonenzymic[e]
$CaCl_2$, 0.5 mM	85	75	99
$MnCl_2$, 1 mM	0	5	0
EDTA, 0.1 mM	0	0	0
Na tripolyphosphate, 0.2 mM	0	0	0
Na sulfite			
1 mM	78	170	0
10 mM	61	274	0
25 mM	56	201	0

[a] All systems contained 0.5 mg protein per mL, 0.12 M KCl and 5 mM histidine at pH 7.1. Temperature of reaction was 6°C.
[b] TBA-reactive substances calculated as malonaldehyde.
[c] The NADH-catalyzed system contained 0.01 mM NADH, 0.1 mM ADP, and 0.015 mM $FeCl_3$ and the NADH regeneration system as described in Fig. 4. The activity of the control was 167 nm MDA per mg protein-hr.
[d] The NADPH-catalyzed system contained 0.4 mM NADPH, 0.1 mM ADP, and 0.05 mM $FeCl_3$. The activity of the control was 13.8 nmol MDA per mg protein-hr.
[e] The non-enzymic system contained 0.05 mM $FeCl_2$. The activity of the control was 43.6 nmol MDA per mg protein-hr.

effects of the additives. Even with this, the rate of the NADPH-catalyzed system was an order of magnitude less than that catalyzed by NADH. Calcium chloride had a slight inhibitory effect on both the NADH- and NADPH-catalyzed reactions, but not the nonenzymic. $MnCl_2$, EDTA and Na tripolyphosphate completely eliminated lipid oxidation in all systems. Sodium sulfite was used at three different concentrations. In the NADH-catalyzed system, it caused a limited inhibition of production of TBA-reactive substances. With the NADPH-cata-

lyzed system, however, the sulfite was stimulatory and displayed a maximum. The sulfite completely inhibited non-enzymic oxidation.

Table 6 shows the effects of several compounds on the NADH-catalyzed lipid peroxidation of flounder microsomes. Propyl gallate, p-hydroxymercuribenzoate and diphenylfuran completely inhibited lipid peroxidation. Superoxide dismutase also severely curtailed the oxidation. Dimethylsulfoxide, thiourea and catalase had more moderate effects in reducing oxidation, and reduced glutathione was slightly stimulatory.

DISCUSSION

END-PRODUCT ANALYSIS and formation of lipid hydroperoxides gave the same general kinetic patterns although on a molar basis, about six times the amount of lipid hydroperoxides were formed compared to either of the breakdown product (Fig. 1). This same similarity in kinetic patterns was observed as a function of temperature (Fig. 2). These results indicated that the rate-limiting step in the reaction occurred prior to the formation of the LOOH. It was likely that this rate-limiting step is abstraction of $H^{\cdot}$. This is generally considered to be the rate-limiting step in nonenzymic lipid oxidations (Simic and Karel, 1980). Since the three analytical techniques gave the same results, the measurement of TBA-reactive substances as the standard was used since it was the easiest to perform. It should be kept in mind, however, that one would not necessarily expect that different measurements of lipid oxidation would produce the same patterns in all systems under all conditions.

The observation indicating that there is a decreasing specific activity of lipid peroxidation in flounder muscle microsomes with increasing protein concentrations is similar to previous observations that were made when storage was carried out in the frozen state (Apgar and Hultin, 1982). One of the suggestions which was made at that time as to the cause of this phenomenon was a limited supply of oxygen in the unfrozen fraction of the medium which would eventually limit the reaction. Although this is still a possibility for the reaction when it is carred out in the frozen state, this appears not to be the cause of the decreasing specific activity when the reaction is carried out at temperatures above 0°C. We have observed in certain other systems, i.e., fish muscle mitochondria, where the rate of activity is greater than that of the microsomal fraction, that there is not a decrease in specific activity with increasing protein concentration (Luo, 1986). Since O_2 is not limiting in a more active system, it does not seem reasonable that it could be the limiting factor in the microsomal system. Other possibilities for this declining specific activity with increasing protein concentration is binding of iron to the membrane and lowering the amount of iron available to the reaction, an increase in the destruction of NADH by membrane-associated NADH oxidase or the presence of an inhibitor in the membrane.

One difference between the fish microsomal system and that of an avian or mammalian system is the relatively high activity of the fish system at low temperatures. There is very little activity in a chicken muscle microsomal system at low temperatures (Player and Hultin, 1977). It is not clear at this point as to what the high activity in the fish muscle system is due. It could be the substrate rather than the enzyme. The high concentration off highly polyunsaturated fatty acids may make the fish system more susceptible to oxidation than the lipids of avian or mammalian systems. Another possibility for the stability or instability of a particular system may be its content of antioxidants such as tocopherols. The high activity of the fish system at low temperatures may have practical importance in terms of the susceptibility of fish muscle tissue to lipid oxidation under refrigerated conditions.

Another point which may be of importance for the functioning of this lipid peroxidation system in postmortem muscle is the low K_M value towards NADH. This was about 10 μM in the absence of ADP and about 1 μM in its presence. Although no data concerning the changes in NAD in winter flounder muscle with postmortem storage time were available, a study in which this question was examined in a different species, i.e., red hake, was carried out. It was found that the initial concentration of NAD was approximately 30–50 μM and even after 14 days, it remained as high as 5–7 μM (Phillippy, 1984). Murata and Sakaguchi (1986) have reported even higher initial values in yellowtail, and even though there was a rapid decrease with time of storage, NAD stayed well above the K_M value observed for the microsomal lipid peroxidation system of flounder *in vitro*. If other factors were available, little diminution in reaction rate over that period of storage would be expected. The kinetic data regarding NADH and the influence of ADP on these parameters indicates again the importance of ADP in the functioning of the system. It is possible that ADP forms a complex with the Fe^{+3} and helps to maintain it in a soluble form which is available to the reaction. Another possibility is that the ADP-Fe^{+3} complex has a more favorable oxidation-reduction potential for the reaction. Floyd (1983) has presented evidence using an electron paramagnetic resonance spin-trapping technique that when Fe^{+3} is added to a solution containing di- and trinucleotides hydroxy free radicals are produced from H_2O_2. This represents another possibility for the role of ADP.

The inorganic phosphate concentration in living muscle is in the range of 3 to 8 mM (Burt et al., 1977; Tanokura and Yamada, 1984). Presumably, the amount of free inorganic phosphate increases with storage time postmortem as organic phosphate compounds are broken down. Inorganic phosphate is a potent inhibitor of the fish muscle microsmal lipid peroxidation system *in vitro* at much lower concentrations than are found in muscle tissue (Tables 2 and 3). Possibly, phosphate interacts with Fe^{+3} to form an insoluble compound. The situation, however, may be more complex. It can be seen in Fig. 6 that increasing the phosphate concentration from 0.5 mM to 1.0 mM does not lead to a corresponding decrease in lipid peroxidation. If the data of Fig. 6 were plotted as their reciprocals, roughly parallel lines are obtained. This is usually indicative of an uncompetitive inhibition, i.e., the inorganic phosphate binds to an enzyme-substrate complex but not to the free enzyme. In this membrane system, however, it is not clear what this might mean. Is the enzyme-substrate the enzyme-Fe^{+3} complex, the enzyme-NAD complex, or the enzyme-lipid complex? It is possible that the iron has to bind to the membrane before it can be tied up by the inorganic phosphate.

The increased lipid peroxidative activity of the fish microsomal system, obtained after adding NADH to the cysteine-Fe^{+3} oxidation system, may indicate that these two systems are independent or that the NADH is a better reductant than is cysteine. The complete inhibition of the oxidation catalyzed by cysteine when ADP is added to the assay medium implies that the ADP-Fe chelate is not available to participate in the reaction. It must be remembered, however, that the same con-

Table 6—Effect of some additives on NADH-catalyzed lipid peroxidation of flounder microsomes

Inhibitor	% of MDA[a] produced compared to control
Propyl gallate, 0.02%	0
p-Hydroxymercuribenzoate, 1.0 mM	0
Reduced glutathione, 0.2 mM	132
Dimethylsulfoxide, 50 mM	78
Thiourea, 20 mM	55
Diphenylfuran, 0.2 mM	0
Superoxide dismutase, 100 μg/mL	11
Catalase, 100 μg/ml	64

[a] TBA-reactive substances calculated as malonaldehyde. The reaction media contained 0.5 mg protein per mL, 0.1 mM NADH, 0.1 mM ADP, 0.015 mM $FeCl_3$, 0.12 M KCl, and 5 mM histidine at pH 7.1. The temperature of incubation was 6°C. The activity of the control was 36.8 nmol MDA per mg protein-hr.

centration of ADP which inhibited the cysteine-Fe-catalyzed oxidation was very stimulating to systems containing NADH and Fe^{+3}.

The lack of added effect when NADH was added to the ascorbate-Fe system might indicate that under the conditions used, the system was operating near maximal activity in the presence of the ascorbate-Fe and that additional reducing equivalents supplied via the NADH-enzyme system did not add much more activity. Unlike the situation with cysteine, ADP did not show significant inhibition with the ascorbate-Fe system. This might be due to the fact that ascorbate formed a strong complex with Fe^{+3}, which might prevent it from interacting with the ADP.

The close correlation between the production of lipid hydroperoxides and later breakdown products (Fig. 1) was not observed when cysteine was added to the system. In this case, a very rapid buildup of the lipid hydroperoxides which reached a peak approximately where the control sample was coming out of its lag phase was observed. Under the conditions of our assays, the lipid hydroperoxides dropped to a very low level within 40 min in the assay medium to which the cysteine had been added. Obviously, cysteine has a major role to play in the breakdown of lipid hydroperoxides. Since it also supports the production of TBA-reactive substances, it is likely that it participates in the breakdown by a mechanism involving free radicals. Sulfur-containing amino acids are susceptible to attack by free radicals (Hultin, 1986).

The reactivity of the system in the presence of various additives may give insight as to the nature of the reaction. EDTA and sodium tripolyphosphate most likely function because they can form complexes with iron ions. One would presume that they form a stronger complex with Fe^{+3} than does ADP. The Fe in these complexes must, however, be unavailable for the oxidation reaction. Svingen et al. (1979) have shown that EDTA-chelated iron participates in the decomposition of lipid hydroperoxides but not in the initiation reaction.

It is important to keep in mind that the order of addition of components may have an important effect on what is observed. For example, many investigators have found that histidine can inhibit lipid oxidation in aqueous systems. We found that this depended on the order of addition. If the Fe^{+3} and the ADP were put into the reaction medium first, histidine had no effect. However, if the iron was added in the presence of histidine but before ADP, the histidine was extremely inhibitory. Presumably, it formed a complex which did not give up the iron to the ADP very readily even though it was incapable of extracting the iron from an ADP-Fe complex already formed.

The effectiveness of propyl gallate indicated that the reaction proceeded via free radical intermediates. That sulfhydryl groups were involved was indicated by the inhibitory action of the p-hydroxymercuribenzoate. Reduced glutathione slightly stimulated the reaction and may behave in a manner similar to that of cysteine, although it was not as effective. The use of active oxygen intermediate scavengers indicated that these compounds could stimulate the reaction. Superoxide dismutase was a scavenger of superoxide anion which was the first reduction product of molecular oxygen; catalase was a scavenger for the two electron-reduced product, H_2O_2. Dimethylsulfoxide and thiourea will remove the hydroxyl free radical, and diphenylfuran is capable of reacting with and removing singlet oxygen.

Based on the observations above, a proposed reaction pathway is suggested in Fig. 9. Some form of reduced iron reacts with molecular oxygen to form an intermediate (here indicated as the $Fe^{+2}O_2$, dioxygen ferrous ion complex, in equilibrium with $Fe^{+3}O_2^-$, preferryl iron). This releases superoxide and Fe^{+3} which forms a complex with ADP whose function is to keep the Fe^{+3} soluble and/or modify its oxidation-reduction potential. On reduction, the Fe^{+2} is released from the ADP complex. The function of the enzyme (*ENZ*) is to reduce the Fe^{+3} to Fe^{+2} using electrons from NADH. This reduction of iron is suggested to be the primary function of the membrane

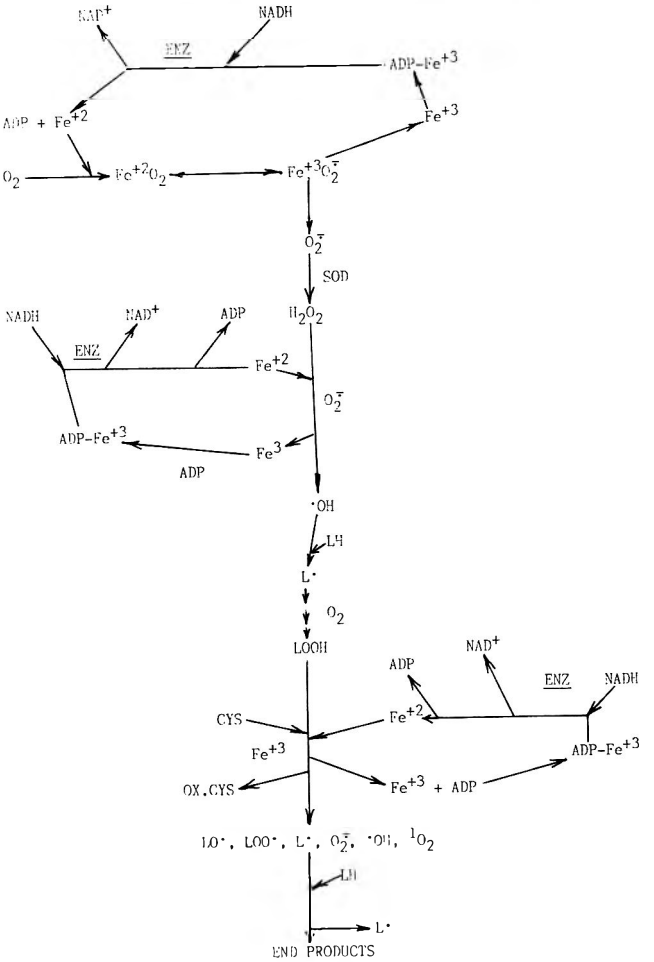

Fig. 9—Scheme of proposed pathway for flounder muscle microsomal lipid peroxidation. ENZ = membrane enzyme system.

enzyme and occurs in two other places in the scheme, indicated by *ENZ*.

Superoxide is dismutated to O_2 and H_2O_2 which can then further react with O_2^- to produce the hydroxy free radical (·OH) via a Fenton-type reaction. Superoxide dismutase inhibited the reaction by removing superoxide to prevent its reaction with the H_2O_2 formed while the inhibitory effect of catalase was due to its ability to remove H_2O_2. The hydroxy free radical may then abstract hydrogen from lipid to initiate lipid oxidation which then proceeds through the usual process to form a hydroperoxide. Hydroxy free radical scavengers destroyed the ultimate initiator, ·OH. The hydroperoxide can be decomposed by ferrous iron or ADP-complexed Fe^{+2} to produce a variety of free radicals and singlet oxygen. The effectiveness of the singlet oxygen scavenger, diphenylfuran, indicated that initiation of the reaction could also be accomplished by singlet oxygen. Cysteine in the presence of Fe^{+3} can also decompose lipid hydroperoxides to produce free radicals. These products may then be involved with further propagation reactions by interacting with other molecules of lipid (LH) to maintain the chain reaction.

The main suggestion of this reaction scheme is that the role of the enzyme is simply to maintain iron in the reduced state. Although we do not have direct proof for this, we have been able to demonstrate a non-enzymic lipid oxidation which occurs very rapidly in the presence of Fe^{+2} and the extent of which (as measured by TBA-reactive substances) is proportional to the amount of Fe^{+2} added to the reaction mixture (Shewfelt and Hultin, 1983). Presumably, in this case, the reaction stops because the enzyme is not available to reduce the Fe^{+3} formed during the reaction to Fe^{+2}.

—Continued on page 27

Functional and Biochemical Changes in Deboned Turkey due to Frozen Storage and Lipid Oxidation

D. M. SMITH

ABSTRACT

The effect of frozen storage and lipid oxidation on protein functional and biochemical properties of mechanically deboned and hand deboned turkey meat was investigated. Turkey meat was evaluated by the 2-thiobarbituric acid test and cook yield. Isolated turkey myofibrils were analyzed for solubility, ATPase activity, gel strength, and gel microstructure. Frozen storage caused protein insolubilization and changes in the biochemical and functional properties in all treatments. Antioxidants prevented some of the biochemical and functional changes in the meat. Myofibril gel microstructure changed from a continuous filamentous matrix to a globular matrix with decreased waterholding capacity during frozen storage of the turkey meat.

INTRODUCTION

FROZEN STORAGE is one of the most important preservation methods for meat and meat products. During frozen storage lower temperatures prevent or minimize many undesirable changes in meat such as microbial growth and metabolic processes; however, some chemical reactions still occur which adversely affect product quality. Changes in texture, water-binding capacity, emulsifying capacity, and cooking yields during frozen storage have been reported in chicken (Khan, 1966; Dhillon and Maurer, 1975; Orr and Wogar, 1979), turkey (Hoke et al., 1968; Johnson et al., 1974), beef (Awad et al., 1968; Sebranek et al., 1979) and fish (Awad et al., 1969). Functional changes during frozen storage have been related to myofibrillar protein insolubilization in the intact muscle of chicken (Khan et al., 1963; Yamamoto et al., 1977), beef (Wagner and Anon, 1986), rabbit (Kang et al., 1983) and fish (Matsumoto, 1980; Shenouda, 1980). The effects of frozen storage on the functionality of turkey meat have not been reported. Likewise, no studies have been found which examined protein denaturation and functionality changes during frozen storage of comminuted meats.

Protein denaturation during frozen storage of meat may be caused by one or more of the following factors: (1) ice crystal damage to cells and membranes, (2) dehydration of protein molecules, (3) increase in solute concentration in the unfrozen water phase, (4) enzymatic activity, (5) reaction of proteins with free fatty acids and other intact lipids, and (6) reaction of proteins with oxidizing lipids (Matsumoto, 1980; Shenouda, 1980). The extent to which these factors interact and contribute to myofibrillar protein denaturation during frozen storage has not been elucidated.

Lipid oxidation occurs extensively during the refrigerated and frozen storage of comminuted turkey meat (Dawson and Gartner, 1983) and may be one cause of myofibrillar protein denaturation. Reactions of meat protein with oxidizing lipid have been studied extensively in model systems (Buttkus, 1967; Jarenback and Liljemark, 1975; Nakhost and Karel, 1983, 1984). Oxidizing lipids cause protein polymerization, insolubilization, polypeptide chain scission, amino acid destruction, and form addition products with protein (Kanner and Karel, 1976; Funns and Karel, 1981; Funns et al., 1982). Lipid-protein interactions alter the functional properties of meat and may cause deleterious changes in final product quality (Sikorski, 1978).

The purpose of this study was to examine the effect of long-term frozen storage and lipid oxidation on changes in the biochemical and functional properties of hand deboned (HDT) and mechanically deboned (MDT) turkey.

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MATERIALS & METHODS

Materials and sample preparation

Fresh, unfrozen MDT and HDT was obtained from a local processor and prepared for storage the same day. HDT was composed of 60% white meat and 40% dark meat. MDT was obtained from turkey racks using a Beehive deboning machine. Both deboned products contained skin. Treatments were (1) HDT, (2) hand deboned turkey meat with antioxidant (HDT + ao), (3) MDT, and (4) mechanically deboned turkey with antioxidant (MDT + ao). Tenox 2® antioxidant (Eastman Kodak) was added at 0.02% of the fat content. Tenox 2 contains butylated hydroxyanisole, propyl gallate, and citric acid. Antioxidant was added while mixing the meat with the paddle attachment of a Kitchen Aid Stand Mixer (Model K5-A, Hobart Corp., Troy, OH) at the lowest speed setting for 10 min.

Meat was packaged in 450g aliquots in mylar-polyethylene film, analyzed at 1 day (unfrozen) or frozen at −20°C and analyzed after 1, 7, 16 and 26 wk of storage. Meat was thawed overnight at 4°C before use in experiments. Proximate composition was determined by AOAC (1980) procedures at the beginning and end of the study.

Lipid oxidation

An extraction method (Salih et al., 1986) was used to measure the development of thiobarbituric acid (TBA) reactive substances. Results were expressed in TBA number (mg malonaldehyde/kg meat).

Preparation of myofibrils

Myofibrils were isolated in 0.1M NaCl, 0.05M K phosphate buffer, pH 7.0 as described by Eisele and Brekke (1981) with some modifications. Myofibrils in 4 volumes of the isolation buffer were stirred for three 1 hr intervals prior to centrifugation. The final myofibril pellet was solubilized in 0.6M NaCl, pH 6.5 to obtain a concentration of 30 mg protein/ml buffer as determined by micro-Kjeldahl (AOAC, 1980).

Indices of protein denaturation

Solubility was determined in 0.6M NaCl, pH 6.5 by centrifuging 10 mL of the myofibril protein solution at 10,000 × *g* for 10 min. Percentage solubility was calculated by dividing the protein content of the supernatant by the total protein content then multiplying by 100. The Ca^{2+}-ATPase activity of the myofibril preparations were assayed as described by Wells et al. (1979).

Electrophoresis

Myofibrils were prepared for electrophoresis as recommended by Porzio and Pearson (1979). Molecular weight standards were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed using a tris-glycine buffer system on 10% gels (Smith and Brekke, 1985).

Gelation characteristics

Gels were prepared by heating 40 mL of the myofibril solution in covered 50 mL centrifuge tubes for 30 min at 70°C. Gels were held overnight at 4°C. Syneresis after gel formation was determined by measuring the volume of free liquid removed from the tubes with a Pasteur pipet. Percentage syneresis was calculated by dividing the volume of free liquid after gelation by 40 mL then multiplying by 100. Gels were equilibrated to 20°C and cut cross-wise into 2.0 cm (height) × 2.5 cm (diameter) cylindrical pieces. An Instron Universal Testing Machine (Model 4202, Canton, MA) was used to compress the gels between two flat parallel surfaces at a crosshead speed of 1 cm/min. Maximum applied force when the gel ruptured (cohesiveness) and the tangential slope (firmness) was calculated from the force-time curve as suggested by Voisey et al. (1975). Gels were prepared for scanning electron microscopy as described by Yasui et al. (1979) and observed with a JEOL scanning microscope (Model JSM-35C) at an accelerating voltage of 15 KV.

Emulsified meat cook test

About 10g turkey meat with or without 2.0% NaCl was emulsified in a tared 50 mL centrifuge tube for 3 min at a speed setting of 3 using a Sorvall Omni-Mixer. The emulsified meat was weighed and cooked for 30 min in a 70°C waterbath. After heating the meat plug was removed and weighed. Percentage cook yield was calculated by dividing the weight of meat after cooking by the weight of raw emulsified meat then multiplying by 100.

Statistics

Results were analyzed using a random block design (Steel and Torrie, 1980). Meat treatment (n = 4) and storage times (n = 5) were the main effects. When the main effects were significant across treatment or storage time, means were separated using Bonferronis test (Kirk, 1982). The predetermined level of probability was 5% for all analyses.

RESULTS & DISCUSSION

Proximate composition

The proximate compositions of MDT and HDT are shown in Table 1. MDT contained more fat and less protein and water than HDT. Addition of antioxidants did not alter meat composition. The proximate composition did not change significantly during 26 wk of frozen storage, indicating physicochemical changes were not caused by product dehydration. The pH of MDT and MDT + ao increased from 6.45 to 6.60, while the pH of HDT and HDT + ao remained constant at 6.05 during frozen storage. Mast and MacNeil (1976) reported a slight decrease in pH of mechanically deboned poultry meat stored at −18°C for 25 wk. Researchers have reported an increase in fish muscle pH (Awad et al., 1969) and no change in beef muscle pH (Awad et al., 1968) during 6 months of frozen storage.

Lipid oxidation

Lipid oxidation as measured by TBA number occurred most rapidly during the first 7 wk of frozen storage in turkey without antioxidant (Fig. 1). The rate of increase in TBA number slowed during the latter part of storage. The TBA test measures malonaldehyde and other TBA reactive substances which often predominate during the early stages of lipid oxidation. Many secondary oxidation products which occur in the later stages of lipid oxidation are not measured by the TBA test, thus, lipid oxidation which occurred in the turkey samples may not have been measured accurately during longer periods of frozen storage.

Table 1—Proximate composition of hand deboned and mechanically deboned turkey[a]

Composition	Treatment[b] HDT	MDT
Moisture (%)	75.6	68.5
Fat (%)	3.8	17.2
Protein (%)	19.2	12.9
Ash (%)	1.0	1.2

[a] Values are the average of triplicate determinations.
[b] HDT: hand deboned turkey; MDT: mechanically deboned turkey.

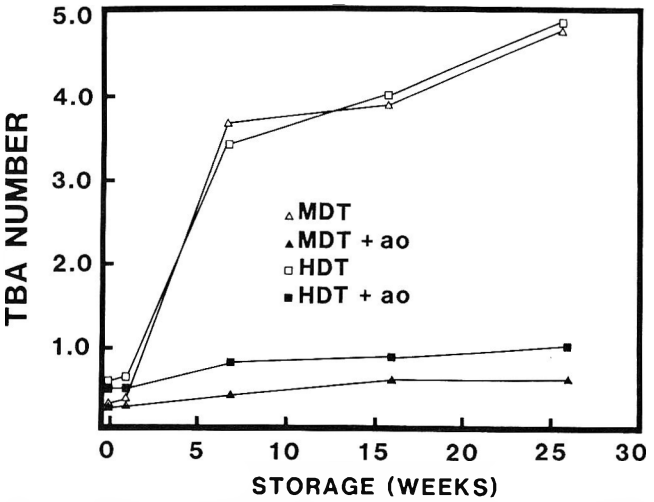

Fig. 1—Influence of frozen storage at −20°C and antioxidant use on mean TBA values of hand deboned turkey and mechanically deboned turkey. TBA measured as mg malonaldehyde/Kg meat (MDT: mechanically deboned turkey; MDT + ao: mechanically deboned turkey with antioxidant; HDT: hand deboned turkey; HDT + ao: hand deboned turkey with antioxidant).

TBA numbers were not significantly different between HDT and MDT at the same storage interval. Lipid oxidation in frozen stored MDT is often more severe than in HDT. MDT usually contains more fat and bone marrow, and is exposed to higher temperatures during deboning than HDT (Dawson and Gartner, 1983). TBA numbers determined by the extraction method used in this study are about half that usually measured by the distillation procedure (Witte et al., 1970; Salih et al., 1986).

Antioxidant helped prevent lipid oxidation in MDT and HDT during frozen storage. The effectiveness of other antioxidants in refrigerated and frozen turkey has been reported previously (Moerck and Ball, 1973; Dawson et al., 1978; Uebersax et al., 1978).

Myofibril denaturation

HDT and MDT myofibril solubility in 0.6M NaCl was ca. 100% before freezing but decreased during frozen storage (Fig. 2). Generally, MDT and MDT + ao had lower solubility than both HDT samples after freezing and throughout the 26 wk storage period. McMahon and Dawson (1976) reported that frozen MDT had a lower percentage of salt-soluble proteins than the percentage found in frozen HDT, and attributed this to protein insolubilization at high temperatures and shear rate during mechanical deboning. Myofibrillar protein solubility decreased during frozen storage in several other meat species (Khan, 1966; Moledina et al., 1977; Matsumoto, 1980; Shenouda, 1980; Wagner and Anon, 1986).

Antioxidants helped prevent losses in protein solubility in both HDT and MDT during frozen storage. After 15 wk of frozen storage MDT and HDT solubility values were 5.0% below the corresponding treatment with antioxidant. After 26 wk of frozen storage the difference in solubility between the same meat type with and without antioxidant increased to 9.0%. Moledina et al. (1977) reported that certain antioxidant combinations helped prevent losses in fish protein solubility during frozen storage.

Myofibrillar Ca^{2+}-ATPase activity of fresh MDT was 4.8% below that of fresh HDT. Ca^{2+}-ATPase activity decreased in all samples during frozen storage (Fig. 3). ATPase activity

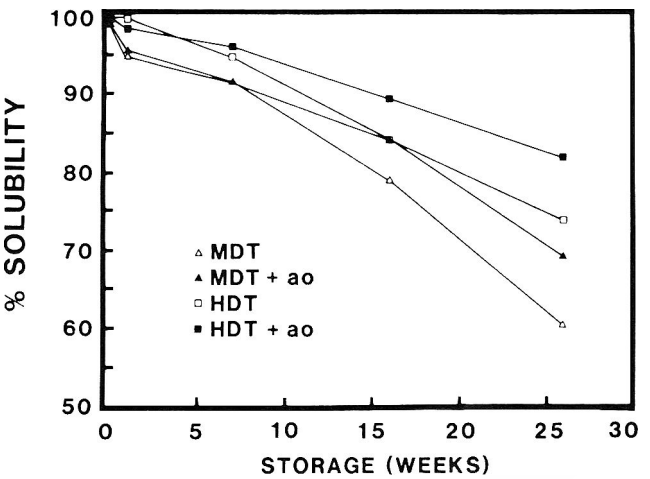

Fig. 2—Effect of frozen storage at −20°C and antioxidant use on the solubility of 3.0% (w/v) mechanically deboned and hand deboned turkey myofibrils in 0.6M NaCl, 0.05M Na phosphate, pH 6.5 (MDT: mechanically deboned turkey; MDT+ao: mechanically deboned turkey with antioxidant; HDT: hand deboned turkey; HDT+ao: hand deboned turkey with antioxidant).

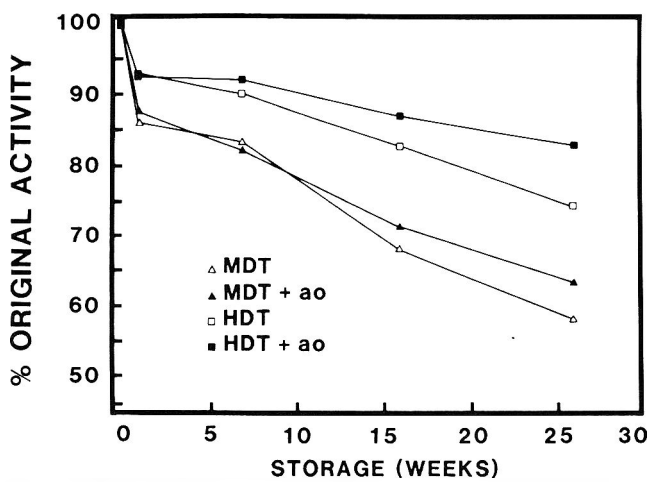

Fig. 3—Effect of frozen storage and antioxidant use on the Ca^{2+}-ATPase activity of hand deboned turkey and mechanically deboned turkey determined in 7.6 mM ATP, 15 mM $CaCl_2$, 150 mM KCl, and 180 mM Tris, pH 7.4 (MDT: mechanically deboned turkey; MDT+ao: mechanically deboned turkey with antioxidant; HDT: hand deboned turkey; HDT+ao: hand deboned turkey with antioxidant).

decreased rapidly during the first week of frozen storage, which was probably due to the slow freezing rate. Wagner and Anon (1985) observed that decreasing the freezing rate of beef muscle increased the loss of ATPase activity. MDT myofibrillar ATPase activity decreased more rapidly during storage than HDT myofibrillar ATPase activity. At 15 wk of storage, HDT and MDT had significantly less ATPase activity than the same meat type containing antioxidant.

Solubility in 0.6M NaCl and ATPase activity are used to measure the extent of myofibrillar protein denaturation (Khan, 1966; Matsumoto, 1980; Wagner and Anon, 1985). Connell (1960) reported that fish myosin was denatured and completely insoluble during frozen storage on reaching 55% of the original ATPase activity. Results of the protein solubility test and ATPase activity measurements indicate that HDT and MDT myofibrillar proteins denature during freezing and frozen storage. Wagner and Anon (1986) reported that denaturation occurred during freezing and frozen storage of beef when the myosin head region unfolded, followed by a weakening of the actin-myosin interaction as indicated by Mg^{2+} and Ca^{2+} ATPase activity losses. These changes caused protein aggregation and decreased solubility.

Oxidized lipid-protein interactions have been reported to cause protein insolubilization, cross-linking, polypeptide chain scission and other deleterious reactions in many food and model systems (Karel et al., 1975; Pokorny, 1977; Nakhost and Karel, 1983, 1984). Jarenback and Liljemark (1975) reported that linoleic acid peroxides were 10 times more effective than unoxidized linoleic acid in decreasing the solubility of cod myofibrillar proteins. Antioxidants prevented some of the myofibrillar protein denaturation during frozen storage of HDT and MDT, as indicated by results of the protein solubility and ATPase tests. Antioxidants prevented lipid oxidation and subsequent oxidized lipid-protein interactions which caused myofibrillar protein denaturation during frozen storage; however, oxidized lipid-protein interactions were not the major cause of protein denaturation during frozen storage. Other factors are involved in turkey myofibril denaturation during storage (Matsumoto, 1980; Shenouda, 1980) and need to be elucidated.

MDT denatured faster than HDT during freezing and frozen storage as indicated by ATPase activity and protein solubility. Differences in denaturation cannot be explained by differences in the extent of lipid oxidation in the samples. Mechanical deboning causes cellular disruption and intermixing of cell components and may have facilitated deleterious solute-protein interactions, ice crystal damage, and reactions with unoxidized lipids to accelerate the rate of protein denaturation in MDT.

Electrophoresis

There were no changes in the electrophoretic patterns of the myofibrillar proteins during frozen storage as observed on SDS-polyacrylamide gels. Results indicate that covalent cross-linking, polypeptide chain scission, and endogenous protease activity were not major factors causing changes in the biochemical and functional properties of turkey during storage. The biochemical and functional changes are probably caused by protein unfolding and non-covalent association to produce high molecular weight aggregates (Matsumoto, 1980; Shenouda, 1980). More research is necessary to establish the specific biochemical changes in turkey caused by frozen storage and lipid oxidation.

Emulsified meat cook yield

MDT emulsions with and without 2.0% salt had lower cook yields than those of HDT throughout storage (Table 2). Several researchers have reported that fresh MDT has lower cook yields than fresh HDT and attributed this to the higher fat content of MDT (McMahon and Dawson, 1976; Froning, 1981). Cook yield decreased by 6-11% in all treatments during 26 weeks of frozen storage. Losses in cook yield during frozen storage have been reported in chicken (Khan and van den Berg, 1967). beef (Awad et al., 1968), and fish (Awad et al., 1969).

There was no significant difference in cook yield in the same meat type with or without antioxidant during storage, except at 16 weeks for HDT and HDT + ao, when 2.0% salt was used in the emulsified meat test. Antioxidants helped prevent decreases in cook yield during frozen storage when emulsions were made without salt. MDT and HDT emulsions made without salt had 5.1% and 4.6%, respectively, lower cook yields than the corresponding meat type with antioxidant after 16 wk of frozen storage.

Heat-induced gelation

Myofibril gels from unfrozen HDT were firmer and more cohesive than gels from unfrozen MDT (Table 3). Myofibrillar protein solubility did not differ between meat types, so differences in gel strength may be caused by differences in meat

Table 2—Influence of frozen storage at −20°C and antioxidant use on the mean emulsified meat cooked yield of hand deboned and mechanically deboned turkey

Storage time (Wk)	Cooked yield (%)[a] Treatment[b] HDT	HDT + ao	MDT	MDT + ao	Rank order (highest to lowest) across treatments[f]
No Salt					
Unfrozen	85.1[c]	86.2[c]	74.0[c]	76.6[c]	A B C D
1	83.1[c]	85.9[c]	73.2[c]	72.8[d]	A B C D
7	81.2[c]	83.7[c,d]	70.7[d]	71.4[d,e]	A B C D
16	77.0[d]	80.6[d,e]	66.5[e]	70.8[d,e]	A B C D
26	74.0[d]	78.6[e]	63.8[e]	68.9[e]	A B C D
2% Salt					
Unfrozen	92.8[c]	92.4[c]	80.9[c]	80.6[c]	A B C D
1	90.9[c]	92.7[c]	79.2[c]	78.9[c,d]	A B C D
7	88.2[d]	90.7[c,d]	78.5[c]	77.8[d]	A B C D
16	84.4[e]	87.8[d,e]	73.2[d]	72.7[e]	A B C D
26	84.3[e]	86.1[e]	73.0[d]	73.8[e]	A B C D

[a] Cooked yield (%) = $\frac{\text{Wt of meat after cooking}}{\text{Wt of raw meat}} \times 100$.

[b] MDT: mechanically deboned turkey; MDT + ao: mechanically deboned turkey with antioxidant; HDT: hand deboned turkey; HDT + ao: hand deboned turkey with antioxidant.

[c-e] Means in the same column within no salt or 2% salt bearing a common superscript do not differ ($p<0.05$).

[f] Means in the same row underscored by a common line do not differ ($p<0.05$).

Table 3—Effect of frozen storage at −20°C and antioxidant use on the characteristics of 3.0% (w/v) hand deboned and mechanically deboned turkey heat-induced myofibril gels prepared in 0.6M NaCl, pH 6.0, 70°C

Storage time (Wk)	Treatment[a] HDT	HDT + ao	MDT	MDT + ao	Rank order (highest to lowest) across treatments[f]
Cohesiveness (N)					
Unfrozen	5.54[b]	5.39[b]	4.55[b]	4.32[b]	A B C D
1	5.71[b]	5.69[b]	4.75[b]	4.54[b]	A B C D
7	4.22[c]	4.31[c]	3.72[b]	3.83[b]	B A D C
16	2.83[d]	2.91[d]	2.62[c]	2.91[c]	B D A C
26	1.30[e]	1.93[e]	0.39[d]	0.55[d]	B A D C
Firmness (N/s)					
Unfrozen	0.29[b]	0.28[b]	0.20[b]	0.21[d]	A B D C
1	0.20[c]	0.31[b]	0.19[b]	0.18[b,c]	B A C D
7	0.21[c]	0.20[c]	0.12[c]	0.11[c]	A B C D
16	0.18[c]	0.16[c]	0.10[c]	0.11[c]	A B D C
26	0.07[d]	0.08[d]	0.02[d]	0.02[d]	B A C D
Syneresis (% of total protein solution)					
Unfrozen	19[b]	19[b]	20[b]	20[b]	nsd
1	24[c]	24[c]	24[b,c]	22[b]	nsd
7	27[c,d]	23[c]	27[c]	26[c]	nsd
16	27[c,d]	27[c]	32[d]	28[c]	nsd
26	30[d]	28[c]	34[d]	35[d]	nsd

[a] MDT: mechanically deboned turkey; MDT + ao: mechanically deboned turkey with antioxidant; HDT: hand deboned turkey; HDT + ao: hand deboned turkey with antioxidant.

[b-e] Means in the same column within cohesiveness, firmness or syneresis bearing a common superscript do not differ ($p<0.05$).

[f] Means in the same row underscored by a common line do not differ ($p<0.05$).

source and composition. Several authors have reported that gel strength varies with the ratio of actin to myosin in the myofibril system (Yasui et al., 1980; Ishiroshi et al., 1983). Asghar et al. (1984) reported the strength of white muscle broiler myosin gels was three times greater than gels from red muscle broiler myosin. MDT which was made from turkey racks probably contained a higher concentration of red muscle when compared to the myosin composition of HDT.

Gel firmness and cohesiveness decreased in all turkey samples during frozen storage. Gel syneresis increased during frozen storage indicating gels gradually lost their ability to bind water. The use of antioxidants did not prevent significant changes in cohesiveness, firmness, or syneresis in the gels during frozen storage. Gels were initially white, shiny, and creamy in appearance, but with storage became tan, flat and grainy. The tan coloration was more pronounced in gels made from turkey which did not contain antioxidant. Oxidized lipid-protein interactions have been reported to cause browning in several model systems (Shenouda, 1980; Pokorny, 1981). Pokorny (1981) reported that antioxidants helped prevent these browning reactions.

Changes in gel microstructure are shown in Fig. 4. There were no differences in microstructure between myofibril gels made from the same meat type stored with or without antioxidant, thus only HDT and MDT gels are shown. The microstructure of HDT and MDT gels prepared from unfrozen meat was filamentous and exhibited an open continuous matrix. HDT gels prepared from unfrozen meat contained thicker protein filaments in a slightly more irregular matrix than gels from unfrozen MDT. The microstructure is characteristic of strong gels with good waterholding capacity. After 26 weeks of frozen storage the microstructure of both HDT and MDT gels was very different from the microstructure of gels made from fresh, unfrozen HDT and MDT samples. The gels were more globular and lacked the regular, filamentous protein network. Large spaces occurred in the gels which were surrounded by highly aggregated protein networks. This microstructure explains the poor gel strength and poor waterholding capacity observed. Other researchers have reported that fish myofibrillar proteins change from a regular, filamentous network to a highly aggregated structure during short periods of frozen storage (Jarenback and Liljemark, 1975; Tsuchiya et al., 1975). Denatured myofibrillar proteins have been reported to produce gels with a more globular microstructure (Ishioroshi et al., 1980). Products made from frozen meat are often reported to have lower cook yields and waterbinding ability than products made from fresh meat. Protein denaturation resulting in a loss of gel matrix integrity and subsequent loss in gel strength may partially explain this phenomena.

Correlations

Correlation coefficients in Table 4 indicate the relationship between indices of turkey myofibrillar protein denaturation and functional properties. Percent protein solubility and myofibrillar ATPase activity were highly correlated ($r^2 = 0.85$). TBA values did not correlate well with the indices of protein denaturation. Thirty-six percent of the variability was due to a correlated response between TBA numbers and solubility ($r = -0.60$, $r^2 = 0.36$), while 27% of the variability was due to a correlated response between TBA numbers and ATPase activity ($r = -0.52$, $r^2 = 0.27$). These results indicate that lipid oxidation is not a major factor causing turkey myofibrillar protein denaturation during frozen storage.

Changes in myofibrillar protein gel characteristics were highly correlated with changes in protein solubility and ATPase activity during frozen storage. Changes in the emulsified meat cook yield during frozen storage were correlated to ATPase activity, but did not correlate as well with protein solubility. ATPase activity may be a simple and rapid method to monitor the functionality of deboned turkey meat during frozen storage.

Gel cohesiveness, firmness, and syneresis were highly correlated to each other. Gel firmness was most closely correlated to emulsified cook yield at 0% and 2% NaCl. This indicates that gel firmness may be a good model system test of emulsified cook yield. This relationship was previously reported by Smith and Brekke (1985).

CONCLUSIONS

LIPID OXIDATION, freezing, and frozen storage caused myofibrillar protein denaturation and decreases in functionality in both MDT and HDT. Antioxidants prevented lipid oxidation and some of the protein denaturation which occurred during storage, although most of the denaturation was a result of other reactions. Protein denaturation and subsequent functionality changes which occur in turkey should be of concern to processors, as MDT is often stored for long time periods. Dena-

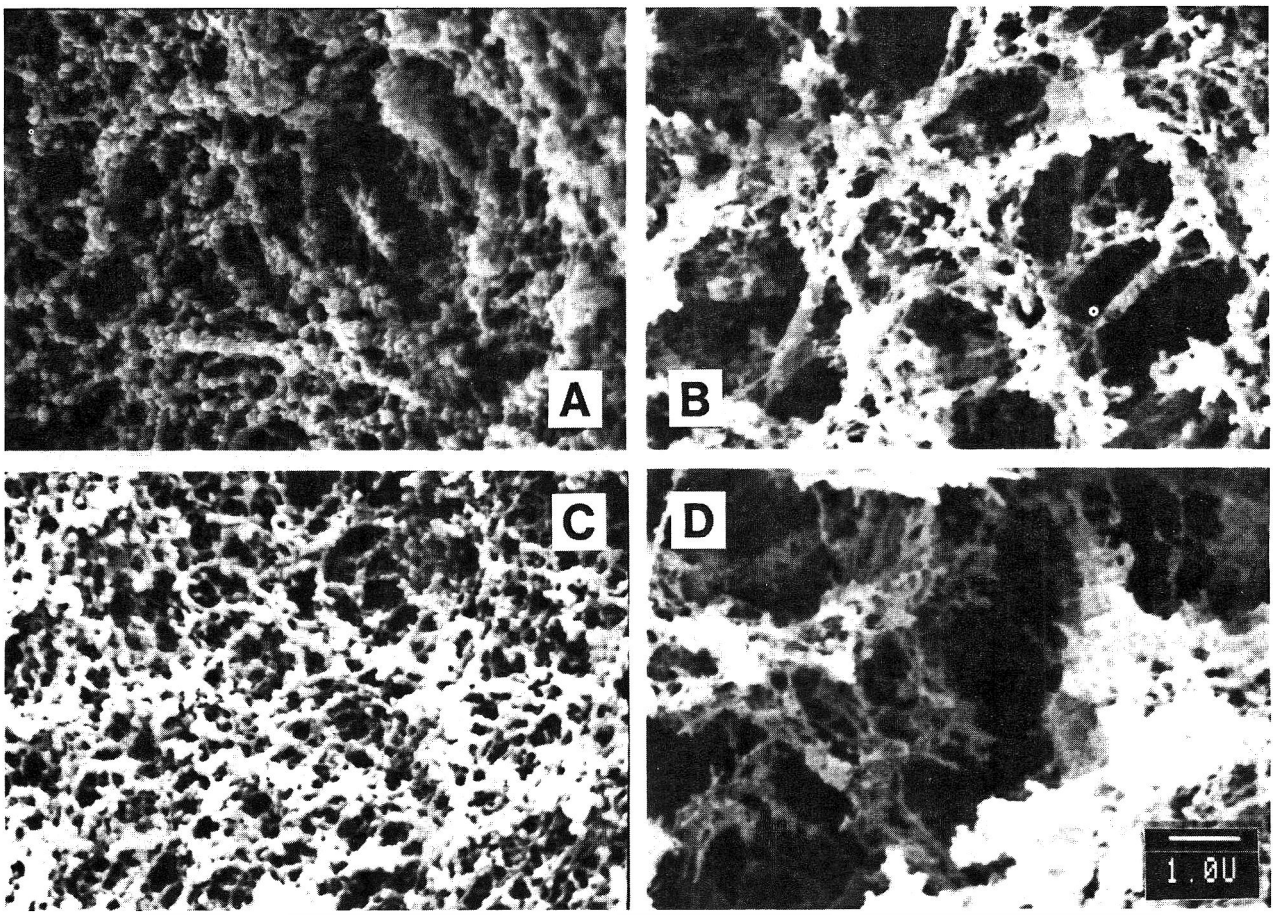

Fig. 4—Scanning electron micrographs of heat-induced 3.0% (w/v) hand deboned and mechanically deboned turkey gels at different times of frozen storage: (A) hand deboned turkey, unfrozen; (B) hand deboned turkey, 26 wk storage; (C) mechanically deboned turkey, unfrozen; (D) mechanically deboned turkey, 26 wk storage.

Table 4—Correlation coefficients between indices of protein denaturation, lipid oxidation and functional tests in frozen stored deboned turkey

	Correlation coefficients						
	Cook yield 0% salt	Cook yield 2% salt	Gel syneresis	Gel firmness	Gel cohesiveness	TBA number	ATPase activity
Protein solubility	0.69	0.60	−0.91	0.86	0.95	−0.60	0.93
ATPase activity	0.80	0.74	−0.93	0.87	0.86	−0.52	
TBA number	−0.41	−0.24	0.59	−0.45	−0.55		
Gel cohesiveness	0.68	0.61	−0.87	0.91			
Gel firmness	0.82	0.75	−0.85				
Gel syneresis	−0.66	−0.59					
Cook yield 2% salt	0.97						

turation caused by freezing and lipid oxidation should be of concern to scientists looking at basic biochemical and functional properties of turkey proteins as the length of storage will influence properties observed.

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Ms received 6/25/86; revised 9/2/86; accepted 10/7/86.

Michigan Agricultural Experiment Station Journal Article No. 12014.

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Ms received 4/18/86; revised 7/21/86; accepted 7/22/86.

This work was supported in part by the Massachusetts Agricultural Experiment Station, by the Graduate School of the Univ. of Massachusetts at Amherst, by the National Fisheries Institute, and by Grant No. I-258-81 from BARD—The United States-Israel Binational Agricultural Research and Development Fund.
We thank Ms. Marcia M. Stevens for editorial assistance.

Effects of Rapid Processing on the Chemical and Sensory Properties of Restructured Steak Made From Bull and Steer Meat

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ABSTRACT

South Devon cattle (10 bulls, 10 steers) were slaughtered to determine effects of sex condition and postmortem temperature conditioning on the chemical and sensory properties of restructured beef steak. Chucks from each left side were boned following a 4 hr delayed chill (DC, 13°C) period, while chucks from right sides were boned following a 48 hr conventional chill (CC. 2°C). Chucks were mechanically tenderized and formulated into restructured steaks. Bulls produced restructured steaks less prone to oxidative rancidity. Restructured steaks from bulls exhibited higher ($P<0.01$) cooking losses and Kramer Shear values. There were no differences between treatments in tenderness, juiciness, flavor, and connective tissue residue ratings; however, DC restructured steaks had lower ($P<0.05$) bind values than CC restructured steaks.

INTRODUCTION

AN INCREASING DEMAND for animal protein by a growing world population makes it imperative that beef production efficiency increase. Presently, the intact male may offer potential for improving production efficiencies. Research indicates (Seideman et al., 1982a) that bulls utilize feed more efficiently, grow faster and produce a leaner carcass with more retail product than steers. However, bull meat is generally less tender than meat from steers (Field, 1971; Gregory et al., 1983). Increasing the value of lower quality cuts, has economically pressured the meat industry to develop the technology of restructured meat products. Restructured processing offers other advantages such as: (1) a controlled portion size; (2) a boneless product; (3) control of fat content; (4) convenience; and (5) intermediate value (Ferren, 1972; Mandigo, 1974; Breidenstein, 1982).

Chunking raw meat materials to form a restructured product works well in the restructuring process (Huffman and Cordray, 1979). The primary advantage of this process is that the final restructured steak product has visual and palatability attributes more nearly resembling intact steaks than restructured steaks made with flake-cut particles (Huffman and Cordray, 1982). Cross and Allen (1982) state that bull meat has low consumer acceptance because cooked meat from young bulls is often less tender than steer beef. Restructuring, via the chunking process, offers an opportunity to increase the tenderness of bull meat, while still presenting the consumer an intermediate cost product, with a steak-like texture.

Hot processing is the removal of bone and trim prior to chilling the edible portion of the carcass. Henrickson (1975) stated that total energy savings could be 50% or more when hot processing is compared to the conventional processing procedures. Solomon and Schmidt (1980) reported that hot processing muscle may offer protein functionality advantages to the restructuring process. The principal disadvantage in the use of hot processed, prerigor muscle for restructured steaks may be a decrease in tenderness due to cold shortening or thaw rigor. However, conditioning sides at near physiological temperatures for a period of 3-6 h postmortem may alleviate tenderness problems associated with hot processed muscle (Henrickson et al., 1974; Falk et al., 1975).

The combination of restructuring and hot processing, in conjunction with carcass temperature conditioning, may provide an excellent process for improving the less palatable cuts from bull carcasses. The purpose of this study was to identify differences, due to sex condition, in the quality of restructured beef steaks and to incorporate hot processing and high temperature conditioning into the processing procedure.

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MATERIALS & METHODS

Steak preparation

South Devon steers ($n = 10$) and bulls ($n = 10$) were slaughtered at 16 mo of age and the chucks from the left sides were boned following a 4 h delayed chill period (13°C) while chucks from the right sides were boned following a 48 hr conventional chill (2°C) period. Boned and trimmed chucks were needle-tenderized (Ross TC 700, Ross Industries, Midland, VA; 2.54 cm advance setting) three times to assure connective tissue breakdown, then ground through a 2.54 cm plate (Hobart Mixer-Grinder; Hobart Manufacturing Co., Troy, OH). The fat source (chuck and plate) was fine flaked (Urschel 3600 Comitrol®, 120 head; Urschel Laboratories, Valparaiso, IN) at a temperature of −5°C prior to mixing. Fat and salt (0.5%) were added during the first 30 sec of blending and batches were mixed in a double-ribbon blender (Leland Food Mixer 100 DA; Leland Detroit Manufacturing Co., Detroit, MI) for 10 min in a 2°C cooler. Each chuck was used to prepare a 9.1 kg batch in which lean tissue was estimated to contain 6–9% fat, thus 10% fat (by weight) was added to the lean in order to approximate a 15% fat content in the final product. Three 1 kg samples were removed from each batch, hand-formed in Cryovac® bags and vacuum packaged. The logs were blast chilled in a −30°C freezer to −3°C, internal temperature, then tempered at −3°C for 12 hr. Frozen logs were pressed at 1.72 MPa (250 lb/in^2) using a cylindrical tube (9 cm diam) and a Carver laboratory press. Pressed logs were sliced (Hobart Commercial Slicer 1712; Hobart Manufacturing Co., Troy, OH) into 2.54 cm thick steaks, vacuum packaged and frozen at −25°C for later analyses.

Chemical analysis

Proximate composition for fat, moisture, protein and ash was determined on raw samples using AOAC (1975) standard procedures. Duplicate samples were used to determine moisture (oven drying, AOAC 1975), fat (soxhlet extraction, AOAC 1975) and protein (Kjeldahl, AOAC 1975). Separate deplicate samples were used to determine percent ash (AOAC 1975). 2-Thiobarbituric acid (TBA) values were determined in duplicate using the procedure of Tarladgis et al. (1960) after one week and 90 days of frozen storage (−25°C).

Cooking procedure

All steaks used for sensory evaluation, cooking loss determination and Lee-Kramer analysis were cooked at 177°C to an internal temperature of 70°C in a convection oven (Toastmaster). Internal temperature was measured with copper-constantan thermocouples inserted into the center of the steaks. Steaks were blotted dry and cooking losses were determined 1 hr after cooking. Total cooking loss was partitioned into evaporative and drip losses. Following cooking loss determination, steaks were used for Lee-Kramer Shear tests. Sensory evaluation samples were held at a constant 50°C following cooking

Table 1—Effect of sex and chill treatment on the chemical composition of chunked and formed beef steaks

	Bulls	Steers	SE	DC[a]	CC[b]	SE
Moisture, %	70.99**	68.95**	0.41	69.58**	70.36**	0.10
Fat, %	10.26**	13.07**	0.53	12.11**	11.23**	0.08
Protein, %	17.91**	17.17**	0.18	17.55	17.54	0.03
Ash, %	1.30	1.28	0.02	1.29	1.29	0.01

[a] DC-Delayed Chill.
[b] CC-Conventional Chill.
** Means in the same row differ significantly ($P<0.01$)

and prior to being served to panelists, using a double boiler system as described by Caporaso (1978).

Lee-Kramer

Steaks were trimmed to a 45 × 45 mm size and weighed. The Lee-Kramer Shear press (Model SP-11) was equipped with a 1365 kg manual dial proving ring with a 20 sec cell speed. Shear force was determined by dividing the peak force by the sample weight and multiplying by 10 (kg force/10g sample).

Color analysis

Restructured steaks were evaluated with a Bausch-Lomb Spectronic 20 equipped with the Color Analyzer Reflectance Attachment. Two steaks from each treatment replication were analyzed at two different surface locations. The reflectance attachment measured a rectangular 2 × 8 mm area on the meat surface. Major fat areas were avoided. A magnesium block was used for 100% reflectance. Objective color measurements were determined using % reflectance at 630 nm minus % reflectance at 580 nm (van den Oord and Wesdorp, 1971). Reflectance at 630 nm is high for oxymyoglobin and low for metmyoglobin; the reverse is true at 580 nm, therefore, a higher numerical (630 nm–580 nm) value indicates a brighter red color.

Sensory evaluation

Panelist selection was based upon interest, availability and consistency of evaluation during ten training sessions. A variety of intact and restructured muscle tissues, cooking times, and temperatures were used to provide examples over the entire scale of characteristics judged. Group discussions were held after each training session to refine sensory impressions of the panelists. Eight individuals were selected to participate on the panel. Panelists were served in individual booths under red lights. Three sessions were held each week with five samples being evaluated at each session. Each sample was evaluated using an 8-point scale, with 8 being extremely tender, juicy, intense flavor, extreme bind or no connective tissue residue and 1 being extremely tough, dry, bland, no bind or abundant in connective tissue residue. Sensory evaluations were conducted within a 90 day period from steak formulation.

Statistical analyses

Analysis of variance was determined using a 2 × 2 (sex condition × chill treatment) factorial arrangement of treatments in a split-plot design as described by Steel and Torrie (1980) with sex condition as the main effect and chill treatments as the subplots. Ten replications were used with replications being represented by individual animals.

RESULTS & DISCUSSION

THE CHEMICAL COMPOSITION of the restructured steaks is presented in Table 1. Restructured steaks from bull meat possessed a higher ($P<0.01$) percent moisture and a lower percent fat than restructured steaks from steer meat. Restructured steaks from conventionally chilled (CC) carcasses displayed a higher ($P<0.01$) percent moisture and a lower percent fat than restructured steaks produced from delayed chill (DC) carcasses (Table 1).

The differences in percent fat of restructured steaks can be partially explained by the fact that the bulls were leaner than steers (Paterson, 1984); however, formulation error is probably the major cause contributing to the proximate analysis differences in fat and moisture. Booren et al. (1981a) reported that higher amounts of inter- and intramuscular fat in the chuck as compared to the round made it more difficult to formulate steaks to a constant fat level when using chucks. Restructured steaks produced from bull meat possessed a significantly ($P<0.01$) higher protein content than steaks produced from steer meat. Lean tissue from bulls has been shown to contain a greater percent protein than that of steers (Jacobs et al., 1977). Percent ash was not affected by sex condition or chill treatment.

Differences in initial TBA values of steaks produced from bull meat or steer meat were not significant (Table 2). DC steaks had significantly ($P<0.01$) lower initial TBA values than CC steaks. It should be noted that DC chucks were held for a shorter time postmortem prior to processing which could have created the difference in TBA values. This would agree with previous (Booren et al., 1981a) who reported that lower TBA values in sectioned and formed beef steaks could be explained by shorter postmortem storage times prior to processing.

Restructured steaks from bull meat possessed significantly ($P<0.01$) lower 90 day TBA values for both chill treatments than restructured steaks from steer meat. This difference could be due to a lower fat content in the restructured steaks from bull meat (Table 1). DC restructured steaks exhibited lower ($P<0.01$) 90 day TBA values than CC steaks. The difference in the 90 day TBA values was similar to that present at one week indicating that fat oxidation proceeded at a similar rate for chill treatments following the initial difference detected at one week.

Restructured steaks produced from bull meat were significantly ($P<0.01$) less tender than steaks produced from steer meat as measured by the Kramer Shear Instrument (Table 2); however, this significant tenderness difference was not expressed by trained sensory panelists (Table 3). Paterson (1984) reported bull meat to be less tender than steer meat as measured by Warner-Bratzler shear values. This inherent tenderness difference may have caused the tenderness differences detected by the Kramer Shear analysis in this study. Perhaps Kramer Shear tests and sensory panelists measure different components of tenderness in restructured steaks.

DC restructured steaks were significantly ($P<0.05$) less tender than CC steaks as indicated by higher peak shear force values. However, sensory panelists did not detect significant tenderness differences between DC and CC restructured steaks (Table 3).

Water is a principal constituent of lean meat and is inversely related to the fat content in a product. Due to the evaporation rate of water and the melting point of fat, a high lean to fat ratio would allow greater losses of water in a product due to cooking (Levick, 1978). Thus it is not surprising that restructured steaks from bull meat exhibited greater ($P<0.01$) total cook losses (Table 2). However, sex condition had no effect when cook losses were partitioned into evaporative and drip loss fractions. Chill treatment did not affect total cook loss or evaporative loss, but DC steaks had higher ($P<0.05$) drip losses than CC steaks.

In another study using these same cattle, Paterson (1984) reported that bulls possessed darker 12th rib lean color scores than steers. This difference in lean color may have attributed to the significantly ($P<0.05$) lower (darker) objective color values received by the restructured steaks from bulls as compared to the restructured steaks from steers (Table 2).

DC steaks received significantly ($P<0.01$) higher (brighter)

Table 2—Effect of sex and chill treatment on TBA values, kramer shear, cook losses and objective color values

	Bulls	Steers	SE	DC[a]	CC[b]	SE
TBA value[c]						
0 Time	0.56	0.63	0.04	0.53**	0.66**	0.01
90 days	1.38**	1.61**	0.06	1.42**	1.57**	0.02
Kramer peak force[d]	6.35**	5.64**	0.12	6.12*	5.88*	0.08
Total cook loss, %	32.87**	30.05**	0.59	31.53	31.39	0.67
Evaporative loss, %	20.25	19.48	0.71	18.99	20.74	0.90
Drip loss, %	12.62	10.57	0.62	12.54*	10.65*	0.62
%R630-%R580[e]	0.14*	0.17*	0.009	0.19**	0.12**	0.007

[a] DC-Delayed Chill.
[b] CC-Conventional Chill.
[c] mg malonaldehyde/kg meat.
[d] kg force/10g sample.
[e] Percent reflectance at 630 nm minus percent reflectance at 580 nm.
* Means in the same row differ significantly (P<0.05);
** Means in the same row differ significantly (P<0.01).

Table 3—Effect of sex and chill treatment on sensory evaluation of chunked and formed steaks[a]

	Bulls	Steers	SE	DC[b]	CC[c]	SE
Tenderness	6.2	6.3	0.09	6.2	6.3	0.07
Flavor	6.3	6.1	0.09	6.1	6.3	0.07
Juiciness	6.2	6.3	0.16	6.2	6.3	0.07
Bind	4.2	4.1	0.12	4.0	4.3*	0.09
Connective tissue residue	6.0	6.2	0.16	6.0	6.2	0.08

[a] One to eight scale with eight being extremely tender, juicy, intense flavor, extreme bind, or no connective tissue residue and one being extremely tough, dry, bland, no bind or abundant connective tissue residue.
[b] DC-Delayed Chill.
[c] CC-Conventional Chill.
* Means in the same row differ significantly (P<0.05)

objective color values than CC steaks. Chucks were boned 4 hr post mortem and stored aerobically prior to processing into DC restructured steaks. This processing step may have created the more desirable color exhibited by the DC steaks. Previous research (Judge and Aberle, 1980) has shown that metmyoglobin formation in prerigor muscle can be reduced by aerobic storage.

Trained sensory panel analysis results (Table 3) indicated that sex condition had no affect on any of the sensory characteristics tested. CC steaks received higher (P<0.05) bind values indicating a more cohesive product than DC steaks. Chill treatment had no other effect on the sensory qualities of the restructured steaks. TBA values (Table 2) indicated that lipid oxidation had occurred to a significant extent by 90 days but not sufficient for detection by the sensory panel. These sensory panel results differ from those of Seideman et al. (1982b) who reported that hot-boned beef resulted in less tender restructured steaks that possessed less desirable flavor ratings than restructured steaks made from beef aged 48 h. However, steaks produced by Seideman et al. (1982b) were sliced and formed restructured steaks and a conditioning period was not included in the processing scheme.

Field (1982) stated that connective tissue is the largest single problem in restructured chunked and formed steaks. Sensory panel scores for connective tissue residue were acceptable (6.0 or higher) for the restructured steaks in this study. It appears that the processing scheme used in the steak formulation eliminated the problems associated with connective tissue.

CONCLUSION

CHUCKS from young bulls can be used as a raw material for producing satisfactory restructured steaks. In addition, hot boning used in conjunction with a carcass conditioning period can be successfully incorporated into the processing scheme of restructured beef steaks.

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Ms received 11/30/84; revised 8/22/86; accepted 8/27/86.

Published as paper number 2025, Journal Series, South Dakota State Univ., Agricultural Experiment Station. Appreciation is expressed to the South Dakota Beef Industry Council for support of this research.

Muscle Protein Structure-Function Relationships and Discrimination of Functionality by Multivariate Analysis

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ABSTRACT

Functional (gel strength, cook loss, emulsifying, fat and water binding capacities) and physicochemical properties of mince and salt-extractable proteins were analyzed. Factor analysis identified three principal components which accounted for over 75% of data variance and distinguished between mechanically and hand-deboned meat and fish samples. Stepwise discriminant analyses classified samples with good, intermediate and poor functionality. Regression analyses yielded equations to predict functionality from physicochemical properties. Generally, samples with superior thermally induced functionality possessed (1) higher protein, lower fat and pH in the mince; (2) higher solubility and sulfhydryl and lower hydrophobicity in unheated salt-extractable proteins; and (3) greater decrease in solubility and increase in hydrophobicity after heating.

INTRODUCTION

THE FUNCTIONAL PROPERTIES of the protein matrix in comminuted meat products deemed important for final product quality have been described in terms of emulsification, binding, and gelation performance (Schmidt et al., 1981). It is generally acknowledged that the binding phenomenon depends on the extraction of salt-soluble myofibrillar proteins from meat particles and subsequent formation of a protein-salt-water matrix which entraps or "emulsifies" fat globules. This system is then stabilized upon gelation of the protein matrix by thermal processing.

In attempts to maintain product quality despite variations in meat ingredient quality, the concept of least-cost, constant quality computer formulation of products was introduced. Various bind constants based on emulsifying capacity, emulsifying stability, soluble protein and/or total protein of the meat ingredients have been suggested (Porteous, 1979; Parks et al., 1985). However, the poor predictability of solubility and emulsifying capacity as indicators of functional performance, as well as the question of whether or not a comminuted meat system can truly be viewed as an emulsion (Lee, 1984), have cast increasing doubt on the reliability of the bind constant system. Comer and Dempster (1981) suggested an alternative approach based on protein quality factors or a functional property test to reflect gelation properties of ingredients. However, in their work, the basis for the protein quality factors was arbitrary, being subjectively chosen to reflect their own experience in the field.

Our recent research has focused on measurement of physicochemical properties of salt-extracted meat proteins which may be related to their functional properties. These studies demonstrated the importance of considering protein hydrophobicity and sulfhydryl group, in conjunction with solubility or dispersibility parameters, to explain emulsifying and fat binding capacities of salt-extractable proteins from beef and rockfish muscle (Li-Chan et al., 1984; 1985). Highly significant multiple regression models were obtained to describe emulsifying and fat binding capacities in terms of hydrophobicity, dispersibility, sulfhydryl and/or their quadratic terms. An optimal balance of these three physicochemical properties rather than high values of the individual parameters was essential for good functionality.

In the present study, further insight into the structure-function relationship was sought by multivariate analyses of data from a variety of hand-deboned and mechanically-deboned samples, including beef, chicken, pork and fish. Functional properties under evaluation included properties of the meat mince (water- and brine-holding capacity, gel strength, cook loss) and of the salt-extracted proteins (gel strength, emulsifying and fat binding capacities). Physicochemical properties included variables related to the mince (protein, salt-extractable protein, fat, moisture and mince-pH) and variables describing salt-extractable protein properties (solubility, dispersibility, hydrophobicity and sulfhydryl group). Principal component analysis, stepwise discriminant analysis and stepwise multiple regression analysis supported the feasibility to use the measured physicochemical properties to predict functional properties and classify samples with good, intermediate and poor functionality.

MATERIALS & METHODS

Materials

Hand-deboned pork (lean ham trim, P), mechanically deboned pork (M), hand-deboned chicken (broiler breast muscle, C), and ling cod fish (F) were obtained fresh from local meat, poultry and fish processors. Hand-deboned chicken (breast muscle) was also obtained from freshly slaughtered, spent laying hens from the University of British Columbia poultry farm. Beef top round (B) was purchased from a local supermarket. Mechanically deboned chicken (backs and necks, m) in unfrozen and frozen blocks were gifts from Western Protein Foods Ltd., Langley, BC. Samples were analyzed in fresh form as well as after varying periods of storage at above freezing (0.5° to 4°C) and/or below freezing (−10° or −20°C) temperatures.

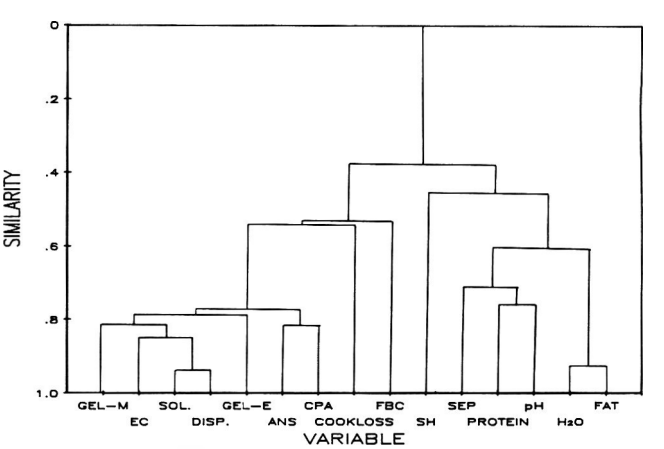

Fig. 1—Dendrogram from cluster analysis of variables (excluding WBC variables).

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Table 1a—Univariate statistics for each functional and physicochemical property (variable) over the entire data set of meat samples (sample size = 230)

Variable	Mean	Std. dev.	Range of values Minimum	Maximum
Gel-M	4.60	5.67	0.00	19.10
Cookloss	3.55	7.27	−1.20	35.40
Gel-E	0.0123	0.0174	0.000	0.0620
EC	40.4	13.2	12.0	75.0
FBC	5.11	3.41	0.90	19.0
Solubility	45	35	5	100
Dispersibility	56	42	5	100
ANS	274	173	66	794
CPA	906	387	299	2056
SH	76.2	12.5	39.9	112.0
S.E.P.	0.96	0.41	0.13	1.64
Moisture	72.7	7.3	59.0	85.7
Fat	6.78	9.50	0.34	25.1
Protein	14.8	4.3	7.0	20.2
Mince-pH	6.18	0.43	5.50	6.95

Table 1b—Univariate statistics for each functional and physicochemical property for unheated and 40°C heated meat samples[a]

Variable	Mean	Std dev.	Range of values Minimum	Maximum
AM_w	0.348	0.331	0.0094	1.221
pH_{aw}	6.29	0.44	5.55	7.04
AM_b	0.279	0.125	−0.0076	0.537
pH_{ab}	6.27	0.42	5.62	6.94
EC	51.1	6.1	39.0	75.0
FBC	6.87	3.65	0.90	19.0
Solubility	77	18	13	100
Dispersibility	95	11	22	100
ANS	146	68	66	387
CPA	622	207	299	1106
SH	80.2	11.3	50.8	112.0

Table 1c—Univariate statistics for each functional and physicochemical property for 40°/80°C and 80°C heated meat samples[b]

Variable	Mean	Std dev.	Range of values minimum	maximum
RM_w	0.382	0.164	0.0658	0.804
pH_{rw}	6.39	0.38	5.69	7.02
RM_b	0.405	0.194	−0.026	0.811
pH_{rb}	6.40	0.35	5.83	6.97
Gel-M	9.27	4.62	2.73	19.10
Cookloss	7.16	9.00	−1.20	35.40
Gel-E	0.0248	0.0172	0.000	0.0620
EC	29.4	8.6	12.0	53.0
FBC	3.32	1.89	1.20	13.8
Solubility	13	11	5	70
Dispersibility	17	20	5	99
ANS	405	148	158	794
CPA	1195	306	421	2056
SH	72.1	12.4	39.9	105.0

[a] Sample size of 58 for AM_w, pH_{aw}, AM_b, and pH_{ab}, and 116 for other variables.
[b] Sample size of 57 for Rm_w, pH_{rw}, RM_b and pH_{rb}, and 114 for other variables.

Table 2—Sorted rotated factor loadings (pattern) from factor analysis of data excluding WBC[a]

	Factor 1	Factor 2	Factor 3
Dispersibility	−0.959	0.0	0.0
Solubility	−0.939	0.0	0.0
ANS	0.864	0.0	0.255
Gel-M	0.862	0.0	0.0
Gel-E	0.853	0.288	0.0
EC	−0.848	0.0	0.0
CPA	0.844	0.0	0.0
FBC	−0.604	0.293	0.0
Cookloss	0.529	0.0	−0.457
Protein	0.0	0.916	0.0
Mince-pH	0.0	−0.852	0.285
S.E.P.	0.0	0.821	0.0
SH	0.0	0.617	0.0
Moisture	0.0	0.0	0.966
Fat	0.0	−0.382	−0.891

[a] The above factor loading matrix has been rearranged so that the columns appear in decreasing order of variance explained by factors. The rows have been rearranged so that for each successive factor, loadings greater than 0.5000 appear first. Loadings less than 0.2500 have been replaced by zero.

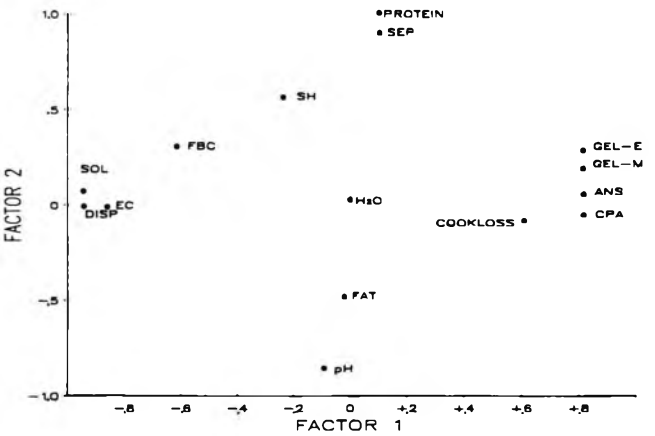


Fig. 2—Factor 2 versus factor 1 plot of rotated factor loadings from principal component analysis of all variables excluding WBC variables.

Table 3—Sorted rotated factor loadings (pattern) from factor analysis of data subset including unheated and 40°C cases[a]

	Factor 1	Factor 2	Factor 3
Protein	0.921	0.0	0.0
ANS	−0.845	0.0	0.0
S.E.P.	0.768	−0.364	0.0
Mince-pH	−0.768	0.450	0.0
Fat	0.0	0.941	0.0
Moisture	0.0	−0.937	0.0
CPA	−0.581	0.626	0.0
SH	0.464	−0.548	0.298
Solubility	0.0	0.0	0.804
Dispersibility	0.0	0.0	0.780
EC	−0.315	0.0	0.660
FBC	0.452	−0.352	0.0

[a] The above factor loading matrix has been rearranged so that the columns appear in decreasing order of variance explained by factors. The rows have been rearranged so that for each successive factor, loadings greater than 0.5000 appear first. Loadings less than 0.2500 have been replaced by zero.

Table 4—Sorted rotated factor loadings (pattern) from factor analysis of data subset including 40°/80°C and 80°C cases[a]

	Factor 1	Factor 2	Factor 3	Factor 4
Fat	−0.863	0.0	−0.267	0.0
Gel-M	0.809	−0.282	0.0	0.0
Moisture	0.785	0.371	0.0	0.406
S.E.P.	0.701	−0.265	0.0	−0.465
Cookloss	−0.696	0.0	0.303	0.0
Mince-pH	−0.687	0.486	−0.369	0.0
Dispersibility	0.0	0.898	−0.298	0.0
Solubility	0.0	0.853	−0.255	0.0
EC	0.0	0.734	0.0	0.0
Protein	0.486	−0.626	0.295	−0.365
SH	0.0	0.0	0.837	0.0
ANS	0.364	0.0	0.841	0.0
CPA	0.0	−0.266	0.792	0.0
Gel-E	0.0	−0.449	0.589	−0.371
FBC	0.0	0.0	0.0	0.926

[a] The above factor loading matrix has been rearranged so that the columns appear in decreasing order of variance explained by factors. The rows have been rearranged so that for each successive factor, loadings greater than 0.5000 appear first. Loadings less than 0.2500 have been replaced by zero.

Preparation of mince and extracts

Preparation of mince and salt extracts from all hand-deboned samples except ling cod were as described previously (Li-Chan et al., 1984), except that the extracting buffer used was 0.01M sodium phosphate (pH 6.5) containing 0.6M NaCl, 1 mM $MgCl_2$, and 0.04% sodium azide. Mechanically deboned samples were obtained in a minced form with a pasty consistency and used directly without further grinding. Ling cod samples were minced by grinding once; the mince was then washed with ice-cold water to remove water-soluble components, essentially as described by Adu et al. (1983). Excess water was removed from the mince by using a press. Half of this mince was frozen while to the other half, a mixture of cryoprotectants (10% sucrose, 0.3% polyphosphate consisting of a 6:4 mixture of sodium pyrophos-

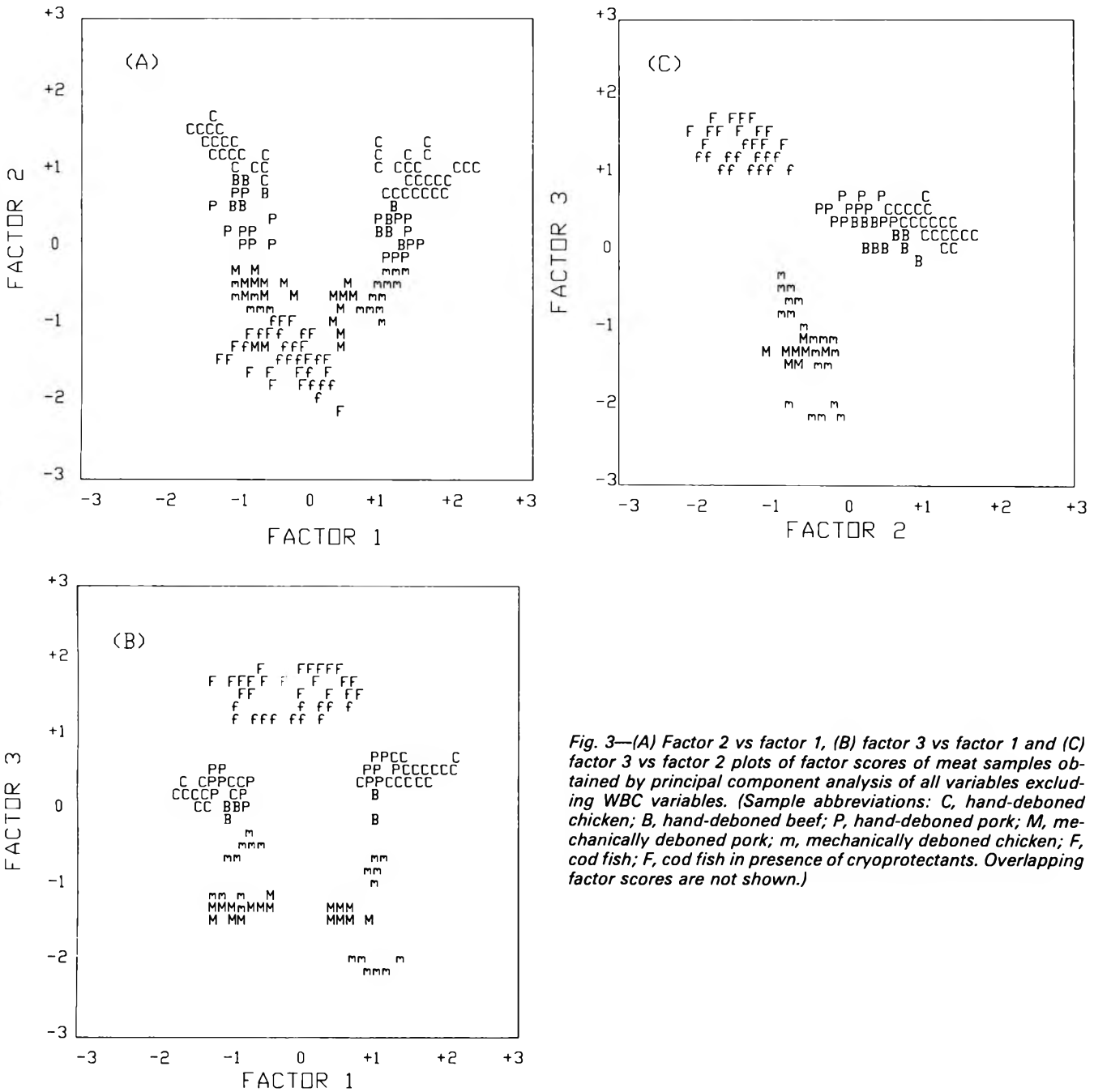


Fig. 3—(A) Factor 2 vs factor 1, (B) factor 3 vs factor 1 and (C) factor 3 vs factor 2 plots of factor scores of meat samples obtained by principal component analysis of all variables excluding WBC variables. (Sample abbreviations: C, hand-deboned chicken; B, hand-deboned beef; P, hand-deboned pork; M, mechanically deboned pork; m, mechanically deboned chicken; F, cod fish; F, cod fish in presence of cryoprotectants. Overlapping factor scores are not shown.)

phate: sodium tripolyphosphate) was added prior to freezing at −10°C. Fish samples were analyzed after various times of frozen storage at this abusive temperature to induce changes in the protein properties.

Heat treatment

Preliminary experiments were performed to determine suitable heating conditions for evaluation of gelation and cook loss properties of samples. These studies indicated that incubation in a 80°C water bath for specified time intervals induced gel formation of both extracts and salted mince samples. Although this temperature is slightly higher than commercial sausage processing temperatures, a survey of the literature supported choice of 80°C or higher temperature (Montejano et al., 1983, 1984; Lanier, 1986). A combined heat treatment (preheating at 40°C followed by heating at 80°C) was also used, based on a recommended process for setting ("suwari") prior to cooking for fish gel formation (Akahane et al., 1981). This two temperature heat treatment was also applied to other species such as chicken, beef and pork to investigate the setting phenomenon observed in certain types of fish.

For heat treatment of the minced meat samples, 50g minced meat was blended with 2.5% salt by weight (30–45 sec, "stir" setting on Osterizer blender), then stuffed into Saran casings (vinylidene chloride/vinyl chloride copolymer, 11 mm diameter, obtained from Kureha Chemical Manufacture Co., Japan) and sealed at both ends to yield 10 cm length. Three heat treatments were used, 40°C 15 min alone, 40°C 15 min–80°C 15 min combination, and 80°C 15 min alone, followed by cooling in an ice-water bath.

For heat treatment of salt-extractable proteins, sample extracts were adjusted to 0.8% protein concentration and pH 6.0. Twenty milliliter aliquots in capped glass vials (2.38 cm i.d.) were de-aerated, heated at 40°C, 30 min alone; 40°C, 30 min then 80°C, 30 min combination; or 80°C, 30 min alone, then cooled.

Physicochemical properties

Mince properties. Moisture of the mince was determined by drying in vacuo at 95–100°C, while crude fat of the dried mince was determined by petroleum ether extraction in a Goldfish apparatus (AOAC, 1980). Protein was calculated from nitrogen determined on a Technicon AutoAnalyzer II system, after micro-Kjeldahl digestion of samples by the method of Concon and Soltess (1973).

Table 5—Sorted rotated factor loadings (pattern) from factor anlaysis of data including AM_w and AM_b[a]

	Factor 1	Factor 2	Factor 3	Factor 4
Fat	0.951	0.0	0.0	0.0
Moisture	−0.832	0.453	0.0	0.0
CPA	0.827	0.0	0.0	−0.277
pHab	0.826	0.460	0.0	0.0
pHaw	0.803	0.469	0.0	0.0
mince-pH	0.747	0.566	0.0	0.0
SH	−0.739	0.0	0.0	−0.265
S.E.P.	−0.605	−0.529	0.0	0.0
AM_w	0.0	0.926	0.0	0.306
ANS	0.0	0.898	0.0	0.0
protein	−0.447	−0.818	0.0	0.0
solubility	0.0	0.0	0.766	0.0
dispersibility	0.0	0.0	0.758	0.0
EC	0.0	0.0	0.574	0.0
AM_b	0.0	0.0	0.0	0.955
FBC	−0.414	−0.351	0.0	0.380

[a] The above factor loading matrix has been rearranged so that the columns appear in decreasing order of variance explained by factors. The rows have been rearranged so that for each successive factor, loadings greater than 0.5000 appear first. Loadings less than 0.2500 have been replaced by zero.

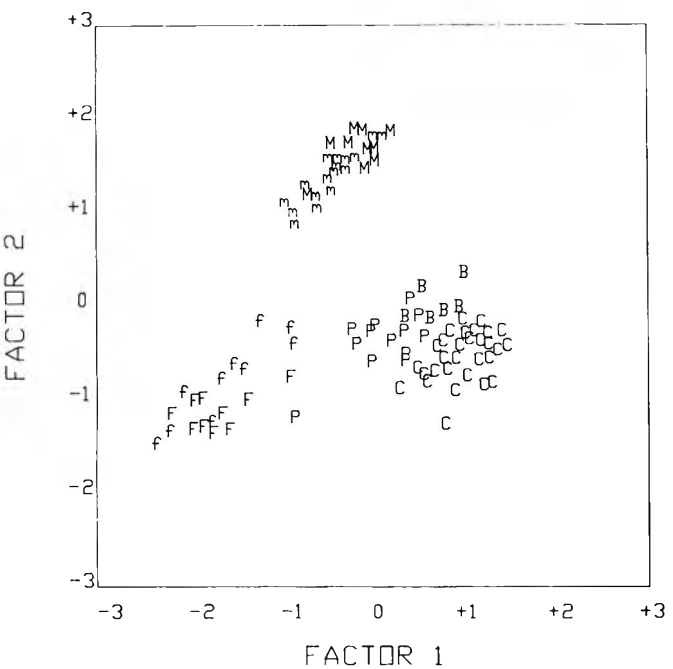


Fig. 4—Factor 2 vs factor 1 plot of factor scores of unheated and 40°C heated meat samples obtained by principal component analysis of all variables excluding WBC variables. (Sample identification as in legend to Fig. 3.)

pH of the mince was determined on a suspension containing 1 g of the mince in 10 ml distilled de-ionized water.

Salt-extractable protein (SEP) in the mince was determined as the % protein in extracts obtained from the mince in a ten-fold volume of phosphate buffer containing 0.6M NaCl. The % protein in the extracts was determined either from nitrogen or by the Biuret-phenol spectrophotometric method using Folin-Ciocalteau reagent (Layne, 1957).

Extract properties. Aliquots of extracts and heated extracts were centrifuged at 27,000 × *g* for 30 min for solubility determination or at 1100 × *g* for 10 min for dispersibility determination. The supernatants and their uncentrifuged controls were analyzed for protein by the Biuret-phenol spectrophotometric method. The % solubility and % dispersibility were calculated as 100 × (protein in supernatant)/(protein in uncentrifuged control).

Protein surface hydrophobicity (S_0) was determined using an aromatic fluorescent probe (1-anilinonaphthalene-8-sulfonic acid or ANS) and an aliphatic fluorescent probe (cis-parinaric acid or CPA), as previously described (Li-Chan et al., 1985).

Sulfhydryl (SH) was determined spectrophotometrically using Ellman's reagent, as previously described (Li-Chan, 1983).

Functional properties

Mince properties. Heated minced meat gels were sliced into sections, each about 3.3 cm in length. Gel strength of the mince (Gel-M) was expressed as peak force in Newtons, calculated from force-deformation curves obtained from action of the shear compression cell with one blade, using the Instron Model 1122 Universal Testing Instrument (500 kg capacity reversible load cell, chart full scale calibrated to 5 or 10 kg, crosshead speed at 100 mm/min).

The % cookloss of the heated mince was determined by weighing the salted mince before cooking, and after cooking and draining of free liquid. The % cookloss was defined as:

$$[(\text{Weight (before - after cooking)}/(\text{Weight before cooking})] \times 100$$

Absorbed moisture and retained moisture after cooking were determined for meat samples in water (AM_w, RM_w) or 1% NaCl brine (AM_b, RM_b), and expressed as g water/g meat sample. These water binding capacity (WBC) parameters were measured essentially by the method of Porteous and Wood (1983), with the following modifications — retained moisture samples were heated at 80°C for 10 min, and centrifugation was carried out at 3000 × *g* for 10 min. In addition, the pH of the supernatants after centrifugation was also determined and designated as pH_{aw}, pH_{rw}, pH_{ab}, and pH_{rb}.

Extract properties. Gel strength of heated extracts in vials (Gel-E) was expressed as peak force in Newtons, obtained from force-deformation curves measured on the Instron Universal Testing Instrument with a 7.9 mm diameter probe (200g capacity compression load cell, chart full scale calibrated to 10 or 20g, crosshead speed at 100 mm/min, recording taken to sample depth of 30 mm).

Emulsifying capacity (EC) was determined on 0.03% protein extracts in buffer using the method of oil titration to the point of phase inversion, as described previously (Li-Chan et al., 1984).

A turbidimetric method (Li-Chan et al., 1985) was used to determine fat binding capacity (FBC) of 0.5% protein extracts.

Statistical analysis

Statistical analyses were performed using the BMDP programs (BMDP Statistical Software, 1985) for factor analysis (P4M), stepwise discriminant analysis (P7M), stepwise regression (P2R) and cluster analysis of variables (P1M). The analyses were carried out using an Amdahl 470 V/8 computer.

Unless otherwise specified, analyses were generally carried out on all of the variables from the data set (n = 230), which included samples after the three heat treatments. Since water-binding capacity variables are by their empirical definition only applicable to the data subsets of unheated (AM_w or AM_b) or 80°C heated (RM_w and RM_b) samples, analyses were performed separately on the data subsets (n = 57 or 58) to include the water-binding capacity variables.

Gel-M, Gel-E, cookloss, RM_w and RM_b properties were measured as functional properties induced after 40/80°C or 80°C thermal treatment. For stepwise discriminant and stepwise regression analyses in which these thermally induced properties were used as the grouping or dependent variables, the properties which were entered as potential independent or predictor variables included not only the physicochemical properties of the heated samples but also those of the samples prior to heating (designated as "property-u" or "unheated") as well as the ratio of heated/unheated property (designated as "property ratio"). Based on results of our previous work (Li-Chan et al., 1984; 1985), quadratic terms including the squared terms of the physicochemical properties and some of their two-factor interactions were also considered as potential independent variables.

RESULTS

Univariate statistics of variables

Variability in both functional and physicochemical properties of the mince and extracts of samples was observed. Table 1a lists some univariate statistics for each property ("variable") over the entire data set, including samples before and after heat treatment. The univariate statistics include mean, standard deviation and range of values for each variable in the data set.

To separate the effects of variability inherent in the unheated samples from the effects of high temperature heating-induced changes, corresponding statistic data were calculated for the variables specific to the data subset containing only unheated

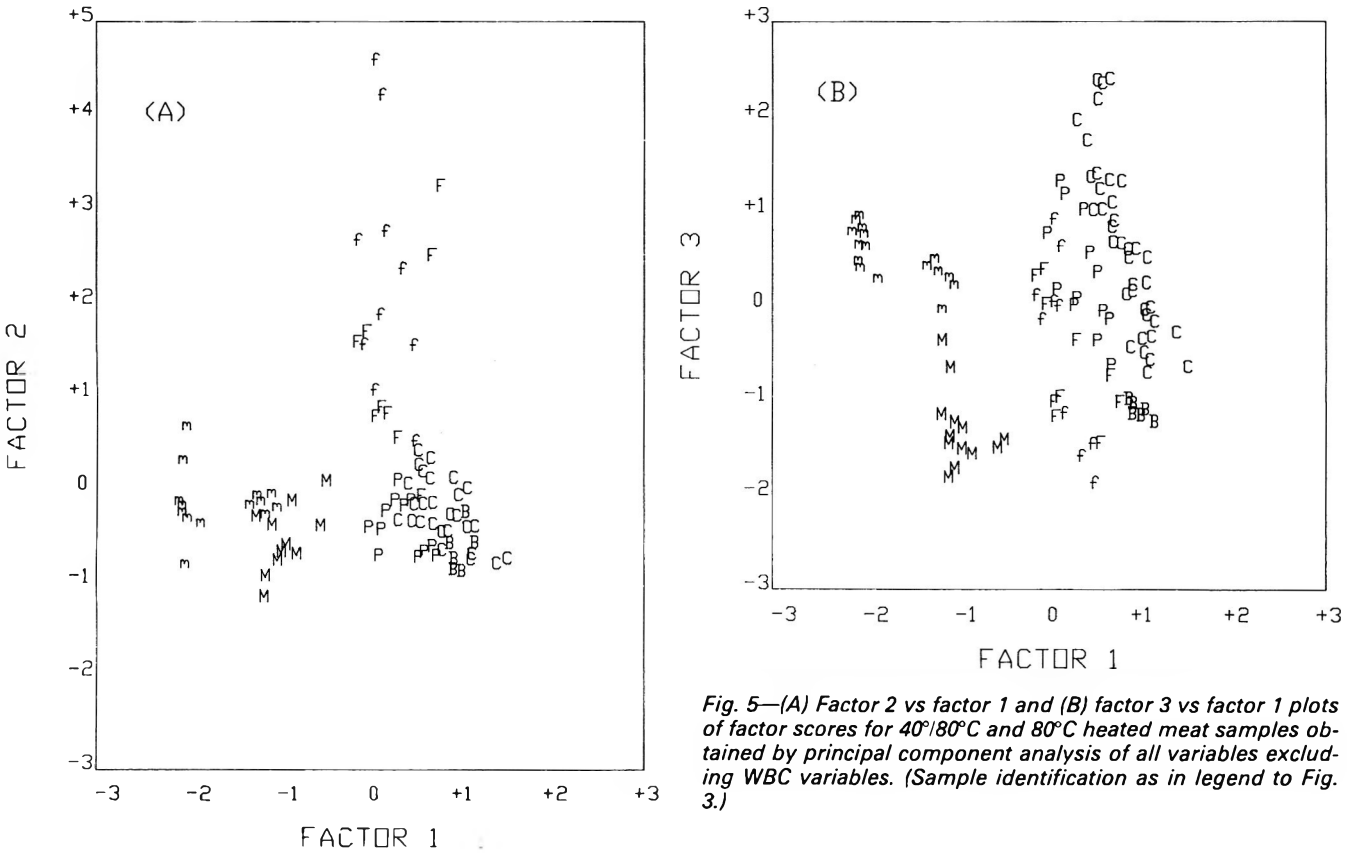


Fig. 5—(A) Factor 2 vs factor 1 and (B) factor 3 vs factor 1 plots of factor scores for 40°/80°C and 80°C heated meat samples obtained by principal component analysis of all variables excluding WBC variables. (Sample identification as in legend to Fig. 3.)

and/or 40°C samples (Table 1b), and the data subset containing only 40°/80°C and/or 80°C samples (Table 1c). These statistics confirmed that even within these two subsets, the samples exhibited considerable variation and range in their properties. For example, Table 1b shows that the functional property AM_w for 58 unheated samples ranged from 0.0094 to 1.221g H_2O/g sample; the mean and standard deviation of 0.348 and 0.331g H_2O g/g sample, respectively, represent a coefficient of variation of 0.95. Table 1c shows that the physicochemical property of protein dispersibility of 114 sample extracts heated at 40°/80°C or 80°C ranged from 5 to 99%, with mean and standard deviation values of 17 and 20%, respectively, representing a coefficient of variation of 1.15.

Cluster analysis of variables

To obtain information on the similarity between the various physical/chemical and functional properties, the data (excluding water binding variables) were analyzed by cluster analysis of variables, using the amalgamation procedure based on single linkage. The measures of similarity were derived by re-coding the absolute values of the correlation matrix of the variables.

The variables fall broadly into two clusters with only slight similarity (38%) (Fig. 1). In the first broad cluster, solubility and dispersibility formed a tight cluster of high similarity (94%), which were further clustered with emulsifying capacity (86% similarity). This cluster of three variables was in turn clustered to variables describing gel strength (Gel-M, Gel-E), which were next combined with the cluster of protein hydrophobicity variables (ANS, CPA). These seven variables formed a cluster of high similarity (77%) which reflected the hydrophobicity/hydrophilicity of the meat proteins and their ability to form a gel. This cluster was linked with cookloss and fat binding capacity variables with moderate similarity (50%).

The second broad cluster encompassed variables generally related to the compositional properties of the mince. It was composed of clusters of protein and pH of the mince with salt-extractable protein content (SEP), a cluster of moisture and fat content, and the variable measuring SH.

Factor analysis

Factor analysis was used for exploratory data analysis to study the inter-relationships between the variables. In this method, variation in the data set was summarized by a minimum number of factors or principal components. Variables with high loadings on the same factor tend to be highly correlated with each other, while those variables with dissimilar loading patterns tended to be less highly correlated.

Factor analysis of all variables excluding water-binding capacity. Factor analysis of the data set (n = 230) using all variables except water-binding capacity variables (as described in "Statistical Analysis" section) extracted three factors which accounted for 76.53% of data variance. The sorted rotated factor loadings pattern (Table 2) shows that factor 1 is a broad factor encompassing the hydrophobic/hydrophilic properties of the salt-extractable proteins and gel strength; factor 2 is a factor describing total and salt-extractable proteins and mince-pH, and factor 3 is a measure of moisture and fat which are highly negatively correlated (−0.913) to each other.

The plot of the rotated factor loadings for factor 2 versus factor 1 is shown in Fig. 2, and indicates clustering of several groups of variables — (1) solubility, dispersibility and EC; (2) Gel-E, Gel-M, ANS and CPA; and (3) total and salt-extractable protein. These groupings were also noted from the results of cluster analysis of the variables.

The plots of factor scores for individual samples are shown in Fig. 3. Identification of individual samples indicated that unheated or 40°C heated samples were located on the negative scale of the factor 1 axis while 80°C and 40°/80°C heated samples were on the positive scale of the factor 1 axis (Fig. 3A and B). Hand-deboned samples (C, P and B or chicken, pork and beef, respectively) had positive factor 2 scores, with the hand-deboned chicken samples (C) having the largest pos-

Table 6—Classification functions for low, medium and high groups of functional properties and coefficients for first two canonical variables

Grouping variable	Variable in classification function	Coefficient for classification functions			Coefficient for	
		Group 1 (low)	Group 2 (medium)	Group 3 (high)	Canonical variable 1	Canonical variable 2
Gel-M	solubility	−1.881	−2.558	−1.373	−0.0898	−0.2961
	SEP	111.6	159.4	151.8	−7.9059	4.6444
	ANS-u	−0.064	0.013	−0.026	−0.0096	0.0137
	CPA-u	0.117	0.094	0.092	0.0055	−0.0012
	solubility²	0.028	0.032	0.021	0.0013	0.0024
	SEP²	−57.94	−77.28	−69.03	2.6867	−3.1357
	moisture²	0.052	0.050	0.048	0.0009	0.0003
	fat²	0.181	0.162	0.152	0.0063	0.0008
	dispersibility-ratio	−25.34	−3.17	−36.82	1.8823	8.6234
	constant	−204.9	−203.2	−189.5		
Cookloss	solubility	−0.045	0.505	0.531	−0.0578	−0.0420
	dispersibility	0.704	−0.732	−1.251	0.185	0.0344
	SEP	951.8	856.4	777.9	15.59	−5.09
	moisture	21.04	21.48	19.13	0.131	−0.428
	mince-pH	248.1	225.4	224.2	2.400	1.725
	ANS-u	0.604	0.454	0.391	0.0200	0.0021
	CPA-u	−0.210	−0.177	−0.152	−0.0052	0.0014
	dispersibility²	−0.027	−0.019	−0.013	−0.0013	0.0003
	SEP²	−405.8	−364.5	−332.1	−6.63	1.94
	fat²	0.329	0.353	0.292	0.0022	−0.0122
	constant	−1879.2	−1689.2	−1478.5		
Gel-E	dispersibility	1.918	1.071	0.931	0.2264	−0.0384
	SH	0.441	0.607	0.653	−0.0473	−0.0134
	ANS-u	0.041	−0.0034	−0.0053	0.0109	−0.0080
	dispersibility²	−0.017	−0.010	−0.0086	−0.0020	0.0002
	ANS-ratio	0.775	0.0934	0.370	0.1197	−0.4647
	constant	−38.22	−27.76	−31.14		
RM_w	dispersibility-μ	6.428	6.140	5.953	0.1277	0.0520
	dispersibility²	0.015	0.008	0.009	0.0021	0.0028
	SEP²	−7.227	−2.949	−1.979	−1.6065	−13.2523
	dispersibility-ratio	−147.5	−82.32	−71.73	−23.7933	−214.5
	constant	−308.2	−291.1	−281.6		
RM_b	CPA	0.028	0.021	0.020	0.0005	−0.0124
	SEP	−28.49	−13.52	−9.082	−1.0685	8.2003
	solubility-u	0.479	0.167	0.034	−0.0256	−0.6550
	dispersibility-u	7.726	7.203	6.678	0.0486	2.5350
	dispersibility²	0.008	0.006	0.005	0.0002	−0.0018
	constant	−374.9	−351.9	−323.1		
AM_w	ANS	0.620	0.639	0.813	−0.0171	−0.0002
	SEP	−8.585	3.693	−20.32	1.1501	−2.2799
	moisture	20.36	22.19	23.55	−0.2698	−0.2627
	protein	−2.564	−4.290	−6.190	0.3100	0.2383
	mince-pH	321.2	317.8	376.3	−4.9557	1.4414
	CPA²	−0.00019	−0.00021	−0.00025	0.00001	0.00000
	fat²	0.248	0.368	0.262	−0.00032	−.02030
	constant	−1683.1	−1801.1	−2231.5		
EC	solubility	0.129	0.152	0.225	− 0.0257	−0.0008
	ANS	0.019	0.036	0.040	−0.0024	−0.0112
	dispersibility²	0.00007	0.00038	0.00099	−0.0002	−0.0001
	CPA²	0.00000	0.00000	−0.00000	0.0000	0.0000
	constant	−7.235	−9.975	−17.30		
FBC	solubility	0.142	0.173	0.162	−0.0164	0.0361
	CPA	0.017	0.016	0.014	0.0013	0.0021
	SH²	0.0012	0.0014	0.0018	−0.00024	−0.00043
	constant	−15.45	−17.08	−18.03		

itive factor 2 scores, reflecting their higher protein and salt-extractable protein. Hand-deboned chicken also showed the largest difference between factor 1 scores of unheated and heated samples, reflecting the high gel strength, large increase in hydrophobicity and decrease in solubility upon heating of samples. Mechanically-deboned samples (M and m) and water-washed cod samples (F anf f) had negative factor 2 scores, indicative of their lower protein and salt-extractable protein; cod samples showed little change in factor 1 scores between unheated and heated samples, reflecting the low gel strength and small changes in hydrophobicity and solubility on heating cod samples.

The plots of factor 3 versus factor 1 (Fig. 3B) and factor 3 versus factor 2 (Fig. 3C) show that the samples could be broadly clustered according to these factors into groups consisting of hand-deboned samples (C, P, B), cod samples (control F and cryoprotectant f groups) and mechanically-deboned samples (M and m).

When only the data subset of unheated or 40°C samples (n = 116) were considered, 3 factors were extracted, accounting for about 73% of data variance. As shown in Table 3, factor 1 has high positive loadings on total and salt-extractable proteins and high negative loadings on ANS and mince-pH; factor 2 has high positive loadings on CPA and fat and negative loading on moisture, while factor 3 has high loadings on solubility, dispersibility and EC variables.

The plots of the factor scores of individual samples for factor 2 versus factor 1 (Fig. 4) shows the clustering into three groups of hand-deboned (C, P, B), mechanically deboned (M, m) and cod (F, f) samples. Hand deboned samples possessed large and positive factor 1 scores and negative factor 2 scores, mechanically deboned samples had high factor 2 scores and interme-

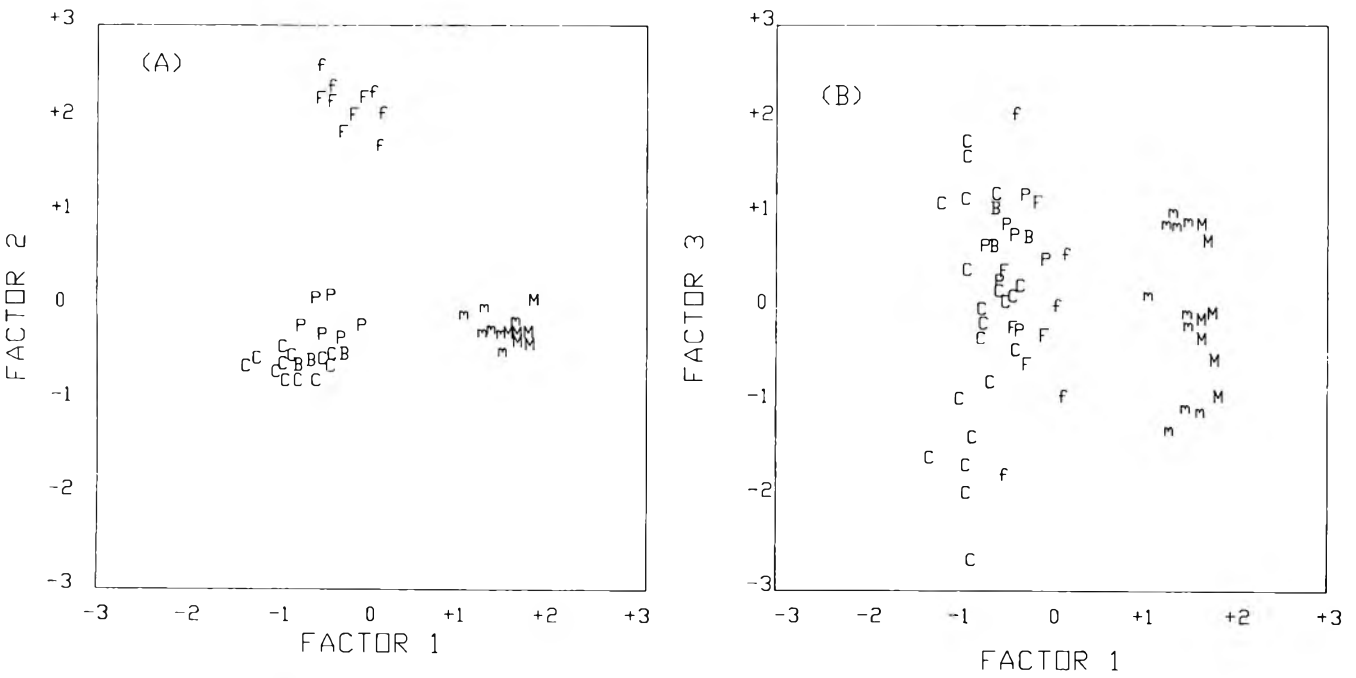


Fig. 6—(A) Factor 2 vs factor 1 and (B) factor 3 vs factor 1 plots of factor scores for unheated meat samples obtained by principal component analysis of variables including AM_w and AM_b.

diate factor 1 scores, while cod samples had low (negative) scores for both factor 1 and factor 2. These reflect the high protein, low mince pH, fat and ANS and CPA hydrophobicity properties of hand-deboned samples, in contrast to the high mince-pH, fat, and ANS and CPA hydrophobicity of mechanically deboned samples. Cod samples had low fat and high mince-pH. ANS hydrophobicity values of the cod samples were much higher than other types of samples, while the CPA hydrophobicity values for cod were intermediate between the other hand-deboned species and mechanically deboned samples.

Factor analysis of the data subset of 40°/80°C and 80°C samples (n = 114) extracted four factors accounting for about 80% of data variance. The sorted rotated factor loadings pattern (Table 4) indicates heavy loadings on mince properties for factor 1 (high loadings on Gel-M, moisture and SEP, and high negative loadings on fat, cook loss and mince-pH). Factor 2 is a component described by dispersibility, solubility and EC of the heated protein extracts, while factor 3 is described mainly by high positive loadings on the heated extract properties of SH, ANS, CPA and Gel-E. Factor 4 is a term related to FBC of the heated extracts.

The plots of factor scores for the heated samples are shown in Fig. 5. Mechanically and hand-deboned samples other than cod could be distinguished by their factor 1 scores, which were negative for the former and positive for the latter, reflecting the lower fat and mince-pH of the latter; little difference in factor 2 scores (solubility, dispersibility, EC variables) were noted between these samples (Fig. 5A).

Although they varied considerably, chicken samples had generally higher factor 3 scores than other species, reflecting the higher values of ANS, CPA and Gel-E of the heated chicken extracts (Fig. 5B). This was observed when comparing hand-deboned and mechanically deboned chicken to the other species.

Cod samples were distinguished by high factor 2 scores (Fig. 5A) and low factor 3 scores (Fig. 5B), reflecting the high dispersibility/solubility and low hydrophobicity/gel strength, respectively, of the heated cod extracts.

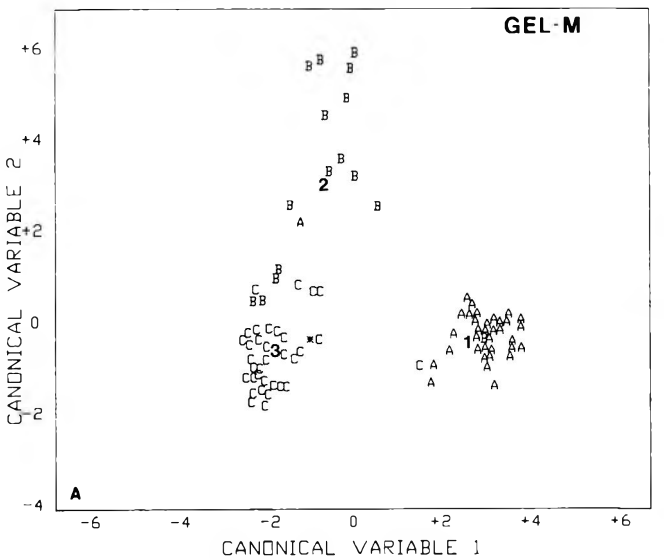
Factor analysis including water binding variables. Factor analysis was performed on the data subset of only unheated samples (n = 58), including absorbed moisture variables, AM_w and AM_b. Four factors accounted for over 77% of the variance in the data. The sorted rotated factor loadings pattern (Table 5) shows that factor 1 has high loadings on fat, CPA and pH variables and negative loadings on moisture, SH and SEP. Factor 2 has high loading on AM_w and ANS hydrophobicity and negative loading on protein. Factor 3 shows heavy loadings on solubility, dispersibility and EC, while factor 4 is described primarily by AM_b.

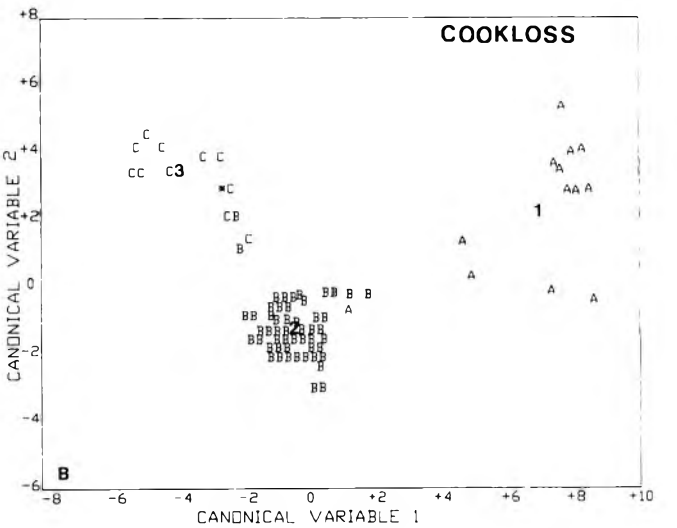
The plots for the factor scores of these samples show that cod samples were easily distinguished by their high factor 2 scores reflecting their high AM_w and ANS hydrophobicity (Fig. 6A). Mechanically-deboned and hand-deboned samples had similar scores for factor 2 but could be distinguished by their factor 1 scores (Fig. 6A), a measure of their mince composition. The plot in Fig. 6B shows that factor 3 could not be used to distinguish between species or type of deboning, indicating the large variability in solubility, dispersibility and EC common to all these types of samples.

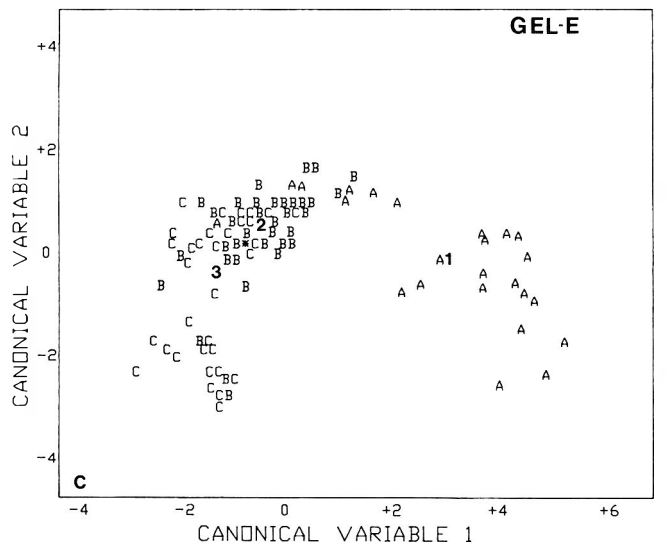
Factor analysis was performed on the data subset (n = 58) consisting of only 80°C heated samples to include retained moisture variables (RM_w, RM_b). Four factors accounted for almost 80% of the total variance in the data, with very similar factor loadings as those from factor analysis on the data subset of all 40°/80°C and 80°C heated samples (Table 4). Plots of the factor scores for the individual samples show the clustering into 3 groups of hand-deboned, mechanically-deboned and cod samples (plots not shown), as already observed in the previously mentioned factor analysis for heated samples.

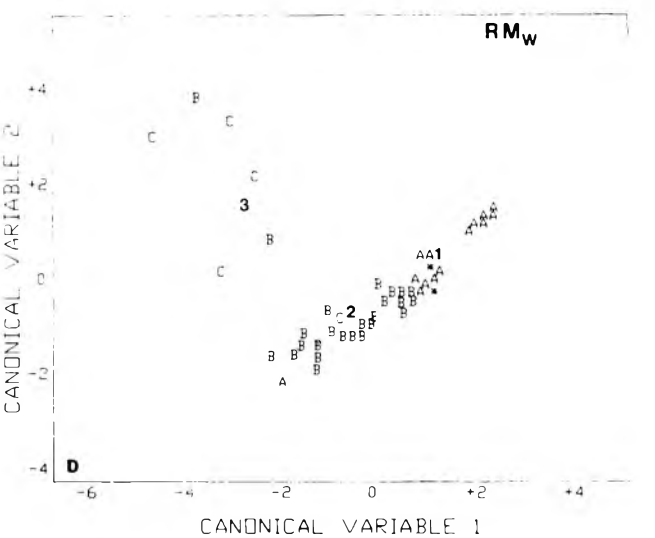
Discriminant analysis

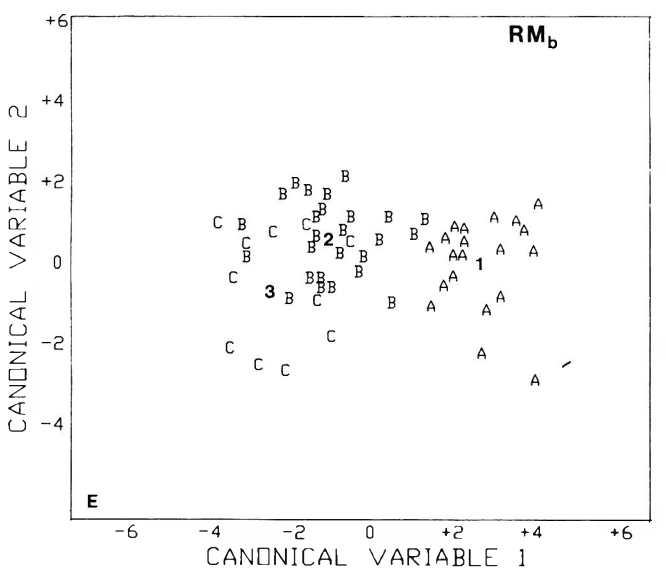
For each functional property, samples were assigned into one of three groups representing low, medium and high functionality. Stepwise discriminant analysis was used to determine the subset of physicochemical properties necessary to describe the classification functions which could best discriminate between the groups. The coefficients for the variables entered into the classification functions for classifying the three groups for each functional property are shown in Table 6. The coefficients of the variables describing the two canonical variables used for visual discrimination of the groups in two-dimensional space are also listed in Table 6. The first canonical variable is the linear combination of variables which best discriminates

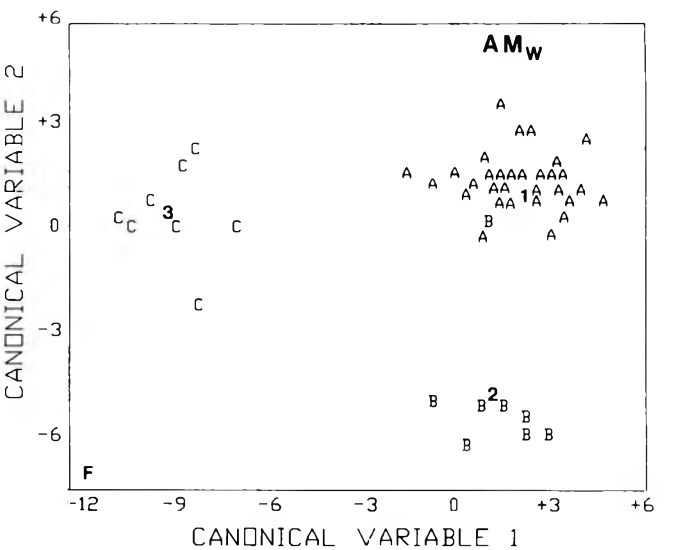

GEL-M
A
CANONICAL VARIABLE 2
CANONICAL VARIABLE 1


COOKLOSS
B
CANONICAL VARIABLE 2
CANONICAL VARIABLE 1


GEL-E
C
CANONICAL VARIABLE 2
CANONICAL VARIABLE 1


RMw
D
CANONICAL VARIABLE 2
CANONICAL VARIABLE 1


RMb
E
CANONICAL VARIABLE 2
CANONICAL VARIABLE 1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CANONICAL VARIABLE 2
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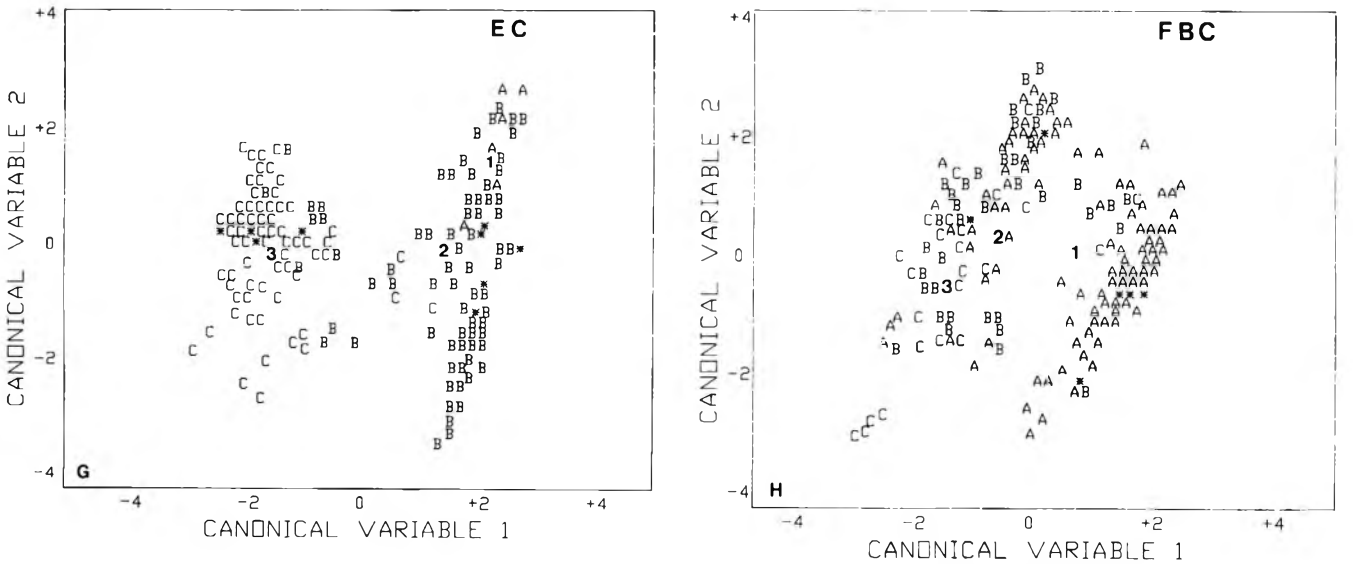

*Fig. 7—Canonical plots of meat samples classified into three groups of low (A), medium (B) and high (C) functionality and the corresponding group means 1, 2 and 3. Overlap of different groups is indicated by *. Canonical variables were computed from the variables selected in stepwise discriminant analyses for each functional property (refer to Table 6).*

Table 7—Relationships between functional properties and physicochemical properties of meat samples[a]

Thermally induced properties	
[1] Gel-M =	−0.029 ANS − 0.38 fat − 3.15 mince pH + 0.17 dispersibility-u + 0.000024 ANS^2 − 2.70 SEP^2 − 0.0018 moisture^2 + 0.0085 solubility·SH + 0.44 SEP·protein + 27.24 (n = 114, R^2 = 0.8344, S.E. = 1.9609, $F_{(9, 104)}$ = 58.21)
[2] Cookloss =	0.010 ANS − 77.3 SEP − 1.61 moisture + 0.80 fat + 30.08 SEP^2 − 0.062 fat^2 − 0.35 mince pH^2 − 0.00065 solubility·CPA + 0.52 CPA·SH + 0.79 SEP·solubility + 173.9 (n = 114, R^2 = 0.8952, S.E. = 3.0525, $F_{(10, 103)}$ = 87.97)
[3a][b] Gel-E =	−0.051 SEP + 0.000024 CPA-u + 0.000012 dispersibility^2 − 0.083 dispersibility-ratio + 0.047 SH-ratio − 0.00000038 solubility·CPA + 0.000000011 ANS·CPA + 0.0033 SEP·protein − 0.027 (n = 114, R^2 = 0.7634, S.E. = 0.0087, $F_{(8, 105)}$ = 42.34)
[3b][b] Gel-E =	−0.00016 ANS-u + 0.000014 CPA-u + 0.00070 SH-u − 0.019 (n = 114, R^2 = 0.5596, S.E. = 0.0116, $F_{(3, 110)}$ = 46.59)
[4] RM_w =	0.015 dispersibility − 0.013 dispersibility-u − 0.00019 CPA-u − 0.00012 dispersibility^2 + 0.088 SEP^2 + 1.50 (n = 58, R^2 = 0.6931, S.E. = 0.0948, $F_{(5, 52)}$ = 23.49)
[5] RM_b =	0.57 SEP + 0.0086 solubility-u −0.018 dispersibility-u + 0.00015 fat^2 −0.00000065 CPA·SH −0.0074 SEP·sol − 0.0087 SEP·protein + 1.13 (n = 58, R^2 = 0.7287, S.E. = 0.1070, $F_{(7,50)}$ = 19.19).
Unheated properties	
[6] AM_w =	0.014 ANS −0.024 protein + 0.39 mince-pH + 0.00012 moisture^2 −0.0000096 CPA·SH −2.06 (n=58, R^2 = 0.8894, S.E. = 0.1152, $F_{(5,52)}$=83.67)
[7] AM_b =	0.002064 CPA −0.0000017 CPA^2 −0.26 (n= 58, R^2 = 0.4390, S.E. = 0.0949, $F_{(2,55)}$=21.52)
General properties	
[8a][b] EC =	0.091 dispersibility −0.024 CPA −0.000046 ANS^2 + 0.0015 moisture^2 + 0.00028 sol·CPA + 0.00040 ANS·CPA + 33.26 (n = 230, R^2 = 0.7740, S.E. = 6.3429, $F_{(6,223)}$= 127.28).
[8b][b] EC =	0.30 dispersibility + 0.00011 ANS·SH + 21.51 (n = 230, R^2 = 0.7411, S.E. = 6.7281, $F_{(2,227)}$= 324.96).
[9a][b] FBC =	−16.2 SEP − 0.00029 solubility^2 + 0.00034 SH^2 + 7.6 SEP^2 + 0.073 SEP·solubility + 8.22 (n = 230, R^2 = 0.5560, S.E. = 2.2981, $F_{(5,224)}$= 56.09).
[9b][b] FBC =	−0.00093 solubility^2 + 0.0017 solubility·SH + 2.19 (n = 230, R^2 = 0.3737, S.E. = 2.7111, $F_{(2,227)}$= 67.73).

[a] F-values for all the regression equations were significant at P<0.001.

[b] For Gel-E, EC and FBC, the functional properties were measured on the salt extracts of proteins after adjustment to specified conditions of protein concentration and pH. Regression equation for these properties were obtained using both mince and extract properties (Equations 3a, 8a and 9a) or using extract properties only (Eq. 3b, 8b and 9b) as the potential independent variables.

among groups along the X-axis, while the second canonical variable discriminates groups along the Y-axis. The canonical plots of the individual samples as well as the means of the three groups are shown for each functional property (Fig. 7 A to H).

Grouping by Gel-M. 40°/80°C and 80°C heated samples were categorized into three groups of Gel-M: (1) 5 or less, (2) 5 to 10, and (3) over 10 Newtons, corresponding to subjective evaluation of weak, medium and strong gels of the heated mince. Nine variables were entered into the classification functions — solubility, SEP, ANS-u, CPA-u, solubility^2, SEP^2, moisture^2, fat^2 and dispersibility-ratio (Table 6). These functions gave 90.5% correct classification of samples into the 3 groups of Gel-M. Figure 7A shows the canonical plot of the samples and the means of the three groups.

Grouping by cookloss. The majority (96.6%) of 40°/80°C and 80°C heated samples were correctly classified into three groups of cookloss (1 or less, 1 to 10, and over 10% cook loss), by classification functions consisting of ten variables — dispersibility, solubility, CPA-u, ANS-u, SEP, mince-pH, moisture, SEP^2, dispersibility^2, and fat^2 (Table 6). Figure 7B shows the canonical plot of the samples and three group means.

Grouping by Gel-E. Discrimination of samples into three groups of Gel-E (0.01 or less, 0.01 to 0.03, and over 0.03 Newtons) was achieved by classification functions described by five variables related to the salt-extracted protein properties — dispersibility, SH, ANS-u, dispersibility^2, and ANS ratio (Table 6). The classification functions gave 70.2% correct classification of the samples into the three groups. The canonical plot of the samples and group means is shown in Fig. 7C.

Grouping by RM_w. Samples were correctly (87.9%) classified into three groups of RM_w (0.3 or less, 0.3 to 0.6, and over 0.6g H_2O/g sample) by classification functions described by dispersibility-u, dispersibility^2, SEP^2, and dispersibility-ratio (Table 6). The canonical plot of the samples and group means are shown in Fig. 7D.

Grouping by RM_b. 82.8% correct classification of samples

into three groups of RM_b (0.3 or less, 0.3 to 0.6, and over 0.6 g H_2O/g samples) was achieved using classification functions described by five variables — SEP, dispersibility-u, solubility-u, dispersibility^2, and CPA (Table 6). Samples were discriminated mainly by canonical variable 1, as shown in the canonical plot of samples and group means (Fig. 7E).

Grouping by AM_w. Only 1 out of 58 samples was misclassified giving 98.3% correct classification into three groups of AM_w (0.3 or less, 0.3 to 0.6 and over 0.6g H_2O/g samples). The classification functions were described by seven variables — ANS, fat^2, moisture, mince-pH, SEP, CPA^2, and protein (Table 6). The canonical plot of samples and group means is shown in Fig. 7F.

Grouping by EC. 73.7% correct classification of samples into three groups of EC (20 or less, 20 to 45, and over 45g oil/30 mg protein) was achieved by classification functions containing four variables describing the protein properties — dispersibility^2, ANS, CPA^2, and solubility (Table 6). The canonical plot in Fig. 7G shows that the majority of misclassification has arisen from the overlapping of samples from the intermediate group (represented as "B") of EC with the other two groups of high EC ("A") and low EC ("C").

Grouping by FBC. Only three variables related to salt-extracted protein properties, namely, solubility, SH^2, and CPA (Table 6) were entered into the classification functions for grouping of samples according to FBC (5 or less, 5 to 10, and over 10 mL oil/g protein). The classification functions yielded 62.5% correct classification of samples. Considerable overlapping of samples between groups was observed in the canonical plot (Fig. 7H).

Stepwise multiple regression

Stepwise multiple regression analyses were carried out for each functional property as the dependent variable to obtain an overview of the general relationship between the functional properties and physicochemical properties of the meat samples. These analyses yielded empirical equations which could be readily used to predict the functional properties of samples based on measurement of their physicochemical properties. Table 7 lists these equations, all of which were highly significant ($P<0.001$) in describing the variability in functional properties using the physical/chemical properties.

DISCUSSION

OUR PREVIOUS WORK has shown that the fat binding and emulsifying properties of salt-extractable proteins from various meat samples can be described by variables representing protein solubility, hydrophobicity, sulfhydryl and/or their quadratic terms (Li-Chan et al., 1984; 1985). In the present work, we have further investigated these relationships as well as that of gel strength of the heated salt-extractable proteins. In addition, functional properties of the minced meat samples rather than their salt extracts were measured, namely, gel strength, cookloss, and water binding properties. The relationship between mince functionality and variables representing mince composition (fat, protein and moisture), pH and salt-extractable protein properties was investigated. Based on these relationships, we have attempted to develop equations to predict functional properties of the samples from their physical and chemical properties.

The results of cluster analysis indicated that variables describing 'hydrophobicity' (ANS and CPA) and 'hydrophilicity' (solubility and dispersibility) were closely correlated with the gelling properties of both mince and salt extract (Gel-M and Gel-E) as well as with emulsifying capacity. On the other hand, a separate cluster was formed consisting of variables describing composition of the mince (protein, salt-extractable protein, fat, moisture and pH).

Great variability between samples was noted in their functional properties as well as the physical and chemical properties (Table 1). Principal component analysis generally indicated that three or four principal components or factors were able to account for over 75% of the variance in the data set. Based on these principal components, three different types of samples could be distinguished clearly: (1) hand-deboned chicken, pork and beef; (2) mechanically deboned chicken and pork; and (3) ling cod.

Hand-deboned chicken samples generally had the highest gel strength of heated extracts and salted mince, as well as the highest water-binding properties (especially RM_b) and fat binding properties. The minced chicken samples had generally higher protein and lower fat than the other samples. In addition, their salt-extractable proteins possessed lower hydrophobicity and higher solubility; after heating, these proteins exhibited the greatest ratio of heated:unheated properties, especially with respect to increase in hydrophobicity and decrease in solubility after heating to form gels. These trends are reflected in the plots of sample factor scores (e.g. Fig. 3 and 5).

Ling cod samples had the highest ANS hydrophobicity for unheated proteins and also the highest water binding capacity for unheated mince (AM_w) of all the samples studied as reflected in their unusually high factor 2 scores in Fig. 6A. The ling cod samples had low protein in the mince, low gel strength of both mince and extract, and small ratios of heated:unheated properties for solubility and hydrophobicity.

Mechanically deboned samples had generally higher hydrophobicity of their salt-extracted proteins than their hand-deboned counterparts, and these proteins showed less increase in hydrophobicity and decrease in solubility after heating. The samples had generally lower gel strength for the heated mince and extracts, as well as lower water binding capacity after heating (RM_e and RM_b). These differences in functional properties of the mechanically deboned samples were due to the differences in physicochemical properties of their salt-extractable proteins as well as their higher fat and pH and lower protein in the mince. Clusters of mechanically deboned samples which were distinct from the other samples were usually easily distinguished in the sample factor score plots (e.g. Fig. 3C, 6A, and 6B). However, even with the mechanically deboned group, the chicken samples showed greater change in salt-extractable protein properties and stronger gels than the pork samples; Fig. 3A shows this as a larger spread in factor 1 scores before and after heating for the mechanically deboned chicken (m) than mechanically deboned pork (P) samples.

The results of stepwise discriminant analyses demonstrated that samples could be classified into groups of low, medium and high functionality, with generally good (62.5–98.3% correct) accuracy of classification. The classification functions shown in Table 6 could be used to predict the category of functional property of unknown samples, based on their properties as represented by the selected variables in the classification functions. Alternatively, the values for the first two canonical variables could be calculated for the unknown samples, using the coefficients for the selected variables in Table 6, and the location of the unknowns on the canonical plots (Fig. 7A to H) could be used to compare the unknown samples' predicted functionality to that of known samples.

For simplicity in using the physical/chemical variables to predict functionality of unknown samples, stepwise multiple regression equations describing the general relationships between the various functional properties and the selected physical and chemical variables were computed (Table 7). It is interesting to note that these equations as well as the classification functions from stepwise discriminant analyses contain quadratic terms of the variables. As suggested in earlier work (Li-Chan et al., 1985), the relationship between functionality and physicochemical properties of proteins does not appear to be linear. An optimum balance of the properties is necessary. The equations for the thermally induced functionality also demonstrate the need to consider not only the protein properties

after heating, but also properties before heating (unheated or "-u"), as well as ratios of the heated/unheated properties.

Structure-function relationship and equations such as those developed in this study could be applied to estimate the functionality of meat ingredients for using in comminuted meat product manufacture. As Parks et al. (1985) suggested, new concepts are needed to estimate the functional contributions of ingredients, particularly water-holding capacity and gelation phenomena. Functionality values obtained by prediction from basic physicochemical properties of the ingredients may be preferred over measurement of individual functional properties themselves, due to easier standardization and reproducibility of methodology to measure the basic properties. In contrast, methodology for functionality measurement is often fraught with inconsistencies due to differences in instruments and conditions, leading to data with high deviations. An additional advantage to using predicted functional values is that a single set of data on basic physicochemical properties can be used to predict diverse functional properties such as gel strength, cook loss, water binding, fat binding and emulsifying properties. Thus, data banks on the basic properties can be used for a more versatile search for suitable ingredients in a blend, depending on the desired functional performance.

Further studies should be undertaken for refinement of the equations obtained in this study, by comparing the predicted functionality to actual experimental functionality for unknown samples. The possibility of deriving more accurate prediction equations by separate analyses of different groups of ingredients (e.g., hand-deboned versus mechanically deboned) should also be investigated. It is anticipated that linear programming for least cost product formulation can be significantly improved by incorporating the limit values of predicted ingredient functionality as additional constraints for product formulation.

GLOSSARY OF ABBREVIATIONS

Physicochemical properties

ANS, hydrophobicity determined using 1-anilinonaphthalene-8-sulfonic acid
CPA, hydrophobicity determined using *cis*-parinaric acid
SEP, salt-extractable protein (%)
SH, sulfhydryl (μM/g protein)
S_o, protein surface hydrophobicity (ANS or CPA)

Functional properties

WBC, water binding capacity (g/g sample)
AM_w, absorbed moisture for sample in water
pH_{aw}, pH of supernatant from AM_w determination
AM_b, absorbed moisture for sample in 1% brine
pH_{ab}, pH of supernatant from AM_b determination
RM_w, retained moisture after cooking for sample in water
pH_{rw}, pH of supernatant from RM_w determination
RM_b, retained moisture after cooking for sample in 1% brine
pH_{rb}, pH of supernatant from RM_b determination
EC, emulsifying capacity (g oil/30 mg protein)
FBC, fat binding capacity (ml oil/g protein)
Gel-M, gel strength of salted mince (Newtons)
Gel-E, gel strength of salt extracts (Newtons)

Samples

C, hand-deboned chicken
B, hand-deboned beef
P, hand-deboned pork
M, mechanically deboned pork
m, mechanically deboned chicken
F, hand-deboned fish
f, hand-deboned fish with added cryoprotectants

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Ms received 3/5/86; revised 6/20/86; accepted 8/23/86.

This work was supported by Agriculture Canada contract OST84-00269. The authors thank Mrs. L. Kwan for skillful technical assistance. Part of this work was presented at the 29th Annual Conference of the Canadian Institute of Food Science & Technology, Calgary, Alberta, June 29-July 2, 1986.

Effect of Water on the Production of Cooked Beef Aroma Compounds

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ABSTRACT

Aroma compounds isolated from cooked fresh ground beef, freeze-dried, defatted and rehydrated to 58% and 17% water, respectively, were less meaty and contained higher relative concentrations of hydrocarbons, ketones, lactones, esters, benzenoids, pyrroles, pyridines and thiazol(in)es (58% H_2O), and of aliphatic ketones, lactones, esters, aliphatic sulfur compounds, pyrroles and pyrazines (17% H_2O) than those obtained from untreated beef. Maximum production of volatiles at water activity (a_w)~0.7, described previously for a model simulated meat flavor system, did not occur. Suggested contributory components to the meatier aroma analyzed from untreated beef were 2-methyl-3-(methylthio)furan, 3-(and 2-)methylcyclopentanones, cyclopent-2-enones and cyclohex-2-enones.

INTRODUCTION

RECENTLY Hartman et al. (1984) reported a study of the effect of water activity (a_w) on the volatiles produced in a model system approximating cooked meat. The system comprised thiamin, ascorbic acid, cystine and glutamic acid heated (135°C/30 min) in a water/propane-1,2-diol mixture of various combinations intended to simulate different a_w environments which may exist in roasting meat. They concluded that the total volatiles produced remained at an approximately constant and low level at a_w 0.0–0.4, then increased very significantly to a clear maximum at a_w 0.72 before declining rapidly at higher a_w levels. This rapid decline was explained as either a dilution effect or an inhibition of condensation reactions at high moisture.

If the same effect applies to a natural meat aroma system, it would perhaps be beneficial to maximize the production of volatile components by using meat of a_w 0.72 or just below. It was decided to test this proposition. If true, trace meat aroma components which have hitherto eluded identification might then be produced in sufficient quantities to facilitate interpretation of their mass spectra.

However, previous work (Galt and MacLeod, 1984; MacLeod and Ames, 1986b) has shown that even when lean fillet steak was used and when all visible fat was removed before cooking, the aroma isolates obtained were dominated by lipid-derived volatiles, e.g., aliphatic hydrocarbons, benzenoids, alcohols, aldehydes and alkan-2-ones, but especially the last three classes. This agrees with observations recently made by Mottram et al. (1982) regarding the headspace volatiles of both beef and pork. These authors also showed that the major precursors of these particular volatiles were the structural phospholipids of the intramuscular fat. Extraction with chloroform/methanol removes both the neutral triglycerides and the phospholipids, and the cooked aroma from such defatted meat is still meaty (Mottram and Edwards, 1983). In view of these findings it was decided to use defatted beef in this study, in the expectation that the gas chromatograms obtained would not be confounded by relatively unimportant volatile components of lipid origin. The use of defatted beef is also more comparable with the model system of Hartman et al. (1984).

The objective of this study was to isolate and analyze the aroma compounds from cooked fresh ground beef which had been freeze-dried, defatted and then rehydrated (before cooking) to different water contents. The volatiles were compared with those generated from untreated fresh cooked ground beef.

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MATERIALS & METHODS

Isolation of aroma compounds

Isolate 1. *Untreated beef.* Using cooking and aroma isolation procedures described previously (MacLeod and Ames, 1986b), the aroma compounds from cooked fresh fillet steak, trimmed of all excess fat and ground to a particle size of 4 mm, were adsorbed on conditioned Tenax TA by N_2 entrainment.

Isolates 2 and 3. *Freeze-dried, defatted and rehydrated beef.* Freeze-drying, defatting and rehydration methods used were based on those of Mottram and Edwards (1983). Fresh fillet steak, trimmed of all excess fat and ground to a particle size of 4 mm, was freeze-dried using a freeze-drier (FTS Systems Inc., Stone Ridge, NY) for~24 hr at a plate temperature at 30°C. Aliquots (6g) were then extracted with $CHCl_3$/MeOH (constant boiling, 87% $CHCl_3$) for 8 hr in a Soxhlet apparatus (Mottram and Edwards, 1983). Longer extraction did not increase the weight of solvent residue. Residual solvent was removed at room temperature (20°C) in a vacuum oven overnight. Replicate extractions were made and the bulked material was stored under N_2 in a desiccator before rehydration to 58% water (isolate 2) or 17% water (isolate 3) using distilled water at 5°C for 24 hr to ensure complete penetration of water. Rehydrated beef was then cooked and the aroma compounds were isolated as described previously (MacLeod and Ames, 1986b). A blank experiment (no meat) was performed before each aroma isolation to monitor adequate cleansing of glassware and N_2 purity.

Capillary gas chromatography

The aroma compounds isolated were analyzed by capillary gas chromatography (GC) as described previously (MacLeod and Ames, 1986b). Peak areas were expressed as a percentage of the total peak area, i.e., relative percentage abundance (RPA).

Capillary gas chromatography — mass spectrometry

Components were identified as far as possible by combined capillary gas chromatography—mass spectrometry (GC-MS) analysis of isolates using the conditions described previously (MacLeod and Ames, 1986b).

Sensory analysis of isolates

The *total* aroma compounds, heat-desorbed from each isolate, were assessed sensorially at an external GC odor port attached to an empty silylated and heated (200°C) short glass GC column (0.3m × 4 mm i.d., 6 mm o.d.) in a Pye Unicam (York St., Cambridge CB1 2PX, England) Series 104 (Model 64) GC. The technique was as described by Galt and MacLeod (1984) and the aroma was described by two assessors experienced in the descriptive sensory analysis of odors.

RESULTS & DISCUSSION

LITERATURE REPORTS agree that for raw beef, an a_w value of 0.72 corresponds with a moisture content of~20% (Taylor, 1961; Saravacos and Stinchfield, 1965; Palnitkar and Heldman, 1971). Our isolate 3 was, therefore, obtained from freeze-dried and defatted beef rehydrated to 17% moisture before cooking in an attempt to maximize the production of volatiles at a_w ~0.7 as described by Hartman et al. (1984) for their model system. Isolate 1 was obtained from untreated beef of

Table 1—Volatile components identified and partially characterized by capillary GC-MS in isolates 1, 2, and 3 (I_1, I_2, I_3)[a]

Component[b,c] and class	GC peak areas[d] I_1	I_2	I_3	RPA[d] I_1	I_2	I_3
INORGANIC COMPOUNDS						
water	9	350	42	tr	0.05	0.05
carbon dioxide	9	420	297	tr	0.05	0.25
hydrogen sulfide	20	tr[g]	tr	tr	tr	tr
sulfur dioxide	32	104	—[h]	tr	0.02	—
	Σ70	874	339	tr	0.12	0.30
ALIPHATIC HYDROCARBONS						
butane	50	—	—	tr	—	—
pentane[e,f]	—	—	25	—	—	0.02
hexane	tr	4180	10	tr	0.75	0.01
heptane	33600	—	104	2.50	—	0.10
octane	55480	12030	tr	4.25	2.00	tr
nonane	1870	3006	80	0.10	0.50	0.10
decane	tr	11870	30	tr	2.00	0.02
undecane	6500	11910	180	0.50	2.00	0.20
dodecane	1696	5300	75	0.10	1.00	0.05
tridecane	5168	5728	—	0.50	0.50	—
methylpropane	40	—	—	tr	—	—
methylbutane	210	860	21	0.02	0.10	0.02
2-methylpentane	tr	373	tr	tr	0.05	tr
3-methylpentane	tr	2353	152	tr	0.50	0.10
2-methylheptane	96	—	—	0.01	—	—
3-methylnonane[e]	—	2187	—	—	0.25	—
*4-methyldecane[e]	—	694	—	—	0.10	—
an octene (M112)	1386	—	—	0.10	—	—
dodec-1-ene	900	tr	—	0.05	tr	—
methylpropene	10	—	—	tr	—	—
penta-1,3-diene	10	3670	—	tr	0.45	—
1,3,6-octatriene	600	—	—	0.05	—	—
unidentified	3919	13000	—	0.26	2.19	—
	Σ 111535	77161	677	8.44	12.39	0.62
ALICYCLIC HYDROCARBONS						
methylcyclopentane	tr	832	—	tr	0.10	—
butylcyclopentane	76	90	—	0.01	0.01	—
cyclohexane	20	tr	tr	tr	tr	tr
methylcyclohexane[e]	—	390	20	—	0.05	0.02
propyl(or *iso*propyl) cyclohexane	260	1800	—	0.02	0.25	—
*a diethyl or dimethyl-ethyl cyclohexane[e]	—	1380	—	—	0.25	—
*a diethyl or dimethyl-ethyl cyclohexane[e]	—	2184	234	—	0.25	0.25
*a diethyl or dimethyl-ethyl cyclohexane[e]	—	255	—	—	0.05	—
*a methylpropylcyclohexane[e]	—	6848	—	—	1.25	—
*a methylpropylcyclohexane[e]	—	2652	tr	—	0.50	tr
*an *iso*propylmethyl-cyclohexane[e]	—	4560	—	—	0.75	—
*a dimethylpropyl-cyclohexane[e]	—	1592	—	—	0.25	—
unidentified	620	6824	—	0.04	1.12	—
	Σ 976	29407	254	0.07	4.83	0.27
TERPENOIDS						
limonene	180	605	170	0.01	0.10	0.20
camphor	434	tr	—	0.02	tr	—
unidentified	1402	418	—	0.12	0.08	—
	Σ 2016	1023	170	0.15	0.18	0.20
ALIPHATIC ALCOHOLS						
ethanol	25	145	—	tr	0.02	—
propan-l-ol	tr	—	—	tr	—	—
pentan-l-ol	17366	1363	50	1.25	0.25	0.05
hexan-l-ol	9150	348	10	0.75	0.05	0.01
heptan-l-ol	10240	3060	80	0.75	0.50	0.10
octan-l-ol	1231	12530	495	0.10	2.00	0.50
oct-l-en-3-ol	13320	—	—	1.00	—	—
2-ethoxyethanol	10	—	120	tr	—	0.10
a nona-2,4-dienol	2400	—	—	0.20	—	—
unidentified	952	—	—	0.06	—	—
	Σ 54694	17446	755	4.11	2.82	0.76
ALIPHATIC ALDEHYDES						
ethanal i.e. acetaldehyde	244	225	115	0.02	0.05	0.10
pentanal	80	—	—	0.01	—	—
hexanal	81540	25480	1005	6.50	4.25	1.00
heptanal	35172	13410	1302	2.75	2.25	1.25
octanal	840	—	865	0.05	—	0.75
nonanal	5205	34088	338	0.50	5.50	0.25
decanal	tr	2447	—	tr	0.50	—
methylpropanal	45	—	—	tr	—	—
2-methylbutanal	119700	2093	154	9.50	0.25	0.10
3-methylbutanal	123200	2684	353	9.50	0.50	0.25
but-2-enal, i.e., crotonal	tr	—	—	tr	—	—
hex-2-enal[e,f]	—	297	100	—	0.05	0.10
a hexenal	62	140	—	tr	0.02	—
2-methylbut-2-enal	1328	tr	—	0.10	tr	—
2-methylpent-2-enal	tr	—	—	tr	—	—
hepta-2,4-dienal	1222	—	—	0.10	—	—
	Σ 368638	80864	4232	29.03	13.37	3.80
ALIPHATIC KETONES						
propanone, i.e., acetone	tr	1170	60	tr	0.20	0.05
butanone	122	—	—	0.01	—	—
pentan-2-one	670	—	—	0.05	—	—
hexan-2-one	2724	1190	220	0.20	0.20	0.20
heptan-2-one	11960	2383	376	1.00	0.50	0.25
octan-2-one	8580	190	840	0.75	0.02	0.75
nonan-2-one	32	2452	1822	tr	0.50	1.75
decan-2-one	10712	3462	1950	0.75	0.50	2.00
undecan-2-one	40	119	—	tr	0.02	—
3-methylpentan-2-one	163	60	—	0.01	0.01	—
4-methylpentan-2-one	655	—	—	0.05	—	—
5-methylhexan-2-one	480	156	—	0.05	0.02	—
hexan-3-one	216	—	—	0.02	—	—
hydroxypropanone	40	620	—	tr	0.10	—
3-hydroxybutanone, i.e., acetoin	23520	15688	tr	1.75	2.50	tr
butanedione, i.e., diacetyl	941	2159	—	0.05	0.25	—
pentane-2,3-dione	40	12905	25	tr	2.25	0.02
pentane-2,4-dione	594	400	—	0.05	0.05	—
pent-3-en-2-one	655	636	—	0.05	0.10	—
pent-1-en-3-one	tr	—	—	tr	—	—
unidentified	2196	—	90	0.20	—	0.10
	Σ 64340	43590	5383	4.99	7.22	5.12
ALICYCLIC KETONES						
cyclopentanone	35	672	—	tr	0.10	—
cyclohexanone	261	370	—	0.02	0.05	—
2-methylcyclopentanone	720	—	—	0.05	—	—
3-methylcyclopentanone	441	80	—	0.02	0.01	—
2-methylcyclopent-2-enone	790	722	45	0.05	0.10	0.05
*3-methylcyclopent-2-enone[e]	—	tr	—	—	tr	—
a trimethylcyclopent-2-enone	1720	tr	—	0.10	tr	—
*a diethyl or dimethylethyl cyclohexanone[e]	—	2771	—	—	0.50	—
4-methylene-3,5,5-trimethyl cyclohex-2-enone	600	816	—	0.05	0.10	—
unidentified	105	234	—	0.01	0.05	—
	Σ 4672	5665	45	0.30	0.91	0.05
ALIPHATIC ACIDS						
acetic acid	1800	20	tr	0.10	tr	tr
propanoic acid	540	tr	—	0.05	tr	—
butanoic acid	112	26	—	0.01	tr	—
pentanoic acid	200	—	—	0.02	—	—
	Σ 2652	46	tr	0.18	tr	tr
LACTONES						
4-hydroxybutanoic acid, lactone, i.e., γ-butyrolactone *or* 4-butanolide	tr	—	630	tr	—	0.50
4-hydroxy-3-methylbutanoic acid, lactone[e]	—	—	446	—	—	0.50
*4-hydroxybut-3-enoic acid, lactone[e]	—	180	9184	—	0.02	9.00
4-hydroxyoctanoic acid, lactone, i.e., γ-octalactone	1144	1040	252	0.10	0.20	0.25
	Σ 1144	1220	10512	0.10	0.22	10.25
ALIPHATIC ESTERS						
ethyl acetate	30	439	—	tr	0.05	—
methyl propanoate	10	—	—	tr	—	—
*methyl butanoate[f]	—	96	—	—	0.02	—
*methyl pentanoate[f]	—	20	1252	—	tr	1.25
methyl hexanoate	10	1152	—	tr	0.20	—
*methyl heptanoate[f]	—	1307	—	—	0.20	—
*methyl octanoate[f]	—	2297	102	—	0.25	0.10
*methyl nonanoate[f]	—	1276	—	—	0.20	—
*methyl decanoate[f]	—	7776	—	—	1.25	—
*methyl pyruvate[e]	—	4200	81	—	0.75	0.10
*methyl but-2-enoate[e]	—	600	—	—	0.10	—
	Σ 50	19163	1435	tr	3.02	1.45

Component[b,c] and class	GC peak areas[d] I_1	I_2	I_3	RPA[d] I_1	I_2	I_3
ALIPHATIC ETHERS						
diethyl ether	tr	—	—	tr	—	—
ethyl vinyl ether	32	—	—	tr	—	—
	Σ 32	—	—	tr	—	—
ALIPHATIC AMINES						
trimethylamine	795	—	10	0.05	—	0.01
	Σ 795	—	10	0.05	—	0.01
ALIPHATIC SULFUR COMPOUNDS						
methanethiol	482	—	203	0.05	—	0.20
carbonyl sulfide	tr	—	—	tr	—	—
methyl propyl sulfide	20	90	tr	tr	0.01	tr
butyl methyl sulfide[e]	—	100	—	—	0.02	—
carbon disulfide	417	201	50	0.02	0.02	0.05
dimethyl disulfide	544	tr	1230	0.05	tr	1.25
dimethyl trisulfide	536	tr	330	0.05	tr	0.25
bis(methylthio)methane	20	—	—	tr	—	—
3-(methylthio)propanal, i.e., methional[e]	—	—	15	—	—	0.01
*dimethyl sulfoxide[e]	—	—	tr	—	—	tr
dimethylsulfone	90	210	72	0.01	0.02	0.05
	Σ 2109	601	1900	0.18	0.07	1.81
CHLORINE COMPOUNDS						
chlorobenzene	48	20	—	tr	tr	—
a dichlorobenzene	675	585	110	0.05	0.10	0.10
	Σ 715	605	110	0.05	0.10	0.10
BENZENOID COMPOUNDS						
benzene	tr	1136	36	tr	0.20	0.02
methylbenzene, i.e., toluene	6490	4399	286	0.50	0.75	0.25
ethylbenzene	2465	793	180	0.20	0.10	0.20
a xylene	1487	1663	600	0.10	0.25	0.50
a xylene	tr	596	135	tr	0.10	0.10
propylbenzene	216	144	98	0.02	0.02	0.10
a trimethylbenzene	2134	1520	704	0.20	0.25	0.75
a C_3 alkyl benzene	123	122	tr	0.01	0.02	tr
a C_3 alkyl benzene	500	tr	80	0.05	tr	0.10
a C_3 alkyl benzene	326	342	66	0.02	0.05	0.05
a C_3 alkyl benzene	40	tr	—	tr	tr	—
a methylpropylbenzene	184	180	96	0.01	0.02	0.10
a tetramethylbenzene	20	182	—	tr	0.02	—
a C_4 alkyl benzene	103	525	42	0.01	0.10	0.05
a C_4 alkyl benzene[e]	—	440	—	—	0.05	—
a dimethyl*iso*propylbenzene	3276	40	—	0.25	0.01	—
a C_5 alkyl benzene[e]	—	210	—	—	0.02	—
vinylbenzene, i.e., styrene	15	210	—	tr	0.02	—
a methylstyrene *or* indan	24000	—	52	1.75	—	0.05
2,6-dimethylstyrene	140	tr	—	0.01	tr	—
4(or 5)-methylindan	528	156	—	0.05	0.02	—
a dimethylindan	270	tr	—	0.02	tr	—
a dimethylindan	32	42	—	tr	0.01	—
a dimethylindan	56	10	—	tr	tr	—
indene	460	tr	—	0.05	tr	—
a methylindene	tr	tr	—	tr	tr	—
naphthalene	6132	777	—	0.50	0.10	—
phenol	tr	10	—	tr	tr	—
benzyl alcohol[e,f]	—	90	170	—	0.01	0.20
benzaldehyde	40920	131308	2187	3.25	21.50	2.25
acetophenone	136	420	tr	0.01	0.05	tr
methyl phenylacetate	50	120	—	tr	0.02	—
unidentified	1167	844	—	0.08	0.15	—
	Σ 92095	146429	4783	7.14	23.86	4.77
FURANS AND DERIVATIVES						
2-methylfuran	tr	21	—	tr	tr	—
2-propylfuran	tr	tr	—	tr	tr	—
2-pentylfuran	190	190	tr	0.01	0.02	tr
2-furanmethanol, i.e., furfuryl alcohol	20	496	—	tr	0.10	—
2-furancarboxaldehyde, i.e., furfural	229	1110	246	0.02	0.20	0.25
5-methylfurfural[e,f]	—	—	16	—	—	0.02
2-methyl-3-(methylthio)furan	1914	—	—	0.10	—	—
4-methyl-2,3-dihydrofuran	236	192	—	0.02	0.02	—
2-methyltetrahydrofuran-3-one	155040	12548	—	12.00	2.00	—
	Σ 157629	14557	262	12.15	2.34	0.26
THIOPHENS						
thiophen	247	—	—	0.02	—	—
2-methylthiophen	640	72	—	0.05	0.01	—
unidentified	1162	—	—	0.09	—	—
	Σ 2049	72	—	0.16	0.01	—

Component[b,c] and class	GC peak areas[d] I_1	I_2	I_3	RPA[d] I_1	I_2	I_3
PYRROLES						
pyrrole	1500	150	923	0.10	0.02	1.00
2-methylpyrrole	416	30	352	0.02	tr	0.25
a methylpyrrole	60	—	1700	tr	—	1.75
2-acetylpyrrole	64	2686	4000	tr	0.50	3.75
2-acetyl-1-methylpyrrole	106	tr	—	0.01	tr	—
unidentified	3285	1477	—	0.22	0.26	—
	Σ 5431	4343	6975	0.35	0.78	6.75
PYRIDINES						
pyridine	3016	4200	9	0.25	0.75	0.01
a methylpyridine	151	2920	435	0.01	0.50	0.50
a methylpyridine	1061	20	—	0.10	tr	—
a methylpyridine	23	tr	—	tr	tr	—
2-ethylpyridine	1200	—	—	0.10	—	—
3-ethylpyridine	40	—	—	tr	—	—
a dimethylpyridine	430	tr	20	0.02	tr	0.02
a dimethylpyridine	550	tr	tr	0.05	tr	tr
a dimethylpyridine	15	30	tr	tr	tr	—
an ethylmethylpyridine	880	—	—	0.05	—	—
3(or 4)-vinylpyridine	160	139	—	0.01	0.02	—
2-acetylpyridine	60	tr	—	tr	tr	—
	Σ 7586	7309	464	0.59	1.27	0.53
PYRAZINES						
pyrazine	62	140	288	tr	0.02	0.25
methylpyrazine	52360	15490	17607	4.00	2.50	17.50
2,3-dimethylpyrazine	7460	2102	153	0.50	0.25	0.10
2,5-(and/or)2,6-dimethylpyrazine	105000	33353	23265	8.00	5.50	22.50
ethylpyrazine	7920	232	—	0.50	0.05	—
trimethylpyrazine	100	26520	1345	0.01	4.50	1.50
2-ethyl-3-methylpyrazine	65700	—	—	5.00	—	—
2-ethyl-5(and/or 6)-methylpyrazine	49358	5674	256	3.75	1.00	0.25
tetramethylpyrazine	26656	1399	198	2.00	0.25	0.20
2,5-dimethyl-3-ethylpyrazine	195	8602	1386	0.02	1.50	1.25
a dimethylethyl *or* a diethyl pyrazine	31200	17203	108	2.50	2.75	0.10
a dimethylethyl *or* a diethyl pyrazine	106	620	—	0.01	0.10	—
a C_4 alkyl pyrazine	440	—	—	0.02	—	—
2-ethyl-3,5,6-trimethylpyrazine	2957	1229	—	0.25	0.20	—
2,3-diethyl-5-methylpyrazine	1200	429	—	0.10	0.05	—
2,5-diethyl-3-methylpyrazine	693	—	—	0.05	—	—
2,6-diethyl-3-methylpyrazine	2948	2120	—	0.25	0.25	—
2-butyl-3,5-dimethylpyrazine	1411	165	—	0.10	0.02	—
a C_4 alkyl dimethylpyrazine	32	459	—	tr	0.10	—
a C_6 alkyl pyrazine	470	150	—	0.05	0.02	—
vinylpyrazine	759	—	—	0.05	—	—
2-methyl-5(and/or 6)-vinylpyrazine	7436	4181	66	0.50	0.75	0.05
2-methyl-5(or 6)-(prop-1-enyl) pyrazine	676	93	42	0.05	0.02	0.05
6,7-dihydro-(5H)cyclopentapyrazine	293	—	—	0.02	—	—
5-methyl-6,7-dihydro-(5H)cyclopentapyrazine	286	63	—	0.02	0.01	—
unidentified	2906	371	—	0.26	0.05	—
	Σ 368624	120595	44714	28.01	19.89	43.75
OXAZOLES	1045	30	—	0.10	tr	—
2,4,5-trimethyloxazole						
unidentified	365	—	—	0.02	—	—
	Σ 1410	30	—	0.12	tr	—
THIAZOL(IN)ES						
thiazole	26	tr	tr	tr	tr	tr
2-methylthiazole[e]	—	360	—	—	0.05	—
4(or 5)-methylthiazole	1350	tr	—	0.10	tr	—
5(or 4)-methylthiazole	620	820	—	0.05	0.10	—
2-acetylthiazole	1980	1893	—	0.20	0.25	—
benzothiazole *or* benzo*iso*-thiazole[e]	—	470	—	—	0.10	—
2-methyl-2-thiazoline	906	242	—	0.05	0.05	—
2-acetyl-2-thiazoline	560	tr	—	0.05	tr	—
	Σ 5442	3785	tr	0.45	0.55	tr

Table 1—footnotes

[a] Isolate 1 was obtained from untreated beef: moisture content ~74% and a_w~0.99 (Lawrie, 1985); isolate 2 was obtained from freeze-dried, defatted and rehydrated beef: moisture content 58%; isolate 3 was obtained from freeze-dried, defatted and rehydrated beef: moisture content 17% and a_w~0.7.

[b] Mass spectra lit. refs are given in MacLeod and Ames (1986b) unless otherwise stated—see footnotes e, f.

[c] Blank isolates (no meat) contained some additional components (artefacts) not listed above; they were concentrated onto the Tenax from the purified N_2 sweep-gas during sampling and consisted mainly of aliphatic, aromatic and alicyclic hydrocarbons and chlorinated compounds (see MacLeod and Ames, 1986b); some of the latter class also originated from the defatting solvent residues.

[d] GC peak areas are given in mV sec^{-1}; relative percentage abundance (RPA) values have been corrected as follows: >5% quoted to nearest 0.5%; 0.25–5% quoted to nearest 0.25%; < 0.25% quoted as 0.25, 0.20, 0.10, 0.05, 0.02, 0.01%.

[e] Mass spectra lit. ref., Anon. (1983).

[f] Mass spectra lit. ref., Jennings and Shibamoto (1980).

[g] tr—indicates that the component was present in trace amount, but no accurate peak area measurement was possible.

[h] "—" indicates that the component was not identified in this isolate.

* Asterisked components have not been reported previously from heated beef (Self et al., 1963; Ching, 1979; Wasserman, 1979; Golovnya et al., 1979; Golovnya and Rothe, 1980; Uralets and Golovnya, 1980; Yamaguchi et al., 1980; MacLeod and Seyyedain-Ardebili, 1981; Lee et al., 1981; Hsu et al., 1982; Mottram et al., 1982; Galt and MacLeod, 1983; Hartman et al., 1983; Galt and MacLeod, 1984; MacLeod and Ames, 1986b).

moisture content~74% and a_w~0.99 (Lawrie, 1985). Isolate 2 was prepared from freeze-dried and defatted beef rehydrated to 58% moisture before cooking, (this value being arbitrarily selected as a meat moisture level intermediate between that used for isolates 1 and 3).

A composite list of all components identified by combined capillary GC-MS is presented in Table 1. Twenty of the compounds (asterisked) have not been reported previously from heated beef (Self et al., 1963; Ching, 1979; Wasserman, 1979; Golovnya et al., 1979; Golovnya and Rothe, 1980; Uralets and Golovnya, 1980; Yamaguchi et al., 1980; MacLeod and Seyyedain-Ardebili, 1981; Lee et al., 1981; Hsu et al., 1982; Mottram et al., 1982; Galt and MacLeod, 1983; Hartman et al., 1983; Galt and MacLeod, 1984; MacLeod and Ames, 1986b). The table also shows that thirty-three components were identified only from the freeze-dried, defatted and rehydrated beef, but on examination, it was unlikely that any of these were prime contributors to cooked beef aroma.

The total GC peak area for isolate 2 was considerably less than that of isolate 1, i.e., 574 × 10^3 vs 1254 × 10^3mV sec^{-1}. The difference was largely accounted for by the expected decreased quantities of the lipid-derived volatiles, e.g., aliphatic hydrocarbons, alcohols, aldehydes, ketones and acids and also by the decrease of all other classes with the exception of the alicyclic hydrocarbons and ketones, lactones, esters and benzenoids. Isolate 3 (a_w~0.7) gave rise to an even smaller total GC peak area of 83 × 10^3 mV sec^{-1}, and in comparison with isolate 2, the only chemical classes that were enhanced in isolate 3 were the lactones, aliphatic sulfur compounds (especially polysulfides) and the pyrroles. Their increased formation was largely explained by the fact that dehydration and condensation reactions were favored under low moisture conditions. However, it should be noted that this effect was not a general one for all volatile products of pyrolysis and Maillard reactions.

The significant decrease in production of total volatile components from 574 × 10^3 mV sec^{-1} (isolate 2) to 83 × 10^3 mV sec^{-1} (isolate 3) does not support the maximum production of volatiles at a_w~0.7 described by Hartman et al. (1984) for their model meat flavor system. It follows, therefore, that results from a specific model system do not necessarily apply to a natural food system. Additionally, Hartman et al. (1984) achieved varying a_w conditions using a solvent containing different proportions of propane-1,2-diol:water. The diol reacted with aldehydes and ketones forming acetals and ketals (MacLeod et al., 1980; Hartman et al., 1984) and was, therefore, not inert. While some reactions are favored by high a_w conditions and others are favored by low a_w levels, in natural meat there is a complex and intertwining network of reactions occurring simultaneously. It would appear that in the meat used for isolate 3, water availability was a limiting factor and was insufficient.

Relative concentrations are more relevant than direct peak area measurements when considering the contribution of various components to the isolated aroma. The aroma desorbed from isolate 1 was described as strongly meaty, roasted, burnt, musty and buttery whereas that from isolate 2 was less meaty with a cereal-like, wet cardboard-like, musty and slightly fruity quality. With regard to chemical composition, isolate 2 contained higher concentrations of all the hydrocarbons (aliphatic, alicyclic and aromatic), ketones, esters, lactones, benzenoids, pyrroles, pyridines and thiazol(in)es, with decreased concentrations of all other classes. The most noticeable difference with respect to any individual compound was for benzaldehyde, which showed an extraordinary increase in concentration from 3.25% in isolate 1 to 21.5% in isolate 2. These results agree — at least in part — with those of Mottram and Edwards (1983) who reported that the processes of freeze-drying, defatting and rehydration gave rise to a less meaty but more roasted aroma which they attributed to higher levels of pyrazines. They also noted a significantly increased concentration of benzaldehyde. In our study, isolate 2 was certainly less meaty than isolate 1 but it did not contain a higher overall concentration of pyrazines and its aroma was certainly not roasted. Some of the individual pyrazines identified in isolate 2 did, however, show enhanced concentrations compared with those of isolate 1, e.g., pyrazine, trimethylpyrazine, 2,5-dimethyl-3-ethylpyrazine, 2-methyl-5(and/or 6)-vinylpyrazine, but the overall manifestation of this did not create a perceived roasted character.

On the other hand, isolate 3 did possess a strongly roasted, burnt odor; its desorbed aroma was described as slightly meaty, roasted, burnt and nutty. In comparison with isolate 2, it possessed higher concentrations of lactones, aliphatic sulfur compounds, pyrroles and pyrazines, with lower concentrations of all other classes. In particular, it was characterized by high RPA levels of 2,5-(and/or) 2,6-dimethylpyrazine (22.50%), methylpyrazine (17.50%), 4-hydroxybut-3-enoic acid lactone (9.00%), and 2-acetylpyrrole (3.75%). The benzaldehyde level on this occasion was as low as 2.25%.

Increased meatiness in the desorbed aroma of isolate 1, compared with all other isolates, cannot be entirely explained from the identifications and concentrations summarized in the table. It is proposed, however, that the identified 2-methyl-3-(methylthio)furan, the 3-(and 2)methylcyclopentanones and the unsaturated alicyclic ketones (e.g., the cyclopent-2-enones and cyclohex-2-enones) are contributory factors. Confirmation of the identity of 2-methyl-3-(methylthio)furan has been reported previously by MacLeod and Ames (1986a), who described it as a potent meaty character impact compound. Nishimura et al. (1980) isolated 3-(and 2)methylcyclopentanone from the reaction products of heated cyclotene/H_2S and described the odor of both compounds as roasted beef-like. Finally, Flament et al. (1978) identified a number of cyclohexenones on heating a meat aroma precursor preparation obtained from raw meat and showed that they possessed meat flavor enhancement properties; furthermore, the use of cyclopent-2-enones (e.g. the 3-methylcyclopent-2-enone of the table) and cyclohex-2-enones in meat flavors has been patented (Flament, 1974; King and Smith, 1976).

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Effect of Inorganic Polyphosphates on Ground Beef Characteristics: Microbiological Effects on Frozen Beef Patties

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ABSTRACT

Microbiological effects of 0.4% sodium tripolyphosphate (STPP), tetrasodium pyrophosphate (TSPP), and three commercial phosphate blends were studied in frozen (90 days, −20°C) and subsequently temperature-abused (24–25°C, 24 hr) beef patties. Phosphates did not significantly ($P>0.05$) reduce mesophilic, psychrotrophic, presumptive *S. aureus* and lactic acid bacterial numbers during frozen storage of the patties, but one of the commercial blends and TSPP inhibited bacterial growth upon subsequent elevated-temperature abuse. However, bacterial inhibition by phosphates during temperature abuse was not sufficient to prevent spoilage of the patties.

INTRODUCTION

SINCE THE ADDITION of various poly- and pyrophosphates to cured and processed meat products was approved (USDA, 1982), their use has gained acceptance within the industry because of possible economic and quality advantages. A large proportion of phosphate-related research, however, has dealt with their physical and chemical effects on meats. Little is known about the possible increase of bacterial susceptibility to freeze injury in phosphate-containing ground beef or other meats.

Phosphates have been shown to be inhibitory to various bacterial types and to enhance the activity of sodium nitrite against *Clostridium* in cooked, vacuum-packaged, refrigerated bratwurst (Molins et al., 1986). Sodium acid pyrophosphate (SAPP) has also been reported to increase nitrite effects against inocualted *Clostridium botulinum* in beef/pork frankfurter emulsions (Wagner and Busta, 1983), while no significant phosphate effect on total bacterial counts was found in chicken frankfurter emulsions kept at 27°C (Nelson et al., 1983). In fresh meats, phosphates were only mildly inhibitory against the natural mesophilic and psychrotrophic bacterial flora of uncooked, bratwurst-type sausage held at 5°C (Molins et al., 1985a), possibly as a result of poly- and pyrophosphate hydrolysis to orthophosphates, as discussed by Awad (1968) and by Sutton (1973). Snyder and Maxcy (1979) examined the effect of 0.5% sodium polyphosphate glassy (SPG) on *Moraxella-Acinetobacter* in ground beef and pork meat, and concluded that the inhibitory activity of that phosphate was due to lowered water activity of the meat. Hoes et al. (1980) found higher total mesophilic microbial counts in phosphate-injected, hot-processed pork loins.

Although it is not anticipated that phosphates will soon be approved as fresh meat additives, the present study was undertaken to determine the possible contribution of 0.4% pure phosphates or phosphate blends to the microbiological quality characteristics of frozen beef patties prepared and stored for use similar to that for commercial fast-food operations.

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MATERIALS & METHODS

Preparation of beef patties

Fresh, refrigerated (2–4°C) beef trim (90/10) and fat (50/50) were obtained from a commercial source, ground through a 3/8 in (0.95 cm) diam plate, mixed for 2 min in a Blonco SS250 mixer (S. Blondheim & Co., Inc., Oakland, CA) in the necessary ratio to obtain 20% fat in the blend, reground through a 1/8 in (0.32 cm) diam bone removal plate and divided into seven, 25-lb batches. Each batch received an appropriate phosphate treatment, either as a powder (to 0.4% of meat weight) or as a 16% (w/v) aqueous solution (to 2.5% of meat weight) to obtain a 0.4% final concentration of phosphate in all batches except one that received no phosphate and was used as control. After mixing for an additional 2 min to thoroughly distribute the added phosphate, the meat was formed into 0.25-lb (114g) patties by means of a Hollymatic 500 patty-making machine (Hollymatic Corp., Park Forest, IL). The patties were interleafed with waxed parchment paper, wrapped in plastic freezer bags in groups of 25, twist-tied, placed in cardboard boxes and labeled according to treatment: (1) control (no phosphates added); (2) Brifisol 414B (added as powder, 0.4% of meat weight); (3) Brifisol 414B (added as 16% aqueous soln., 2.5% of meat weight); (4) Brifisol 414P (added as powder, 0.4% of meat weight); (5) Brifisol 614P (added as powder, 0.4% of meat weight); (6) sodium tripolyphosphate (STPP) (added as powder, 0.4% of meat weight); (7) tetrasodium pyrophosphate (TSPP) (added as 16% aqueous soln., 2.5% of meat weight).

Brifisol phosphate blends (unspecified composition) were provided by BK-Ladenburg Corporation (Cresskill, NJ). Sodium tripolyphosphate (STPP) and tetrasodium pyrophosphate (TSPP) were commercially available (Monsanto, St. Louis, MO).

The boxed patties were frozen in an air-blast freezer and held at −20°C for the duration of the study (90 days). Freezing was considered to be complete after 48 hr. Four replications of the experiment were initiated at 1 wk intervals.

Sampling

Microbiological differentiations, pH, and soluble orthophosphate determinations were performed on fresh patties the day of manufacture (day 0) and on frozen patties 2 days after manufacture, which was considered to be the first day of frozen storage at −20°C. Thereafter, sampling was performed on days 7, 30, 60 and 90 of frozen storage.

Duplicate samples were obtained for microbiological evaluation on each sampling day. Approximately 15g were aseptically taken from each patty, weighed to make a 30g composite sample, placed in a sterile plastic bag containing 270 ml of sterile 0.1% peptone water and blended in a Stomacher 400 Lab Blender (Tekmar Co., Cincinnati, OH) for 2 min. Serial dilutions were prepared from the resulting extract according to standard methods and used for microbiological assays and soluble orthophosphate determinations. Trypticase soy agar (TSA, BBL) was the medium used to enumerate total mesophilic (30°C, 48 hr) and psychrotrophic (5°C, 7–10 days) bacteria. Lactic acid bacteria were recovered in lactobacilli selective agar (LBS, BBL) and counted after incubation at 30°C for 48 hr (Rogosa et al., 1951). Baird-Parker agar base (Difco) was used to identify and enumerate presumptive *Staphylococcus aureus* (incubation at 37°C for 24 and 48 hr; Baird-Parker, 1962). To determine viable anaerobic spore counts, 10 mL of the initial extract were pipetted into sterile tubes, heat-shocked in a water bath at 80°C for 20 min, allowed to cool at room temperature (24–25°C) and plated with TSA containing 0.1% soluble starch using the plastic pouch method (37°C, 48 hr) of Bladel and Greenberg (1965).

Determinations of pH were made with a Radiometer 28 meter (Radiometer, Copenhagen, Denmark), equipped with an Orion 9163 probe (Orion Research, Cambridge, MA) after the remaining portions of the

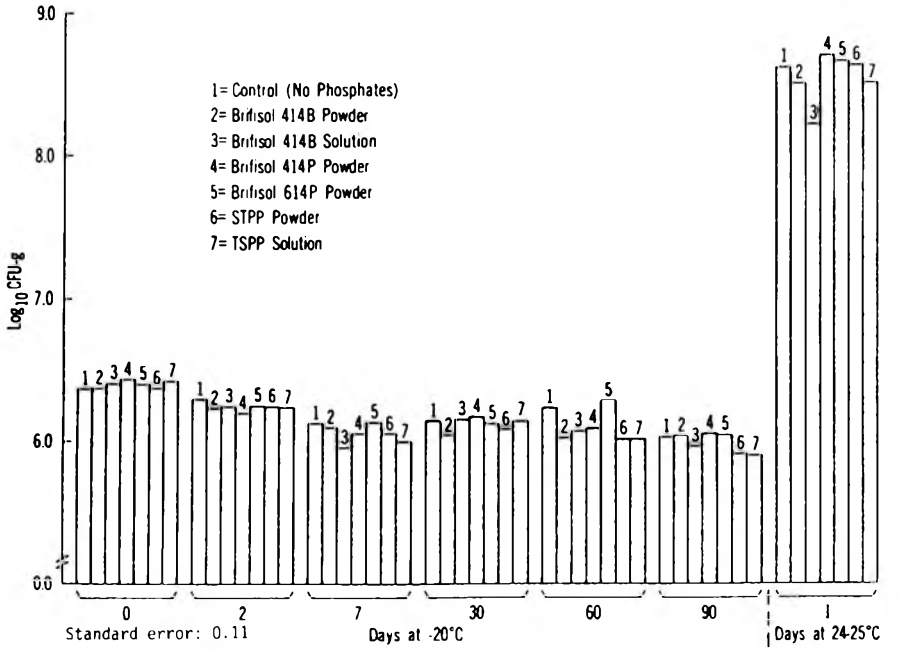

Fig. 1—Effect of 0.4% phosphates on mesophilic bacteria in beef patties.

Table 1—Mean pH values of beef patties (std error : 0.01)

Days at −20°C	Control[a]	414B powder[b]	414B soln.[bc]	414P powder[bc]	614P powder[d]	STPP powder[c]	TSPP soln.[e]
0	5.95	6.10	6.20	6.17	6.31	6.23	6.50
2	5.97	6.10	6.19	6.15	6.30	6.22	6.45
7	5.96	6.09	6.20	6.12	6.30	6.21	6.47
30	5.94	6.09	6.20	6.14	6.28	6.21	6.43
60	6.02	6.12	6.21	6.16	6.31	6.24	6.47
90	5.99	6.08	6.19	6.15	6.30	6.25	6.46
Days at 24–25°C							
1	5.86	5.99	6.17	6.06	6.25	6.12	6.41

[a,b,c,d,e] Treatments with the same superscript letter are not significantly different (P>0.05).

Table 2—Mean soluble orthophosphate content (μg/g of meat) or beef patties (std error : 16)

Days at −20°C	Control[d]	414B powder[c]	414B soln.[c]	414P powder[bc]	614P powder[b]	STPP powder[a]	TSPP soln.[b]
0	817	904	836	952	1075	1186	1010
2	922	1084	1060	1090	1151	1292	1183
7	837	1042	1106	1037	1185	1307	1126
30	809	1017	1043	1109	1082	1206	1148
60	891	1167	1157	1065	1211	1250	1232
90	775	1152	1220	1094	1179	1301	1226
Days at 24–25°C							
1	829	1816	2255	1708	1681	1842	2325

[a,b,c,d] Treatments with the same superscript letter are not significantly different (P>0.05).

patties were thawed 4 hr at room temperature (24–25°C). Analysis of orthophosphates was accomplished by the method of Dick and Tabatabai (1977) as adapted to meats by Molins et al. (1985b).

For comparative purposes, the remaining portions of the patties analyzed on day 90 of frozen storage at −20°C were repackaged in aluminum foil and kept at 24–25°C for 24 hr. Analyses were performed for bacterial numbers, pH and soluble orthophosphates as described previously. This was done to evaluate effects of phosphates on "temperature-abused" products.

Microbiological data were transformed into logarithms and all data from the four replications were analyzed using a Statistical Analysis System (SAS, 1982) computer program with analysis of variance and general linear model options. Comparison of means was based on Duncan's multiple range test.

RESULTS & DISCUSSION

NO SIGNIFICANT (P>0.05) microbiological effect could be attributed to phosphate addition upon freezing the beef patties or throughout the 90-day storage period at −20°C. All bacterial counts decreased by less than one logarithmic cycle from day 0 to 90 (Fig. 1–4) for all treatments. Similarly, phosphate addition had no effect on anaerobic spore viability (data not shown) or on presumptive *S. aureus* counts (Fig. 4) after 90 days of storage at −20°C.

When the patties were temperature-abused at 24–25°C for 24 hr after the 90-day frozen storage period, however, 0.4% TSPP and Brifisol 414B added to the meat as a 16% (w/v) aqueous solutions exhibited significant (P<0.05) inhibitory properties against lactic acid microorganisms (Fig. 3). Brifisol 614P resulted in the highest numbers of psychrotrophic and second highest numbers of mesophilic bacteria when compared with all other Brifisol treatments. After 24 hr at 24–25°C, total bacterial counts in all patties, regardless of treatment, were sufficiently high (10^8–10^9/g) to indicate spoilage.

Because of its implications in terms of processing, it must also be noted that adding the phosphates in solution resulted in lower numbers of lactic acid bacteria than incorporating the

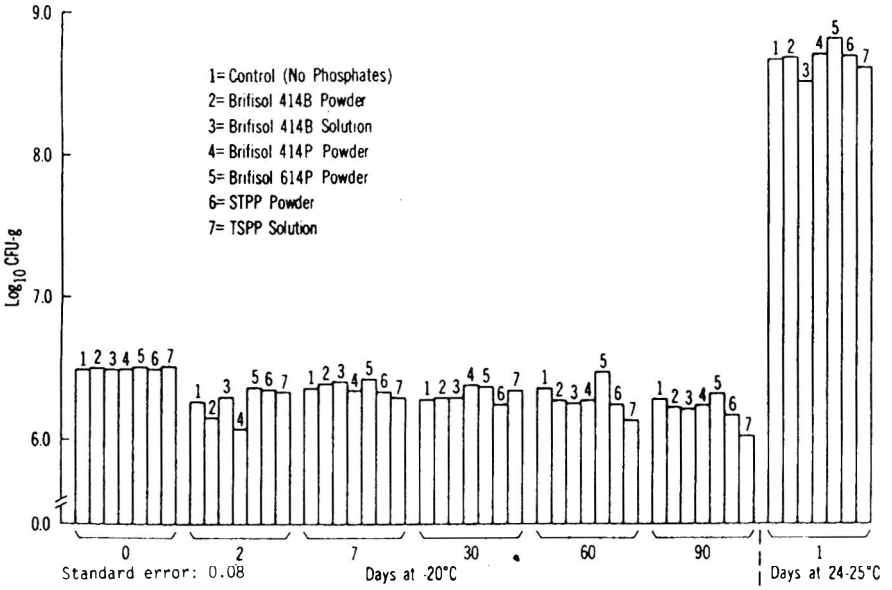

Fig. 2—Effect of 0.4% phosphates on psychrotrophic bacteria in beef patties.

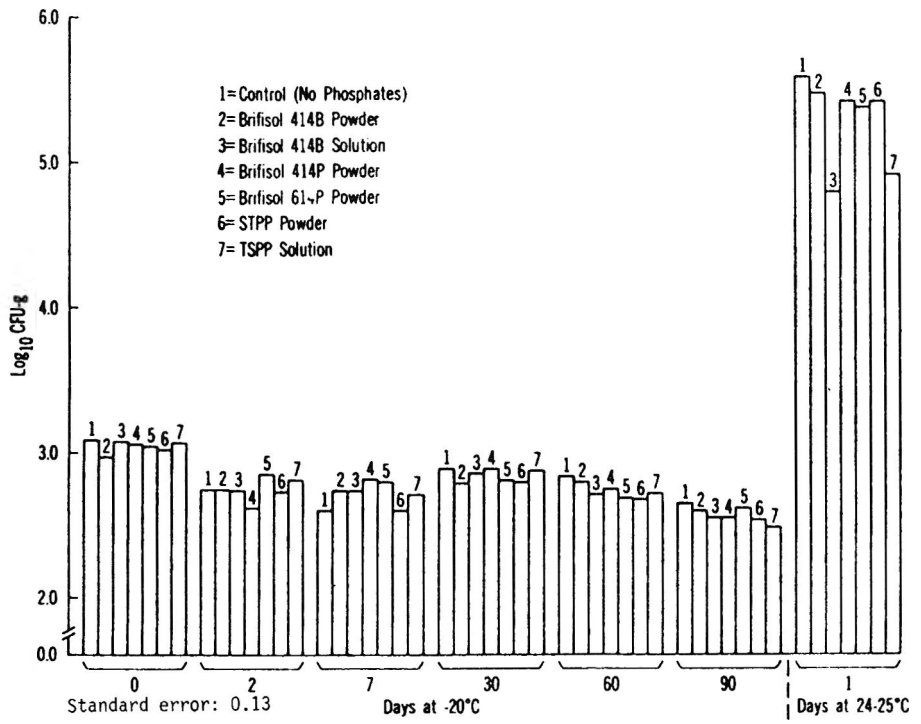

Fig. 3—Effect of 0.4% phosphates on lactic acid bacteria in beef patties.

phosphates in powder form. This result might be related to improved distribution and diffusion of the added phosphate solution through the meat mass. Addition of phosphate in powder form may cause accumulation of the polyphosphates at the immediate surface of contact, complexing with protein and resulting in slower rates of migration (Tenhet et al., 1981). However, it would be desirable to add phosphates in a dry form in order to avoid the dissolving step and eliminate water addition.

The pH values of beef patties throughout the experimental period (including the final 24 hr under temperature-abuse conditions) are shown in Table 1. TSPP increased the pH of the meat more than any other phosphate or blend tested, followed by Brifisol 614P>STPP>Brifisol 414B in solution>Brifisol 414P>Brifisol 414B powder>control.

Table 2 shows that there was also an immediate increase in the level of soluble orthophosphates present in the meat after addition of all phosphates. The increase was highest in patties treated with TSPP and was maintained in all patties containing phosphates during the experimental storage period, indicating that hydrolysis of the condensed phosphates proceeded even at temperatures as low as −20°C. The rate of phosphate hydrolysis markedly increased when the patties were held at 24–25°C for 24 hr. In agreement with the results of previous studies (Molins et al., 1986), those phosphates that contributed the highest soluble orthophosphate levels also affected bacterial growth the most when the meat was temperature-abused (24–25°C), although the nature of a possible relationship between these two effects has not yet been established.

CONCLUSIONS

ADDITION of 0.4% levels of the pure or blended phosphates tested did not significantly ($P>0.05$) reduce numbers of me-

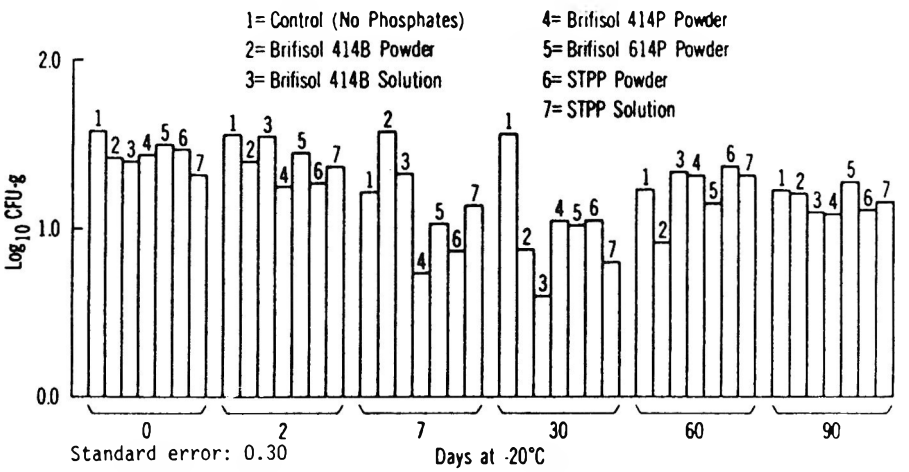

Fig. 4—Effect of 0.4% phosphates on presumptive Staphylococcus aureus.

sophilic, psychrotrophic, lactic acid bacteria, presumptive *Staphylococcus aureus* or viable anaerobic spores in beef patties stored at −20°C for up to 90 days.

Phosphates did not prevent spoilage in patties subjected to 24 hr temperature abuse at 24–25°C. Numbers of lactic acid bacteria were significantly ($P<0.05$) lower when Brifisol 414B and TSPP were added to the meat in solution. The most effective phosphate tested in terms of antimicrobial activity was Brifisol 414B (solution).

All phosphates studied increased the pH and the soluble orthophosphate content of beef patties. Hydrolysis of phosphates added to ground beef occurred even during frozen storage at −20°C.

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Ms received 1/16/86; revised 8/28/86; accepted 8/28/86.

Journal Paper No. J-12105 of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA. Project No. 2252. The authors thank BK-Ladenburg Corporation for partial support of the study. The authors also thank Diane B. James for the technical assistance provided and Bev Allfree for preparation of the manuscript.

Mention of any company or product name does not constitute endorsement.

Effect of Inorganic Polyphosphates on Ground Beef Characteristics: Some Chemical, Physical, and Sensory Effects on Frozen Beef Patties

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ABSTRACT

Chemical, physical, and sensory effects of 0.4% sodium tripolyphosphate (STPP), tetrasodium pyrophosphate (TSPP) and three commercial phosphate blends were studied in frozen beef patties over a 90-day storage period at −20°C. Addition of phosphates significantly ($P<0.05$) increased pH, soluble orthophosphates, Hunter color a values, cook yields and overall acceptability scores. Phosphate addition did not affect ($P>0.05$) proximate analysis, texture, and flavor scores. Hunter L (lightness) and b (yellowness) values were also unaffected by phosphates. Overall quality of patties, as measured by thiobarbituric acid (TBA) numbers and cooking yields, was improved by all phosphates. There were indications that phosphates interfered with the distillation TBA test.

INTRODUCTION

ECONOMIC AND QUALITY ADVANTAGE, such as improved water retention and subsequent higher cook yields, increased binding, and improved palatability scores, have been reported extensively to result from alkaline phosphate inclusion in the formulation of frankfurters (Hargett et al., 1980) and bologna (Swift and Ellis, 1956). Phosphates have also resulted in higher-quality products when used in ham curing brine (Siegel et al., 1978; Vollmar and Melton, 1981), or when added to beef rolls (Moore et al., 1976) or to frozen pork patties (Keeton, 1983). Phosphates improved the physical, chemical and microbiological quality of chicken meat when used in solutions for chilling chicken carcasses (Klose et al., 1963; Landes, 1972; Shults and Wierbicki, 1973) or presoaking chicken parts (Chen et al., 1973). Quality improvements from phosphate addition were also reported by Knipe (1982) to occur in meat emulsions. Hot-processed pork loins injected to 110% of weight with a 5% solution of a sodium polyphosphate glassy (SPG) and tetrasodium pyrophosphate (TSPP) resulted in juicier, more tender, heavier pork chops (Hoes et al., 1980). However, treated chops exhibited more thaw loss and less browning upon cooking than pork containing no added phosphate.

Little information is available on the effects of phosphates in frozen ground beef. Although it is not anticipated that phosphates will soon be approved as fresh meat additives, the present study was undertaken to determine the possible contribution of 0.4% pure phosphates or phosphate blends to some physical, chemical and sensory characteristics of frozen beef patties prepared and stored for use similar to that for commercial fast-food operations.

MATERIALS & METHODS

Preparation of beef patties

Beef patties were prepared as for previous studies (Molins et al., 1987).

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Sampling

Soluble orthophosphate and pH determinations were performed on the day of manufacture (day 0) for fresh patties while TBA numbers, Hunter color values and sensory evaluations began on frozen patties 2 days after manufacture, considered to be the first day of frozen storage at −20°C. Sampling was continued on days 7, 30, 60, and 90 of frozen storage.

After the patties were thawed at room temperature (24–25°C) for 4 hr, pH determinations were made on duplicate samples from each treatment. A Radiometer 28 (Radiometer, Copenhagen, Denmark) pH meter equipped with an Orion 9163 probe (Orion Research, Cambridge, MA) was used.

Analysis of soluble orthophosphates was completed using the method of Dick and Tabatabai (1977) as adapted to meats by Molins et al. (1985). Lipid oxidation was determined by the thiobarbituric acid (TBA) test according to the method of Tarladgis et al. (1960). Color changes in phosphate-treated patties were monitored throughout the frozen storage period by determining Hunter L, a and b values on patties thawed at 24–25°C for 4 hr.

Proximate analysis (moisture, fat and protein) was performed on the first day of frozen storage (48 hr after manufacture) using AOAC (1980) methods.

Sensory evaluation of the treatments included juiciness, texture, flavor, and overall acceptability. On each sampling day, 10 patties per treatment were broiled on a gas grill set at 400°F (204°C) for 2.5 min on one side, turned, and cooked for additional 2 min to simulate a normal fast-food restaurant cooking operation procedure. Cooking losses were determined by weighing 5 of the 10 patties before and after cooking and recording the difference. All patties were cut into nine portions and presented on plates around tables to an untrained, taste panel consisting of an average of 35 college students. All treatments were sampled at each sensory panel. Water for rinsing between samples was provided. Flavor, texture, juiciness and overall acceptability were evaluated on a 7-point hedonic scale ranging from "like extremely" (score = 7) to "dislike extremely" (score = 1).

Data from four replications were analyzed using a Statistical Analysis System (SAS, 1982) computer program with analysis of variance and general linear model options. Comparison of means for treatment effects over the 90-day frozen storage period was based on Duncan's multiple range test.

RESULTS & DISCUSSION

MEAN pH VALUES of beef patties throughout the experimental period are listed in Table 1. TSPP increased the pH of the meat more than any other phosphate or blend tested, followed by Brifisol 614P>STPP>Brifisol 414B in solution>Brifisol 414P>Brifisol 414B powder>control.

Table 2 shows that there was also an immediate increase in the level of soluble orthophosphates present in the meat after the addition of all the phosphates tested. This increase was greatest in patties treated with TSPP and continued in all patties containing phosphates throughout the experimental period. This indicated that hydrolysis of the condensed phosphates proceeded even at a temperature as low as −20°C. Similar hydrolysis of STPP was reported by Sutton (1973) in cod and beef muscles.

There seemed to be a pattern corresponding to soluble orthophosphate content and pH such that higher orthophosphate levels in the patties paralleled higher pH values. This observation corroborates the findings of Awad (1968), who theorized that the ability of condensed phosphates to increase the

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Table 1—Mean pH values of beef patties (std error: 0.01)

Days at −20°C	Control[a]	414B powder[b]	414B soln.[bc]	414P powder[bc]	614P powder[d]	STPP powder[c]	TSPP soln.[e]
0	5.95	6.10	6.20	6.17	6.31	6.23	6.50
2	5.97	6.10	6.19	6.15	6.30	6.22	6.45
7	5.96	6.09	6.20	6.12	6.30	6.21	6.47
30	5.94	6.09	6.20	6.14	6.28	6.21	6.43
60	6.02	6.12	6.21	6.16	6.31	6.24	6.47
90	5.99	6.08	6.19	6.15	6.30	6.25	6.46

[a,b,c,d,e] Treatments with the same superscript letter are not significantly different (P>0.05).

Table 2—Mean soluble orthophosphate content (μg/g of meat) of beef patties (std error: 16)

Days at −20°C	Control[d]	414B powder[c]	414B soln.[d]	414P powder[bc]	614P powder[b]	STPP powder[a]	TSPP soln.[b]
0	817	904	836	952	1075	1186	1010
2	922	1084	1060	1090	1151	1292	1183
7	837	1042	1106	1037	1185	1307	1126
30	809	1017	1043	1109	1082	1206	1148
60	891	1167	1157	1065	1211	1250	1232
90	775	1152	1220	1094	1179	1301	1226

[a,b,c,d] Treatments with the same superscript letter are not significantly different (P>0.05).

Table 3—Mean TBA numbers of beef patties expressed as mg malonaldehyde/100g tissue (std error 0.01)

Days at −20°C	Control[a]	414B powder[b]	414B soln.[b]	414P powder[b]	614P powder[b]	STPP powder[b]	TSPP soln.[b]
2	0.96	0.28	0.30	0.34	0.30	0.30	0.25
7	1.19	0.23	0.28	0.26	0.26	0.27	0.26
30	1.11	0.21	0.25	0.30	0.26	0.27	0.24
60	1.20	0.29	0.33	0.33	0.34	0.35	0.33
90	1.29	0.30	0.37	0.39	0.32	0.35	0.31

[a,b] Treatments with the same supercript letter are not significantly different (P>0.05).

Table 4—Mean Hunter L, a, b values of beef patties (std error: 0.2 Hunter L; 0.1 Hunter a; 0.1 Hunter b)

Days at −20°C	Hunter value	Control[d]	414B powder[cd]	414B soln.[ab]	414P powder[ab]	614P powder[a]	STPP powder[ab]	TSPP soln.[bc]
2	L	39.1	39.0	39.7	39.7	40.4	39.9	40.3
	a	13.8	15.0	16.0	17.0	16.8	16.6	15.7
	b	9.9	10.1	10.4	10.5	10.4	10.5	10.0
7	L	41.4	40.6	40.2	39.8	40.4	40.6	41.0
	a	11.7	11.6	13.2	12.4	12.5	12.6	12.2
	b	9.6	6.9	7.4	9.5	9.5	9.5	9.4
30	L	41.0	41.1	40.5	39.4	40.9	41.1	41.0
	a	11.3	11.6	12.5	12.0	13.1	13.2	12.4
	b	9.7	10.0	9.8	9.6	9.8	10.3	10.2
60	L	40.6	41.7	40.1	40.1	39.9	41.2	40.9
	a	10.4	10.5	10.3	11.0	11.6	10.5	10.9
	b	8.6	9.0	8.6	8.7	8.9	8.4	9.0
90	L	39.3	37.9	38.2	37.6	39.5	38.3	38.3
	a	10.4	10.8	11.3	11.8	11.8	11.1	10.6
	b	9.3	9.7	9.5	9.5	9.7	9.8	9.6

[a,b,c,d] Treatments with the same superscript letter have no significantly different (P>0.05) Hunter a values. There are no treatment Hunter L and b value differences.

Table 5—Mean percent cook loss scores of beef patties (std error: 0.3 for cook loss)

Days at −20°C	Control[a]	414B powder[d]	414B soln.[cd]	414P powder[bc]	614P powder[bc]	STPP powder[bc]	TSPP soln.[cd]
7	21.9	13.7	18.4	16.0	18.4	18.9	15.4
30	26.5	19.8	19.4	21.9	20.2	24.3	21.6
60	25.2	16.4	18.0	21.1	19.9	20.9	18.1
90	25.0	16.1	18.7	19.4	20.0	23.1	18.2

[a,b,c,d] Means with the same superscript letter are not significantly different (P>0.05).

Table 6—Mean percent juiciness score of beef patties (std error: 0.04 for juiciness scores)

Days at −20°C	Control[d]	414B powder[a]	414B soln.[abc]	414P powder[bc]	614P powder[abc]	STPP powder[c]	TSPP soln.[ab]
7	4.0	4.7	4.5	4.4	4.5	4.3	4.7
30	4.0	4.8	4.6	4.6	4.7	4.1	4.8
60	3.7	5.0	4.8	4.2	4.4	4.3	4.6
90	3.9	5.0	4.6	4.5	4.6	4.5	5.1

[a,b,c,d] Juiciness scale: extremely juicy = 7, extremely dry = 1. Means with the same superscript letter are not significantly different (P>0.05).

Table 7—Mean overall acceptability scores of beef patties (std error: 0.03)

Days at −20°C	Control[b]	414B powder[a]	414B soln.[a]	414P powder[ab]	614P powder[a]	STPP powder[a]	TSPP soln.[a]
7	3.9	4.2	4.2	4.0	4.6	4.4	4.4
30	4.1	4.4	4.4	4.5	4.5	4.3	4.4
60	3.8	4.5	4.7	4.0	4.2	4.2	4.2
90	3.9	4.6	4.5	4.4	4.5	4.5	4.7

[a,b] Overall acceptability scale: like extremely = 7, dislike extremely = 1. Treatments with the same superscript letter are not significantly different ($P>0.05$).

pH of meat is related to the extent to which a particular phosphate is degraded to orthophosphates.

Thiobarbituric acid (TBA) values were significantly ($P<0.05$) lower in all phosphate-containing patties than in controls (Table 3). However, it is important to observe that TBA numbers in patties treated with phosphates were lower than in control patties since the first determination on day 0 of frozen storage, indicating that phosphate interference with the analytical method of Tarladgis et al. (1960) may have occurred. This result is in agreement with those of Koniecko (1979). It is not known whether the lower TBA values observed with phosphate addition were due to inhibition of further lipid oxidation during the distillation phase of the analysis, as observed by Rhee (1978) with other chelating agents like propyl gallate and ethylenediaminetetraacetic acid (EDTA) or otherwise.

Although the data are not presented, no differences ($P>0.05$), in proximate analysis could be attributed to any phosphate treatment.

Hunter L (lightness) and Hunter b (yellowness) values indicated that phosphates did not cause any significant ($P>0.05$) discoloration of beef patties (Table 4). Redness, measured by Hunter a values, was significantly ($P<0.05$) higher in all phosphate-treated patties. Frozen storage ($P<0.05$) affected color by causing lightness of the patties to increase and redness to decrease after seven days regardless of treatment.

Cooking losses were ($P<0.05$) higher in control samples than in any phosphate-containing patties, but no such differences existed among phosphate treatments (Table 5). Best cook yields were achieved with Brifisol 414B (powder). Storing the patties for seven days at −20°C resulted in ($P<0.05$) the lowest cooking losses in comparison to those held from 60 to 90 days. Frozen storage for 30 days resulted in the largest decrease in cook yields. Scores for juiciness (Table 6) were inversely related to those of cooking loss.

Phosphates did not significantly ($P>0.05$) improve texture or flavor scores. Data on texture and flavor scores are not presented.

Overall, treating beef patties with phosphates resulted in significant ($P<0.05$) improvements for overall acceptability (Table 7).

CONCLUSIONS

ALL PHOSPHATES studied increased the pH and the soluble orthophosphate content of beef patties. Hydrolysis of phosphates added to ground beef occurred even during frozen storage at −20°C.

Addition of phosphates appeared to significantly ($P<0.05$) reduce the development of oxidative rancidity in frozen beef patties, but there were indications that phosphates interfered with the TBA analytical method.

The phosphates studied did not affect proximate analysis of frozen beef patties but produced significantly ($P<0.05$) higher Hunter a values, cook yields and overall acceptability scores.

Cooking yield of frozen beef patties was best improved with addition of Brifisol 414B in powder form to a level of 0.4% (w/w) of meat weight.

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Ms received 1/16/86; revised 8/28/86; accepted 8/28/86.

Journal Paper No. J-12106 of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA Project No. 2252. The authors thank BK-Ladenburg Corporation for partial support of the study. The authors also thank Dianne B. James for the technical assistance provided and Bev Allfree for preparation of the manuscript.

Mention of any company or product name does not constitute endorsement.

Determination of Ascorbic Acid, Erythorbic Acid, and Uric Acid in Cured Meats by High Performance Liquid Chromatography

MARK A. KUTNINK and STANLEY T. OMAYE

ABSTRACT

A paired-ion reversed-phase high-performance liquid chromatographic procedure with amperometric detection was applied to the determination of ascorbic acid (AA), erythorbic acid (EA), and uric acid (UA) in 5% meta-phosphoric acid extracts of beef bologna, beef frankfurters, bacon, and ham cured with AA or EA. AA concentrations (mg/g meat) ranged from 0.008 in EA-cured beef frankfurters to 0.584 in AA-cured bacon. EA concentrations ranged from 0.149 in EA-cured beef frankfurters to 0.545 in EA-cured bacon. UA was quantified only in EA-cured bologna and frankfurters with 0.013 and 0.010 mg/g, respectively. Correlations of total ascorbate (AA + EA) by HPLC with fluorometric and colorimetric procedures were 0.992 and 0.988, respectively.

INTRODUCTION

ASCORBIC ACID (AA) and erythorbic acid (EA) are added to a wide variety of processed meats to inhibit nitrosamine formation, ensure proper color development and protect against fading (Fiddler et al., 1973; Mirvish et al., 1972; Mills et al., 1958). Structurally, EA and AA differ only in the orientation of the C-5 hydrogen and hydroxyl group. However, EA has only 5% of the vitamin activity of AA (Berk, 1976). Other differences in their properties have been reviewed elsewhere (Borenstein, 1965). Most nonchromatographic methods for determining ascorbate in foods, biological tissues, or pharmaceuticals are unable to distinguish between isomeric forms. However, several high-performance liquid chromatographic (HPLC) methods which can resolve these two isomers have recently been developed. Most are based on either weak anion exchange (Bui-Nguyen, 1980; Geigert et al., 1981) or paired-ion reversed-phase chromatography (Finley and Duang, 1981; Tsao and Salimi, 1982; Coustard and Sudraud, 1981).

We have applied a paired-ion reversed-phase HPLC method developed for AA and EA determinations in human plasma (Kutnink et al., 1985) to the determination of these components in meat products cured with AA or EA. The method uses an electrochemical detector, which is sensitive to sub-nanogram quantities of both isomers. Uric acid (UA) is also resolved, and, because it is also electrochemically active, can be quantified under the same chromatographic conditions used for AA and EA analysis. Urate has been shown to inhibit ascorbate oxidation *in vitro*, and may also protect tissues against oxidative damage *in vivo* (Lam et al., 1984; Ames et al., 1981).

The purpose of the present study is to examine the feasibility of using this procedure to determine AA, EA, and UA levels in meats cured with AA or EA and to compare the results with those obtained by colorimetric and fluorometric procedures for total ascorbate (AA + EA).

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MATERIALS and METHODS

Samples and sample preparation

Vaccum-sealed packages of beef frankfurters, sliced beef bologna, sliced bacon, and imported sliced ham cured with AA or EA were selected at random in local retail grocery stores. They were kept refrigerated, unopened, until the day of analysis. Extractions were made before product pull-dates expired. A 4g to 10g sample from the center part of internal slices was minced with scissors and divided into two portions of equal weight. For bacon and ham slices, only the lean parts were used. Each portion was immediately ground for 3 to 4 min in a mortar with 15 mL of a cold solution containing 5% (w/v) metaphosphoric acid (MPA; J.T. Baker Chemical Co., Phillipsburg, NJ) and 0.1 mg/mL disodium ethylenediaminetetraacetic acid (EDTA; Sigma Chemical Co., St. Louis, MO) with and without the addition of AA (Hoffmann-La Roche, Inc., Nutley, NJ), EA (ICN Nutritional Biochemicals, Cleveland, OH), and UA (Fisher Scientific Co., Pittsburgh, PA) for recovery determinations. Spike concentrations were as follows: AA-cured products (bacon, ham) were extracted with an MPA-EDTA solution containing 40 μg/mL AA and 1 μg/mL UA; EA-cured products (bologna, frankfurters, bacon) were extracted with an MPA-EDTA solution containing 3 μg/mL AA and UA and 50 μg/mL EA. The extracts were then centrifuged at 1500g for 10 min. Prior to HPLC analysis, supernatants were diluted 10-fold with mobile phase (see below for composition) or 40- to 100-fold with mobile phase containing 0.5% (w/v) MPA and filtered through Acro-LC13 disposable 0.2 micron filter assemblies (Gelman Sciences, Ann Arbor, MI). For fluorometric and colorimetric analyses, the supernatants were diluted with 5% and 2.5% MPA, respectively.

HPLC standards and standard preparation

AA and EA stock solutions were made daily by dissolving 10 mg of each isomer in 100 mL cold mobile phase (see below for composition) containing 0.5% (w/v) MPA. A UA stock solution was made by dissolving 10 mg UA in 50 mL water containing 0.25 mL 2N NaOH, and bringing the volume to 100 mL with water. The stock standard solutions were diluted with the cold mobile phase-MPA solution to make four working standard solutions such that 10 μL contained 2, 5, 10, or 20 ng AA and/or EA and 2, 5, 10, or 20 ng UA. These solutions were filtered through disposable 0.2 micron filters prior to HPLC analysis.

Chromatography

A detailed description of the chromatographic procedure has been given elsewhere (Kutnink et al., 1985). Briefly, the stationary phase was a 4.6 mm by 25 cm Altex Ultrasphere ODS (C18) analytical column and a 3 cm Brownlee RP-18 guard column (Rainin Instrument Co., Woburn, MA). Particle size of both columns was five microns, spherical. The aqueous mobile phase contained 0.04M sodium acetate (Mallinckrodt, Inc. Paris, KY), 0.005M tetrabutylammonium phosphate (TBAP, Waters Associates, Milford, MA), 0.2 mg/mL disodium EDTA, and was titrated to pH 5.25 with glacial acetic acid.

Analyses were performed with a 1084B liquid chromatograph (Hewlett-Packard, Santa Clara, CA) and an LC4B amperometric controller with a glassy-carbon electrode (Bioanalytical Systems, West Lafayette, IN). Elution was isocratic with continuous recycling of the detector-cell effluent into the mobile phase reservoir. For samples and standards known to contain only AA and/or UA a flow rate of 0.8 mL/minute was used, otherwise the flow rate was 0.4 mL/minute. The applied electrode potential was +0.6 volts (vs Ag/AgCl reference), with a sensitivity range of 100 nanoamps. The column temperature was 30°C. Under these conditions, the baseline offset current

STANDARDS

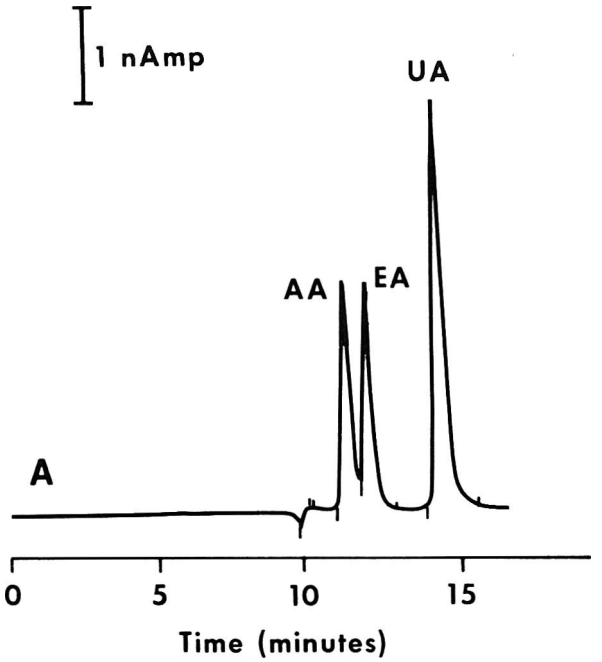

Figure 1A

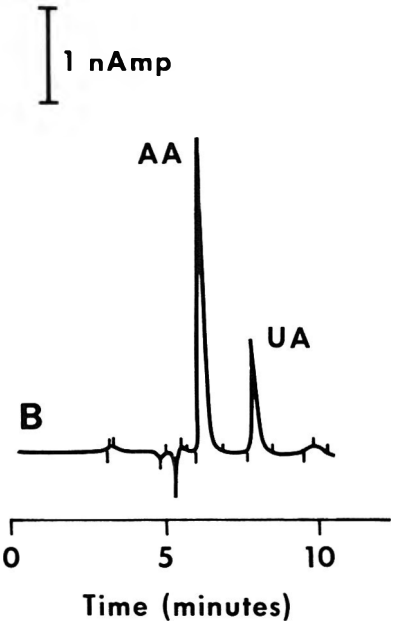

Figure 1B

Fig. 1—(A) Chromatogram of a 10 μL injection containing 2.5 nanograms each of AA, EA, and UA, standards. Mobile phase flow rate is 0.4 mL/min; (B) Chromatogram of a 10 μL injection containing 2.0 nanograms AA and 0.5 nanograms UA standards. Mobile phase flow rate is 0.8 mL/min.

Table 1—Ascorbic, erythorbic, and uric acid concentrations in cured meats by HPLC

	Ascorbic acid mg/g meat		Erythorbic acid mg/g meat		Uric acid mg/g meat	
	Within extract	Between extracts	Within extract	Between extracts	Within extract	Between extracts
Imported sliced ham (AA-Cured)	0.117(3.0)[a]	0.108(8.6)	—	—	—	—
Beef frankfurters (EA-Cured)	0.009(4.6)	0.008(0.8)	0.171(0.8)	0.160(9.3)	0.010(0)	0.010(0.7)
Sliced beef bologna (EA-Cured)	0.011(0.7)	0.011(4.4)	0.182(0.4)	0.194(9.5)	0.013(1.1)	0.013(3.8)

[a] Mean (%C.V.), n = 2

Table 2—Ascorbic and erythorbic acid concentrations in cured meats by fluorometric, colorimetric, and HPLC methods

	HPLC		Fluorometric	Colorimetric
	AA mg/g meat	EA mg/g meat	(AA + EA) mg/g meat	(AA + EA) mg/g meat
Imported sliced ham (AA-Cured)	0.101	—	0.103(1.6)[a]	0.084(1.0)
Beef frankfurters (EA-Cured)	0.008	0.149	0.150(2.7)	0.117(1.1)
Sliced beef bologna (EA-Cured)	0.012	0.207	0.216(1.4)	0.217(0)
Sliced bacon (AA-Cured)	0.584	—	0.560(0.9)	0.544(1.3)
Sliced bacon (EA-Cured)	0.023	0.545	0.630(1.4)	0.632(0.8)

[a] Mean (%C.V.), duplicate analyses of same extract.
For HPLC, n = 1.

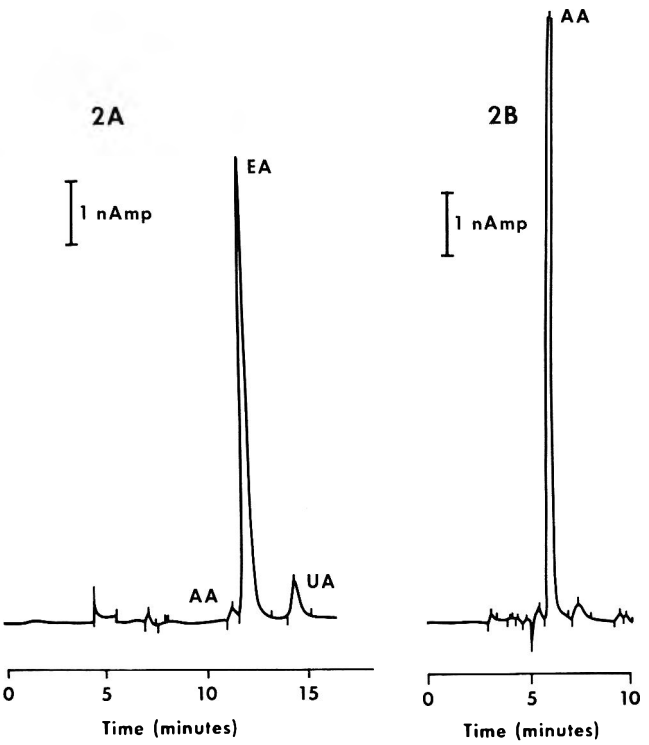

Fig. 2—(A) Chromatogram of 10 μL of a 100-fold diluted EA-cured beef frankfurter extract. Mobile phase flow rate is 0.4 mL/min; (B) Chromatogram of 10 μL of a 40-fold diluted AA-cured ham extract. Mobile phase flow rate is 0.8 mL/min.

was 2 to 6 nanoamps. A 10 μL injection volume was routinely used for both standards and diluted meat sample extracts.

Fluorometric and colorimetric procedures

Meat sample extracts preserved in 5% MPA were analyzed for total ascorbate (AA + EA) by a fluorometric procedure based on the oxidation of ascorbate to dehydroascorbic acid and the condensation of this compound with o-phenylenediamine to form fluorescent quinoxaline (Omaye et al., 1979).

Extracts were also analyzed for total ascorbate (AA + EA) by an automated continuous-flow procedure based on the reduction by ascorbate of 2,6-dichloroindophenol (DCIP, E. H. Sargent and Co., Skokie, IL) at pH 3.5 (Sauberlich et al., 1976). The dye reduction was accompanied by a color change monitored at 505 nm.

RESULTS & DISCUSSION

HPLC standard curves

Calibration curves obtained by least square linear regression for HPLC determinations of AA, EA and UA standards at both flow rates were linear over the range of 2 to 20 nanograms per injection, with correlations of nanograms versus peak area ranging from 0.9994 to 1.000.

At the 0.4 mL/min flow rate, peak area reproducibility, expressed as percent coefficient of variation (C.V.) for each component at all standard levels (2, 5, 10, and 20 nanograms), varied from 1.7 to 6.7 for six standard curves run on six separate days over a 4 wk interval. Variations in AA, EA, and UA retention times (%C.V.) were 1.2, 1.3, and 1.0, respectively. At the 0.8 mL/min flow rate, peak area variations for AA and UA at the 2, 5, and 10 nanogram levels ranged from 5.6 to 10.7%; retention time variations were 1.7 and 1.0% for AA and UA, respectively.

The presence of EA in standards or samples required a flow rate of 0.4 mL/min for improved resolution of the two isomers. Typical chromatograms of AA, EA, and UA standards at these flow rates are shown in Fig. 1A and 1B. Although the pen deflection was off-scale for high standard levels, the detector-integrator response remained linear and within the 100 nanoamp sensitivity setting for all three components. A higher attenuation would allow peak heights rather than areas to be used for quantitative purposes.

Processed meat samples

Although the chromatographic procedure allowed direct and simultaneous determination of AA, EA, and UA standards, processed meat sample extracts usually required analysis at two dilutions: a tenfold dilution for analysis of UA and endogenous AA in EA-cured meats and a 40-fold or 100-fold dilution for determination of the ascorbate isomer added in processing. Injection volumes were kept small and constant (10 μL) to avoid exposing the system to more MPA than necessary. Chromatograms of an EA-cured beef frankfurter extract and an AA-cured ham extract are shown in Fig 2. An MPA peak with a retention time of about 25 min is not shown in these chromatograms. Beef bologna extract chromatograms were similar to those of beef frankfurter extracts.

The extracts of ham and bacon cured with AA contained small amounts of a substance which eluted in a broad peak slightly ahead of, and as a leading shoulder to, added UA. The chromatogram of this bacon extract also had a small peak which appeared slightly behind and as a trailing shoulder to added EA. The identities of these substances are not known. Their concentrations in the meat extracts are not high enough to allow stopped-flow ultraviolet scans of their peaks to be made.

Table 1 shows AA, EA, and UA levels found in imported sliced ham cured with AA and EA-cured beef frankfurters and beef bologna. Within-extract concentrations are for duplicate HPLC analyses of the same extract. Between-extract concentrations are for HPLC analyses of extracts from two packages made on different days. The HPLC method determined the concentration of the reduced form of the isomers only.

The colorimetric and fluorometric procedures do not distinguish between AA and EA. The isomer concentrations determined by HPLC are combined for comparison with these methods. For the five products shown in Table 2, the correlation between colorimetric and HPLC procedures was 0.988, while the correlation between fluorometric and HPLC procedures was 0.992. The fairly good agreement of ascorbate levels measured by two methods which do not detect dehydro forms (HPLC and colorimetric) with levels obtained by a third method which does (fluorometric) suggests that most of the ascorbate in the samples was in the reduced forms.

AA recoveries were 90.7 and 94.4% for AA-cured ham and bacon, respectively. In the three EA-cured products, AA recoveries were 85.0, 88.8, and 87.7% for beef frankfurters, beef bologna, and bacon, respectively, while their respective EA recoveries were 90.6, 92.4, and 94.0%. UA recoveries in the EA-cured frankfurters and bologna were 90.3 and 92.7%, respectively.

A small, late-eluting peak present in all samples and standards was associated with the preservative, MPA. By synchronizing injections so that the MPA peak from the previous analysis was eluted in the early part of the next chromatogram, analysis time was decreased by about 50%.

Because the electrode system often required several hours to re-stabilize after being shut-off, it was more efficient and economical to recycle the mobile phase by directing the electrode flow-cell effluent back into the mobile phase reservoir, which was slowly stirred with a magnetic stir-bar. Continuous recycling of 1L of mobile phase for 3 to 4 days had no effect on the chromatographic baseline or column pressure.

On two new Ultrasphere ODS columns, AA, EA, and UA were not adequately resolved with TBAP as the ion-pairing agent. This was corrected by using an agent with a longer carbon chain (Sood et al., 1976).

We have obtained good resolution of all three components at a 1 mL/min flow rate with dodecyltriethylammonium phos-

phate (Regis Chemical Co., Morton Grove, IL) at a concentration of 1.5 mM in a mobile phase containing 0.04 M sodium acetate, 0.54 mM disodium EDTA, 7.5% methanol, and titrated to pH 4.75 with glacial acetic acid. UA is eluted well before AA and EA under these conditions. This ion-pairing agent is compatible with MPA.

However, some ion-pairing agents (n-octylamine, n-decylamine, hexadecyl-trimethylammonium bromide) formed insoluble complexes with MPA when the mobile phase was used to dilute the MPA extracts prior to analysis. Addition of methanol to the mobile phase did not prevent this. Similar results have been observed with the ion-pairing agent tridecylammonium formate and MPA extracts of fruits and vegetables (Watada, 1982).

The results demonstrate the applicability of HPLC to the determination of AA and EA in meats cured with these substances. In addition, the sensitivity of the electrochemical detector allows endogenous AA to be quantified in extracts of EA-cured meats. The determination of EA concentrations may be of nutritional interest because this substance has little vitamin activity, but is a common food additive.

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Ms received 11/18/85; revised 6/19/86; accepted 7/5/86.

The authors thank Debra Huerta and Dennis O'Connor for conducting the colorimetric and fluorometric ascorbate analyses.

Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

COOKED BEEF AROMA COMPOUNDS. . .From page 45

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We are grateful to W.G. Gunn and A.E. Cakebread for operating the GC-MS and data processing system, and also to the Proctor and Gamble Company, USA for financial support.

Cholesterol Oxides in Swedish Foods and Food Ingredients: Fresh Eggs and Dehydrated Egg Products

JAFFAR NOUROOZ-ZADEH and LARS-ÅKE APPELQVIST

ABSTRACT

Cholesterol oxides were enriched from lipid extracts of fresh and dehydrated egg yolk products by chromatography on Lipidex and TEAP-Lipidex. Appropriate fractions from TEAP-Lipidex were analyzed by capillary GLC as their TMS derivatives. At the detection limit level, ca 0.2 ppm in total lipids, no cholesterol oxides could be detected in fresh egg yolk. Similarly, spray dried egg yolk powder contained only traces of cholesterol oxides when fresh or stored for 2 months at 4°C. Prolonged storage gave lipid extracts which contained variable levels (0.2–12 ppm) of known oxidation products, viz., cholest-5-ene-3β, 7α-diol, cholest-5-ene-3β, 7β-diol, 5,6α-epoxy-5α-cholestan-3β-ol, 5,6 β-epoxy-5 β-cholestan-3β-ol, cholest-5-ene-3β, 20α-diol, 3 β-hydroxycholest-5-en-7-one. Traces or small quantifiable amounts of cholest -5-ene-3β, 25-diol and 5α-cholestane-3β, 5, 6β-triol were observed in some samples at longer storage periods.

INTRODUCTION

CHOLESTEROL easily undergoes autoxidation and a wide array of reaction products are formed. In a comprehensive treatise covering the literature up to 1980, the complete or partial structure of some 70–80 products are recorded (Smith. 1981). Several of these compounds have been found to possess undesired biological effects, recorded at the enzymic, cellular or the tissue level of experimental animals given a concentrate of cholesterol oxides or single pure compounds. Such effects are: feedback inhibition of cholesterol biosynthesis at the 3-hydroxy-3-methylglutaryl-Coenzyme A (HMG-CoA) reductase level, cytotoxicity, angiotoxicity and mutagenicity (Kandutsch, 1980; Smith, 1981; Peng and Taylor, 1983). There appear to be at least two separate groups of biological effects which have been suggested to result in two classes of disorders, viz., atherosclerosis and cancer.

Exogenous 25-hydroxycholesterol is resorbed in mammals and causes defects in the aortic surface as revealed by scanning electron microscopy (Peng et al., 1982). Several hydroxylated cholesterol derivatives are more or less potent inhibitors of HMG-CoA reductase in the aortic cells. A high reduction in cholesterol biosynthesis causes cell death because of membrane dysfunction (angiotoxicity, cytotoxicity). The dead cells could be the primary area for lipid infiltration leading to atherosclerosis. The deleterious effects of the hydroxy-derivatives are discussed in several recent papers (Peng and Taylor, 1983; Jacobson et al., 1985; Peng et al., 1985; Parish et al., 1986).

The possible association between dietary intake of 5,6α-epoxy-5α-cholestan-3β-ol and development of cancer, although supported by only cytotoxicity studies and indirect relations, has caused considerable concern among food scientists. The carcinogenicity of cholesterol epoxides has been implicated in a few toxicological studies (Chan and Chan, 1980; Sevanian and Peterson, 1984).

Although the presence of individual cholesterol oxides in various foods has been reported (Smith, 1981; Finocchario and Richardson, 1983), no data were available in the literature on the quantities of the most important cholesterol oxides in food or food ingredients produced with well-defined technologies and stored under controlled conditions at the initiation of our studies. The present paper is the first in a series which deals with cholesterol oxides in Swedish foods and food ingredients. The aim of this study was to obtain data on the level of some important oxidation products of cholesterol in fresh eggs and some dehydrated egg products. A preliminary account of part of this work has recently been presented elsewhere (Appelqvist and Nourooz-Zadeh, 1986).

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MATERIALS & METHODS

Food samples

Fresh eggs were purchased from a local supermarket and freeze-dried egg yolk powder was prepared from fresh egg yolk in the laboratory. Spray-dried egg-yolk powder and egg-yolk mix powder (Petit choux) were received from Källberg Industrier AB (Töreboda, Sweden; company A in Table 1) and AB Svenska Äggprodukter (Helsingborg, Sweden; company B in Table 1). The samples were kept in sealed plastic bags in a dark room at a temperature of +4°C until analysis.

Reagents

5α-cholestane, cholest-5-ene-3β-ol(cholesterol), 5,6α-epoxy-5α-cholestan-3β-ol(5α-,6α-epoxycholestanol), 3β-hydroxycholest-5-en-7-one(7-ketocholesterol) and 5α-cholestane-3β,5,6β-triol were purchased from Sigma Chemical Co., St. Louis, MO; cholest-5-ene-3β,7α-diol(7α-hydroxycholesterol),cholest-ene-3β,7β-diol (7β-hydroxycholesterol), cholest-ene-3β,20α-diol(20α-hydroxycholesterol) and cholest-5-ene-3β,25-diol(25-hydroxycholesterol) were obtained from Steraloids, Inc. (Wilton, NH). 5,6β-Epoxy-5β-cholestan-3β-ol (5β-,6β-epoxy-cholestanol) was a gift from Professor Peter Eneroth, Karolinska Hospital, (Stockholm, Sweden). Hexane, methanol, 1,2-dimethoxyethane, chloroform, epi-chlorhydrin, boron trifluoride, and triethylamine were purchased from E. Merck (Darmstadt, FRG). 1,2-Dichloroethane was obtained from Fischer Scientific. Tri-Sil was obtained from Pierce Chemical, Rock Ford, IL, and Lipidex 5000 was purchased from Packard Instrument Company, Inc., (Downers Grove, IL).

Triethylaminohydroxypropyl-Lipidex (TEAP-Lipidex) synthesis

TEAP-Lipdex is an ion exchange derivative of Lipidex 5000 and was prepared as described by Axelson et al. (1977) and Tetsuo et al. (1980). An appropriate amount of Lipidex 5000 was freed from the storage solvent, ethanol, dried at 18°C and allowed to swell in dichloromethane (6 mL/g gel) for 30 min under careful stirring. Boron trifluoride (0.15 mL/g gel) and epichlorhydrin (0.64 mL/g gel) were slowly added under stirring for a further 30 min. The gel was subsequently washed on a Buchner funnel with 1.0L 75% ethanol, 1.0L chloroform and 1.0L 100% ethanol and was dried at 18°C. A 15% substitution was obtained as judged from weighing.

The gel was allowed to swell in isopropanol (3.5 mL/g gel) under stirring for 30 min. Water (3.5 mL/g gel) was added and the mixture stirred for another 30 min. Solid NaOH (0.45 g/g substituted gel) was dissolved in water (5 mL/g total gel) and an equal volume of isopropanol was added. This mixture was pooled with the gel slurry and the stirring was continued for 30 min. Triethylamine (4 mL/g gel) was slowly added under stirring. The temperature was increased to 55°C and the mixture stirred for 4 hr. The gel was transferred into a Buchner funnel and freed from the solvent. It was washed with 1L 0.5M NaOH in 72% ethanol and then with 20% ethanol until neutral (pH 5), followed by 50% and 100% ethanol. The ion exchange capacity was 0.6 meq/g gel as judged by titration (Axelson et al., 1976). The gel was

Table 1—Cholesterol in fresh eggs, dehydrated eggs and egg mixes

Company[a]	Product	Storage time	Cholesterol oxides, ppm in lipids[b]							
			5α-, 6α-epoxy	5β-, 6β-epoxy	7-keto	7α-hydroxy	7β-hydroxy	20α-hydroxy	25-hydroxy	5,6-dihydroxy
–	Fresh egg yolk	0	ND[c]	ND	ND	ND	ND	ND	ND	ND
–	Freeze dried egg yolk	0	ND	ND	TR[d]	ND	ND	ND	ND	ND
A	Dehyd egg mix	2 months	ND	ND	ND	ND	ND	ND	ND	ND
A	Dehyd egg mix	6 months	ND	ND	ND	ND	ND	ND	ND	ND
A	Dehyd egg mix	12 months	0.3	2.7	0.6	0.7	0.2	ND	ND	ND
A	Dehyd egg mix	18 months	0.4	3.2	1.5	1.2	1.0	ND	ND	ND
A	Dehyd egg yolk	2 months	ND	ND	TR	TR	TR	ND	ND	ND
A	Dehyd egg yolk	12 months	2.3	9.8	2.4	7.6	9.8	0.9	ND	TR
A	Dehyd egg yolk	8 years[e]	9.4	ND[f]	5.7	27.5	46.8	6.6	10.4	27.6
B	Dehyd egg yolk	0	TR	ND	ND	TR	TR	ND	ND	ND
B	Dehyd egg yolk	2 months	ND	ND	TR	TR	TR	ND	ND	ND
B	Dehyd egg yolk	6 months	1.3	6.5	3.5	2.2	2.5	0.8	ND	ND
B	Dehyd egg yolk	12 months	2.5	12.0	2.9	8.9	9.4	0.7	ND	TR

[a] A = AB Svenska Äggprodukter, Helsingborg, Sweden; B = Källberg Industrier AB, Töreboda, Sweden.
[b] Means of duplicate analysis (extraction, enrichement and GLC).
[c] Not detected, detection limit ca 0.2 ppm in lipids.
[d] Traces (ca 0.1–0.2 ppm).
[e] Unrecorded conditions (18°C or +4°C or −20°C for different time periods).
[f] Interferences made the quantitation impossible.

converted into the acetate form by washing with 1L 10% acetic acid in 72% ethanol, 20% ethanol until neutral, and subsequently with 50% and 100% ethanol. Finally, it was stored at -20°C in 100% ethanol.

Lipid extraction

Six egg yolks or a 5g egg sample (dehydrated egg yolk, dehydrated egg yolk mix) was placed in a separatory funnel, 90 mL hexane/isopropanol (3/2, v/v) (HIP) was added and the funnel was shaken vigorously (Hara and Radin, 1978). The suspension was filtered through a sintered glass Buchner funnel which was washed three times with 15 mL of HIP (3/2). The combined filtrate was transferred to another separatory funnel and 60 mL 0.47M Na_2SO_4 was added. The upper layer was transferred into an evaporation flask and the lipid was taken to dryness at a temperature of 30°C in a rotary evaporator. Egg yolk lipid (100—150 mg) was weighed and stored in hexane at 4°C. Prior to loading the Lipidex 5000 column, the hexane solution was taken to dryness under a stream of nitrogen and the lipid redissolved in 0.3 mL of hexane/1,2-dichloroethane (9/1,v/v).

Gel filtration

An amount of 10g Lipidex 5000 was transferred to a Buchner funnel and freed from the storage solvent, ethanol. The gel was washed consecutively with 20%, 50%, and 100% ethanol at 70°C in a beaker with continuous stirring and then washed again on the Buchner funnel with 100% ethanol and with hexane/1,2-dichloroethane (9/1,v/v) at four times the gel volume. The gel was allowed to swell in the actual chromatographic solvent for 2 hr. The slurry was then poured into two 50 × 1.0 cm columns which were allowed to settle through sedimentation at 4°C. The column was washed with several column volumes of the solvent and nitrogen pressure (1.0 kg/cm^2) was applied to achieve a uniform packing. The different lipid classes eluted as shown in Fig. 1. The cholesterol oxides were collected between 3.4 and 4.5 column volumes. This was slightly different from the figures reported by Appelqvist et al. (1986). The lipid was taken to dryness at a temperature of 30°C in a rotary evaporator. The residue was dissolved in hexane/1,2-dichloroethane (3/7,v/v), transferred into a glass-stoppered test tube and kept at 4°C.

Ion exchange chromatography

An amount of 5–7g of TEAP Lipidex gel was poured into a Buchner funnel and freed from the storage solvent, ethanol. The gel was washed with several portions of 0.3M NaOH in 72% ethanol. It was subsequently allowed to swell in hexane/1,2-dichloroethane (3/7,v/v) for 30 min. The slurry was poured into two 20 × 0.45 cm columns with a solvent reservoir where the gel was allowed to settle by gravity. The gel was washed with several column volumes of the actual solvent for chromatography and nitrogen pressure (0.75 kg/cm^2) was applied to achieve a compact packing. Shortly before loading onto the column, the sample was dried under a nitrogen stream and then dissolved in 0.1 mL hexane/1,2-dichloroethane(3/7, v/v). The cholesterol oxides were collected in fractions as illustrated in Fig. 1.

Thin layer chromatography

Thin layer chromatography (TLC) was performed using silica gel 60 F_{254}-HPTLC plates (E. Merck. Darmstadt, FRG). The samples were applied by an automatic applicator, CAMAG Linomat III, together with standards. The plate was developed in diethyl ether/cyclohexane (9/1,v/v), dried at 18°C and viewed under UV light (254 nm) to detect the fluorescence-quenching compound, 7-ketocholesterol. Then it was sprayed with 50% H_2SO_4 and heated at 110°C for 5 min for color development. The cholesterol oxides were identified by their position and the color on the TLC plate (Smith et al., 1967).

Saponification

The cholestane-triol fraction was dried with a rotary vacuum evaporator at 30°C. The sample was dissolved in diethyl ether and transferred into glass-stoppered test tubes. Cholestane (0.2 μg) was added and the solvent dried under a stream of nitrogen. The sample was then dissolved in 1 mL 2M NaOH in 95% ethanol and saponified by refluxing for 1 hr, cooled and diluted with 2 mL water. The triol was extracted three times with 4 mL diethyl ether. The combined extracted was washed twice with water and the ether extract was concentrated under a nitrogen stream.

Derivatization of sterols to TMS ethers

The cholesterol oxide fractions from TEAP-Lipidex were transferred from the evaporation flask to glass-stoppered test tubes and cholestane (0.2 μg) was added as an internal standard. The solvent was dried under a stream of nitrogen, Tri-Sil (100 μL) was added and the sample was dissolved in the TMS reagents by shaking, and heated at 60°C for 30 min. Subsequently, the TMS reagents were removed under nitrogen and the residue was dissolved in hexane (100 μL). One-two microliters were injected into the gas chromatograph.

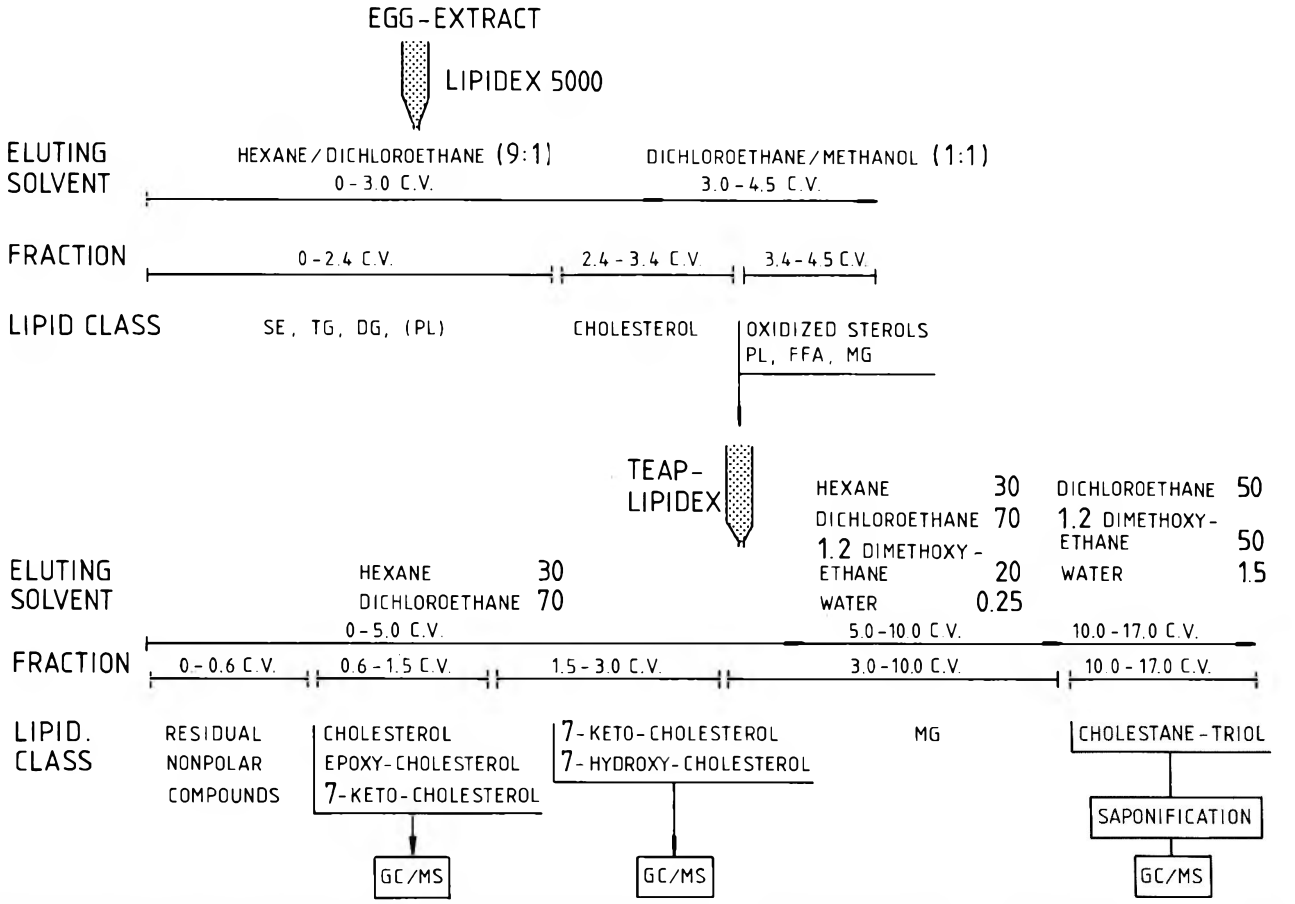


Fig. 1—Enrichment and isolation of cholesterol oxides. Abbreviations: C.V. = column volumes; SE = sterol esters; TG = triacylglycerols; DG = diacylglycerols; MG = monoacylglycerols; PL = phospholipids; FFA = free fatty acids

Gas chromatography

Gas chromatography (GLC) was carried out in a Varian 3700 equipped with a flame ionization detector (FID) and falling needle injector. The cholesterol oxides were separated as TMS ethers on a cross-linked methyl silicone column, Ultra hp, 25m × 0.33 mm, film thickness 0.35μ. Helium was used as carrier gas. Operating conditions were column temperature 280°C, detector temperature 310°C, flow rates, helium 1.80 mL/min, air 300 mL/min, hydrogen 30 mL/min, make-up 30 mL/min. The peaks were recorded by Hp 3390 A Computing Integrator (Hewlett Packard, Avondale, PA).

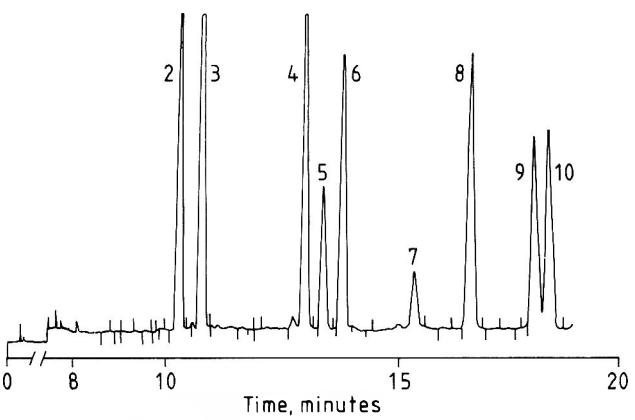
Gas chromatography-Mass spectrometry

Gas chromatography-mass spectrometry (GC-MS) was performed with a Finnigan 4000 GC-MS MAT (San Jose, CA), equipped with a falling needle injector. The column was chemically bonded methyl silicone, 25m × 0.2 mm, film thickness 0.2 μ (Quadrex Corp., New Haven, CT). Helium was used as carrier gas. The spectra were obtained using electron impact ionization at 40 eV and scan rate 1 sec/scan. Operating conditions were column temperature 260°C, interface temperature 260°C, ion source temperature 260°C.

RESULTS & DISCUSSION

Isolation and identification of fractions rich in cholesterol oxides

The complete separation by capillary GLC of all relevant cholesterol oxides is not easily achieved. Almost baseline separation of eight important oxidation products, namely those presented in Fig. 2, was obtained after extensive elaboration of the gas chromatographic parameters. The very good separation obtained was noteworthy, since it has been often claimed that GLC analysis of TMS derivatives of cholesterol oxides may result in an incomplete separation with one or other critical pairs overlapping. In the paper in which the methodology was established (Appelqvist et al., 1986), it was found that cholest-5-ene-3β,7α-diol was not fully separated from cholest-5-ene-3β-ol (cholesterol). Another overlapping pair was reported by Fischer et al. (1985), who could not resolve the very important isomeric forms of 5,6-epoxides. It appears that such



2- Cholest-5-ene-3β,7α-diol (7α-hydroxycholesterol)
3- Cholest-5-ene-3β-ol (cholesterol)
4- Cholest-5-ene-3β,7β-diol (7β-hydroxycholesterol)
5- 5,6β-Epoxy-5β-cholestan-3β-ol (5β-,6β-epoxycholestanol)
6- 5,6α-Epoxy-5α-cholestan-3β-ol (5α-,6α-epoxycholestanol)
7- Cholest-5-ene-3β,20α-diol (20α-hydroxycholesterol)
8- 5α-Cholestane-3β,5,6α-triol (cholestane-triol)
9- 3β-Hydroxycholest-5-en-7-one (7-ketocholesterol)
10- Cholest-5-ene-3β,25-diol (25-hydroxycholesterol)

Fig. 2—Gas chromatogram of TMS derivatives of synthetic cholesterol oxides.

a complete separation as reported by us has only been reported previously for cholesterol oxides in food by Missler et al. (1985).

When subjecting an egg powder extract to a slight modification of the procedure for isolation of cholesterol oxides developed by Appelqvist et al. (1986), a gas chromatographic pattern such as the one presented in Fig. 3 was obtained.

The identification of cholesterol oxides was based on RRT values of sample TMS ethers compared to those of synthetic standards and with comparison of the mass spectra of standards to those of the relevant GLC fractions. The mass spectral identification of the peaks in a chromatogram such as the one presented in Fig. 3 was deemed necessary for each product type to establish full identity. Since difficulties have been found in the separation of the 5α,6α- and 5β, 6β-isomers of epoxides and the α-isomer is suspected to be carcinogenic, particular emphasis was placed on the complete identification of the α-epoxy compound. A comparison of the higher mass portions of the spectra of synthetic α-epoxide and synthetic β-epoxide to those of the compounds from an egg yolk extract with the identical RRT values amply demonstrates that the compounds of the egg yolk extracts are identical to those of the standards (Fig. 4).

Figures given for the quantitative composition of the cholesterol oxides were based on a comparison of the peak areas obtained in a gas chromatograph equipped with a flame ionization detector. No correction for possible differences in response factors was made. Park and Addis (1985) reported relative response factors for TMS ethers of some cholesterol oxides to be 0.98 to 1.13 with that of cholesterol = 1.00. Since the mass spectra were recorded for all peaks of importance from all sample extracts and found to be identical to those of corresponding standards, we are confident that the predominant, if not the total mass recorded under the peak with the FID represents the TMS derivative of the sterol oxides indicated in Tables 1 and 2.

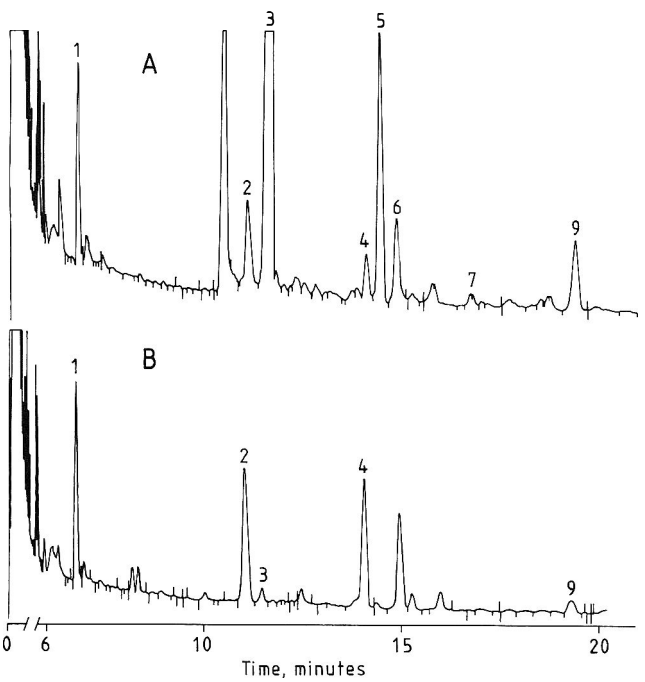


Fig. 3—Gas chromatogram of TMS derivatives of cholesterol oxides isolated from dehydrated egg yolk. A = TEAP-Lipidex fraction number 2; B = TEAP-Lipidex fraction number 3.

Tsai and Hudson (1985) reported a specific difference in the mass spectral pattern of the nonderivatized isomeric epoxides. Under these conditions, using TMS derivatives, no obvious differences between the mass spectra of isomers of the epoxides could be found.

Quantitative results

Using the method discussed above, a number of samples including fresh egg yolk and egg yolk lyophilized in the laboratory as well as various commercial samples was analyzed. As a reference, an 8-year-old sample of egg yolk powder, known to yield high amounts of high cholesterol oxides, was used. No quantifiable levels of any of these eight typical cholesterol oxidation products, which were identified in the 8-year-old sample (Table 1), were found when analyzing fresh egg yolk. The minimum level of quantitative measurement was about 0.2 ppm in the lipids, which was established by spiking with known standards. Freeze-drying of egg yolk in the laboratory caused the formation of only trace amounts of 3β-hydroxycholest-5-en-7-one.

Analysis of dehydrated egg yolk from the two different manufacturing plants in Sweden and one type of dehydrated egg mix indicated that fresh products and those stored for only 2 months contained traces of a few of the oxidation products (Table 1). The Petit-choux mix, which contained substantial amounts of vegetable oil in addition to lipids from egg yolk powder, was free from oxidation products at 2 and 6 months of storage. The patterns at 12 and 18 months were dominated by the epoxides and the levels at 18 months were slightly higher than at 12 months. No side-chain hydroxylated derivatives could be observed with the Petit-choux mix. The ratio of β- to α-epoxide was ca 10 to 1.

The storage of egg yolk powder in closed plastic bags at 4°C in the laboratory caused an elevation of the level of several of the oxidation products. The levels at 12 months were rather similar to the products from the two Swedish companies. With this product type, the 7-hydroxycholesterols were the major components, followed by the epoxides. These powders also had quantifiable amounts of 20 α-hydroxycholesterol. The ratios of β-epoxide to α-epoxide were approximately 5 to 1. The levels at 6 months were lower than at 12 months but no conclusion can be drawn on the progression of the formation of cholesterol oxides in egg yolk powder when stored at 4°C.

Presently, egg yolk powders are stored by the manufacturer at ambient temperature for several months, up to a year. Storage under refrigeration conditions, however, could also be considered. A few samples stored in the egg powder manufacturing plant were analyzed but no samples from a planned storage experiment were available.

The 3-month-old, spray-dried, egg yolk powder stored in the plant at 5–8°C had measurable levels of four compounds, considerably more than the 2-month-old sample stored at 4°C (compare Tables 1 and 2). Two different batches of 14-month-old yolk powder, manufactured on the same day, were available. These had been stored for 3 months at ambient temperature (ca 22°C) and then for 11 months at 5–8°C and contained similar levels of cholesterol oxides. The similarities between the results, representing duplicates in extraction, liquid chromatography and GLC analysis, may be taken as an indication of the precision of the method. The levels were lower than those of the 12-month-old samples stored at 4°C in the laboratory. It is noteworthy that the ratio of β-epoxides to α-epoxides and the ratio of 7β-hydroxy- to 7α-hydroxycholesterol was higher in lab-stored samples compared to those stored under industrial conditions. The differences noted between industrial storage and laboratory storage may be a reflection of differences in package size and type as well as moisture levels during storage.

Samples stored at ambient temperature for 3 and 5 months had substantial levels of oxidation products (Table 2). Com-

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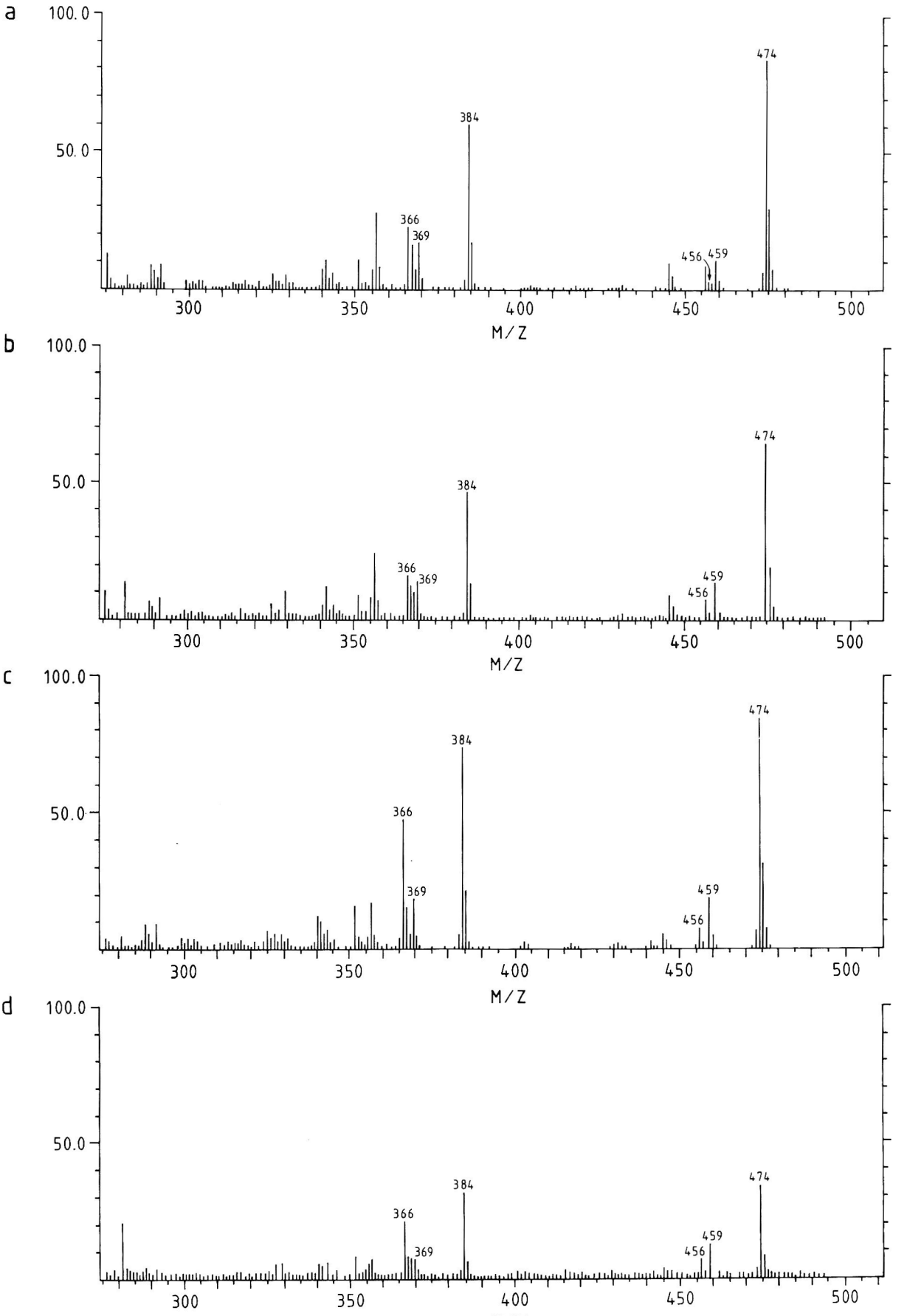

Fig. 4—Mass spectra of TMS derivatives of (a) synthetic 5β-, 6β- epoxycholestanol; (b) compound isolated from dehydrated egg yolk; (c) synthetic 5α-,6α-epoxycholestanol; (d) compound isolated from dehydrated egg yolk.

Table 2—Cholesterol oxides in dehydrated egg yolk stored in a manufacturing plant

Storage conditions	Cholesterol oxides, ppm in lipids[a]							
	5α-, 6α-epoxy	5β-, 6β-epoxy	7-keto	7α-hydroxy	7β-hydroxy	20α-hydroxy	25-hydroxy	5,6-dihydroxy
3 months at 5–8°C	TR[b]	1.0	1.5	2.5	0.8	ND[c]	ND	ND
3 months at 18–22°C	0.4	1.4	2.2	2.9	1.4	ND	ND	ND
5 months at 22°C	2.4	4.2	3.0	5.5	3.0	1.0	TR	TR
14 months at 22/5–8°C[d]	2.4	7.0	3.0	6.7	5.1	0.3	0.3	TR
14 months at 22/5–8°C[d]	2.1	6.1	2.0	6.6	5.2	0.4	TR	TR

[a] Means of duplicate analysis (extraction, enrichment and GLC)
[b] Traces (ca 0.1–0.2 ppm)
[c] Not detected, detection limit ca 0.2 ppm in lipids
[d] Stored for 3 months at 22°C followed by 11 months at 5–8°C

paring storage at 5–8°C to that at 22°C for 3 months, it is obvious that a delay in the oxidation can be accomplished by lowering the storage temperature (Table 2).

Reliability of quantitative data on cholesterol oxides in food

There are several potential errors involved in the quantitative determination of cholesterol oxides in composite foods. One is the generation of oxides during the analytical procedure, another is the breakdown of such compounds during analysis.

As regards the breakdown it is especially important to observe the formation of 5α-cholestane-3β,5,6β-triol which is a spontaneous breakdown product of the two epoxy compounds. Some earlier papers reporting quantitative data on cholesterol oxides in foods do not consider the cholestane-triol (Csiky, 1982; Tsai and Hudson, 1985) and therefore, no assessment of the possible breakdown of the epoxides in their procedures can be made. Another error involves the breakdown of cholesterol oxides during saponification. Separate reports of losses of single cholesterol oxides are presented in the literature. Tsai et al. (1980) reported a loss of ca 75% of the α-epoxide after saponification according the *Official and Tentative Methods* (AOCS, 1971). On the other hand, Maerker and Unruh (1985) reported no losses of the isomeric epoxides but a substantial loss of 7-ketocholesterol during hot saponification according to two different official methods.

Since no saponification step was used, except for the cholestane-triol fraction, and the analyses of cholestane-triol was made, it is obvious that the breakdown of cholesterol oxides during our analytical procedure was at a minimum. Recovery studies of ^{3}H labelled cholestane-triol was ca 95% in the analytical system. Furthermore, it is noticeable that the 8-year-old sample contained a large amount of cholestane-triol, together with monoacyl and diacylglycerol. These later contaminants could, however, be successfully removed by saponification.

The other risk in this type of analysis was the generation of oxides during the workup and separation. Fischer et al. (1985) reported that pure cholesterol in their system gave ca 10 ppm epoxides when saponification was omitted and ca 20 ppm when the saponification step was included. Maerker and Unruh (1986) found that saponification with hot alkali of pure cholesterol in triolein generated cholesterol oxides despite precautions taken to minimize oxidation.

The lowest level of quantification reported in the literature seems to be the one reported by us, about 0.2 ppm in the lipids. Therefore, it is of great interest to note that our fresh egg yolk and the freeze-dried egg yolk had no quantifiable amounts of any of the oxidation products. Tsai and Hudson (1984) also reported the absence of 5,6a-epoxy-5a-cholestan-3β-ol and 5,6β-epoxy-5β-cholestan-3β-ol but their level of detection was about 0.3 ppm in the products, which means 0.5 ppm in the yolk lipids. The procedure used by them and by us would probably be similarly protective against the generation of artifacts. In this study dim light was used. The entire procedure from extraction to silylation of the TEAP-Lipidex fraction was conducted in one long working day (12 hr). On the other hand, Fischer et al. (1985) reported ca 3 ppm epoxycholesterols and about 5 ppm of each of the two epimeric cholest-5-ene-3β,7α-diol and cholest-5-ene-3β,7β-diol in freeze-dried eggs. However, they used a saponification step as part of their isolation.

Cholesterol oxides in fresh and stored products

These data seem to be the first in the literature to cover all the important oxidation products in dehydrated egg yolk. Missler et al. (1985) recently reported similar patterns for two samples of 5-year-old egg mixes. They reported the presence of all the eight mentioned oxidation products except the 20α-hydroxycholesterol. It was noteworthy that the level of the suspected carcinogenic compound 5α,6α-epoxycholestanol was five times that of the 5β,6β-epoxycholestanol in egg mixes stored in cans for five years. Tsai and Hudson (1984) found about 5 times as much of the β-compound as of the α-compound. All of our samples were dried by indirect spray drying, and therefore one would not expect to find such high levels of the epoxy compounds as those in some of the samples analyzed by Tsai and Hudson (1985). Fisher et al. (1985) reported the sum of the two epoxides. In the view of the unsettled questions of the carcinogenicity of the α-epoxy compound, it is of great important to report the two isomers separately.

In the storage of the samples at 4°C, which is lower than that generally used in the industry, the dehydrated egg mix reached quantifiable levels of oxidation products only after 12 months and the level rose during storage up to 18 months. The dehydrated egg yolk had quite substantial levels at 6 months storage, and this was elevated for both manufacturing companies in their samples stored for 12 months at 4°C. A 12-month storage period for egg powder is reported not to be unusual in the food industry. It is noted in Table 1 that the 5,6β-epoxy-5β-cholestan-3β-ol is the major compound, together with the cholest-5-ene-3β,7β-diol and that their respective isomer and epimer are present in slightly lower amounts. The 7-ketocholesterol, which in some other products was the major oxidation product, was present in lesser amounts. The side chain hydroxylated compounds were represented only by the 20 α-hydroxycholesterol after rather long storage periods. At 12 months there was also a trace of the cholestane-triol.

The predominant part of cholesterol in egg yolk is present in free form and values of at least 90 % have been reported (Tattrie, 1972). There are no reported analyses of esterified cholesterol oxides in egg powder. However, Korahani et al. (1982) have studied the rate of autoxidation of cholesterol stearate, oleate, linoleate and linolenate in solid form and in aqueous dispersions and found the relative rates to vary with the fatty acid moiety esterified.

Our data are generally lower than those of Fischer et al. (1985) using saponification and thereby including also esterified cholesterol oxides. It must be determined whether the difference between their data and ours is mainly due to artifact formation during saponification or an extremely high extent of autoxidation of the small amounts of cholesterol esters present in egg yolk.

—Continued on page 67

Texture and Microstructure of Cooked Whole Egg Yolks and Heat-Formed Gels of Stirred Egg Yolk

S. A. WOODWARD and O. J. COTTERILL

ABSTRACT

Hardness, cohesiveness, and springiness of heated intact egg yolks were determined by an Instron compression test. Hardness increased as temperature was increased from 75° to 90°C and as time of heating was increased from 10 to 30 min. Cohesiveness and springiness increased as temperature was increased from 75° to 80°C and as time was increased from 10 to 30 min. Egg yolks from cooked shell eggs were lower in all textural parameters than gels formed from stirred egg yolk. Low scores for cohesiveness and springiness were indicative of the crumbliness of yolk from cooked shell eggs; crumbliness was attributed to structural characteristics. The microstructure consisted of adjoining polyhedral grains, which showed no evidence of crosslinking. Grains appeared to be equivalent to yolk spheres, ranging in size from 40 to 100 μm. The structure of a stirred yolk gel consisted of a highly crosslinked protein matrix containing spheres (5–35 μm) and granules (1–2 μm).

INTRODUCTION

ONE OF THE IMPORTANT functional properties of egg yolk (EY) is its ability to coagulate upon heating. The familiar texture of cooked EY is the crumbly, mealy texture found in hard-cooked shell eggs. This texture contrasts with that expected of heat-induced protein gels, which is solid with a certain degree of elasticity and cohesiveness (Flory, 1974). When yolk membranes are ruptured and the stirred yolk liquid is heated, a firm, rubbery gel is formed (Hawley, 1970, 1971). The basis for the crumbly texture of cooked EY and the cause of the texture change induced by disruption of the yolk are not known but may be related to yolk microstructure.

No micrographs of cooked EY have been published to date. However, the microbodies of raw liquid yolk have been well characterized. Raw yolk is composed of concentric white and yellow yolk layers, each of which consists of continuous and discontinuous phases similar to an emulsion (Robinson, 1979). The continuous phase is a dispersion consisting of low-density lipoproteins and livetins (Bellairs, 1961; Chang et al., 1977). Spheres 4–150 μm and granules 0.3–2.0 μm in diameter comprise the discontinuous phase (Bellairs, 1961; Robinson, 1979). Spheres are thought to be minor constituents of yolk compared to other particles (Chang et al., 1977), while granules make up 11.5% of EY (Burley and Cook, 1961). Spheres are composed of the same basic structure as the rest of the yolk, containing granules suspended in a continuous fluid phase (Bellairs et al., 1972; Robinson, 1979).

The purposes of this study were (1) to investigate the texture and microstructure of cooked EY and (2) to compare the texture and microstructure of EY cooked intact with those of EY stirred before cooking. Since the texture of a food system depends to a high degree on its microstructure (Stanley and Tung, 1976), a study of the structural characteristics of cooked EY should provide a basis for understanding its textural characteristics.

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MATERIALS & METHODS

YOLKS were separated from day-old eggs (ca 64g each) and rolled on damp cheesecloth to remove adhering albumen. Individual intact yolks were heated in sealed beakers (2.5 cm i.d.) in a water bath at various temperatures (75–100°C) and times (10–50 min). This produced 15 samples in a 5 × 3 factorial design; the experiment was replicated three times. The resulting samples shall be designated as beaker-cooked egg yolk (BCY) to distinguish them from other treatments. Samples were cooled to 20°C, removed from the beakers as cylinders 2.5 cm in diameter, cut to 2.5 cm in height and subjected to the Instron double compression test (50% compression) described by Woodward and Cotterill (1986). Hardness, cohesiveness and springiness were calculated from texture profile analysis curves, and results were evaluated by analysis of variance and Duncan's multiple range test using the Statistical Analysis System (SAS, 1982).

In a second experiment, the texture of BCY was compared to that of yolk from cooked shell eggs (ShCY), of stirred cooked EY (StCY) gels and of a commercially processed yolk product. StCY was prepared by separating yolks from albumen, removing vitelline membranes and stirring the fluid yolk with a magnetic stirrer. BCY and StCY in sealed beakers (2.5 cm diam) and shell eggs were then heated at 85°C for 30 min. A frozen "long egg" product was obtained from Ralston Purina Co. (St. Louis, MO) to provide a comparison to a commercially prepared, cooked EY product. According to the patents for this product (Hawley, 1970, 1971), liquid EY was heated to form a rubbery gel, which was ground to form a crumbly texture. The ground particles were then formed into a casing which was placed in a tube containing liquid egg albumen. The entire tube was heated to coagulate the albumen, followed by freezing. The "long egg" was obtained in frozen form and thawed to 20°C. ShCY, BCY, StCY and "long egg" yolk (LY) were cut with a wire cutter or a sharp knife into cubes measuring 2.0 cm on a side and subjected to the 50% compression test.

EY samples were prepared for scanning electron microscopy as follows: ShCY, StCY and LY were cut into 1.5 × 4 × 10 mm pieces and prepared by glutaraldehyde fixation and osmium-tannic acid-uranyl acetate post-fixation according to the procedure described by Woodward and Cotterill (1985). Because ShCY samples were crumbly, they were encased in agar prior to fixation to prevent sample disintegration during handling (Woodward and Cotterill, 1986). After fixation, samples were dehydrated in ethanol, defatted in chloroform, and critical point dried with CO_2 as the transition fluid. All samples were mounted, sputter coated with gold-palladium, and observed in a JEOL-JSM 35 scanning electron microscope at 20 kV.

RESULTS & DISCUSSION

Effects of time and temperature of heating

Significant time × temperature interactions occurred for hardness, cohesiveness, and springiness, indicating that the effects of time and temperature on the texture of BCY were not independent. At 10 and 30 min of heating, hardness increased significantly as temperature was increased from 75 to 90°C (Fig. 1). When BCY was heated for 50 min, no significant differences in hardness occurred as the temperature was increased. When heating time was extended from 10 to 30 min over the temperature range of 75 to 85°C, BCY hardness increased significantly. No differences occurred between treat-

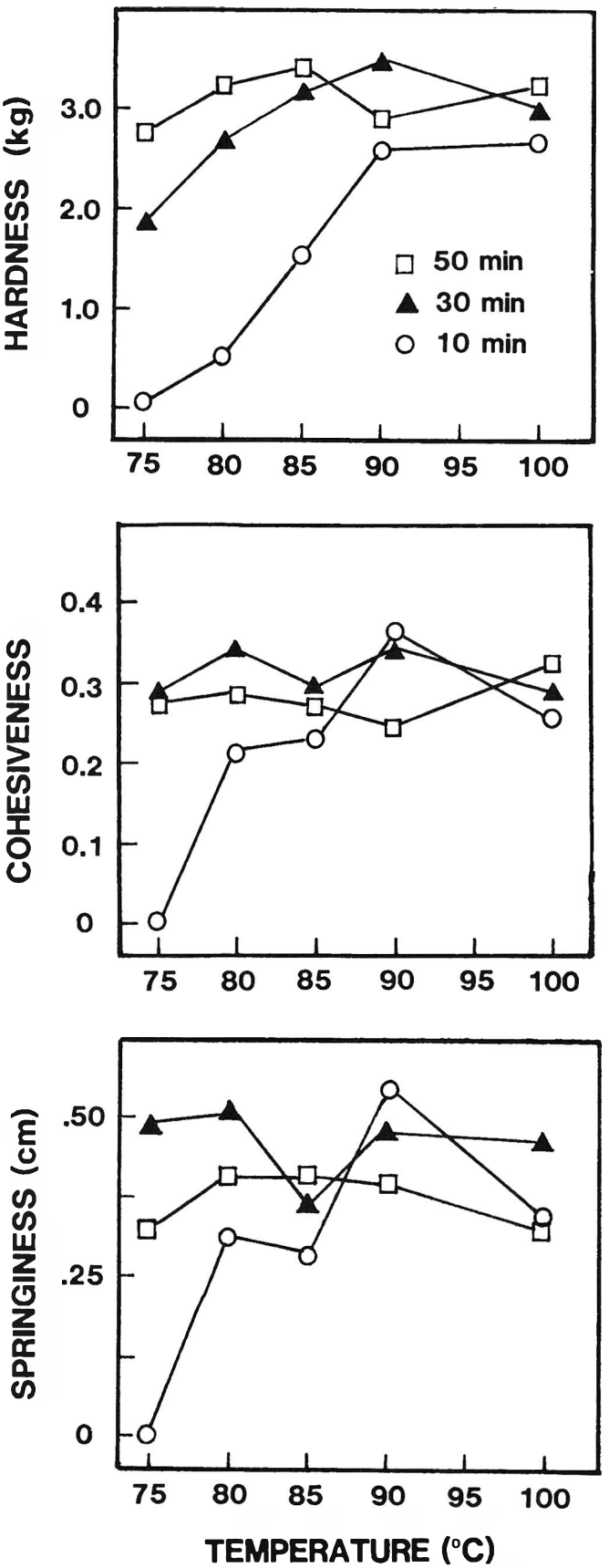

Fig. 1—Texture profile analysis of beaker-cooked egg yolk (BCY) heated at various temperatures and times in the shape of 2.5 cm cylinders.

ments of 30 and 50 min. Both cohesiveness and springiness increased significantly as temperature was increased from 75° to 90°C at 10 min of heating and as time was extended from 10 to 30 min at 75°C. No differences occurred when temperature was increased at 30 and 50 min of heating.

Romanoff and Romanoff (1949) reported that yolk begins to thicken at 65°C, ceases to flow at about 70°C and coagulates at 85°C. Dixon and Cotterill (1981) found that no egg yolk proteins were electrophoretically mobile when fluid EY was heated to 81°C, indicating that protein aggregation had occurred. Nakamura et al. (1982) reported that solution of low density lipoprotein isolated from yolk started to become rigid when heated at 65°C and increased in rigidity as temperature was increased to 85°C. In this study, BCY heated for 10 min in a 75°C bath was still fluid, and a higher temperature or heating time was required to solidify yolk samples.

The key to understanding the textural data for BCY was the combination of hardness with cohesiveness and springness. It was expected that BCY would become harder as time and temperature were increased, and it did up to 30 min at temperatures between 85 and 90°C. The cohesiveness and springiness of BCY also increased with time and temperature; however, internal crosslinking was limited as all BCY samples were fractured under compression. These data may be compared with texture profiles of EY gels prepared under similar conditions (Woodward, 1984). Hardness data for EY gels were similar to those for BCY, with the same trends of increasing hardness with temperature increases from 75 to 90°C and time from 10 to 30 min. The crumbly texture for BCY was apparent from cohesiveness and springiness data, which were maximal at 0.365 (ratio) and 0.55 cm, respectively (data from Fig. 1). By comparison, maximal cohesiveness and springiness for gels (Woodward, 1984) were 0.558 (ratio) and 1.10 cm, respectively, or 153% and 200% of the values for BCY.

Texture profile of various cooked EY products

A comparison of ShCY, LY, BCY, and StCY indicated that there were major differences in textural properties, with StCY gels scoring highest and ShCY lowest in all parameters (Table 1). ShCY fractured readily due to its low degree of internal bonding, as indicated by its low hardness and cohesiveness. LY had a grainy mouthfeel similar to that of ShCY, though textural scores for LY were significantly higher. Its relatively low hardness and cohesiveness were expected, since this product was designed to simulate the crumbly texture of ShCY. However, LY was almost as springy as StCY gels, possibly because it was formed initially from stirred yolk which had been gelled. BCY had textural properties intermediate between those of the crumbly ShCY and the firm, rubbery, cohesive gels of StCY. Shaping intact yolks into cylinders prior to cooking had a pronounced effect on the ultimate textural properties of the cooked intact yolk. This observation implied that the textural data from experiment 1 (BCY) did not directly apply to the texture of yolk cooked in the intact shell egg. Stirring native EY caused major textural changes in the subsequently cooked yolk in comparison to ShCY, allowing the formation of a firm, rubbery gel during heating in place of the crumbly, fragile yolk of an undisturbed cooked egg.

Table 1—Hardness, cohesiveness and springiness means[a] for ShCY, LY, BCY and StCY[b]

Treatment	Hardness (kg)	Cohesiveness (Ratio)	Springiness (cm)[c]
ShCY	0.47 g	0.137 g	0.350 g
LY	1.39 f	0.256 f	0.674 e
BCY	2.13 e	0.440 e	0.618 f
StCY	3.59 d	0.563 d	0.749 d

[a] Means (n = 12) within columns followed by different letters differ significantly ($P < 0.05$).

[b] ShCY = Shell cooked egg yolk, LY = "long egg" yolk, BCY = beaker cooked egg yolk, StCY = stirred, cooked egg yolk.

[c] Distance sample rebounded following 1.00 cm compression.

Microstructure

Major differences were apparent in the gross structures of ShCY, LY, and StCY (Fig. 2). ShCY was made up of adjoining polyhedrons ranging in size from 40 to 100 μm (Fig. 2A). Because of their coarse, grainy appearance, these polyhedral structures henceforth will be referred to as grains. They corresponded in size but not in shape to yolk spheres routinely isolated from liquid EY (Romanoff and Romanoff, 1949; Bellairs, 1961). Fujii et al. (1973), by fixing an undisturbed, intact yolk rather than a yolk suspension, were able to observe spheres from raw yolk in the form of polyhedral "crystals." They suggested that yolk spheres may be packed tightly together in natural conditions, accounting for the unusual shape. When free of external pressure in the liquid state, these grains would likely become spherical. Cooking of the intact yolk in the present study made permanent the polyhedral shape.

By comparison, the StCY gel had a smooth, flat surface, with spheres from 5–35 μm in diameter embedded in a protein matrix (Fig. 2B). An examination of the total surface of specimens in the scanning electron microscope revealed that spheres 25 μm or larger in diameter were only 6% as numerous in StCY gels as grains in the surface of ShCY. Apparently most grains (spheres) were ruptured by stirring. The remaining spheres were much smaller in size than the majority of ShCY grains. LY was characterized by a relatively smooth surface containing voids and fracture lines (Fig. 2C). The inclusions probably resulted from the product being incompletely compacted together after grinding, and they probably contributed to the crumbly texture and mouthfeel of the product.

Figures 2D-F show various aspects of the ShCY surface. A single grain 60 × 70 μm (Fig. 2D) had a few extraneous particles on its otherwise smooth surface. Small spherical particles from 0.5 to 5 μm in diameter were nestled at the junction of three grains (fig. 2E). Several long threadlike filaments were spread across the surface of two of the grains. These may have been remnants of a membrane of a yolk grain. A 3.5 μm sphere was found in the cavity of a grain surface (Fig. 2F). It was attached to the interior of the grain by several projecting filaments that appeared to be protein strands. The small sphere may have been exposed by the removal of lipid from the surface of the grain.

Surfaces and interiors of various spheres and grains from both ShCY and StCY were compared to determine their structural relationships (Fig. 3). The outer surface of a ShCY grain (Fig. 3A) consisted of round globules ca 0.1 μm in diameter compacted tightly together. Several larger particles, ranging from 0.3 to 1.0 μm in diameter, were scattered over the surface; they appeared to be loosely attached. These may have been yolk granules based on their size and shape. The smooth outer surface of a sphere from a StCY gel (Fig. 3B) was made up entirely of globules 0.1 to 0.2 μm in diameter fitting closely together with few void spaces. This surface was similar in appearance to that of the ShCY grain, except it was free of extraneous particles. The surface of a 70 μm-diameter sphere from a StCY gel prepared for SEM by freeze-drying (Woodward and Cotterill, 1985) is shown in Fig. 3C. The voids in the surface were probably formed by the removal of lipid-containing particles. The surface consisted of a random array of granules (0.3–0.5 μm) and smaller globules (0.1 μm) in a protein matrix. Protein strands were interconnected and also were attached to surface particles.

A ShCY grain with its interior exposed is shown in Fig. 3D. Micrographs of the interior structure of this grain are shown

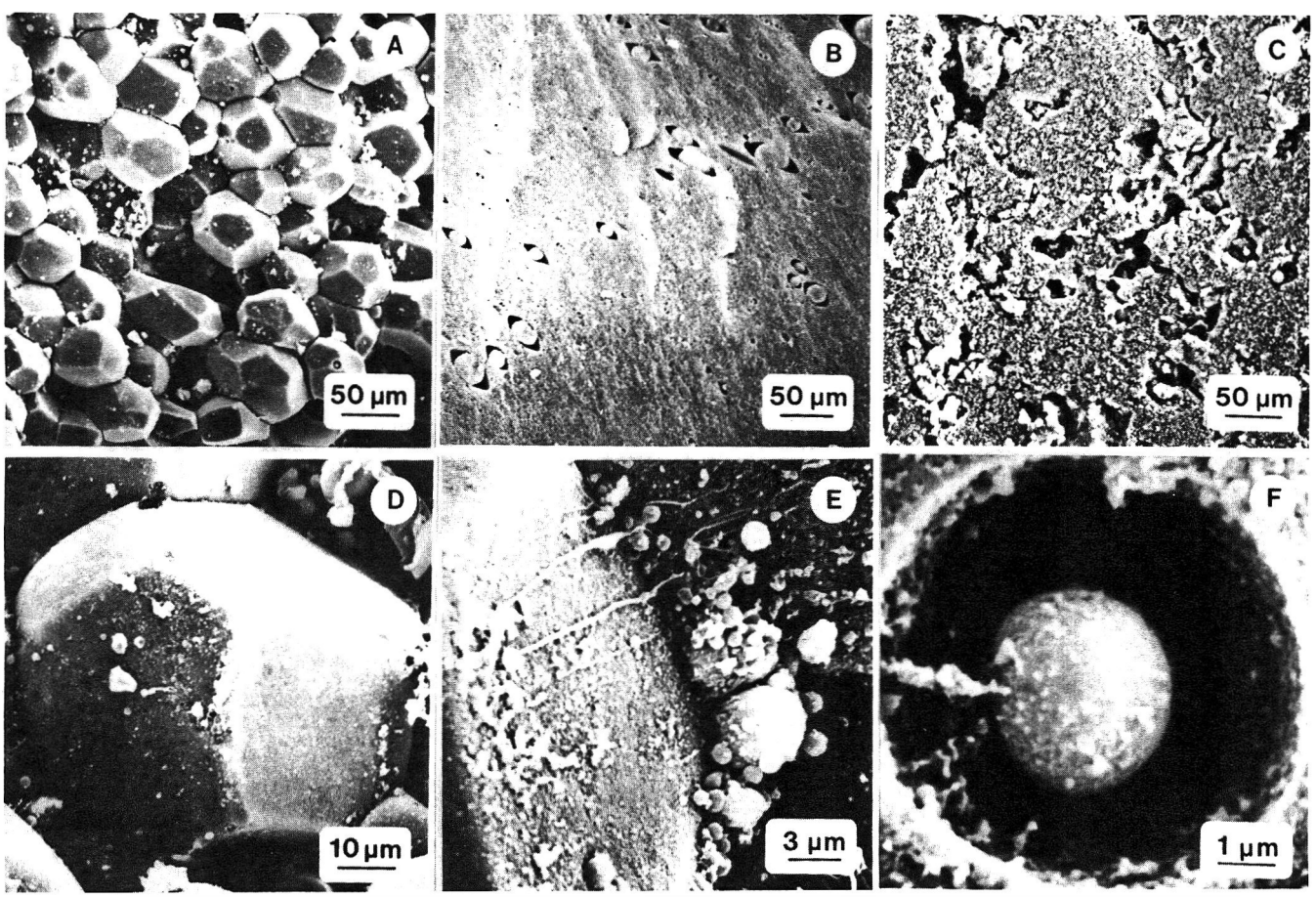

Fig. 2—Scanning electron micrographs of shell-cooked egg yolk (ShCY) compared to those of a stirred, cooked yolk gel (StCY) and a commercially processed "long egg" yolk (LY). (A) ShCY; (B) StCY; (C) LY; (D) a single ShCY grain; (E) junction of three ShCY grains; (F) sphere in ShCY surface cavity.

at higher magnification in Fig. 3E and F. Two types of structures were present. In Fig. 3E, numerous spherically shaped structures 0.2–0.3 μm in diameter cluttered a background which consisted mostly of 0.1 μm particles. In Fig. 3F, several granules from 0.5 to 1.5 μm in diameter were held in a background of the small globules. The interior of a sphere from an StCY gel (Fig. 3G) contained all the elements of the two previous micrographs with granules (0.5–1.5 μm) inset in a background of globules (0.1–0.2 μm). The continuous phase of an StCY gel (Fig. 3H) contained particles similar in size and appearance to those in the interior of the grain and the sphere. However, this surface (Fig. 3H) was not a part of any yolk sphere.

This series of micrographs (Fig. 3) suggested that the grains of ShCY were equivalent to the spheres of StCY. The surface structures were comparable, and the interiors of both were consistent with the structures present in the continuous phase of StCY. It was evident from the present study that the current theory of EY microstructure was incomplete. ShCY consisted almost exclusively of 40–100 μm polyhedral grains. Calculations indicated that approximately 3.2×10^7 grains 100 μm across would be required to fill the volume of a yolk from a large (57 g) egg. These grains were equivalent to spheres in size and in structure, but not in shape. It was probable that most structures in EY were originally contained in grains (spheres). Gentle stirring disrupted 90 to 95% of the grains larger than 25 μm across, leaving the remaining spheres as isolated structures in a continuous phase consisting of the contents of disrupted grains. Bellairs (1961) documented the existence of two types of spheres in liquid yolk containing various sizes and numbers of subdroplets. These subdroplets, or granules, were present in a fluid phase which was identical with the continuous phase of EY (Bellairs, 1964). It was likely that the granules became a part of the discontinuous phase upon rupture of grains, while the fluid phase of the grains became the continuous phase of the yolk fluid.

The theory that native EY consists entirely of polyhedral grains was first proposed by Fujii et al. (1973); however, their work has received little prior recognition. This theory is supported by an observation made by Maurice (1952). He noted that intact egg yolk had very low conductivity and that stirring the yolk increased the conductivity by 40 times. He explained that yolk must contain an internal structure, such as a continuous solid phase, which is easily broken down to yield a continuous aqueous phase. This theory of yolk microstructure is further supported by the paradoxically slow rate of water diffusion into the yolk from albumen in the shell egg. The vitelline membrane acts as a minimal barrier to water diffusion (Needham, 1931), yet water does not diffuse readily from albumen (88% water) to yolk (50% water). Shenstone (1968), in a review of the organization of egg yolk components, concluded that the rate of water diffusion into the yolk from albumen was governed by some structural arrangement in the

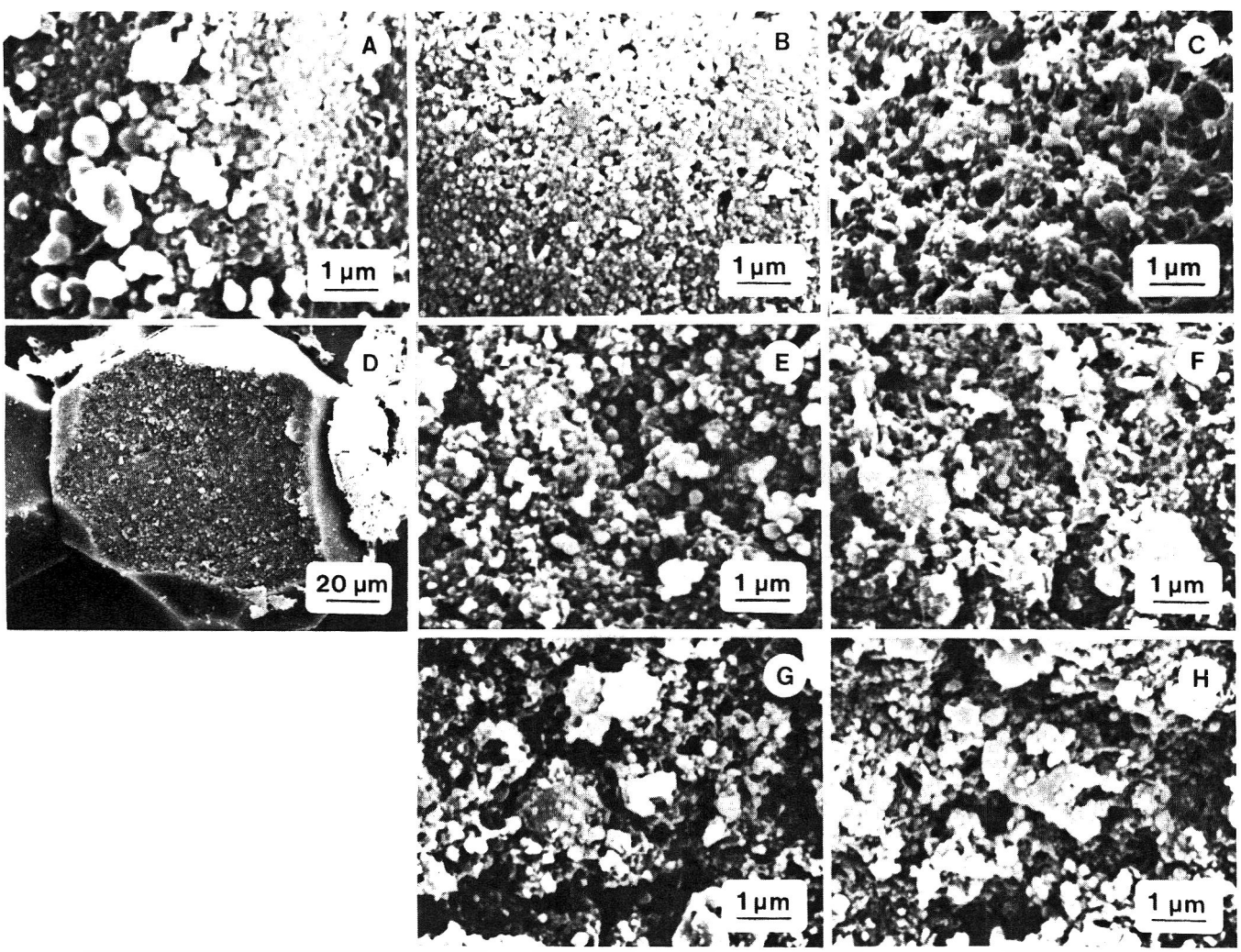

Fig. 3—Comparison of surface and interior structures of shell-cooked egg yolk (ShCY) and stirred, cooked yolk (StCY). (A) outer surface of ShCY grain; (B) outer surface of sphere from StCY gel; (C) outer surface of 70 μm sphere from StCY gel; (D) interior of ShCY grain; (E) interior of ShCY grain at higher magnification; (F) granules in interior of ShCY grain; (G) interior of sphere from StCY gel; (H) background matrix of StCY gel.

yolk. It is proposed that the grains in the yolk function as that continuous solid phase which limits conductivity and acts as a barrier to water diffusion.

The question of whether yolk spheres are surrounded by membranes has been asked for decades. Grodzinski (1951) suggested they were surrounded by semi-permeable membranes. The ease of sphere rupture by gentle stirring in the present sutdy indicated that whatever held the spheres intact had weak structural integrity. This fact was observed as long ago as 1839; Schwann (1839) inferred that yolk spheres "possess a superficial pellicle very soft however and delicate." Fujii et al. (1973) observed a wall of "viscous substances" surrounding yolk spheres and suggested the probability of a membrane-like structure; however, Bellairs et al. (1972) were unable to observe membranes on yolk spheres.

The presence of discrete grains contributed to the crumbly texture of ShCY. These individual packages of yolk components were each capable of gelling. Their disruption allowed the formation during heating of a three-dimensional protein network with a hard, cohesive, rubbery texture. A knowledge of the structure responsible for the crumbly texture of ShCY should be useful to those hoping to duplicate its texture while using a stirred EY liquid.

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Ms received 9/26/85; revised 8/28/86; accepted 8/28/86.

Missouri Agricultural Experiment Station Journal Series No. 9949.

This work was supported in part by the American Egg Board, by a Ralston Purina Food Science Fellowship, and by the Missouri Egg Merchandising Council.

Mention of a specific product does not imply endorsement of that product over others not mentioned.

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Ms received 3/28/86; revised 8/28/86; accepted 8/28/86.

Financial support from the Swedish Council for Agriculture and Forestry Research is acknowledged.

Professor Jan Sjövall, Dept. of Physiological Chemistry, Karolinska Institutet, Stockholm, is thanked for generous hospitality and effective guidance to the second author (L.-Å.) during methodological research preceding this study.

Acknowledgement is made to MR Ulf Bondesson, Research Laboratory, Ulleråaker Hospital, Uppsala, Sweden, for generous practical and intellectual support in carrying out the GLC-MS analysis, and to Svenska Äggprodukter AB, Helsingborg, Sweden and Kallberg Industrier AB, Töreboda, Sweden, for providing samples.

Texture Profile Analysis, Expressed Serum, and Microstructure of Heat-Formed Egg Yolk Gels

S. A. WOODWARD and O. J. COTTERILL

ABSTRACT

Egg yolk gels were formed by heating previously stirred egg yolk, having various pHs, protein concentrations, and NaCl levels, at various times and temperatures. Texture profiles were obtained by an Instron double-compression test, and serum was expressed from frozen-thawed gels. Microstructures of selected gels were observed by scanning electron microscopy. Gel hardness generally increased with increasing pH, protein or salt concentration, temperature, and length of heating time. The amount of serum expressed from gels decreased as gel hardness increased. Cohesiveness and springiness of gels increased with time and temperature. Gel microstructure consisted of spheres (4-50 μm) and granules (0.3–1.0 μm) dispersed in a continuous matrix. Spheres were physically disrupted by dilution and by addition of salt.

INTRODUCTION

THE COAGULATING or gelling ability of egg yolk (EY) is well recognized but has received surprisingly little attention in scientific literature. Cotterill (1970) commented that heat coagulation might become the most important functional property of EY. Yolk from hard-cooked eggs has a crumbly texture, yet when the yolk is physically disrupted by stirring prior to heating, a firm rubbery gel is formed upon the application of heat (Hawley, 1970). Woodward and Cotterill (1987) reported that gels formed from stirred yolk were harder, more springy, and more cohesive than cooked whole yolks.

Romanoff and Romanoff (1949) reported that EY began to thicken at 65°C, lost fluidity at 70°C, and coagulated at 85°C. Nakamura et al. (1982) found that the gelation of low density lipoprotein (LDL) isolated from EY was dependent on temperature of heating, pH, and salt (NaCl) concentration. An LDL solution lost fluidity at 65°C and formed a gel which increased in rigidity as temperature was increased to 85°C. Stable gels were formed from pH 4 to 9, with minimal rigidity at pH 6–7 and maximal rigidity at pH 4 and 9. The addition of 0.02 to 0.05M NaCl caused a tenfold increase in rigidity of LDL gels, but rigidity did not increase further when NaCl concentration was increased from 0.05 to 0.10M. Kojima and Nakamura (1985) reported the isoelectric point of LDL was in the pH range of 6.5 to 7.3, which corresponded to the pH range of minimal gel strength.

Gossett and Baker (1983) observed that the water-binding ability of coagulated whole egg mixtures was improved as the concentration of EY in the mixture was increased; they concluded that the water-binding ability of EY was much greater than that of egg white. While no other studies on the water-binding ability of EY have been reported, in general this property is influenced by the same factors which control gel textural properties, namely, pH, heating temperature, protein level, and ionic strength (Hermansson and Lucisano, 1982; Woodward and Cotterill, 1987).

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The microstructure of a food system is an important determinant of its textural properties (Stanley and Tung, 1976). Consequently, knowledge of food microstructure is important in the interpretation and understanding of food texture. The presently accepted theory of yolk microstructure is that uncooked yolk is an emulsion-type fluid with a continuous phase (plasma) and a discontinuous phase composed of spheres and granules (Chang et al., 1977; Robinson, 1979). However, Fujii et al. (1973) observed spheres from raw yolk in the form of polyhedral crystals. They suggested that yolk spheres may be packed tightly together in natural conditions, which would account for the unusual shape. Woodward and Cotterill (1987) proposed that native yolk consists entirely of "spheres" in the shape of polyhedral grains. They reported that gentle stirring disrupted 90 to 95% of these grains, releasing plasma as the continuous phase fluid and granules as a part of the discontinuous phase. They concluded that this change in the microstructural components released proteins into solution and was responsible for the gelling ability of stirred EY.

The major objective of this study was to determine the textural properties of EY gels as affected by several variables, including pH, time and temperature of heating, and protein and salt concentration. A second objective was to determine the influence of these variables on other properties of gels, such as water-binding ability and microstructure.

MATERIALS & METHODS

DAY-OLD EGGS were obtained from one strain of Single Comb White Leghorns. Yolks were separated from albumen and rolled on damp cheesecloth to remove adhering albumen and chalazae. Yolks were pooled and stirred with a glass rod until the mixture was homogeneous. Total solids were determined by drying 5g samples in a forced draft air oven at 105°C for 24 hr. Protein content was calculated as 31.1% of solids using the data of Cotterill and Glauert (1979).

Three factorial experiments were designed to study the effects of pH, time and temperature of heating, protein concentration, and added NaCl (Table 1). The overall design of this study was the same as that reported by Woodward and Cotterill (1986) for egg white gels, in order to make comparisons between the gelation properties of these two protein systems. Batches of EY were first diluted with a small amount of water to facilitate pH adjustment. Samples (ca 50 mL each) were then adjusted from an initial pH of 6.0 using 1.0M HCl or NaOH to a final pH ranging from 5 to 9, followed by final dilution to 11% protein (exp. 1 and 3) or 9–13% protein (exp. 2). NaCl was stirred into liquid EY (exp. 3) to bring solutions to final concentrations of 0.01, 0.03, 0.10, 0.32, or 1.00M NaCl. Weighed samples (ca 50g) were heated in foil-covered 50-mL beakers, which had been spray coated with a nonstick oil spray (PAM®), in a pre-heated water bath

Table 1—Research Design

	Treatments	Levels	Design
1.	Temperature × Time × pH	75, 80, 85, 90°C 10, 30, 50 min 5, 6, 7, 8	4 × 3 × 4 factorial, 2 replicates
2.	Protein × pH	9, 10, 11, 12, 13% 5, 6, 7, 8, 9	5 × 5 facctorial, 3 replicates
3.	Salt (NaCl) × pH	0.01, 0.03, 0.10, 0.32, 1.00 M 5, 6, 7, 8, 9	5 × 5 factorial, 3 replicates

for 30 min at 85°C (exp. 2 and 3) or 10–50 min at 75°C to 90°C (exp. 1). Samples were then removed from the water bath, cooled to 20°C, and refrigerated overnight at 4°C.

Temperatures of sample gels were monitored during a separate experiment by centering thermocouples in covered beakers of EY. Beakers were then heated in a water bath at either 75°, 80°, 85°, or 90°C.

Texture profile analysis of gels

Gel cylinders, having diameters of 3.8 cm and heights of 4.0 cm, were equilibrated to 20°C and removed from beakers. Cubes with sides 2.5 cm long were cut from gel centers with a wire cutter. Gels were subjected to a double-compression test (50% compression) at 12.7 cm/min on an Instron Universal Testing Machine (Model 1132). Hardness, springiness, and cohesiveness as defined by Bourne (1978) were computed from force-distance curves generated for each sample. All samples were then frozen at −24°C for 18–24 hr and thawed at 20°C. Serum was expressed in a Carver Press by the procedure described by Feiser and Cotterill (1982). Expressed serum (ES) was calculated as the percentage of serum removed from the gelled sample (Woodward and Cotterill, 1986). ES and textural data were evaluated by analysis of variance and Duncan's multiple range test using programs available in the Statistical Analysis System (SAS, 1982).

Serum electrophoresis

Serum proteins were separated by polyacrylamide gel electrophoresis using the procedure described by Feiser and Cotterill (1982). For each serum sample, 10 μL serum containing 20–240 μg protein were mixed in the sample gel. Unheated EY (400 μg protein) was run as a control. Serum protein content was determined by the Bio-Rad protein assay (Bio-Rad, 1979), which is based on protein dye binding.

Scanning electron microscopy

Selected EY gels, which were prepared from membrane-free yolk, were prepared for scanning electron microscopy by fixation in glutaraldehyde followed by the osmium-tannic acid-uranyl acetate post-fixation procedure as described by Woodward and Cotterill (1985). Gel pieces were then dehydrated in ethanol, defatted in chloroform, critical point-dried with CO_2 as the transition fluid, mounted, sputter-coated with gold-palladium, and examined in a JEOL JSM-35 SEM at 20 kV.

Two micrographs were obtained from EY gels which had been freeze-fractured to expose the inner structures of yolk spheres. EY gel strips were immersed in liquid nitrogen, fractured, freeze-dried, defatted in chloroform, and critical point dried with CO_2 as the transition fluid (Woodward and Cotterill, 1985).

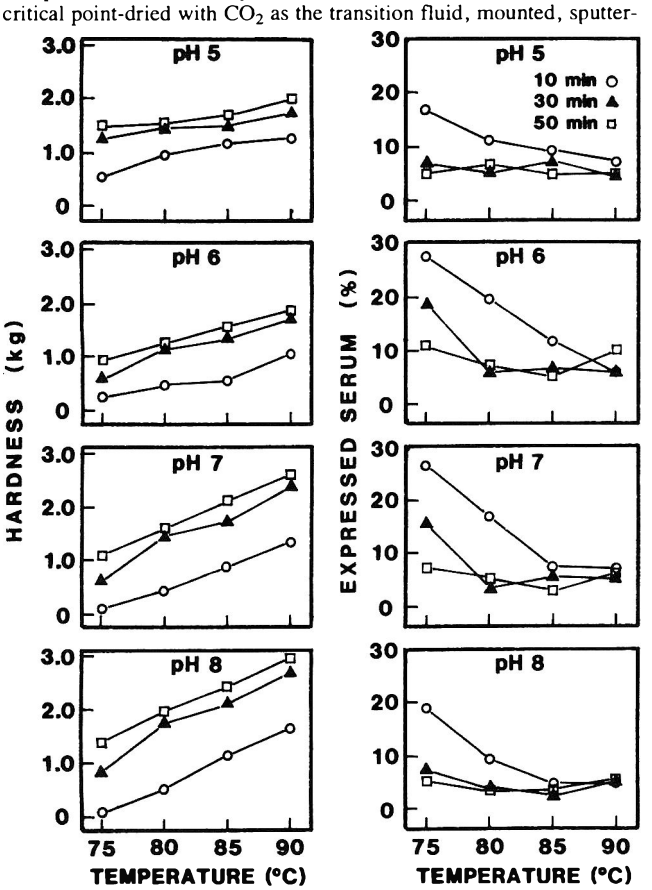

Fig. 1—Hardness and expressed serum of egg yolk gels heated at various pH's, water bath temperatures, and times of heating.

RESULTS & DISCUSSION

Exp. 1: pH × Temperature × Time

EY temperatures rose rapidly during the first 4 min of heating, but when EY reached 68°C to 70°C, the temperatures leveled off as gelation was initiated for the next 3 to 6 min. EY temperatures increased slowly thereafter, and reached within 0.5°C of the water bath temperature at 15 min for gels in the 75°C and 80°C baths and at 20 min for gels in the 85°C and 90°C baths. Although no gels had reached the bath temperature within 10 min, all samples removed at 10 min were no longer liquid and were able to hold their own shape.

This relatively long come-up time had two probable causes. The first was the large sample size and distance required for heat transfer (1.9 cm to the gel center). The second was the reduced rate of temperature increase due to the initiation of gelling. Johnson and Zabik (1981) reported that solutions of egg albumen proteins decreased in temperature when coagulation was initiated. They concluded that the aggregation of polypeptides proceeded endothermically, absorbing energy from the system.

Significant ($P < 0.05$) pH-temperature and pH-time interactions occurred for gel hardness in this experiment (Fig. 1). Hardness of EY gels increased with increasing temperature and time, but the rates of change in hardness varied with pH. When EY was heated at 75°C for 10 min, soft gels (hardness = 0.6 kg) were formed at pH 5, while gels of minimal hardness (0.1 to 0.2 kg) were formed at pH 6 to 8, indicating that gelation was initiated more rapidly at pH 5. Nakamura et al. (1982) similarly reported that heated LDL solutions formed gels more rapidly at pH 4–5 than at pH 6–8. Since this rapid gel formation occurred only at pH values on the acidic side of the isoelectric point, the authors hypothesized that LDL may be more susceptible to heat denaturation of the acidic side of pI than on the alkaline side. Data from this experiment suggested that EY followed that pattern.

As temperature was increased from 75°C to 90°C, hardness

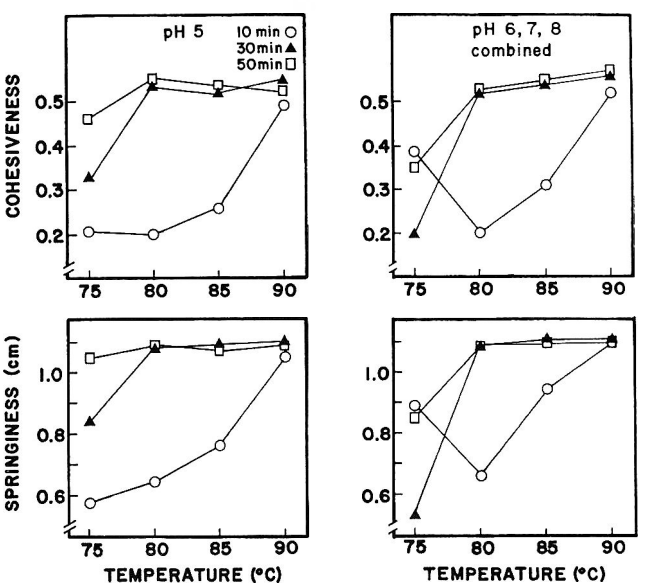

Fig. 2—Cohesiveness and springiness of egg yolk gels heated at various pHs, water bath temperatures, and times of heating. Data for pH 6, 7, and 8 were combined since no significant differences ($P > 0.05$) were present.

of gels increased significantly at all pH values and times of heating, but the rates of increase were greatest at pH 7 and 8. Extending heating time from 10 to 30 min caused large increases in hardness; these were probably related to the time lag involved in heat transfer from the water bath to the sample. Much smaller differences in hardness occurred as time was increased from 30 to 50 min.

Significant ($P < 0.05$) pH-temperature and time-temperature interactions occurred for serum expressed from gels (Fig. 1). The percentage of ES decreased as temperature was increased, but the rate of decrease varied according to the pH or length of heating time. EY samples heated for 10 min at 75°C were highest in ES, ranging from 17 to 28%, due to the incompleteness of gelation related to slow heat transfer. ES declined steadily as temperature was increased to 85°C for gels heated 10 min. Gels heated for 30 or 50 min achieved minima of ca 5% ES at 80°C, and did not change significantly in ES with increases in temperature. Gels formed at pH 5 and 8 generally were lowest in ES over the entire experiment, while those at pH 6 were highest. By comparison, Woodward and Cotterill (1986) reported that egg white gels prepared under the same conditions of pH, temperature, heating time, and protein concentration had ES from 50 to 80%.

Cohesiveness and springiness data reflected the development of internal bonding in a three-dimensional gel network, and generally increased with increasing temperature and length of heating time (Fig. 2). No significant differences ($P > 0.05$) occurred among gels at pH 6, 7, and 8; therefore, data were combined for these pH values, and data for pH 5 were plotted separately (Fig. 2). Significant ($P < 0.05$) pH-time and time-temperature interactions occurred for both cohesiveness and springiness. The pH-time interaction indicated that gels developed at different rates due to pH. After 10 min of heating at 75°C, cohesiveness and springiness were minimal at pH 5, but were unexpectedly high at pH 6, 7, and 8. Since samples at pH 6–8 were very soft gels at this stage of heating, initial compression did not cause irreversible deformation; hence, the values obtained in the second compression were high relative to the first, resulting in higher than expected ratios. Cohesiveness and springiness were significantly greater for gels at pH 5 than those at pH 6–8 which were heated for 30 or 50 min at 75°C, suggesting more rapid gelation for gels at pH 5 at 75°C. However, at higher temperatures there were no differences in cohesiveness and springiness due to pH. Values were increased from minima by increasing temperature above 80°C at 10 min of heating, and by extending length of heating time from 10 to 30 or 50 min at 75°C and 80°C; these textural changes were probably related to the time lag involved in heat transfer. Cohesiveness and springiness maxima were obtained by heating EY for 10 min at 90°C or for 30 min at 80°C. These time-temperature conditions also resulted in maximum water-binding ability, according to ES data (Fig. 1).

Serum electrophoresis. Proteins in EY serum (Fig. 3) were identified by comparison to electrophoretic patterns for EY reported by Chang et al. (1970), McBee and Cotterill (1979), and Dixon and Cotterill (1981). Serum in quantities large enough for analysis were obtained from only 17 of the 48 EY gels, and protein content of the serum ranged from 2 mg/mL at 90°C to 24 mg/mL for serum from gels heated 10 min at 75°C. Traces of a lipoprotein band were found in serum from gels heated 10 min at 75°C, but no lipoproteins were apparent at more severe heating conditions. The γ-livetin bands were present in serum from gels heated for 10 min at pH 7 and 8 at temperatures up to 85°C. Thus, γ-livetin was more stable to heat at pH 7 and 8 than at pH 5 and 6. Dixon and Cotterill (1981) noted that γ-livetins were affected by heat at 60°C and were totally absent in electrophoretograms of samples heated 3.5 min at 75°C. Several β-livetins were present in serum from gels at all pH values and heating conditons up through 85°C and 10 min. Dixon and Cotterill (1981) observed that β-livetins

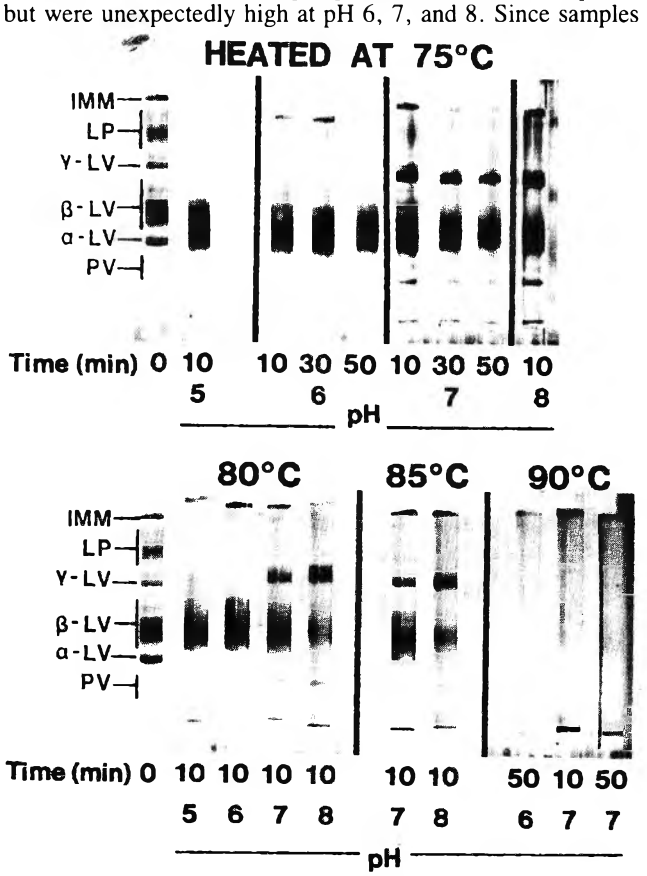

Fig. 3—Polyacrylamide gel electrophoresis of serum expressed from egg yolk gels prepared at various pHs, water bath temperatures, and times of heating. Key to protein bands: IMM = immobile, LP = lipovitellins, LV = livetins, PV = phosvitin.

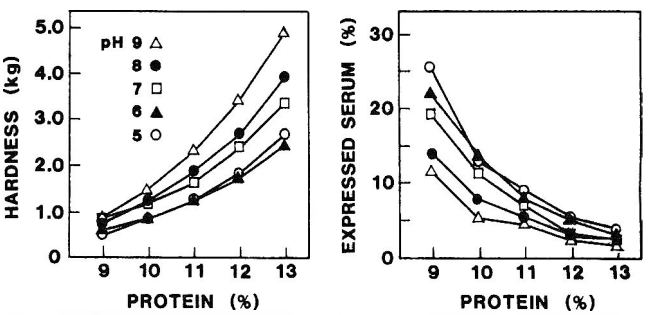

Fig. 4— Hardness and expressed serum of egg yolk gels at various pHs and protein concentrations.

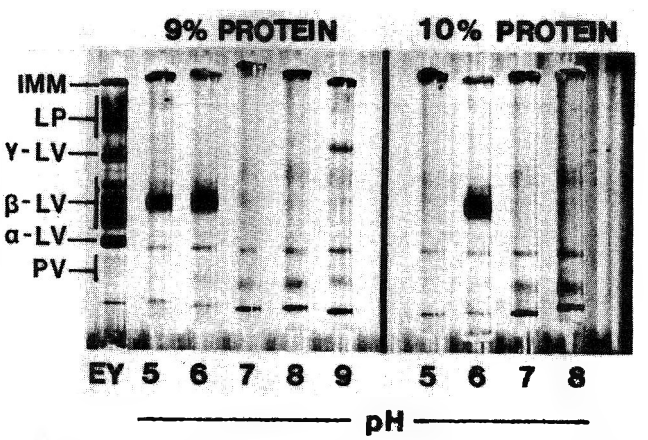

Fig. 5—Polyacrylamide gel electrophoresis of serum from egg yolk gels at various pHs and protein concentrations. Key to protein bands: IMM = immobile, LP = lipovitellins, LV = livetins, PV = phosvitin.

lost their electrophoretic mobility after EY was heated for 3.5 min at 81°C. A band of α-livetin was present in serum from samples treated at pH 7 and 8, 75°C, and 10 min. At 90°C no distinct protein bands were present. The reduction in quantities of soluble proteins in serum with increasing temperature and length of heating time indicated that those proteins were incorporated into the gel either by denaturation or aggregation (Matsuda et al., 1981; Woodward and Cotterill, 1986). Discrepancies between the stability of various EY proteins reported in this study and that of Dixon and Cotterill (1981) were probably due to differences in rates of heat transfer, based on differences in EY sample size.

Exp. 2: pH × Protein concentration

Significant ($P < 0.05$) pH-protein interactions were present in the data for hardness and ES (Fig. 4). As protein concentration was increased, gel hardness increased logarithmically, but the rates of increase were pH dependent. Gels were softest at pH 5 and 6 and hardest at pH 9; this response to pH was similar to that reported for LDL gels (Nakamura et al., 1982). ES values declined curvilinearly as pH and protein level increased. ES and gel hardness were significantly negatively correlated. A minimum of 2–5% ES was obtained at 13% protein; egg white gels at 13% protein had an average of 47% ES (Woodward and Cotterill, 1986). Cohesiveness of gels averaged 0.55, and only the gel containing 9% protein at pH 5 (cohesiveness = 0.43) was significantly different from others (data not shown). Springiness of gels, averaging 87% of the initial deformation, was not significantly affected by either pH or protein concentration. The lack of treatment effects on cohesiveness and springiness was probably due to the completeness of gelation under the heating conditions of this experiment (30 min at 85°C); maximum values were achieved at 80°C and 30 min in the previous experiment (Fig. 2).

Serum electrophoresis. Serum, containing 2–5 mg/ml protein, was available in quantities large enough for analysis only from gels containing 9 or 10% protein (Fig. 5). Since gels in this experiment were heated for 30 min at 85°C, it was expected that little or no protein would be present in serum based on results obtained in the first experiment. A γ-livetin band was present in serum from the pH 9, 9% protein gel. Two β-livetins were present at pH 5 and 6, 9% protein, and at pH 6, 10% protein. One α-livetin band was present in serum at all pH values for both 9% and 10% protein gels. A phosvitin band was present at pH 7, 8 and 9.

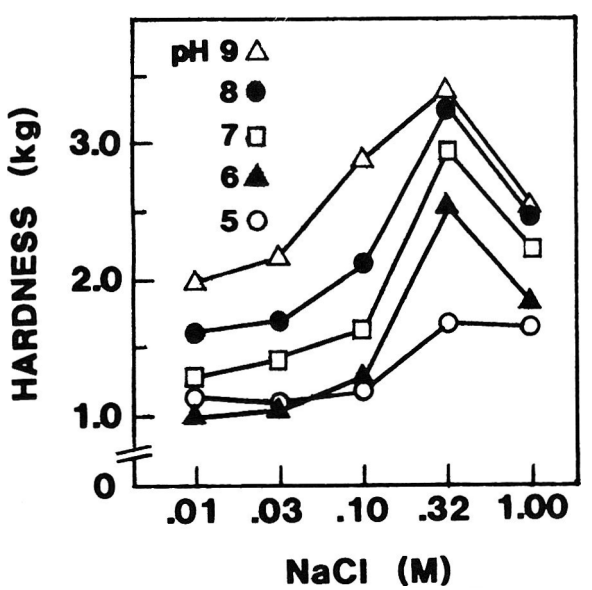

Fig. 6—Hardnes of egg yolk gels at various pHs and NaCl concentrations.

Exp. 3: pH × NaCl

The addition of 0.32M NaCl produced an increase in gel hardness from 150 to 200% over control gels at each pH (Fig. 6). The effect was less pronounced at pH 5 than at pH 6–9. At pH 8 and 9 significant increases in hardness occurred at 0.10M NaCl. This might have been attributed to the ionic effect of the NaOH which was used to adjust the pH of the liquid EY; in a titration of EY with NaOH, the concentrations of NaOH were 0.04M in pH 8 EY and 0.07M in pH 9 EY. Increasing NaCl concentration from 0.32 to 1.00M caused a weakening of gels.

Hermansson (1979) stated that salt generally caused enhanced aggregation in protein gels. Addition of salt to egg white gels promoted aggregation and reduced gel strength (Woodward and Cotterill, 1986). However, Nakamura et al. (1982) reported that the rigidity of LDL gels was enhanced by the addition of 0.05M NaCl. They also evaluated ovalbumin and bovine serum albumin, reporting that those proteins formed gels of maximum rigidity at 0.03M NaCl, with decreased rigidity at 0.05M NaCl. In the present study, it was likely that EY gels did not respond to low levels of NaCl because of the initial ionic strength of EY; Grodzinski (1951) reported that yolk is isotonic with 0.16M NaCl solutions. The response of EY gels at 0.32M NaCl was probably caused by the rupturing of yolk granules by salt (Burley and Cook, 1961). Granules make up 11.5% of yolk and account for 25% of total yolk protein; the proteins in granules include 70% lipovitellins, 16% phosvitin, and 12% LDL (Burley and Cook, 1961). Upon the disruption of granules, proteins would have been released which then could have been incorporated into the gel matrix.

No pH-NaCl interactions occurred for ES and cohesivenss data, so the results were reported as main effects (Table 2). ES decreased significantly with the addition of 0.32 or 1.00M NaCl to EY. ES also decreased with increasing pH to a minimum of 3.13% at pH 9. Cohesivensss was greater for gels formed at 0.32 and 1.00M NaCl than at lower salt levels. In response to pH, cohesiveness increased significantly as pH was increased from 5 to 9. Springiness of gels was not affected by either pH or salt concentration, averaging 88% of the initial deformation (data not shown).

Electrophoresis for this experiment was not possible since the amount of serum collected was negligible.

EY gels in the present study had similarities to egg white gels (Woodward and Cotterill, 1986), particularly in the responses of gel hardness and ES to changes in pH, temperature, length of heating time, and protein concentration. However, the addition of NaCl had opposing effects on egg white and EY gels. Salt weakened egg white gels and reduced their water-binding ability (Woodward and Cotterill, 1986). However, NaCl disrupted spheres and granules in EY, releasing protein for incorporation into the gel network. Apparently the disruption

Table 2—Expressed serum and cohesiveness[a] of egg yolk gels as affected by NaCl concentration[b] and pH[c]

NaCl (M)	Expressed serum (%)	Cohesiveness (Ratio)
0.01	6.47 d	0.476 e
0.03	6.68 d	0.477 e
0.10	5.96 d	0.488 e
0.32	3.31 e	0.535 d
1.00	4.33 e	0.525 d
pH		
5	7.90 d	0.474 e
6	6.12 e	0.495 de
7	5.17 ef	0.501 de
8	4.43 f	0.510 de
9	3.13 g	0.524 d

[a] Means for each treatment within columns with different superscripts are significantly different ($P < 0.05$).

[b] Means at each NaCl concentration were averaged from data at five pH treatment, since no significant NaCl × pH interaction was present ($P > 0.05$).

[c] Means at each pH were averaged from data at five NaCl concentrations, since no significant NaCl × pH interaction was present ($P > 0.05$).

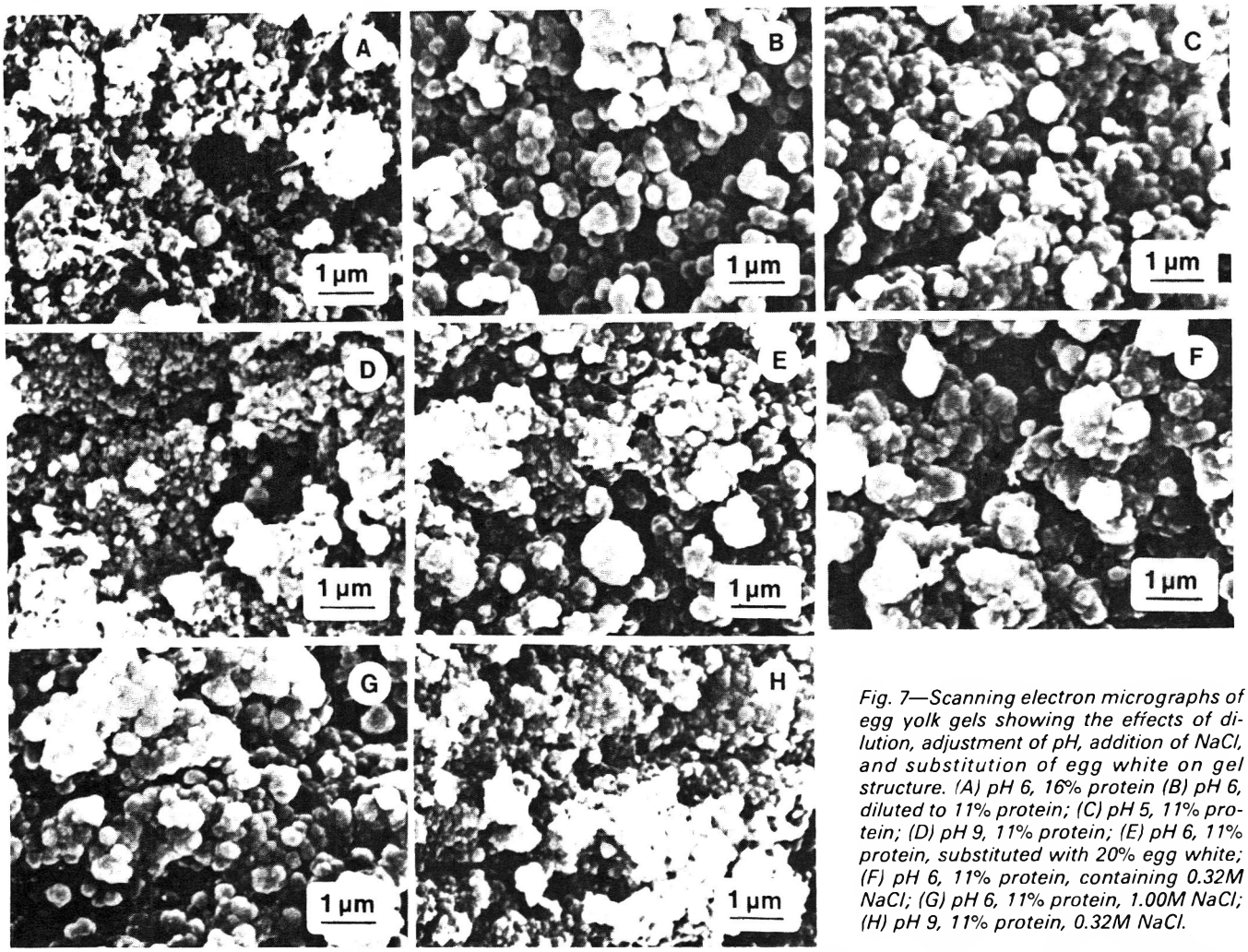

Fig. 7—Scanning electron micrographs of egg yolk gels showing the effects of dilution, adjustment of pH, addition of NaCl, and substitution of egg white on gel structure. (A) pH 6, 16% protein (B) pH 6, diluted to 11% protein; (C) pH 5, 11% protein; (D) pH 9, 11% protein; (E) pH 6, 11% protein, substituted with 20% egg white; (F) pH 6, 11% protein, containing 0.32M NaCl; (G) pH 6, 11% protein, 1.00M NaCl; (H) pH 9, 11% protein, 0.32M NaCl.

of spheres and granules overcame the aggregation effects of salt and resulted in harder gels with enhanced water-binding ability.

Egg yolk gel microstructure

A gel formed from stirred, undiluted EY had a continuous surface with no pore openings, and was composed of discrete particles ranging from 0.1 to 1.0 μm in diameter (Fig. 7A). Small, interconnected globules formed a uniform, crosslinked matrix which contained small spherical structures, presumably granules.

Dilution of EY resulted in the loss of about 90% of the large spheres which had been present in stirred fluid yolk. This was consistent with the finding of Grodzinski (1951) that dilution of yolk spheres caused their walls to burst. After dilution the crosslinking protein strands were no longer apparent, and no fine protein structure was evident (Fig. 7B). The gel structure was composed almost entirely of spherical bodies ca 0.5 μm in diameter, the identities of which were unknown. They appeared to be cemented together, but the nature of the cementing material was not apparent. The gel adjusted to pH 5 (Fig. 7C) was similar in appearance to the pH 6 gel (Fig. 7B), with spherical bodies ranging in size from 0.2 to 0.7 μm in diameter.

As pH was increased to 9, EY gels became more translucent, probably due to the rupture of most of the remaining spheres and disruption of some of the granules. The gel structure (Fig. 7D) was composed of much smaller particles than at pH 6, with the majority ranging in size from 0.05 to 0.1 μm in diameter. The size reduction of these particles was probably related to the high net charge and the reduced tendency for protein aggregation at this pH (Hermansson, 1979).

A gel was formed containing the approximate proportions of EY and egg white found in commercially available liquid yolk (Fig. 7E). Its microstructure differed from that of the pH 6 EY gel (Fig. 7B) in that its particle size was less uniform, varying from 0.1 to 1.0 μm. Some of the large particles appeared to be aggregates of small particles, reflecting the tendency of egg white to aggregate at pH 6 (Woodward and Cotterill, 1986).

The addition of 0.32M NaCl to diluted EY apparently disrupted all spheres 25 μm or larger, and also reduced the number of granules compared to the salt-free gels. Gels at pH 6 containing 0.32 or 1.00M NaCl were composed of irregularly shaped clusters up to 3 μm across (Fig. 7F and 7G). The high degree of aggregation probably was due to the low net charge of the proteins at pH 6 and to the enhancement of aggregation by NaCl.

The addition of 0.32M NaCl to EY at pH 9 increased the firmness of the gel, but had very little effect on the microstructure (Fig. 7H); the gel matrix was similar to that of the pH 9 gel without salt (Fig. 7D).

Bellairs (1961) reported the existence of both "rough" and "smooth" spheres containing large and small "subdroplets", respectively; examples of those types of spheres were observed in EY gels. A "rough" sphere (Fig. 8A) had spherically shaped glubules or granules up to 1 μm across protruding from its surface. The interior of a freeze-fractured "rough" sphere (Fig. 8B) contained several granules and small spheres 1–4 μm in diameter embedded in a protein matrix. This matrix was apparently formed from the "continuous phase" fluid of the sphere (Bellairs et al., 1972). The crater of a "rough" sphere (Fig. 8C) contained various-shaped particles up to 3.0 μm in diamter; these were probably remnants of a sphere which was burst by the effects of dilution and/or salt. The surface of a "smooth"

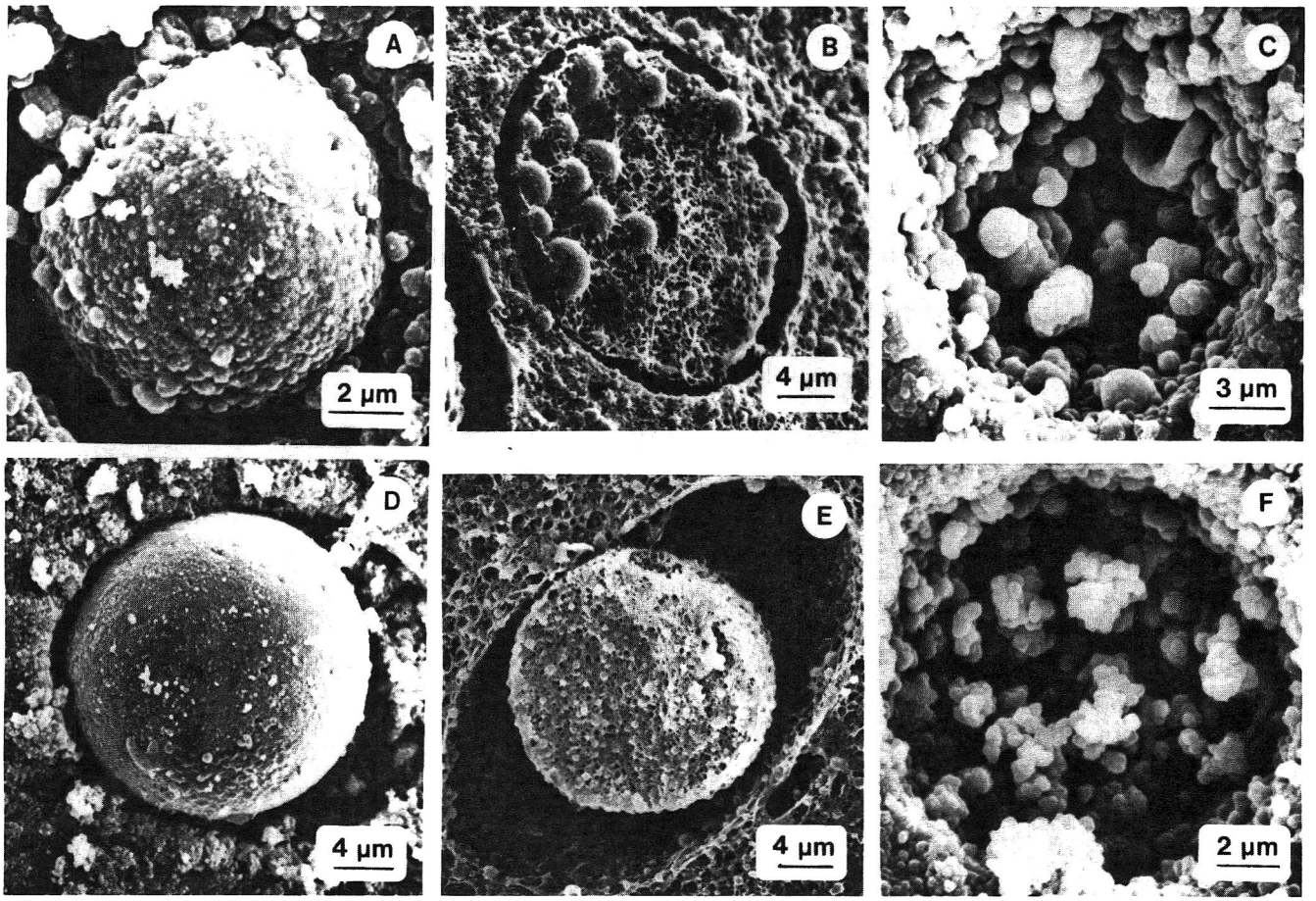

Fig. 8—Scanning electron micrographs of whole and broken spheres from egg yolk gels. (A) rough sphere from gel at pH 6, 0.32M NaCl; (B) freeze-fractured sphere from gel at pH 6; (C) crater of disrupted sphere from gel at pH 6, 0.32M NaCl; (D) smooth sphere from gel at pH 6, 0.32M NaCl; (E) freeze-fractured smooth sphere from gel at pH 6; (F) crater of disrupted sphere from gel at pH 6.

sphere (Fig. 8D) was apparently related to the small, uniform size of its internal particles. In a freeze-fractured "smooth" sphere, these particles appeared to be bound in a protein matrix (Fig. 8E). The crater of a "smooth" sphere (Fig. 8F) contained particles remaining from a 12 μm sphere, which were of the same size range (0.4 to 0.8 μm) as those in the fractured "smooth" sphere (Fig. 8E).

Several partially broken spheres and their surface structures were observed in EY gels. The surface of a "smooth" sphere (Fig. 9A) was disrupted by cracks and tears, and the surface layer had pulled away from the sphere. A sphere with a rough-appearing surface (Fig. 9B) consisted of a smooth inner core surrounded by a fractured outer shell, which was made up of globules 0.1 to 0.2 μm in diameter. A "rough" sphere (Fig. 9C) containing slightly larger surface particles (0.3 to 0.4 μm in diameter) had a rough-textured inner core and a fractured outer shell.

A structure which appeared to be the remnant of a sphere covering or membrane was observed in an EY gel containing 1.00M NaCl (Fig. 9D); it was filmlike with small particles and strands attached to it. Near it on the same gel was a sphere which had no outer covering (Fig. 9E); it was partially disrupted and spread across the gel in a three-dimensional network. Based on the sizes and proximity of these two structures, the covering probably came from the sphere. Another sphere from a gel containing 0.32M NaCl was surrounded by partially attached globular material (Fig. 9F). Both the sphere and its surrounding material may have come from a larger disrupted sphere. The structural damage may have been caused by dilution, high pH, and/or the addition of salt. Grodzinski (1951) reported that spheres placed in hypotonic solutions became swollen and ruptured. Those placed in hypertonic solutions began to shrink and became turbid; their "superficial membranes" began to unravel in filaments and released the "free fat drops", now known as granules, from their interior.

These micrographs suggested that some spheres in EY may be surrounded by an outer structural layer. Grodzinski (1951) concluded from his studies of the osmotic properties of yolk spheres that they were surrounded by semi-permeable membranes. Fujii et al. (1973), studying spheres by scanning electron microscopy, observed that they were surrounded by walls made of viscous substances. Bellairs (1961) examined yolk spheres by transmission electron microscopy and observed three types of surfaces on spheres, but concluded that these surfaces differed from true cell membranes. In a later study, Bellairs et al. (1972) were unable to observe membranes on yolk spheres.

CONCLUSION

THE PRESENT STUDY has indicated that the gelation of EY was influenced by various treatments. Hardness increased and ES decreased as pH, temperature, length of heating time, protein concentration, and NaCl concentration were increased. Cohesiveness and springiness of gels increased to maxima as heating conditions were increased to 80°C and 30 min; beyond that temperature-time combination, only minor changes occurred. Increasing cohesiveness and springiness were related to reduced ES; as gels developed a three-dimensional network, the enhancement of protein crosslinking was reflected in an improved ability of EY gels to bind water.

Changes in the microstructure of gels and EY spheres occurred with changing conditions of dilution, pH, and salt concentration. Spheres were disrupted first by stirring to allow gelation to occur. Dilution of EY caused increased disruption of spheres and appeared to increase aggregation of gels. Adjustment of pH to 8 or 9 disrupted both spheres and granules;

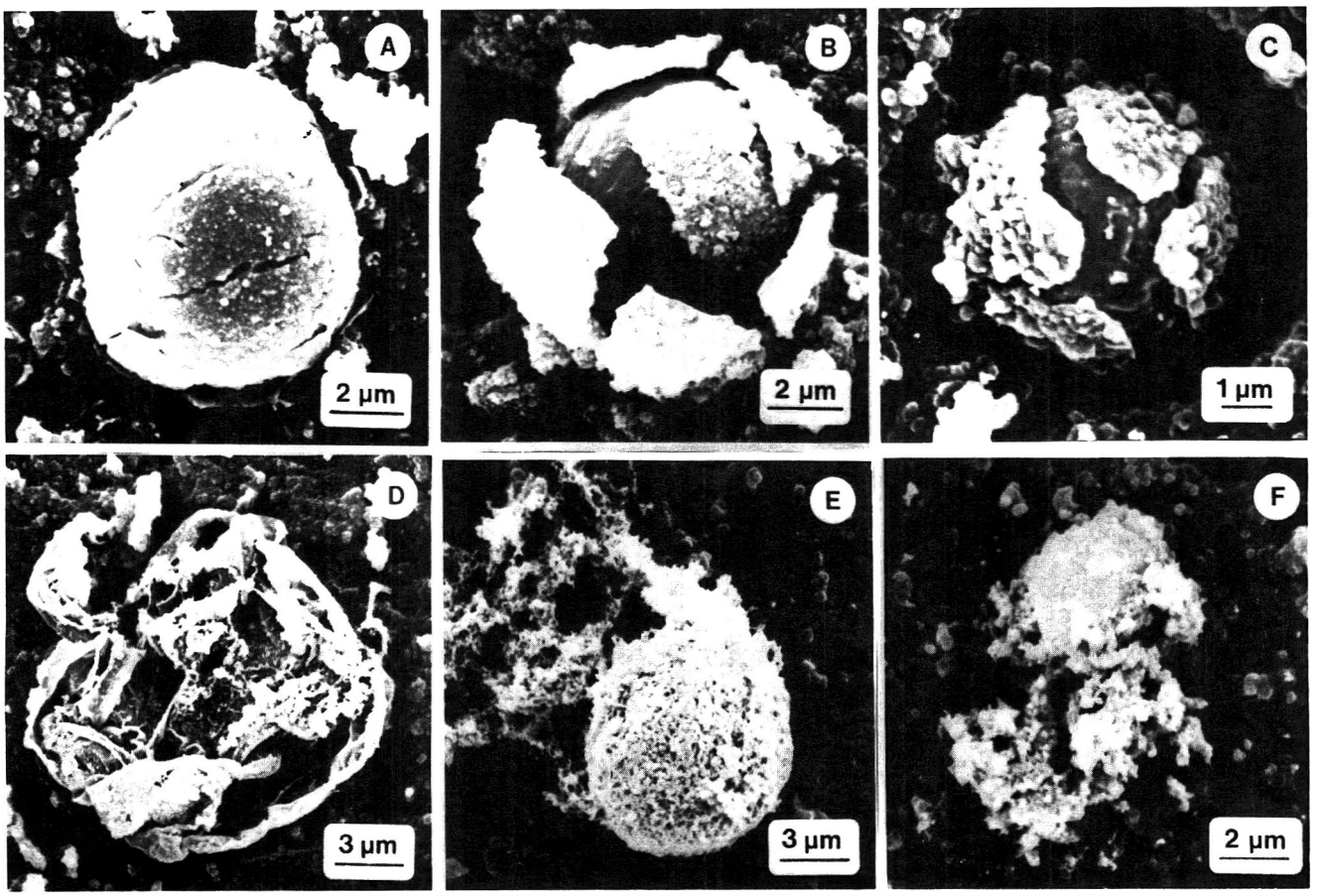

Fig. 9—Micrographs of spheres with fractured or disrupted surfaces, from egg yolk gels. (A) smooth sphere with fractured surface, from gel at pH 9, 0.32M NaCl; (B) rough sphere with fractured surface, from gel at pH 6; (C) rough sphere with fractured surface and rough inner core, from gel at pH 6, 1.00M NaCl; (D) membrane-like structure from gel at pH 6, 1.00M NaCl; (E) disintegrating sphere found adjacent to membrane-like structure in (D); (F) small sphere surrounded by globular protein, from gel at pH 9, 0.32M NaCl.

pH also played a major role in controlling aggregation. Large aggregates were formed in gels at pH 5 and 6, while the gel at pH 9 was uniform and fine-featured. The aggregated gel structure probably contributed to the reduction of hardness and water-binding ability of gels. Salt had much less apparent effect on aggregation than either pH or dilution, but contributed to gel properties by breaking down spheres and granules. It was concluded that these microstructural changes are major determining factors in the textural properties of EY gels.

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Ms received 2/26/86; revised 6/19/86; accepted 8/29/86.

Missouri Agricultural Experiment Station Journal Series No. 10035.

This work was supported in part by the American Egg Board, by a Ralston Purina Food Science Fellowship, and by the Missouri Egg Merchandising Council.

Heat-Stability of Milk-Clotting Enzymes in Conditions Encountered in Swiss Cheese Making

PASCALINE GARNOT and DANIEL MOLLE

ABSTRACT

Chymosin is inactivated in gelled milk at pH 6.50 at 53°C according to a biphasic kinetic. Both phases appear to follow first-order kinetics. The D-value for the first phase is 30 min. In the same medium, the *Mucor miehei* and *Mucor pusillus* proteases are much more stable ($D_{53°C}$ > 100 min) while bovine pepsin, a heat-labile *Mucor miehei* protease and the *Endothia parasitica* protease are rapidly inactivated ($D_{53°C}$ < 10 min). pH appears to be the most important parameter for heat stability. Protein and calcium concentrations affect the resistance to heat treatment. A residual activity of chymosin in Swiss-type curd will be very weak at the most unless the curd is cooked at pH < 6.50.

INTRODUCTION

THE ACTIVITY of the milk-clotting enzyme remaining in the curd is important for the ripening of some cheeses such as Cheddar, Camembert or Gouda (Green and Foster, 1974; Gripon *et al.*, 1975; Visser, 1977). For Swiss-type cheeses, the role of the milk-clotting enzyme is still unclear. Most of the activity is lost as the curd is cooked in the whey at 50–55°C (Matheson, 1981). However, due to the lack of a very sensitive method, it is not possible to rule out the presence of a low residual activity which could play a role during long ripening periods. In other respects, the available data on the heat stability of the common milk-clotting enzymes recently reviewed by Garnot (1986) cannot be extrapolated to what occurs in the particle of curd as the experimental conditions were very different from those encountered in Swiss-type cheese making. Most researches were conducted on the resistance of these enzymes to pasteurization or similar heat treatment, in the whey (Harper and Lee, 1975; Thunell *et al.*, 1979).

The purpose of this study is (1) to investigate the heat stability of different milk clotting enzymes in whey and in milk, under conditions commonly used for Swiss-type cheese-making i.e. in non acidified medium, at 53°C; (2) to investigate the effect of slight variations of pH or temperature; and (3) to estimate the level of enzyme activity which could remain in the Swiss-cheese curd.

MATERIALS & METHODS

Enzymes

Chymosin was purified by two ion-exchange chromatographies from a rennet powder (Boll, Arpajon, France) (Garnot and Mollé, 1982). The freeze-dried enzyme was dissolved (1 $mg \cdot mL^{-1}$) in a 2.10^{-2} M Na acetate buffer, pH 5.7. The bovine pepsin extract (Bovipep), (> 1700 $mg \cdot l^{-1}$ of bovine pepsin), and the *Mucor miehei* protease (MM protease) (Mucagel 1:10,000) were supplied by Granday (Beaune, France). A heat-labile *Mucor miehei* protease, Rennilase XL (MMXL protease) (14 Kilo Novo rennet units per mL) was supplied by Novo (Paris, France). The *Mucor pusillus* protease (MP protease) (Noury, 1:10,000) came from Eurozyme (Paris, France) and the *Endothia parasitica* protease (EP protease) (Suparen, 1:10,000) from Pfizer (Orsay, France).

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Heating media

The milk from an INRA experimental farm was skimmed before adding 0.5% sodium azide. The pH was adjusted with 2% lactic acid or 1M NaOH. The whey was recovered after clotting the milk with bovine pepsin, centrifugation (2000 × *g*), filtration and pepsin inactivation by heating (90 min, 37°C, pH 8). Then the pH was adjusted with 1M HCl.

The whey protein solutions were prepared using a whey protein concentrate (Armor protéines, St Brice-en-Cogles, France) obtained by ultrafiltration and containing more than 75% protein.

The caseinate was prepared from skim milk by two precipitations at pH 4.7, solubilized in water at pH 7 and freeze-dried. For the experiments, 3% caseinate solution was prepared in 0.05M phosphate buffer pH 6.50. The permeate was obtained by ultrafiltration of milk with a hollow-fiber concentrator (Amicon, Paris, France).

Heat treatments

The enzyme solutions were diluted in the medium in order to get an activity high enough to measure a 95% reduction in activity. The dilution for chymosin was 1:150, for bovine pepsin 1:100 and for the microbial coagulants 1:200. Milk used for enzyme inactivation was previously cooled to 6°C to avoid gelation. This allowed distribution of 2 mL fractions of the mixture to glass tubes (0.9 cm diameter) closed with caps. The tubes were kept at 30°C for 30 min before heating in a water bath at 51°C, 53°C or 55°C. Zero time was taken after the 1 min interval required for the temperature elevation. The inactivation was stopped at different intervals by cooling the tubes in cold water. The activity was measured in the supernatent obtained after breaking the curd and centrifuging (1400 × *g*, 15 min). When other media were used, the inactivation was carried out directly without the first step. The activity was determined without centrifugation. After heating the pH was not changed. Each result is the mean of 3 determinations. For each trial, a reference sample was treated in the same way but without heating.

From the kinetics of inactivation we determined two parameters classically used to characterize the heat stability of enzymes or microorganisms: the D-value which is the heating time necessary to inactivate 90% of the enzyme and the z-value which is the temperature increment required to reduce the D-value by 90%.

Enzyme assay

Activity was determined as the reciprocal of the flocculation time measured at 30°C when 0.1 mL enzyme solution was added to 1 mL 0.2% κ-casein solution in a 5.10^{-2} M Na_3 citrate-HCl, 0.075M NaCl buffer, pH 5.3 (Douillard and Ribadeau Dumas, 1970).

Analytical determinations

The protein content (N×6.38) was determined according to the Kjeldhal method, using an automatic apparatus (Kjeltec Tecator, Humeau, La Chapelle/Erdre, France). The calcium content was determined using atomic absorption equipment (AA 1275, Varian, Orsay, France) according to Brulé *et al.* (1974).

RESULTS & DISCUSSION

Kinetics of thermal inactivation in milk

Some of the kinetic measurements carried out in milk are shown in Fig. 1. The logarithm of the residual activity is represented as a function of the incubation time. For the slowest or fastest inactivations, first order kinetics were observed (as

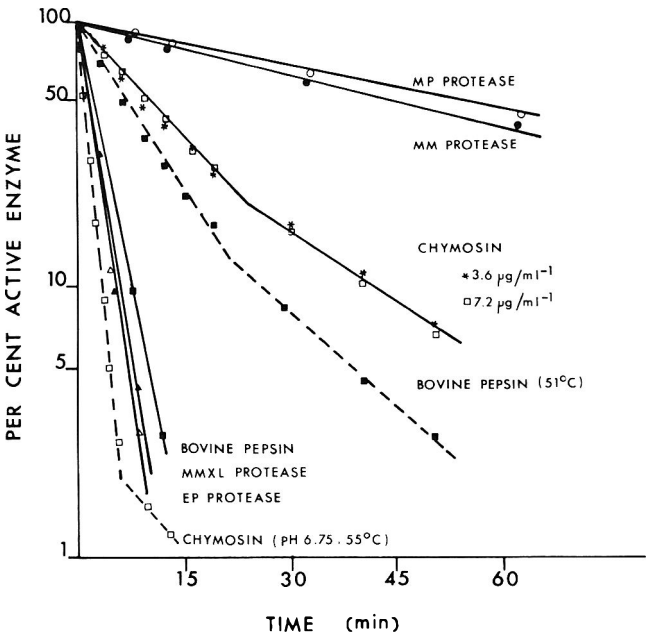

Fig. 1—Kinetics of inactivation of milk clotting enzymes by heating in milk at pH 6.50, 53°C, except: □—□ heating in milk at pH 6.75, 55°C; ■—■ heating in milk at pH 6.50, 51°C.

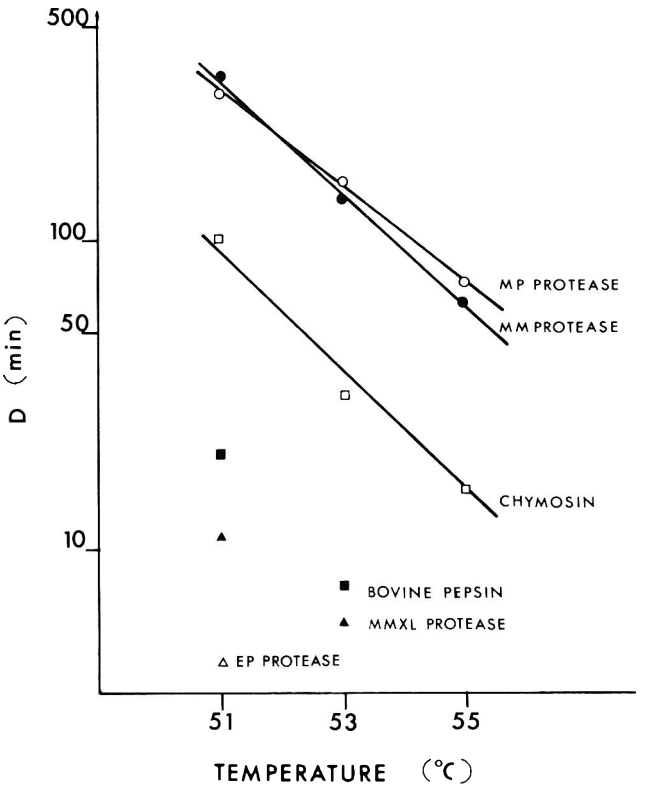

Fig. 2—D-values measured in milk (pH 6.50) between 51° and 55°C (first phase of denaturation)

for MM protease or bovine pepsin in milk, pH 6.5, 53°C). However, for the fastest inactivations one or two of the last measurements sometimes deviated from single stage kinetics (chymosin in milk, pH 6.75, at 55°C). Most often this was not detected because of the very low residual activity. Between these extremes the loss of activity was biphasic. The two phases appeared as two first-order reactions. This was confirmed by the similar rate constants observed for both phases with two different concentrations of chymosin (Fig. 1).

According to the conditions, a given enzyme did or did not exhibit a biphasic loss of activity, as shown for bovine pepsin. In identical conditions, MM and MP proteases were the most stable. Bovine pepsin, EP and MMXL proteases were considerably less stable. The stability of chymosin was intermediate between these two groups.

The first-order kinetics of the heat inactivation of the milk clotting enzymes are in agreement with the results of Harper and Lee (1975), Hyslop *et al.* (1979) and Daemen (1981). However, we have observed a biphasic inactivation when the enzyme half-life was between 5 and 20 min, regardless of the enzyme or the medium. The initial presence of other enzymes in the solutions can be excluded since purified chymosin and bovine pepsin were used. It is probable that a structural rearrangement leading to a still active and more heat-resistant molecule would occur. Indeed under our conditions (51–55°C), the hydrophobic interactions are strengthened by temperature (Scheraga, 1963) and would stabilize a modified enzyme molecule, while electrostatic interactions and hydrogen bonds are weakened. This hypothesis requires experimental confirmation which is beyond the scope of this work; nevertheless, it is in agreement with results concerning thermostable enzymes which often undergo a conformational change around 55°C (Singleton and Amelunxen, 1973). In connection with this, Mozhaev and Martinek (1984) have obtained a more heat-stable structure of trypsin by refolding it at 50°C after incubation in urea. A more fundamental study (Privalov *et al.*, 1981), has shown that pepsin is denatured in two different ways occurring at different temperatures. This was explained by a different stability of the two lobes characteristic of the tridimensional structure of the aspartyl proteases (Andreeva and Gustchina, 1979).

Because of the two stages of inactivation, the D-values appear useless for extrapolating the residual activity at times longer than those tested. Similarly, it seems doubtful to extrapolate from z-values determined at very different temperatures, as indicated by Zale and Klibanov (1983). However, it is noticeable that, using D-values graphically determined from the first-phase of inactivation, we obtained estimations of z-values between 5.2 and 6.3 (Fig. 2) which is of the same magnitude as the values measured by Hyslop *et al.* (1979) at 70°C. This may be because the mechanism of inactivation at 53°C during the first stage is the same as that occurring at a higher temperature.

Figure 2 also allows comparison of the heat stabilities of all the available milk-clotting enzymes in conditions identical to those encountered in Emmental cheese making. Beginning with the most heat stable enzyme the range of heat stability appears to be: MM protease, MP protease > chymosin > bovine pepsin, MMXL protease > EP protease. This is in agreement with other partial data on all of these enzymes except the destabilized MM protease (Harper and Lee, 1975; Hyslop *et al.*, 1979; Thunell *et al.*, 1979).

Influence of conditions of inactivation

In dairy plants, milk pH at renneting may vary from 6.5–6.75 and the cooking temperature ± 2°C around 53°C. To visualize the effect of such changes, we determined the ratios of residual activities, measured with one parameter varying each time, for chymosin and MM protease (Fig. 3). The higher the ratio, the greater was the effect of the variation tested on heat stability.

For the MM protease the activity ratios were, for almost all variations tested, below 1.5, indicating a 50% decrease in residual activity, at most. The effect was greater on chymosin stability which decreased with heating times greater than 12 min because of the biphasic kinetics of inactivation. The residual activity was reduced by ~90% after 30 min as the pH or medium was changed. The greatest change was observed when the pH increased 0.25 unit. In this pH zone, the chymosin stability decreased when pH increased slightly (Foltman, 1959). In agreement with Struble and Sharp (1940), we observed that the heat treatment emphasized this pH effect.

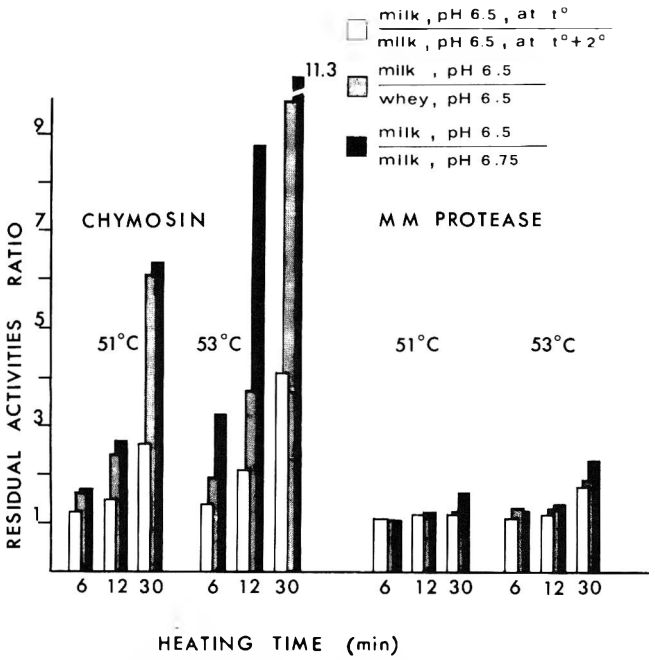

Fig. 3—Influence of variations of pH, medium, and temperature on the residual activity (R.A.) of chymosin and MM protease after different heating times.

Table 1—Influence of protein and calcium contents on $D_{53°C}$-values

Medium	N × 6.38 Total (g L^{-1})	Calcium content (g L^{-1})	$D_{53°C}$-values[b] (min)
Sodium caseinate[a]	29	0	7.8
Whey proteins[a]	26	0	10.3
Milk permeate	2	0.33	10.7
Whey proteins + $CaCl_2$, 10^{-2}M	26	0.40	41

[a] Na-caseinate and whey proteins were dissolved in a phosphate buffer, $6 \cdot 10^{-2}$M, pH 6.50.

[b] $D_{53°C}$-values are the times required to reduce activity of chymosin by 90% at 53°C.

The effect of the type of medium is also shown in Fig. 3. This may be explained based on differences in protein content between whey (9 g·L^{-1}) and milk (29 g·L^{-1}) since the stabilizing effect of protein was shown for chymosin using whey and concentrated whey (Verhey, 1973; Harper and Lee, 1975; Daemen, 1981). However whey and milk differ not only in their protein contents, but also in the protein type and mineral composition. Therefore, we have tried to assess the respective importance of these parameters for chymosin using different media whose protein and calcium contents were known (Table 1). The proteins or the calcium had about the same protective effect on chymosin. The additive effect of these two factors enhanced the heat-stability of chymosin. The components of the soluble phase (permeate) were also very effective. The effects of lactose and NaCl were already known (Van den Beek and Gerlsma, 1969). We show that calcium is also a stabilizing factor for chymosin which was already observed with metalloproteases (Mayerhofer *et al.*, 1973; Barach *et al.*, 1976) or thermophilic enzymes (Mozhaev and Martinek, 1984). The binding of the calcium ion stabilized the molecular structure (Dahlquist *et al.*, 1976). At the pHs studied (6.5, 6.75), the variation of 0.25 unit did not affect the calcium solubility sufficiently to be taken into account in explaining the effect of pH.

From our data, we have tried to estimate the residual rennet activity remaining in the curd and in the whey at pressing when the temperature is below 50°C. Our results show that about 6% of the initial chymosin activity was still present after 60 min at 53°C in milk at pH 6.5, which is about the time corresponding to the heat treatment of the curd during Swiss-cheese making (Gilles *et al.*, 1983). From data of Holmes *et al.* (1977) one can assume that at most, 20% of the chymosin added to milk remained in the curd. Therefore, no more than 1.2% of the initial enzymic activity added to the milk should remain in the curd, which is almost negligible compared to other cheeses (Garnot *et al.*, 1987). If the pH is higher than 6.5, one can reasonably assume that there will be no residual activity. This is in agreement with the data of Matheson (1981) who could not detect any residual activity in Swiss cheese. If a bovine pepsin extract or EP protease is used instead of rennet, these enzymes will be totally inactivated before the cooking of the curd has ended. This explains the development of the use of EP protease in spite of its proteolytic activity. On the other hand the MM and MP proteases are too heat stable; the activity remaining in the curd would be too great and would affect the course of ripening. The destabilized MM protease has a lower heat stability than rennet and could be used for Swiss cheese making. The chymosin remaining in the whey of Swiss cheese was inactivated at the end of cooking without any further heating. Thus, pasteurization of whey is useful only to avoid acidification. Troubles due to residual activity should only arise with MM or MP proteases.

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Effects of Commercial Food Grade Enzymes on Free Fatty Acid Profiles in Granular Cheddar Cheese

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ABSTRACT

Commercial food grade enzymes (Neutrase, calf lipase and NaturAge) were incorporated into cheese at various concentrations and ripened at 7° and 13°C. Gas chromatographic analysis indicated that free fatty acid (FFA) production increased significantly ($p<0.05$) with lipase and high level NaturAge. Neutrase had little effect on FFA production but showed synergistic effects with lipase. The short-chain FFA profiles were similar among control, Neutrase- and NaturAge-treated cheeses. Significant correlations ($p<0.05$) between C_8 and C_{14}, C_{16} or $C_{18:1}$ and total free fatty acids were observed in all tested samples. Total concentrations of C_4 and C_6 may be a good indicator of flavor development during cheese ripening. Cheese with a medium level of NaturAge, ripened at 13°C for 4 wk, showed profiles of FFA similar to those of the control cheese ripened at 7°C for 12 wk.

INTRODUCTION

FREE FATTY ACIDS (FFA) are known to play an important role in flavors of many varieties of aged cheese. They were reported to constitute the backbone of Cheddar cheese flavor (Patton, 1963). Two major sources are generally considered for FFA in aged cheese. These are (1) the direct breakdown product of milk fat by lipolysis and (2) the end products of carbohydrates and proteins metabolized by bacteria. Cheeses made from skim milk have considerably lower levels of FFA (in chain length C_4 or greater) than those made from whole milk, indicating that lipolysis is the principal contributor in the production of FFA over protein or carbohydrate breakdowns (Patton, 1963; Ohren and Tuckey, 1969; Foda et al., 1974; Dulley and Grieve, 1974). To accelerate flavor development during ripening, therefore, researchers attempted to promote lipolysis and proteolysis by adding enzymes to cheese curd (Law, 1980). Richardson et al. (1971) showed that better flavors were developed when gastric lipase preparations were included in Cheddar and Provolone cheese manufacturing. Kosikowski and Iwasaki (1975) and Sood and Kosikowski (1979a, b) reported that fungal acid proteinases and bacterial decarboxylases did not contribute to cheese flavor development, but controlled additions of small amounts of lipase and neutral protease produced strong flavor in Cheddar cheese slurries in a relatively short time without causing distinctive flavor defects. Law and Wigmore (1982) found that typical cheese flavor was accelerated when neutral proteinase was added to Cheddar cheese curds. Recently, Arbige et al. (1986) reported an insoluble lipase enzyme preparation (Flavor Age) that could be incorporated into milk during Cheddar cheese manufacturing for accelerated cheese flavor development. Their studies indicated that FFA profiles identical to those in controls and total quantities of short-chain FFA (C_4 through C_8) were important for developing typical Cheddar cheese flavors during accelerated cheese ripening.

The objectives of the present work were to investigate the effects of commercial food grade enzymes (calf lipase, neutral protease, and culture/enzyme mixture), singly or in combination, on the liberation of individual FFA and on the degree of lipolysis in cheese at regular and elevated ripening temperatures and to establish various statistical relationships between the quantitative changes of individual FFA and lipolysis.

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MATERIALS & METHODS

Cheese sample preparation

Granular Cheddar cheese was manufactured in the Dept. of Animal Sciences and Industry dairy plant at Kansas State Univ. Standard procedures were followed in cheese making using 600 gallon pasteurized milk and Hansen's Redi-Set DVS starter culture. Enzymes were added to the granular curds using salt (2.5%) as a vehicle before hooping and pressing. After pressing overnight, cheeses were cut to pound-size blocks, vacuum packaged, and stored at 7° and 13°C for 16 wk.

Enzymes

Commercial food grade enzymes including *Bacillus subtilis* neutral proteinase (Neutrase 1.5G, Novo Co., Wilton, CT), lipase (Calf Lipase #600, Miles Laboratories, Inc., Madison, WI), and bacterial culture/enzyme mixture (NaturAge, Miles Laboratories, Inc., Madison, WI) were incorporated into cheeses at various levels as shown in Table 1. Cheese samples without added enzyme preparations served as controls.

Chemical compositional analysis

All cheese samples were analyzed on the second day after vacuum packaging for moisture, fat and protein using the *Standard Methods for the Examination of Dairy Products* (Case et al., 1985).

Analysis of free fatty acids

One gram samples of maturing cheese were removed periodically (0, 4, 8, 12 and 16 wk), minced and extracted with diethyl ether and hexane for 2 hr and eluted through a 10 mm i.d. glass column containing neutral alumina according to the method described by Deeth et al. (1983). The column containing alumina with adsorbed FFA then was dried under vacuum and transferred to a stoppered glass tube. One milliliter isopropyl ether containing 6% formic acid was added and mixed thoroughly with the alumina and then centrifuged at 2000 rpm for 5 min. A 1 μL aliquot of the supernatant was injected into a

Table 1—Commercial food grade enzymes incorporated into cheese sample preparations

Samples	Enzymes and types	Commercial sources	Amount[a]
N_1[b]	Neutrase - neutral protease	Novo Co.	0.48
N_2[c]	Neutrase - neutral protease	Novo Co.	2.40
N_3[d]	Neutrase - neutral protease	Novo Co.	4.80
L_1	Calf Lipase #600 - lipase	Miles Lab.	3.00
L_2	Calf Lipase #600 - lipase	Miles Lab.	6.00
CM_1[e]	Neutrase & Calf Lipase - protease & lipase	Novo & Miles	3.48
CM_2[f]	Neutrase & Calf Lipase - protease & lipase	Novo & Miles	8.40
NA_1	NaturAge® - culture/enzyme mixture	Miles Lab	5.00
NA_2	NaturAge® - culture/enzyme mixture	Miles Lab	10.00
NA_3	NaturAge® - culture/enzyme mixture	Miles Lab	20.00

[a] Amount of enzyme applied was g/40 pounds cheese curd.
[b,c,d] Levels of Neutrase were determined as 2, 10, and 20 units of proteolytic activity, respectively, using the Hide Powder Azure method (Law and Wigmore, 1982).
[e] Combination of N_1 and L_1.
[f] Combination of N_2 and L_2.

Hewlett-Packard Model 5880A Gas Chromatograph (GC) equipped with a flame ionization detector. A GC Terminal (Level Four) integrator (Hewlett-Packard, Palo Alto, CA) was used for the analysis of FFA including C_4, C_6, C_8, C_{10}, C_{12}, C_{14}, C_{16}, C_{18}, $C_{18:1}$, $C_{18:2}$, and $C_{18:3}$. The FFA separations were achieved using a 91 cm × 2 mm i.d. glass column packed with 10% SP-216-PS on 100/120 Supelcoport (Supelco Inc., Bellefonte, PA). The GC was operated with nitrogen carrier gas flow at 40 mL/min, hydrogen gas at 29 mL/min, and air at 410 mL/min. The column oven was programmed at three temperature levels: initial holding for 2 min at 90°C, first level heating to 110°C at 5°C/min, holding for 2 min; second heating to 150°C at 8°C/min, holding for 5 min; third heating to 180°C at 5°C/min, holding for 8 min. Both injector and detector temperatures were 230°C. All quantitative analyses were done by relating the peak area of individual FFA to that of valeric acid added as an internal standard. Each FFA was identified by the retention time of a standard mixture.

Lipolysis

The total degree of lipolysis was measured by the Acid Degree Value method as modified by Dulley and Grieve (1974).

Data analysis

Statistical analyses including analysis of variance (ANOVA), multiple comparisons, and correlation were employed for treating all data (Snedecor and Cochran, 1980).

RESULTS & DISCUSSION

Chemical composition

Both experimental and control cheese samples used for analysis were similar in moisture (36.03–37.13%), fat (31.5–32.0%), and protein (23.51–24.65%). These compositional ranges were not significantly different ($p > 0.01$).

Production of free fatty acids

For both control and enzyme-treated samples ripened for 16 wk, the production of individual FFA (C_4 to $C_{18:3}$) was accelerated more at 13°C than at 7°C. Cheese samples with lipase (L_1, L_2) and lipase/protease combinations (CM_1, CM_2) showed a significant ($p<0.01$) increase in the production of individual FFA at both ripening temperatures when compared with either control or Neutrase- and NaturAge-treated cheese. The effects of various levels of enzyme treatment and ripening temperatures on the production of FFA are illustrated in Fig. 1 through Fig. 6, with C_4 representing the short-chain FFA and C_{16} the long-chain FFA. Neutrase (protease) had little effect on the production of individual FFA within each ripening temperature used (Fig. 1 and 4). When lipase and Neutrase were combined at the same levels as used individually (CM_1, CM_2), however, they promoted a greater degree of lipolysis than either one (Fig. 2 and 5). This synergistic effect in FFA production not only may be significant in understanding the behavior of added enzymes in cheese flavor development, but also may aid in explaining flavor development in conventional cheese ripening. Cheese with NaturAge had lipolytic activities similar to those of the control samples in the production of individual FFA at both ripening temperatures (Fig. 3 and 6), except samples with the high level (NA_3) showed pronounced acceleration in the production of short-chain FFA at 13°C (Fig. 3). The production of long-chain FFA (Fig. 6) was somewhat less affected by higher ripening temperature; however, NA_3 still produced a larger amount within each ripening temperature.

Short-chain FFA profiles

The ratios between short-chain FFA (C_4, C_6, C_8, C_{10}) and total free fatty acids (TFFA) of control and experimental cheeses ripened at 7 and 13°C for 16 wk are shown in Tables 2 and 3, respectively. Statistical analyses indicated that cheese treated with lipase (L_1, L_2) and lipase/protease combination (CM_1, CM_2) showed significant ($p<0.05$) increase in the ratio of short-chain FFA and TFFA as compared to control, Neutrase- or Natur Age-treated cheese at both ripening temperatures. To further illustrate the effects of various enzymes on production of individual short-chain FFA, a break-down was made for high and low ratio groups, and a multiple comparison test was

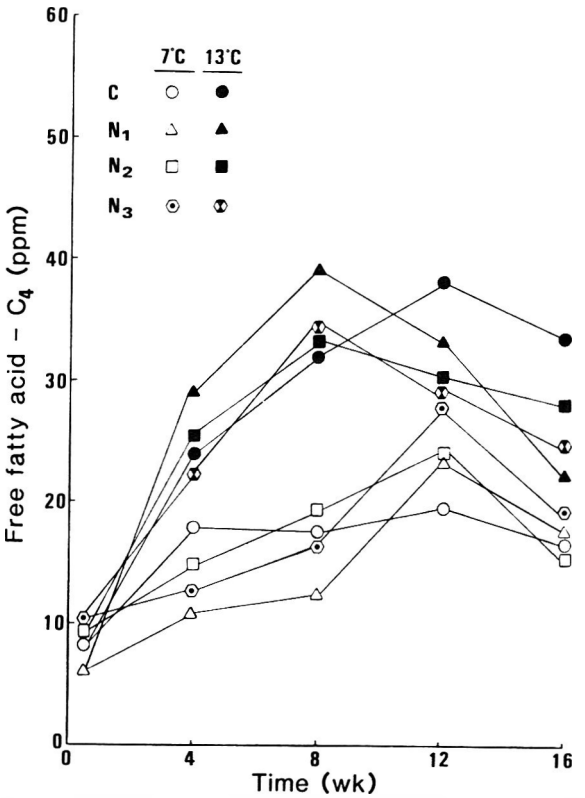

Fig. 1—Production of C_4 (ppm) in control (C) and Neutrase (N_1, N_2, N_3)-treated cheese ripened at 7° and 13°C for 16 wk.

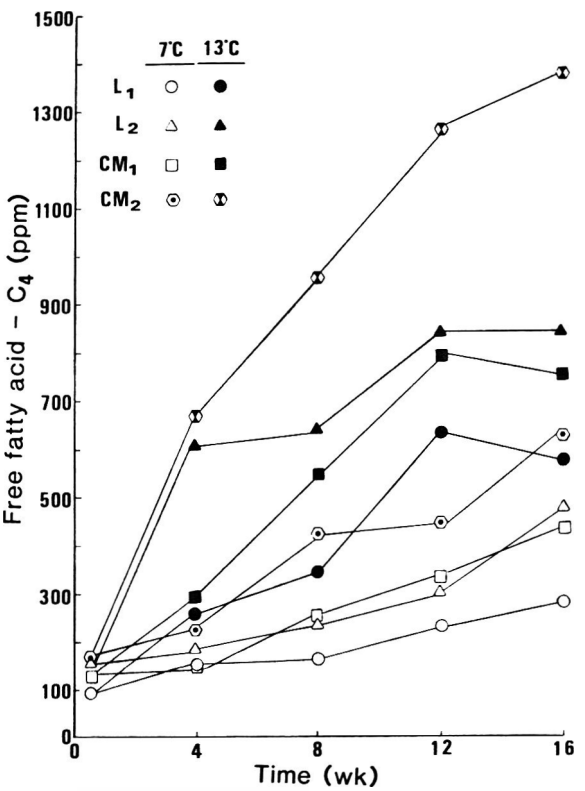

Fig. 2—Production of C_4 (ppm) in lipase- (L_1,L_2) and lipase/protease combination (CM_1, CM_2)-treated cheese ripened at 7° and 13°C for 16 wk.

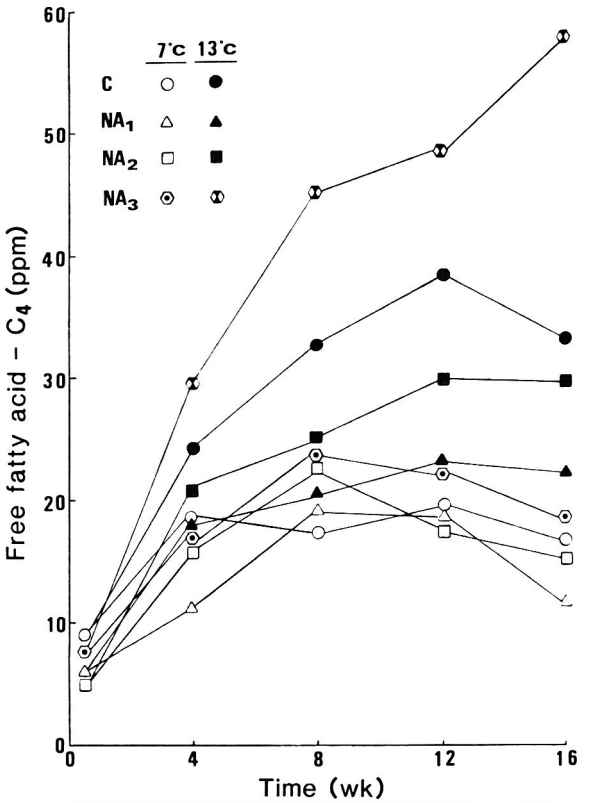

Fig. 3—Production of C_4 (ppm) in control (C) and NaturAge (NA_1, NA_2, NA_3)-treated cheese ripened at 7° and 13°C for 16 wk.

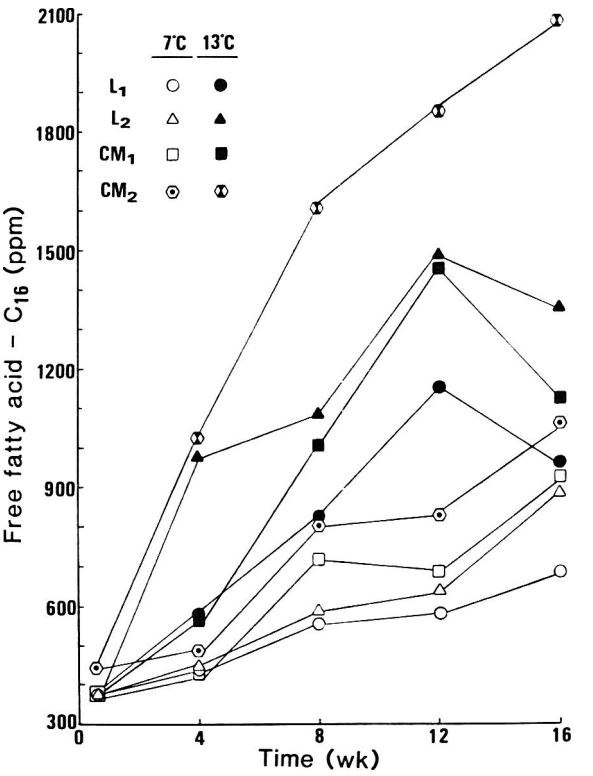

Fig. 5—Production of C_{16} (ppm) in lipase- (L_1, L_2) and lipase/protease combination (CM_1, CM_2)-treated cheese ripened at 7° and 13°C for 16 wk.

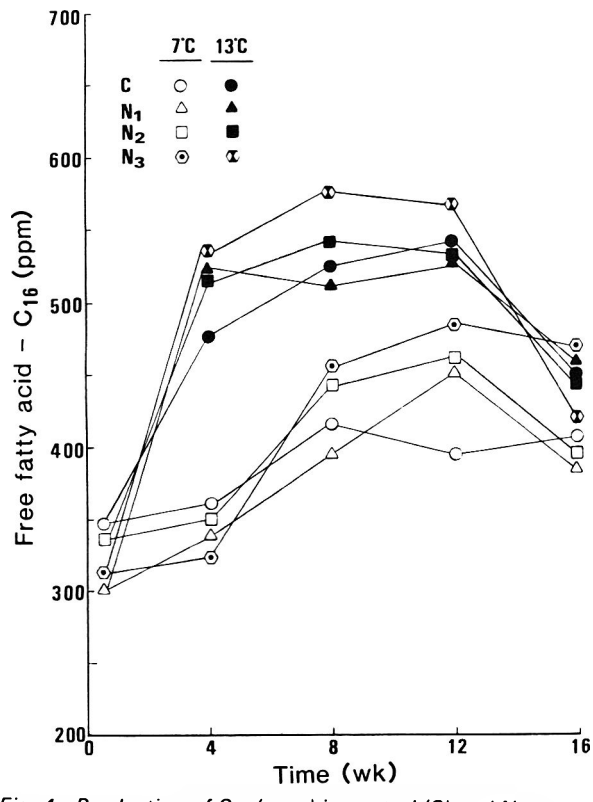

Fig. 4—Production of C_{16} (ppm) in control (C) and Neutrase (N_1, N_2, N_3)-treated cheese ripened at 7° and 13°C for 16 wk.

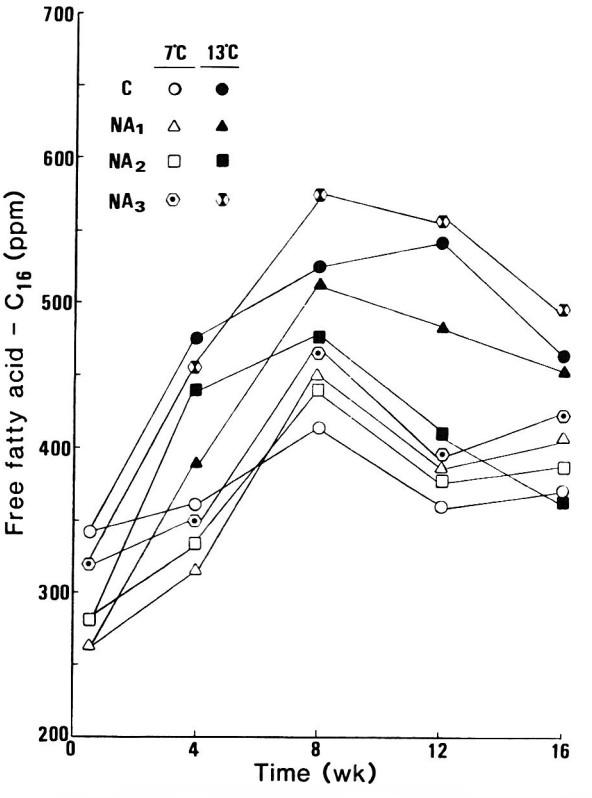

Fig. 6—Production of C_{16} (ppm) in control (C) and NaturAge (NA_1, NA_2, NA_3)-treated cheese ripened at 7° and 13°C for 16 wk.

performed (Table 4). High level lipase (L_2) and lipase/protease combination (CM_2) were significantly different ($p<0.05$) in their ratios from L_1 and CM_1, except for the ratio C_{10}/TFFA of CM_1 and CM_2 at 13°C. Control samples ripened at 7°C were similar to cheese treated with all levels of Neutrase and NaturAge; only the ratio of C_4/TFFA showed a significant ($p<0.05$) difference from low level Neutrase-treated cheese

Table 2—Ratio[a] between short-chain free fatty acids (FFA, C_4, C_6, C_8, C_{10}) and total free fatty acids (TFFA) of control and enzyme-treated granular cheese samples ripened at 7°C for 16 wk[b]

Sample[c]	C_4/TFFA		C_6/TFFA		C_8/TFFA		C_{10}/TFFA	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Control	1.65	0.17	1.05	0.21	0.74	0.05	2.79	0.19
N_1	1.33	0.29	1.08	0.38	0.63	0.14	2.72	0.27
N_2	1.58	0.23	1.22	0.48	0.68	0.12	2.67	0.24
N_3	1.50	0.21	1.09	0.42	0.72	0.06	2.74	0.20
L_1	8.51	0.81	3.79	0.29	2.03	0.26	5.57	0.31
L_2	10.15	0.68	4.56	0.29	2.34	0.18	6.37	0.42
CM_1	9.45	0.84	4.22	0.33	2.19	0.27	6.06	0.55
CM_2	11.13	0.91	4.84	0.34	2.54	0.18	6.85	0.43
NA_1	1.41	0.35	1.27	0.60	0.69	0.13	2.69	0.27
NA_2	1.61	0.09	1.02	0.37	0.74	0.08	2.74	0.17
NA_3	1.74	0.30	1.17	0.41	0.70	0.13	2.70	0.22

[a] Ratio = short-chain FFA/TFFA × 100.
[b] Two replicates were measured at each 4 wk interval.
[c] Sample preparations refer to Table 1.

Table 3—Ratio[a] between short-chain free fatty acids (FFA, C_4, C_6, C_8, C_{10}) and total free fatty acids (TFFA) of control and enzyme-treated granular cheese samples ripened at 13°C for 16 wk[b]

Sample[c]	C_4/TFFA		C_6/TFFA		C_8/TFFA		C_{10}/TFFA	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Control	2.23	0.37	1.28	0.29	0.77	0.06	2.90	0.29
N_1	2.02	0.36	1.33	0.50	0.82	0.13	2.91	0.20
N_2	2.06	0.31	1.47	0.61	0.82	0.08	2.87	0.27
N_3	1.73	0.28	1.14	0.41	0.74	0.11	2.73	0.12
L_1	10.90	0.90	4.66	0.31	2.31	0.20	6.30	0.45
L_2	12.88	0.72	5.41	0.26	2.58	0.13	6.75	0.38
CM_1	12.24	1.24	5.18	0.42	2.53	0.22	6.76	0.32
CM_2	13.45	0.65	5.75	0.15	2.79	0.12	7.09	0.13
NA_1	1.62	0.27	1.22	0.38	0.71	0.06	2.76	0.10
NA_2	2.05	0.56	1.27	0.20	0.79	0.09	2.97	0.23
NA_3	3.39	0.88	2.38	0.71	1.02	0.16	3.39	0.31

[a] Ratio = short-chain FFA/TFFA × 100.
[b] Two replicates were measured at each 4 wk interval.
[c] Sample preparations refer to Table 1.

Table 4—Multiple comparisons[a] of the production ratio of short-chain free fatty acids (C_4, C_6, C_8 and C_{10}) to total free fatty acids (TFFA)[b]

Sample[c]	Ripening temperature: 7°C				Ripening temperature: 13°C			
	C_4/TFFA	C_6/TFFA	C_8/TFFA	C_{10}/TFFA	C_4/TFFA	C_6/TFFA	C_8/TFFA	C_{10}/TFFA
L_1	c	c	c	c	c	c	c	c
L_2	b	a	a,b	b	a,b	b	b	b
CM_1	b	b	b,c	b	b	b	b	a
CM_2	a	a	a	a	a	a	a	a
Control	d,e	d	d	d	e	e	e,f	e,f
N_1	f	d	d	d	e,f	e	e	e,f
N_2	d,e	d	d	d	e,f	e	e	e,f
N_3	d,e,f	d	d	d	f	e	e,f	f
NA_1	e,f	d	d	d	f	e	f	e,f
NA_2	d,e	d	d	d	e,f	e	e,f	e
NA_3	d	d	d	d	d	d	d	d

[a] Multiple comparisons were made by protected LSD method; alpha = 0.05; columns with a different letter represent significant difference at $p<0.05$ (Snedecor and Cochran, 1980).
[b] Data refer to Tables 2 and 3.
[c] Sample preparations refer to Table 1.

(N_1). When ripened at 13°C, NA_3 showed a significantly ($p<0.05$) higher production rate than control, Neutrase-, and low and medium level of NaturAge (NA_1, NA_2)-treated cheese (Table 4). These results suggested that NaturAge at low or medium concentrations in ripened cheese, yielded short-chain FFA profiles similar to those of the control samples. However, high level NaturAge (NA_3) at 13°C promoted a higher production rate, which may be important for the flavor development of accelerated cheese ripening. Among Neutrase-treated cheeses, although no significant differences ($p>0.05$) were observed in the production rate of each short-chain FFA, N_3 showed the least production at 13°C. This phenomenon may be related to the high rate of proteolysis (data not shown), which might have interfered with the lipolytic activities. Law and Wigmore (1982) reported that Neutrase had a statistically significant effect on the intensity of Cheddar cheese flavor development. However, our Neutrase-treated samples, which were similar in short-chain FFA profiles to the control samples, were not significantly different in the total concentrations of C_4, C_6 and C_8. It has been suggested that the total concentration of C_4, C_6 and C_8 is an important factor for flavor development during cheese ripening (Arbige et al., 1986). In addition to the importance of short-chain FFA profiles, the total free amino acids and peptides produced by protease also impart flavor to the total flavor development. In fact, many studies have indicated that strong correlations exist between total free amino acids and flavor development in Cheddar cheese (Aston et al., 1983a, b and c; 1985).

Adding lipase to cheese could not only significantly promote production of total FFA but also influence the rate of individual FFA production (Tables 2 and 3). Control samples produced the largest quantities of C_{10} among short-chain FFA, whereas C_4 was a predominant FFA in the lipase and lipase/protease combination-treated cheeses (L_1, L_2, CM_1, and CM_2). Similar results were observed in producing large quantities of C_4 when lipases were incorporated into either butter cream or cheeses

Table 5—Correlation coefficients (r) of selected free fatty acids (FFA) and total FFA (TFFA) in control and enzyme-treated granular cheeses at 7° and 13°C for 16 wk

FFA	Ripening temperature: 7°C					Ripening temperature: 13°C				
	Control	N[a]	L[b]	CM[c]	NA[d]	Control	N	L	CM	NA
C_4/Low[e]	0.924	0.943	0.999	0.999	0.933	0.980	0.956	0.999	0.999	0.985
C_4/High[f]	0.914	0.940	0.999	0.999	0.931	0.974	0.956	0.999	0.999	0.984
C_4/C_{12}	0.700	0.846	0.982	0.977	0.534	0.757	0.855	0.987	0.990	0.804
$C_4/C_{18:1}$	0.697	0.824	0.966	0.967	0.613	0.730	0.714	0.963	0.984	0.460
C_6/Med[g]	0.984	0.991	0.999	0.999	0.993	0.986	0.994	0.999	0.999	0.998
C_6/C_{14}	0.723	0.883	0.944	0.981	0.842	0.889	0.811	0.990	0.992	0.697
C_6/C_{16}	0.453[h]	0.843	0.962	0.970	0.800	0.843	0.709	0.986	0.991	0.547
$C_6/C_{18:1}$	0.489[h]	0.734	0.976	0.980	0.598	0.650	0.562	0.970	0.985	0.472
C_8/C_{14}	0.838	0.928	0.965	0.983	0.861	0.956	0.940	0.989	0.995	0.749
C_{10}/C_{12}	−0.165[h]	0.832	0.994	0.997	0.750	0.841	0.922	0.997	0.999	0.851
C_{10}/C_{16}	−0.041[h]	0.890	0.956	0.962	0.908	0.770	0.911	0.979	0.997	0.650
C_{16}/TFFA	0.904	0.969	0.983	0.988	0.984	0.979	0.983	0.997	0.998	0.950
$C_{18:1}$/TFFA	0.919	0.969	0.996	0.997	0.942	0.965	0.965	0.992	0.996	0.933
Low/High	0.996	0.998	0.999	0.999	0.998	0.997	0.999	0.999	0.999	0.999

[a] Neutrase-treated cheese samples.
[b] Lipase-treated cheese samples.
[c] Combination of Neutrase- and lipase-treated cheese.
[d] NaturAge-treated cheese samples.
[e] Low = $C_4 + C_6$.
[f] High = $C_4 + C_6 + C_8$.
[g] Med = $C_6 + C_8$.
[h] Not significant ($p>0.05$); all others significant at $p<0.05$.

(Harper, 1957; Nelson, 1972; Arnold et al., 1975; Law and Wigmore, 1985; Arbige et al., 1986). Jensen (1964) suggested that this was due to the preferential release of C_4 as a low molecular weight fatty acid when triglycerides were hydrolyzed by certain lipolytic enzymes. Law and Wigmore (1985) indicated that typical cheese flavor could not be developed in lipase-treated cheese because of the flavor imbalance produced by high levels of C_4. However, Arbige et al. (1986) showed that controlled production of C_4 and accelerated cheese flavor development could be achieved by using selected fungal lipase in conjunction with fungal protease. Examination of the ratios between short-chain FFA and TFFA (Tables 2 and 3) indicates some similarities, such as those between 7°C L_2 and 13°C L_1, 7°C CM_2 and 13°C CM_1, and 7°C NA_2 and 13°C NA_1. This phenomenon suggests that FFA production rate can be accelerated at higher ripening temperatures with less enzyme.

Correlation of FFA

As shown in Table 5, some significant correlations existed among the FFA of enzyme-treated samples. Lipase (L_1 and L_2) and lipase/protease combination-treated cheese (CM_1 and CM_2) showed higher correlation coefficients than other samples at both ripening temperatures. Among individual FFA, C_8 and C_{14} were correlated most. However, Low ($C_4 + C_6$) and High ($C_4 + C_6 + C_8$) combinations showed the best correlations ($r=0.996$ or better) in all tested samples. According to the correlation, the concentrations of C_4 and C_6 may substitute for the combination of C_4, C_6 and C_8, which has been reported to be responsible for the total flavor development (Arbige et al., 1986), as a flavor indicator during accelerated cheese ripening. The high correlation coefficients between either C_{16} or $C_{18:1}$ and TFFA ($r=0.904$ - 0.997) indicated that these two long-chain FFA were produced in a consistent ratio under lipolysis with or without added enzymes at both ripening temperatures.

Similarity of FFA profiles

Examination of the composition of the FFA produced in all treatments indicated that cheese samples with the medium level of NaturAge (NA_2), ripened at 13°C for 4 wk, were similar in FFA profiles to the control samples, which were ripened at 7°C for 12 wk (Table 6). Each individual FFA showed the same, or very similar, concentrations, except that C_{16} and C_{18} congeners appeared to be higher with NA_2. However, statistical analysis of paired t-test indicated no significant difference ($p>0.05$) between these two FFA profiles. This similarity of FFA profiles between control and NaturAge-treated samples may be an important contributor to accelerated cheese flavor development, as reported by Arbige et al. (1986). In addition, cheese with a high level of NaturAge (NA_3) ripened at 13°C showed significant increases in the production of each short-chain FFA when compared with control samples (Tables 3 and 4). These observations suggested that further efforts on combinations of time, ripening temperature, and enzyme concentration would be needed to produce large concentrations of short-chain FFA, which might be a key factor for developing an accelerated cheese ripening process applicable in the dairy industry.

Lipolysis

Acid degree values (ADV) of control and enzyme-treated cheeses at both ripening temperatures are shown in Table 7. Significantly higher ($p<0.05$) ADV readings were observed at the initial measurement (0 wk) of L_1, L_2, CM_1 and CM_2 samples over other samples. This could have resulted from lipase activities on butterfat during the time of incorporating enzymes, pressing curds and vacuum packaging, which were done at room temperature. Control, Neutrase- and NaturAge-treated samples all showed slow but consistent increases in ADV, except that N_1 and N_2 at 7°C and some samples at 13°C had a slight drop in ADV. Samples containing lipase (i.e., L_1, L_2, CM_1 and CM_2) showed rapid increases in ADV after 4 wk at 13°C and 8 wk at 7°C and yielded pronounced rancid flavor. The increase of ADV during ripening was considered mainly coming from the production of C_4 and greater FFA in Cheddar cheese, because cheese made with skim milk showed no measurable increase of ADV and only contained fair amounts of acetic acid (Dulley and Grieve, 1974). Deeth and Fitz-Gerald (1975) reported that an inverse relationship existed between ADV and flavor scores in Cheddar cheese.

For a better understanding of the role of each individual FFA in ADV and rancid flavor development, further statistical analyses were made. Table 8 shows the correlation coefficients of ADV and selected FFA of control, lipase- and lipase/protease combination-treated cheeses at both ripening temperatures for 16 wk. Significant correlations between ADV and selected FFA were observed, with the exception of L_2 at 13°C and control samples at either temperature used. The combinations of either 2 or 3 short-chain FFA (Low, Med, and High) and TFFA did not yield significantly better correlations between ADV and short-chain FFA. Although Marsili (1985) reported that lipolysis in Cheddar cheese could be predicted

Table 6—Free fatty acids (FFA) composition (μg/g cheese) of control (7°C, 12 wk) and NA_2-treated cheese (13°C, 4 wk)

Sample	C_4	C_6	C_8	C_{10}	C_{12}	C_{14}	C_{16}	C_{18}	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
Control[a]	19.5	12.1	7.9	30.1	45.8	138.6	371.3	116.1	291.7	27.3	11.0
(S.D.)	0.4	1.4	1.0	0.9	1.1	13.5	26.3	0.4	7.4	0.3	1.2
NA_2[a]	20.6	11.1	7.9	30.1	45.2	134.8	385.1	134.5	312.1	41.3	11.4
(S.D.)	0.5	1.2	1.1	4.5	5.8	14.0	37.0	9.3	12.9	10.7	1.1

[a] The mean value of duplicates.

Table 7—Acid degree values (ADV) of control and enzyme-treated granular cheese samples ripened at 7° and 13°C for 16 wk[a]

	Ripening Temperature: 7°C					Ripening Temperature: 13°C				
Sample[b]	0 wk[c]	4 wk	8 wk	12 wk	16 wk	0 wk[c]	4 wk	8 wk	12 wk	16 wk
Control	0.44	0.64	0.70	0.71	0.72	0.44	0.66	0.72	0.76	0.77
N_1	0.47	0.67	0.70	0.68	0.62	0.47	0.63	0.73	0.81	0.74
N_2	0.53	0.67	0.69	0.71	0.67	0.53	0.61	0.71	0.79	0.80
N_3	0.53	0.66	0.68	0.73	0.79	0.53	0.61	0.76	0.81	0.76
L_1	0.71	1.02	1.14	1.38	1.47	0.71	1.19	1.47	3.11	2.32
L_2	0.79	1.17	1.32	1.65	1.78	0.79	2.32	2.29	3.26	3.61
CM_1	0.72	0.88	1.33	2.03	2.12	0.72	1.35	2.17	3.50	3.07
CM_2	0.75	1.41	1.75	2.23	2.41	0.75	1.96	3.65	4.96	5.49
NA_1	0.45	0.60	0.65	0.74	0.78	0.45	0.63	0.69	0.85	0.64
NA_2	0.45	0.62	0.65	0.75	0.80	0.45	0.66	0.68	0.87	0.81
NA_3	0.55	0.64	0.76	0.78	0.85	0.55	0.68	0.71	0.84	0.79

[a] Data represent mean of duplicates.
[b] Sample preparations refer to Table 1.
[c] ADV was measured on the third day after cheese making.

Table 8—Correlation coefficients (r) of acid degree values (ADV) and selected free fatty acids (FFA) of control (C), lipase- (L_1, L_2) and enzyme combination (CM_1, CM_2)-treated granular cheese samples ripened at 7° and 13°C for 16 wk

Ripening temp (°C)	Sample[a]	Correlation coefficients (r) between ADV and FFA						
		C_4	C_6	C_8	Low[b]	Med[c]	High[d]	TFFA[e]
7	C	0.900	0.336[f]	0.520[f]	0.763	0.401[f]	0.758	0.480[f]
7	L_1	0.966	0.977	0.959	0.971	0.973	0.971	0.938
7	L_2	0.897	0.911	0.921	0.902	0.915	0.906	0.915
7	CM_1	0.947	0.939	0.929	0.945	0.937	0.943	0.914
7	CM_2	0.930	0.938	0.950	0.933	0.944	0.937	0.940
13	C	0.933	0.688	0.654	0.871	0.702	0.856	0.680
13	L_1	0.931	0.902	0.854	0.923	0.888	0.916	0.861
13	L_2	0.878	0.561[f]	0.718	0.886	0.618[f]	0.888	0.805
13	CM_1	0.988	0.972	0.988	0.986	0.979	0.987	0.975
13	CM_2	0.994	0.989	0.984	0.996	0.988	0.996	0.992

[a] Sample preparations refer to Table 1.
[b] Low = $C_4 + C_6$.
[c] Med = $C_6 + C_8$.
[d] High = $C_4 + C_6 + C_8$.
[e] Total free fatty acids.
[f] Not significant ($p>0.05$); all others significant at $p<0.05$.

statistically by applying C_{10}, C_{14} and C_{16} to the regression equation, our results indicated that short-chain FFA were better correlated with ADV than individual medium- or long-chain FFA (data not shown) in lipase-added cheese samples. It is interesting that although no rancid flavor was detected in the control cheese at either ripening temperatures for 16 wk, high correlations ($r=0.90$ at 7°C; $r=0.93$ at 13°C) were observed only between butyric acid and ADV. Rancid flavor in milk is due to the lipolysis of milk fats. It was reported to be due to the combination of C_{10} and C_{12} fatty acids (Al-Shabibi et al., 1964) or the combination of C_4 through C_{12} (Scanlan et al., 1965). However, the relationship between rancid flavor in cheese and short-chain FFA is not clear in the literature, although rancid Cheddar cheese had been reported to contain two or three times more of C_{10}, C_{12} and C_{14} than fine quality cheeses (Ohren and Tuckey, 1969). It was also reported that a cheese ADV of about 3.0 or above was both rancid and butyric (Deeth and Fitz-Gerald, 1975). We feel that if rancid flavor is the result of the combination of C_4, C_6 and C_8, the ADV may not be a good indicator for detecting rancidity in cheese. However, if C_4 is mostly responsible for rancid flavor, the ADV can be used as an indicator, since we found good correlations between C_4 and ADV in both control and lipase-treated rancid samples. Further studies are under way in our laboratory for better understanding of the relationship between ADV, individual FFA and rancidity in cheese.

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Milk Curdling by Rennet under High Pressure

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ABSTRACT

The effect of pressure on milk curd formation initiated by rennet was studied under high pressures. The milk used was a 20% solution of skim milk powder in 0.02M $CaCl_2$. After holding the milk with rennet at 35°C for given periods under high pressure, up to 1300 kg/cm^2, the extent of proteolytic digestion and the curd tension were measured under atmospheric pressure. Rennet and milk protein were not denatured by pressure under the experimental conditions used. The primary phase reaction of milk curdling by rennet (hydrolysis of casein) was not affected by compression. The secondary phase reaction (core formation of casein micelle aggregates) was delayed under high pressure and the tertiary phase reaction (milk curd formation) was accelerated.

INTRODUCTION

SINCE MILK COAGULATION by rennet is the essential step in cheese manufacture, the coagulation mechanism has been studied in the past (Niki, 1980; Kaminogawa and Shimizu, 1983). The primary phase is known to be hydrolysis of κ-casein, located on the surface of casein micelle. The peptide bond between residues 105 (phenylalanine) and 106 (methionine) in κ-casein is specifically hydrolyzed by chymosin (purified rennet). This reaction separates the extremely soluble hydrophilic glycomacropeptide from the molecule leading to destabilization of the casein micelle in the presence of calcium ions. The secondary phase of coagulation is the formation of cores for the aggregation of destabilized casein micelles. This core formation was elucidated from the dramatic increases in viscosity and from microscopic examinations of micelle size (Green et al., 1978). The cores then grow to form milk curd. This tertiary phase of coagulation is associated with an increase in the rigidity of the curd (Yun et al., 1981). In this phase, the tension of curd formed by immobilized rennet increased linearly with increase of incubation time at 35°C, even though the immobilized enzyme was removed from the renneted milk system just before the initiation of coagulation (Yun et al., 1981). This indicates that the secondary and tertiary phases are nonenzymatic reactions. However, all these experiments were carried out under atmospheric conditions, and there has never been an experimental trial to see whether milk coagulation can develop under higher pressure. The object of this report was to study milk coagulation by rennet and curd formation under high pressures up to 1300 kg/cm^2. Measurements were made with a curd tension meter and the results are discussed in terms of activation volume changes accompanied by rennet milk coagulation at each phase of milk curdling.

MATERIALS & METHODS

Materials

Skim milk powder (Snow Brand Milk Products Co., Japan) was dissolved in 0.02M $CaCl_2$ solution at a concentration of 20% (pH 6.4–6.5) to obtain the curd tension values measurable with our curd meter. This solution was used as the test milk in all trials. Hansen's Danish Rennet Tablets were dissolved at a given concentration in tris hydroxymethylaminomethane-HCl buffer (Tris, 5 mM, pH 7.0) and dialyzed against a large amount of the same buffer at 5°C overnight. The dialysate was centrifuged at 8000 × g for 15 min and the supernatant was used as the rennet solution. All the other reagents were of analytical grade.

Methods. Clotting time of the test milk, degassed under reduced pressure, was determined by detecting curd flecks around a red glass rod in a test tube at 35°C after the addition of the rennet solution (Iwasaki et al., 1967). The amount of rennet was controlled to initiate coagulation of the test milk (10 mL) in 20 min. The test milk was clotted by rennet in a small test tube (5 × 50 mm). Curd tension or rigidity was determined with a curd tension meter (M-301AR, Iio Electric Co., Japan) and was calculated as follows (Iio, 1969):

$$\text{Curd tension} = \frac{F}{S} g \ (\text{dyne/cm}^2),$$

where F = reading of curd tension meter at the time at when the surface texture of the curd was just broken by a curd sensor (curd knife); S = surface area of the curd sensor; and g = acceleration of gravity. The curd meter was sensitized with a highly sensitive spring because only the smaller curd sensor with 2 mm diameter rather than the regular one with 20 mm diameter was available.

The extent of proteolysis of milk protein by rennet was determined according to a following equation,

$$\text{Extent of proteolysis (\%)} = \frac{E - C}{T - C} \times 100$$

where E = the amount (mg/mL) of 5% trichloroacetic acid (TCA) soluble nitrogenous compounds in the test milk after enzymatic reaction; C = the amount (mg/mL) of 5% TCA soluble nitrogenous compounds in the test milk before enzymatic reaction; and T = the amount (mg/mL) of total nitrogenous compounds in the test milk. The amounts of the nitrogenous compounds in the test milk or the coagulum solubilized in 5% TCA solution were estimated according to the Lowry-Folin method (Lowry et al., 1951).

A Bridgman-type high-pressure apparatus utilizable up to 6000 atm (Kobe Steel Co., Japan) was employed to treat the samples under high pressure. The hydrostatic pressure was generated through silicone oil with a commercial hand pump. The pressure was measured with a bridge-type pressure gauge (Model SS-80-E, Toyo Baldwin Co., Japan). The temperature of the sample, controlled by circulating water of a given temperature through the jacket surrounding the exterior of the pressure vessel was measured with a digital thermometer (Model D221, Takara Kogyo Co., Japan), equipped with a sensor imbedded in the jacket. Temperature fluctuations in the equilibrated vessel were within 0.1°C. The times for inserting and taking out the sample under high pressure were about 5 min and were included in the press time. The mixtures of test milk and rennet solution were transferred into the small test tubes described above. Each tube was covered with a plastic film (Parafilm, American Can Co.), set in the pressure vessel and then compressed at a given pressure for a given period. The rigidity (curd tension) of the sample was determined under atmospheric pressure immediately after decompression at room temperature (20°C).

RESULTS & DISCUSSION

THE CURD TENSION value of the coagulum prepared by soluble rennet was 4 × 10^4 dyne/cm^2 after 90 min incubation at 35°C under atmospheric pressure. This value was used as a standard to compare the curd tensions of other samples. Thus, all the curd tension values shown in this report are presented as the percentage of this standard value.

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The effect of pressure on the proteolytic activity of rennet on the test milk protein was studied. The mixture of the test milk and rennet was kept under a pressure of 1300 kg/cm^2 at 35°C for a given period, and the extent of proteolysis after the decompression of the samples (Fig. 1) was determined. The extent of proteolysis increased up to about 2% after 70 min incubation. This proteolytic profile was very similar to that of control experiments performed under atmospheric pressure, suggesting that the proteolytic activity of rennet was negligibly affected by the compression at 1300 kg/cm^2. The residual proteolytic activity of rennet which had been compressed at 1300 kg/cm^2 for 60 min was measured against the test milk protein under atmospheric pressure. The extent of proteolysis by rennet with and without the pressure treatment was also very close for the same reaction period. This result suggested that proteolytic activity of rennet was negligibly affected by pressure, i.e., rennet was insignificantly inactivated during incubation for 60 min at 35°C under 1300 kg/cm^2.

The rigidities of the rennet curd formed with the test milk which had been compressed at 1300 kg/cm^2 and of the native rennet having no pressure treatment were determined. As shown in Fig. 2, the milk clotting time (about 20 min) and the rate of rigidity increase of the rennet curd of the compressed test milk were very similar to those of noncompressed test milk. The effects of pressure on the milk protein were further tested by checking digestibility of the compressed milk protein by native rennet. The test milk which had been compressed at a pressure of 1300 kg/cm^2 for 60 min at 35°C was hydrolyzed by native rennet under atmospheric pressure to the same extent as the noncompressed skim milk. The degree of hydrolysis by rennet of both milk proteins with and without pressure treatment was around 2% after 60 min reaction, indicating that the pressure treatment did not affect the digestibility of milk protein by rennet.

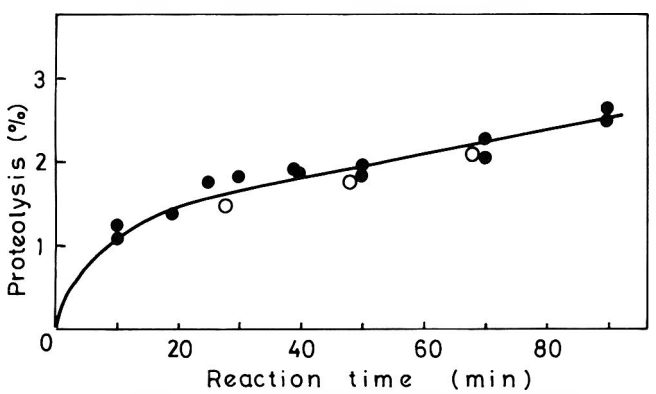

Fig. 1—Time dependence of proteolysis of the test milk by rennet at 35°C. Atmospheric pressures (- ● -); 1300 kg/cm² (- ○ -).

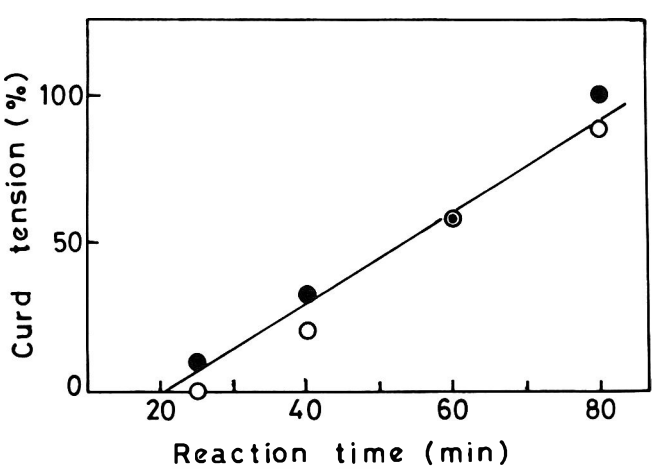

Fig. 2—Time dependence of tension of rennet curd prepared with noncompressed rennet and test milk. Compressed at 1300 kg/cm² for 60 min at 35°C (- ○ -); noncompressed (- ● -). Curd formation and determination of curd tension were performed under atmospheric pressure.

Curd tension values of rennet-treated skim milk were determined after various incubation periods at 35°C under pressures of 400, 800 and 1300 kg/cm^2 (Fig. 3). The milk clotting and curd formation of these samples were carried out under high pressure immediately after addition of rennet to the test milk, i.e., the mixtures were compressed before the initiation of coagulation. Plots of curd tension values vs compression time (min) resulted in linear curves in all cases (Fig. 3). The slope of a curve obtained under compression at 400 kg/cm^2 was 1.3-fold the value obtained under atmospheric pressure. However, the slopes of curves obtained under pressures of 800 and 1300 kg/cm^2 were 0.9-fold and 0.8-fold the slope obtained under atmospheric pressure, respectively. This indicated that the increasing rates of curd tension might be depressed with an increase of pressure in these pressure regions, although the rate was increased at a pressure of 400 kg/cm^2. Each curve (Fig. 3) was extrapolated to find the intersection points on the abscissa, which may be regarded as the initiation times for milk coagulation. The times estimated were 20, 20, 40, and 175 min under pressures of 1, 400, 800, and 1300 kg/cm^2, respectively, suggesting that the initiation time seemed to be delayed according to the increase in pressure, although lower pressures, such as 400 kg/cm^2, may not affect the initiation of milk clotting.

Figure 4 shows the effect of pressure on the rigidity (curd tension values) of the milk curd. The samples were kept under their respective pressures for 60, 90 and 120 min immediately after the addition of rennet to the test milk. The curd tension value increased slightly when the sample was compressed at 200–400 kg/cm^2 for 60 min. However, the value decreased when a pressure higher than 600 kg/cm^2 was applied, and the test milk was not coagulated at all by rennet when pressed for 60 min at 1000 kg/cm^2. In the case of the 90 min incubation, compressions at 200 kg/cm^2 and 400 kg/cm^2 increased the curd rigidity obtained under atmosphere by 20% and 10%, respectively, indicating that compression at 200 kg/cm^2 gave maximum rigidity of the rennet curd. Further increase in pressure lowered curd rigidity, and the test milk could be slightly coagulated at 1000 kg/cm^2. However, additional 30 min incubation allowed formation of curd under the compression at 1000 kg/cm^2 and required a high compression at 1300 kg/cm^2 to inhibit curd formation.

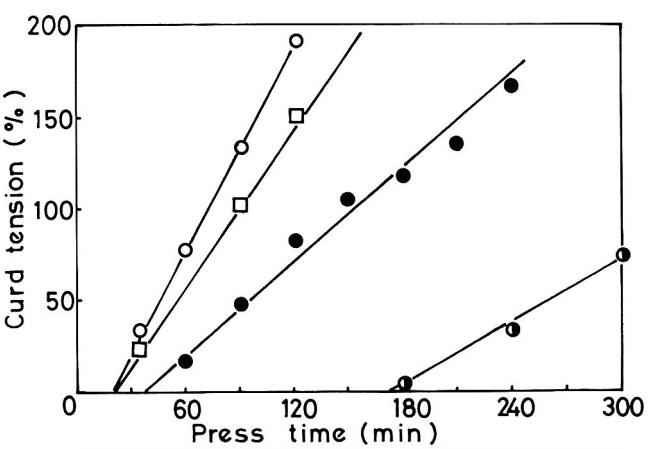

Fig. 3—Effect of compression time and pressure on tension of rennet curd. Atmospheric pressure (- □ -); 400 kg/cm² (- ○ -); 800 kg/cm² (- ● -); and 1300 kg/cm² (- ◑ -) at 35°C. All the samples were pressurized immediately after addition of rennet to the test milk, indicating that the pressure was applied to samples before the initiation of milk clotting.

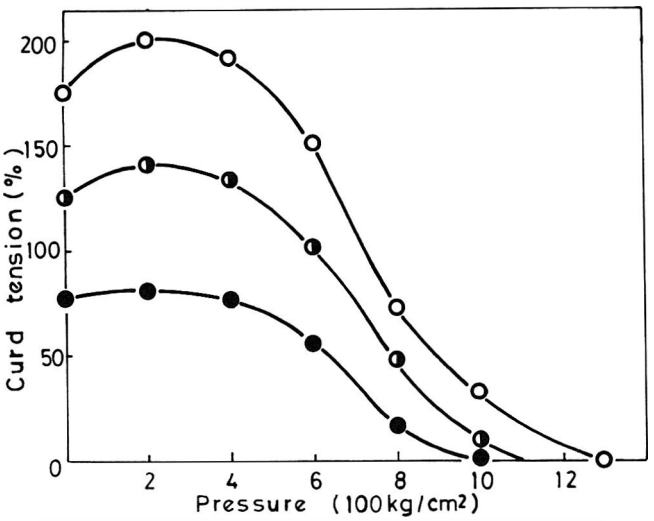

Fig. 4—Pressure dependence of tension of rennet curd at various pressures. 60 min(- ● -); 90 min(- ◑ -); 120 min(- ○ -). Curd tension was determined at atmospheric pressure.

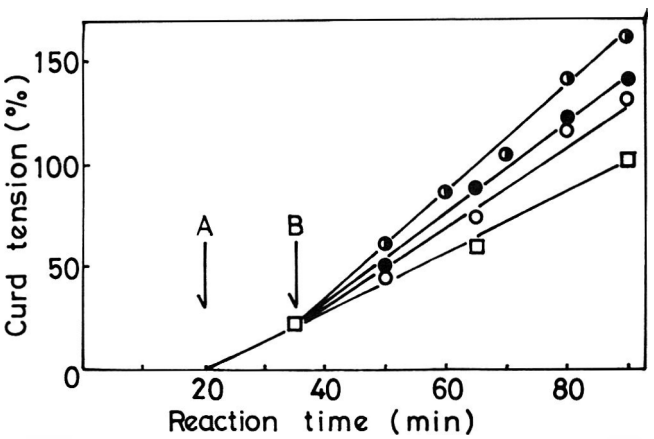

Fig. 5—Time dependence of rennet curd tension. Atmospheric pressure (- □ -); 400 kg/cm²(- ○ -); 800 kg/cm²(- ● -); 1300 kg/cm²(- ◑ -) at 35°C. Samples were pressurized after 15 min milk clotting. Arrows indicate the initiation times for milk coagulation (A) and milk compression at a given pressure (B).

According to Fig. 3 and 4, curd rigidity increases with an increase in incubation time of the samples under any pressure, but the relative rate of rigidity increase decreased with an increase in pressure. These results suggested that curd formation of milk by rennet could proceed slowly even under high pressure.

When high pressure was applied to the sample 15 min after onset of clotting, the tension values increased with increase of both compression time and pressure (Fig. 5). The relative rates of rigidity, estimated from the ratio of slopes of these curves (Fig. 5), increased 1.3, 1.4, and 1.6 under pressures of 400, 800 and 1300, kg/cm^2, respectively. These data indicated that increase in curd rigidity could be accelerated by increasing the pressure when the pressure was applied to the sample 15 min after initiation of clotting under atmospheric pressure.

Due to the technical difficulties of operating a curd tension meter under compressed conditions, all the tension values of the samples tested were determined under atmospheric pressure after decompression. We assumed that milk curdling caused by rennet under compression changed negligibly during the few minutes required for analysis under atmospheric pressure. If this was the case, degradation of milk protein (the primary phase of milk clotting) by rennet would not be affected by pressure as high as 1300 kg/cm^2 because of the insignificant changes in the proteolytic action of rennet and casein properties due to degradation by rennet (Fig. 1 and 2). Therefore, the amount of rennet used in this experiment was controlled to get negligible changes in curd ridigity until the final measurement. Insignificant denaturation of rennet and milk protein by compression at 1300 kg/cm^2 is very reasonable since a super high pressure of 4000–6000 kg/cm^2 is required to induce denaturation of enzymes and other proteins such as α-amylase (Suzuki and Kitamura, 1963), trypsin (Miyagawa and Suzuki, 1963), Taka-amylase (Miyagawa and Suzuki, 1964), γ-globulin (Suzuki and Miyosawa, 1965) and egg albumin (Suzuki, 1958).

Aggregation of casein micelles by rennet action is well known to be a secondary phase of milk coagulation by rennet. This phase seems to be significantly surpressed by compression as shown in Fig. 3, in which formation of curds with measurable rigidity required longer compression time at higher pressure such as 600–1300 kg/cm^2. One of the reasons for the delay of the secondary phase development may be a suppression of collision between casein micelles which were destabilized by the partial degradation of κ-casein by rennet. Similar phenomena were also reported in the cases of the nucleation or core formation found in collagen fibril formation (Gekko and Koga, 1983) and aggregations of myosin (Davis, 1981) and of microtubes (Salmon, 1975; Engelborghs et al., 1976).

Soon after the formation of cores of rennetted casein micelles, curd formation rapidly developed as observed by electron microscopy and viscometry (Green et al., 1978). This is also well known as the tertiary phase reaction of milk curdling, which was obviously enhanced by a pressure higher than atmospheric (Fig. 5). Therefore, the reaction in the third phase might be very different from the one in the secondary phase. These differences were also observed by electron microscopy, namely, that the core formed in the secondary phase was aggregated further to a long fibrous structure (Kalab and Harwalkar, 1973).

Based on our observations, the increase in curd tension under a pressure of 200–400 kg/cm^2 (Fig. 4) could be explained by the fact that the reactions in the primary and the secondary phases proceeded normally under a pressure lower than 400 kg/cm^2 (Fig. 3, 4) and that the tertiary phase was enhanced under such pressure. At a pressure higher than 600 kg/cm^2, the secondary phase was a rate-limited process that delayed the initiation of the tertiary phase, resulting in a suppression of milk curdling.

According to the hydration theory of protein proposed by Low and Somero (1975), the aggregation of protein particles will possibly induce an increase in activation volume (ΔV^*) as the result of the release of water hydrated around protein particles. Needless to say, the reactions within volume increase can be suppressed by compressing the reaction systems under pressure. The reactions with the volume decrease are expected to be enhanced by compression. According to this theory, the casein degradation reaction as the primary phase in milk curdling by rennet may not result in volume change ($\Delta V^* = 0$) due to the negligible effect on the casein degradation. Core formation for initiating milk curdling, i.e., the secondary phase in milk clotting, may be one of volume increasing reactions ($\Delta V^* > 0$) as revealed by the remarkable delay of micelle aggregation. Curd formation, i.e., the tertiary phase in milk curdling, may be a volume decreasing reaction ($\Delta V^* < 0$) because of the enhancement of curd rigidity under compression. This may be the first report describing the acceleration of milk curd formation by rennet under pressure lower than 400 kg/cm^2 and its suppression under high pressure at a range of 600–1300 kg/cm^2.

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Ms received 1/3/86; revised 9/11/86; accepted 9/12/86.

This work was supported in part by a grant-in-aid for Food Research (59360013) from Ministry of Education, Science and Culture of Japan.

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Part of this work has been supported by the "Institut Technique du Gruyère." The authors acknowledge Dr. John Burton for his help with translating.

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Ms received 5/27/86; revised 9/6/86; accepted 9/12/86.

Contribution No. 86-463-J, Dept. of Animal Sciences & Industry, Kansas Agricultural Experiment Station, Kansas State Univ., Manhattan, KS 66506.

Partial Purification of an Antioxidizing Component in Raw Cow Milk

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ABSTRACT

Gel filtration was used to partially purify the antioxidizing component of a centrifuged serum of raw cow milk, and its properties were studied. The antioxidizing effect did not decrease after dialysis or heating. The finding of no change after dialysis suggested that the compound was of high molecular weight, estimated at 38,000 by gel filtration. The antioxidizing effect decreased with irradiation, accompanied by a parallel decrease in the riboflavin content. The antioxidizing component of this serum fraction may be a protein-bound riboflavin.

INTRODUCTION

ANTIOXIDANTS such as butylated hydroxyanisole and butylated hydroxytoluene are used to prevent lipid peroxidation, but their applications as food additives are confined to a narrow range because of undesirable effects on the human body (Ito et al., 1985; Ponder and Green, 1985; Selvan and Rao, 1985; Takahashi et al., 1985; Wattenberg 1972; Witschi and Morse, 1985). The demand for tocopherol as a replacement for these antioxidants has increased. Many natural antioxidizing substances besides tocopherol are known, but their potential harm to the human body hinders their use as food additives. Components of common foods are less likely to cause unexpected health problems than newly synthesized compounds. In a preliminary experiment (unpublished), we found an antioxidizing component in a serum of cow milk. The purpose of this study, was to isolate and identify the component, and investigate its suitability as a food additive.

MATERIALS & METHODS

Sample preparation

Raw milk was obtained from a dairy farm in Nara Prefecture. About 10 liters were collected twice a month in the morning from a single Holstein cow given alfalfa hay, a concentrate of rolled barley, rolled milo, molasses, beet pulp, wheat, and mixed feed. Crude serum in the sample was obtained by centrifugation at 5000 × *g* for 30 min. The cream layer was removed and the crude serum centrifuged at 80,000 × *g* for 60 min, giving a serum fraction. This was pipetted into containers and stored at −30°C in the dark for later use.

Dialysis, heat, and light irradiation of serum

Serum (300 mL) was dialyzed with a tube-shaped dialysis membrane (diameter, 27 mm; wall thickness, 0.0203 mm; pores, 24 Å; Visking Co., USA) at 4°C against 1500 ml of either 40 mM Tris-HCl buffer, pH 8.0, or 40 mM sodium phosphate buffer containing 0.154M NaCl, pH 7.4, for 36 hr. Different serum samples (300 mL) were heated in a water bath for 30 min at 60° and 90°C. Separately, samples (300 mL) were stirred during irradiation at a wavelength of 450 nm at 4°C and 500 or 10,000 lux for 30 min. The antioxidizing effects of these samples were measured.

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Column chromatography with diethylaminoethyl (DEAE) cellulose and Sephadex G-150 gel filtration

The serum fractions were separated by DEAE-cellulose (DE52, Whatman, Ltd., Great Britain) column chromatography as follows. A DEAE-cellulose column (3.8 × 54 cm) was equilibrated with 50 mM Tris-HCl buffer (pH 8.0), washed with the same buffer, and developed in a linear gradient made with 300 mL of this buffer and 300 mL of the same buffer containing 0.6M NaCl. The flow rate was 30 mL/hr and fractions of 10 mL were collected. Each of the resulting fractions was desalted with Sephadex G-25 (Pharmacia Fine Chemicals, Sweden) and concentrated by ultrafiltration (Toyo Ultrafilter UK-10, Japan). The resulting concentrate was further purified by gel filtration with a 2.3 × 58 cm column packed with Sephadex G-150 (Pharmacia) and equilibrated with a 40 mM sodium phosphate buffer, pH 7.4 (temperature, 4°C; flow rate, 10 mL/hr; fraction volume, 5 mL). The protein concentration of each fraction was measured by absorbance at 280 nm.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis of fraction A′ was done by the method of Davis (1964) under the following conditions. The gels were run at 2 mA/column for 80 min. After electrophoresis, each band

Table 1—Antioxidant activity in cow milk serum after dialysis, heat, and light

Treatment	Antioxidant activity (%)[a]
None	100
Dialysed against:	
Tris-HCl buffer, pH 8.0	96
NaCl-phosphate buffer, pH 7.4	92
Heated for 30 min at 60°C	75
Heated for 30 min at 90°C	70
Irradiated for 30 min at 500 lux	13
Irradiated for 30 min at 10,000 lux	6

[a] Mean of three measurements, each made in duplicate.

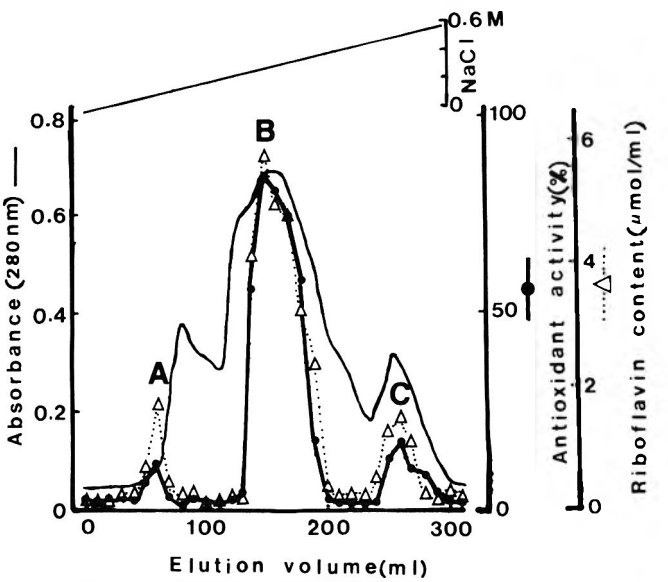

Fig. 1—DEAE-cellulose column chromatography of an antioxidizing component in a serum.

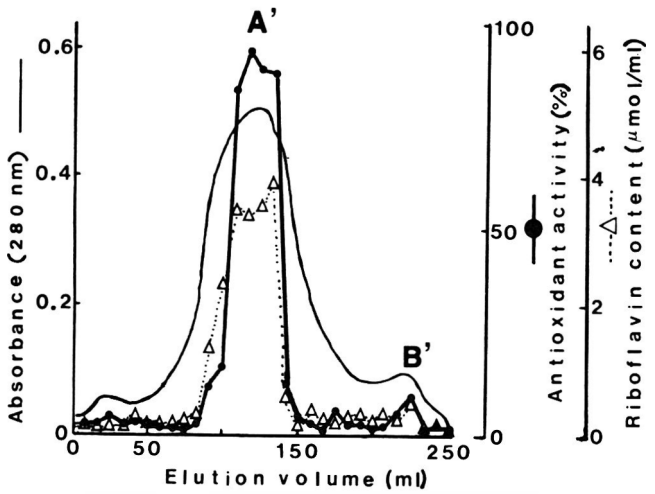

Fig. 2.—Sephadex G-150 gel filtration of the antioxidizing component in fraction B.

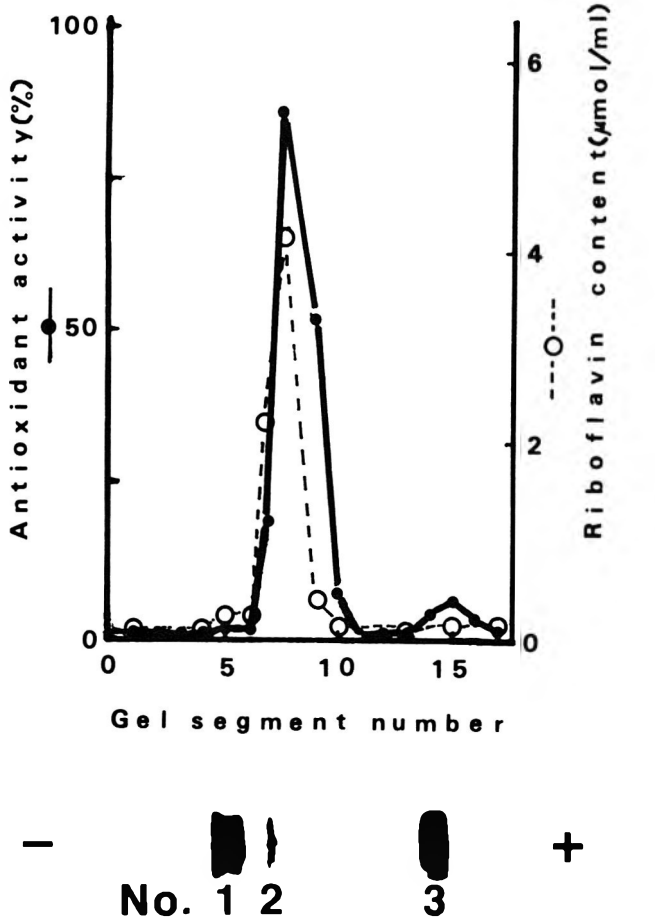

Fig. 3—Polyacrylamide gel electrophoresis of fraction A' and antioxidant activity in eluates from gel segments.

from the polyacrylamide gel was isolated in a Canalco (U.S.A.) Prep-Disc apparatus as described in the manual. A constant voltage (350 V) was applied to this system for about 3 hr at between 20 and 35 mA. The samples were dialyzed and lyophilized.

The protein concentration of samples of recovered bands from polyacrylamide gels were measured with a total protein kit (Sigma Chemical Co., U.S.A.).

Molecular weight estimation

The molecular weight of the antioxidizing component was estimated using the method of Andrews (1965). A standard protein kit for molecular weight measurement (Sigma; alkaline phosphatase, 115,000; ovalbumin, 45,000, and cytochrome c, 13,000) was used. The elution volume for the alkaline phosphatase was found by the method of Garen and Levinthal (1960), for ovalbumin by absorbance at 280 nm, and for cytochrome c by absorbance at 410 nm.

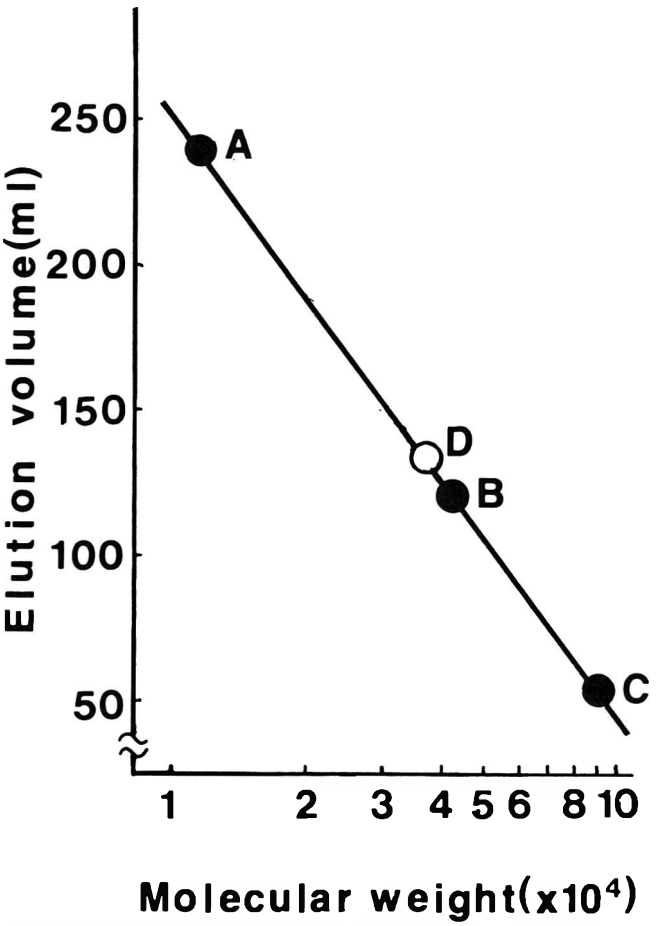

Fig. 4—Estimation of molecular weight of the antioxidizing component by gel filtration on Sephadex G-150. Extracts from gel segments No. 6–11 from Fig. 3 and standard solutions were put on a column (2.1 × 51 cm) equilibrated with 40 mM sodium phosphate buffer (pH 7.4), and elution was done with the same buffer. A, cytochrome c; B, ovalbumin; C, alkaline phosphatase; D, antioxidizing component.

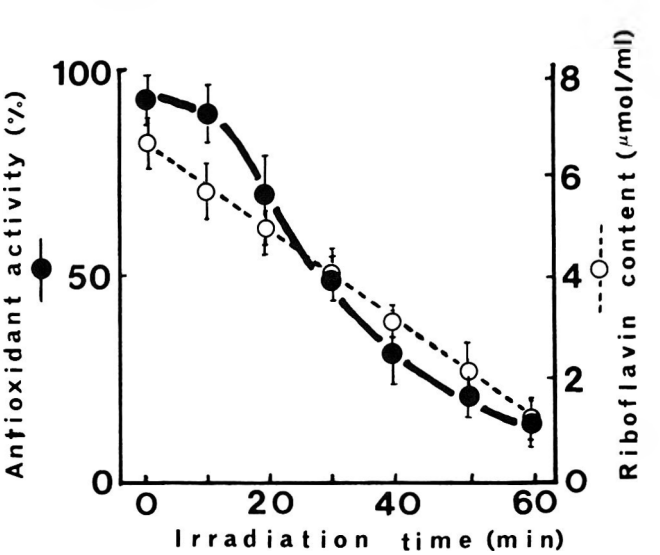

Fig. 5—Effects of light on the antioxidant activity and riboflavin content of the antioxidizing component in fraction A'. Each point represents the mean of three trials, given with the standard deviation.

Assay of antioxidizing action

The serum sample and fractions of the gel filtration were tested for antioxidizing effects against linoleic acid autoxidation by the method of Mitsuda et al. (1966) with 2×10^{-3}M linoleic acid, 0.1M sodium phosphate buffer (pH 7.0), and 12 μM $FeCl_3$. The total reaction volume was 5.0 mL, and the mixture was shaken at 37°C for 1 hr. After incubation, the generation of linoleic acid hydroperoxide was measured by the method of Cathcart et al. (1984). The amount of dichlorofluorescein (DCF) was measured by monitoring of the fluorescence intensities at 470 nm (excitation) and 550 nm (emission) with a 605-40 fluorescence spectrophotometer (Hitachi, Ltd., Japan). The antioxidant activity remaining, with the control activity (with linoleic acid only) as 100%, was calculated from the equation:

$$\text{Antioxidant activity remaining (\%)} = 100 - \left\{ \frac{\text{Amt of DCF (pmol) in } a - \text{Amt of DCF (pmol) in } c}{\text{Amt of DCF (pmol) in } b - \text{Amt of DCF (pmol) in } c'} \times 100 \right\}$$

where a = test serum or fraction from gel filtration plus linoleic acid, b = control sample (linoleic acid only), and c and c' = a and b, respectively, before incubation.

Measurement of riboflavin

Samples were prepared by the method of Ashoor et al. (1983). The samples were filtered through a 0.22-μm membrane filter and riboflavin was assayed by high-performance liquid chromatography (HPLC) as described elsewhere (Toyosaki et al. 1986). Riboflavin standard solutions and samples (10 μL) were assayed on separate columns of Cosmosil ($5C_{18}$; 4.6 × 150 mm, Nakarai Chemicals, Ltd., Japan).

RESULTS & DISCUSSION

WE FOUND A FRACTION of milk serum with antioxidizing activity (data not shown). The results of various treatments of the serum before fractionation are shown in Table 1. Dialysis did not decrease antioxidant activity, so the antioxidizing component was of high molecular weight. Heated serum retained more than 70% of its activity, so the component was not an enzyme. Protein itself has weak antioxidizing activity (Kajimoto and Yoshida, 1972, 1974; Tappel, 1955; Yukami, 1972). However, heat did not destroy most of the antioxidizing activity, of the serum as it would if the component were entirety protein. The component was sensitive to light, and since riboflavin in milk is sensitive to light (Toyosaki et al., 1983, 1984), we thought that riboflavin might be related to the antioxidizing effects of the fraction.

DEAE-cellulose column chromatography of the serum, done to isolate the antioxidizing component gave three fractions with antioxidant activity, A, B, and C. The elution pattern, antioxidant activity, and riboflavin content of each fraction are shown in Fig. 1. All three fractions contained riboflavin; we chose fraction B, with the most riboflavin, for further separation by gel filtration on Sephadex G-150. Two fractions with antioxidizing activity, A′ and B′, were obtained, and their elution patterns, antioxidant activity, and riboflavin content are given in Fig. 2. Fractions A and C were not examined further.

The mean riboflavin content of the raw milk was 1.39 μg/mL, and 1.30 μg/mL was recovered in the serum. In general, riboflavin is bound to protein (Kanno and Kanehara, 1985). Thus, protein-bound riboflavin is probably the antioxidizing substance in question.

Fraction A′ was separated by polyacrylamide gel electrophoresis, and the antioxidizing activity of the three or four resulting irregular bands was measured (Fig. 3). Band 2 had the strongest antioxidizing action and contained riboflavin; its molecular weight was estimated by gel filtration to be about 38,000 (Fig. 4). This band was not identified but it is, we think, a protein that contains riboflavin. In a separate experiment, we irradiated fraction A′ with light at 500 lux for 60 min (Fig. 5). Antioxidizing activity tended to decrease with longer irradiation, with a parallel decrease in riboflavin content. These findings correspond with the results shown in Table 1; that is, the antioxidizing action of fraction A′ was degraded rapidly by light. Based on these results, we concluded that riboflavin is probably related to the antioxidizing activity of the serum component.

Miyazawa et al. (1983a, b) found that riboflavin has antioxidizing effects *in vivo*. Ohama and Yagi (1969) found that riboflavin tetrabutylate has antioxidizing action *in vitro* and that it inhibits peroxidate production. On the other hand, Totani et al. (1975) and Aoyama et al. (1985) report that riboflavin has little antioxidizing action. Whether riboflavin is antioxidizing *in vivo, in vitro*, or both is unsettled. Protein that probably contained riboflavin was antioxidizing *in vitro* in this study. The antioxidizing effects of the serum component reported here may involve interaction between riboflavin and protein bound together.

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Ms received 2/12/86; revised 8/29/86; accepted 9/5/86.

Effect of Alkaline Treatment on the Dispersibility of Soy Protein Isolates and Properties of Milk Clots formed from Nonfat Milk and Treated Soy Protein Mixtures

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ABSTRACT

Dispersibility of soy protein isolates was improved by adjusting pH to 12 and acidifying to pH 6.5. Alkaline treated isolates added to reconstituted nonfat milk reduced rennet induced coagulum firmness and syneresis more than did untreated soy protein isolates. Treated soy protein added to reconstituted nonfat dry milk reduced coagulum firmness and syneresis more than untreated soy protein. Soy protein interference with coagulum firmness and syneresis increased with an increase in pH becoming most severe at pH values above 11. Adjusting the pH of reconstituted NFDM in the absence of soy protein to pH 8 and holding for 30 min before adjusting it back to pH 6.5 had no effect on coagulum firmness or syneresis. However, higher pH treatments (pH 10 and above) caused reduction in coagulum firmness and in syneresis.

INTRODUCTION

SOY PROTEIN has been incorporated into cow's milk in the form of soy milk (Del Valle et al., 1984; Metwalli et al., 1982a, b; Abou El-Ella, 1980; Abou El-Ella et al., 1978; Hang and Jackson, 1967), soy flour (Dordevic and Carcic, 1969) and soy protein extracts or fractions thereof (Lee and Marshall, 1979, 1981) for making rennet-induced curd and/or cheese. To study the effect of soy protein on the coagulation properties of casein, soy protein isolates (SPI) are the products of choice since they contain at least 90% protein and are commercially available.

Adding soy protein isolates to milk systems without further treatment is the most practical way of using the isolates. To determine how soy protein isolates will function in a given system, the isolates should be added to the food system to be tested (Aoki et al., 1984; Kinsella, 1979; Hermansson, 1979; Hermansson and Akesson, 1975; Mattil, 1971; Johnson, 1970). In preliminary work when soy protein isolates were added to milk before coagulation, a precipitate formed, indicating that some of the soy protein did not stay in suspension.

Soy protein isolates vary in their solubility or dispersibility depending upon source and processing conditions (Kinsella, 1979; Hermansson, 1979; Shen, 1976a). The solubility or dispersibility may vary considerably among SPI. Variations in test conditions such as pH, temperature, ionic strength, concentration, time and rate of stirring and time, temperature and centrifugation force also result in variations in reported solubility and/or dispersibility (Kinsella, 1979; Shen, 1976a,b; Johnson, 1970; Van Megen, 1974; Pour-El, 1976; Hutton and Campbell, 1977).

Soy protein solubility increases as pH is changed on either side of the isoelectric point (Shen, 1976a; Kinsella, 1979; Pour-El, 1975; Hermansson, 1979; Van Megen, 1974; Mattil, 1971). Also, alkali treatment affects soy protein dispersibility by causing unfolding and dissociation into subunits (Kinsella, 1979; Thanh and Shibasaki, 1979; Ishino and Kudo, 1979, 1980; Hermansson, 1978; Kelley and Pressey, 1966; Draper and Catsimpoolas, 1978).

Most of the methods used for measuring protein solubility, such as nitrogen solubility index (NSI) or protein dispersibility index, depend on determining the nitrogen content of the supernatant after centrifugation (Johnson, 1970; Pour-El, 1975; Hermansson, 1972; AACC, 1976) without reference to the residual protein obtain after centrifugation. Shen (1976a) indicated that a single measurement of NSI at a given pH and ionic strength is insufficient to characterize the solubility of soy protein. The objectives were to study the effect of pH on dispersibility of SPI and the effect of alkaline treated soy protein on nonfat milk coagulation by rennet.

MATERIALS AND METHODS

THREE SPI (PRO-FAM S-970, S-640 and S-955) were provided by Grain Processing Corporation (Muscatine, IA) and another three (Purina-Protein Supro-620, 660 and 710) were obtained from Ralson Purina Company (St. Louis, Mo). The approximate chemical composition and some of the functional properties of the isolates as given by the companies are summarized in Table 1. Grade A low heat NFDM was obtained from Mid America Dairymen, Inc. (St. Paul, MN). A single lot was used. Antifoam FG-10 emulsion was provided by Down Corning Corporation (Midland, MI).

pH adjustment and protein dispersibility measurement

A 2% solution of isolate was prepared by stirring it into water. A few drops of antifoam were added and the mixture was stirred for 30 min. Aliquots (50g), taken while stirring into 100 mL beakers, were separated into "pH-adjusted" and "pH adjusted-readjusted" samples. For "pH-adjusted" samples, 1N NaOH or 1N HCl was added to bring the pH to 6, 8, 10, 11, 11.5, or 12. For "pH adjusted-readjusted" samples, the pH was readjusted to pH 6.5 immediately upon reaching the desired pH. The pH was monitored using an Orion 601A pH meter standardized between pH 7 and 10. The amount of added NaOH or HCl was recorded; then water was added to make a total added volume of 10 mL. While the mixture was being stirred, three 10 mL aliquots were taken from each beaker and transferred into 15 mL graduated centrifuge tubes. All 36 tubes were centrifuged at 1000 rpm (250 × *g*) for 10 min at room temperature (20–25°C) using an International Centrifuge Model PRJ. The centrifugation force was kept very low in an attempt to predict how the isolates would behave when added to a liquid system like milk.

The volume of the precipitate (or sediment) in the bottom of the tube was recorded as ml precipitate/10 mL sample. A light behind the centrifuge tubes was used to help read the volume of the precipitate, because of occasional cloudiness in dispersions. Each experiment was replicated at least three times for each SPI and the averages were used for plotting the data. Since the degree of variability in all the samples was within the same range, the standard deviation was represented by the bars in one of the graphs only. All of the isolates tested had a pH range of 6.5 to 7.3 in a 2% dispersion (Table 1).

Spray drying of "pH-adjusted-readjusted" isolates

A slurry (<10%) of SPI was adjusted to pH 12, then immediately readjusted to pH 6.5 and spray dried using a Niro Utility Model spray

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Table 1—Approximate chemical composition and some characteristics of soy protein isolates

Typical chemical analysis (%)	S-970 (A)	S-640 (B)	S-955 (C)	620 (D)	660 (E)	710 (F)
Protein (N × 6.25 moisture-free basis)	92	92	92	91.5	91.5	92
Moisture	6.0 (max)	6.0 (max)	6.0 (max)	5.5	5.0	5.6
Fat (PE extract)	0.5	0.5	0.5	0.5	0.5	0.3
Fiber (crude)	...	...	...	< 0.2	0.2	0.1
Ash	5.5 (d.b. max)	5.5 (d.b. max)	5.5 (d.b. max)	3.8	3.8	3.8
Calcium	0.15	0.15	0.16	0.2	0.2	0.17
Phosphorus	0.8	0.8	0.8	0.8	0.8	0.8
Potassium	0.15	0.15	0.15	0.09	0.08	0.08
Sodium	1.2	1.2	1.2	1.2	1.0	1.2
pH	7.05–7.75	6.25–6.95	6.25–9.95	7.0	6.8	6.9
pH of 2% sol[a]	7.32	6.35	6.65	6.99	6.78	6.80
Nitrogen solubility Index (minimum)	70	50	20	...	...	...
Bulk density	18–26 lb/cu ft	18–25 lb/cu ft	16–35 lb/cu ft	Standardized	Standardized	Standardized
Characteristics	bland, highly soluble, high water absorption, excellent thermal stable gels, etc.	bland, intermediate solubility, disperses easily in water, moderate water binding	bland, low solubility, very low water absorption, wets easily in water, does not increase in functionality with heating	bland, highly functional protein, highly digestible	bland, instantized, easily dispersed with excellent suspension properties. highly digestible	bland, highly digestible, highly functional protein
Suggested Application	Imitation dairy products and milk replacers, etc.	Infant formulas, nutritional supplements, etc.	High protein tablets, baked and pasta products, etc.	For food products where gel formation, emulsification and emulsion stability are required	Ideal for dietary supplements	Where low viscosity is important, liquid coffee whitener, etc.

[a] pH of 2% solution prepared in the lab. Results are averages of three runs.

Table 2—Reconstituted nonfat dry milk-soyprotein mixture pH values 30 min after adding NFDM and before adjusting the mixtures to pH 6.5

pH to which soyprotein was adjusted	pH after 30 min of adding NFDM[a]
5.5	6.47 ± 0.02
6.5	6.55 ± 0.01
9.0	6.60 ± 0.01
11.0	6.82 ± 0.04
11.5	7.14 ± 0.03
12.0	9.72 ± 0.17

[a] NFDM = nonfat dry milk

dryer (Niro Atomizer Ltd., Copenhagen, Denmark). The inlet temperature was maintained at 200 ± 5°C and the outlet temperature was maintained at 100 ± 5°C.

pH adjustment of soy protein, nonfat dry milk addition and coagulum formation

To prepare a rennet-induced coagulum. the amounts of water and NFDM to make a 10% solids reconstituted NFDM were measured. Then a predetermined amount (5% of the NFDM used) of SPI S-970 was stirred into part of the water. A few drops of antifoam were added and the mixture was stirred for 30 min. The pH was adjusted to the desired pH (5.5, 6.5, 9, 11, 11.5 and 12) using 1N NaOH and 1N HCl. Immediately thereafter, a preweighed amount of NFDM was added, and the mixture was stirred for another 30 min. The pH was checked and adjusted to pH 6.5, and the remaining water (minus the amount of HCl and NaOH) was added. The mixture was filtered through cheese cloth to remove any extraneous matter and put into a water bath for 30 min to bring the temperature to 32.2°C. Rennet addition, coagulum formation, whey syneresis and textural properties (curd tension, hardness, rate of firming and areas under the curve) were obtained from force-distance curves of an Instron as described by Mohamed (1985).

pH adjustment of reconstituted nonfat dry milk and coagulum formation

Enough NFDM to make a 10% total solids reconstituted product was weighed and stirred into part of the required amount of water. Antifoam was added as in the previous experiment. The mixture was stirred for 30 min and the pH then adjusted to the desired level [5.5, 6.5 (control), 8, 10 or 10.75]. Samples were mixed for 30 min and pH was adjusted again to pH 6.5. The remaining water (minus the amount of NaOH and HCl) was added. Then the mixture was treated as above. Each experiment in this section and in the previous section was conducted at random and in triplicate. Mean values were used for plotting data. The standard deviation for all the samples was within the same range; accordingly, the standard deviation is indicated by the bars on the control samples only.

RESULTS & DISCUSSION

Effect of pH on dispersibility of soy protein isolates

The precipitate volumes from SPI dispersions pH-adjusted to 6, 8, 10, 11.5 and 12 are shown in Fig. 1. The data from samples of the same isolates pH-adjusted to the above pH values and readjusted to 6.5 are presented in Fig. 2.

In the pH-adjusted experiment, as pH was increased from 6, the precipitate volume increased up to a maximum which varied depending on the isolate (Fig. 1). Then the amount of the precipitate decreased to a minimum around pH 11.5 to 12 for all of the isolates except C. In a separate experiment (data not shown) the volume of the precipitate for isolate C at pH 12.5 was similar to that of the other isolates at pH 12. All of the isolate suspensions became clear and yellowish with an increase in pH.

The initial increase in precipitate volume with increased pH may be attributed to (1) an increase in the negative charge of the protein leading to an increase in the inter and intramolecular repulsive forces which, in turn, results in unfolding and expansion of the polypeptides (Tanford, 1968) and (2) an increase in the ability of protein to hold more water and swell (Pour-El, 1975; Hermansson, 1972; Mattil, 1971). However, the decrease in the volume of the precipitate after a certain maximum may be attributed to unfolding and dissociation of soy protein into subunits at pH 10 to 12 (Ishino and Okamoto,

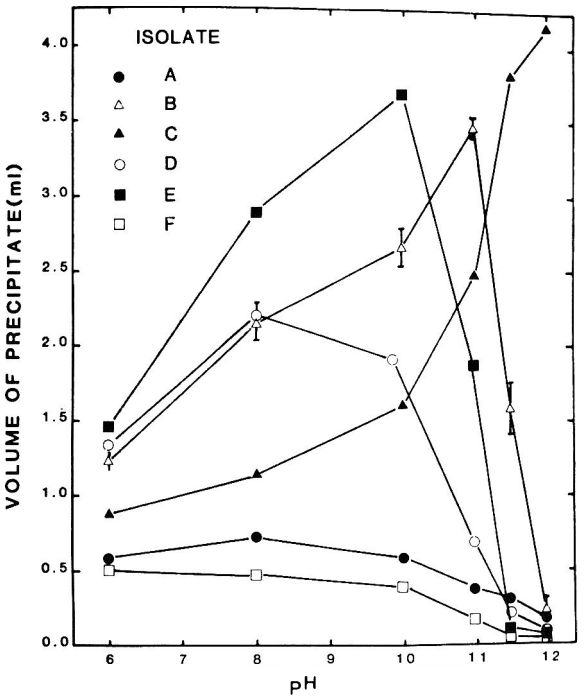

Fig. 1—Volume of precipitate (mL) per 10 mL soy protein isolate suspensions treated at a specified pH and centrifuged at 1000 rpm for 10 min.

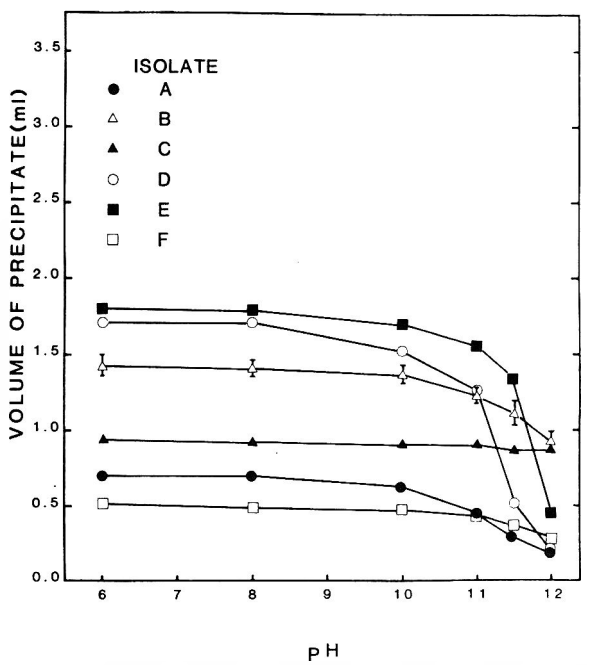

Fig. 2—Volume of precipitate (mL) per 10 mL soy protein isolate suspensions treated at a specified pH and adjusted immediately to pH 6.5 and centrifuged at 1000 rpm for 10 min.

1975; Ishino and Kudo, 1979; Hermansson, 1978; Draper and Catsimpoolas, 1978).

As shown in Fig. 2, with the pH adjusted-readjusted experiment, the volume of precipitates for all of the SPIs remained similar to that observed at pH 6.0 up to about pH 10 and then decreased to a minimum at pH 12 with the exception of SPI C. For all of the SPIs the pH at which precipitate volume started to decrease (Fig. 2) corresponded to a similar pH in the experiment reported in Fig. 1 at which precipitate volume reached a maximum and started to decrease. Perhaps, some irreversible changes occurred at these pH values because of

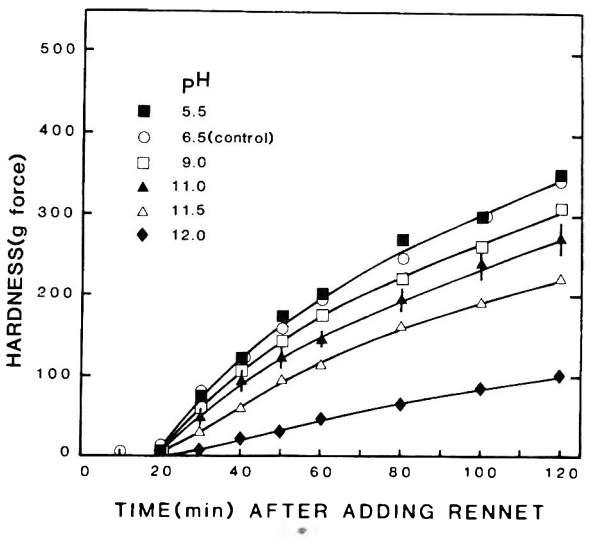

Fig. 3—Effect of adjusting pH of soy protein isolate (A) before the addition of nonfat milk on coagulum hardness (g force) with time after rennet addition. Rennet was added at pH 6.5 for all treatments.

unfolding and dissociation of the protein. Reversible dissociation reactions of soy protein fractions (7S and 11S) are possible up to pH values of 10 to 11.5, depending on the experimental conditions, after which they become irreversible (Kinsella, 1979; Kelley and Pressey, 1966; Ishino and Okamoto, 1975; Thanh and Shibasaki, 1979; Ishino and Kudo, 1980; Kitamura et al., 1977).

From data in Fig. 1 and 2, it is obvious that for "pH adjusted-readjusted" SPI, except C, adjustment to pH 12 followed by immediate readjustment to pH 6.5 resulted in a minimum precipitate volume. To determine the effect of spray drying on the dispersibility of "pH adjusted-readjusted" SPI, isolates A and E were adjusted to pH 12, then immediately adjusted back to pH 6.5 and spray dried. The redried isolates designated AR and ER were treated similarly to the isolates referred to in Fig. 1 and 2. Both of the redried isolates gave similar results to those reported in Fig. 1 and 2 (Mohamed, 1985).

Thanh and Shibasaki (1979) showed that adjusting the pH of a protein solution from pH 2 to 12 results only in a small change in the ionic strength. Mattil (1971) pointed out that determining solubility in water may result in misleading solubility values and that it should be determined in the ionic environment of the food system. Accordingly, NFDM (enough to make 10% solids) was added to pH-adjusted soy protein suspensions before they were readjusted to 6.5 and then the precipitate volume was determined. Precipitation behavior trends similar to those observed in Fig. 2 were observed.

Effect of alkaline pH of soy protein isolate (A) before the addition of nonfat dry milk on coagulum formation

Based on the previous experiment apparently the soy protein underwent some physicochemical changes with the increase in pH. Because of possible physicochemical changes, we postulated that many active groups were exposed to the surface and that may adding NFDM to pH-adjusted soy protein some interaction between soy protein and milk protein might result in better clot formation. The pH range used in this experiment was 5.5 to 12. The results shown in Fig. 3 were opposite to what was hypothesized. As the pH of soy protein was increased the coagulum strength at any given time after rennet addition decreased. The difference in hardness increased with time after adding rennet, and very large differences were observed between the pH 11.5 and 12 curves (Fig. 3). Similar trends were

obtained by measuring the areas under the curves and from curd tension (Mohamed, 1985).

The rate of firming at any given pH (Fig. 4) reached a maximum at about 50 min after adding rennet, then started to level off or decrease. It decreased drastically with an increase in pH, and the greatest effect was at pH 12.

As the pH of soy protein was increased, the ability of the coagulum to undergo syneresis decreased, especially above pH 11 (Fig. 5). Perhaps soy protein absorbed more water at higher pH values or soy protein interfered more with casein aggregation as pH was increased, resulting in a softer curd that was able to hold more water than the control.

When the pH of the soy protein suspension was adjusted to pH 12, then immediately readjusted to pH 6.5 before the addition of NFDM, no considerable effect on syneresis or coagulum strength was observed (syneresis and hardness curves were similar to the pH 6.5 (control) curve in Fig 3 and 5, respectively). This is contrary to the results of Lee and Marshall (1984) who, after treating soy protein with 5N NaOH to pH 13 for 1, 10 and 100 min and neutralizing with 5N HCl within one min, found that the soy protein treated for 1 and 10 min gave a stronger coagulum than the untreated control or the one treated for 100 min. They attributed their results to less interference from alkali treated proteins with inter-micellar bond formation. They also indicated that the amount of protein lost in the whey was higher with larger exposure time. Their results suggested that some hydrolysis of protein occurred during these severe alkaline treatments.

Lowering the pH of soy protein to pH 5.5 had no significant effect on coagulum firmness (Fig. 3) or rate of firming (Fig. 4). However, syneresis (Fig. 5) was increased.

To show the effect of pH more clearly, the data obtained at 60 min after rennet addition are plotted in Fig. 6. Results of curd tension, hardness and area under the curve as well as syneresis are almost parallel to each other. All of these parameters decreased slightly with an increase in pH up to pH 11 (pH at which most unfolding and dissociation of soy protein starts to take place) after which they decreased considerably.

The decrease in coagulum strength with an increase in the pH of soy protein may be attributed to the following. First, as pH of soy protein was increased, its negative charge increased and unfolding and dissociation occurred. As pH was lowered to 6.5 by the addition of NFDM and HCl, the soy protein as it was going through the process of renaturation (refolding) may have interacted with casein chemically (may have bound Ca^{2+} or have undergone reactions between exposed amino acid residues) and/or physically (due to the increase in the surface area occupied by unfolded and dissociated soy protein), thus interfering with casein micelle aggregation and coagulum formation. The degree of interference increased with pH and reached a maximum at pH 12 where maximum unfolding and dissociation is known to occur. Second, as pH was increased, the soy protein was able to absorb more water (Hermansson, 1972), thus preventing casein micelles from getting closer together. Third, when NFDM was added to soy protein solutions

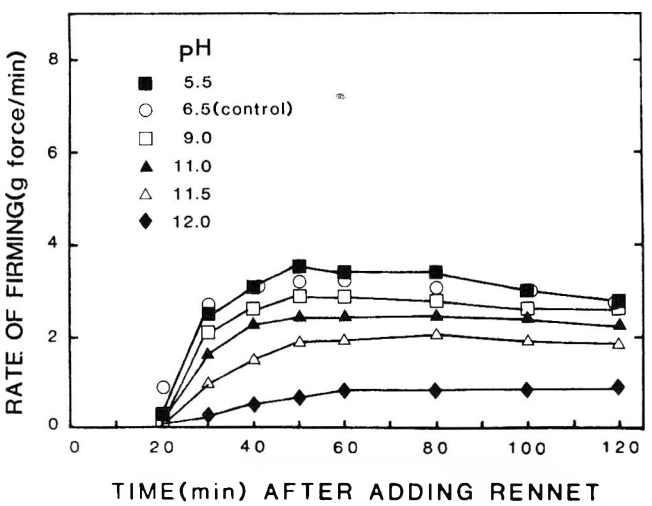

Fig. 4—Effect of adjusting pH of soy protein isolate (A) before the addition of nonfat milk on the rate of firming (g force/min) of rennet-induced coagulum with time after rennet addition. Rennet was added at pH 6.5 for all treatments.

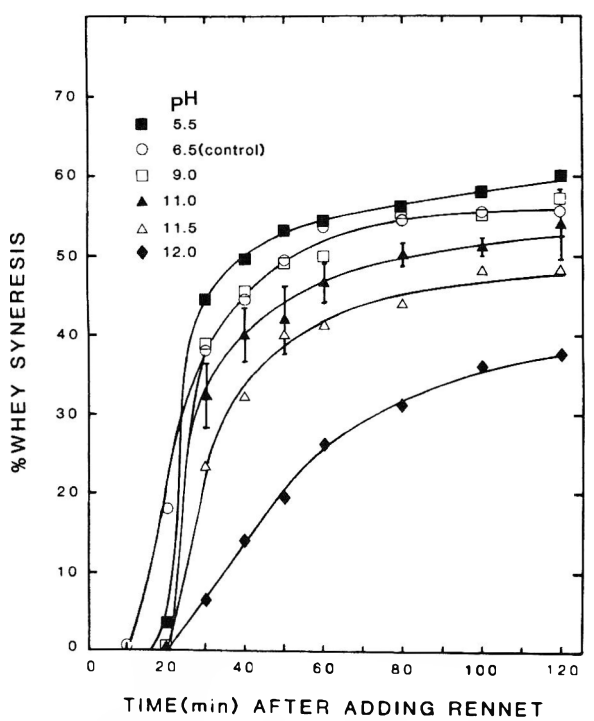

Fig. 5—Effect of adjusting pH of soy protein isolate (A) before the addition of nonfat milk on percent whey syneresis of rennet-induced coagulum with time after rennet addition.

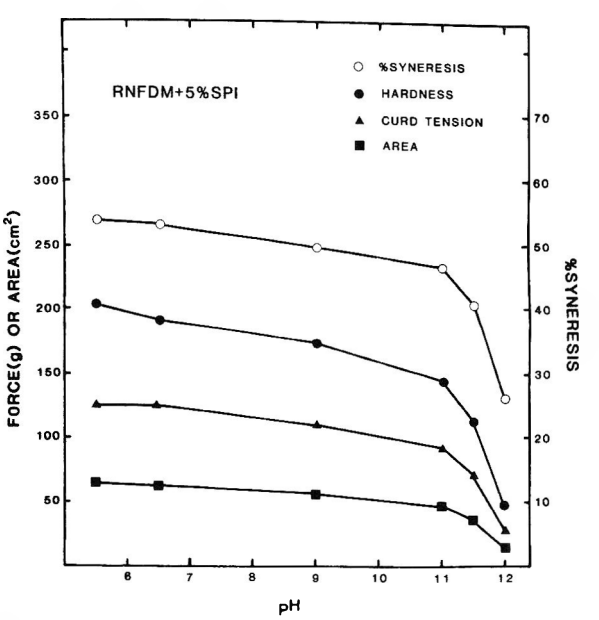

Fig. 6—Effect of adjusting pH of soy protein isolate (SPI) (A) before the addition of reconstituted nonfat dry milk (RNFDM) on rennet-induced coagulum strength [as measured by curd tension (g force), hardness (g force), area under the curve (cm²)] and percent whey syneresis at 60 min after adding rennet.

at alkaline pH, some of the casein properties may have been altered.

As NFDM was added to alkaline treated soy protein solutions, it was first exposed to high pH, then the pH was lowered due to the high buffering capacity of milk protein. Table 2 shows the pH values obtained 30 min after adding NFDM to pH adjusting soy protein solution. After the addition of NFDM, the pH of the soy protein solution dropped from pH 11.5 to pH 7.14 or less, whereas the pH of the soy protein solution at pH 12 was lowered to only pH 9.72.

Effect of adjusting the pH of reconstituted nonfat dry milk from pH 5.5 to 10.75, holding for 30 min. and readjusting back to pH 6.5 on coagulum formation

Coagulum firmness (strength) as determined by hardness data (Fig. 7), curd tension (Fig. 8) and area under the force-distance curve (Fig. 9) was not affected by adjusting pH of RNFDM to 8 and readjusting it back to pH 6.5, while adjusting pH to 10 and readjusting it back to pH 6.5 caused a reduction in coagulum firmness. The effect generally became more pronounced with time after rennet addition. Further increases in pH to 10.75 and readjusting back to 6.5 impaired coagulum firmness and resulted in a very soft coagulum which did not clot until about 45 min after rennet addition. Possibly casein properties were altered irreversibly, affecting the chymosin sites and/or the functions responsible for aggregation and coagulum formation. The casein micelle integrity may have been destroyed. In fact, when the pH was adjusted to about pH 11 and readjusted back to pH 6.5, coagulation was inhibited completely and no clotting was observed even several hours after adding rennet. The effect of pH on casein seemed to depend on time, because when the pH of RNFDM was adjusted to pH 10.9 and immediately adjusted back to 6.5, it formed a firmer coagulum than when adjusted to pH 10.75 for 30 min, then readjusted to pH 6.5 (data not shown).

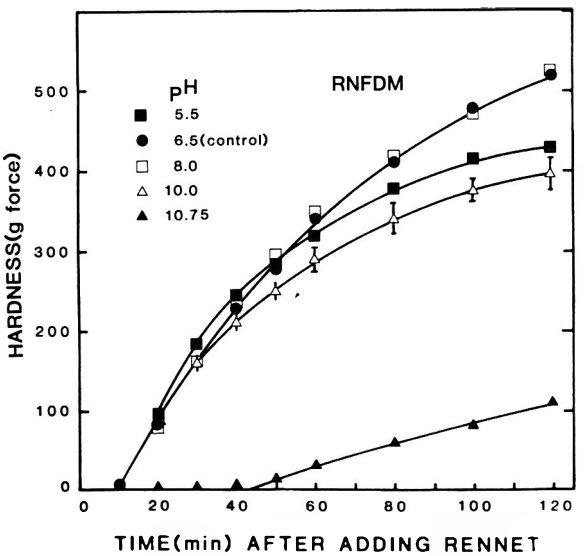

Fig. 7—Effect of adjusting pH of reconstituted nonfat dry milk (RNFDM) to 5.5–10.75 for 30 min then adjusting it back to pH 6.5 on rennet-induced coagulum firmness (hardness) with time after rennet addition.

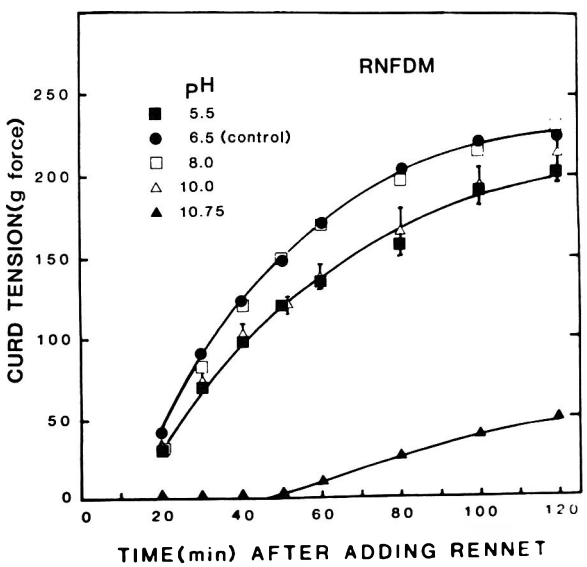

Fig. 8—Effect of adjusting pH of reconstituted nonfat dry milk to 5.5 – 10.75 for 30 min and adjusting it back to pH 6.5 on curd tension (g force) of rennet-induced coagulum with time after rennet addition.

Adjusting pH of RNFDM to 5.5 and readjusting to pH 6.5 resulted in a firmer coagulum than the control up to 50 min after adding the rennet, after which it decreased as compared to that of the control (Fig. 7). The coagulum strength, in fact, was weaker than that of the control at any given time after rennet addition, as shown by curd tension (Fig. 8) and area under the curve (Fig. 9) results. The coagulum, when cut, was found to have a shiny surface with some obvious aggregates or particles present, indicating that some irreversible aggregation or precipitation had occurred at pH 5.5, forming weak points in the coagulum. The shapes of the force-distance curves obtained by the Instron under normal conditions (Mohamed, 1985) were different for the sample treated at pH 5.5, having irregular trace lines between the curd tension peak and the hardness peak, and high hardness peaks at the end, making it difficult to draw slope lines for hardness. This confirms previous observations (Mohamed, 1985) that curd tension, area under the curve and hardness data may measure different textural parameters of the coagulum and/or the same parameter to different degrees.

The effect of lowering the pH to 5.5 may be attributed to solubilization of colloidal calcium phosphate and a decrease in the stability of casein micelles (Jenness and Patton, 1959; Walstra and Jenness, 1984). The extent of solubilization is more pronounced in the pH range 6.0 to 5.0 (Rajput et al., 1983) and the casein starts to precipitate at pH 5.2 – 5.3 (Jenness and Patton, 1959). Some of these changes in this study obviously had occurred after adjusting the pH to 5.5. However, the extent of reversibility of these changes by increasing the

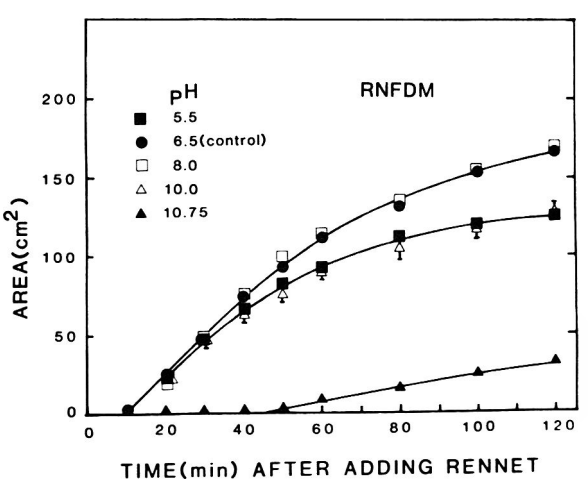

Fig. 9—Effect of adjusting pH of reconstituted nonfat dry milk (RNFDM) to 5.5–10.7 and readjusting it back to pH 6.5 on rennet-induced coagulum strength (area under the curve) with time after rennet addition.

pH to 6.5 is not known and may require a longer equilibration time than 30 min. Lowering the pH below 6.0 causes a reduction in curd firmness (Marshall et al., 1982; Jen and Ashworth, 1970) which seems to be irreversible under the conditions of this study.

The ability of the coagulum to undergo syneresis is shown in Fig. 10. The coagulum from the pH 5.5 treated milk exhibited more whey synersis than did the control even though it was weaker in structure, which may indicate the presence of weak points and the lack of a continuous, uniform network structure. The ability of the coagula from milk treated at pH 8 and 10 to undergo syneresis was similar to that of the control, while that from milk treated at pH 10.75 was very low. Even though the coagulum obtained from pH 10 treated milk was similar in firmness to that from the pH 5.5 treatment as indicated in Figs. 8 and 9, the ability to exhibit syneresis was higher at pH 5.5 which may indicate tht the two gels were different in their structure.

As shown in Fig. 11, measurements of curd tension, hardness and area obtained 60 min after rennet additions all showed similar trends when plotted against change in pH. The most pronounced effect occurred at pH 10 and above. The syneresis curve did not follow the trend of the other curves initially, but above pH 10 it followed the same trends as the other parameters. Thus, good correlation exists between coagulum firmness and syneresis. Lower syneresis in the presence of alkali treated soy protein (Fig. 5) may have been due to the formation of soft coagulum rather than to an increase in absorption of water by the soy protein.

This experiment was done to provide information as to whether or not previous results (obtained in the experiment where NFDM was added to pH adjusted soy protein) were influenced by the effect of alkaline pH on milk proteins. Results obtained in this section indicate that the addition of NFDM to soy protein adjusted up to pH 11.5 had no effect on the milk protein, since the pH of the mixtures was less than 8 after adding NFDM (Table 2). However, adding NFDM to soy protein adjusted to pH 12 may have affected the properties of the milk protein since the pH of the mixture only reached pH 9.72 after 30 min

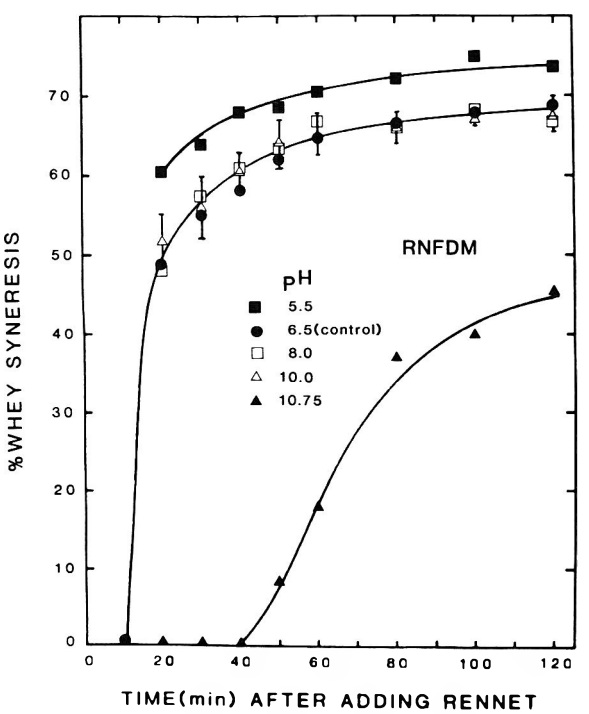

Fig. 10—Effect of adjusting pH of reconstituted nonfat dry milk (RNFDM) to 5.5–10.75 and readjusting it back to pH 6.5 on percent whey syneresis of rennet-induced coagulum with time after rennet addition.

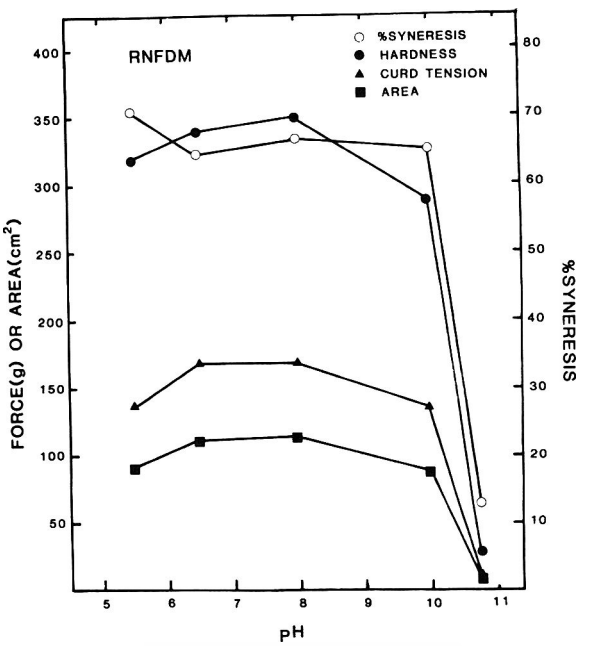

Fig. 11—Effects of adjusting pH of reconstituted nonfat dry milk (RNFDM) to various pH values from 5.5 to 10.75 and readjusting the pH back to pH 6.5 on coagulum strength [as measured by curd tension (g force), hardness (g force), and area (CM²) under the curve obtained from the Instron force-distance curves] and percent syneresis at 60 min after rennet addition.

(Table 2), considering that the NFDM was exposed to higher pH values before it reached equilbrium at pH 9.72. This observation supports the idea that the effect of pH adjusted soy protein up to pH 11.5 is due mainly to interactions between soy protein and milk protein and rules out the pH effect on milk protein. However, the greater exposure of milk protein to alkaline conditions when NFDM was added to soy protein solutions at pH 12 may have had an effect on coagulum properties additional to that of alkaline treated soy protein. This may explain the large difference in coagulum strength between the pH 11.5 and pH 12 treatments (Fig. 3).

The effect of alkaline pH on the physicochemical properties of proteins has been less well studied in milk than in soy proteins. Colloidal calcium phosphate increases, calcium ion activity decreases at alkaline pH values (Waltra and Jenness, 1984; Rajput et al., 1983), and the casein micelles become less stable and disintegrate (Walstra and Jenness, 1984; Rajput et al., 1983; Swaisgood, 1982). The action may be irreversible at high pH values. Casein expands at high pH and low Ca^{2+} activity (Walstra and Jenness, 1984) and viscosity increases and reaches a maximum at pH around 9 to 11 depending on concentration (Roeper, 1977; Hayes and Muller, 1961 Hermansson, 1975). One of the severe effects of alkaline pH on milk protein probably is the liberation of inorganic phosphate from casein and the destruction of some of the serine (Creamer and Matheson, 1977; Manson and Carolan, 1972). Creamer and Matheson (1977) found that treating casein solutions at above pH 10 for 60 min and 60°C resulted in an increase in the quantity of phosphate released, lysinoalanine formation and a reduction in serine and lysine contents.

Whey proteins such as β-lactoglobulin may undergo reversible conformational changes around pH 7.5 (Tanford et al., 1959; Swaisgood, 1982); at high pH molecular expansion and dissociation may occur (Swaisgood, 1982) and the proteins can be altered irreversibly at pH 11 and above (Tanford et al., 1959). All of these changes might have been involved to a certain extent when milk proteins were exposed to pH 10.75 for 30 min.

When the RNFDM control (pH 6.5) data (Figs. 7 and 10)

were compared with the RNFDM-soy protein control (pH 6.5) data (Figs. 3 and 5), it was clear that the addition of soy protein caused a reduction in coagulum firmness and in percent whey syneresis in agreement with previous observations (Mohamed, 1985).

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Ms received 8/26/85; revised 9/26/86; accepted 9/26/86.

Published as paper No. 14,587 of the Scientific Journal series of the Minnesota Agricultural Experiment Station on research conducted under Minnesota Agricultural Experiment Station Project No. 1879, supported in part by Hatch funds and in part by Al-Fateh University, in Tripoli, Libya.

Flavor Protein Interactions: Characteristics of 2-Nonanone Binding to Isolated Soy Protein Fractions

TIMOTHY E. O'NEILL and JOHN E. KINSELLA

ABSTRACT

THE ABSORPTION COEFFICIENTS at 280 nm of 1% solutions of pure soy protein, β-conglycinin, glycinin, the acidic and basic subunits of glycinin were 6.04, 4.4, 8.04, 7.18, and 8.8, respectively. Using equilibrium dialysis the binding affinities of these proteins for the model flavor compound 2-nonanone were determined. On an equivalent weight basis soy protein, β-conglycinin and glycinin had approximately 5,2 and 3 primary binding sites per 100,000 daltons and affinity constants (K) of 570, 3050 and 540 m^{-1}, respectively, i.e., β-conglycinin showed a fivefold greater affinity for nonanone than the other soy protein. The acidic and basic subunits showed binding behavior similar to that of glycinin.

INTRODUCTION

THE PROBLEM of off-flavors associated with soy protein preparations has been a significant barrier to their widespread use and acceptance in conventional and fabricated foods (Kinsella, 1979). These off-flavors (alkanones, alkanals, alcohols) result from lipoxygenase initiated peroxidation of the unsaturated fatty acids in the soybean (Kalbrenner et al., 1974; Wolf, 1975; Rackis et al., 1979; Kinsella and Damodaran, 1980). Their subsequent binding by proteins has been implicated in the persistence of these off-flavors (Arai et al., 1970; Beyeler and Solms, 1974; Gremli, 1974; Kinsella, 1979).

The binding of various nonpolar compounds to soy proteins has been studied in solution (Arai et al., 1970; Beyeler and Solms, 1974; Gremli, 1974; Franzen and Kinsella, 1974; Damodaran and Kinsella, 1981a, 1981b), and in the dry powdered state (Aspelund and Wilson, 1983). Damodaran and Kinsella (1981a, 1981b) using an equilibrium dialysis method, concluded that in aqueous systems the binding of aliphatic carbonyls to soy proteins is hydrophobic in nature and that the β-conglycinin component of soy protein may be the protein fraction responsible for the off-flavor binding by soy proteins. Wilson (1985) concluded that flavor binding to soy proteins in nonaqueous systems involved hydrogen binding. Intepretation of binding data is complicated by the heterogenous nature of soy proteins (Kinsella et al., 1985; Nielsen 1985a, b). This study was undertaken to characterize the binding behavior of individual purified soy protein fractions, to ascertain if β-conglycinin was the major binding component and to determine the binding behavior of the acidic and basic subunits of glycinin.

MATERIALS & METHODS

Materials

Whole soy protein, β-Conglycinin and glycinin fractions were prepared from defatted, low heat-treated soy flakes (Central Soya, Fort Wayne, IN). Spectral grade isooctane was purchased from Fischer Scientific Co. (Fairlawn, NJ). 2-Nonanone (99+%) was obtained from Aldrich Chemicals (Milwaukee, WI). Concanavalin A-Sepharose-4B was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). All other chemicals used were reagent grade. Distilled, deionized water was used in all experiments.

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Isolation and fractionation of soy proteins

Whole soy protein was prepared from finely ground, defatted, low heat-treated soy flakes as described (Damodaran and Kinsella, 1981a, b). Crude soy glycinin and B-conglycinin were isolated from ground soy flakes using the method of Thanh and Shibasaki (1976) and were further purified by Con A- Sepharose 4B affinity chromatography using the procedure of Kitamura et al. (1974). One hundred milliliters Con A-Sepharose 4B in 0.1M acetate buffer, pH 6, was degassed and poured as a slurry into a 2.0 by 60 cm glass column. The column was then equilibrated with 20 mM Tris HCl buffer, pH 7.1, containing 0.5M NaCl, 0.02% sodium azide, and 10 mM 2-mercaptoethanol (standard buffer). The crude glycinin and β-conglycinin (0.6g) in standard buffer were applied to the column separately. The glycinin was eluted with 800–1000 mL of standard buffer at a flow rate of 25 mL/hr. The adsorbed material, i.e., conglycinin, was then eluted with 800–1000 mL standard buffer containing 0.25M β-methyl D-mannoside at a rate of 25 mL/hr. The eluted pure β-conglycinin and glycinin were collected in 10 mL fractions and their concentrations were determined by measuring absorbance at 280 nm. The glycinin acidic and basic subunits were prepared by the method of Kella et al. (1986). All protein preparations were dialyzed and freeze-dried. The absorbance of each soy protein preparation was measured at 280 nm using a Cary double beam spectrophotometer and quartz cells of 1 cm path length. The protein concentrations of these solutions were confirmed by the microbiuret method (Bailey, 1961) using purified soy glycinin protein as a standard. Absorption coefficients for the other soy protein fractions were then calculated relative to the absorption coefficient of glycinin protein.

Electrophoresis

The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method of Laemmli (1970) was used to assess the purity of the soy protein preparations as described by Utsumi and Kinsella (1985). Gels were fixed in 10% acetic acid and 30% methanol, stained in 0.10%. Coomassie Brilliant Blue R, 30% methanol, 10% acetic acid, 3% sulfosalicyclic acid and destained in 10% acetic acid and 10% methanol. The gels were scanned using an E-C densitometer (EC Co. St. Petersburg, FL) interfaced with a Hewlett-Packard computing integrator.

Measurement of binding

For binding studies, protein solutions were made in 30 mM Tris-HCl buffer, pH 8.0, containing 10 mM 2-mercaptoethanol and 0.2% sodium azide. All protein solutions were passed through a Millipore (Bedford, MA) type AP prefilter to remove undissolved material. The concentrations of whole soy protein, β-conglycinin, glycinin, glycinin acidic and glycinin basic subunit solutions were estimated using the absorption coefficients for 1% solutions at 280 nm (Table 1). For binding studies all protein solutions were made at 1% concentration except in the case of whole soy protein which was made as though the absorption coefficient for a 1% solution were identical to that of glycinin protein to duplicate experimental conditions used by Damodaran and Kinsella (1981a). The binding of 2-nonanone, a represent-

Table 1—Absorption coefficients of soy protein fractions

Protein fraction 1% solution	Absorption coefficient at 280 nm
whole soy	6.04
purified β-conglycinin	4.40
glycinin fraction	8.04[a]
glycinin acidic subunits	7.18
glycinin basic subunits	8.79

[a] From Koshiyama (1972).

ative volatile flavor carbonyl to soy proteins, was studied using an equilibrium dialysis method (Damodaran and Kinsella, 1981a, b). Acrylic cells of equal volume, separated by a membrane and bolted together, were used. One milliliter of protein solution was placed on ne side of the membrane, and 1 mL of buffer containing only the flavor ligand was placed in the other compartment. The cells were then shaken for 20–40 hr at 25°C to attain equilibrium. Aliquots were then removed from each side of the membrane, placed in vials with equal volumes of isooctane and shaken to extract the ligand into the isooctane phase. The concentration of the ligand in the isooctane phase was determined by gas chromatography (Damodaran and Kinsella, 1981a, b). The difference between the concentrations of ligand in the respective compartments represented the amount of ligand bound by the protein. Using the amount of ligand bound, the quantity of protein present and the molecular weight of the protein, the number of moles of ligand bound per mole of protein was calculated. The most commonly used graphical method for the interpretation of binding data is the Scatchard plot (Scatchard, 1948). The equilibrium between a ligand, L, and a protein may be expressed as follows:

$$P + L = PL$$

The association constant, K, is defined as $K = (PL)/[(P)(L)]$,

so: $$(PL) = K(P)(L).$$

Since $P(total) = PL + P$, then $(PL) = K(L)[P(total) - (PL)]$,

or $$\frac{(PL)}{P(total)} = \frac{K(L)}{1 + K(L)} \text{ then } \frac{(PL)}{P(total)} = V,$$

where V is the number of moles of ligand bound per mole of total protein.

Thus: $$V = \frac{K(L)}{1 + K(L)}$$

which is a statement of the law of mass action. If there are *n* independent, binding sites, the equation for the extend of binding is *n* times that for a single site with the same instrinsic binding constant, K. hence

$$V = \frac{nK(L)}{1 + K(L)}$$

Thus, plotting V/L vs V gives the Scatchard plot. The above equation may be rearranged to give the Klotz equation:

$$\frac{1}{V} = \frac{1}{nK(L)} + \frac{1}{n}$$

The binding data obtained from equilibrium dialysis were analyzed using the double reciprocal Klotz equation where V is the number of moles of ligand bound per mole of protein, [L] is the free ligand concentration, *n* is the total number of binding sites on the protein molecule and K is the intrinsic binding constant (Klotz and Urquhard, 1949).

RESULTS & DISCUSSION

THE SDS-PAGE electrophoresis indicated that the purified glycinin and the glycinin acidic and basic subunit protein fractions contained negligible amounts of contaminants (Fig 1). The whole soy protein contained roughly equal parts of β-conglycinin and glycinin substituents with some minor contaminates. The purified β-conglycinin preparation contained a small amount of glycinin. Densitometric scans of the whole soy, crude β-conglycinin, and purified β-conglycinin protein lanes are shown in Fig. 1B. Assuming that all protein constituents have equal binding affinity for Coomassie Brilliant Blue, a progressive enrichment of the β-conglycinin component from 40 to 55 to 90% of the total protein was obtained during its purification in going from whole soy protein to crude β-conglycinin to the purified β-conglycinin fraction, with concomitant decreases in the glycinin component from 49 to 36 to <10%. Notwithstanding the genetic variability normally found in soybean protein content and composition, the composition of the whole soy protein prepared in this study is consistent with that determined previously (Nielsen, 1985a, b). Wolf et al. (1962) reported that whole soy protein contains 37% β-conglycinin and 31% glycinin while Thanh and Shibasaki (1976) found the conglycinins and glycinin constitute 48% and 35% of the total protein, respectively.

The absorption coefficients of 1% solutions of soy protein fractions at 280 nm are shown in Table 1. The absorption coefficient for the β-conglycinin fraction is in close agreement with the value of 4.16 reported by Thanh and Shibasaki (1978). This value was significantly lower than the absorption of the glycinin, which is consistent with the much higher tryptophan of glycinin (Kinsella et al., 1985; Nielsen, 1985a, b). The absorption coefficient of whole soy protein was intermediate between the absorption coefficients of the β-conglycinin and glycinin which indicated that the whole soy protein preparation consisted of approximately equal mixtures of these proteins. The arithmetic mean of the absorption coefficients for the glycinin acidic and basic subunits gave a value of 7.99, which agreed very closely with the absorption coefficient of native glycinin. This indicated that the absorption of ultraviolet light by the acidic and basic subunits of glycinin was not altered by their dissociation from the native oligomeric structure and that the subunits underwent little conformational change as a result of this dissociation.

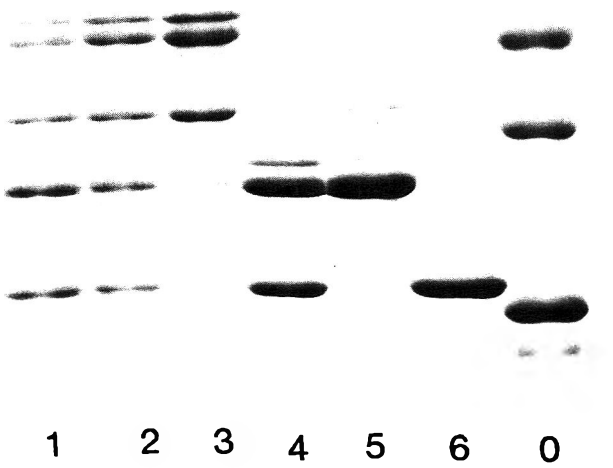
Binding of 2-nonanone to soy proteins

Double reciprocal plots for the binding of 2-nonanone to whole soy protein is shown in Fig. 2. The protein concentrations used in obtaining these data were adjusted to give an absorbance of 8.04 at 280 nm. The intercept corresponded to 5.5 binding sites for 2-nonanone based on a protein molecular weight of 100,000, while the slope of this plot indicated a binding affinity of 570 M^{-1}. These values were based on the assumption that a solution of whole soy protein which had an absorbance of 8.04 at 280 nm corresponded to a 1% protein concentration.

Damodaran and Kinsella (1981a, b) reported a value of between 4 and 5 binding sites per 100,000 molecular weight. Using a value of 4 binding sites a binding constant, K, of 930 M^{-1} was calculated while a value of 744 m^{-1} for K (which is close to the value observed in the present study) was obtained assuming n equalled five. The differences in K obtained between these studies could be caused by differences in the composition of the soy proteins used and because an inappropriate absorption coefficient was used for estimating protein concentration in the previous study (Damodaran and Kinsella, 1981a, b). Thus, based on our current data, the use of an absorption coefficient of 8.04 for a 1% solution of whole soy protein resulted in the preparation of a solution with an actual protein concentration of 1.33% rather than 1% as assumed by Damodaran and Kinsella (1981a, b). This error would result in overestimation of the number of moles of 2-nonanone bound per 100,000 g of protein at any given ligand concentration.

Double reciprocal plots for the binding of 2-nonanone to purified β-conglycinin and glycinin are shown in Fig. 3. The binding parameters determined from these plots, calculated on the basis of both actual molecular weights and on the hypothetical molecular weight of 100,000, are shown in Table 2. β-Conglycinin exhibited a binding constant of about 3050 M^{-1} for 2-nonanone which is higher than that reported for bovine serum albumin (Kinsella and Damodaran, 1980). β-lactoglobulin (O'Neill and Kinsella, 1986) and twofold greater than the value previously reported for crude conglycinin by Damodaran and Kinsella (1981a, b). In addition, the two binding sites per 100,000 molecular weight were lower than reported previously. Using a molecular weight of 160,000 three binding sites per β-conglycinin protein molecule were obtained. Whether these binding sites exist entirely within the structure of the individual subunits or are formed as a result of the association of the subunits into β-conglycinin, is not known.

Damodaran and Kinsella (1981a, b) reported a very low



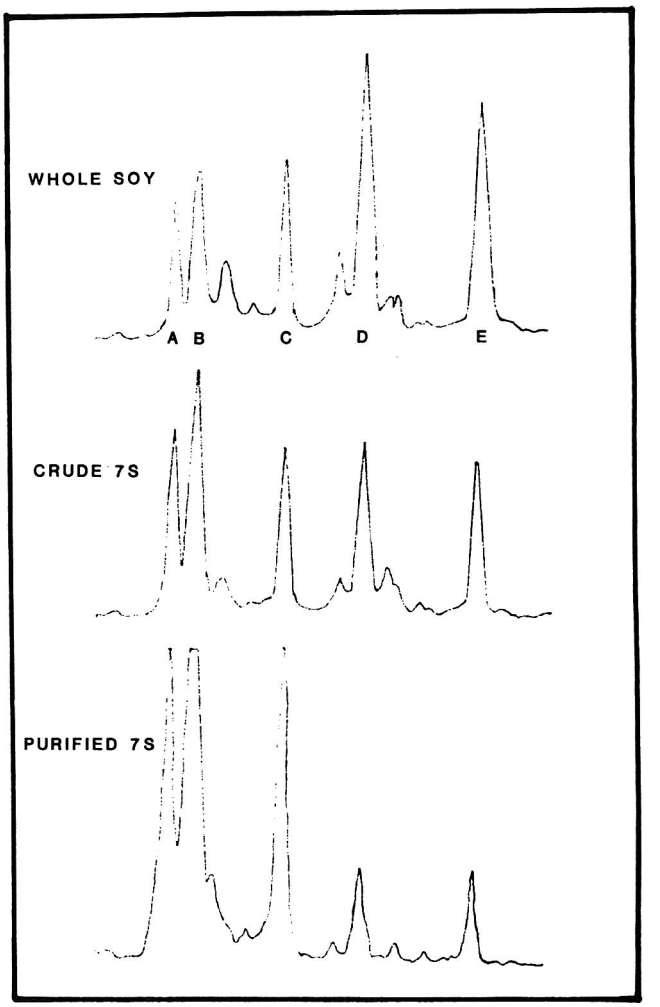


Fig. 1a above—Electrophoretic SDS-PAGE patterns of soy protein fractions. (1) Whole soy protein; (2) crude β-conglycinin; (3) purified β-conglycinin, prepared by Con A-Sepharose 4B affinity chromatography; (4) glycinin purified by Con A-Sepharose 4B affinity chromatography; (5) glycinin acidic polypeptides; (6) glycinin basic polypeptides; (O) molecular weight standards: ∂ lactalbumin, 14,300, β lactoglobulin, 18,400, ovalbumin, 43,000 and bovine serum albumin, 67,000. Linear gradient slab gels were prepared and run as described in methods. Fig. 1B (right)—Densitometric scans of SDS-PAGE gel electrophoresis of soy protein fractions. (A) Whole soy protein; (B) crude β-conglycinin protein; (C) pure β-conglycinin, prepared using Con A-Sepharose 4B affinity chromatography.

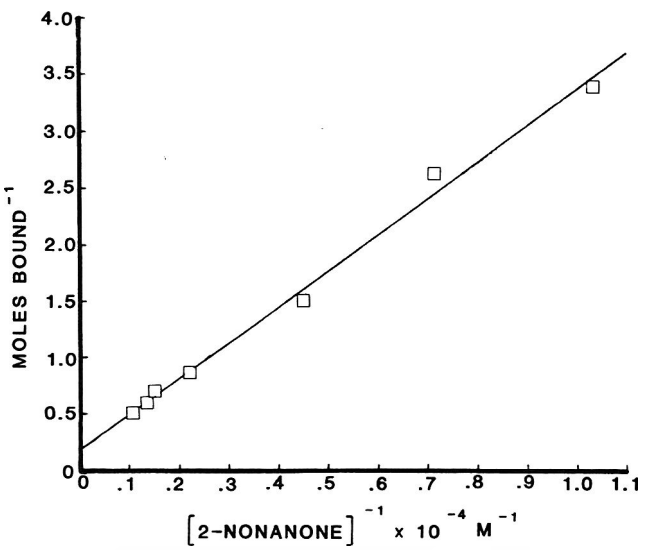


Fig. 2—Double reciprocal plot of the binding of 2-nonanone to whole soy protein. Protein concentration was adjusted to give an absorption of 8.04 at 280 nm.

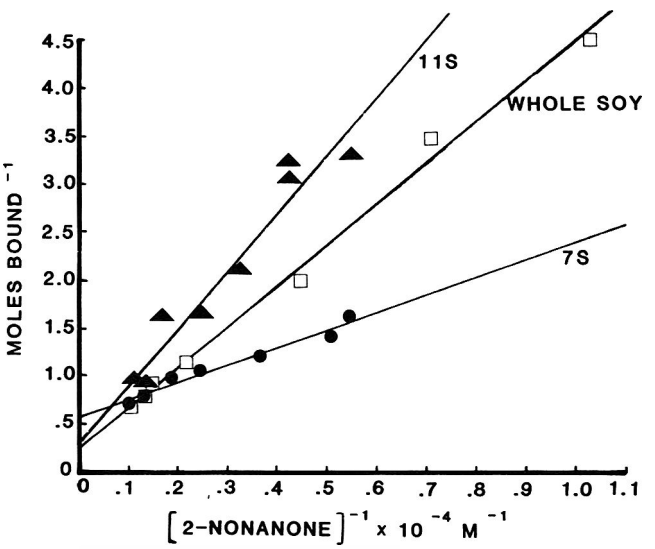


Fig. 3—Binding of 2-nonanone to whole soy protein isolate, β-conglycinin and glycinin. β-Conglycinin and glycinin fractions were purified by Con A-Sepharose 4B affinity chromatography.

affinity of 2-nonanone for crude glycinin. The data obtained in this study also indicate a relatively low binding affinity for glycinin, i.e., the plot (Fig. 3) indicates a binding constant of 540 M^{-1} and about 10 binding sites per 320,000 daltons. This suggested that there might be some exposed nonpolar surface area on the glycinin molecule not suitable for high affinity binding of 2-nonanone. The value of nK determined for whole soy protein, 3140 M^{-1}, fell between the corresponding values for glycinin and β-conglycinin, suggesting that the binding behavior of the whole soy protein was an approximate average of the binding behavior of the β-conglycinin and glycinin constituents. Thus, the β-conglycinin protein contributed mostly to the binding of 2-nonanone by whole soy protein.

The binding parameters determined in this study clarify the

Table 2—Binding parameters for the interaction of 2-nonanone with soy proteins

Protein	Mol. wt basis	*n*	K(M^{-1})	nK
whole soy	100,000	5.5	570	3140
β-conglycinin	100,000	1.8	3050	5490
β-conglycinin	160,000	2.8		8780
glycinin	100,000	3.1	540	1670
glycinin	320,000	9.9	—	5340

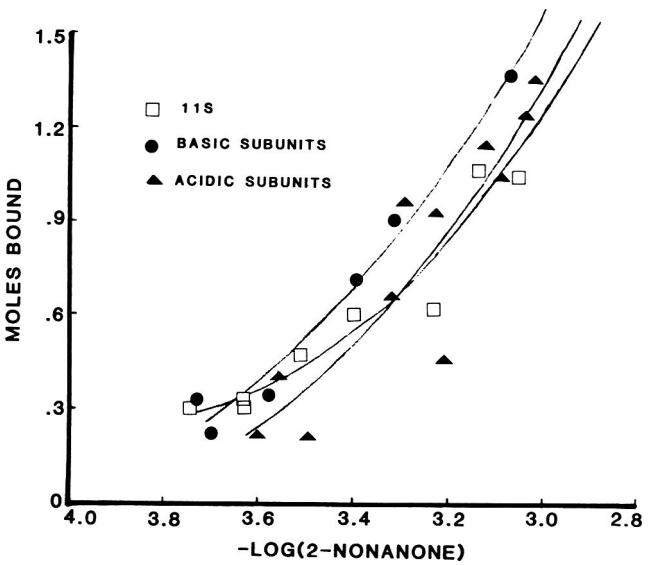


Fig. 4—Binding isotherms for 2-nonanone with native soy glycinin, glycinin and isolated acidic and basic subunit glycinin fractions.

inconsistencies reported earlier by Damodaran and Kinsella (1981a) showing that the double reciprocal plots for the binding of 2-nonanone to whole soy protein and crude β-conglycinin were very similar. Since the glycinin had low binding, they concluded that β-conglycinin accounted for most of the flavor binding of the whole soy protein. However, the present data demonstrated that the binding characteristics of pure β-conglycinin were not equivalent to soy protein isolate. This discrepancy is partly explained by the fact that the absorption coefficient of 6 used earlier to estimate the β-conglycinin protein concentration was based on the absorption coefficient for whole soy rather than on the true value as 4.4 determined in this study. This in addition to the presence of contaminants in the β-conglycinin preparation in the previous study resulted in an overestimation of carbonyl binding to the β-conglycinin.

Binding to acidic and basic subunits of glycinin

Binding isotherms for the interaction of 2-nonanone with native glycinin, glycinin acidic and basic polypeptides are shown in Fig. 4. When examined on the basis of equal molecular weights, the basic subunits appear to have a slightly higher affinity for 2-nonanone than the acidic subunits, which is consistent with the higher hydrophobicity of the basic subunits (Kinsella et al., 1985). The three protein fractions exhibited only small differences in affinity for 2-nonanone binding. Apparently, the binding of 2-nonanone by the acidic and basic subunits was unaffected by their association into the oligomeric glycinin form under conditions used. This may suggest the hydrophobic surfaces which are responsible for the binding of 2-nonanone are not involved in the association of the acidic and basic subunits which forms the native quaternary structure of soy glycinin protein. However, this does not exclude the possibility that hydrophobic forces play a role in the stabilization of the native quaternary structure of soy glycinin.

The data presented in this study demonstrated that the β-conglycinin was responsible for most of the flavor binding by soy protein in aqueous solution. The β-conglycinin fraction apparently has three binding sites for aliphatic carbonyls which may correspond to the presence of three subunits in this protein (Thanh and Shibasaki, 1976). The binding affinity of these sites is relatively high by comparison to other proteins which have been studied, e.g., K (M^{-1}) of 2,440 and 820 for β-lactoglobulin and bovine serum albumin, respectively. This suggests that off-flavor problems in soy protein isolates may result from the selective binding of lipid oxidation products by the β-conglycinin fraction. Further research is needed to examine more closely the factors which affect flavor binding and release by β-conglycinin to develop methods to control or minimize off-flavor problems in soy proteins.

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Ms received 4/21/86; revised 8/11/86; accepted 8/13/86.

Supported in part by NSF grant #CB 8506243 and a special award from General Foods Foundation to J.E. Kinsella.

Bioavailability of Iron in Fermented Soybeans

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ABSTRACT

Hemoglobin depletion-repletion assay was used for determining iron bioavailability. The relative biological value (RBV) of iron in boiled nonfermented soybeans was the lowest among the products evaluated. The RBV of iron increased from 60.1% in boiled nonfermented soybeans to 86.7% by lactic acid producing microorganisms and to 87.5% by *Rhizopus oligosporus* fermentation ($FeSO_4$ = 100%). Results of this research suggested that fermentation by lactic acid producing organisms and *Rhizopus oligosporus* increased the RBV of iron.

INTRODUCTION

SOYBEANS contain appreciable amounts of iron but the availability is poor. Minerals from plant sources, particularly those that arise from plant seed, are less effectively utilized than those from animal sources (O'Dell et al., 1972). This is due, in part, to phytic acid and fiber present in the plant.

Degradation of phytate and hydrolyzing the complex form and/or insoluble form by the enzymes of microorganisms should liberate the minerals and increase their availability. Fermentation is an ageless process. Utilization of this method of processing is likely to increase because of the interest of young adults who are interested in new foods. Also there is the need of a growing world population for more foods (Hesseltine, 1983). Lopez et al. (1983) observed that natural lactic acid fermentation decreased the phytic acid in cornmeal due to phytase production by microorganisms. Also, active phytase was demonstrated by *Rhizopus oliosporus* when used in tempeh fermentation (Sudarmadji and Markakis, 1977).

Fermentation has a potential for decreasing the phytate content of soybean meal and increasing the bioavailability of iron; therefore, the objective of this study was to evaluate and compare the effects of fermentation using lactic acid producing organisms and *R. oliosporus* on soybean meal. Also, two methods of heat treatment were also used to test the influence of heat on iron bioavailability. The bioavailability of iron was measured by a rat bioassay.

MATERIALS & METHODS

Source of sample

A 1983 crop of certified Williams soybean seeds was obtained from the Missouri Seed Improvement Association. Soybeans were processed in November 1984.

Sample cleaning

Soybeans were cleaned by sieving through a 3.35-mm sieve (The W.S. Tyler Co., Cleveland, OH) to remove unwanted materials. The cleaned soybeans were then heated by one of two methods: boiling for 30 min or autoclaving at 121°C for 30 min.

Autoclaved soybeans

One kilogram cleaned soybeans was washed and divided into four portions of 250g. Each portion was placed in an aluminum pan (25 × 10 × 7.5 cm) and autoclaved for 30 min at 121°C. After 30 min, the soybeans were removed and dried in an air flow oven (Freas Model 385, Precision Scientific, Chicago, IL) at 55°C for 20 hr. The dried soybeans were then ground in a Wiley Laboratory Mill (Model 4, Arthur H. Thomas Co., Philadelphia, PA) using 1-mm screen. The yield of ground autoclaved soybeans flour was 800g. The fat in the ground autoclaved soybeans was removed using ethyl ether (Fisher Scientific Co., Fair Lawn, NJ). Into each of four 600-mL Pyrex beakers, 200g autoclaved soybean flour were placed, 200 mL of ether was added, and the mixture was stirred for 30 min. The liquid was decanted and the remaining solids from each of the four portions were spread in an aluminum tray (40 × 25 × 7.5 cm). The tray was placed under a fume hood and allowed to stand overnight for evaporation of remaining ether.

Boiled soybeans

One kilogram cleaned soybeans was divided into four portions of 250g, and each portion was placed in an aluminum pan (25 × 10 × 7.5 cm). Tap water (iron was considered to be nil) was added (ca 600 mL) to each portion, and the beans were allowed to soak overnight at 25°C. The water was then drained and the beans were rinsed once with tap water. Each portion of soybeans was placed in a 2-L Pyrex beaker with enough tap water added to cover the beans (ca 800 mL) which were then boiled for 30 min. After the four portions of beans were removed from the heat, the water was drained, and the beans were spread in an aluminum tray for drying in an air flow oven at 55°C for 20 hr. The dried beans were ground by a Wiley Laboratory mill using a 1-mm screen. The ground, boiled soybeans were defatted using the method described previously for defatting autoclaved soybeans.

The preparation of autoclaved defatted soybean flour and boiled defatted soybean flour was replicated three times. The products obtained from the three replications were mixed well and stored at 4°C (maximum of 6 wk). This product was designated as the non-fermented food sample for diets for animal feeding.

Fermentation

Into each of four 1000-mL Pyrex beakers, 100g of autoclaved defatted soybean flour was placed. Ten grams sugar and 400 mL tap water (to simulate home or a commercial procedure) were added. The mixture was stirred, and 10 mL of liquid from fermented corn (17 hr inoculum of a fermented slurry of corn with a 1:4 ratio of solids to water) was added. The mixture was then stirred well. The same procedure was applied to the boiled defatted soybean flour, except that 12.5g of sugar were added instead of 10g as used with the autoclaved soybeans. These eight beakers were covered with aluminum foil and placed in an incubator (Aloe Scientific, St. Louis, MO) and incubated at 30°C for 4 days.

Titratable acidity and pH were measured initially and followed every 24 hr until the end of the fermentation by a Fisher Accumet pH meter (Model 600, Fisher Scientific Co., Pittsburgh, PA). Titratable acidity was determined by titrating 10 mL of sample with 0.0984 N sodium hydroxide. The titration was terminated when the sample pH was 8.4. At the end of the fermentation, the pH of one-half of the fermentation product was adjusted with baking soda (Host Favorite, North American Food Service Corp., Chicago, IL) to the original pH to ensure acceptance by some of the rats. The autoclaved fermented food was adjusted to pH 6.5, while the boiled fermented food was adjusted to pH 6.7. The other half of the fermented product was left at the acidic pH 4.2, the pH which occurred during fermentation. The fermented products were then transferred into aluminum pans and were dried in an air flow oven at 55°C for 48 hr. The dried sample was reground by a Wiley laboratory mill with a 1-mm screen. The product treated with baking soda was designated as the fermented food sample with pH 6.5 and 6.7. The other product was designated as the fermented food sample with pH 4.2. The fermentation procedure was replicated three times, and the products obtained from the three replications of

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the fermentation were mixed well and stored at 4°C (maximum of 4 weeks) for making diets for animal feeding.

Rhizopus oligosporus fermentation (Tempeh)

Tempeh was prepared by a method that is practiced in Indonesia. Six hundred grams cleaned soybeans were divided into three portions of 200g. Each portion was placed in an aluminum pan and was soaked in about 500 mL of tap water overnight at 25°C. The water was drained, and the soybeans were rinsed once with tap water. Each portion was placed in a 2-L Pyrex beaker, tap water was added to cover the beans (ca 700 mL), and the beans were boiled for 15 min. The skins were then removed from the beans by hand under running water. The peeled soybeans were boiled for another 30 min with water just covering the beans. After the soybeans were removed from the heat, the water was drained, and the peeled, cooked soybeans were allowed to cool at 25°C before inoculating with *Rhizopus oligosporus*, NRRL 2710 (ATCC 22959, American Type Culture Collection, Rockville, MD). One standard slant of *Rhizopus oligosporus* was suspended with 3 mL of double distilled water and was mixed with 300g of peeled cooked soybeans. The mixture was then packed in a polyethylene bag (15 × 14 × 2 cm). The bag was perforated for air supply and incubated at 30°C for 26 hr. After 26 hr incubation, the fresh, white tempeh was sliced (3 to 5 mm thick) and placed in an aluminum plate for drying. The tempeh was dried using an air flow oven at 55°C for 20 hr. The dried tempeh was ground with a Wiley laboratory mill using a 1-mm screen and stored at 4°C. The preparation of tempeh was replicated three times, and tempeh obtained from the three replications of fermentation was mixed well and stored at 4°C (maximum of 4 wk) for diets for animal feeding.

Rats

Male sprague Dawley rats, 21 days old, were obtained from the Sasco Company in Omaha, NE and housed individually in stainless steel cages. Temperature and humidity were maintained at 25°C and 60%, respectively. Lighting was regulated automatically to provide 12 hr of light and 12 hr of darkness. Food and double distilled water were provided *ad libitum*.

Diets preparation

Composition of the basal diet (purified diet AIN, 1977) for the rats is listed in Table 1. The mineral mix was formulated with salts obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ). The mineral mixture was that recommended by the American Institute of Nutrition (AIN mineral mixture AIN, 1977), as listed in Table 2. After the salts were weighed and combined, the mixture was passed through a Wiley mill to insure homogeneity.

The vitamin premix was purchased from U.S. Biochemical Corp. (Cleveland, OH), and contained the following vitamins in mg per kg of premix: thiamin-HCl, 600 mg; riboflavin, 600 mg; pyridoxine-HCl, 700 mg; nicotinic acid, 3.0g; D-calcium pantothenate, 1.6g; folic acid, 200 mg; D-biotin, 20 mg; cyanocobalamin, 1.0 mg; retinyl palmitate, 800 mg; dl-tocopheryl acetate, 20g; cholicalciferol, 2.5 mg; menaquionine, 5.0 mg; and sucrose, 972.9g.

Table 1—Composition of basal diet, iron deficiency diet and standard diets[a]

Ingredient	%	g/kg diet
Casein[b]	20.0	200.0
Corn oil[c]	5.0	50.0
Fiber[d]	5.0	50.0
Mineral mix[e]	3.5	35.0
Vitamin mix[e]	1.0	10.0
DL-Methionine[f]	0.3	3.0
Choline bitartrate[f]	0.2	2.0
Glucose[g]	65.0	650.0

[a] Basal diet for rats consisted of the following: casein, 20%, DL-methionine, 0.30%; glucose, 65%; fiber, 5%; corn oil, 5%; mineral mix, 3.50%; vitamin mix, 1% and choline bitartrate, 0.2%. Iron deficiency diet as above without added iron. Standard diets as above with added iron. Iron ($FeSO_4 \cdot 7H_2O$) added at graded levels to achieve: 7.5, 15 and 25 μg per g of diet.
[b] Contained 3.75 μg iron per g.
[c] Mazola corn oil, with 0.01% BHT and 0.01% BHA Best Foods, Chicago, IL.
[d] Hydrolyzed-acid washed cellulose, trade name Celufil, U.S. Biochemical Corp., Cleveland, OH.
[e] Composition of mineral and vitamin mix as recommended by The American Institute of Nutrition (AIN, 1977).
[f] U.S. Biochemical Corp., Cleveland, OH.
[g] Glucose monohydrate trade name Clintose, J.M. Swank, Co., Iowa City, IA.

Table 2—Composition of mineral mixture[a]

Ingredient	g/kg of mixture
Calcium phosphate dibasic ($CaHPO_4$)	500.00
Sodium chloride (NaCl)	74.00
Potassium citrate, monohydrate ($K_3C_6H_5O_7 \cdot H_2O$)	220.00
Potassium sulfate (K_2SO_4)	52.00
Magnesium oxide (MgO)	24.00
Manganous carbonate (43–48% Mn)	3.50
Ferric citrate (16–17% Fe)	---
Zinc carbonate	1.60
Cupric carbonate (53–55% Cu)	0.30
Potassium iodate (KIO_3)	0.01
Sodium selenite ($Na_2SeO_3 \cdot 5H_2O$)	0.01
Chromium potassium sulfate ($CrK[SO_4]_2 \cdot 12H_2O$)	0.55
Cellulose	to make 1,000

[a] Salt obtained from J.T. Baker Chem. Co. (Phillipburg, NJ).

Table 3—Composition of test diets[a]

Diet identification[b] and soybean treatment	Soybean product	Casein	Corn oil	Glucose monohydrate
Autoclaved nonfermented	16.80	12.48	2.46	58.26
Boiled nonfermented	26.98	7.11	0.36	55.55
Autoclaved fermented (pH 6.5)	17.94	11.79	2.21	58.06
Autoclaved fermented (pH 4.2)	16.87	12.38	2.30	58.45
Boiled fermented (pH 6.7)	29.15	6.00	0.27	54.58
Boiled fermented (pH 4.2)	29.07	5.66	0.18	55.09
Tempeh[c]	17.26	10.66	—	62.08

[a] Iron level in each diet was 20 μg per g of diet derived from each test sample used.
[b] All diets contained 5% fiber, 3.5% mineral mix (as listed in Table 3), 1.0% vitamin mix, 0.3% DL-methionine, 0.2% choline bitartrate.
[c] Iron in this diet was 10 μg per g of diet.

Diets

Composition of the iron deficiency diet is listed in Table 1. Three kinds of standard diets were made by adding three graded levels of iron as $FeSO_4 \cdot 7H_2O$ to achieve 7.5, 15.0, and 25 μg per g of diet. The composition of standard diets is listed in Table 1.

Seven test diets were prepared by supplementing with seven kinds of test food: autoclaved nonfermented, boiled nonfermented, autoclaved fermented pH 6.5, autoclaved fermented pH 4.2, boiled fermented pH 6.7, boiled fermented pH 4.2 and tempeh. Each of these diets, except the one prepared with tempeh, contained 20 μg iron per g of diet. The iron was derived from the supplement and for this reason the amount of soy product added to the diets (Table 3) varied. For the diet made with tempeh, the level of iron was 10 μg per g of diet. This provided an equal level of fat in all diets. Also, the amounts of casein and corn oil in the diets containing test foods were adjusted so that the % protein and % fat were equal in all diets after chemical analyses.

Design of experiment

Sixty male weanling rats weighing 40–50g (mean 46.4 +/− 3.67) were used. In the first 4 wk, the rats were fed the iron deficiency diet (Table 1). At the end of the 4-wk depletion period, the blood that was taken from the tail by cutting 1 cm from the tail tip was analyzed for hemoglobin, and the body weights were recorded.

Rats were divided into 10 groups of six based on the value of hemoglobin g per 100 mL (dL) times body weight (g), so that the means of hemoglobin times body weight (Hb × Wt) in all groups were equal. Each diet was assigned to a group in a random manner. Three groups were fed standard diets with $FeSO_4 \cdot 7H_2O$ added at levels of 7.5, 15.0 and 25.0 μg per g of diet. Another seven groups were fed seven kinds of test diets: autoclaved nonfermented, boiled nonfermented, autoclaved fermented pH 6.5, autoclaved fermented pH 4.2, boiled fermented pH 6.7, boiled fermented pH 4.2, and tempeh. After 14 days on the test diet, the rats were weighed and then were

sacrificed by decapitation. The blood flow from the neck was taken for analysis of hemoglobin.

Determination of hemoglobin

The blood taken from the tail of each animal at the end of the depletion period and the blood taken after decapitation at the end of repletion period were analyzed for hemoglobin using a cyanmethemoglobin method described by Crosby et al. (1954). Twenty microliters of whole blood were delivered with a Sahli pipette into exactly 5.0 mL of Drabkin's solution (1.0g sodium bicarbonate, 0.2g potassium ferricyanide, and 0.05g potassium cyanide in 1,000 mL of double distilled water which contained 0.5 mL 30% Brij-35 solution). The pipette was rinsed repeatedly so that all of the blood was delivered. The blood and solution were mixed by swirling and allowed to stand for 15 min. The absorbance was then read at 540 nm on a Spectronic-21 spectrophotometer. Hemoglobin was determined using a standard curve based on analyses of serial dilutions of the cyanmethemoglobin standard. The cyanmethemoglobin standard was prepared by diluting a hemoglobin standard (lyophilized human methemoglobin, 36 mg) with 50 mL of Drabkin's solution, and the solution was allowed to stand for 30 min before using. All reagents and the standard used in the hemoglobin assay were purchased from Sigma Chemical Co., St. Louis, MO.

Regression analysis

The change in the value obtained from the hemoglobin level (g per dl) times body weight in g (Hb × Wt) was used to calculate relative biological value (RBV) of endogenous iron in the test diet (Gordon and Chao, 1984). Iron intake was calculated by multiplying food intake (g) times actual concentration of iron in the diet. A regression equation was calculated by the method of least squares (Snedecor and Cochran, 1980) for each response measurement from animals fed standard diets with increasing levels of iron (7.5, 15.0 and 25.0 μg per g of diet supplied as $FeSO_4 \cdot 7H_2O$). Each response observed in the animals fed the test diet was substituted in the regression equation so that the corresponding iron intake could be determined. RBV was calculated by dividing this value by the actual iron consumed by the animal × 100.

Iron bioavailability

As described previously, regression analysis was used to analyze the values of iron bioavailability. Analysis of variance, one way classification, was used to evaluate total food composition, body weight, and hemoglobin level among groups of animals. The means of total food consumption, body weight, hemoglobin level, and RBV were compared using the least significant difference rule (Snedecor and Cochran, 1980).

Determination of total iron

The total iron was determined using the method described by Gordon and Robert (1977). First, samples were wet digested with concentrated nitric acid and 70% perchloric acid, and then the total iron was analyzed using atomic absorption spectrophotometry.

One gram of sample was placed in a 250-mL digestion flask. Twelve milliliters of reagent grade nitric acid (Fisher Scientific Co., Fair Lawn, NJ) were added, and the sample was allowed to stand overnight. Then, the sample was placed on a hot plate (Thermolyne model: HP-A2245 M, Dubuque, IA) at low heat (100°C) for 1 hr, after which heat was increased to 200°C. After heating for 3 hr, the sample was allowed to cool at room temperature. Two milliliters 70% perchloric acid (Fisher Scientific Co., Fair Lawn, NJ) were added to the digestion flask which was heated first at low heat (100°C) with a gradual increase to 400°C. The digestion was continued until the volume was reduced to approximately 2.0 mL. The sample was again allowed to cool, 40.0 mL of double distilled water were added, the solution was brought to boil and then allowed to cool. This sample solution was quantitatively transferred into a 100-mL volumetric flask and brought to volume with double distilled water for analysis by atomic absorption spectrophotometry.

A Perkin-Elmer Model 2380 spectrophotometer with hollow cathode lamps and an air-acetylene flame was used for determining total iron. There are specific standard operation conditions for iron. The calibration of the instrument for iron is described in the operator's manual, Publication 303-0152 (Perkin-Elmer, Norwalk, CT). The standard solutions were prepared from a standard stock solution (Fisher Scientific Co., Fair Lawn, NJ).

All samples were wet digested and diluted to the desired volume, depending on the element and the anticipated amount of this element in the sample. Each sample was aspirated through the nebulizer, and a reading was taken after 5 sec. Each reading was the average of three determinations automatically taken 3 sec apart by the spectrophotometer. The instrument has a microprocessor which allowed the automatic conversion of absorption into concentration. A standard sample was read between each test sample to insure the proper calibration of the instrument.

RESULTS & DISCUSSION

Titratable acidity and pH of fermented soybeans by a mixed lactic acid microflora

The initial pH of boiled soybeans (pH 6.7) was slightly higher than the pH of autoclaved soybeans (pH 6.5). The pH of both products dropped rapidly during the first day of fermentation, after which the pH reached the same pH of 4.2.

Concomitantly, with the drop in pH, there was a rise in the titratable acidity of both the boiled and autoclaved soybeans. The titratable acidity increased sharply on the first day and continued to rise slowly up to the fourth day of fermentation. This pattern is characteristic of a natural lactic acid fermentation (Zamora and Fields, 1979).

The depletion-repletion method used in this study was as described by Gordon and Chao (1984). Actual food iron intake, weight gain, change in hemoglobin, and food consumption during the 14 days repletion period of rats are reported in Table 4. There was no significant difference in weight gain among the groups of rats fed the soybean products. Using ferrous sulfate as a standard, equal to 100%, the RBV of iron in soybeans products are reported in Table 5.

Nonfermented soybeans

The RBV of iron in autoclaved nonfermented soybeans was high (95.8%) which was higher than that found in boiled soybeans (60.1%) (Table 5). Steinke and Hopkins (1978), using the depletion-repletion method with rats, found that autoclaving isolated soybean protein at 108.4°C for 30 min improved the RBV of iron over that of the unheated samples (106% vs 64%). Rotruck and Luhrsen (1979) found that RBV of iron in protein isolates ranged between 82 to 102% and from 71 to 102% in processed soybean protein. Estimation of RBV by the in vitro method showed that the baking process did not alter the RBV of nonheme iron in breads and meals containing different iron fortification sources (Schricker and Miller, 1982).

The lower RBV of iron in boiled nonfermented soybeans compared to autoclaved nonfermented soybeans (Table 5) was in agreement with results observed by Ashworth et al. (1973) with human subjects. Using whole body counting techniques with 42 infants, it was shown that the mean absorption of iron from baked soybeans was three times greater than from boiled soybeans. The low RBV of iron in boiled non-fermented soybeans in the study reported herein may have been due to the binding of iron in protein, phytate or cellulose. Camire and Clydesdale (1981) found that boiling caused the binding of iron to cellulose, lignin, and wheat bran.

Lactic acid fermented soybeans

Lactic acid fermentation increased the RBV of iron both in autoclaved and boiled soybeans (Table 5). A similar trend was found by Derman et al. (1980). Iron absorption from maize and sorghum beer was 12 fold greater than that from a gruel made from the constituents used to prepare the beer. Iron absorption from a solution of $FeCl_3$ containing 2 mL lactic acid per L was four fold greater than the same solution adjusted to the same pH (2.5) with HCl. It was suggested that lactic acid acted as a chelator which enhanced iron absorption from the beer.

The amino acid, lysine, also was reported to enhance iron

Table 4—Dietary iron concentration, initial body weight, weight gain, hemoglobin change, and total food consumption and total iron intake during 14-day iron repletion period

Diet	Iron concentration		Body weight[a,b]		Hemoglobin[a,b]		Total food consumption	Total iron intake
	Added	Actual[c]	Initial	Gain	Initial	Gain		
	μg/g diet		g		g/dL		g	γ
$FeSO_4 \cdot 7H_2O$	7.5	12.50	160.5	50.4	3.94	2.30	205.7	2571.3
$FeSO_4 \cdot 7H_2O$	15.0	20.50	154.9	77.6	3.98	5.37	246.2	5047.1
$FeSO_4 \cdot 7H_2O$	25.0	32.30	161.6	80.5	3.84	8.25	256.4	8281.7
ANF[d]	20.0	20.50	153.4	83.2	4.01	4.07	234.6	4809.3
BNF[e]	20.0	22.00	150.8	70.1	3.83	2.56	240.5	5291.0
AF[f] (pH 6.5)	20.0	22.00	164.4	83.4	3.75	4.92	235.6	5183.2
AF (pH 4.2)	20.0	21.25	156.1	77.1	3.61	4.55	237.7	5051.1
BF[g] (pH 6.7)	20.0	20.50	155.6	67.8	3.64	4.23	239.8	4915.9
BF (pH 4.2)	20.0	23.75	151.7	74.7	3.96	4.18	229.6	5453.0
Tempeh	10.0	15.50	154.1	66.4	3.85	2.33	215.4	3338.7

[a] N = six animals.
[b] At start and completion of repletion period, 50 and 64 day (animal age), respectively.
[c] Mean value of duplicate determination.
[d] Autoclaved nonfermented.
[e] Boiled nonfermented.
[f] Autoclaved fermented.
[g] Boiled fermented.

Table 5—Relative biological value of iron

Sample	Relative biological value (%)
Standard ($FeSO_4$)	100.0
Autoclaved non-fermented	95.8[a,b]
Boiled non-fermented	60.1[d]
Autoclaved fermented (pH 6.5)	101.9[a]
Autoclaved fermented (pH 4.2)	93.6[a,b,c]
Boiled fermented (pH 6.7)	86.7[b,c]
Boiled fermented (pH 4.2)	81.1[c]
Tempeh	87.5[b,c]

[a-d] Where letters differ within a column, values differ significantly ($P < 0.05$) from each other.

absorption (Van Campen and Gross, 1969; Van Campen, 1973). Available lysine increased significantly in lactic acid fermentation of corn (Hamad, 1978; Au and Fields, 1981; Umoh and Fields, 1981; Tongnual et al., 1981; Kratochvil, 1984).

Based on the studies cited above, it may be hypothesized that the increase in the RBV of iron by lactic acid fermentation, as observed in this study, may be caused by three factors: (1) Iron is released from complexes by enzymes, such as proteases and phytases, produced by lactic acid microorganisms. (2) Lactic acid, produced by microorganisms, acts as a chelator of iron. (3) Other chelating agents, such as lysine, are produced by lactic acid microorganisms.

Tempeh

Rhizopus oligosporus fermentation increased the RBV of iron in tempeh (Table 5). This was probably due to the action of enzymes, such as proteases or phytases, by the mold. These enzymes release iron from the binding components, such as protein or phytate. Although lysine was not measured, it may have increased and acted as a chelator. Murata et al. (1967) observed that available lysine in tempeh was 42 times greater than in non-fermented soybeans.

CONCLUSION

FERMENTATION by either lactic acid producing bacteria or *R. oliosporus* increased the RBV of iron in soybeans. Alternatively, boiling may reduce the RBV of iron compared to other treatments evaluated in this study. The RBV of iron in autoclaved nonfermented soybeans was higher than that of boiled nonfermented soybeans.

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Ms received 3/21/86; revised 6/19/86; accepted 7/17/86.

Contribution from the Missouri Agricultural Experiment Station. Journal Series No. 10048.

Effects of Germination on the Proximate Composition and Nutritional Quality of Winged Bean (*Psophocarpus tetragonolobus*) Seeds

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ABSTRACT

Winged beans were germinated using the between paper technique. The nonprotein nitrogen was found to increase gradually and the protein nitrogen content to decrease. A slight decrease in the lipid content was also observed. Trypsin inhibitor activity was uneffected by germination. However, lipoxygenase activity was found to decrease; 77% of the original activity was lost after 120 hr. These changes are compared to the changes in proximate composition of other legumes during germination. Following a decrease in the amino acid composition of the seeds after 48 hr of germination, significant increases in the concentrations of cysteine, aspartic acid, and histidine were found after 72 hr incubation.

INTRODUCTION

GERMINATION has often been proposed as a means by which the nutritional quality of legume seeds might be improved. Many aspects of the compositional changes taking place as a result of the high metabolic activity within the seed during germination have been examined, and are extensively reviewed by Finney (1983).

The protein content of legumes has been shown to generally increase during germination (Kylen and McCready, 1975; Hsu et al., 1980) but after allowing for the loss of solids there is no net synthesis of protein. The depletion of oil in germinating legumes has been reported by Kylen and McCready (1975) for lentils, mung beans, and soybeans.

The stored carbohydrates in seeds are utilized during the early stages of germination (Abrahamen and Sudia, 1966). Several investigators (Silva and Luh, 1979; Hsu et al., 1973; East et al., 1972) have shown that the oligosaccharides of the raffinose family are hydrolyzed during germination. Recently it has been shown that this is also the case in winged beans (Puwastien and King, 1984).

Germination has also been investigated as a means of reducing antinutritional factors such as trypsin inhibitors in soybeans (Desikachar and De, 1950; Bates et al., 1977) and in navy beans (Kakade and Evans, 1966).

The objective of this study was to examine the effects of germination on the proximate composition of winged beans and on some aspects of the nutritional quality of the seeds.

MATERIALS & METHODS

Winged beans

Dry winged beans (*Psophocarpus tetragonolobus* (L).D.C. variety RAJBURI), were supplied by The Institute of Scientific and Technological Research, Thailand. The beans were two years old and had a moisture content of 10.7%. The beans were inspected and any foreign matter removed, then they were randomly divided into six aliquots for each experiment and kept at 4°C in screw-cap bottles until required.

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Germination of beans

Germination was accomplished using the "between paper" technique, according to the method of The International Rules for Seed Testing as previously described (Puwastien and King, 1984). Samples were incubated for 0, 24, 48, 72, 96, and 120 hr.

Preparation of winged bean flour

Ungerminated and freeze-dried germinated seeds were pulverized in a coffee grinder so that the resulting flour passed through a 60 mesh sieve. The full fat flours were stored at 4°C in closed containers until required.

Total solids

The moisture content of the winged bean flour from germinated and nongerminated seeds was determined by drying ground samples at 105°C to constant weight. Total solids and solid loss resulting from germination were calculated.

Protein content

The total nitrogen (TN) content of the winged bean flour was estimated by Kjeldhal digestion (Egan et al., 1981) followed by colorimetric determination of the ammonia in the digest using the Berthelot reaction in an autoanalyser. Nonprotein nitrogen (NPN) was determined by the method of Becker et al. (1940). The protein nitrogen was then calculated by difference (TN-NPN).

Determination of fat

The winged bean flour was extracted with petroleum ether (40–60% °C BP) for 6–8 hr in a soxhlet extracter. The percentage lipid was estimated after removing the solvent and drying at 80°C to constant weight.

Protein solubility

Protein solubility of the winged bean flours was determined using a modified procedure of Betschart (1974), (King and Puwastien, 1984).

Trypsin inhibitor activity

The trypsin inhibitor activity of winged bean flours was determined using a modified procedure of Kakade et al. (1974), (Smith et al., 1980).

Lipoxygenase activity

The lipoxygenase activity of the winged bean flour was determined on phosphate buffer extracts using the procedure of Ben Aziz et al. (1970).

Amino acid analysis

Samples (0.5g) were hydrolyzed in 20 mL 6M hydrochloric acid at 110°C for 24 hr under nitrogen, the hydrolysates were stored frozen in a screw-cap bottle until required. The hydrolysates were diluted so that the protein concentration was 0.3g/mL. Cystine and methionine were determined using the method of Moore (1963).

The amino acids in the hydrolysates were determined by ion-exchange chromatography using a Bio-tronic LC 5000 Automatic Amino Acid Analyzer, fitted with a single column packed with sulphonate

polystyrene beads cross linked by divinyl benzene. Stepwise elution with sodium citrate buffers, pH 3.30, 3.85, 4.30, 5.20, and 10.1 was used to separate the amino acids. The amino acids were detected using ninhydrin, the absorbance being measured at 570 and 440 nm. A standard, 0.1 μmol/mL, was run every seventh sample. The amounts of the amino acids recovered were adjusted according to the recovery of a norleucine internal standard and are expressed as g/16g nitrogen (by Kjeldhal analysis) in the original sample.

The amino acid profile of the seeds was measured at regular intervals during germination. The data were subjected to a one-way analysis of variance and the Student's t-test applied to compare the mean concentration of each amino acid at each interval of germination with the mean value in ungerminated seeds.

RESULTS & DISCUSSION

NUMEROUS METHODS have been used for germinating legume seeds. In this study the "between paper" technique was employed because of the low levels of microbial contamination usually experienced with this technique, the low levels of dry matter lost (2.5% after 24 hr and up to 6.1% after 96 hr of germination), and the high rate of germination (19% after 48 hr and 96% after 120 hr) (Puwastien and King, 1984), compared to other techniques (El-Mahdy and El-Sebaiy, 1982). However, this technique cannot be considered as a practical method for germinating seeds for consumption. During germination, degradation of storage protein and the synthesis of new proteins and other nutrients takes place. Germination of the winged bean seeds resulted in a small decrease in the protein nitrogen content during the 120 hr of incubation (Fig. 1). The nonprotein nitrogen content during this time showed a gradual increase. Similar changes have been observed with peas, fenugreek seeds (Chen and Thacker, 1978) cowpea and green-gram (Kumar and Venkataraman, 1975). The increase in NPN might be due to an increase in free amino acid content as a result of increased proteolytic activity (Chen and Thacker, 1978) or an increase in the nucleic acid content resulting from the increased metabolic activity. The solubility of the protein during germination was followed (Table 1). Since high proteolytic activity within the germinating seeds might be expected, an increase in the protein solubility resulting from hydrolysis of the storage proteins would not be unreasonable. With the winged bean seeds studies there was, however, no significant change in protein solubility. Trypsin inhibitor levels

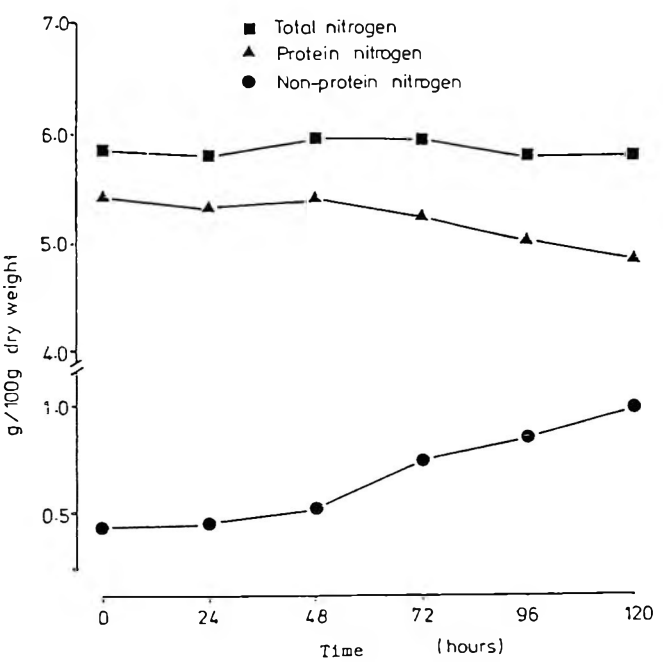

Fig. 1—Changes in nitrogenous constituents during germination.

Table 1—Effect of germination on the fat content, lipoxygenase activity, trypsin inhibitor level and protein solubility of winged beans[a]

Time of incubation (hr)	Total fat content (%)	Lipoxygenase activity (units/mg of sample)	Trypsin inhibitor activity (units/mg of sample)	Protein solubility (%)
0	19.9 ± 0.14	150.8 ± 4.87	74.7 ± 1.31	72.3 ± 0.71
24	19.4 ± 0.07	149.4 ± 7.79	74.1 ± 1.60	74.1 ± 1.02
48	19.4 ± 0.06	107.8 ± 2.31	75.8 ± 1.59	73.6 ± 0.62
72	19.6 ± 0.04	63.6 ± 3.74	75.0 ± 1.41	74.3 ± 0.71
96	19.3 ± 0.11	60.6 ± 5.29	74.0 ± 1.58	72.8 ± 0.71
120	19.1 ± 0.11	35.4 ± 2.84	73.7 ± 2.07	71.3 ± 0.40
SED(b)	0.14	6.82	2.28	1.09
Variance Ratio	9.10	102.67	0.24	2.14

[a] Each value is the mean ± standard error of the mean
[b] Standard error of difference between any two means

were measured and found not to change significantly during 120 hr of germination (Table 1). This result is in accord with the results reported for soybean seeds (Desikachar and De, 1950). Workers using other legumes reported a reduction in the trypsin inhibitor content on germination (Bates et al., 1977).

A small, but significant, decrease in the total fat content was observed. This is not unexpected as the fat forms a major energy source for the developing embryo. The depletion of oil in germinating seeds has been reported (Abrahamsen and Sudia, 1966; Holman, 1948). The bulk of the fatty acids are probably broken down by β-oxidation. Oxidative degradation of unsaturated fatty acids is also possible as lipoxygenase is present in winged bean seeds (King and Puwastien, 1984). During germination the lipoxygenase activity in the seeds decreased rapidly after 24 hr of incubation when the seeds were rehydrated; about 77% of the original activity was lost during the 120 hr incubation (Table 1). Even though the activity of lipoxygenase activity was reduced during germination. The gradual decrease in lipoxygenase activity of winged beans during germination together with the development of off-flavors in the flours prepared from the seeds has previously been reported (Throung et al., 1982).

No significant changes occurred to the concentrations of threonine, proline, methionine, tyrosine and phenylalanine during the 120 h germination period studied (Table 2). Glutamic acid, lysine, isoleucine, valine and alamine were only slightly affected by germination. The amino acids cystine, aspartic acid, arginine, serine and leucine were affected. With the exception of cysteine and lysine a decrease in the amino acid content was observed after 48 h of incubation when the radicle started to emerge. Cystine increased with a high level of significance ($P > 0.001$) and remained at that level throughout the period of study. As germination progressed the levels of aspartic acid and histidine increased significantly compared to those in ungerminated seeds.

The levels of the essential amino acids in ungerminated seeds and seeds germinated for 72 hr or longer compare well with the FAO (1973) provisional amino acid scoring pattern, valine and tyrosine being lower. A change in the amino acid profile is to be expected due to the turnover in protein during germination. The increase in histidine and cystine, common constituents of many enzymes, might be expected during germination when a considerable amount of enzyme synthesis occurs (Young and Varner, 1959). There are few reports where amino acid profiles have been examined at regular intervals during germination. Most workers have compared ungerminated seeds with those germinated for a given period; this period has varied considerably between reports making comparison of data difficult. A marked increase in amino acid content of pea seeds was observed after 5 days germination (Kakade and Evans, 1966). Hsu et al. (1980) reported little change in the essential amino acid content of yellow pea and faba bean after 4 days of germination.

The highly significant increase in cysteine and histidine, an

Table 2—Amino acid composition[a] of winged bean seeds germinated for different periods of time (g/16g of nitrogen)

Amino acid	Germination periods (hr) 0	24	48	72	96	120	SED	Variance ratio[b]
Lysine	7.2	7.8	6.8	6.9	6.9	6.9	0.23	5.68*
Histidine	2.7	2.9	2.6	2.7	2.9	3.0	0.05	16.24**
Arginine	6.1	6.4	5.4	5.8	5.6	5.7	0.15	10.54**
Aspartic acid	10.5	11.0	9.8	11.1	11.1	12.5	0.20	41.74***
Threonine	4.3	4.6	4.2	4.2	4.4	4.4	0.17	1.60
Serine	5.6	6.0	5.2	5.7	5.9	5.6	0.13	8.35*
Glutamic acid	13.3	14.3	12.1	13.2	13.2	12.8	0.40	5.97*
Proline	6.1	6.2	5.3	5.4	5.8	5.8	0.25	3.82
Glycine	4.1	4.2	3.9	3.9	3.9	3.9	0.12	5.45*
Alanine	4.2	4.2	3.7	3.9	4.1	3.9	0.13	4.58*
Cystine	1.4	1.3	1.7	1.7	1.7	1.7	0.04	44.18***
Valine	4.8	5.0	4.4	4.7	4.7	4.7	0.11	4.97*
Methionine	1.1	1.0	1.0	1.1	1.1	1.2	0.05	1.97
Isoleucine	4.2	4.3	3.9	4.1	4.0	4.0	0.09	5.59*
Leucine	8.2	8.2	7.2	7.5	7.7	7.7	0.18	7.79*
Tyrosine	4.4	4.4	4.3	4.2	4.1	4.0	0.13	3.62
Phenylalanine	4.6	4.7	4.4	4.6	4.5	4.7	0.16	1.38

[a] Average concentration of two replicate samples.
[b] Degree of significant difference: *: $0.05 \geq P \geq 0.01$, **: $0.01 \geq P \geq 0.001$, ***: $P < 0.001$

essential amino acid for infants, can be considered as a substantial improvement in the protein quality of winged bean seeds particularly for the preparation of weaning foods. Taking methionine and cystine together the total amount of S-containing amino acids after 72 hr of germination was 2.8 g/16g N. Previously, it was reported that the raffinose family oligosaccharides responsible for flatulence were significantly reduced after 72 hr of germination (Puwastien and King, 1984). This period coincides with an improvement in the amino acid content of the beans and so should be considered as the minimum period of germination for improvement in nutritional value.

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Ms received 3/14/86; revised 6/25/86; accepted 7/17/86.

P. Puwastien thanks the United Nations University for a fellowship which enabled her to undertake this study.

Isolation and Partial Characterization of Phytic Acid-Rich Particles from Great Northern Beans (*Phaseolus vulgaris* L.)

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ABSTRACT

Phytic acid-rich particles were isolated from a combined density fraction (CDF) of Great Northern beans. CDF contained more than 80% of the total phytic acid, and 70% of the total crude protein and substantial amounts of certain minerals. CDF had phytic acid in both water soluble and water insoluble forms. An isolate containing phytic acid-rich particles was prepared from CDF using a Tris-base solution. The phytic acid-rich isolate contained 26.6% phytic acid, 34.3% protein, 30.0% total carbohydrates, 0.6% calcium, 2.1% magnesium, and 0.3% potassium. Chemical analysis and fractionation of CDF indicated that the water insoluble form of phytic acid in Great Northern beans was present as a salt of calcium-magnesium-potassium in association with proteins.

INTRODUCTION

PREVIOUS WORK on Great Northern beans has involved the characterization of bean proteins, functionality of proteins, protein quality, preparation of protein isolates and concentrates, and methods for quick-cooking of beans (Rockland and Nishi, 1979; Deshpande and Cheryan, 1984; Sathe et al., 1984a,b; Deshpande et al., 1984; Chang and Satterlee, 1982). The nutritional quality of bean proteins is low unless the proteins are subjected to various types of processing and heat treatments. Use of bean flour in processed foods is limited because the flour contains several antinutrients and unwanted components (Sathe et al., 1984a; Sathe and Salunkhe, 1984). Phytic acid (myoinositol 1,2,3,4,5,6-hexakis dihydrogen phosphate) is one of the antinutrients in dry beans and serves as the main phosphorus reservoir (Reddy et al., 1982; Wise, 1983; Graf, 1983). It accounts for up to 80% of the total phosphorus in dry beans and occurs as a complex with mono- and divalent cations in discrete regions of the bean (Reddy et al., 1982). Recent studies (Griffiths, 1982; Deshpande et al., 1982; Reddy et al., 1978) have indicated that most of the phytic acid in dry beans is located in the cotyledons and not in the seed coat. Working with dry peas, Ferguson and Bollard (1976) found that 99% of the phytic acid is in the cotyledons and 1% in embryo axis. Concern about the presence of phytic acid in dry beans and their derived fractions and products arises from the evidence that phytic acid decreases the bioavailability of essential minerals (calcium, magnesium, zinc and iron) and proteins by forming complexes, i.e., phytic acid-protein, phytic acid-mineral-protein and other related complexes (Cheryan, 1980; Reddy and Salunkhe, 1981; Graf, 1983; Wise, 1983; Knuckles et al., 1985; Davies and Olpin, 1979; Oberleas and Harland, 1981). Under physiological conditions, these complexes are insoluble and may be unavailable for absorption in humans and animals. Further, the presence of phytic acid in dry beans introduces a series of complicating factors in the production of bean protein concentrates and isolates, the isolation and purification of homogenous proteins from beans, and the bioavailability of essential minerals from beans. The presence of phytic acid can also significantly influence the functional and nutritional properties of the bean proteins (Cheryan, 1980). It has been reported that phytic acid can inhibit enzymes such as pepsin, alpha-amylase and trypsin (Camus and Laporte, 1976; Cawley and Mitchell, 1968; Sharma et al., 1978; Singh and Krikorian, 1982; Deshpande and Cheryan, 1984).

Current literature suggests that phytic acid primarily occurs as potassium-magnesium salt in rice (Ogawa et al., 1975), wheat (Tanaka et al., 1973) and broad beans (Lott and Buttrose, 1978). The chemical form in which phytic acid occurs in dry beans such as Great Northern, pinto, navy, California small white, mung and garbanzo beans is not known. An understanding of the chemical form(s) in which phytic acid occurs in beans and its associated components will help food technologists and nutritionists in developing methods for production of low-phytate bean-based food products.

The objective of this study was to isolate and partially characterize phytic acid-rich particles from Great Northern beans. The chemical composition of major density fractions and isolated phytic acid-rich fraction from Great Northern beans was also determined.

MATERIALS & METHODS

Great Northern beans

About 7 kg of dry, mature whole Great Northern beans (GNB) were purchased from a local retailer. Whole dry beans were ground to a 60 mesh flour in a Mikro Pulverizer (Pulverizing Machinery Co., Summit, NJ). The GNB flour was stored in a glass container at 4°C.

Fractionation of GNB Flour

The GNB flour was fractionated by differential centrifugation using nonaqueous media (Tanaka et al., 1973; Sobolev et al., 1977) to obtain six density gradient fractions (Fig. 1). One hundred fifty grams of GNB flour were suspended in 400 mL acetone and carbon tetrachloride ($\rho = 1.27$) mixture at room temperature (21°C) and stirred for 10 min. The suspension was centrifuged at 2,600 × *g* for 15 min. The supernatant was mixed with equal volume of acetone and centrifuged at 5,800 × *g* for 15 min to obtain fraction I (Fig. 1). The residue at $\rho = 1.27$ was further fractionated by resuspension and centrifugation using mixtures of acetone and carbon tetrachloride at various densities ($\rho = 1.28$, $\rho = 1.36$, $\rho = 1.42$ and $\rho = 1.45$) as shown in Fig. 1. All six density gradient fractions (Fractions I, II, III, IV, V, and VI) were washed twice with acetone and air dried. Preliminary protein and phytic acid data on these six density fractions indicated that the density fractions IV and V contained most of the phytic acid and protein.

In a separate experiment, 150g of GNB flour was fractionated to obtain two density fractions IV and V and these two fractions were combined together, washed with acetone twice, air dried and referred to as combined density fraction (CDF) in the subsequent text. The CDF was used for isolating phytic acid-rich particles.

Chemical analysis

Moisture and total protein (Kjeldahl N × 6.25) of GNB flour, fractions IV, V and VI, and CDF were determined by AOAC (1981) methods. For mineral analysis, GNB flour, fractions IV, V and VI,

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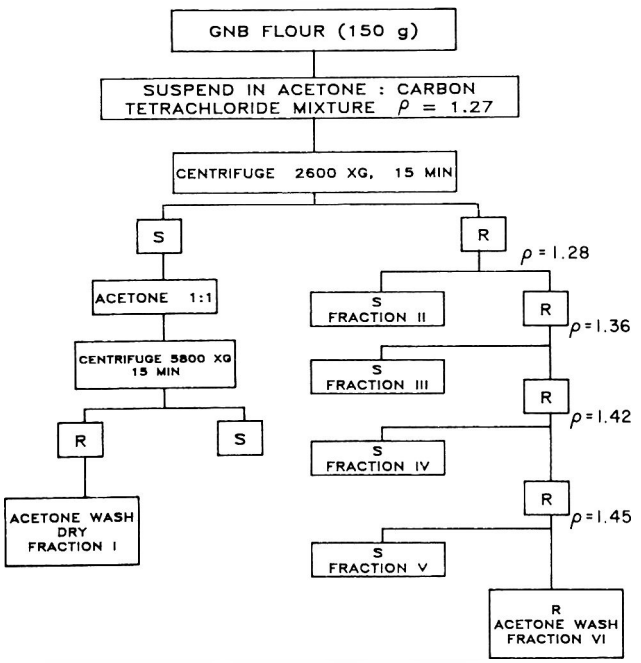

Fig. 1—Fractionation of Great Northern Bean (GNB) Flour. R = Residue, and S = Supernatant.

and CDF were dry ashed according to the AOAC (1981) method. The ashed samples were dissolved in dilute hydrochloric acid and made to known volume with distilled water. Calcium, magnesium, iron, zinc, copper, sodium and potassium were determined by atomic absorption spectrophotometry (AOAC, 1981).

To determine total carbohydrates, GNB flour, fractions IV, V and VI and CDF were hydrolyzed according to the method of Bittner et al., (1980). Total sugars in the hydrolyzates were estimated by the method of Dubois et al. (1956). Glucose was used as a standard.

Phytic acid of GNB flour, various density fractions and CDF was determined by a combination of three methods. Extraction and precipitation of phytic acid were performed according to method of Wheeler and Ferrel (1971). The conversion of ferric phytate to ferric hydroxide was carried out by Makower's (1970) procedure. Ferric iron (Fe^{+++}) was determined by the AOAC (1981) method using a o-phenanthroline reagent. Phytic acid was calculated on the assumption that it contained 28.20% phosphorus by weight.

To determine amino acids, samples (50–100 mg) of GNB flour, fractions IV and V and CDF were hydrolyzed with 20 mL of 6.0N HCL for 24 hr at 110°C in evacuated Teflon screw-capped test tubes. To the resultant hydrolyzate, 10 mL of citrate buffer (0.2M, pH 2.20) were added, adjusted to pH 2.20 with dilute NaOH (0.1N) or HCL (0.1N) and made up to known volume (50 ml). Analyses were conducted by injecting up to 100 µL samples into a Beckman model 118 Amino Acid Analyzer. Norleucine was used as an internal standard.

Isolation of phytic acid-rich particles

Four different methods were used to isolate phytic acid-rich particles from CDF. In Method 1, CDF was fractionated by two isolation procedures to obtain phytic acid-rich particles. These two isolation procedures were based on the method of Ogawa et al. (1975). In the first procedure, 20g CDF were suspended in 100 mL aqueous polymer two-phase solution (8% dextran 500 and 6% polyethylene glycol 6000 in 0.01M tricine-KOH buffer, pH 8.0 containing 0.5M sucrose and 0.01M sodium chloride) and fractionated by differential centrifugation. The fraction was washed with water and acetone and dried.

In another procedure, 20g CDF were homogenized in 50 mL of 0.05M Tris-HCL buffer, pH 7.80 in a homogenizer. The homogenate was centrifuged at 10,000 × *g* for 30 min. The pellet was further fractionated by differential centrifugation using an aqueous polymer two-phase solution. This method yielded a fraction, which was washed with water and acetone and dried.

In Method 2, CDF was fractionated by homogenization in 0.5M sucrose solution and differential centrifugation to obtain phytic acid-rich particles (Sharma and Dieckert, 1975). The differential centrifugation yielded two fractions, which were washed with water and acetone and dried.

CDF was fractionated into two fractions using glycerol and differential centrifugation in Method 3. Ten grams CDF were suspended in 100 mL glycerol and stirred for 15 min at room temperature. The suspension was centrifuged at 2,000 × *g* for 20 min. The residue was washed with ethanol and acetone and dried (Fraction I). The supernatant at 2,000 × *g* was further centrifuged at 40,000 × *g* for 20 min and the residue was collected, washed with ethanol and acetone and dried (Fraction II).

Method 4, as modified by Lui and Altschul (1967), was used for isolation of phytic acid-rich particles from CDF. The isolation details are presented in Fig. 2.

All the isolated fractions from the above four methods were analyzed for phytic acid and protein. The isolated fraction from Method 4 contained highest amount of phytic acid. The isolated phytic acid-rich fraction from Method 4 was analyzed for mineral and carbohydrate content. The CDF and phytic acid-rich fraction from Method 4 were also subjected to EDX-ray analysis for confirmation of certain minerals.

RESULTS & DISCUSSION

GREAT NORTHERN BEANS (GNB) used in this study contained 2.7% phytic acid (Table 1), which accounted for about 80% of total phosphorus. Literature values for phytic acid of GNB range from 0.6-2.1% (Lolas and Markakis, 1975; Iyer et al., 1980; Reddy et al., 1982; Elkowicz and Sosulski, 1982; Deshpande et al., 1982; Sathe et al., 1983; Deshpande and Cheryan, 1984). The wide variations observed in phytic acid of the GNB may be due in part to differences in cultivars used and methods of phytic acid determination and presentation. GNB flour was fractionated into six density fractions I, II, III,

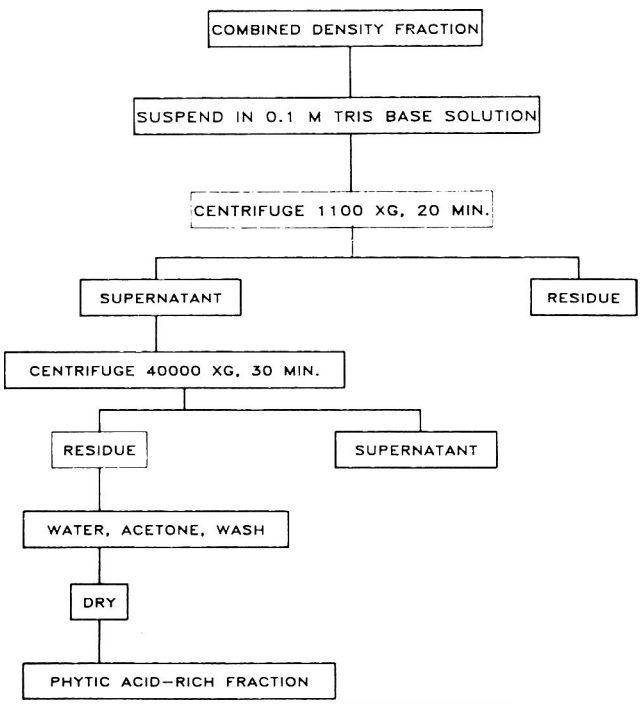

Fig. 2—Fractionation of phytic acid-rich particles from combined density fraction.

Table 1—Protein, carbohydrate, and phytic acid and yields of various density fractions from Great Northern bean flour[a]

	Protein (%)	Carbohydrate (%)	Phytic acid (mg/g)	Yield (g)
Great Northern bean flour	25.7	47.1	26.6	150.0
Fraction I	42.7	—	33.4	2.8
Fraction II	58.0	—	40.2	1.0
Fraction III	53.6	—	42.9	1.9
Fraction IV	53.3	25.7	65.5	31.0
Fraction V	56.6	43.5	42.9	27.0
Fraction VI	7.0	87.6	8.3	75.5
Combined density fraction	54.3	33.0	55.2	65.0

[a] All values are expressed on a dry weight basis

IV, V, and VI using differential centrifugation and various density mixtures of acetone and carbon tetrachloride (Fig. 1). The total yields of density fractions I, II and III were less than 10%. Fractions IV and V yields accounted for 39% of the total. Fractions I, II, III, IV, V and VI respectively had 3.3, 4.0, 4.3, 6.6, 4.3, and 0.8% phytic acid (Table 1). Additionally, fractions IV and V contained more than 80% of total phytic acid and 70% of total crude protein. Fraction VI contained mostly starch granules (confirmed by scanning electron microscopy) and small amounts of protein and phytic acid.

In a separate experiment, fractions IV and V were prepared from GNB flour and combined together for isolating phytic acid-rich particles. The combined density fraction (CDF) had 5.5% phytic acid, 54.3% protein and 33.0% total carbohydrates (Table 1). Mineral content of major density fractions IV, V and VI, CDF and GNB flour are presented in Table 2. CDF has about an average mineral content to that of fractions IV and V. Fraction VI contained 72, 34, 38, 30, 31, 45 and 25%, respectively, of the original calcium, magnesium, iron, zinc, copper, sodium and potassium of GNB flour. On the other hand, CDF had 14, 65, 67, 77, 73, 57 and 77%, respectively, of the original calcium, magnesium, iron, zinc, copper, sodium and potassium of GNB flour. The phytic acid:zinc molar ratio ranged from 38.1 to 99.9 between fractions IV, V and VI, CDF and GNB flour (Table 2). The CDF had a higher phytic acid:zinc molar ratio than GNB flour and fraction VI. A phytic acid:zinc molar ratio of above 20 is reported to be associated with clinical or chemical zinc deficiency (Oberleas, 1975; Davies and Olpin, 1979; Morris and Ellis, 1980; Oberleas and Harland, 1981), while a phytic acid:zinc molar ratio of 20 or less is considered to be adequate in providing dietary zinc. Further, some of these molar ratios can be used to predict bioavailability of zinc from various food products (Oberleas and Harland, 1981). Zinc may not be readily available from GNB flour, CDF and major density fractions since these have high phytic acid:zinc molar ratio.

The amino acid profile of GNB flour, fractions IV and V and CDF is presented in Table 3. The amino acid content of fraction VI was not determined because it had low amounts of protein, i.e., 7.0%. CDF had higher amounts of amino acids compared to GNB flour. In CDF, certain amino acids are twice the amount in GNB flour (Table 3).

Isolation of phytic acid-rich particles

In many seeds, phytic acid is concentrated in electron dense regions termed globoid crystals (Sobolev, 1966; Lui and Altschul, 1967; Ogawa et al., 1975; Prattley and Stanley, 1982; Lott, 1980, 1984) which are located within the proteinaceous matrix of protein bodies. However, some seeds may lack globoid crystals and still contain phytic acid. For example, Lott et al. (1984) reported that protein bodies in peas lack globoid crystals and contain phytic acid within the protein body. EDX-ray and chemical analysis of globoids revealed that phytate deposits commonly contain phosphorus and potassium along with calcium and/or magnesium (Buttrose, 1978; Lott, 1984; Prattley and Stanley, 1982). Based on the available information we attempted to isolate phytic acid-rich particles from GNB flour using CDF. The CDF was subjected to four differential centrifugation methods using four different aqueous media.

Method 1 removed starch granules, cell debris and other soluble compounds. Further, it yielded a fraction which did not contain phytic acid. Most of phytic acid from CDF was lost during solubilization and differential centrifugation with aqueous polymer two-phase solution. Ogawa et al. (1975) used aqueous polymer two-phase solution successfully to isolate phytic acid-rich particles from rice bran.

In Method 2, 0.5M sucrose solution was used to dissolve the proteinaceous matrix of protein bodies and other soluble compounds. Solubilization of CDF in 0.5M sucrose solution and subsequent differential centrifugation yielded two fractions. These two fractions contained traces of phytic acid. More than 75% phytic acid was detected in the discarded aqueous supernatant. The presence of phytic acid in aqueous supernatant confirmed the water soluble nature of phytic acid in CDF. Sharma and Dieckert (1975) isolated phytic acid-rich globoids from aleurone grains of peanut using a 0.5M sucrose solution.

In Method 3, CDF was suspended in glycerol to rupture the external membrane of protein bodies and thus facilitate the liberation of globoids and simultaneous dissolution of the amorphous zone. Fractionation of the suspended material by differential centrifugation yielded two fractions. These two fractions appeared to be similar in appearance (observed by scanning electron microscopy). Fraction I contained 43.7 mg/g phytic acid, while the other fraction had 54.6 mg/g phytic acid. Equal amounts of protein and minerals were present in these two fractions.

Solubilization of CDF in 0.1M Tris-base solution (pH 7.0) and subsequent fractionation (Fig. 2) yielded a phytic acid-rich fraction in Method 4. The scanning electron microscopic observations indicated that phytic acid-rich particles had small rod-like structures with rough surfaces. The isolated phytic acid-rich particles of GNB were different in appearance to those isolated from oil seeds (cotton seed, soybean, peanuts and castor seeds) and rice bran (Lui and Altschul, 1967; Ogawa et al., 1975; Sharma and Dieckert, 1975; Sobolev et al., 1977; Prattley and Stanley, 1982). The chemical composition of phytic acid-rich particles is presented in Table 4. The phytic acid-rich particles contained 26.6% phytic acid, 34.3% protein, 30% carbohydrates, 0.6% calcium, 2.1% magnesium and 0.3% potassium. The presence of calcium, magnesium, potassium and phosphorus in the phytic acid-rich particles was further confirmed by EDX-ray analysis (Fig. 3). The isolated phytic

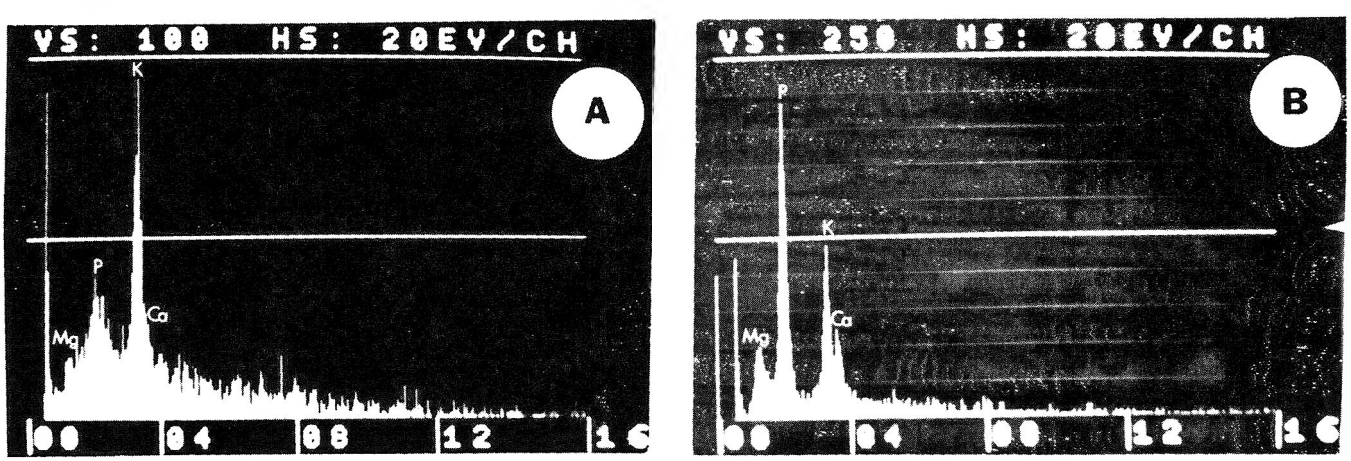

Fig. 3—Energy dispersive X-ray analysis spectra of (A) combined density fraction, and (B) isolated phytic acid-rich particles.

Table 2—Mineral content of Great Northern Bean flour and major density fractions[a]

Component		Great Northern bean flour	Fraction IV	Fraction V	Fraction VI	Combined density fraction
Calcium	(mg/g)	3.4	0.5	1.5	4.8	1.1
Magnesium	(mg/g)	1.8	2.6	2.6	1.2	2.8
Iron	(μg/g)	84.4	129.9	119.8	63.3	130.4
Zinc	(μg/g)	36.4	65.0	59.3	21.6	64.5
Copper	(μg/g)	9.6	18.6	14.2	5.8	16.2
Sodium	(μg/g)	31.6	61.0	38.0	27.9	41.2
Potassium	(mg/g)	1.6	3.2	2.6	0.8	2.9
Phytic acid/Zinc Molar ratio		72.4	99.9	71.7	38.1	84.8

[a] All values are expressed on a dry weight basis

Table 3—Amino acids of Great Northern bean flour and major density fractions[a]

Amino acid	Great Northern bean flour	Fraction IV	Fraction V	Combined density fraction
Aspartic acid	25.4	55.4	46.4	45.7
Threonine	7.8	20.0	18.6	16.8
Serine	11.9	29.7	28.7	25.1
Glutamic acid	35.5	70.0	61.4	67.0
Proline	9.7	17.0	10.9	11.4
Glycine	8.1	18.0	12.8	15.6
Alanine	5.4	19.1	23.3	17.0
Valine	17.3	28.7	39.9	32.2
Methionine	2.7	4.8	3.8	4.4
Isoleucine	9.6	21.6	16.5	18.4
Leucine	17.3	30.2	39.4	34.0
Tyrosine	6.4	15.5	11.5	13.1
Phenylalanine	19.1	21.9	32.4	28.0
Histidine	7.0	15.2	8.7	12.9
Lysine	15.3	23.8	30.3	28.7
Arginine	14.3	20.5	33.5	27.5
Total protein (%) (Kjeldahl N X 6.25)	25.7	53.3	56.0	54.3

[a] All the values are expressed in mg/g on a dry weight basis

Table 4—Chemical composition of phytic acid-rich particles[a]

Component		
Protein (Kjeldahl N × 6.25) (%)		34.3
Carbohydrate (%)		30.0
Phytic acid	(mg/g)	265.8
Minerals:		
Calcium	(mg/g)	6.4
Magnesium	(mg/g)	20.5
Potassium	(mg/g)	2.6
Iron	(μg/g)	527.8
Zinc	(μg/g)	133.2
Sodium	(μg/g)	157.2
Copper	(μg/g)	Trace
Phytic acid/zinc molar ratio		197.7

[a] All values are expressed on a dry weight basis

acid-rich globoids from soybeans also had large amounts of phytic acid, calcium, magnesium and potassium (Prattley and Stanley, 1982). Further, Prattley and Stanley (1982) concluded that phytic acid-rich globoids from soybeans contained insoluble form of phytate, i.e., as a salt of calcium-magnesium-potassium. Since the phytic acid-rich particles from GNB flour contained 20% original phytic acid, it was evident that the remaining 80% of phytic acid might be present in water soluble form in GNB. This was confirmed by extracting phytic acid from CDF by water and phytic acid determination. About 75% of phytic acid in CDF was water soluble. Prattley and Stanley (1982) reached similar conclusions based on their work with soybeans and its isolated phytic acid-rich globoids. In contrast, Lolas and Markakis (1975) indicated, that 99.6% of total phytic acid in beans (*Phaseolus vulgaris* L.) is in a water-soluble form. Lott et al. (1984) suggested that most of the phytic acid in pea cotyledons is water soluble and present as K-phytate.

Most of the methods used in this investigation have also been employed in the isolation of phytic acid-rich particles from oil seeds and rice bran (Lui and Altschul, 1967; Ogawa et al., 1975; Sharma and Dieckert, 1975; Sobolev et al., 1977; Prattley and Stanley, 1982). The phytic acid-rich particles isolated from oil seeds and rice bran contain the water-insoluble form of phytic acid. Based on the methods used in this study, Method 4 may be most effective in isolating the water-insoluble form of phytic acid from other beans and grains. However, Method 1 may be useful in isolating phytic acid-rich particles from bran of cereals.

It may be concluded from this investigation that phytic acid in Great Northern beans was present in water soluble form as well as water insoluble form as a salt of calcium-magnesium-potassium in association with porteins. Further studies related to removal of phytic acid and characterization of water soluble form of phytic acid from Great Northern beans are underway.

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Relationships Between Sensory and Objective Measures of Postharvest Quality of Snap Beans as Determined by Cluster Analysis

A. V. A. RESURRECCION, R. L. SHEWFELT, S. E. PRUSSIA, and W. C. HURST

ABSTRACT

Seventeen sensory, physical and chemical measurements of postharvest quality were obtained from 72 snap bean samples during four separate experiments. Cluster analysis was used to determine relationships between the 17 variables and to reduce the data into a smaller set of underlying structures without appreciable information loss. Four clusters, representing 79% of the total variance, were obtained and named 'acceptability measures', 'objective measures', 'ascorbic acid', and 'appearance measures' according to the variables within each group. Acceptability, mouthfeel and flavor were the most important subjective measures while hue angle and moisture were the most important objective measures of postharvest quality.

INTRODUCTION

SNAP BEANS are one of the more important vegetable crops in the United States (Lewis, 1958). Much of the research work on snap beans has been directed at beans for processing (Freeman and Sistrunk, 1978; Godwin et al., 1978; Gould, 1951; Woodroof et al., 1962; Powers et al., 1977; Robinson et al., 1964; Sistrunk and Gonzales, 1983). Although fresh beans were used as a reference sample for evaluating processed beans in some cases (Powers et al., 1977), desirable characteristics for the fresh market are not necessarily identical to those for processing.

The eating quality of vegetables may be measured directly by subjective sensory methods or indirectly through chemical and physical measurements. Substantial effort has been expended to develop sound objective tests for the quality evaluation of food in an effort to replace sensory evaluations (Martens, 1985).

One of the most important indices of snap bean quality is maturity (Robinson et al., 1964; Moss and Muirhead, 1983). Maturity has been described by sieve size or percentage of seeds. Other criteria were used such as "seed index" based on the product of seed weight by length (Silbernagel and Drake, 1978) and weight-to-length ratio (W/L) (Ramaswamy et al., 1980). Mouthfeel was reported by Godwin et al. (1978) as one of the most important determinants of quality next to flavor in processed green beans.

Watada and Morris (1966) found that during storage at temperatures below 4–5°C, nine cultivars of snap beans developed chilling injury. Significantly higher retail losses were noted by Ceponis and Butterfield (1984) when beans were merchandized in bulk (14.9%) as compared to prepackaged (3.8%). These authors concluded that desiccation was the leading cause of retail loss. They also found that consumer-grade samples held for 3 days at 5–6°C to simulate home storage conditions were higher for the prepackaged (10.5%) compared to samples procured from bulk displays (8.2%), mostly as a result of mechanical and physical injuries. Russeting or discoloration occurred in both water sprinkled and nonsprinkled beans held at −17.2°C (1°F) or −15.0°C (5°F) for 5 or 10 days then for 1 day at room temperature, but was more pronounced in sprinkled beans particularly after 10 days storage. The nonsprinkled beans however, became flabby or dry, lost weight and were unattractive after 1 day at room temperature (Lewis, 1958).

The applications of multivariate analytical techniques such as factor and cluster analysis have been used to help with treatment and interpretation of data from sensory evaluations and analysis of flavor components. Studies employing these multivariate statistical methods are gradually increasing (Martens, 1983). These have been used in the study of cabbage (Martens, 1985), wines (Wu et al., 1977), and tomatoes (Resurreccion and Shewfelt, 1985). Powers et al. (1977) and Godwin et al. (1978) related sensory and objective measures of canned and frozen green beans using factor and cluster analysis. Fox and Kramer (1966), on the other hand, found little or no correlation between instrumental measurements and sensory perceptions of quality of snap beans.

The objectives of this study were: (1) to use cluster analysis to determine relationships between the sensory and objective measures of postharvest quality of snap beans for the fresh market and (2) to identify the most important measures of postharvest quality in snap beans.

METHODS & MATERIALS

Experimental design

Bean samples were collected during four separate periods. In the first study (Test Set 1), samples from each of three retail outlets were obtained and analyzed. In the second study (Test Set 2), samples from two wholesale dealers at the Atlanta Terminal Market were obtained and analyzed (0 days storage) or stored at three different conditions, room (21°C, 70% RH) storage representing abuse conditions found in smaller retail outlets and produce stands, refrigerated (5°C, 80% RH), or chilled (1°C, 80% RH) temperatures. All samples were held for 4, 7, 11, and 14 days and then analyzed. The third study (Test Set 3) involving sampling of ten lots (1–2 pallets shipped from the same location) of beans at five wholesale locations (produce dealers or food chain warehouses) in the Atlanta area over a two-day period and placed under room (21°C, 70% RH) storage for either 6 or 12 days, while in the fourth study (Test Set 4), beans were obtained from three different sources, tracked through the postharvest system and sampled upon leaving simulated warehouse conditions (3 days at 5°C, 80% RH) and subsequent storage under either refrigerated (5°C, 80% RH) or room (21°C, 70% RH) temperatures for 6 days. Objective and sensory measures of postharvest quality were obtained on beans representing periods prior to and after storage. A total of 72 samples was evaluated.

Objective measures

Chemical and physical measurements were performed on the samples as described previously by Shewfelt et al. (1986).

Sensory evaluation

Duplicate samples for sensory evaluation were prepared by placing 400g of trimmed whole snap beans in containers with 50 mL boiling water. The beans were heated in a steam box for 20 min and then thoroughly mixed and heated for an additional 20 min. Samples of whole beans were assigned a random three digit code number and presented to the judges in random order on a white plate. Sensory

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evaluations were carried out in isolated booths in the sensory evaluation laboratory of the Dept. of Food Science at the Georgia Experiment Station. Two sessons were conducted each day between 9:00 to 10:00 a.m. and 2:00 to 3:00 p.m. Except for test set 1 where replicates were tested simultaneously, replicates were tested during another session on the same day.

Sensory panel

The judges were selected from a pool of students and staff members of the Dept. of Food Science at the Georgia Experiment Station, who had previous experience on sensory panels. Judges were selected on the basis of results of analysis of variance procedures using responses collected during test set 1 to determine consistency of responses in scoring of each descriptor. Two judges who had inconsistent scoring patterns were not used in succeeding tests. Furthermore, their responses were excluded from the analyses.

The final panel of 12 judges evaluated the samples of beans during each session for affective attitudes toward appearance, color, flavor, mouthfeel and acceptability using a nine-point hedonic scale ranging from 1 for 'like extremely' to 9 for 'dislike extremely' and four descriptor scales (Powers et al., 1977). The descriptor scales ranging from 0 to 8 were used in scoring attributes such as appearance—'not uniform' to 'uniform'; color—'brown or off-shade' to 'green'; flavor—'off-flavor' to 'pleasant vegetable flavor'; and texture—'fibrous' to 'tender'. To delineate the attribute scores from hedonic scores in the following sections, the attributes for appearance, color, flavor, and texture will hereafter be referred to as 'uniform', 'green', 'vegetable' and 'tender', respectively. This procedure is an adaptation of that used by Godwin et al. (1978). Each bean sample was evaluated 24 times (12 judges × 2 replicates).

Statistical analyses

A data matrix consisting of a total of 72 samples and 17 mean sensory scores and objective measures for each sample was analyzed using principal component cluster analysis (VARCLUS). This procedure was used to group the 17 variables into clusters representing the underlying structures in the data set (Martens, 1983) and to generate reliable scales to represent each of the resulting underlying structures or clusters in the data set. A scoring procedure was then used to effectively reduce the data set originally composed of 72 samples with 17 variables (72 × 17 matrix) into a smaller data set with fewer variables. Cluster scores were used in subsequent analyses of variance using the GLM procedure. All statistical procedures used were those given in SAS (1982).

RESULTS & DISCUSSION

MULTIVARIATE DATA ANALYSIS methods such as cluster analysis allow a reliable and efficient method fo simplification and interpretation of many different variables simultaneously. In this study, principal component cluster analysis was used to establish relationships among 17 different subjective and objective measures of postharvest quality of snap beans. The 17 variables in the study included sensory scores for the attributes 'uniform' (flavor), 'green' (color), 'vegetable' (flavor), and 'tender' (texture), and hedonic scores for appearance, color, flavor, mouthfeel and acceptability; and objective measures of color ($\tan^{-1}$ b/a, ΔE and L), moisture, shear values, percent seed, blender fiber and ascorbic acid.

Cluster analysis grouped all 17 variables into a smaller number of non-overlapping clusters or neighborhoods representing underlying tendencies-of-variation (Martens, 1983). In this study, four clusters that explain 79% of the total variance were generated by the principal components cluster analysis procedure. The cluster structure is presented in Table 1. Also listed are the squared inter-cluster correlations of variables within each cluster, the loadings (correlation coefficients) of each variable for each of the 4 clusters, and the proportion of variance explained by each cluster. Only one variable, ascorbic acid, is in cluster III.

The sensory variables acceptability, flavor, 'vegetable' (flavor), 'tender' (texture), and mouthfeel were grouped with moisture and hue angle ($\tan^{-1}$ b/a) in cluster I. By definition, this cluster is the first principal component and explains 54% and the greatest amount of the variance in the data. It is named the 'Acceptability measures' cluster defined by the heaviest loading (0.97, 0.96 and 0.93) sensory variables, therefore the most important variables in the cluster namely acceptability, mouthfeel and flavor, respectively. Hue angle ($\tan^{-1}$ b/a) and moisture are likewise grouped in cluster I and, based on their loadings of 0.78 and 0.75, are the most important objective measures in the cluster. Cluster II is composed of the variables ΔE, L, percent seed, blender fiber and shear value. Cluster II explains 14% of the variance and is named the 'Objective measures' cluster because it is defined by objective measures only, and none of the sensory variables are highly correlated with the cluster. Cluster III is named 'Ascorbic acid' after the sole variable in the cluster. Cluster IV is named the 'Appearance measures' cluster and is composed of the sensory variables appearance, 'uniform' (appearance), color and 'green' (color). Cluster III and IV explain 6% and 5% of the variance, respectively.

To illustrate the extent of data reduction by cluster analyses, and to provide a basis for comparison, Tables 2 to 4 list mean sensory scores and objective measures for the 17 variables as grouped in clusters I through IV. Mean sensory scores of the seven variables in cluster I are listed in Table 2. Significant differences were found ($P < 0.0001$ to $P < 0.05$) in test sets 1 through 4, in the variables acceptability, flavor, mouthfeel and hue angle. The variables, moisture, 'vegetable' (flavor) and 'tender' (texture), were not different for the 4 test sets. Table 2 also lists sensory scores and objective measures for snap beans stored for the five storage periods, 0, 4, 6-7, 11-12, and 14 days. Acceptability, hue angle and 'vegetable' flavor measures were significantly different ($P < 0.0001$ to $P < 0.005$) for the different storage periods. Mean scores and measures obtained by snap beans stored under the three conditions, refrigerated storage (5°C, 80% RH), storage at chilling temperatures (1°C, 80% RH) and at room (21°C, 70% RH) temperature are likewise listed in Table 2. Beans held at room temperature had lower scores for acceptability ($P < 0.001$), flavor ($P < 0.001$), mouthfeel ($P < 0.001$), 'vegetable' flavor ($P < 0.001$) and 'tender' texture ($P < 0.01$). Among the objective measures included in this cluster, moisture was significantly lower for the snap beans held at room temperature and hue angle was lower but not significantly different. Interpretation of the data in this table is difficult because of the large number of variables, and there appears to be no consistent pattern in the different measures.

The data in Table 2 become more meaningful only after comparison with the loadings for cluster I listed in Table 1. Acceptability, mouthfeel and flavor are the most important sensory attributes, while the most important objective measures are hue angle and moisture, as determined by their high loadings. Similar results, obtained by Godwin et al. (1977), found flavor and mouthfeel to be the most important determinants of acceptability of processed green beans.

Table 3 lists the mean values for the objective measures, ΔE, L, percent seed, shear value and blender fiber, that were grouped under cluster II. Although significant differences existed in test sets, there seemed to be no readily apparent pattern. On the other hand, the cluster pattern in Table 1 indicated that the most important variables for cluster 2 were L and ΔE, while the variables blender fiber, shear value and percent seed were of lower and decreasing importance as determined from the loadings in Table 1. This group of objective measures explained an additional 14% of the variance and together with the variables in cluster 1 explained a total of 68% of the variance in the data. The negative signs before the ΔE and percent seed loading indicated a 'reflected' or inverse relationship.

Means scores for the variables, ascorbic acid values together with appearance, color, 'green' color, and 'uniform' appearance are listed in Table 4. Ascorbic acid values were not significantly different. Only appearance and color scores for test set 1 were different ($P < 0.0001$) from all other test sets, while

Table 1—Cluster structure of 17 sensory and objective measures of postharvest quality of snap beans

Cluster	Variable	Type of measurement[a]	Cluster loadings[b] I	II	III	IV	$(R)^2$
Cluster I — Acceptability measures:							
	Acceptability	S	97	−54	17	82	0.94
	Flavor	S	93	−53	18	66	0.87
	Moisture	O	75	−48	−14	54	0.57
	Mouthfeel	S	96	−52	14	77	0.91
	Hue angle ($\tan^{-1}$ b/a)	O	78	−59	24	64	0.61
	'Vegetable' flavor	S	91	−51	18	63	0.83
	'Tender' texture	S	87	−43	10	71	0.77
Cluster II — Objective measures:							
	ΔE	O	45	−94	−1	8	0.88
	L	O	−52	96	−4	−14	0.89
	Percent seed	O	−28	61	10	−8	0.37
	Shear value	O	−78	77	2	−53	0.60
	Blender fiber	O	−40	88	8	−14	0.77
Cluster III — Ascorbic acid							
		O	15	3	100	13	1.00
Cluster IV — Appearance measures:							
	Appearance	S	69	−24	15	94	0.88
	Color	S	79	−35	20	95	0.90
	'Green' color	S	79	−24	12	96	0.91
	'Uniform' appearance	S	58	2	2	86	0.77
Proportion of variance explained			0.54	0.14	0.06	0.05	
Cumulative proportion of variance explained			0.54	0.68	0.74	0.79	

[a] O = objective measure; S = sensory score
[b] Loadings are multiplied by 100 and rounded.

Table 2—Mean values[a] for seven variables included in cluster I grouped according to test set, storage period and storage condition

Variable	1 Acceptability	2 Flavor	3 Moisture	4 Mouthfeel	5 Hue angle ($\tan^{-1}$ b/a)	6 'Vegetable' Flavor	7 'Tender' Texture
Test set							
1	4.54[bc]	4.70[bc]	90.60	4.38[b]	124.6[a]	5.51	4.36
2	4.90[ab]	5.04[ab]	89.59	5.12[a]	123.3[a]	4.76	4.83
3	4.08[c]	4.25[c]	88.27	4.43[b]	120.7[b]	4.12	4.40
4	5.11[a]	5.30[a]	90.16	5.29[a]	122.3[a]	5.51	5.08
F	6.27	4.69		4.85	3.26		
P ≤	0.005	0.01	NS	0.005	0.05	NS	NS
Storage time (days)							
0	5.28[a]	5.27	90.15	5.44	123.9[a]	5.30[a]	5.17
4	5.24[a]	5.19	89.07	5.25	123.9[a]	5.05[a]	5.12
6–7	4.26[bc]	4.51	89.45	4.57	121.4[b]	4.37[b]	4.57
11–12	3.82[c]	4.01	87.61	4.12	119.5[c]	3.65[c]	3.92
14	4.35[b]	5.15	89.16	5.05	121.9[b]	4.88[a]	4.82
F	8.06				5.19	5.89	
P ≤	0.0001	NS	NS	NS	0.005	0.001	NS
Storage condition							
Refrigerated	5.19[a]	5.27[a]	90.79[a]	5.41[a]	123.8	5.21[a]	5.21[a]
Chilled	5.05[a]	5.28[a]	89.87[a]	5.24[a]	123.0	4.93[a]	4.88[ab]
Room	4.11[b]	4.29[b]	88.23[b]	4.40[b]	121.0	4.16[b]	4.33[b]
F	11.07	10.17	5.98	9.85		7.98	5.69
P ≤	0.0001	0.001	0.005	0.001	NS	0.001	0.01

[a-c] All values with a different letter superscript are significantly different as determined by Duncan's Multiple Range Test.

color scores and 'green' color were lower ($P < 0.05$) for the beans held at room temperature. Significant differences were observed in all variables included in cluster IV for the five storage periods.

The cluster analysis procedure also resulted in weights (scoring coefficients) for each variable for each of the 4 clusters. By following the analysis with a scoring procedure, four cluster scores for each sample were calculated from each of the original 17 variables. As a result, considerable reduction of the data set from a 72 × 17 data matrix resulted in a 72 × 4 matrix.

Table 5 lists as a basis for comparison with Tables 2 through 4 the mean scores for clusters I through IV for snap beans grouped according to test set, storage condition, and storage time. The means for cluster I summarize the information contained in Table 2 without appreciable loss of information, likewise the scores for clusters II, III and IV summarize all the information contained in Tables 3 and 4, respectively. Quality of snap beans, as defined by acceptability measures or cluster I scores, was not different for any of the test sets. Scores decreased ($P < 0.01$) with the length of storage from 0 and 4 days of storage to 11–12 days. When data were grouped according to storage condition, cluster I scores for snap beans held at refrigeration and chilling temperatures were significantly higher ($P < 0.001$) than those held at room temperature. Cluster II scores were significantly different only for test set 3, while, no significant differences were found in cluster III scores. Cluster IV scores were significantly different for different test sets, were significantly lower in beans after 11–12 and 14 days of storage, and when stored at room temperature.

Interpretation of results of each of the 17 quality variables in Tables 2 through 4 was tedious and difficult and did not take into consideration the relative importance of each variable in defining quality of snap beans. Analysis and interpretation

Table 3—Mean values[a] for five variables included in cluster II grouped according to test set, storage period and storage condition

Variable	ΔE	L	Percent seed	Shear value	Blender fiber
Test set					
1	28.03[b]	39.69[b]	8.31[b]	63.4[b]	1.69[c]
2	30.04[a]	37.51[c]	7.84[b]	68.5[b]	0.91[d]
3	25.08[c]	43.56[a]	16.82[a]	90.4[a]	2.44[a]
4	28.36[b]	39.38[bc]	6.70[b]	84.5[a]	2.05[b]
F	15.57	16.08	2.91	94.5	111.61
P ≤	0.0001	0.0001	0.05	0.0001	0.0001
Storage time (days)					
0	27.74	40.11	11.81	74.2[c]	1.86
4	29.50	38.03	7.84	63.5[d]	0.91
6–7	27.16	41.01	11.61	82.9[b]	1.91
11–12	26.64	41.98	13.45	91.1[a]	1.86
14	30.92	36.68	7.84	70.9[cd]	0.91
F				27.6	
P ≤	NS	NS	NS	0.05	NS
Storage condition					
Refrigerated	28.96	38.66	7.60	68.3[b]	1.41
Chilled	30.85	36.64	7.16	66.1[b]	0.89
Room	26.29	42.07	14.16	88.3[a]	2.05
F				125.3	
P ≤	NS	NS	NS	0.0001	NS

[a-d] All values with a different letter superscript are significantly different as determined by Duncan's Multiple Range Test.

Table 4—Mean values[a] for five variables included in clusters III and IV grouped according to test set, storage period and storage condition

	Cluster III	Cluster IV			
Variable	Ascorbic acid	Appearance	Color	'Green' color	'Uniform' appearance
Test set					
1	5.79	2.93[b]	3.12[b]	4.70	5.24
2	7.68	5.17[a]	5.35[a]	4.56	4.53
3	7.55	4.72[a]	4.69[a]	4.34	4.89
4	8.14	5.13[a]	5.53[a]	5.19	5.14
F		9.53	11.84		
P ≤	NS	0.0001	0.0001	NS	NS
Storage time (days)					
0	8.13	5.60[a]	5.70[ab]	5.22[ab]	5.39[a]
4	8.13	5.59[a]	5.86[a]	5.43[a]	5.13[a]
6–7	7.94	4.76[ab]	4.96[b]	4.59[b]	4.77[ab]
11–12	6.12	4.08[bc]	4.01[c]	3.58[c]	4.27[b]
14	7.87	3.82[c]	4.08[c]	3.64[c]	3.64[c]
F		8.89	9.15	4.92	4.85
P ≤	NS	0.0001	0.0001	0.01	0.01
Storage conditions					
Refrigerated	7.99	5.07	5.35[a]	5.01[a]	5.01
Chilled	6.69	5.26	5.45[a]	4.56[ab]	4.48
Room	7.63	4.67	4.68[b]	4.33[b]	4.78
F			4.47	3.20	
P ≤	NS	NS	0.05	0.05	NS

[a-c] All values with a different letter superscript are significantly different as determined by Duncan's Multiple Range Test.

of the data set resulting from cluster analysis followed by scoring procedures were more accurate and considerably more efficient.

SUMMARY & CONCLUSIONS

IN DETERMINING postharvest quality of snap beans using several subjective and objective measures of quality, interpretation of the data becomes increasingly difficult as the number of variables increase. Furthermore, all variables do not have identical weights and should not be treated as equally important. It is possible through the use of cluster analysis to reduce the large data matrix that results from such a study into a smaller data set without appreciable loss of information while

Table 5—Mean scores[a] for clusters 1, 2, 3, and 4 for test set, storage period and storage condition

Variable	Cluster I 'Acceptability measures'	Cluster II 'Objective measures'	Cluster III 'Ascorbic acid'	Cluster IV 'Appearance measures'
Test set				
1	34.15	6.43[b]	5.79	4.27[b]
2	34.02	5.19[b]	7.68	5.27[a]
3	32.86	10.55[a]	7.55	5.00[ab]
4	34.25	6.52[b]	8.14	5.64[a]
F		13.44		3.86
P ≤	NS	0.0001	NS	0.05
Storage time (days)				
0	34.50[a]	7.56	8.13	5.88[a]
4	34.25[ab]	5.33	8.13	5.92[a]
6–7	33.32[c]	8.10	7.94	5.12[a]
11–12	32.35[d]	8.98	6.12	4.28[b]
14	33.73[bc]	4.84	7.87	4.08[b]
F	3.86			7.08
P ≤	0.01	NS	NS	0.0001
Storage conditions				
Refrigeration	34.54[a]	5.49[a]	7.99	5.49[a]
Chilled	34.15[a]	4.53[a]	6.69	5.31[a]
Room	32.90[b]	9.25[b]	7.63	4.95[b]
F	9.65			3.57
P ≤	0.001	NS	NS	0.05

[a-d] All values with a different letter superscript are significantly different as determined by Duncan's Multiple Range Test.

increasing the efficiency of interpretation of results. In this study involving 17 subjective and objective measures of snap beans sampled during four test periods, held for up to five storage periods under one to three storage conditions, reduction of the various quality measures to four underlying structures or clusters was demonstrated. This procedure resulted in increased efficiency in the interpretation of postharvest quality changes in a total of 72 samples obtained over four test periods.

In conclusion, our results indicated that the most important measure of postharvest quality of snap beans for the fresh market was acceptability, mouthfeel and flavor while hue angle and moisture were the most important objective measures. Other objective measures including ΔE, L, percent seed, shear values and fiber explained only an additional 14% of the variance. Ascorbic acid, appearance scores and sensory judgments of color were not as important in determining postharvest quality of snap beans.

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Effect of Adenosine-Nucleotides and Their Derivatives on the Denaturation of Myofibrillar Proteins *in vitro* during Frozen Storage at −20°C

SHANN-TZONG JIANG, BAO-SHYUNG HWANG, AND CHING-YU TSAO

ABSTRACT

To investigate the effect of adenosine-nucleotides and their derivatives on the denaturation of myofibrillar proteins, 235 nmoles/mL adenosine-5′-diphosphate (ADP), adenosine-5′-monophosphate (AMP), inosine-5′-monophosphate (IMP), inosine (HxR), or hypoxantine (Hx), was added to 3 mg/mL actomyosin (AM) solution suspended in 0.10M KCl solution and stored at −20°C for 12 wk. The AM was extracted from milkfish dorsal muscle. Protein denaturation was evaluated by measuring solubility, Ca-ATPase and Mg(EGTA)-ATPase activity of AM, by analyzing changes in electrophoretic profiles and transmission electron microscopy. Inosine and hypoxanthine accelerated protein denaturation compared to control samples. Infrared spectrum analyses indicated that negatively charged groups of these nucleotides interacted with amino or imino groups on AM after addition. ADP, AMP, and IMP had a protective effect on denaturation of AM during frozen storage at −20°C.

INTRODUCTION

FREEZING, considered as one of the best methods for fish preservation, has been employed to an increasing extent both on shore and on board fishing vessels. Nevertheless, marked undesirable quality changes in the products frequently occur after prolonged storage, even at a temperature as low as −20°C. These deteriorative changes are considered to be due to undesirable reactions occurring in lipids and proteins (Sikorski, 1978, 1980; Matsumoto, 1979, 1980; Suzuki, 1981; Noguchi, 1982). Deteriorative changes in texture as a consequence of long-term storage are recognized as resulting predominantly from severe alterations of muscle proteins, usually termed denaturation-aggregation (Dyer, 1951; Sikorski et al., 1976; Sikorski, 1978, 1980; Matsumoto, 1979, 1980; Shenouda, 1980; Andou et al., 1980; Noguchi, 1982). Most of the alterations occur in the myosin-actomyosin system (Sikorski et al., 1976). Early observations showed that myosin, upon standing in solution or frozen solutions, tended to aggregate almost exclusively side-to-side, to form dimers, trimers, and high molecular weight polymers (Lowey and Holtzer, 1959; Connell, 1959, 1960, 1962, 1963; Buttkus, 1970).

Some studies indicated that the myofibrillar proteins of fish muscle aggregated into a high molecular weight polymers during frozen storage (King, 1966; Anderson and Ravesi, 1970; Childs, 1973; Jarenback and Liljemack, 1975; Jiang, 1977, 1984; Jiang and Lee, 1985). The interactions between myofibrillar proteins and other components have been investigated to elucidate the mechanism of protein denaturation during storage or frozen storage (Andou et al., 1979, 1980, 1981a, b; Braddock and Dugan, 1973; Braun and Radin, 1969; Childs, 1973, 1974; Fullington, 1969; Hironaka et al., 1976; Jarenback and Liljemark, 1975; Karel et al., 1975; King et al., 1962; Kostuch and Sikorski, 1977; Noguchi, 1974; Noguchi et al., 1976; Ooshiro et al., 1976; Robenson, 1966; Takama, 1974a, b; Jiang, 1984). However, little is known about the roles of some muscle constituents such as nucleotides, on various protein denaturation (Shenouda, 1980; Jiang and Lee, 1985). Previously Jiang et al. (1986a) found that the content and composition of adenosine-nucleotides in the fish muscle were related to the denaturation of frozen fish muscle. High levels of inosine and hypoxanthine were related to the instability, while the high levels of ATP, ADP, AMP, and IMP were related to the stability of myofibrillar proteins during frozen storage. The purpose of this study was to investigate the effect of adenosine-nucleotides on protein denaturation and the interactions between these nucleotides and myofibrillar proteins in vitro during frozen storage at −20°C.

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MATERIALS & METHODS

Preparation of the actomyosin

Actomyosin was extracted according to Noguchi (1974). Ten grams milkfish dorsal muscle (*Chanos chanos*) was blended in a Waring Blendor, subjoined with a baffle plate, for 2 min with 90 mL chilled 0.6M KCl solution (pH 7.0). The extract was centrifuged at 5000 X*g*, 0°C, for 20 min. Actomyosin was precipitated by diluting the extract with three volumes chilled distilled water and collected by centrifuging at 5000 × *g*, 0°C, for 20 min. The actomyosin was then suspended in chilled 0.10M KCl solution (pH 7.0) and homogenized with a magnetic stirrer for 1 hr at 0°C. The homogenized actomyosin was diluted to 300 mL with chilled 0.10M KCl solution for the following test, resulting in a concentration of 3 mg/mL.

To investigate the effects of adenosine-nucleotides on protein denaturation, each nucleotide, except for adenosine-5′-triphosphate (ATP), which will be degraded into ADP by actomyosin, was added to an actomyosin solution. Each actomyosin solution had a final concentration of 235 nanomoles nucleotides per ml, a concentration almost equivalent to that in fish muscle. Aliquots (20 mL) of the mixtures were put in plastic test tubes, stoppered, and stored at −20°C for 12 wk. At definite time intervals samples were removed. One tube of each sample, without thawing, was directly freeze-dried using a freeze-dryer (Refrigeration For Science, Inc., New York) at a plate temperature of 0°C. The rest of each sample was thawed at room temperature (25°C), and subjected to the following measurements.

Determination of the composition of adenosine-nucleotides

The nucleotides were extracted according to Ehira et al. (1974) and the composition analyzed using ion exchange chromatography (resin: AG 1, X4, Cl type; mesh: 200–400) according to Kato et al. (1973). The elute was passed through a UV-Detector (Single Path Monitor UV-1, Pharmacia Fine Chemicals) and collected using a Fractional Collector (FRAC-100, Pharmacia Fine Chemicals). The concentration of nucleotides was expressed automatically on the base of peak area.

Infrared spectrum analyses

Two mg of freeze-dried protein powder was ground with pestle and mortar, 200 mg potassium bromide was then added. This mixture was ground to a very fine particle size of 200–300 mesh. The mixture was placed on a tablet former with a vacuum pump providing a pressure of 0.1 Torr, and pressed into a transparent disc (13 × 1 mm) under 600 Kg/cm pressure for 30 sec.

The infrared spectrum of the samples was recorded from 400 to 4000 cm^{-1} using an Infrared Spectrophotometer (Hitachi Model 260-30, Hitachi Ltd., Tokyo, Japan). The changes in Amide I and II were

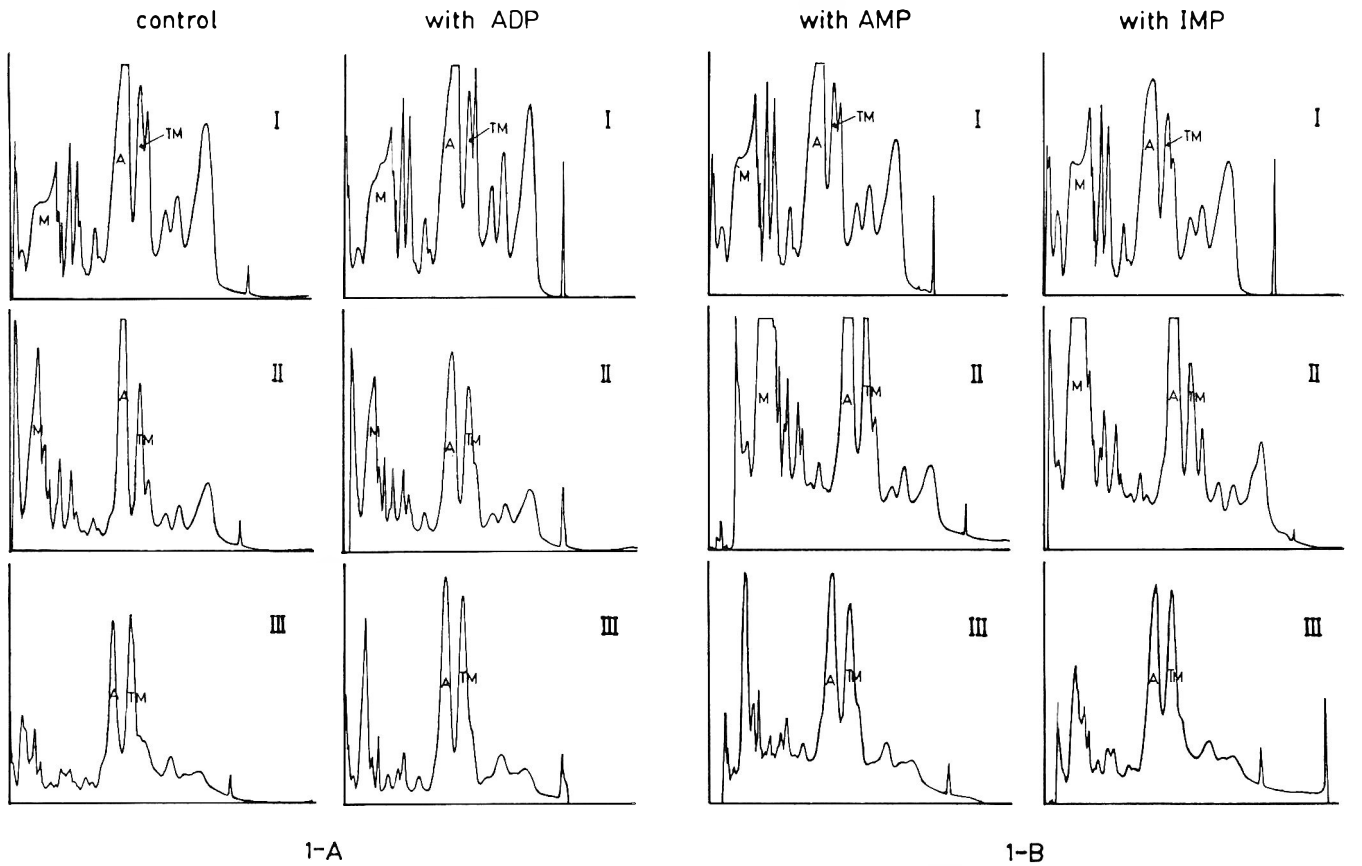


Fig. 1—Effect of adenosine-nucleotides and their derivatives on the electrophoretic profile of actomyosin extracted from milkfish dorsal muscle during frozen storage at −20°C (1-A: control sample and samples with added ADP; 1-B: samples with added AMP and IMP; 1-C: samples with added HxR and Hx; I: samples before freezing; II: samples before precipitating denatured proteins after 12 wk storage; III: soluble proteins after 12 wk storage; M: myosin heavy chain which was mentioned in text as MHC; A: actin; TM: tropomyosin).

used for evaluating the conformational changes of protein molecule during frozen storage.

Solubility

Potassium chloride solution (1.2 M) was added to the freeze-thawed suspension before homogenization to bring the salt concentration to 0.6M KCl. After homogenizing with a magnetic stirrer for 30 min at 0–5°C, the resultant suspension was centrifuged at 5000 × *g*, 0°C for 20 min. The supernatant was used for solubility, Ca-ATPase and Mg(EGTA)-ATPase (EGTA: ethyleneglycol-bis(2-aminoethylether)-tetraacetic acid) activity analyses.

The protein concentration of these supernatants was determined using the Biuret Method modified by Umemoto (1966). The solubility was expressed as the ratio of the quantity of soluble protein to that of original quantity of actomyosin.

Ca-ATPase activity

To 1 mL actomyosin solution (0.3–3 mg/mL), 0.5 mL 0.5M Tris-maleate buffer (pH 7.0), 0.5 mL 0.1M $CaCl_2$, 7.5 mL distilled water and finally 0.5 mL 20 mM Na_2-ATP solution (pH 7.0) were added in turn. The Ca-ATPase activity was measured according to Arai (1974). The rate of release of inorganic phosphate at 25°C during 3 min reaction with ATP was determined after the addition of 5 mL 15% trichloroacetic acid to stop the reaction. The Ca-ATPase specific activity was shown as micromoles inorganic phosphate liberated per mg protein within one min reaction at 25°C.

Mg(EGTA)-ATPase activity

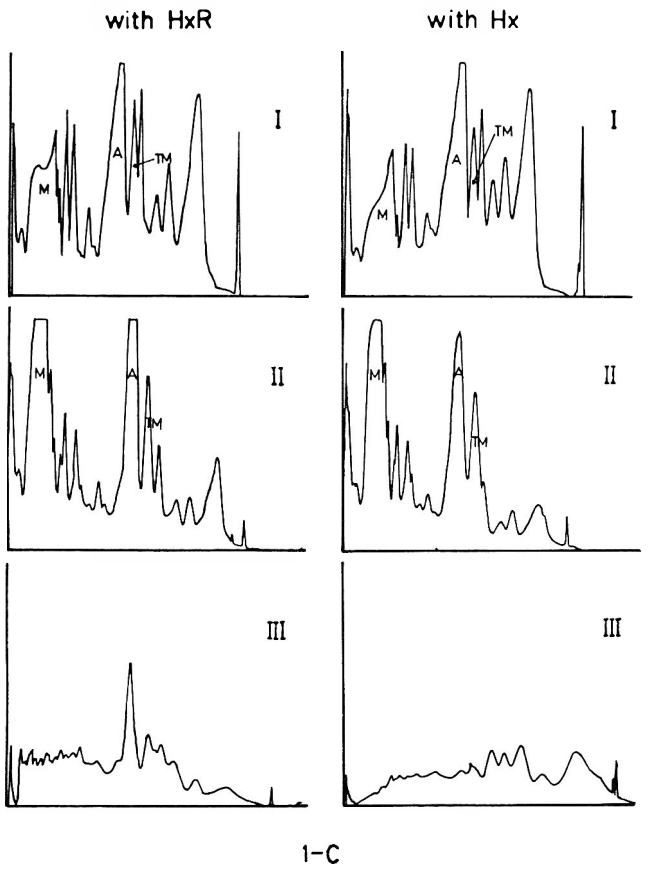
To 1 mL actomyosin solution (0.3–3 mg/mL), 1.0 mL 0.02M $MgCl_2$, 1.0 mL 0.005M EGTA, 1.0 mL 0.2M Tris-maleate buffer (pH 7.0), 5.0 mL distilled water, and 1.0 mL 20 mM Na_2-ATP were added in this order. After the addition of 5 mL 15% trichloroacetic acid to stop the reaction, the rate of release of inorganic phosphate at 25°C during 3 min reaction with ATP was determined according to Arai (1974). The Mg(EGTA)-ATPase specific activity was shown as micromoles of inorganic phosphate release per milligram of actomyosin within 1 min for the reaction at 25°C.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analyses

To investigate the changes in electrophoretic separation of actomyosin during frozen storage, the SDS-polyacrylamide gel electrophoresis was performed according to Weber and Osborn (1969). The ionic strength of freeze-thawed samples was adjusted to 0.6 using a 1.2M KCl solutions. Those protein solutions contained both native and denatured proteins. To remove the denatured proteins, those samples were centrifuged at 5000 × *g* for 20 min at 0°C. Both protein solution before and after centrifugation were dialyzed overnight at 5°C against 0.01M sodium phosphate buffer containing 0.1% SDS (pH 7.2). Those dialyzed samples were then incubated at 40°C for 2 hr in a solubilizing solution consisting of 0.01M soldium phosphate buffer, 1% SDS, 25% glycerin and 2% mercaptoethanol (pH 7.2). About 0.01 mL 0.05% bromo-phenol-blue and 0.06 mL of the solubilized protein sample were dropped on top of the gel with a micropipet.

The polyacrylamide gel was prepared basically as described by Weber and Osborn (1969). The concentration of polyacrylamide was 7.5%. After the electrophoretic run in 0.1% SDS-0.1M sodium phosphate buffer, the gels were stained overnight with 0.12% Coomassie blue and 50% methanol in 9.2% acetic acid solution. They were destained with 50% methanol in 7.5% acetic acid solution for 8–10h, as recommended by Seki (1974).

Distance scanning at 585 nm, which was the maxium absorbance of the staining solution, was employed to analyze the bands of gel, using a UV-VIS Microprocessor-Controlled Spectrophotometer System 2600 (Gilford Instrument Lab. Inc.). The patterns of electrophoretic separations of protein solutions before and after precipitating the denatured proteins were used for evaluating the microstructural changes of protein molecules during frozen storage.



1-C

Table 1—Changes in adenosine-nucleotides and their derivatives after being added into actomyosin solution and during frozen storage at −20°C

Additives[a]	Storage time (wk)	Levels of nucleotides (nanomoles/ml)[b] ATP	ADP	AMP	IMP	HxR	Hx
Control	0	–	–	–	–	–	–
	6	–	–	–	–	–	–
	12	–	–	–	–	–	–
ADP	0	–	–	–	232	–	–
	6	–	–	–	227	–	–
	12	–	–	–	230	–	–
AMP	0	–	–	–	230	–	–
	6	–	–	–	225	–	–
	12	–	–	–	227	–	–
IMP	0	–	–	–	229	–	–
	6	–	–	–	229	–	–
	12	–	–	–	231	–	–
HxR	0	–	–	–	–	227	–
	6	–	–	–	–	230	–
	12	–	–	–	–	229	–
Hx	0	–	–	–	–	–	230
	6	–	–	–	–	–	228
	12	–	–	–	–	–	233

[a] The quantity added was 235 nanomoles/mL actomyosin solution. ATP: adenosine-5'-triphosphate; ADP: adenosine-5'-diphosphate; AMP: adenosine-5'-monophosphate; IMP: inosine-5'-monophosphate; HxR: inosine; Hx: hypoxanthine.

[b] The protein concentration was 3 mg/mL, thus the level of nucleotides was nanomoles/3 mg protein.

Electron microscopy

All protein solutions, after thawing in running water (20°C) to a temperature of 0°C and adjusting the ionic strength to 0.6, were negatively stained with 2% phosphotungstic acid (PTA) solution (Huxley, 1963; Higashi and Tooyama, 1976) and observed in a Hitachi H-600 type Electron Microscope.

Table 2—Effect of adenosine-nucleotides and their derivatives on the solubility[a] of actomyosin in vitro *during frozen storage at −20°C*

Additives[b]	Weeks at −20°C 0	3	6	9	12
Conrol	1.323 f[c] (44.1)[d]	1.080 d (36.0)	0.588 bc (19.6)	0.630 b (21.0)	0.564 c (18.8)
ADP	2.733 a (91.1)	2.349 a (78.3)	1.563 a (52.1)	1.584 a (52.8)	1.455 a (48.5)
AMP	2.586 b (86.2)	2.046 b (68.2)	1.572 a (52.4)	1.503 a (50.1)	1.371 b (45.7)
IMP	2.391 c (79.7)	1.800 c (60.0)	1.467 a (48.9)	1.512 a (50.4)	1.524 a (50.8)
HxR	1.650 e (55.0)	0.972 e (32.4)	0.603 b (20.1)	0.552 c (18.4)	0.345 d (11.5)
Hx	2.118 d (70.6)	1.026 de (34.2)	0.570 c (19.0)	0.489 d (16.3)	0.312 e (10.4)

[a] The solubility was expressed as mg of protein/mL.

[b] See Table 1 for definitions.

[c] Values in the same column bearing unlike letters differ significantly ($P<0.01$).

[d] Values in parentheses are the percentage ratio relative to the original concentration of actomyosin.

Table 3—Effect of adenosine-nucleotides and their derivatives on the Ca-ATPase specific activity[a] of actomyosin in vitro *during frozen storage at −20°C*

Additives[b]	Weeks at −20°C 0	3	6	9	12
Control	0.278 c[c] (48.2)[d]	0.273 b (45.6)	0.203 c (35.3)	0.108 b (18.8)	0.081 b (14.1)
ADP	0.444 a (77.0)	0.423 a (73.5)	0.394 b (68.4)	0.339 a (58.9)	0.331 a (57.4)
AMP	0.482 a (83.6)	0.463 a (80.4)	0.445 a (77.2)	0.369 a (64.1)	0.353 a (61.2)
IMP	0.476 a (82.6)	0.430 a (74.7)	0.427 a (74.2)	0.364 a (63.2)	0.344 a (59.8)
HxR	0.313 b (54.3)	0.193 c (33.5)	0.137 e (23.7)	0.084 bc (14.5)	0.065 b (11.3)
Hx	0.329 b (57.1)	0.297 b (51.6)	0.197 d (34.2)	0.104 b (18.1)	0.068 b (11.8)

[a] The Ca-ATPase specific activity were expressed as micromoles of released phosphate/mg protein within 1 min reaction at 25°C.

[b] See Table 1 for definitions.

[c] Values in the same column bearing unlike letters differ significantly ($P<0.01$).

[d] Values in parentheses are the percentage ratio relative to the original activity.

Statistic analyses

Duncan's multiple range test was used for statistical analyses.

RESULTS & DISCUSSION

Changes in adenosine-nucleotides and their derivatives

The ADP and AMP, after addition to AM solution, were degraded into IMP, and then no change in IMP content occurred during frozen storage. The added HxR and Hx did not change after addition and during frozen storage at −20°C (Table 1). The degradation of ADP and AMP into IMP after addition was considered to be due to the high instability of ADP and AMP.

Effect of adenosine-nucleotides and their derivatives on the denaturation of actomyosin during frozen storage

The solubility of actomyosin with added ADP, AMP, and IMP was significantly higher ($P<0.01$) than that of samples with added HxR and Hx, and control sample immediately after freezing (Table 2). The Ca-ATPase specific activity of acto-

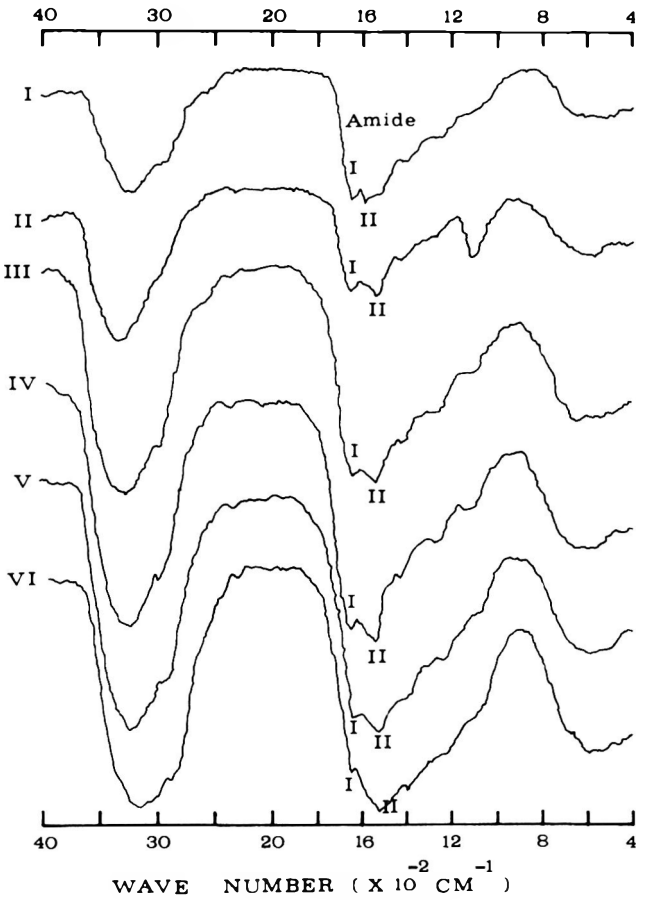


Fig. 2—Effect of adenosine-nucleotides and their derivatives on the infrared spectrum profile of actomyosin extracted from milkfish dorsal muscle (I: control sample; II: actomyosin after the addition of ADP; III: actomyosin after the addition of AMP; IV: actomyosin after the addition of IMP; V: actomyosin after the addition of HxR; VI: actomyosin after the addition of Hx).

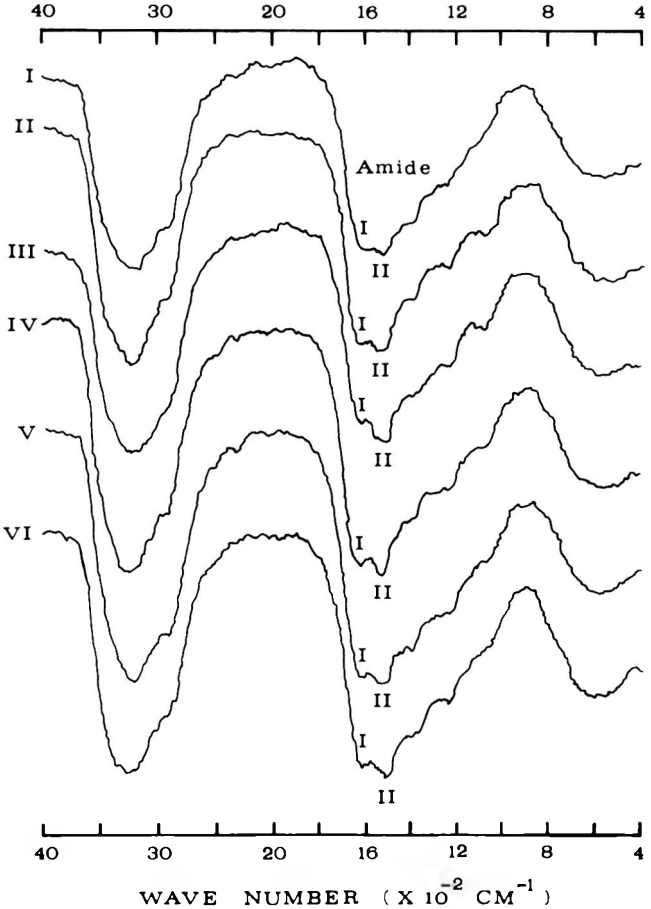


Fig. 3—Effect of adenosine-nucleotides and their derivatives on the infrared spectrum of actomyosin extracted from milkfish dorsal muscle after 12 wk storage at −20°C (I: control sample; II: with added ADP: III: with added AMP; IV: with added IMP; V: with added HxR; VI: with added Hx).

myosin after extraction was 0.577 μmoles phosphate liberated/mg protein/min at 25°C. However, that of samples with and without the addition of adenosine-nucleotides and their derivatives decreased significantly ($P<0.01$), compared to the unfrozen actomyosin. The Ca-ATPase activity of samples with ADP, AMP, and IMP was significantly higher ($P<0.01$) than that of control sample and with added HxR and Hx, after freezing (Table 3). After 12 wk storage, samples with ADP, AMP, and IMP revealed higher Ca-ATPase activity than that of control sample and samples with HxR and Hx (Table 3).

Considering the change rate of the solubility and Ca-ATPase specific activity of actomyosin, samples with added HxR denatured faster than control sample during storage, whereas the samples with Hx had almost the same change rate as control samples (Table 4). Thus, ADP, AMP, and IMP showed a protective effect on the denaturation of actomyosin during frozen storage.

Actomyosin is recognized as the protein group responsible for gel-strength, or ashi, of minced fish products. Several cryoprotectives have been developed for increasing the stability of frozen surimi (or frozen minces). Sucrose and sorbitol are the most commonly used in frozen surimi processing (Arai et al., 1970; Okada et al., 1974). Sodium glutamate and glutamic acid are also used for protecting the denaturation of frozen surimi (Matsumoto, 1979, 1980). According to these data (Table 1, 2, 3, and 4), IMP will be potentially useful in frozen surimi processing as a cryoprotective substitute for sucrose or sorbitol. It is well known that 5–10% sucrose and sorbitol used in frozen surimi results in an oversweet taste, which consequently limits the utilization of these compounds. More detailed experiments for use IMP are being done in our laboratory now.

The Mg(EGTA)-ATPase activity of those samples with and without adenosine-nucleotides and their derivatives were low during early stage of storage. It increased rapidly in the control sample after 6 weeks, and in samples with HxR and Hx after 3 weeks storage, respectively (Table 5). The Mg(EGTA)-ATPase activity of samples with added ADP, AMP, and IMP was at a low level during 9 wk storage, and rapidly increased after 12 wk storage (Table 5). This rapid increase in Mg(EGTA)-ATPase activity suggested that loss in Ca-sensitivity occurred on myofibrillar proteins (Seki et al., 1979). Thus, the addition of ADP, AMP, and IMP had a desirable effect on retarding the loss of Ca-sensitivity of myofibrillar proteins during storage.

The activity of native tropomyosin contributes to the Ca-sensitivity of myofibrillar proteins (Ebashi et al., 1968). The loss of Ca-sensitivity is considered to be due to the filamentation of myofibrils caused by hydrolysis of protease (Tokiwa and Matsumiya, 1969). According to the studies by Seki and Hasegawa (1978), Seki and Iwabuchi (1978), Shitamura and Seki (1978), and Seki et al. (1979), the loss of Ca-sensitivity of myofibrillar proteins is not due to the hydrolysis of tropomyosin and troponin by protease, but is due to the modification of actin-myosin interaction by oxidation of the thiol groups of myosin moiety. Accordingly, the rapid increase in Mg(EGTA)-ATPase activity suggested that the oxidation of sulfhydryl groups into disulfide bonds occurred on actomyosin molecules, and

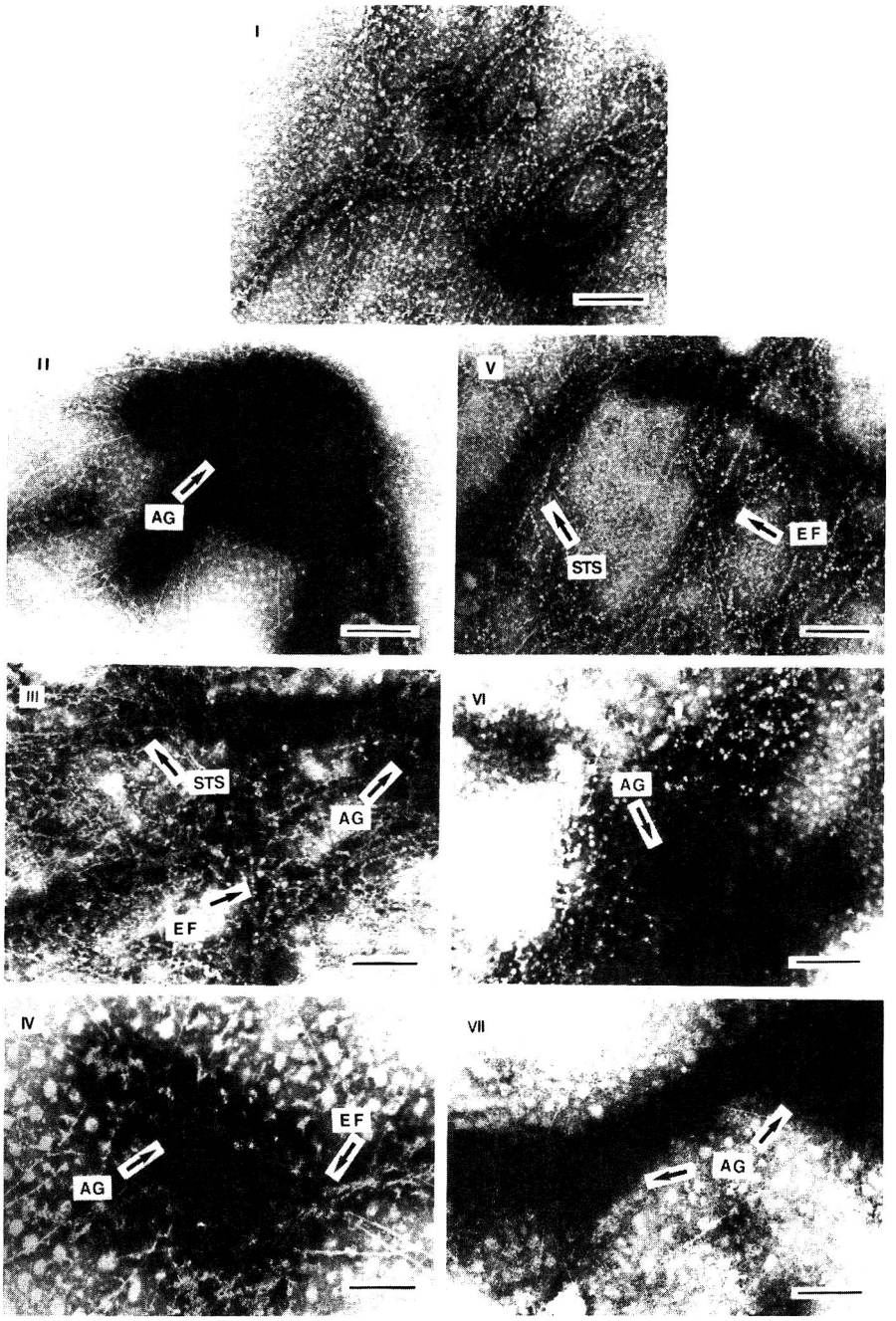


Fig. 4—Effect of adenosine-nucleotides and their derivatives on the transmission electron micrography of actomyosin suspended in 0.10M KCl solution during frozen storage at −20°C (I: unfrozen actomyosin; II: actomyosin after 12 weeks storage; III: actomyosin with ADP after 12 wk storage; IV: actomyosin with AMP after 12 wk storage; V: actomyosin with IMP after 12 wk storage; VI: actomyosin with HxR after 12 wk storage; VII: actomyosin with Hx after 12 wk storage). In all electron micrographs the bars represent 0.2 μm; AG: aggregated filament; EF: entangled filament; STS: side-to-side aggregation).

consequently caused protein denaturation during storage. This can also explain the previous studies by Jiang et al. (1984, 1986b).

Effect of adenosine-nucleotides and their derivatives on the electrophoretic profiles of actomyosin during frozen storage

No distinct changes in electrophoretic separation of actomyosin was observed after the addition of adenosine-nucleotides and their derivatives (I in Fig. 1-A, B, and C). As compared to the electrophoretic profiles obtained after the addition of adenosine-nucleotides and their derivatives, the band size of light chains (24,000–17,000 daltons) in electrophoretic profiles of all samples before precipitating the denatured proteins decreased after 12 weeks storage (II in Fig. 1-A, B, and C). However, the percentage of myosin heavy chain (MHC) on electrophoretic separation of the soluble protein (III in Fig. 1-A, B, and C) was slightly decreased in samples with the addition of ADP, AMP, and IMP, and more severely in control samples and samples with HxR and Hx after 12 wk storage at −20°C.

The electrophoretic separation of samples before precipitating the denatured proteins after 12 weeks storage revealed almost the same subunit composition as that of samples before freezing (I and II in Fig. 1-A, B, and C). However, the electrophoretic separation of the soluble proteins of control sample and samples with HxR and Hx after 12 wk storage were considerably different from that of unfrozen samples. The MHC in soluble proteins of control and samples with added HxR and Hx disappeared and subsequently formed many unknown components with molecular weights around 250,000–90,000 daltons. The actin in soluble proteins with HxR and Hx markedly decreased after 12 weeks storage (III in Fig. 1-C). There were no distinct changes in tropomyosin between protein samples before and after precipitation of the denatured material in the

Table 4—Effect of adenosine-nucleotides and their derivatives on quality change of actomyosin during frozen storage at −20°C

Additives[a]	Rate of change: Solubility (mg/ml/wk)	Rate of change: Ca-ATPase specific activity (μmole Pi/min/mg protein/wk)
Control	−0.273	-45.92×10^{-3}
ADP	−0.154	-22.83×10^{-3}
AMP	−0.156	-19.58×10^{-3}
IMP	−0.174	-20.33×10^{-3}
HxR	−0.276	-52.08×10^{-3}
Hx	−0.276	-45.75×10^{-3}

[a]See Table 1 for definitions.

Table 5—Effect of adenosine-nucleotides and their derivatives on the Mg(EGTA)-ATPase specific activity[a] of actomyosin in vitro *during frozen storage at −20°C*

Additives[b]	Weeks at −20°C: 0	3	6	9	12
Control	0.078 c[c]	0.083 c	0.283 a	0.278 a	0.181 b
ADP	0.084 c	0.089 c	0.094 bc	0.109 b	0.311 a
AMP	0.082 c	0.073 d	0.105 b	0.109 b	0.298 a
IMP	0.086 c	0.091 b	0.099 b	0.104 b	0.284 a
HxR	0.074 d	0.283 a	0.277 a	0.210 b	0.165 c
Hx	0.089 d	0.297 a	0.285 a	0.204 b	0.178 c

[a] The Mg(EGTA)-ATPase specific activity was expressed as micromoles of released phosphate/mg protein within one minute reaction at 25°C.

[b] See Table 1 for definitions.

[c] Values in the same row bearing unlike letters differ significantly ($P<0.01$).

control and samples with added ADP, AMP, and IMP after 12 wk storage. The band size of tropomyosin in soluble proteins with HxR and Hx, however, was considerably decreased after 12 wk storage, compared to that of samples before precipitation. This kind of change suggested that, in comparison with that of control samples, the addition of HxR and Hx caused the denaturation not only of MHC and actin, but of tropomyosin during frozen storage (II and III in Fig. 1-A and C).

From the electrophoretic analyses it appears that the myofibrillar proteins of control samples denatured mainly on MHC and actin, while those with HxR and Hx denatured mainly on MHC, actin and tropomyosin during frozen storage. This might be due to both dissociation and aggregation. The addition of ADP, AMP, and IMP, actually IMP because ADP and AMP degraded immediately after addition (Table 1), protected against protein denaturation. This is because the proteins interacted with those compounds which have 3, 2, and 2 negatively charged groups, and consequently increased the net negative charge of protein molecules. This interaction would intensify the repulsion force among protein molecules and subsequently prevent protein denaturation.

Changes in infrared spectrum of actomyosin after the addition of adensine-nucleotides and their derivatives during frozen storage

The frequencies at 1650 and 1580 cm^{-1} in the infrared spectrum of actomyosin after extraction were assigned to amide I and amide II, respectively (I in Fig. 2). The amide II in samples after the addition of ADP, AMP, IMP, HxR and Hx were shifted to lower frequencies: 1540, 1540, 1540, 1520, and 1520 cm^{-1}, respectively. Amide I and II are mainly caused by the $-C=O$ stretching and $-N-H$ bending, respectively (Fraser and Suzuki, 1970; Susi, 1972; Wallach and Oseroff, 1974). The amide II shift to a lower frequency in these samples, compared to the initial one, suggested that the negatively charged groups of ADP, AMP, IMP, HxR and Hx interacted with $-N-H$ group on actomyosin after addition (I, II, III, IV, V, and VI in Fig. 2).

After 12 wk storage, amide I and II of all samples shifted to lower frequencies. This suggested the hydrophilic interaction of both $-C=O$ and $-N-H$ groups of protein molecule occurred during frozen storage, and caused protein aggregation-denaturation (I, II, III, IV, V and VI in Fig. 3). This explanation can be supported by the decrease in solubility and Ca-ATPase specific activity of these samples.

Electron microscopic observation of actomyosin and actomyosin with the addition of ADP, AMP, IMP, HxR, and Hx during frozen storage

As depicted in Fig. 4-(I), the actomyosin of myofibrils could be seen clearly by transmission electron microscopy (TEM) before freezing. Many filaments extending linearly were observed in this plate. The filaments were approximately 0.8–5 μm long and 15–25 nm wide, and revealed the arrowhead structures specific to actomyosin filaments (Oguni et al., 1975). After 12 wk storage at −20°C, some filaments in samples with the addition of ADP, AMP, and IMP were aggregated with each other side-to-side and cross-wise, and further entangled, but their arrowhead structures were still maintained (III, IV, and V in Fig. 4). However, protein solutions of control samples and with added HxR and Hx showed massive aggregation formations after 12 weeks storage (II, VI and VII in Fig. 4).

To summarize the present results, the negatively charged groups of ADP, AMP, IMP, HxR, and Hx interacted with the amino or imino groups of protein molecules after the addition of these nucleotides and their derivatives. The ADP, AMP, and IMP prevented the denaturation of myofibrillar protein during frozen storage, whereas the HxR and Hx had no effect on, or accelerated, protein denaturation during frozen storage.

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This research work was supported by National Science Council, Republic of China, under grant No. NSC 74-0409-B019-03.

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Appreciation is expressed to Bob Flewellen, Sue Ellen McCullough, and Robert Ryals for technical assistance.

Determination of the beta-Lactoglobulin, alpha-Lactalbumin and Bovine Serum Albumin of Whey Protein Concentrates and Their Relationship To Protein Functionality

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ABSTRACT

The β-lactoglobulin, α-lactalbumin and bovine serum albumin of eight whey protein concentrates (WPC) were determined by reversed-phase HPLC and by sodium dodecyl sulfate polyacrylamide gel-electrophoresis (SDS-PAGE). The total protein as determined by HPLC was in good agreement with the soluble protein of the WPC. Total protein determined by SDS-PAGE agreed with the total protein of the WPC. The β-lactoglobulin determined by HPLC was significantly correlated with emulsion capacity, protein solubility, whipped topping overrun, gel strength, and free sulfhydryl of the WPC.

INTRODUCTION

WHEY PROTEIN CONCENTRATES (WPC) are a potential source of nutritional and functional proteins for the food industry. One reason that their utilization has failed to reach their full potential is the sample-to-sample variability in functionality that occurs in WPC (Harper, 1984). The reasons for this variability are not understood, but deWit (1984) has suggested that a more complete study of the effects of heating during processing might aid in this matter.

Differential scanning calorimetry (DSC) has been utilized to study the effects of heating on whey proteins (deWit and Swinkels, 1980; deWit and Klarenbeek, 1981; Ruegg et al., 1977). Generally, two regions of heat absorption are observed—one near 70°C and one near 130°C. These have been generally attributed to the denaturation and unfolding of residual protein structure of β-lactoglobulin. The β-lactoglobulin pattern of heat absorption is virtually identical to that seen with WPC (deWit, 1981).

The extent of protein denaturation due to heating has been studied by electrophoresis (Sawyer, 1968; Hillier, 1976; Hillier et al., 1979). the rate and extent of whey protein denaturation has been shown by these studies to be affected by temperature, pH and mineral composition. More recently reversed-phase HPLC has been utilized to estimate the changes in whey protein concentration that occur during processing (Pearce, 1983; Morr, 1985). To gain a better understanding of whey protein functionality, these changes must be related to the functional properties of the WPC. The object of this study was to determine the β-lactoglobulin, α-lactalbumin and bovine serum albumin (BSA) in a variety of WPC by gel electrophoresis and by reversed-phase HPLC and to relate the compositional data to the functionality of the WPC in model food systems.

MATERIALS & METHODS

Sweet WPC were produced by the New Zealand Dairy Research Institute as described by Matthews (1978). Process temperatures used to prepare the WPC are shown in Table 1.

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Chemical methods

Nitrogen was determined by the method of Scales and Harrison (1920). Nonprotein nitrogen (NPN) was defined as the Kjeldahl nitrogen soluble in 12.5% TCA. True protein was defined as total Kjeldahl nitrogen—NPN × 6.38. Protein solubility was determined by the procedure of Morr et al. (1985). Free sulfhydryls were determined by the DTNB procedure of Ellman (1959), as modified for milk proteins by Patrick and Swaisgood (1976).

HPLC

All chromatography was performed with a 300A pore C4 bonded silica column (Vyadec 214TP, The Sep/a/ra/tions Group, Hesperia, CA; 250 × 4.6 mm). Proteins were chromatographed at room temperature (22°C) at a flow-rate of 1 mL/min using a Waters High-Performance Chromatography system consisting of 2 M6000A pumps, Wisp auto injector, 490 multiple wavelength detector and an 840 data system. A nonlinear gradient (Curve 8) was run from 30 to 45% acetonitrile containing 0.1% trifluoroacetic acid (TFA) for 15 min. All solvents were HPLC grade. Samples of WPC were dissolved (1% w/v) in double-distilled, demineralized water containing 0.1% TFA. Several concentrations of each standard protein were prepared by the same procedure for construction of standard curves. Proteins were monitored simultaneously at 210, 230, 250 and 280 nm. Quantitation was based on peak areas of whey proteins and external standards for each protein. The coefficient variations for the procedure were 5.87% for BSA, 4.39% for α-lactalbumin and 6.29% for β-lactoglobulin.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Whey samples (0.4% w/v) and appropriate amounts of each standard protein were dissolved in Tris-HCl buffer, pH 6.7 containing 2% SDS and 1% mercaptoethanol. The samples were denatured for 5 min at 100°C and a few drops of Bromophenol Blue were added. Electrophoresis was initially at 1.0 mA/tube for 15 min. After the proteins had entered the gel, the current was increased to 2.5 mA/tube until the tracking dye migrated to the bottom of the tube. The gels were stained with Coomassie Blue R250 and destained by diffusion. Gels were scanned with a Model SL-504-XL Soft Laser Densitometer (Biomed Instruments, Inc., Fullerton, CA). A standard curve was constructed for each protein on samples electrophoresed and stained at the same time as the WPC. The concentration of unknown proteins was read from the standard curves. The average coefficient of variations of the procedure was 11.7%.

Functional properties

Emulsion capacity was determined on 3g of protein. The WPC was dissolved in 75 mL of cold (4°C) distilled water, the pH was adjusted to 6.0 with 0.1N HCl/NaOH, and the solution was stirred for 30 min at 4°C. Cold 1M NaCl (225 mL) was added. Aliquots (50 mL) were transferred to a Waring Blendor and titrated with soybean oil containing Oil Red 0 until inversion occurred. The oil was delivered into the blender from a rapid delivery pipet through a hole in the lid of the blender jar at a rate of 45 mL/min. The rate of delivery was decreased to a dropwise addition about 2 mL before the emulsion endpoint.

Whipped topping overrun was determined in a system containing 30% fat as described by Peltonen-Shalaby and Mangino (1986).

Gel strength was determined on solutions containing 10% protein adjusted to pH 8.0. Triplicate 10 mL aliquots were placed into 10 × 100 mm screw-top test tubes and heated in a 90°C circulating water-bath for 15 min. The test tubes were cooled for 5 min in an ice-water

Table 1.—Process temperatures used in production of whey protein concentrates.

	Milk				Whey			Retentate	
Sample	Preheat	Pasteurization[a]	Set	Cook	Preheat	Pasteurization	Cool	Ultra-filtration	Pasteurization
4116	32	None	32	38	55	72°C	20	50	72°C
4117	32	72°C	32	38	55	72°C	20	50	None
4118	32	None	32	38	55	72°C	20	50	None
4120	55	72°C	32	38	37	None	20	50	None
4123	55	72°C	32	38	55	72°C	20	50	72°C
4124	32	None	32	38	37	None	20	50	72°C
4125	55	72°C	32	38	37	None	20	50	72°C
4126	32	None	32	38	37	None	20	50	None

[a] Pasteurization (72°C/15 sec)

Table 2.—Regression line for each standard curve of α-lactalbumin (ALAC), β-lactoglobulin (BLG) and bovine serum albumin (BSA)

Protein	Wavelength (nm)	Slope	Intercept	r
ALAC	210	1521.85	− 7.262	0.9979
	230	610.45	− 2.180	0.9976
	250	57.05	− 0.438	0.9939
	280	142.95	− 1.002	0.9973
BLG	210	851.70	86.400	0.9902
	230	389.00	8.946	0.9986
	250	23.72	1.006	0.9938
	280	60.14	1.924	0.9961
BSA	210	1414.20	− 4.910	0.9989
	230	417.30	− 3.750	0.9970
	250	23.38	− 0.246	0.9961
	280	45.62	− 0.410	0.9974

Table 3.—Bovine serum albumin content[a] in sweet whey protein concentrates (g/g) monitored at 280, 210, 230, and 250 nm

	Bovine serum albumin			
Sample	280	210	230	250
4116	0.164 ±0.0036	0.071 ±0.0006	0.096 ±0.0012	0.138 ±0.0006
4117	0.188 ±0.0012	0.086 ±0.0035	0.111 ±0.0017	0.173 ±0.0006
4118	0.167 ±0.0065	0.076 ±0.001	0.104 ±0.0021	0.152 ±0.0017
4120	0.167 ±0.0038	0.076 ±0.0026	0.104 ±0.0015	0.148 ±0.0046
4123	0.165 ±0.0032	0.071 ±0.0083	0.095 ±0.0015	0.138 ±0.0044
4124	0.156 ±0.0032	0.071 ±0.0061	0.095 ±0.0026	0.138 ±0.0035
4125	0.168 ±0.0067	0.076 ±0.0035	0.104 ±0.0035	0.148 ±0.0069
4126	0.156 ±0.0035	0.071 ±0.0040	0.098 ±0.0012	0.139 ±0.0023

[a]Average of three determinations

bath and allowed to stand at room temperature for 1 hr before gel strength was measured by the Instron procedure of Dunkerley and Hayes (1980), using a 6mm circular probe. Gel strength was defined as the height of the first peak on the chart.

RESULTS & DISCUSSION

DATA on the regression lines for standard curves for BSA, β-lactoglobulin and α-lactalbumin are found in Table 2. Comparison of the slopes obtained at each wavelength showed that monitoring at 210 nm instead of at 280nm increased the sensitivity by approximately tenfold for α-lactalbumin, fourteen fold for β-lactoglobulin and thirtyfold for BSA. The linearity was good for all of the proteins at each of the wavelengths.

Tables 3 to 5 give the content of BSA, α-lactalbumin and β-lactoglobulin in sweet whey protein concentrates. Standard errors for these determinations were typically in the range 3 to 5%. The values obtained for α-lactalbumin and β-lactoglobulin were fairly consistent regardless of the wavelength used for

Table 4—α-Lactalbumin content[a] in sweet whey protein concentrates (g/g) monitored at 280, 210, 230, and 250 nm

	α-Lactalbumin			
Sample	280	210	230	250
4116	0.080 ±0.0040	0.080 ±0.0015	0.089 ±0.0023	0.082 ±0.0025
4117	0.087 ±0.0036	0.084 ±0.0029	0.092 ±0.0015	0.086 ±0.0067
4118	0.087 ±0.0051	0.084 ±0.0025	0.092 ±0.0012	0.086 ±0.0081
4120	0.074 ±0.0038	0.076 ±0.0026	0.082 ±0.0025	0.080 ±0.002
4123	0.081 ±0.0042	0.081 ±0.0046	0.092 ±0.0015	0.084 ±0.0053
4124	0.080 ±0.0038	0.081 ±0.0055	0.089 ±0.0044	0.084 ±0.0029
4125	0.062 ±0.0056	0.059 ±0.0043	0.079 ±0.0044	0.077 ±0.0023
4126	0.086 ±0.0031	0.085 ±0.0017	0.093 ±0.0053	0.086 ±0.0052

[a] Average of three determinations

Table 5.—β-Lactoglobulin content[a] in sweet whey protein concentrates (g/g) monitored at 280, 210, 230, and 250 nm

	β-lactoglobulin			
Sample	280	210	230	250
4116	0.37 ±0.0306	0.37 ±0.0100	0.36 ±0.0252	0.36 ±0.0361
4117	0.41 ±0.0153	0.41 ±0.0379	0.41 ±0.0100	0.42 ±0.0208
4118	0.41 ±0.0153	0.41 ±0.0520	0.41 ±0.0100	0.41 ±0.0153
4120	0.41 ±0.0306	0.41 ±0.0153	0.40 ±0.0208	0.41 ±0.0231
4123	0.38 ±0.0351	0.38 ±0.0173	0.36 ±0.0153	0.33 ±0.0208
4124	0.38 ±0.0300	0.38 ±0.0115	0.36 ±0.0153	0.33 ±0.0153
4125	0.40 ±0.0153	0.40 ±0.0379	0.38 ±0.0058	0.40 ±0.0115
4126	0.40 ±0.0173	0.40 ±0.0173	0.38 ±0.0208	0.40 ±0.0252

[a] Average of three determinations

Table 6—Protein distributin[a] (%) in sweet whey protein concentrates determined by RP-HPLC monitored at 210 nm

1 Sample	2 Protein (Kjeldahl)	3 Blg[b]	4 ALAC*	5 BSA[d]	6 total (HPLC)	7 RP-HPLC / Kjeldahl	8 Soluble protein
4116	48.9	37	8.0	7.1	52.1	0.70	57.7
4117	63.3	41	8.4	8.6	58.0	0.76	62.87
4118	69.8	41	8.4	7.6	57.0	0.77	66.45
4120	54.7	41	7.0	7.6	56.2	0.76	66.89
4123	65.3	38	8.1	7.1	53.2	0.71	57.22
4124	72.3	38	8.1	7.1	53.2	0.72	57.40
4125	65.1	40	5.9	7.6	53.5	0.72	59.16
4126	65.3	40	8.5	7.1	55.6	0.75	66.22

[a] Average of Three Determinations
[b] β-Lactoglobulin (mg/100g)
[c] α-Lactalbumin (mg/100 g)
[d] Bovine serum albumin. (mg/100 g)

Table 7—Protein distribution[a] (%) in sweet whey protein concentrates determined by SDS-PAGE

1 Sample	2 Protein (Kjeldahl)	3 β-Lactoglobulin	4 α-Lactalbumin	5 BSA	6 TOTAL
4116	74.5	41	5.4	2.5	48.9
4117	76.3	47	12.2	4.1	63.3
4118	74.0	56	10.7	3.1	69.8
4120	74.4	44	8.9	1.8	54.7
4123	74.5	51	11.0	3.3	65.3
4124	73.4	57	12.2	3.1	72.3
4125	74.7	53	8.8	3.3	65.1
4126	74.4	53	8.8	3.5	65.3

[a] Average of three determinations

Table 8—Compositional analysis of whey protein concentrates[a]

WPC	% Protein	% NPN[b]	% Lipid	% Ash	% Moisture	μM/g Disulfide	μM/g Free Sulfhydryl
4116	74.5	0.98	6.7	3.65	5.20	79.0	13.5
4117	76.3	1.05	4.6	3.43	6.68	86.1	22.45
4118	74.0	1.21	7.4	3.32	5.41	83.6	24.5
4120	74.4	1.16	6.7	3.82	5.78	88.3	23.5
4123	74.5	1.30	6.0	3.57	6.70	101.7	19.0
4124	73.4	1.13	7.1	3.88	4.63	91.5	10.0
4125	74.7	1.19	5.4	3.88	5.10	87.6	22.9
4126	74.4	1.16	6.6	3.30	4.17	89.8	20.5

[a] Average of three determinations
[b] Nonprotein nitrogen

measurement. However, the values obtained for BSA varied by a factor of two when those obtained at 280 nm were compared with those obtained at 210 nm. BSA is capable of binding a variety of small molecules and the absorbancy of these molecules may account for the differences observed. The binding of molecules which have substantial extinction coefficients at 280 nm would have much less impact on the values obtained at 210 nm when compared to 280nm because of the higher extinction coefficient for the proteins at 210nm as compared to 280 nm (Table 2). Similar phenomena may limit the usefulness of using 280 nm monitoring to determine proteins in many other food systems. Food systems containing high levels of phenolics, common in many plant-derived foods, may be particularly vulnerable to this type of interference.

Table 6 presents the composition of the WPC as determined at 210 nm. The total protein as determined by HPLC comprised from 70 to 77% of the values obtained by Kjeldahl analysis. The values obtained by HPLC analysis were in much better agreement with the data for total soluble and thus potentially functional protein than were the values based on total nitrogen. This probably reflects the fact that only soluble protein is resolved by the HPLC procedure. The insoluble protein is probably removed in the sample purification procedure.

Protein as determined by SDS-PAGE is presented in Table 7. With the exception of sample 4116, the total protein values were in much closer agreement with the Kjeldahl figures than were the HPLC data. This would suggest that the SDS-PAGE was a better indicator of total rather than soluble protein of the WPC. This is reasonable since SDS is capable of solubilizing denatured proteins and once solubilized they would not be separated from the native protein. Thus, the HPLC procedure gives values that are in good agreement with the soluble protein content while the SDS procedure measures both native and denatured proteins and agrees better with total protein.

Commonly cited literature values for β-lactoglobulin, α-lactalbumin and BSA are 3.3, 1.2, and 0.3 g/L of whey, respectively (deWit and Klarenbeek, 1984). When converted to percent total whey protein, the values obtained for β-lactoglobulin were in excellent agreement with this figure. The values obtained for α-lactalbumin are on the average 30% lower but well within the range reported by Eigel et al. (1984). The values obtained for BSA by HPLC were more than double those reported in the literature. This may well reflect the presence of UV absorbing molecules in the BSA from the WPC which were absent from the commercial protein standards utilized for the construction of the standard curve. The values obtained for α-lactoglobulin by SDS-PAGE were also in excellent agreement with literature values as were those obtained for BSA. It would thus appear that any molecules bound to the BSA in the WPC did not interfere with its ability to interact with Coomassie Blue R250. The values obtained for α-lactalbumin by SDS-PAGE were quite variable. The highest values observed by SDS-PAGE were higher than those found by HPLC but in agreement with the data of deWit and Klarenbeek (1984). In general, the data obtained by SDS-PAGE were more variable than those obtained by HPLC.

The chemical composition of the WPCs is presented in Table 8 and their functionalities are recorded in Table 9. The correlation coefficients for β-lactoglobulin, as determined by HPLC and various functional properties, are listed in Table 10. No other protein was significantly correlated with any functional property, and β-lactoglobulin as determined by SDS-PAGE did not correlate with any functional property.

The strong correlation between β-lactoglobulin and WPC free sulfhydryls is expected as β-lactoglobulin is known to be the main source of these groups in milk (Eigel et al., 1984). Peltonen-Shalaby and Mangino (1986) have shown that sulfhydryl groups are one of the best predictors of overrun of whipped toppings made with β-lactoglobulin and thus β-lactoglobulin would be expected to correlate well with this functional property. β-Lactoglobulin comprises over 60% of the protein of WPC and thus would be expected to be highly correlated with its solubility characteristics. Aoki et al. (1981) have reported that the emulsifying properties of proteins are related to the extent of protein denaturation that has occurred. Thus, the major contribution made by β-lactoglobulin to WPC protein solubility would explain its significant correlation with emulsion capacity. Mangino et al. (1986) have noted that β-lactoglobulin is the most important predictor of WPC gel strength at pH 8.0 but not at pH 6.5 or 4.6. They hypothesized that at pH 8.0 sulfhydryl-disulfide interchange made an important contribution to the formation of a gel matrix. As previously noted, β-lactoglobulin is the major source of sulfhydryl groups in WPC and thus this correlation is reasonable.

The lack of correlation of other whey proteins with functionality probably reflects their lesser contribution to the composition of WPC. The lack of correlation of the SDS-PAGE data probably reflects the fact that SDS solubilizes otherwise insoluble protein (Wycoff et al., 1977), and that functional,

Table 9—Physical properties[a] of sweet whey protein concentrates

Property	Whey protein concentrates							
	4116	4117	4118	4120	4123	4124	4125	4126
Whipped topping Overrun (%)	213	259	261	270	229	248	264	
Gel Strength (N)	1.0	3.5	3.3	3.6	1.5	2.0	2.5	2.8
Emulsion Capacity (%)	52	53	53	53	52	52	52	53
Se[b]	222	287	238	291	243	239	242	260
SO[c]	446	556	521	574	467	485	507	555
Solubility (%)	77.4	82.4	89.8	89.9	76.8	78.2	79.2	89.0

[a] Average of three determinations
[b] Exposed hydrophobicity (μM/g)
[c] Surface hydrophobicity (μM/g)

Table 10—Correlation coefficients for β-lactoglobulin as determined by RP-HPLC and selected properties of sweet whey protein concentrates

Property	r	P
Whipped topping overrun	0.929	0.0008
Gel strength	0.976	0.0001
Emulsion capacity	0.833	0.0102
Exposed hydrophobicity (Se)[a]	0.712	0.0475
Surface hydrophobicity (So)[b]	0.899	0.0024
Solubility	0.775	0.0239
Free sulfhydryl content	0.920	0.0012

[a] Exposed hydrophobicity (μM/g)
[b] Surface hydrophobicity (μM/g)

as well as non-functional, proteins are measured. The HPLC procedure described was relatively simple to perform and appeared to give a good indication of the amount of functional protein present in WPC.

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Ms received 5/5/86; revised 9/20/86; accepted 9/20/86.

Journal article no. 55-86.

Salaries & research support provided by State & Federal funds appropriated by The Ohio State Agricultural Res. & Dev. Center, The Ohio State University. This work was part of a cooperative research effort with The Commonwealth Scientific Industrial Research Organization, Australia, The New Zealand Dairy Research Institute, New Zealand, and Clemson Univ. SC, and was supported in part by NSF Grants INT8211388 & INT8413635.

Surface Active Properties of Food Proteins: Effects of Reduction of Disulfide Bonds on Film Properties and Foam Stability of Glycinin

S.H. KIM and J.E. KINSELLA

ABSTRACT

Reduction of soy glycinin with 5 mM and 10 mM dithiothreitol resulted in molecular conformational changes which enhanced molecular hydrophobicity and increased viscosity. These changes were associated with significant improvement increases in surface active properties of the reduced proteins. The surface yield stress and elasticity of surface films were increased. The stability of foams as reflected in drainage time was improved especially at pH 6 and 7.0.

INTRODUCTION

INTERFACIAL FILM FORMATION is an essential event in the development of foams and emulsions. In many food systems amphipathic proteins are the principal surface active agents (Kinsella 1981, Halling 1981). In foaming systems the formation of strong cohesive viscoelastic films relatively impermeable to air and possessing appropriate viscoelastic and mechanical properties to resist shocks is desirable (Graham and Phillips, 1976, 1979; Cumper, 1953; Kinsella, 1981, 1986; Halling, 1981). Several phenomena occur during formation of an interfacial film. The protein diffuses and adheres to the interface, some segments of the polypeptide may unfold to a certain extent and spread owing to the favorable thermodynamic situation at the interface. The polypeptide residues in the interface may interact with the residues of the neighboring protein molecules and associate via electrostatic and hydrophobic interactions and hydrogen bonding to form a continuous network or film. The hydrophobic and polar hydrophilic residues orient toward the apolar (air) and polar aqueous phase respectively while, depending upon the protein, some of the protein molecule occupies the interface (Graham and Phillips, 1976; Kinsella, 1981, 1986; Halling, 1981). These dynamic events are influenced by the nature of the protein and the prevailing environmental conditions; e.g, less ordered flexible proteins like α-casein rapidly forms films whereas the extensively cross-linked, stable, compact lysozyme has limited film-forming properties (Graham and Phillips, 1979). The stability of the tertiary structure of proteins affect film formation and properties of the resultant film i.e according to Graham and Phillips (1976, 1980a, b) some molecular flexibility is conducive to the formation of cohesive films.

Native soy glycinin, or 11s, because of its compact tertiary structure which is stabilized by disulfide cross-linking has limited foaming properties. However, reduction of some disulfide bonds may improve their foaming ability (German et al., 1985) by allowing greater conformational flexibility.

There are a few conflicting reports concerning the effects of reducing disulfide bonds on the foam stability of proteins. Peter and Bell (1930) reported that sulfite treatment of whey proteins and egg albumin improved foam stability whereas Musselwhite (1968) noted that reduction of BSA in presence of 8M urea led to a more stable foam than the native protein. However, when glutelin was dissociated by breaking inter- and intrasubunit disulfide bonds, its superior foam stability in presence of 3M urea was lost (Mita et al., 1978).

It is possible that the controlled reduction of disulfide bonds can improve the surface active and the rheomechanical properties of glycinin interfacial film by allowing greater reorientation at the interface and facilitating greater protein:protein interaction to give stronger films. Controlled reduction of glycinin with dithiothreitol (DTT) causes conformational changes in glycinin (Kim and Kinsella, 1986) and the present study was conducted to determine the effects of cleavage of disulfide bonds on surface active, film and foaming properties of glycinin.

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MATERIALS & METHODS

CHEMICAL REAGENTS were purchased from Sigma Co., (St. Louis, MO) and Eastman Kodak (Rochester, NY). Doubly distilled water was used in all solutions.

Preparation of soy glycinin

Soy glycinin (11s) was prepared from defatted, low heat treated soy flour (Central Soya, Chicago, IL; #4 Bank White Flakes) by the procedure of Thanh and Shibasaki (1976). The defatted flour (50g) was suspended in 1L 0.03M Tris-HCl buffer, pH 8.0, containing 2 mM 2k-mercaptoethanol. The solution was stirred for 1 hr at 24°C and then centrifuged at 15,000 × *g* for 20 min at 20°C. The supernatant was adjsuted to pH 6.4 with 2N HCl and centrifuged at 15,000 × *g* for 20 min at 2°C. The glycinin precipitate was washed with Tris-HCl buffer pH 6.4 and then dispersed in Tris-HCl buffer, pH 8.0. Aliquots of 2N NaOH were added while stirring until the protein was fully dissolved (pH 8.0). The protein solution was dialyzed against water at pH 8.0 at 4°C and lyophilized. This preparation contained 95% glycinin as determined by gel electrophoresis (Thanh and Shibasaki, 1976).

Reduction of glycinin

The glycinin was reduced using two levels (5 and 10 mM) of dithiothreitol (DTT) and the sulfhydryl (SH) groups were blocked with iodoacetamide to prevent subsequent disulfide interchange as described previously (Kim and Kinsella, 1986). The number of disulfide bonds in the proteins were determined using 2-nitro-5 thiosulfobenzoic acid (NTSB) by the procedure of Kella et al. (1986 a, b). Aliquots of the protein solution (10% w/v) were mixed with the NTSB assay solution (1:1 v/v) held at 25°C in the spectrophotometer (Cary 219) and the absorbance read at 412 nm after 20 min. For converting the absorbance to disulfide bond concentration an extinction coefficient at 13,600 $M^{-1}cm^{-1}$ at 412 nm was used.

The viscosity, net hydrophobicity, UV fluorescent spectra and tyrosine titration were determined by established methods as described by Kim and Kinsella (1986). In this paper surface active properties include surface activity as measured by surface pressure development, surface viscosity, yield stress and elasticity of films and foam stability.

Surface activity

The surface tension (surface pressure) of glycinin and modified glycinin was determined using the Wilhelmy platinum plate Cahn electrobalance procedure as outlined (Kim and Kinsella, 1985; Waniska and Kinsella, 1985).

Surface viscosity

The surface viscosity (n_s) and surface yield stress (f_s) which reflect the cohesiveness and strength of protein films were determined using a cylindrical rotator attached via torsion wire to a Brookfield viscometer as described by Kim and Kinsella (1985).

Film elasticity

The responsiveness of surface tension to changes in surface area of interfacial films, i.e, film elasticity is an important criterion for foam stability (Graham and Phillips, 1976, 1980a, b). In the present study film elasticity was estimated using tensiolaminometry as described by German et al. (1985).

Foam stability

Foams were made using the procedure of Waniska and Kinsella (1979). The rate of drainage of liquid from foams is a reliable index of foam stability (Halling, 1981). Plots of the log of the volume of liquid remaining in a foam versus time (log volume vs t) yields an apparent first order relationship from which the rate constant of drainage or half life of liquid in the foam can be determined (Kim and Kinsella, 1985). All data represent the mean of triplicate analyses and variability was less than 10% within each determination.

RESULTS & DISCUSSION

THE EFFECTS of reduction on some molecular properties of glycinin are summarized in Table 1. Reduction with 5 mM DTT cleaved an average of 6 disulfide bonds mostly the intersubunit disulfide bonds linking the acidic and basic polypeptides but also some of the intramolecular disulfide bonds (Kim and Kinsella, 1986). This was consistent with the observation of Kella et al. (1986b) who showed that some intramolecular bonds were reduced before all intermolecular bonds were cleaved by sulfite. All disulfide bonds were reduced with 10 mM DTT. Analyses of molecular properties indicated extensive changes in molecular conformation, i.e increased exposure of apolar groups previously buried in the interior of the molecules, unfolding of the tertiary structures as reflected by the increased viscosity and a marked increase in hydrophobicity (Kim and Kinsella, 1986). These changes should result in improved surface active properties of the modified glycinin.

The surface pressure of native and reduced soy 11S globulin after 5 minutes of adsorption at pH 6, 7, and 8 is shown (Fig. 1). Reduction of intersubunit disulfide bonds (5 mM DTT) caused a sharp increase in surface pressure after 5 min of adsorption while there was no further appreciable change following reduction of intrasubunit disulfide bonds (10 mM DTT). Whereas pH affected surface pressure development of native glycinin it had a limited effect on the reduced glycinin.

The rate of surface pressure development is directly affected by the rate at which protein molecules diffuse to, collide with and become adsorbed to the interface. As segments of the protein molecules accumulate at the interface and spread, the surface pressure progressively decreases. The diffusion coefficient depends upon the molecular size and shape of the protein (Edsall, 1953) (and probably the chemical nature of the protein surface) while the probability of a molecular collision with the interface resulting in adsorption is affected by electrostatic phenomena (MacRitchie and Alexander, 1963) and may also be influenced by the hydrophobicity and molecular flexibility of the molecules (Kato and Nakai, 1980; Tornberg, 1978a, b). In addition, the nature of molecules i.e net charge already at the interface may affect successful adsorption (MacRitchie, 1978). In the present study the reduction of intersubunit disulfide bonds of glycinin caused dissociation of the oligomeric glycinin into acidic and basic subunits (Damodaran and Kinsella, 1982). This may have enhanced the rates of diffusion of the smaller subunits, particularly the acidic subunits of glycinin to the interface. However, this may have been counteracted to some extent by the increased molecular drag as reflected in the increased viscosity of the reduced molecules. The increased hydrophobicity of the reduced glycinin may have resulted in more facile adsorption at the interface while the increased molecular 'flexibility' may have facilitated protein:protein interaction in the interface to form an interfacial film. The low rate of surface pressure development by native glycinin is consistent with the observation of that the adsorption of soy isolate at an interface was quite slow (Tornberg, 1978a,b).

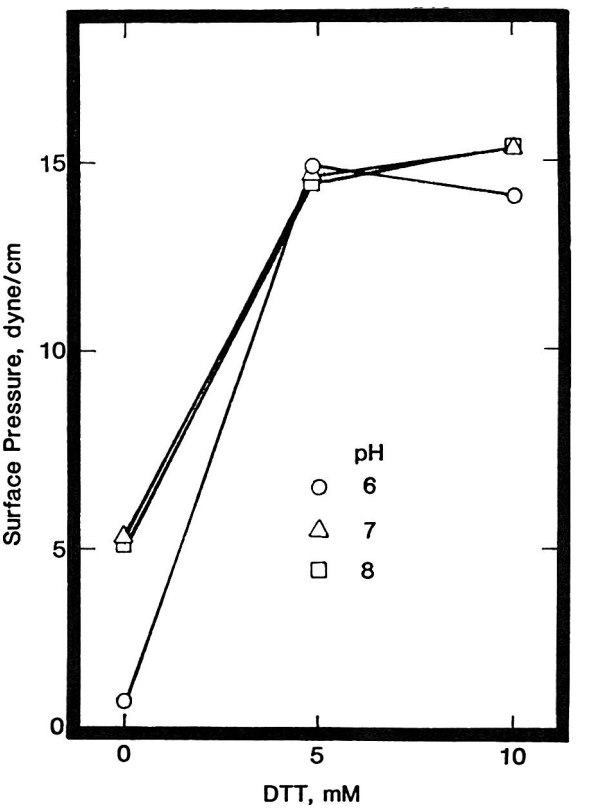

Fig. 1—Surface pressure after 5 min of adsorption of native and reduced glycinin. Protein concentration was 5 × 10^{-3} (w/v) in 20 mM phosphate buffer (pH 6, 7, and 8). Reduction of disulfide bonds was performed with dithiothreitol (DTT) (5mM and 10mM).

Table 1—Summary data showing the effects of disulfide reduction with dithiothreitol on some molecular properties of glycinin

Properties	Glycinin		
	Native 20 SS Bonds	Reduced 13 SS Bonds	Fully Reduced 0 SS Bonds
Relative hydrophobicity	220	900	870
Specific viscosity × 10^{-3}	4.2	8.0	7.3
UV absorbance (272 nm)	0.90	0.65	0.79
Tyrosine ionized at pH 11.5%	20	55	50
Relative fluorescence at 336 nm	46	52	57

The rheological properties of an interfacial film greatly affect the stability of foams because they indicate the capacity of films to withstand shock and rupture under gravity (MacRichie, 1978). Films with mechanical strength as reflected in enhanced surface viscosity and surface yield stress improve stability and retard drainage of foams (Graham and Phillips, 1976; Bikerman, 1973). The surface yield stress of reduced glycinin increased with extent of reduction (Fig. 2) and, with the exception of native glycinin, decreased with increasing pH. The surface yield stress is the non-Newtonian component of surface viscosity of the film and may be more related to the strength and stability of foams than the surface viscosity per se (Buckingham, 1972). The values for the surface yield stress observed in this study are comparable with

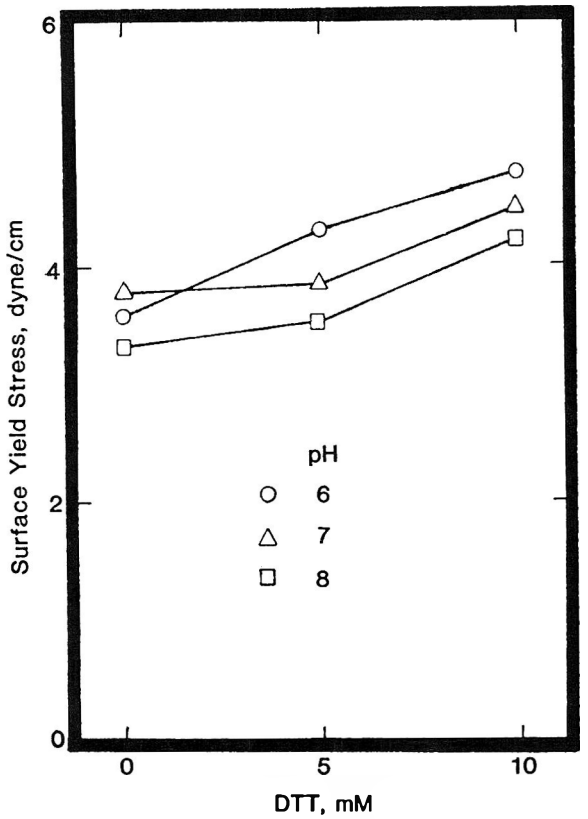

Fig. 2—The surface yield stress of native and reduced glycinin. Protein concentration was 0.1% (w/v) in 20 mM phosphate buffer.

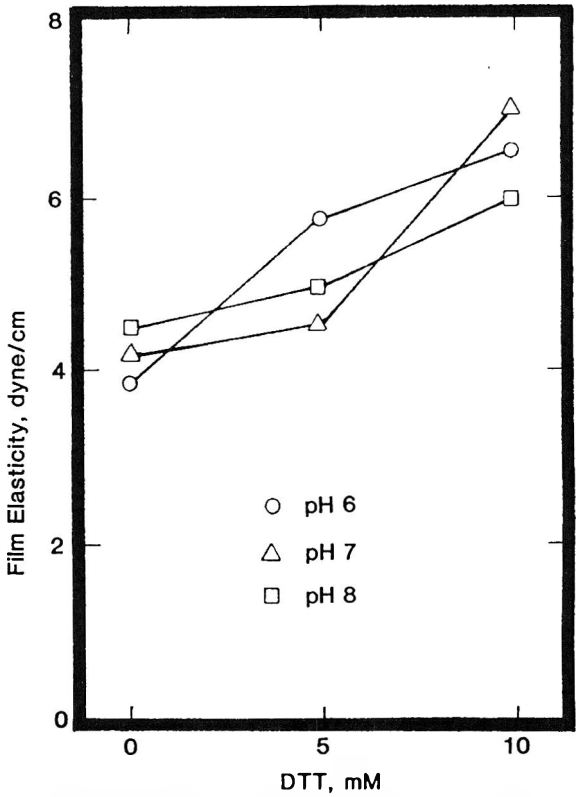

Fig. 3—Film elasticity of native and reduced soy glycinin. Protein concentration was 0.1% (w/v) in 20 mM phosphate buffer (pH 6, 7, and 8).

those observed for bovine serum albumin by Kim and Kinsella (1985) but are substantially higher than those reported by Buckingham for BSA but lower than those reported for films of ribulose bis-phosphate carboxylase (7–11 dyne/cm) (Barbeau and Kinsella, 1986). The disparity in these values may reflect differences in protein concentration and differences in molecular behavior of these proteins in the interface.

Higher surface yield stress reflects greater intermolecular interactions in the interface per se. Proteins with more flexible polypeptide segments should facilitate a greater degree of interdigitation and enhances association via hydrophobic and electrostatic interactions and hydrogen bonding, which in the aggregate strengthen the film matrix. Reduction of the disulfide bonds by improving molecular flexibility may thereby enhance protein:protein association to form a stronger more condensed film at the interface. The importance of electrostatic interactions in intramolecular association in the film is indicated by the increased yield stress of films formed at lower pH where net electrostatic repulsion is reduced. This observation is consistent with previous data obtained for other proteins (MacRitchie, 1978; Kim and Kinsella, 1985; Graham and Phillips, 1979; Waniska and Kinsella, 1985).

Dynamic film elasticity is an important prerequisite which determines the capacity of a film to expand and contract in response to localized applied stresses and directly relates to the stability of foams (Graham and Phillips, 1979; MacRitchie, 1978; Halling, 1981). The dynamic film elasticity of glycinin progressively increased with the extent of reduction of disulfide bonds (Fig. 3). The high film elasticity probably reflects greater molecular interaction to form a more cohesive film that possessed extensive overlapping of coiled polypeptides which enabled it to expand upon the application of a stress.

These studies indicate that the reduction of the disulfide bonds of glycinin by increasing the molecular flexibility of the protein components improved the formation and the properties of interfacial films. The interfacial films formed from proteins may be considered as thin layers of protein gel, the rheological properties of which reflect the conformation of the component molecules; and the extent of protein:protein interactions within the film per se. Generally molecules which possess flexible polypeptide moieties but retain some tertiary structure tend to give rise to stronger films which are more resistant to mechanical deformation, i.e have a higher surface viscosity, shear elastic and dilatational moduli (Phillips, 1977).

Several authors have suggested that these properties are important in determining the stability of foams made from such proteins. To assess this, stability of foams made from these proteins was measured. The foam stability expressed as the half-life time of liquid in the foam, i.e drainage stability, showed a progressive increase with reduction of the disulfide bonds of glycinin (Fig 4). The foams made from the fully reduced glycinin were more stable than those formed from native or partially reduced glycinin and foam stability was higher when the net charge on the component proteins were lowest. This suggests that maximum protein:protein interaction to form thick condensed films with high elasticity and yield stress gave more stable foams.

In summary, these observations indicate a relationship between the molecular 'flexibility' of proteins, film properties and foam stability. However, they do not establish a clear relationship between any of these parameters. Presumably the rate of surface pressure development, the viscosity and elasticity of the films formed all contribute to characteristics of foams made from different proteins. It is generally accepted that maximum protein:protein interaction to yield a condensed film containing sufficient residual tertiary structure may impart elasticity and ensure a more stable foam (Joly, 1972a, b; Graham and Phillips, 1976; Halling, 1981). The data from this study are consistent with those observed for bovine serum albumin, beta-lactoglobulin, and ribulose bis-phosphate carboxylase, respectively (Kim and Kinsella, 1985; Waniska and Kinsella, 1985; Barbeau and Kinsella, 1986).

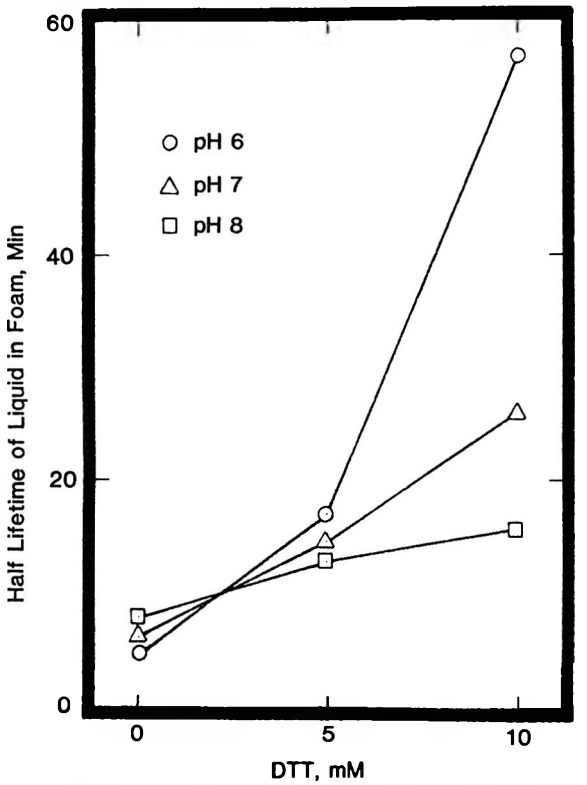

Fig. 4—The foam stability of native and reduced glycinin. Protein concentration was 0.1% (w/v) in 20mM phosphate buffer (pH 6, 7, and 8). Foams were formed as described in methods.

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Ms received 5/2/86; revised 8/11/86; accepted 8/22/86.

Supported in part by American Soybean Association and a special award to J.E. Kinsella from General Foods Foundation.

Volatile Compounds of the Wax Gourd (*Benincasa hispida*, Cogn) and a Wax Gourd Beverage

CHUNG-MAY WU, SHU-ER LIOU, YUNG-HO CHANG, and WENCHANG CHIANG

ABSTRACT

The volatile compounds of the wax gourd (*Benincasa hispida*, Cogn) and a wax gourd beverage were isolated by the Likens-Nickerson method and identified by Kovats indices and mass spectra. The major compounds identified in the gourds were E-2-hexenal, n-hexanal and n-hexyl formate; however, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine, 2,3,5-trimethylprazine, 2-methylpyrazine, and 2-ethyl-5-methylpyrazine were the major compounds in the wax gourd beverage.

INTRODUCTION

THE WAX GOURD (*Benincasa hispida*, Cogn), also known as winter melon or gourd melon which originated in China, can serve as a vegetable in diets and also as raw material to produce a beverage, candy or jam. There is little information on the chemical composition of the beverage which is popular in Taiwan, and to date, no reports on the volatile components of the vegetable as well as the beverage have been published. The aroma of the wax gourd and the beverage are completely different. Information on the aroma differences between wax gourd and beverage will be useful to food processors. The object of this study was to determine the volatile components of the wax gourd and the beverage.

MATERIALS & METHODS

Materials

Wax gourd was obtained from the local market. Commercial sugar from Taiwan Sugar Company was used in the wax gourd beverage preparation. n-Pentane, diethyl ether and CaO were reagent grade obtained from E. Merck Company (Darmstadt, W. Germany). The solvents were purified by Vigreaux column distillation. Pyrazines used as authentic compounds in the identification of volatile components were purchased from Pyrazine Specialties Company (Atlanta, GA.).

Preparation of wax gourd beverage

Wax gourd was peeled, the central soft part removed and cut to approximately 45 × 4.5 × 4.5 mm slices. To 1 kg strips, 50g CaO and 750 mL water were added in a stainless container, and the slices were soaked in the liquid. The container was covered with a single layer of cheesecloth. After aging at room temperature (17–25°C) for 18 hr, the slices and liquid were blended in a blender and filtered through a double layer of cheesecloth. The filtrate was settled to obtain a clear upper layer. For every mL clear filtrate, 1g sugar was added and the solution heated with occasional stirring. Heating was continued until the liquid temperature reached around 115°C. The time needed was 6–8 hr; then, the mixture was cooled and solidified. The solid product obtained was a concentrated form of wax gourd beverage which could be sold commercially. Water is added to dissolve the solid product to prepare a drink. Usually, water is added 5–7 times the amount of the solid product.

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Sample preparation

Two types of samples were prepared: (1) 2 kg wax gourd, after blending with 1200 mL distilled water: (2) wax gourd beverage solid product which was produced from 2 kg wax gourd, with 1000 mL distilled water added. Volatile components of the sample were extracted for 2.5 hr in a Likens-Nickerson apparatus (Romer and Renner, 1974). Redistilled pentane and ether (1:1) were used as extracting solvent. n-Nonanal was added as an internal standard. The volatile extracts were dried with anhydrous Na_2SO_4 and concentrated to about 0.5 mL by using a spinning band distillation apparatus (Kontes Co., NJ). The concentrate was then transferred to an open glass capillary tube with one end sealed and heated in a water bath at 40°C to volume of about 50 μL.

Gas chromatography (GC)

GC was carried out on a Hewlett-Packard 5880A gas chromatograph, equipped with dual flame ionization detectors. Two 50m × 0.2 mm fused silica columns individually coated with Carbowax 20 M and OV-1 (Chrompack International, B.V.) were used as dual columns. The samples were divided equally and introduced into two columns after injection. The oven temperature was held at 50°C for 5 min programmed linearly from 50°C to 200°C at 1°C/min and then held at 200°C for 35 min. Carries gas was hydrogen at a flow rate of 20 mL/min.

The peak area reported by the dual flame ionization detectors was calculated by a dual built-in integrator (Hewlett-Packard 5880A GC

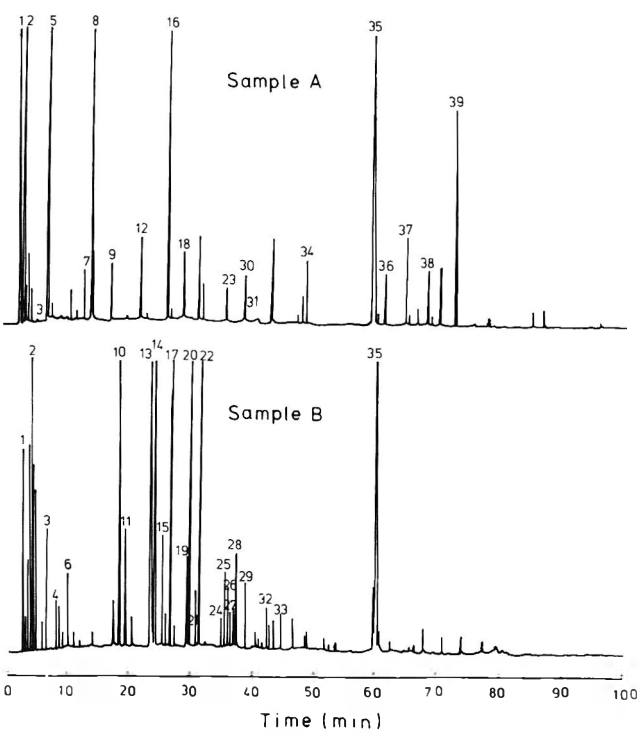

Fig. 1—Gas chromatogram of volatile compounds of wax gourd beverage samples by Carbowax-20M column. Sample A, was wax gourd; sample B, was wax gourd beverage, final product. Compounds are identified by numbers shown in Table 1.

terminals). The linear retention indices of the volatile components were calculated using n-parafffin (C_8-C_{25}) (Alltech Associates, Canada) as references (Majlat et al., 1974).

Gas chromatography-mass spectrometry (GC-MS)

GC-MS was carried out on a Hewlett-Packard 5985B system, and operational parameters were as follows: carrier gas, helium; ionization voltage, 70 eV; electron multiplier voltage, 2400 V; ion source temperature, 200°C.

RESULTS & DISCUSSION

FIGURE 1 shows the gas chromatogram of the isolated volatiles of the wax gourd and wax gourd beverage. Table 1 shows the composition of volatiles identified. Most of the compounds were identified by comparing the Kovats indices and mass spectra (Carbowax-20M and OV-1 columns) of the components with authentic compounds. The major compounds identified in wax gourd were (E)-2-hexenal, n-hexanal and n-hexyl formate. (E)-2-Hexenal had a powerful green fruity, pungent vegetable like odor, pungent in high concentrations, almost acrylic-sharp, but pleasant fruity and fresh-green in dilutions below 0.1%. n-Hexanal had a very powerful, penetrating, fatty green, grassy odor while n-hexyl formate had a strongly penetrating, ethereal-fruity odor, refreshingly green, and reminiscent of unripe plums and apples (Arctander, 1969). However, 2.5-dimethylpyrazine, 2,6-dimethylpyrazine, 2,3,5-trimethylpyrazine, 2-methylpyrazine, and 2-ethyl-5-methylpyrazine were the major compounds in the wax gourd beverage. Pyrazines have been characterized as significant contributers to the unique flavor and aroma associated with the roasting or toasting of numerous foods. Naturally occurring pyrazines have also been isolated from food systems that have not undergone heat treatment (Maga and Sizer, 1975). However, in many food systems, pyrazine compounds play a minor role, but in the wax gourd beverage volatiles were over 75% pyrazine compounds. The aroma of the wax gourd and the beverage were completely different.

Wax gourd contains 0.5g glucose and 0.5g fructose per 100g edible portion, but no sucrose (Wills et al., 1984). However, sucrose was added in the processing. Wu et al. (1984) reported wax gourd contained 132 mg% free amino acids, but after 18 hr CaO aqueous solution soaking, amino acids increased to 208 mg%, further increased to 229 mg% after sugar addition and initial heating, and then decreased to 67 mg% in the final solid form product. The above values were on wet weight basis of wax gourd. Figure 2 shows the changes in pH and moisture during wax gourd beverage processing. Before heating, pH was higher than 11; however, after 330 min heating, the value

Table 1—Volatiles identified in wax gourd and wax gourd beverage

Peak No.[c]	Compound	MW	Ik[d] CW-20M	Ik[d] OV-1	Sample A[e]	Sample B[e]	I.D.
1	solvent						
2	ethyl acetate	88	883		4.96	12.00	a
3	3-methylbutanal	86	917		1.01	3.53	b
4	2,3-pentanedione	100	1044	681	–[g]	2.33	b
5	n-hexanal	100	1079	788	31.40	–	a
6	2,3-heptanedione	128	1138	816	–	3.27	b
7	(Z)-3-hexenal	98	1176	834	1.79	–	a
8	(E)-2-hexenal	98	1189	817	48.71	–	a
9	isoamyl alcohol	88	1231	728	2.20	–	a
10	2-methyl pyrazine	94	1266	800	–	30.67	a
11	unk[f]-45(100), 43(59.5) 88(11.5), 29(7.4)	–	1281	–	–	7.33	
12	(E)-2-heptenal	112	1286	859	3.60	trace	a
13	2,5-dimethylpyrazine	108	1325	884	–	121.13	a
14	2,6-dimethylpyrazine	108	1331	888	–	48.53	a
15	2,3-dimethylpyrazine	108	1341	892	–	9.87	a
16	n-hexyl formate	130	1347	967	22.98	–	a
17	unk[f]-96(100), 67(87.7). 53(56.3), 39(38.0)	–	1359	828	–	19.07	
18	(E)-2-hexen-1-ol	100	1361	841	3.19	–	a
19	2-ethyl-6-methylpyrazine	122	1367	986	–	6.73	a
20	2-ethyl-5-methylpryazine	122	1386	971	–	29.67	a
21	2-ethyl-3-methylpyrazine	122	1392	972	–	1.57	a
22	2,3,5-trimethylpyrazine	122	1404	974	–	37.93	a
23	1-octen-3-ol	128	1407	957	1.80	–	a
24	2-ethyl-3,6-dimethylpyrazine	136	1408	1051	–	2.08	a
25	2-ethyl-3,5-dimethylpyrazine	136	1446	1056	–	4.68	a
26	furfural	96	1449	814	trace	4.37	a
27	2,5-diethylpyrazine	136	1456	1048	–	2.57	a
28	2,6-diethylpyrazine	136	1462	1052	–	7.27	a
29	2,3,5,6-tetramethylpyrazine	136	1478	1058	–	4.62	a
30	(E,E)-hepta-2,4-dienal	110	1480	–	2.69	–	a
31	benzaldehyde	106	1489	964	0.38	trace	a
32	3.5-diethyl-2-methylpyrazine	150	1491	1133	–	3.15	a
33	2.3-diethyl-6-methylpyrazine	150	1515	1135	–	2.33	a
34	(E,E)-nona-2,4-dienal	136	1548	1185	3.71	–	a
35	n-nonanol	144	1632	1162	I.S.[h]	I.S.	a
36	10-undecen-1-ol	170	1664	–	3.18	–	b
37	unk[f]-57(100), 43(51.8), 82(47.6), 41(43.6)	–	1695	–	5.07	–	
38	2-hexylfuran	152	1712	–	4.37	–	b
39	(E,E)-deca-2,4-dienal	152	1765	1541	14.45	–	a

[a] By comparison of retention time and mass spectrum with that of authentic compound.
[b] The mass spectrum or retention time was consistent with that of published data (De Brauw et al., 1981; Heller and Milne, 1980) (tentative identification).
[c] Number refers to Fig. 1.
[d] Calculated Kovats' indices.
[e] Sample A, wax gourd; sample B, wax gourd beverage, final product. Unit is 10^{-6} g/100g by wet basis according to wax gourd.
[f] Mass spectra data: M/Z (relative intensity).
[g] Not detected.
[h] Internal standard.

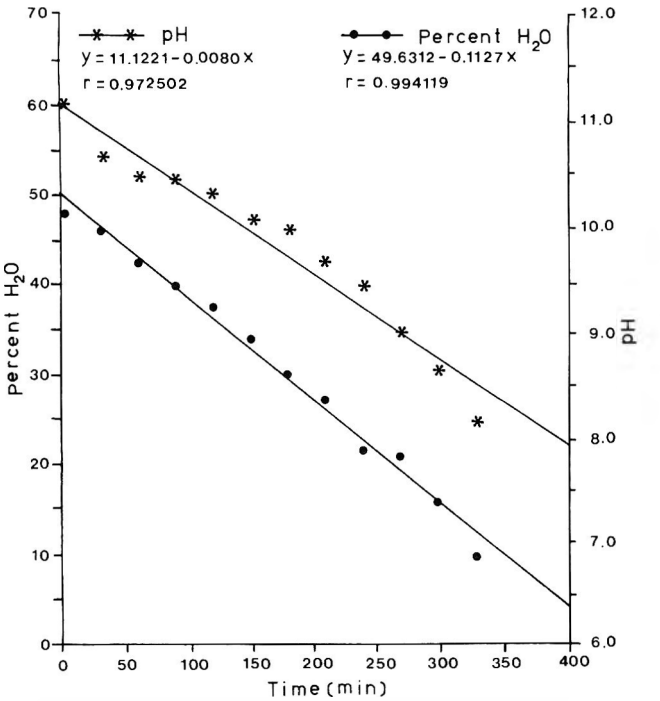

Fig. 2—Changes in pH value and moisture content during wax gourd beverage processing.

decreased to around 8. When water (5–7 times) was added to prepare a drink, the pH value decreased to neutral range.

In the presence of free amino acids, sugars and alkaline pH, Maillard reactions occur readily after heating. Koehler et al. (1969) used radioisotopic labeling techniques to establish the source materials for pyrazine formation and concluded that the carbon in pyrazine compounds came from the sugar and the nitrogen from the amino acids. Shibamoto and Bernhard (1976) reported addition of hydroxide ion increased total pyrazine formation in model systems. Pyrazine formation pathways in foods have been studied intensively using model reactions, mainly with amino acids and sugars as reactants (Newell et al., 1967; Koehler and Odell, 1970; Shibamoto and Bernhard, 1976). From the volatile compounds identified (Table 1), it is postulated that the formation of volatiles of wax gourd beverage followed a pathway similar to the model reactions described above. The pyrazine compounds likely were formed mainly from monosaccharides and amino acids of the wax gourd during processing of the beverage.

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Grateful acknowledgment is made for financial support from the Council for Agricultural Planning & Development (CAPD) of the Republic of China.

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Presented at the 45th Annual Meeting of the Institute of Food Technologists, Atlanta, GA, June 9–12, 1985.

The authors gratefully acknowledge the assistance of Mr. S.R. Pulusani (Dept. of Food Science & Animal Industries, Alabama A&M Univ., Normal, AL) in amino acid analyses and Mr. C.H. Price (VPI & SU) in EDX-ray analyses spectra.

Amino Acid Profiles of Common Cultivated Mushrooms Including the Identification of *N*-(*N*-γ-L-Glutamyl-3-Sulfo-L-Alanyl)Glycine in *Flammulina velutipes*

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ABSTRACT

Amino acid profiles of nonprotein nitrogen (NPN) and protein nitrogen (PN) fractions of common cultivated mushrooms were determined. Free amino acids in the NPN fractions were generally characterized by the predominant occurrence of alanine and amino acids metabolically related to the glutamic acid family and also by the common presence of unique nonprotein amino acids, such as saccharopine, cystathionine and ethanolamine. A novel glutathione analog, *N*-(*N*-γ-L-glutamyl-3-sulfo-L-alanyl)glycine, was found in *Flammulina velutipes*. The proportion of the NPN to the total nitrogen was relatively high and large amounts of the NPN were made up of free amino acids and related compounds. Protein amino acids in the NPN and PN fractions accounted for about 65% of the total nitrogen, suggesting that a practical nitrogen-protein conversion factor for the mushrooms may be considered to be about 4 on the average.

INTRODUCTION

BY THE DEVELOPMENT of the mushroom industry in Japan, many kinds of cultivated mushrooms have acquired popularity as common food in addition to usual use as condiments. The fruiting bodies, actively growing parts of the mushrooms, contain a number of unusual nitrogenous compounds, especially free amino acids and related compounds (Chilton, 1982), which are predominant constituents of the nonprotein nitrogen (NPN) fractions that should be considered from the nutritional view point as a promising food source (Crisan and Sands, 1978; Lampe, 1983).

The NPN of the common cultivated mushrooms, *Agaricus bisporus* (Oka et al., 1981) and *Pleurotus ostreatus* (Oka et al., 1984), consists primarily of free amino acids and related compounds which account for a large part of the nitrogen. The purpose of this study was to obtain more detailed data, especially amino acid profiles of the NPN and protein nitrogen (PN) fractions, including the occurrence of a novel glutathione analog in *Flammulina velutipes*, to increase the very limited information on the nitrogen profiles of the cultivated mushrooms.

MATERIALS & METHODS

Materials

Freshly harvested fruiting bodies of the cultivated mushrooms (edible stage, see Table 1), *Flammulina velutipes* (Enokitake), *Lyophyllum ulmarium* (Shirotamogitake), *Pholiota nameko* (Nameko), were obtained from commercial growers; *Grifolia frodosa* (Maitake) was kindly supplied by Dr. Taniguchi (Takara Central Research Institute, Otsu, Japan). *N*-(*N*-γ-L-glutamyl-3-sulfo-L-alanyl)glycine used as the analytical standard was prepared by performic acid oxidation of glutathione according to Calam and Waley (1962). The other chemicals and reagents in this study were the same as described in the previous papers (Oka et al., 1981; Oka et al., 1984).

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Preparation of NPN and PN fractions

Pooled fruiting bodies (about 1 kg) of the mushrooms were homogenized and extracted with three volumes of ethanol with continuous stirring for 1 hr at 25°C. The extraction with 75% (v/v) ethanol was repeated following centrifugation at 8,000 × *g* for 20 min, and the combined extracts (degignated as the NPN fractions in this experiment) were used for the preparation of the amino acid fractions as described earlier (Oka et al., 1981). The 75% (v/v) ethanol-insoluble fractions (designated as the PN fractions) were hydrolyzed with 6N HCl at 105°C for 24 hr and subjected to amino acid analyses.

Analyses

The amino acids and related compounds were determined with a Hitachi KLA-5 automatic amino acid analyzer equipped with an analytical system for physiological fluids (Oka et al., 1981). Unusual ninhydrin-positive compounds detected in the amino acid fractions were isolated chromatographically and subjected to chemical and spectroscopic analyses (Oka et al., 1981; Oka et al., 1984). Nitrogen and moisture were determined according to standard methods (AOAC, 1980).

RESULTS & DISCUSSION

Occurrence of *N*-(*N*-γ-L-glutamyl-3-sulfo-L-alanyl)glycine in *Flammulina velutipes*

An unidentified ninhydrin-positive compound (FX-1) found in the acidic amino acid fraction (Oka et al., 1981) of *F. velutipes* was purified by preparative paper chromatography and electrophoresis in a similar manner as described previously (Oka et al., 1979). The chromatographically pure FX-1 became ninhydrin-negative by spraying Cu^{2+} reagent (Offord, 1969) on paper chromatograms and gave equimolecular amounts of 3-sufoalanine (cysteic acid), glutamic acid and glycine by acid hydrolysis (6M HCl, 100°C for 20 hr), while glutamic acid was liberated even under mild hydrolytic condition (1M HCl, 100°C for 2 hr). This fact and the result of Sanger's degradation with 2,4-dinitrofluorobenzene (Frankel-Conrat et al., 1958) suggested that glutamic acid was the N-terminal amino acid and that FX-1 was a γ-glutamyl peptide (Ogawa, 1974) having a sequence of glutamyl-3-sulfoalanylglycine. The 3-sulfoalanine moiety was determined to have the L-configuration by the chromatographic method of Manning (1972) and the glutamic acid moiety was proved to be of the L-configuration by complete degradation with L-glutamic acid decarboxylase (EC 4.1.1.15) (Guilbault, 1976). The structure, *N*-(*N*-γ-L-glutamyl-3-sulfo-L-alanyl)glycine, was further confirmed by comparison of the chromatographic behavior with that of the standard compound.

F. velutipes also contains small amounts of glutathione disulfide (oxidized glutathione, see Table 1), which did not undergo further oxidation during purification and analyses. Although the compound identified here has been reported as an artificial product from glutathione exposed to hydrogen peroxide during food processing (Finley et al., 1981) and to cryotherapeutic drugs (Au[III] compounds) (Witkiewicz and Shaw, 1981), this is the first report of the occurrence of a unique glutathione analog in nature. Recently, it was shown that this compound markedly increased sensory test scores of some con-

Table 1—Free amino acids and related compounds in cultivated mushrooms

Compounds[b]	Mushroom[a]			
	F. velutipes	*L. ulmarium*	*P. nameko*	*G. frondosa*
	(Amount, μmol/g fruiting bodies, fresh weight)[c]			
N-(*N*-γ-L-Glutamyl-3-sulfo-L-alanyl)glycine	0.25	—	—	—
Phosphoserine	tr[h]	0.14	tr	tr
Phosphoethanolamine	tr	tr	0.04	tr
Urea	12.51	11.79	0.22	tr
Aspartic acid	1.61	0.36	2.04	0.12
D_s-*erythro*-2-Amino-3,4-dihydroxybutanoic acid[d]	—[h]	2.15	—	—
D_s-*erythro*-2-Amino-4-ethoxy-3-hydroxybutanoic acid[e]	—	0.14	—	—
Threonine	0.70	1.21	1.52	0.50
Serine	1.30	2.62	1.35	1.11
N-(γ-L-Glutamyl)ethanolamine	0.41	0.06	0.02	tr
Asparagine	1.11	1.24	1.15	0.25
Glutamic acid	10.04	9.42	6.30	2.08
Glutamine	5.00	9.04	2.62	1.04
Glutathione disulfide	0.01	—	—	—
Saccharopine lactam[f]	0.08	—	—	—
N^{δ}-Acetyl-L-ornithine	—	0.21	—	—
α-Aminoadipic acid	0.06	0.12	0.09	0.01
Proline	0.14	0.55	0.78	0.50
Glycine	0.83	1.54	1.86	0.64
Alanine	4.54	4.12	3.57	2.60
Citrulline	0.08	tr	tr	tr
α-Amino-*n*-butyric acid	0.04	0.10	0.05	tr
Valine	0.59	1.54	1.50	0.84
Saccharopine	1.59	0.22	0.78	0.09
Cystine	0.05	0.59	0.21	0.09
Methionine	0.16	tr	0.06	0.06
Cystathionine	0.23	0.60	0.16	tr
Isoleucine	0.38	0.83	0.90	0.29
Leucine	0.70	1.16	1.31	0.17
Tyrosine	0.63	0.48	0.25	0.41
β-Alanine	0.47	tr	tr	0.02
Phenylalanine	0.58	0.96	0.46	0.26
γ-Aminobutyric acid	1.07	0.79	0.22	0.18
Ornithine	2.05	7.83	0.96	1.29
Ethanolamine	0.06	0.08	0.03	0.05
Ammonia[g]	1.59	0.66	0.59	0.37
Lysine	1.72	1.59	0.03	0.26
Histidine	1.31	0.66	0.40	0.52
Arginine	1.01	9.41	0.55	1.05

[a] Average fresh weight of a fruiting body at edible stage; *F. velutipes*, 0.4g; *L. ulmarium*, 2.8g; *P. nameko*, 2.3g; *G. frondosa*, 247g.
[b] Listed according to the elution positions on the amino acid analyzer (Oka et al., 1981).
[c] All the values are average of the duplicate determination of pooled samples corresponding to about 1 kg of the fruiting bodies.
[d] From Ogawa et al. (1984).
[e] From Ogawa et al. (1985).
[f] Identified according to Nabeta et al. (1973), but whether the lactam derivative is a natural constituent must be determined because of the easy conversion of saccharopine to its lactam (Nabeta et al., 1973).
[g] The values in 75% (v/v) ethanol extracts were adopted.
[h] tr, trace (less than 0.01 μmol/g fruiting bodies, fresh weight); —, not detected.

Table 2—Amino acid composition of the protein nitrogen fraction of cultivated mushrooms

Amino acid	Mushroom					
	F. velutipes	*L. ulmarium*	*P. nameko*	*G. frondosa*	*A. bisporus*[a]	*P. ostreatus*[b]
	(μmol/g fruiting bodies, fresh weight)[c]					
Aspartic acid	21.60	12.80	9.79	16.78	8.98	16.33
Threonine	12.82	6.60	9.17	9.94	5.68	11.52
Serine	12.67	6.13	9.45	12.01	5.41	6.25
Glutamic acid	19.58	12.06	13.47	18.56	9.87	17.16
Proline	3.13	1.52	3.75	8.51	6.27	8.36
Glycine	16.91	9.70	13.55	16.45	7.29	14.73
Alanine	16.74	10.98	12.02	16.31	7.22	15.89
Valine	11.93	7.24	12.38	10.66	4.64	17.50
Cystine	tr	0.60	0.47	0.49	0.31	1.27
Methionine	0.67	1.54	0.98	1.28	0.69	2.05
Isoleucine	9.20	5.66	6.28	8.44	4.03	5.20
Leucine	16.17	10.15	9.32	14.19	6.60	10.41
Tyrosine	3.98	2.12	1.85	3.83	2.07	2.99
Phenylalanine	7.63	4.28	4.00	6.67	3.49	4.76
Lysine	7.21	12.36	4.75	11.62	7.10	15.89
Histidine	3.44	1.68	2.19	3.61	1.88	3.60
Arginine	2.52	4.92	3.57	11.87	3.84	10.91
Glucosamine[d]	13.83	14.72	20.36	11.87	7.79	17.60

[a] From Oka et al. (1981).
[b] From Oka et al. (1984).
[c] All the values are the average of duplicate determinations of pooled samples corresponding to about 1 kg of the fruiting bodies.
[d] See the text.

Table 3—Nitrogen and protein of cultivated mushrooms

	Mushroom					
	F. velutipes	*L. ulmarium*	*P. nameko*	*G. frondosa*	*A. bisporus*[a]	*P. ostreatus*[b]
	(g/100g fruiting bodies, fresh weight)					
Moisture[c]	88.7	89.2	90.1	91.7	91.3	91.0
Nitrogen (N)						
Total-N[c]	0.52	0.51	0.34	0.47	0.62	0.62
N in NPN fraction	0.20 (38)[d]	0.17 (33)	0.14 (41)	0.07 (15)	0.39 (63)	0.28 (45)
N in PN fraction	0.32 (62)	0.33 (65)	0.20 (59)	0.40 (85)	0.23 (37)	0.34 (55)
Total protein amino acid-N[e]	0.30 (59)	0.32 (61)	0.24 (71)	0.33 (70)	0.35 (57)	0.40 (64)
Protein amino acid-N in NPN[f]	0.06 (12)	0.13 (25)	0.05 (15)	0.02 (4)	0.19 (31)	0.12 (19)
Protein amino acid-N in PN[g]	0.24 (47)	0.19 (36)	0.19 (56)	0.31 (66)	0.16 (26)	0.28 (45)
Protein						
I[h]	2.1	2.0	1.4	1.9	2.5	2.5
II[i]	2.1	1.8	1.5	2.0	1.8	2.1
III[i]	2.7	3.3	1.1	3.7	3.9	3.3

[a] From Oka et al. (1981).
[b] From Oka et al. (1984).
[c] Values are the average of the duplicate determinations of pooled samples corresponding to about 1 kg fruiting bodies.
[d] Values in parentheses are percentages of total nitrogen.
[e] Calculated from Tables 1 and 2.
[f] Calculated from Table 1.
[g] Calculated from Table 2.
[h] Total-N × 4.
[i] Sum of the residual weights of whole protein amino acids.
[j] Cited in *Standard Table of Food Composition in Japan* (1982).

diments containing sodium glutamate and 5′-nucleotides (Mori et al., 1985).

Amino acid profiles of the NPN and PN fractions of the mushrooms

The free amino acids and related compounds in the NPN fractions of the fruiting bodies of the cultivated mushrooms are listed in Table 1. Alanine, glutamic acid and glutamine were found to be the predominant protein amino acids (used to express the amino acids found in normal proteins; Fowden, 1970). Cystathionine, saccharopine, ornithine, γ-aminobutyric acid and ethanolamine were commonly observed as nonprotein amino acids (used to express the amino acids other than protein amino acids; Fowden, 1970) in all the mushrooms examined as well as in *A. bisporus* (Oka et al., 1981) and *P. osteratus* (Oka et al., 1984). Among them, abundance of the amino acids metabolically related to the glutamic acid family, such as glutamic acid, glutamine, γ-aminobutyric acid, ornithine and arginine, were shown to be characteristic of the free amino acid profiles of cultivated mushrooms. Similar observation has been reported with other mushrooms (Sato et al., 1985). The values in the table show the average of duplicate determinations of pooled samples of a given mushroom (about 1 kg). Although there are no comparable data on the amino acid profiles, it is noted that intrinsic compositional variability may arise among different samples of a given species if the strain or the compost is changed (Crisan and Sands, 1978).

During the course of the studies on the profiles of the ninhydrin-positive compounds in the mushrooms, it was shown that an identification of an amino acid based solely on the particular elution position on the chromatograms of the amino acid analyzer would sometimes lead to error. For example, on the chromatograms of the amino acid analyzer, equipped with the standard system for physiological fluids (Oka et al., 1981), elution peaks of *N*-(γ-L-glutamyl)glycine in *A. bisporus* and D_s-*erythro*-2-amino-3,4-dihydroxybutanoic acid in *L. ulmarium* overlapped that of aspartic acid; *N*-(γ-L-glutamyl)ethanolamine in several mushrooms, that of serine; and saccharopine, that of cystine in all the mushrooms tested. Especially, D_s-*erythro*-2-amino-3,4-dihydroxybutanoic acid, which occurs in *L. ulmarium* at relatively high concentrations, has probably been determined as aspartic acid. When the free amino acid fraction of *L. ulmarium* was further treated with a column of Dowex 1 (acetate form) to remove acidic amino acids, the fraction that passed through the column still contained sizable amounts of a ninhydrin-positive compounds emerging at the elution position of aspartic acid. Also the peak area of cystine was laregly reduced in size, indicating the removal of saccharopine which occurs in the mushrooms as a common nonprotein amino acid. These facts suggest that group separation of the free amino acid fractions is required, at least, prior to analyses with the automatic amino acid analyzer as described previously (Oka et al., 1981).

The amino acid profiles of the hydrolyzates of the PN fractions are shown in Table 2, together with those of *A. bisporus* (Oka et al., 1981) and *P. ostreatus* (Oka et al., 1984). Mushroom proteins are relatively abundant in essential amino acids except for sulfur amino acids and are considered to be nutritionally comparable to some common legumes and vegetables as noted by Crisan and Sands (1978). In the PN fractions, glucosamine was found as the only ninhydrin-positive compound except for the protein amino acids and ammonia; glucosamine may be derived from chitin-like polysaccharides and is a predominant source of nonprotein nitrogen in the PN fractions.

Contribution of amino acids to the nitrogen profiles of mushrooms

The nitrogen profiles of the cultivated mushrooms are summarized in Table 3. The total nitrogen of the mushrooms at the edible stage ranged from 0.34g in *P. nameko* to 0.62g in *A. bisporus* per 100g of fresh fruiting bodies. While the NPN of the nitrogen varied from 15% in *G. frondsa* to 63% in *A. bisporus*, significant proportions were made up of free amino acid nitrogen, which accounted for about 60% of the NPN on the average. These NPN fractions would have an important role in providing the protein amino acids in the mushrooms. Protein amino acid nitrogen in the PN fractions to total nitrogen, calculated from the data in Table 2, ranges between a low of 26% for *A. bisporus* and a high of 65% in *G. frondosa*. However, nitrogen of whole protein amino acids occurring in either free or bound forms accounted for 56–71% (63% on the average) of the total nitrogen obtained by the Kjeldahl method (Table 3). Since the Kjeldahl method does not distinguish between protein and nonprotein nitrogen, the above mentioned value, 63%, can be used to evaluate the protein of the mushrooms. Based on this assumption, a practical nitrogen-protein conversion factor for the cultivated mushrooms may be considered to be about 4 on the average. This value is consistent with that previously reported by Crisan and Sands (1978) who suggested that crude protein calculated as Kjeldahl nitrogen × 4.38 may be better indication of nutritive value when consid-

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Sensory, Acoustical, and Force-Deformation Measurements of Potato Chip Crispness

ZATA M. VICKERS

ABSTRACT

The auditory (sound only) and oral (normal biting and chewing) crispness of five different potato chip products, each adjusted to two moisture contents, were measured. Instrumental acoustical and force-deformation measurements were made on these same samples and compared with the sensory crispness measurements. Oral crispness judgments appeared to be made on the basis of both auditory and oral tactile sensations, since auditory crispness was not highly correlated to oral crispness. A combination of acoustical and force-deformation measurements provided an excellent measure of oral chip crispness.

INTRODUCTION

EFFORTS to understand the sensation of cripsness and efforts to develop instrumental measures of crispness are mutually supportive. Knowledge of the sensations of crispness provides the basis for selecting appropriate instrumental measures of the stimuli, whereas useful instrumental measurements provide clues to the nature of the sensations.

The sensation of crispness is currently hypothesized to be based on the vibrations produced by a food as it is bitten or chewed (Christensen and Vickers, 1981). These vibrations may be perceived by both auditory and oral tactile senses. If this hypothesis is true one would expect judgments of crispness made on only the sounds of foods being bitten and chewed to closely match the crispness judgments made when subjects actually bit and chewed the foods. However, the two studies that have examined the relationships between auditory only and oral judgments of crispness have not found the two sets of judgments to be closely matched (Vickers, 1981; Edmister and Vickers, 1985). Both of these studies examined a variety of food products in an effort to reach general conclusions about crispness that would apply to all food products; this variety may have blurred an otherwise large correlation. The failure of auditory and oral judgments of crispness to match closely also suggests that there are some non-auditory components of the crispness sensation. This in turn supports the suggestion of Mohamed et al. (1982) that a combination of acoustical and force-deformation instrumental measurements could potentially provide a better measurement of sensory crispness than either type of measurement alone.

Few scientists have published their attempts to measure the crispness of potato chips instrumentally. Iles and Elson (1972) placed potato crisps conditioned to various relative humidities on a supporting ring and broke them with a probe desending through the ring. They found that deformation to rupture decreased significantly as crispness increased. Bourne et al. (1966) used a similar test method and found that the slope of the force-deformation curve appeared to increase with increasing crispness. They also noted that the area under the resulting force-deformation curve, a measure of work, was greater for the chewier potato chip than either the very crisp potato chip or the wet chips. Katz and Labuza (1981) used a snap test to measure crispness in potato chips, but concluded that their analyses did not produce any useful quantitative information for indicating crispness intensity. They found that potato chips, due to irregular size, shape, curvature, and inconsistent fracturing pattern, did not produce a consistently shaped force-deformation curve.

Although there are no reported cases of acoustical measurements of potato chip crispness, Edmister and Vickers (1985) and Mohamed et al. (1982) have measured crispness in other food products using acoustical techniques. Both found that measures indicating the loudness of the sounds correlated most closely with crispness.

One objective of the present study was to compare auditory and oral judgments of crispness made only on a single product type, potato chips, to determine whether the two types of crispness judgments were more closely related when only one type of product, not a wide variety, was studied. A second objective of this research was to develop an instrumental measurement of crispness for potato chips.

MATERIALS & METHODS

Sample preparation

The following five potato chip products were purchased at a local supermarket during March 1985: Pringles regular chips, Pringles light chips, Fritolay Ruffles, Fritolay O'Grady's extra thick and crunchy, and Fritolay Lays Regular Potato chips. These chips were used for all the sensory and acoustical testing. Another group of the same five potato chip products were purchased in July 1985 for the Instron tests. The potato chips from each type were divided into two lots. One lot was held for 2 wk in a desiccator containing a saturated solution of LiCl (for a water activity of 0.11); the other lot was held in a desiccator over a saturated solution of K_2CO_3 (for a water activity of 0.44). These moisture adjusted chips were then held in airtight containers until used. All potato chips used were unbroken; otherwise no attempt was made to select chips according to size or contour.

Preparation of tape recording

Each of four different subjects (2 men and 2 women) bit and chewed twice five chips from each of the ten potato chip samples. During both the biting and chewing, a microphone (Bruel and Kjaer 4133) was held against the outer ear immediately above the opening of the ear canal. Sounds were recorded on Scotch Brand 227 Audio Recording Tape using a Nagra IV-SJ tape recorder (tape speed 19 cm/sec). A Bruel and Kjaer 2619 pre-amp and a QSJA-BK microphone amplifier were connected between the microphone and the tape recorder. The four best (free of extraneous noise) bite and two-chew sounds for each potato chip from each subject were removed and used for the final tapes. Four different tapes were prepared. Each tape contained forty "bite and two-chew" sounds (four sounds from each of the ten potato chips). The four sounds for a specific product on a single tape were chosen randomly from the pool of sixteen sounds (four sounds × four subjects) and grouped together on the tape. The ten four-sound groups were ordered randomly on the tapes. Individual sounds on all the tapes were separated by segments of non-magnetic tape.

Subjects

The 20 subjects were students and staff from the St. Paul Campus of the Univ. of Minnesota (14 females, 6 males, ages 20-35 years). They were paid for their participation.

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Sensory testing procedure

Each subject was tested separately by an experimenter who was present throughout the test session to give instructions, serve the samples, and operate the tape recorder. Subjects evaluated the crispness intensity of the foods and tape recorded sounds using a 170 mm line scale labelled "not crisp" at the left end and "extremely crisp" at the right end. When judging the potato chip samples, subjects were asked to bite the chips and chew them at least twice. When judging the tape recorded sounds, subjects were told they would be listening to tape recorded sounds of other people biting and chewing potato chips. They were asked to listen to all four sounds for a specific chip before making their crispness judgment. Half the subjects judged the sounds first; half judged the chip samples first. All judgments were replicated, meaning each subject judged 20 groups of four sounds (one tape followed by a second tape) and 20 chip samples (a randomly ordered set of ten followed by a second randomly ordered set of ten).

Acoustical analysis of the sounds

Individual food sounds were displayed on a Tektronix 5111 storage oscilloscope. The following parameters were measured on the "bite" portion of each of the 16 sounds for each of the ten products: (1) the number of peaks or sound occurrences, (2) the mean height of all the peaks in volts, and (3) the duration of the sound. Only those peaks with heights equal to or greater than the arbitrarily chosen level of 0.05 volts were counted.

Instron analysis of the chips

This test consisted of 'biting' the chip between two horizontal bars made with 2 mm diameter steel rods about 100 mm long connected to aluminum bars 25 mm high. The chip rested (partially held by hand) on the stationary lower bar while the parallel upper bar was driven downward at 500 mm/min. This test was chosen because it imitates the bite which has been shown in other studies (Christensen and Vickers, 1981; Vickers, 1985) to be important for judging crispness. Measures of (1) peak force, (2) slope of the force-deformation curve at its steepest point, and (3) area under the force-deformation curve from the time the upper bar contacted the food to the deformation at which the peak force occurred, were made on ten samples of each product at each water activity. The upper bar descended to about 0.5 mm above the lower bar before reversing direction. All the chips except the O'Grady's had broken and fallen away from the bars at that point.

Analysis of data

The sensory scores were determined by measuring the distance in mm from the "not crisp" end of the scale to the subject's mark. Only the scores from each judge's second replicate were used for the analysis. Although the correlations between the mean scores of the two replicates were 0.95 (oral crispness) and 0.88 (auditory crispness), the first replicate was considered as practice. Regression analyses were used to analyze mean values from the instrumental measurements and combinations of these values for their ability to predict mean sensory crispness scores.

RESULTS

Auditory vs oral crispness

Oral and auditory crispness scores are shown in Table 1, and the relationship between the scores is shown in Fig. 1. The correlation coefficient for this relationship is 0.85, slightly higher than correlation coefficients shown earlier by Vickers (1981) (r = 0.082), and considerably higher than that shown by Edminster and Vickers (1985) (r = 0.65). However, it is not large enough to suggest that auditory crispness of potato chips could be used to predict oral crispness.

Table 1—Mean crispness scores[a] of potato chip samples

Potato chip	Oral crispness	Auditory crispness
Ruffles $a_w = 0.11$	115	127
Ruffles $a_w = 0.44$	83	108
O'Gradys $a_w = 0.11$	107	125
O'Gradys $a_w = 0.44$	63	87
Lays $a_w = 0.11$	120	128
Lays $a_w = 0.44$	88	89
Regular Pringles $a_w = 0.11$	107	120
Regular Pringles $a_w = 0.44$	79	76
Light Pringles $a_w = 0.11$	109	124
Light Pringles $a_w = 0.44$	85	75

[a] Expressed as mm from the "not crisp" end of the 170 mm lines.

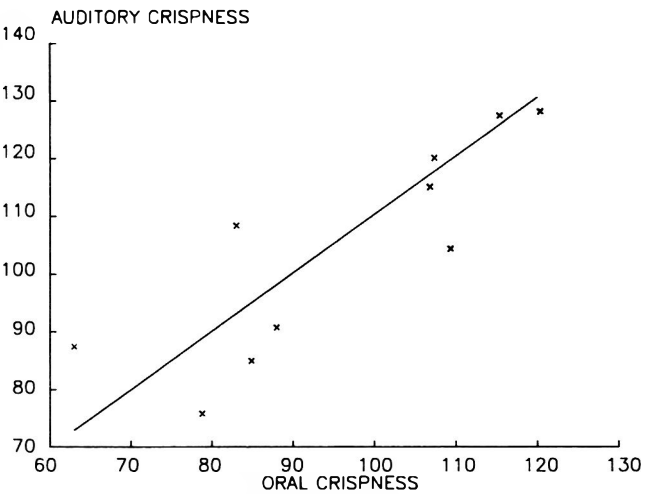

Fig. 1—Auditory crispness (sensory judgments made on tape recorded sounds) vs oral crispness (sensory judgments made by normally biting and chewing) of potato chips. The line shown is the regression line (r = 0.85) for the first equation in Table 2. N = 20 subjects.

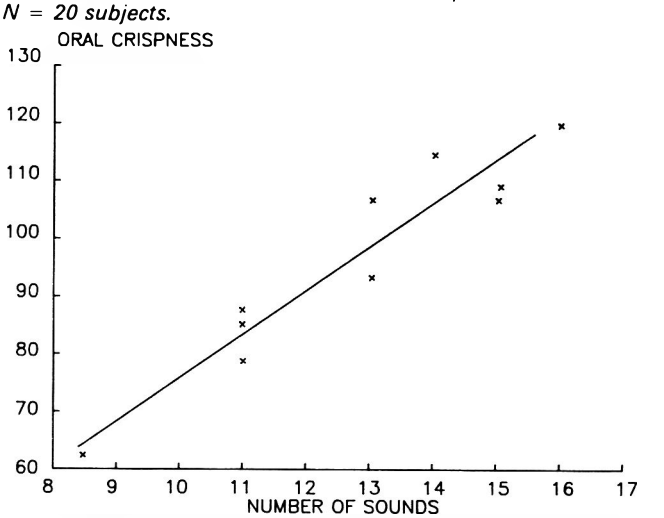

Fig. 2—Relationship between sensory crispness of potato chips and the number of sounds produced during biting (number of peaks) r = 0.92; n = 20 subjects.

Since oral judgments of crispness appear to be made on the basis of both auditory and oral tactile sensations, it might be possible to correct for the lack of oral tactile information by supplementing the auditory crispness scores with force-deformation information provided by the Instron testing. Regression equations relating oral crispness to auditory crispness and one of the three Instron measurements had correlation coefficients ranging from 0.96 to 0.97, a definite improvement over r = 0.85. The equations, shown in Table 2, are similar (because all three of the Instron measurements were highly correlated to one another). All show the Instron measures to be inversely related to crispness when combined with the auditory crispness scores. What this may mean to a person eating these foods is that force-deformation sensations of hardness, toughness, etc. may partly counteract the auditory sensations responsible for crispness.

Instrumental measures of crispness

The single best instrumental measure of oral chip crispness was the number of sounds produced during biting (r = 0.92, Fig.2). The

Table 2—Equations relating oral crispness to auditory crispness

Oral Crispness = 19.3 + 0.72 Auditory Crispness (0.16)[a]		(r = 0.85)
Oral Crispness = 25.4 + 0.75 Auditory Crispness (0.09)	− 10.5 Slope (2.4)	(r = 0.96)
Oral Crispness = 29.2 + 0.69 Auditory Crispness (0.09)	− 1.96 Area (0.49)	(r = 0.96)
Oral Crispness = 27.2 + 0.72 Auditory Crispness (0.08)	− 4.23 Peak Force (0.90)	(r = 0.97)

[a] Standard deviation of coefficient.

Table 3—Correlation matrix for some sensory and instrumental measures of crispness

	Oral crispness	Auditory crispness	Slope	Peak	Area	Duration	No. of sounds	MHP
Auditory crispness	0.85							
Slope	0.38	0.07						
Peak	0.46	0.01	0.97					
Area	0.51	0.09	0.86	0.96				
Duration	0.87	0.76	0.40	0.52	0.61			
No of sounds (NP)	0.92	0.84	0.38	0.49	0.58	0.98		
Mean height peaks (MHP)	0.12	0.43	0.75	0.77	0.73	0.17	0.09	
NP × MHP	0.71	0.88	0.35	0.27	0.17	0.52	0.59	0.75

duration of the biting sound (which was very highly correlated to the number of sounds) was also a good predictor of oral crispness (r = 0.87). The mean height of the sound bursts, a parameter that correlated well with sensory crispness in the Edminster and Vickers' (1985) study, correlated very poorly with either oral or auditory crispness. Correlation coefficients for these and other instrumental measurements are shown in Table 3.

The three measurements from the Instron were inversely related to oral crispness and unrelated to auditory crispness. Efforts were made to improve the correlation between oral crispness and instrumental measurements by combining more than one of the instrumental measurements. When adjusted for degrees of freedom, combinations of three parameters, (1) number of sounds (NP), (2) mean height of peaks (MHP), and (3) peak or slope of force-deformation curve, produced higher correlations with oral crispness than the number of peaks alone. The highest correlation, r = 0.99, was for the following equation:

$$\text{Oral Crispness} = -15.6 + 5.35(\text{NP}) + 133(\text{MHP}) - 6.21\ (\text{Peak})$$

(Standard deviations of coefficients) (0.54) (17.2) (0.94)

All combinations of NP, MHP and one of the force-deformation parameters had correlation coefficients of 0.98 or 0.99.

Since the correlation of 0.99 is higher than 0.92 obtained using the best instrumental parameter, it provides evidence for the idea that combinations of acoustical and force-deformation measurements can be more useful than either type of measurement alone.

The above equation also shows that crispness is positively related to the number of sound occurrences and the amplitude of these sounds. These two parameters together indicate the total amount of sound produced by biting the chip. The third parameter, peak force, is negatively related to crispness. Since the force measured by a bite test cell may indicate hardness or toughness, this may mean that such hardness or toughness counteracts the auditory sensations. The idea that other textural sensations could diminish crispness is in line with observations in other senses such as taste and smell that show counteractions among different odorants or tastants.

CONCLUSION

THE HYPOTHESIS that crispness is a vibratory sensation needs to be modified to allow for the influence of nonvibratory sensations. This study of potato chip product crispness suggests that these non-vibratory sensations may be detracting from or counteracting crispness. The use of a combination of acoustical and force-deformation measurements can produce an excellent prediction of potato chip crispness.

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Ms received 5/27/86; revised 9/8/86; accepted 9/26/86.

Paper No. 14,933 of the Scientific Journal Series of the Minnestoa Agricultural Experiment Station on research conducted under Minnesota Agricultural Experiment Station Project No. 18-60, supported by Hatch and GAR funds.

The author thanks Judith Dudziak for her technical assistance.

Rheological Properties of Tomato Concentrates as Affected by Particle Size and Methods of Concentration

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ABSTRACT

Shear rate-shear stress data were obtained on tomato concentrates made from juices that were produced using finisher screen openings (FSO): 0.020, 0.027, 0.033, and 0.045 in. In general, the apparent viscosity of the concentrates at a shear rate of 100 sec^{-1} increased with increase in FSO. However, concentrates made from juice using a 0.027 in FSO had the highest apparent viscosity. Magnitudes of yield stress of concentrates increased in direct proportion to FSO. Apparent viscosities of concentrates made by evaporating tomato juice were lower than those obtained either by evaporating the serum or by reverse osmosis concentration of the serum.

INTRODUCTION

THERE IS a considerable volume of literature in which the viscosity or consistency characteristics or other physical properties of tomato juice and concentrates have been related to processsing conditions (Kertesz and Loconti, 1944; McColloch et al., 1950; Davis et al., 1954; Whittenberger and Nutting, 1957, 1958; Kopelmann and Mannheim, 1964; Mannheim and Kopelmann, 1964). In most cases, however, the viscosities or consistencies reported were the results of single-point measurements; that is, a single viscosity or consistometer time was given for each sample. Few studies have taken the non-Newtonian nature of tomato concentrates into consideration.

The Bostwick consistometer values of tomato concentrates are related to the insoluble solids by an exponential relationship (Marsh et al., 1977) that become very small (<1 cm) as the concentration increases so that they cannot be determined for solids concentration more than about 15%. Tomato juice characteristics might be expected to depend partly on the structure of the original cells and cell walls (Hand et al., 1955). Kertesz and Loconti (1944) found that the size and shape of suspended cell wall particles reflected the severity of mechanical stresses applied by finisher (or screen).

Particle size distribution is a major contributor to the viscosity of tomato juice (Surak et al., 1979). When tomato juice is passed through a fine finishing screen, small particles are incorporated in the juice and a high proportion of the particles remain spherical. The diameter of the holes in the finisher screen affects the particle size and the juice viscosity (Kattan et al., 1956; Smit and Nortje, 1958). The speed (rpm) of operation of the paddle finisher also has an effect on juice viscosity (Whittenberger and Nutting, 1957). At higher operating speeds there is an increase in the number of particles produced and the particles tend to be more elongated and the juice has higher viscosity (Hand et al., 1955).

The quantity, configuration, and characteristics of the suspended particles also influence the viscosity of the product (Tanford, 1961; Hand et al., 1955; Robinson et al., 1956; Luh et al., 1954). However, Luh et al. (1956) found no correlation between cellulose content, which is the major component in cell walls, and the consistency of tomato juice. It appears that not only the quantity of particles affects consistency but their characteristics, such as shape and size have a more direct effect on consistency.

Microscopic examination of samples indicated that tomato products finished with a screen with large holes did not consist of uniformly large particles but rather of a mixture of small particles along with suspended shreds of tissue, the amount of the latter increasing with the screen size (Kattan et al., 1956). Kertesz and Loconti (1944) reported that under the microscope and with proper staining many of these suspended particles exhibit torn, ragged shape.

The use of fine screens for the reduction of particle size would enhance oxidation because of relatively large surface exposed to air and metal. Therefore, increasing the screen size increased the retention of color and ascorbic acid (Kattan et al., 1956).

Harper and El Sahrigi (1965) presented a relationship between apparent viscosity at 500 sec^{-1}, concentration and temperature for a sample obtained by direct evaporation of tomato juice. The shear rate ranged from 500 to 800 sec^{-1} and concentration ranged from 12.8 to 30% total solids. They also removed the insoluble solids in tomato juice by centrifugation, concentrated the serum by evaporation to 65° Brix, and reconstituted the components. Harper and El Sahrigi (1965) found that the apparent viscosities of the reconstituted samples were only about one-third of those of the corresponding original concentrates. A similar result was observed by Mannheim and Kopelmann (1964).

Rao et al. (1981) studied the rheology of tomato concentrates from four different cultivars and arrived at a viscosity relationship similar to that of Harper and El Sahrigi (1965). For the cultivars studied, the magnitude of the concentration exponent was found to be 2.4 to 2.6.

The yield stress refers to the stress that must be exerted to just move one fluid layer past another (Charm, 1962). On this basis the yield stress has been related to the strength of the coherent network structure as the force per unit area required to breakdown the structure, followed by a rupture of the network bonds or linkages connecting the flow units (Dzuy and Boger, 1983).

Because of the small magnitudes of yield stress of many foods, it is difficult to determine its value experimentally. Therefore, it has been found convenient to fit the experimental shear stress-shear rate data to one or more of the constitutive equations proposed by Herschel and Bulkley (1926), Casson (1959), and Mizrahi and Berk (1972). The application of these models to tomato concentrates was studied by Rao et al. (1981) and Rao and Cooley (1983).

The overall objective of the present investigation was to study the rheological properties of hot break tomato juice and concentrates that contained different sized pulp particles and that were concentrated in different manners. For this purpose, concentrates were prepared from juices that were produced using finisher screens with different hole diameters. Using juice from one finisher screen, concentrates were also produced by three methods: (1) by evaporation of tomato juice, (2) from

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evaporated tomato serum, and (3) from serum that was concentrated by reverse osmosis.

MATERIALS & METHODS

Preparation of tomato juice

One hundred sixty kilograms of fresh tomatoes, cultivar FM6203, were hand-picked from commercial fields in Erie county, New York, stored in a 21.1°C room overnight and processed in the Experiment Station's pilot plant. The tomatoes were sorted, washed, and crushed in a hammer mill (W.J. Fitzpatrick Co., Chicago, IL) and the macerate was heated immediately in a steam-jacketed kettle to 98°C. The time interval between crushing the tomatoes and for the macerate to reach 98°C was approximately 1–2 min. The macerate was held at 98°C for 4 min and 5 sec as calculated using a z-value of 8.33°C (Nelson and Tressler, 1980), and using t_0 of 45 sec at 103.9°C (Birnbaum et al., 1977). The macerate was transferred to a finisher (fabricated in the Dept. of Food Science & Technology) that was operated at 1000 rpm and with various screen openings (FSO): 0.020, 0.027, 0.033, and 0.045 in. (or 0.508, 0.686, 0.838, and 1.143 mm, respectively). Juice from the finisher was boiled in a steam-jacketed kettle and hot-filled into No. 303 cans. The cans were sealed, rolled for 3 min, and spin cooled in cold water. They were stored in a −3.9°C room until experimentation. Seventy-six cans of tomato juice from 0.033 in. screen and two cans each of the juice from 0.027 and 0.045 in. screens were produced.

Preparation of tomato concentrates by evaporation of juice (JE)

Lots of juice from the finisher equipped with different FSO were transferred to a steam-jacketed vacuum kettle described in detail by Saravacos and Moyer (1967). The kettle was operated at 132.4 kPa (26–27 in. vacuum). The concentrates from 0.033 in screen were taken out periodically at the approximate total solids (T.S.) of 10, 15, 20, 25, and 28%; in addition, a sample with the highest concentration of about 30% T.S. was obtained. Other concentrates, from 0.020, 0.027, and 0.045 in. screens, were taken when the concentrations were about 30–40% T.S. The tomato concentrates were canned and stored as described for the juice.

Preparation of tomato concentrates by evaporation of serum (SE)

Canned tomato juice from 0.033 in. screen described earlier was centrifuged at 11,700 × *g* at 20°C for 45 min (Sorvall RC-5, Ivan Sorvall, Inc., Norwalk, CT), and the volume of serum was measured and transferred to a beaker. The pulp was scraped from the centrifuge tubes and transferred into a plastic bottle and stored in a refrigerator. The serum was concentrated in a steam-jacketed kettle to various °Brix. When the concentrated serum was cooled to room temperature, it was proportionally combined with the separated pulp to obtain concentrates (150 mL) of 10, 12, 14, 16, 18, 22, and 28 °Brix.

A 25 °Brix serum concentrate was prepared to study the effect of heat applied to serum on the rheological properties of the reconstituted concentrates. It was diluted with distilled water to obtain serum samples at 22, 20, 18, 16, 14, 12, and 10 °Brix. The diluted serum samples were proportionally combined with the separated pulp to obtain 100 mL samples of concentrates. All SE concentrates were allowed to rehydrate overnight in the refrigerator. The portions of the concentrates that were not used on the next day were stored at −3.9°C.

Preparation of tomato concentrates by reverse osmosis concentration of serum (SRO)

Serum and pulp were separated from canned tomato juice in the manner described for SE concentrates. Instead of evaporation, reverse osmosis was used to concentrate the serum using a cellulose acetate membrane (type S-97 CAB) (Osmonics, Inc., Minnetonka, MN) in a batch type reverse osmosis unit with a volumetric capacity of 200 mL at 63.1 MPa (900 psig) (Abcor Inc., Cambridge, MA). Continuous agitation of the serum above the membrane surface was provided by means of a magnetic stirrer. The concentrated serum was collected periodically at various °Brix and then proportionally combined with the separated pulp. The SRO concentrates were allowed to rehydrate in the refrigerator overnight before use in experiments. Portions of the concentrates that were not used in the experiment on the next day were stored at −3.9°C.

Natural tomato soluble solids determination

An AO ABBE Refractometer (American Optical Corp., Buffalo, NY) was used to determine the natural tomato soluble solids (NTSS) in °Brix. In the case of a very concentrated samples, only its serum portion was used for NTSS determination because the presence of the pulp in large amounts obscured the reading.

Total solids determination

A sample was weighed in an aluminum pan and dried in a vacuum oven (Central Scientific Co., Chicago, IL) operated at 21.1°C (70°F), 64.9 kPa (28 in. vacuum) for 48 hr. The dry sample was cooled in a desiccator for at least 2 hr before its weight was measured.

Determination of particle size distribution

The wet sieving technique proposed by Kimball and Kertesz (1952) was employed to determined weighted average diameter of particles in tomato juice and concentrate samples. A set of five U.S.A. Standard sieve series (Newark Wire Cloth Co., Newark, NJ) with 20, 40, 60, and 100, and 140 mesh openings were used. For the particles retained on the sieve with the largest openings, the average effective particle size (diameter) was assumed to be 50% over the diameter of the openings. For particles which passed through one sieve but not the next one, an average effective particle diameter half way between the diameters of the openings of the two sieves was assumed. All experiments were replicated and the weighted average diameters of the particles were calculated.

Rheological measurements

Flow properties of the concentrates were determined with a concentric cylinder viscometer (Haake RV2, Haake Inc., Saddle Brook, NJ) as described earlier (Vitali and Rao, 1984) at five temperatures: 10°, 25°, 40°, 55°, and 70°C for the concentrates processed from FSO of 0.045, and 0.033, and 0.027 in. For concentrates from 0.020 in. screen, whose rheological behavior was determined first, the temperatures employed were 5°, 15°, 25°, 35°, and 45°C. Yield stress of samples was determined using the relaxation technique described by Van Wazer et al. (1963).

RESULTS & DISCUSSION

Rheological properties of tomato concentrates and tomato juice

Flow curves consisting of log shear rate ($\dot{\gamma}$) against log shear stress (τ) of tomato concentrates from the three different concentration processes and from the four different screen sizes, as well as concentrated serum, showed power-law behavior.

$$\tau = K\dot{\gamma}^n \quad (1)$$

Linear regression analysis was performed on the data resulting in values of slopes (n), intercepts (K), and correlation coefficients. The correlation coefficients were in the range 0.97 to 1.00. The flow behavior index n of the tomato concentrates was found to vary from 0.266 to 0.444. With values of n being less than 1, tomato concentrates are shear thinning fluids. The flow behavior index showed no definite trends with concentration, temperature, or methods of concentration in accordance with the findings by Harper and El Sahrigi (1965) and Rao et al. (1981). Using magnitudes of K and n, apparent viscosities of the concentrates were calculated from the relationship:

$$\eta_{a,100} = K\,(100)^{n-1} \quad (2)$$

Effect of temperature. The effect of temperature on the apparent viscosity of the concentrates at 100 sec^{-1} was described well by the Arrhenius relationship:

$$\eta_{a,100} = \eta_\infty \exp\,(E_a/RT) \quad (3)$$

Magnitudes of the activation energy (E_a) of the concentrates ranged from 2.0 to 3.0 kcal/mole and were within the range of values reported by others (Harper and El Sahrigi, 1965; Rao et al., 1981).

Effect of concentration. The relationships between apparent viscosity and concentration were of the power type. The exponents did not vary much with either screen sizes or temperatures (Table 1). At 25°C, for all screen sizes the exponent of the power relationship was 2.24 with a correlation coefficient of 0.954; this magnitude is in the range of values: 2.5 and 2.0 reported by Rao et al. (1981) and Harper and El Sahrigi (1965), respectively.

Effect of screen size

Shear rate-shear stress data of a 20% T.S., JE concentrate are shown in Fig. 1. From the data, it can be ascertained that in general smaller FSO (theoretically smaller particle size distribution) yielded lower apparent viscosities. However, the concentrates from 0.027 in. screen had the highest apparent viscosity among the four screen sizes. Similar results were obtained for tomato juice (Somsrivichai, 1986). It is interesting to note that particle size distributions of samples from 0.027 in. and 0.045 in. screens were similar to each other on one hand (Fig. 2) and those using 0.020 in and 0.033 in. were similar to each other on the other hand (Fig. 3).

The observed influence of screen size may be explained in that small screens reduce the size of the particles. However, at the same time they remove some of the large particles from the finished products resulting in tomato concentrates with narrow particle size distribution and a small amount of large particles. Based on theories of suspension rheology (Jinescu, 1974), small suspended particles may give high viscosity due to their greater surface area. Large particles contribute to high viscosity also. Therefore, small screen sizes can affect the gross viscosity of tomato concentrates in two opposite manners: one is enhancing gross viscosity due to large surface area of small particles and the other one is diminishing the gross viscosity due to the exclusion of large particles. Screen size of 0.020 in may produce tomato concentrates with too small particle size distribution and very small amount of large particles resulting in small magnitude of viscosity while 0.027 in. screen may produce small particles as well as allow some of the large particles to be in the tomato concentrates. It may be that using 0.027 in. screen resulted in tomato juice and concentrates with appropriate particle sizes which yielded the highest viscosity.

Effect of methods of concentration

Effect of methods of concentration on η_{100} of tomato concentrates with 16% T.S. from three different processes: juice-evaporation, serum-evaporation, and serum-reverse osmosis, can be seen in Fig. 4. At low concentrations, apparent viscosities of SRO and SE concentrates were not significantly different. At higher concentrations, concentrates from serum-evaporation were exposed to heat for longer periods of time, therefore, their apparent viscosities were less than that of concentrates from serum-reverse osmosis. Nevertheless, both SRO and SE concentrates showed higher apparent viscosities than that of JE concentrates at the same concentrations.

From data in Fig. 4 it appears that concentrating tomato serum by means of evaporation or reverse osmosis does not have significant effect on apparent viscosity of reconstituted concentrates with unheated pulp. When heat is applied to the whole tomato juice, both serum and pulp are subjected to heat. Structure of pulp may be affected by heat. Particle sizes or volume of the pulp may be reduced during the heat treatment. Moreover, concentrating tomato juice and tomato serum by heating to the same °Brix requires different heating time because tomato juice has lower heat transfer coefficient than the serum (Kopelman and Mannheim, 1964).

Kopelman and Mannheim (1964) found that SE concentrates had much lower viscosity than JE concentrates. They concluded that lower consistency in SE may be attributed to the centrifugation during serum separation (which was not specified in their publication) which led to crushing of the cells and the disruption of the solid suspension structure of the juice. However, their tomato concentrates were made by cold break method (60°C). Pectic enzymes may still have been active in the concentrates resulting in subsequent loss of consistency.

Effect of heat in concentration step. It has been known for a number of years that when tomato concentrates are diluted to lower concentration, the diluted products have lower viscosity than if they are concentrated straight from the juice. In the present study, this effect was first observed for a JE concentrate. Figure 5 contains the apparent viscosities of two 16% T.S. JE tomato concentrates: one prepared by straight concentration of juice and the other by dilution of a concentrate with 41% total solids. It is clearly seen that the straight concentrate had higher apparent viscosity than the diluted concentrate.

Figure 6 shows that SE tomato concentrates prepared from dilution also have lower apparent viscosity than the straight concentrates. In this case, only the serum experienced heat. Structure of the pulp should be the same in both concentrates, diluted and straight, only the nature of the serum was different. Heat alters the structure of pectic substances by means of hydrolysis. Colloidal properties of serum may be altered by heat resulting in lower apparent viscosity of reconstituted tomato concentrates with unheated pulp. In this respect, Caradec and Nelson, (1985) reported that viscosity of tomato juice serum decreased with heat treatment. The observation of Caradec and Nelson (1985) is in agreement with the present results in that heat treatment reduces the viscosity of serum and juice.

Marsh et al. (1977) found that pulp lost bound water as a result of the physical forces that developed as concentration progressed and the loss altered their ability to influence consistency. Therefore, water removal by means of evaporation may irreversibly affect the rheological properties of the final products.

Labuza (1977) suggested that the apparent loss of consistency or viscosity was most likely due to the failure of the macromolecular polymeric substances, comprising the water insoluble solids, to resorb to their maximum extent. Pectic substances and other long-chair carbohydrate polymers can be hydrolyzed by heat (Kertesz, 1951) resulting in smaller molecules. Colloidal properties exhibited by pectic substances are changed. Cell wall materials become less rigid and smaller in size when heat is applied.

Yield stresses of tomato concentrates

Yield stresses of tomato concentrates from juice evaporation process using four FSO were determined over the concentra-

Table 1—Slope of the plot ln (η_{100})[a] versus ln (total solids) of tomato concentrates from different processes

Process	Screen size (in.)	Temperature (°C)								
		5	10	15	25	35	40	45	55	70
Juice evaporation	0.020	2.29	—	2.63	2.64	2.82	—	2.85	—	—
Juice evaporation	0.027	—	2.55	—	2.46	—	2.50	—	2.64	2.82
Juice evaporation	0.033	—	2.86	—	2.94	—	2.91	—	3.08	2.97
Juice evaporation	0.045	—	2.77	—	2.80	—	2.77	—	2.84	3.16
Serum evaporation	0.033	—	—	—	2.36	—	—	—	—	—
Serum reverse osmosis	0.033	—	—	—	2.82	—	—	—	—	—

[a] η_{100} is apparent viscosity at a shear rate of 100 sec^{-1}.

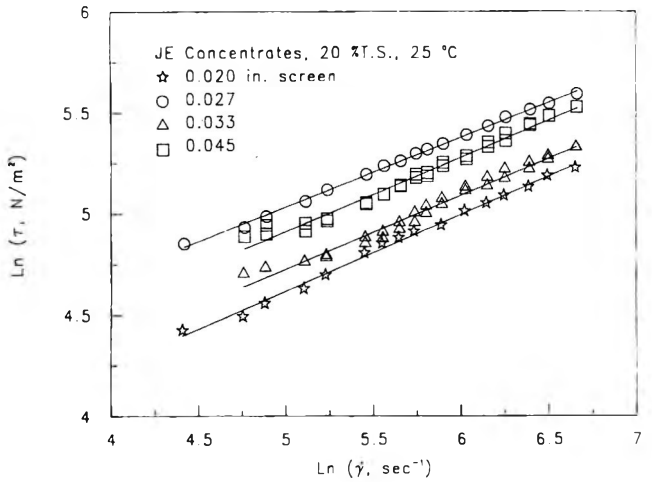

Fig. 1—Shear rate ($\dot{\gamma}$)-shear stress (τ) data at 25 °C of 20% juice evaporated (JE) concentrates that were made using tomato juices from different finisher screens.

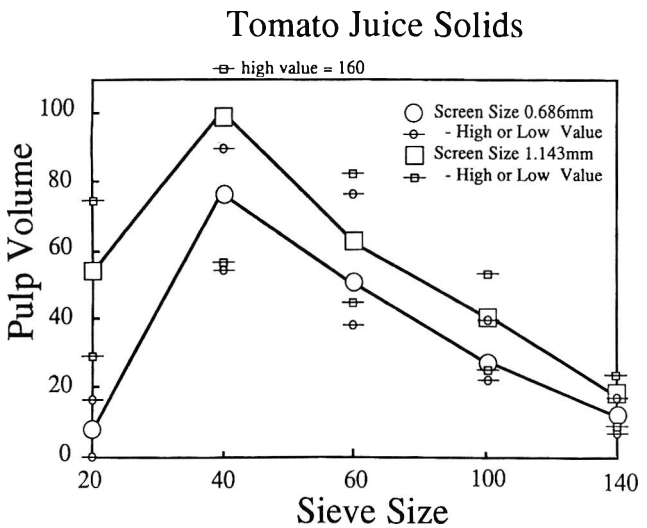

Fig. 2—Volume of pulp retained on sieves for juice samples from finisher screens of 0.027 in. (0.686 mm) and 0.045 in. (1.143 mm).

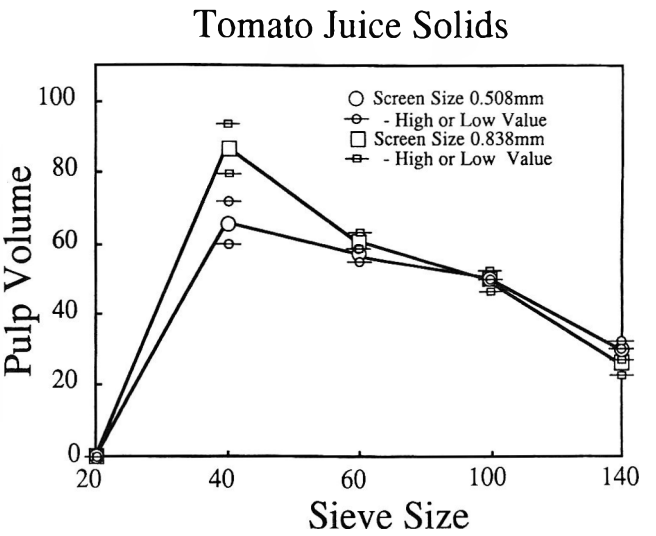

Fig. 3—Volume of pulp retained on sieves for juice samples from finisher screens of 0.020 in. (0.508 mm) and 0.033 in. (0.838 mm).

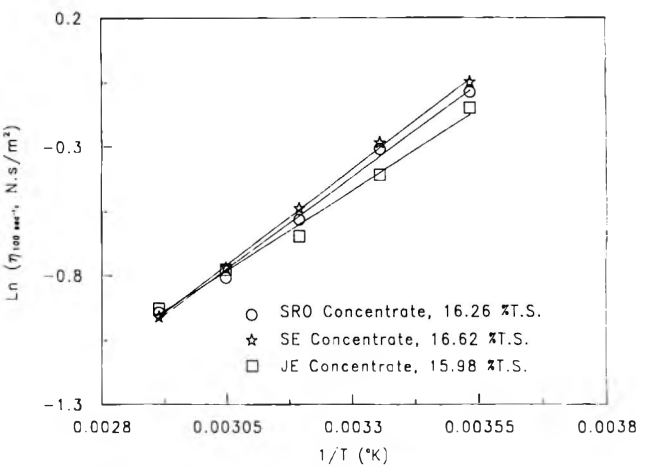

Fig. 4—Apparent viscosity at 100 sec^{-1} (η_{100}) as a function of temperature of 16% concentrates made by evaporation of juice, evaporation of serum, and reverse osmosis concentration of serum.

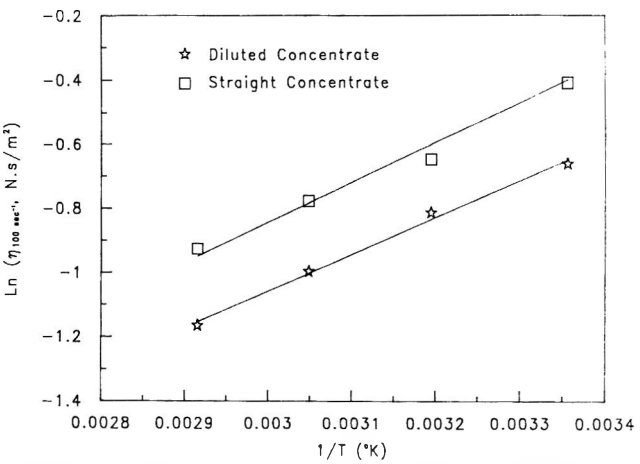

Fig. 5—Apparent viscosity at 100 sec^{-1} (η_{100}) as a function of temperature of straight and diluted, 16% total solids, juice evaporated concentrates, from 0.033 in. screen.

tion range 9 to 14%. These concentrations were selected in order that the most sensitive torque measuring head (50 g-cm) of the viscometer could be used. Their magnitudes, shown in Fig. 7, depend on total solids of the concentrates as well as on FSO. Regression analysis of concentration versus ln of yield stress resulted in quadratic equations as found earlier by (Rao et al., 1981). The use of larger finisher screens resulted in concentrates with higher yield stress. Also, as total solids of the concentrates increased, the magnitude of yield stress increased.

When values of yield stress are determined for a fluid, another flow model containing the yield stress term, such as that of Herschel-Bulkley (Eq. 4), must be employed to fit the viscometric data.

$$\tau = \tau_{OH} + K_H \dot{\gamma}^{n_H} \quad (4)$$

In Eq. (4), τ is the shear stress, $\dot{\gamma}$ is the shear rate, τ_{OH} is the yield stress, K_H is the consistency index, and n_H is the flow behavior index.

It should be pointed out that magnitudes of rheological parameters obtained from this analysis in which yield stress was included will be somewhat different from the analysis based on the simple power law model (Eq. 1). However, apparent viscosities will be the same in both analyses, only the flow

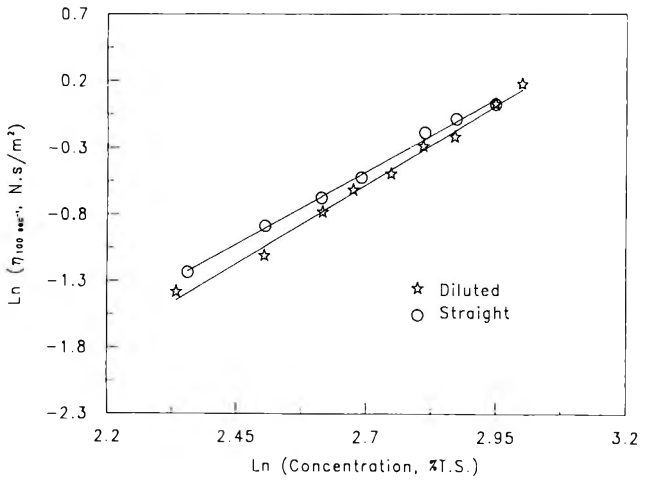

Fig. 6—Plot of ln concentration (% total solids) versus ln apparent viscosity (Pa.s) at 25 °C of serum evaporated straight and diluted concentrates from 0.033 in. screen.

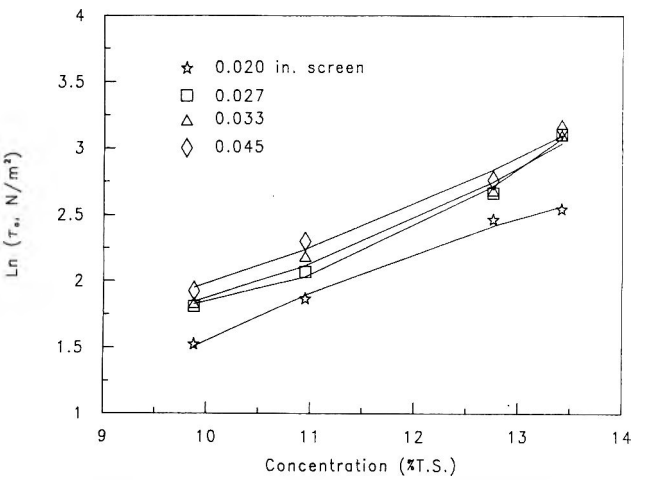

Fig. 7—Yield stress (N/m²) of juice evaporated (JE) tomato concentrates from different finisher screens as a function of concentration (% total solids).

behavior index, n, and the consistency index, K, will be different.

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Ms. received 4/18/86; revised 8/22/86; accepted 8/22/86.

One of us (TS) was the recipient of a scholarship from the Anandha Mahidol Foundation, Bangkok, Thailand. This work also was supported by funds from the Hatch act.

Based on a paper presented at the 46th Annual Meeting of the Institute of Food Technologists, June 15–18, Dallas, TX.

Effect of Moisture on the Thermal Behavior of Strawberries Studied using Differential Scanning Calorimetry

YRJÖ H. ROOS

ABSTRACT

Differential scanning calorimetry was used to determine the thermal transitions in fresh and freeze-dried strawberries and the moisture dependence of these transitions. The freeze-dried strawberry samples had a glass transition at 30–60°C, and the melting endotherm of the dried products was similar to that for freeze-dried sugars. The glass transition temperature of humidified samples was a linear function of the water activity; it decreased with increasing moisture content. Ice was found to melt at a moisture content of 21.4% or above. The melting of ice in strawberries was similar to that in sugar solutions and fruit juices.

INTRODUCTION

THE THERMAL BEHAVIOR of carbohydrate materials at low temperatures has been the subject of studies of freeze-drying behavior of fruits, fruit juices, and sugar solutions. The thermal transitions of these materials are often related to the loss of structure and to aroma retention during freeze-drying, and to the overall quality of the freeze-dried product (MacKenzie, 1975), but they may also be related to the stability of the dried products (To and Flink, 1978).

During the freezing of food materials water is crystallized as almost pure ice, whereas the solution becomes more concentrated. When carbohydrate materials are frozen, the concentration of the solution results in the formation of a concentrated amorphous solution (CAS) (Bellows and King, 1972). In such systems no eutectic behavior is observed, and the collapse during freeze-drying is related to the decrease in the viscosity of the solution. Ito (1970, 1971) showed that solutions which exhibit eutectic behavior collapsed if they were freeze-dried above their eutectic temperature. MacKenzie (1975) pointed out that the structural transitions during freeze-drying were a result either of eutectic melting or collapse. Eutectic melting was found to occur in the frozen part of the material being freeze-dried, while collapse was reported to occur at the freeze-drying interface or in the partially dry material.

Various thermal transitions are found during the phase transitions of frozen sugar solutions (Luyet and Rasmussen, 1968; Rasmussen and Luyet, 1969; MacKenzie, 1975). These transitions include glass transition, ante-melting, recrystallization, incipient melting, and melting, and they are detectable by differential thermal analysis (DTA) or differential scanning calorimetry (DSC). Simatos et al. (1975) also reported similar transitions in rehydrated freeze-dried plasma. Materials which are of very low moisture usually only exhibit glass transition, but the glass transition temperature is dependent on the moisture (Parducci and Duckworth, 1972; Simatos et al., 1975). To and Flink (1978) reported thermal transitions in freeze-dried sugars above freezing temperatures.

Differential scanning calorimetry, as a related method to differential thermal analysis, gives a lot of information about the thermal transitions of food materials and, according to Rey (1960), the results may be used to optimize the freeze-drying process. The purpose of this study was to investigate the thermal transitions of strawberries, both in the fresh and freeze-dried rehydrated state, and to determine the moisture dependence of these transitions.

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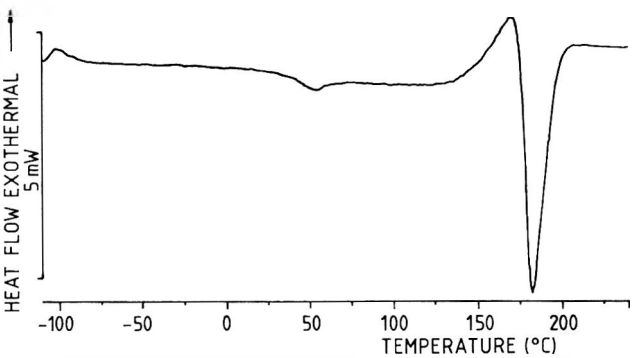

Fig. 1—A typical DSC thermogram for a dried strawberry powder. The heating rate was 5°C/min.

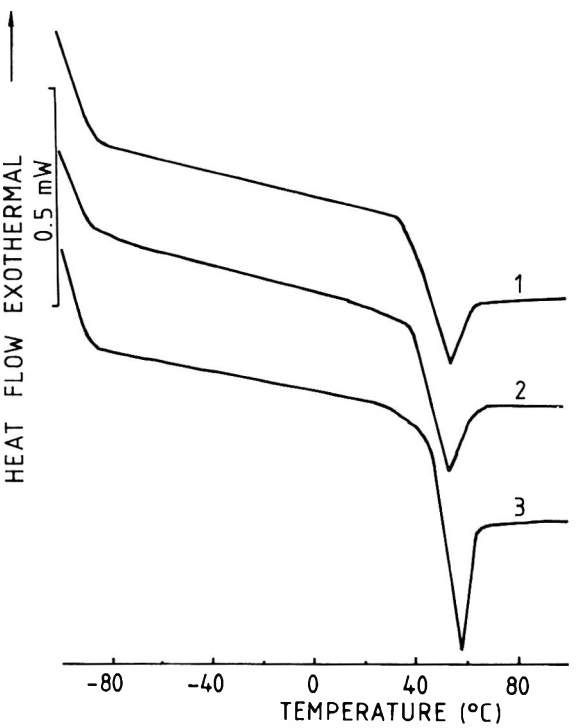

Fig. 2—Glass transition of strawberry powders freeze-dried at different surface temperatures. The heating rate was 5°C/min. 1 = freeze dried at 20°C; 2 = freeze-dried at 40°C; 3 = freeze-dried at 60°C.

MATERIALS & METHODS

Strawberries

The strawberries used were of the variety Senga Sengana, obtained from a local producer. The strawberries were hulled and prepared for the experiments the same day as they were harvested.

Table 1—Thermal transitions of dry strawberry powders freeze-dried at different surface temperatures[a]

Surface temp (°C)	Glass transition temperature		Melting temp	Heat of fusion
	Onset (T_{g1},°C)	End (T_{g2},°C)	(T_f,°C)	(ΔH_f, J/g)
20	36.3 ± 2.4	52.8 ± 1.3	181 ± 2.3	220 ± 22
40	39.5 ± 1.1	53.9 ± 1.6	172 ± 3.2	239 ± 21
60	47.8 ± 1.0	57.6 ± 0.6	183 ± 2.4	191 ± 28

[a] Average of six measurements ± standard deviation

Table 2—Saturated salt solutions used for humidification of the freeze-dried strawberry powders, and their moisture contents after equilibration

Salt[a] solution	Water activity (a_w^{25})	Equilibrium water content[b]	
		%	g/100g dry matter
LiCl	0.12	1 ± 1	1 ± 1
CH_3COOK	0.23	2.6 ± 0.1	2.6 ± 1.0
$MgCl_2 \times 6H_2O$	0.33	6.1 ± 0.6	6.5 ± 0.7
K_2CO_3	0.44	9.1 ± 1.3	10.0 ± 1.6
$Mg(NO_3)_2 \times 6H_2O$	0.52	11.5 ± 1.2	13.0 ± 1.5
NaCl	0.75	23.3 ± 0.5	30.3 ± 0.8
$Li_2SO_4 \times H_2O$	0.85	32.1 ± 1.1	47.2 ± 2.4
KNO_3	0.94	51.1 ± 2.1	104 ± 8.6

[a] Pro analysis (J.T. Baker Chemicals B.V.; Merck)
[b] The moisture content is an average of six samples ± standard deviation

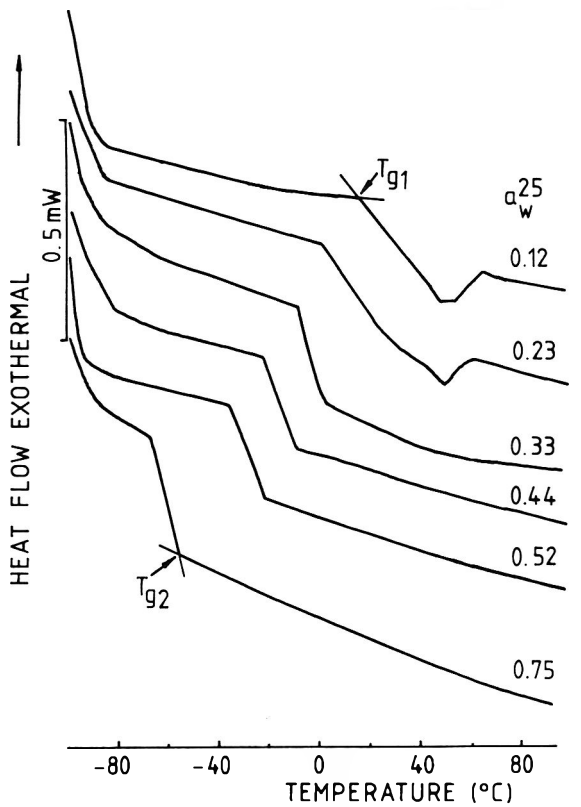

Fig. 3—Glass transition of strawberry powders humidified to different water activities. The heating rate was 5°C/min. T_{g1} = onset of glass transition; T_{g2} = end of glass transition.

Freezing and freeze-drying

Whole strawberries were frozen on trays on the shelves of a freeze-drier (Edwards EF 10/10) at −40°C. The freezing time was 2 hr. After freezing the freeze-drying chamber was evacuated, and the strawberries were freeze-dried for 24–48 hr. Three platen temperatures, 20°C, 40°C, and 60°C, were used to study the effect of dehydration temperature on the thermal behavior of the freeze-dried material. The freeze-drier was loaded with 10 kg strawberries, and the highest vacuum pressure during freeze-drying was 20 Pa. Two homogenized strawberry samples (3–5 g) were freeze-dried with each batch for determinations of the residual moisture and the moisture of fresh strawberries. After freeze-drying the vaccum was broken with nitrogen (AGA, 99.9% N_2), and the freeze-dried strawberries were immediately packed in aluminum laminate bags under nitrogen. The freeze-dried products were stored for 5 months at room temperature before analysis.

Table 3—Glass transition temperatures (T_{g1} and T_{g2}) of freeze-dried strawberry powders humidified at different water activities

Water activity (a_w^{25})	Glass transition temperature (°C)[a]	
	Onset (T_{g1})	End (T_{g2})
0.12	21.4 ± 4.6	46.7 ± 2.1
0.23	2.3 ± 2.3	42.0 ± 3.5
0.33	−10.3 ± 2.9	4.5 ± 2.4
0.44	−23.3 ± 2.4	−9.1 ± 2.3
0.52	−34.4 ± 3.1	−20.2 ± 1.0
0.75	−65.3 ± 0.8	−54.3 ± 1.6

[a] The glass transition temperatures are averages of six determinations ± standard deviation

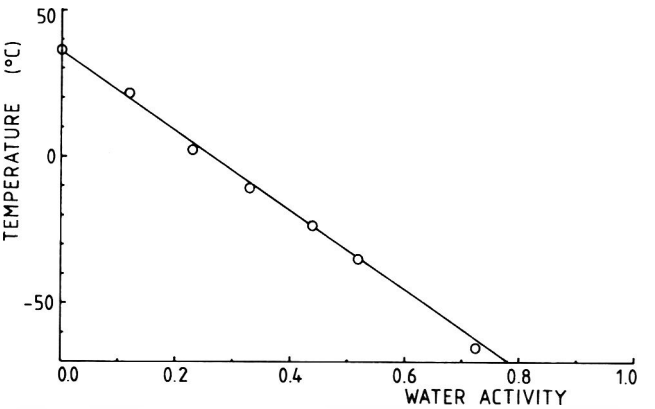

Fig. 4—Glass transition (T_{g1}) of strawberries as a function of water activity (a_w^{25}). The correlation coefficient was r = 0.999, and the regression equation $T_{g1} = -135.49(a_w^{25}) + 35.79$.

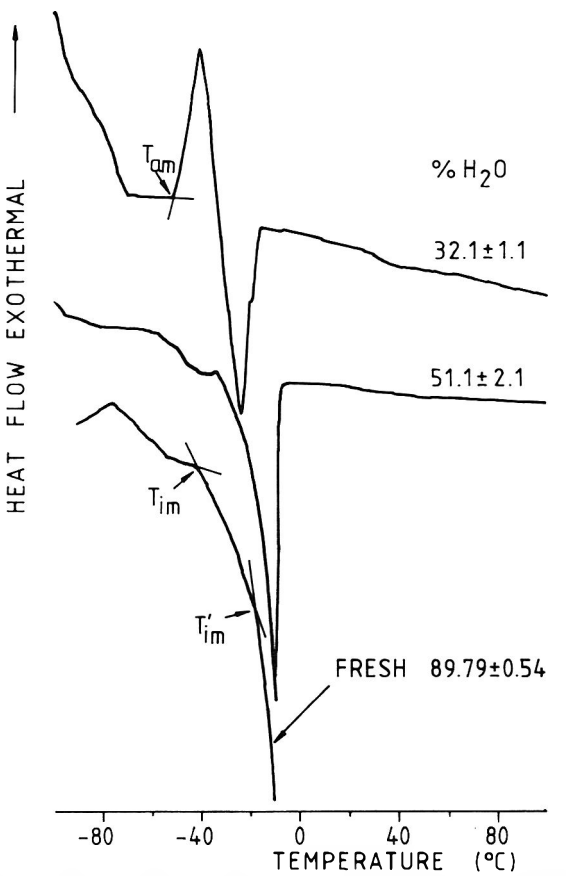

Fig. 5—Melting of ice in strawberry samples with different moisture contents as detected using DSC. The heating rate was 5°C/min. T_{im} = incipient melting point; T'_{im} = incipient intensive melting point; T_{am} = ante-melting.

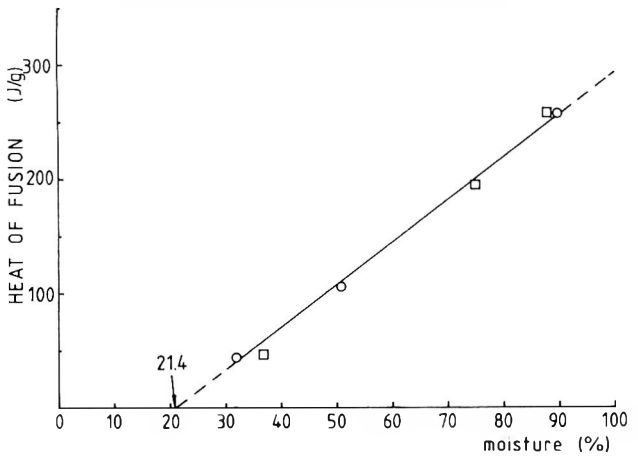

Fig. 6—Latent heat of melting of ice (ΔH_m) in strawberry samples with different moisture contents, and the unfreezable water content determined from the regression equation. ○ = strawberries in this study, correlation coefficient r = 0.999, and regression equation $\Delta H_m = 3.74$(moisture %) − 80.25; □ = ΔH_m of carrots, reindeer meat and white bread (Roos, 1986).

Sample preparation and moisture determination

The thermal behavior was determined for fresh and freeze-dried strawberries rehydrated to different moistures. The moisture of the fresh strawberries was determined by placing the freeze-dried homogenized samples in an air oven at 105°C for 2 hr, and the total weight loss was determined.

The samples for the determination of the thermal behavior of fresh strawberries were prepared with a small knife to get a thin slice of the flesh (5–10 mg). The samples were placed in DSC pans (40 μL, Mettler) made of aluminum immediately after preparation, and the pans were hermetically sealed and weighed for DSC.

The freeze-dried strawberries were powdered, and only seedless powder from the flesh was used. This powder was further dried on P_2O_5 at room temperature in a vacuum desiccator for 1–4 wk. The dried powder was analyzed, and samples made of strawberry powder freeze-dried at 20°C were humidified in DSC pans on saturated salt solutions (Table 2). The moisture uptake was determined at room temperature by weighing the samples before and after humidification. The moisture content of the dried powder was considered to be negligible. The weight of the humidified samples was 2–5 mg, and the time used for humidification was 23–35 hr, which was found to be suitable for full equilibration. After humidification the sample pans were enclosed and hermetically sealed for DSC.

Differential scanning calorimetry

The differential scanning calorimeter used was a Mettler TA 3000 system with a DSC 30 measuring cell. The apparatus was calibrated as reported by Roos (1986). An empty aluminum pan was used as a reference sample, and the heating rate was 5°C/min in all experiments. A nitrogen gas (AGA, 99.9% N_2) flow of 20–30 mL/min was used to avoid water condensing in the measuring cell.

The fresh strawberry samples were scanned from −100°C to 100°C. The DSC thermogram was typical of food materials with a large endothermic peak of melting of ice. The melting curve was integrated to determine the latent heat of melting (ΔH_m). The enthalpy (ΔH) of the samples was determined from −60°C to 20°C. ΔH_m and ΔH were used to calculate the unfreezable water content of fresh strawberries (Roos, 1986). A ΔH_m of 325 J/g and ΔH of 550 J/g were used for water. The incipient melting temperature (T_{im}) and the incipient intensive melting temperature (T'_{im}) of fresh strawberries were determined as shown in Fig. 5 and reported by Rey (1960). The onset temperature of melting was determined as in a previous study (Roos, 1986). The results were given as the average of 18 samples ± standard deviation.

The freeze-dried powdered strawberry samples were scanned from −120°C to 250°C. The curves differed from each other depending on the moisture content, but the main transitions determined were the onset of glass transition (T_{g1}), end of glass transition (T_{g2}), T_{im}, ante-melting (T_{am}) (Fig. 5), and the melting temperature of the dry powder (T_f) (Fig. 1). Ante-melting and incipient melting were presumed to be transitions related with the viscosity changes in the concentrated amorphous phase (MacKenzie, 1975). When the melting of ice was detected, the melting curve was integrated to determine ΔH_m. The melting curve of the dry powder was integrated to determine the heat of fusion (ΔH_f). The results were given as an average for 6 samples ± standard deviation.

Regression analysis was used to relate ΔH_m and T_{g1} with the moisture or water activity at 25°C (a_w^{25}) of different strawberry samples.

RESULTS

DSC of fresh strawberries

The moisture content of the fresh strawberries was 89.8 ± 0.6% and the pH 3.45, which were typical values for the Senga Sengana variety (Skrede, 1980). The DSC thermogram of the fresh strawberries was typical of food materials, and similar to those of foods with high moisture contents (Roos, 1986). The ΔH_m determined from the peak area under the curve was 257 ± 9 J/g. The unfreezable water content of fresh strawberries determined from latent heat of melting was 10.7% of the total weight (105g unfreezable water/100g dry matter). The ΔH was 460 J/g, and the unfreezable water content determined by enthalpy was 6.2%. (60.7g unfreezable water/100g dry matter). The T_m of fresh strawberries was found at −3.5 ± 0.3°C.

DSC of dry strawberry powders

A thermogram of a dried strawberry powder is shown in Fig. 1. Glass transition was found between 30°C and 60°C (Fig. 2). This transition became steeper, and the peak temperature increased with increasing freeze-drying temperature. Above 100°C there was an exothermal transition, which was probably related to the thermal browning of the dried material. This transition was followed by a sharp melting endotherm of the carbohydrates. The transition temperatures and the energy changes determined are given in Table 1.

DSC of humidified strawberry powders

The humidified strawberry powders were all freeze-dried at 20°C. The moisture contents of the humidified samples are given in Table 2. The DSC thermograms were similar to those for the dry powders up to an equilibirum moisture content of 0.75 a_w^{25}, above which melting of ice was observed. However, the glass transition temperatures decreased with increasing moisture (Fig. 3). The T_{g1} was found to be a function of a_w^{25} as shown in Fig. 4. The T_{g1} and T_{g2} of the samples are given in Table 3. The T_f of the humidified samples occurred at a lower temperature when the moisture content was increased, and was covered by the evaporation curve of water at high moisture contents. The ΔH_f also increased with increasing moisture content.

Melting of ice in fresh and humidified samples

The melting of ice was found in samples humidified at 0.85 a_w^{25} or above (Fig. 5). The glass transition observed in samples with a lower moisture content was not found in the temperature range studied. The T_{im} of the fresh strawberries was found to be −41.8 ± 1.5°C, and T'_{im} to be −18.2 ± 1.4°C (Fig. 5). The T'_{im} was not found in the DSC curves of the humidified samples. The samples humidified at 0.94 a_w^{25} had a glass transition at about −55°C, which was followed by a small endothermal transition at −40.0 ± 1.6°C, and then by rapid melting at −32.4 ± 1.2°C. The samples humidified at 0.85 a_w^{25} showed exothermal behavior with onset at −50.6 ± 2.7°C, and a peak temperature of −40.3 ± 2.1°C. The onset temperature was considered to be ante-melting (T_{am}), and the peak temperature to be incipient melting (T_{im}). The latent heat of melting of the different samples was found to be a function of the moisture content as shown by the regression line in Fig. 6. The regression equation gave a moisture content of 21.4% to be the lowest detectable with DSC.

DISCUSSION

MOST CARBOHYDRATE MATERIALS are found to be difficult to freeze-dry because of the collapse of the physical structure during sublimation. The carbohydrate content of strawberries is high, and fresh Senga Sengana strawberries contain about 9.2% soluble solids (Skrede, 1982). Skrede (1982) reported the main sugars in this variety to be fructose (1.81%), sucrose (1.75%) and glucose (1.52%). MacKenzie (1966) reported on the collapse temperatures during freeze-drying of these sugars, and found that the collapse temperature of individual sugars may be increased by the addition of materials with a higher collapse temperature. Also, the structure of a solid food may increase its collapse temperature (Rey, 1964). In this study the strawberries freeze-dried at 20°C were found to be of high quality, but the strawberries freeze-dried at 60°C were slightly different in appearance and taste. This was probably due to the collapse of the soluble solids, and to their reactions at the higher temperatures of the dried surface during freeze-drying.

The melting of the ice in fresh strawberries was found to resemble that in other foods (Roos, 1986), and the melting of the ice in the humidified strawberry powders was found to be similar to that for sugar solutions and fruit juices (Maltini, 1977). Maltini (1975) reported that most fruit juices collapse at about −40°C, which is about the same temperature as the incipient melting temperature of strawberries determined in this study. Rey (1964) pointed out that the sublimation temperature for the ice in most biological materials in the freeze-drying process is between T_{im} and T'_{im}. Therefore the sublimation temperature of whole strawberries should be below −18.2°C, and that of strawberry juice about −40°C, because the juice has no solid structure and the viscosity of the material decreases with increasing temperature, thus promoting collapse.

The ante-melting and recrystallization temperatures of sugar solutions are related to viscosity changes in the CAS (Bellows and King, 1972), and usually appear at a slightly lower temperature than T_{im} (MacKenzie, 1975). The freeze-dried strawberry powders showed similar behavior to the concentrated sucrose solutions with equal moisture contents (Maltini, 1977), but only T_{im} was found in the melting curve of fresh strawberries. However, the incipient melting temperature was not found to depend on the moisture content, which is typical of most materials (MacKenzie, 1975; Simatos et al., 1975; Rasmussen and Luyet, 1969). These results indicated that naturally occurring food materials with high carbohydrate content behave similarly to most sugar solutions in the freezing and melting processes, and the collapse of these materials in the freeze-drying process is due to the same physical phenomena.

To and Flink (1978) found freeze-dried sucrose to have a glass transition at 52°C. The glass transition of the dried strawberry powders was found to be in the same temperature range, and to depend on their thermal history in the freeze-drying process. In this study it was shown that the glass transition temperature was dependent on the moisture content of the sample, and the T_{gl} was a function of the water activity at a constant temperature. It was also found that the glass transition was related to those found in sugar solutions (Maltini, 1977), plasma (Simatos et al., 1975), and real foods (Parducci and Duckworth, 1972). According to these results the determination of T_{gl} could be used as an indirect method to determine the water activity of freeze-dried foods. It is also possible that the glass transition and collapse of the dried layer of a freeze-drying material occur at equal moisture content.

The percentage of unfreezable water in foods varies depending on the moisture content but the amount of unfreezable water should be constant when expressed on the dry weight basis. Therefore a moisture content above which the latent heat of melting is a linear function of the moisture content can be found (Fig. 6). The moisture content of strawberries in which the water remained unfrozen was found to be 21.4% or 27.2g H_2O/100g dry matter, which was close to the value obtained previously using different foods (Roos, 1986). The unfreezable water content determined using ΔH_m for fresh strawberries was 105 g/100g dry matter. Roos (1986) used the enthalpy change in the determination of the unfreezable water content, and obtained lower values than when using the latent heat of melting. These results were comparable to the value of 27.2 g/100g obtained for strawberries in this study. However, the unfreezable water content of fresh strawberries determined using enthalpy change was 60.7 g/100g dry matter, which was much higher than the moisture content needed for the melting peak of ice to appear. However, the results showed that the unfreezable water content may be determined using ΔH_m by plotting the moisture content of samples against the latent heat of melting (Fig. 6), and that the water activity below water remained unfrozen was about 0.75 a_w[25].

In this study it was shown that the thermal behavior of a freeze-dried natural carbohydrate material was strongly moisture dependent. The freeze-dried samples had a glass transition indicating an amorphous state of the freeze-dried materials. The transitions reported for strawberries were also found in freeze-dried fruits and vegetables, and may be used in the evaluation of the behavior of these materials in different processes. However, more work should be done to relate these transitions to the sorption isotherms and to the collapse of the materials under different conditions, and to the behavior of ice and of the already dried material during sublimation in the freeze-drying process. It was also shown that the measurements are useful for the determination of unfreezable water and water activities of freeze-dried materials.

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Ms received 3/31/86; revised 7/14/86; accepted 7/30/86.

This research was supported by the Academy of Finland, grant no. 01/787.

A Rapid Direct Extraction-Derivatization Method for Determining Sugars in Fruit Tissue

A.R. LONG and G.W. CHISM, III

ABSTRACT

Trimethylsilyl (TMS) derivatives of sucrose and TMS oxime derivatives of fructose and glucose were made directly from samples of freeze-dried fruit tissues without a preliminary extraction of sugars and quantified using gas chromtography. The sugars were identified by a combination of gas chromatography-mass spectrometry (GC-MS) and gas chromatographic retention times of authentic compounds. Results were quantitative and reproducible for all samples except tomatoes which had values for sucrose which were significantly more variable than those obtained for fructose and glucose. Hydrolysis of sucrose was minimized and extraction of free sugars was consistent when a 50:1 ratio of oxime reagent (μL) per mg of dry tissue was used.

INTRODUCTION

SUGARS are nonvolatile compounds found in many plant tissues. The determination of sugars has been used to assess ripening and optimum quality of various fruits (Wrolstad and Shallenberger, 1981; Marriot et al., 1981). Sugar composition of different fruit tissues has been experimentally determined utilizing numerous methods, the most recent of which are high-pressure liquid chromatography (HPLC) (Dunmire and Otto, 1979; Hurst et al., 1979; Palmer and Brandes, 1974) and gas liquid chromatography (GLC) (Akhavan and Wrolstad, 1980; Kline et al., 1970). HPLC and GLC have been shown to be valuable in determining free fructose, glucose and sucrose in a variety of plant tissues.

HPLC in addition to being less sensitive than GLC when utilizing a refractive index (RI) detector, also requires an extraction and clean-up step prior to detection. The traditional extraction and clean-up step (AOAC, 1984) is time consuming due to extended refluxing times with aqueous ethanol solutions and may also contribute to acid hydrolysis of sucrose if careful neutralization of the reluxing solution is not observed.

GLC utilizing flame-ionization detector (FID) offers greater sensitivity but requires derivatization of the sugars prior to detection. Trimethylsilyl (TMS) derivatives of sugars have been shown to give reliable, near complete derivatization products with recoveries approaching 100% utilizing internal standardization (Laker, 1980). Reducing sugars such as fructose and glucose are generally analyzed as trimethylsilylated oxime (TMS/OX) derivatives while sucrose is analyzed as its TMS derivative. Oximes of reducing sugars eliminate tautomeric forms of TMS derivatives which may give rise to multiple peaks when analyzed. GLC provides a means of measuring these sugars more specifically than titration and polarization techniques and, is more sensitvie than RI detection with HPLC.

The object of this study was to develop a GLC procedure which was rapid, reproducible and quantitative for the determination of free fructose, glucose and sucrose, in freeze-dried fruit tissues without extensive sample preparation or tedious extractions of the sugars from tissues.

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MATERIALS & METHODS

APPLES, bananas and nectarines were purchased from a local supermarket; tomatoes were obtained from the Ohio Agricultural Research & Development Center (OARDC), Wooster, OH. Fruit were cut into approximately 0.5cm-thick slices, weighed and freeze-dried. The freeze-dried tissue was weighed and immediately placed into freezer bags, wrapped in aluminum foil and placed in a freezer (−15°C) until sugar determinations were made.

Silylation-grade pyridine and STOX (oxime-internal standard reagent; 25mg/ml hydroxylamine hydrochloride and 6mg/ml phenyl-β-D-glucopyranoside in pyridine), were purchased from Pierce, Rockford, IL. Fructose, glucose, sucrose and bis-(trimethylsilyl)trifluoroacetamide (BSTFA) were purchased from Sigma Chemical Co., St. Louis, MO.

A 0.1g sample of freeze-dried fruit tissue was ground to a uniform powder at −15°C with a chilled mortar and pestle or by hand-crushing in a plastic freezer bag. Replicate samples (2–5 mg) of the ground powder were weighed into scrupulously cleaned, oven-dried Teflon-capped reaction vials (1.5 mL Wheaton). STOX (75 μL) and an equal volume of silylation grade pyridine (75 μL) was added. The mixture was heated for 40 min at 80°C (Minitherm BLOK Heater, Reliance Glass Works, Inc., Bensenville, IL.) and gently swirled at 10, 20, and 30 min. At the end of 40 min, 150 μL of BSTFA were added and the mixture was heated for 10 min at 80°C.

Derivatized sample solutions were refrigerated until aliquots (1 μL) were injected into a Hewlett-Packard HP5890A gas chromatograph equipped with a 2% OV-17 on 100-120 Mesh Chromasorb W-H.P. glass column (0.32 cm o.d. × 91 cm) and flame-ionization detector (FID). Chromatographic conditions were temperature programmed from 160° to 260°C at 15/min with a 3-min delay, unless otherwise indicated. Carrier gas (N_2) flow-rate was 34 mL/min. Injection port and detector temperatures were set at 280°C. Standard fructose, glucose, and sucrose were dried over phosphorous pentoxide under vacuum and stored desiccated. Response factors for standards were determined utilizing a Hewlett-Packard Model HP3392A integrator. Standard fructose, glucose and sucrose were derivatized as described above and analyzed to determine response factors compared to the internal standard phenyl-β-D-glucopyranoside prior to each assay. Response factors were linear over the ranges evaluated.

Mass spectral analysis was done on a Finnegan Model 9610 gas chromatograph fitted with a silanized glass column (2 mm i.d. × 1.5m) containing 3% OV-17 linked to a Finnigan Model 4021 Mass Spectrometer. A 70 ev ionizing potential with a 2S/Mass Decade Scan was used for all spectra. Spectra were recorded and analyzed using a Data General DS 5OS/NOVA 4 computer (Data General Corp., Westboro, MA.).

RESULTS & DISCUSSION

RESULTS (Table 1) show the simultaneous extraction and derivatization of fructose and glucose from a solid matrix to be reproducible with a coefficient of variation (cv) from 2.7 to 6.9 for five replicates. The sucrose values for tomato fruit were more variable (cv 15.7 to 17.9) than values for apple, banana and nectarine (cv 1.6 to 5.3). The small amount of sucrose present in tomatoes could, in part, account for some variability; also, nonhomogenous distribution within the sample matrix could add to the variability observed. Total sugar (TS), defined as the sum of fructose, glucose and sucrose, of the samples analyzed showed a cv of 1.7 to 5.9, indicating uniform extraction and derivatization of free sugars from the matrix.

Figure 1 shows representative gas chromatograms of TMS/

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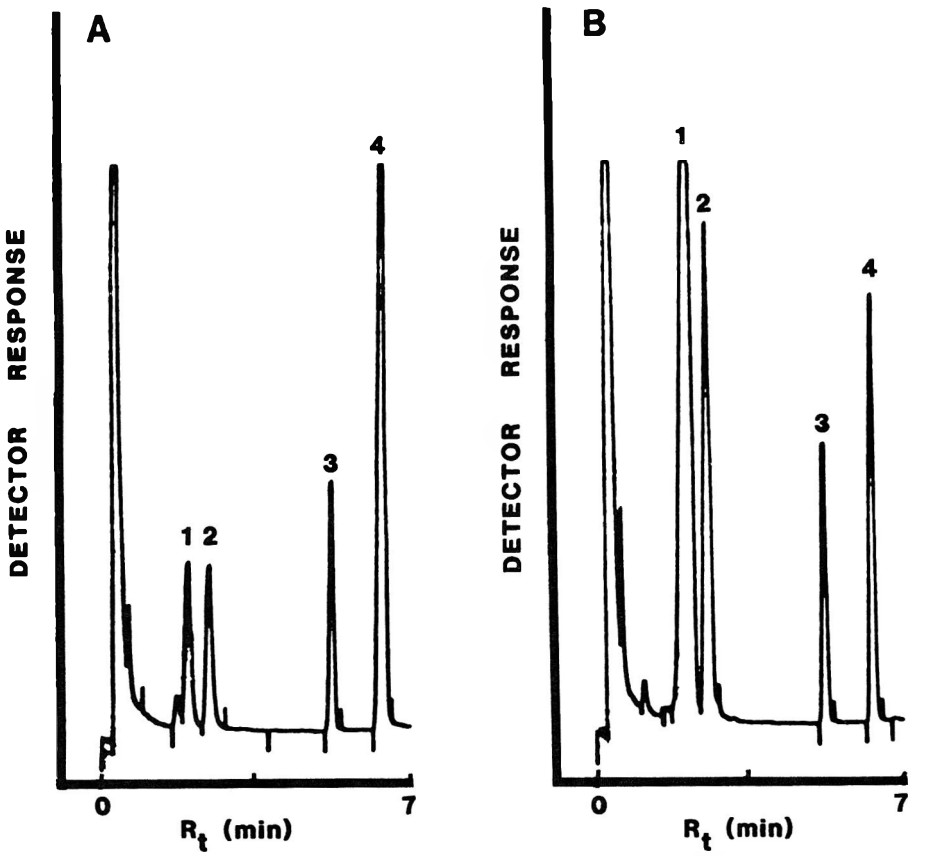
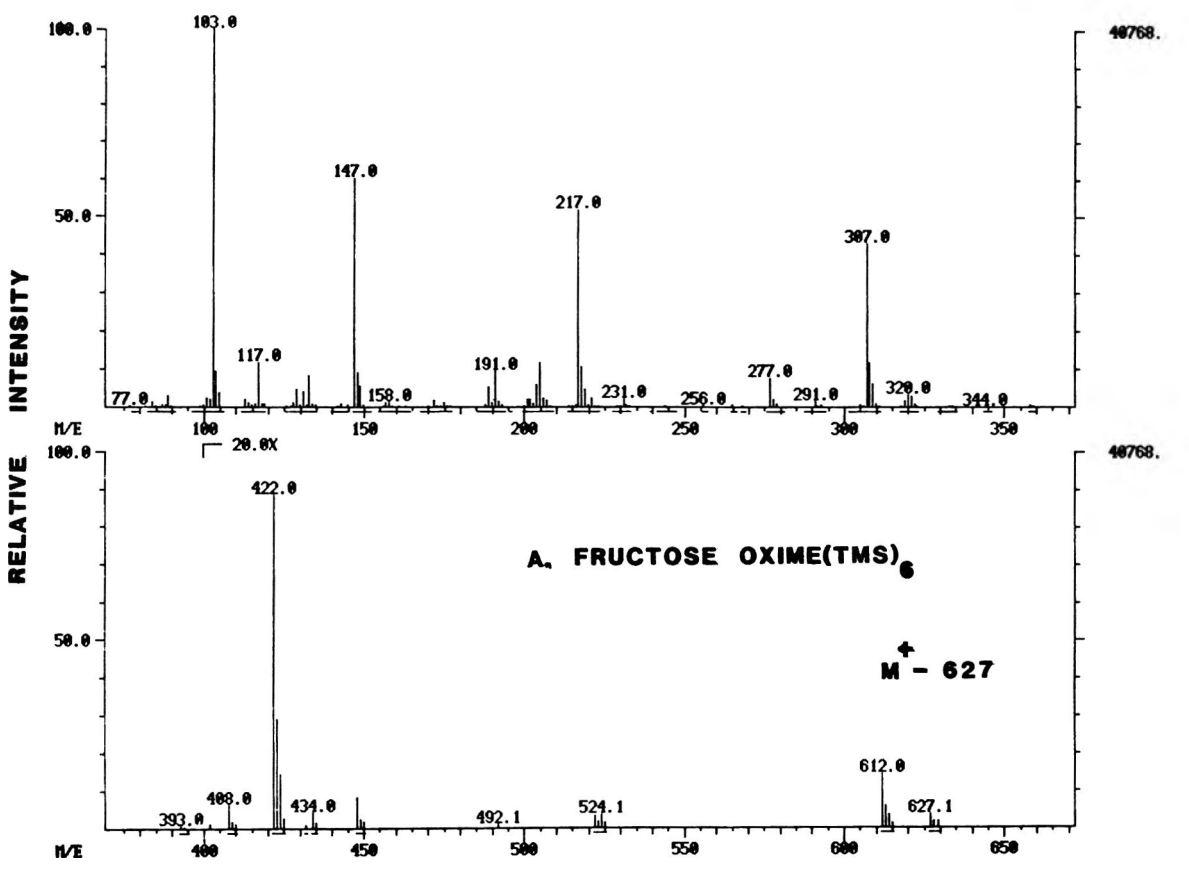


Fig. 1—Gas chromatograms of sugars from banana (A) and Red Delicious apple (B). Order of elution was fructose oxime $(TMS_6)^1$, glucose oxime $(TMS_6)^2$, phenyl-β-D-glucopyranoside $(TMS_4)^3$, and sucrose $(TMS_8)^4$.



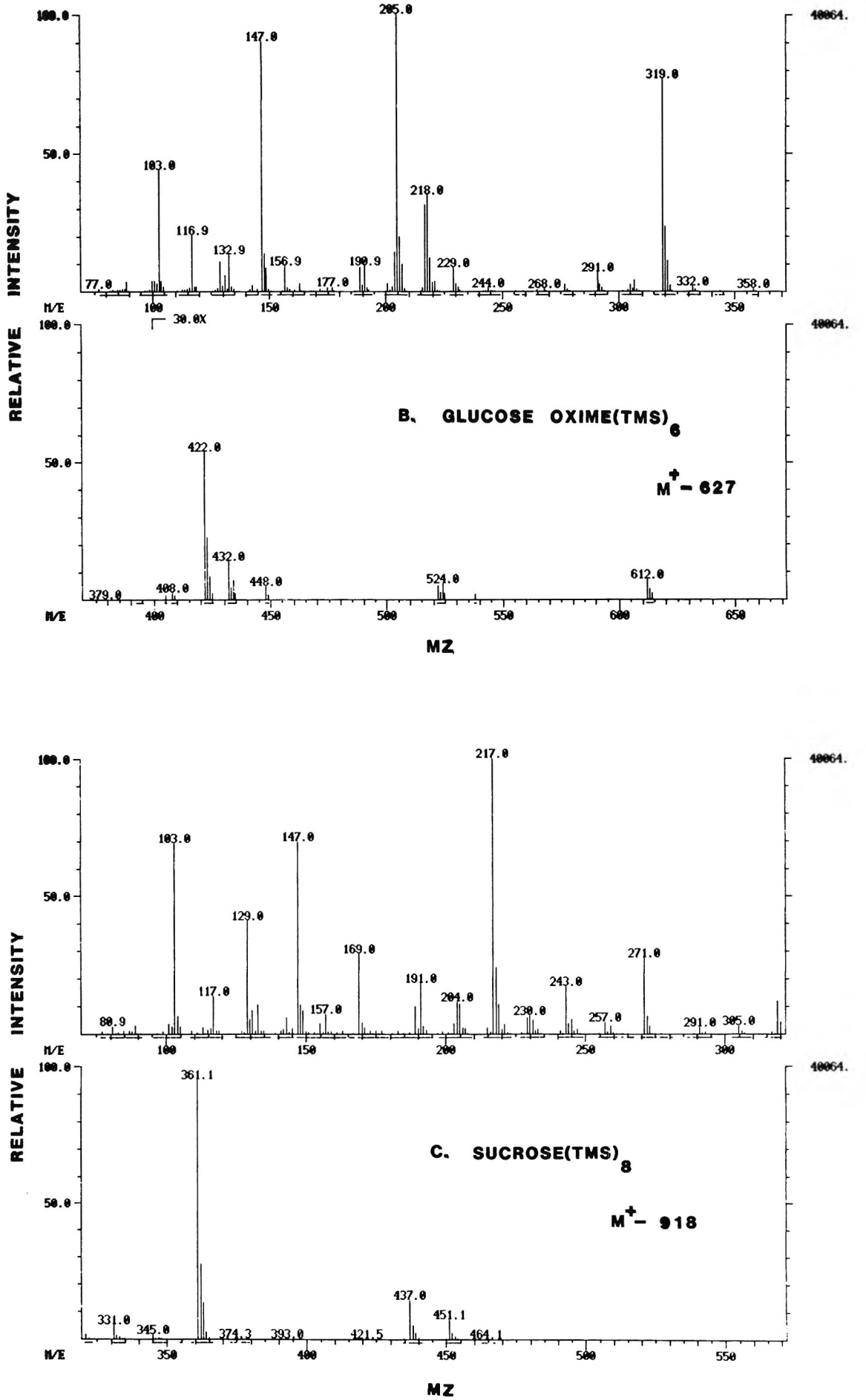


Fig. 2—Mass spectra of: (A) fructose oxime (TMS_6); (B) glucose oxime (TMS_6); and (C) sucrose (TMS_8) from Red Delicious apple.

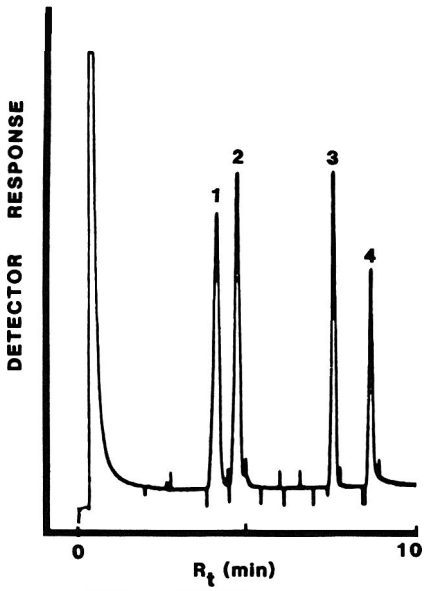


Fig. 3—Gas chromatograms of derivatized standards. Order of elution is fructose oxime (TMS_6)[1], glucose oxime (TMS_6)[2], phenyl-β-D-glycopyranoside (TMS_4)[3] and sucrose (TMS_8)[4]. Chromatographic conditions as described in Methods except the temperature program was from 150° to 260°C at 10°C/min with a 2-min delay.

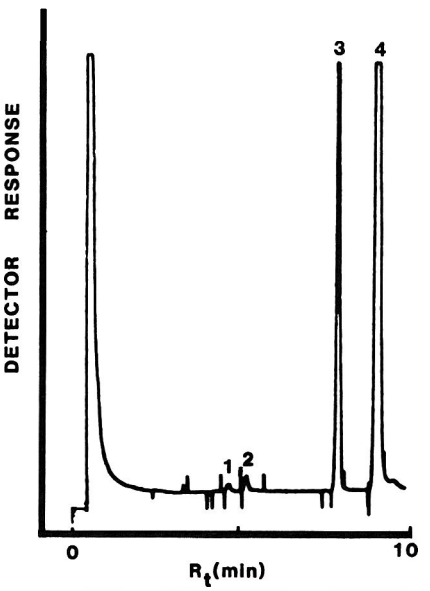


Fig. 4—Gas chromatograms showing partial hydrolysis of standard sucrose when subjected to the derivatization procedure. Order of elution was fructose oxime (TMS_6)[1], glucose oxime (TMS_6)[2], phenyl-β-D-glucopyranoside (TMS_4)[3] and sucrose (TMS_8)[4]. Chromatographic conditions were as described in Methods except the temperature program was from 150-260°C at 10°C/min with a 3-min delay.

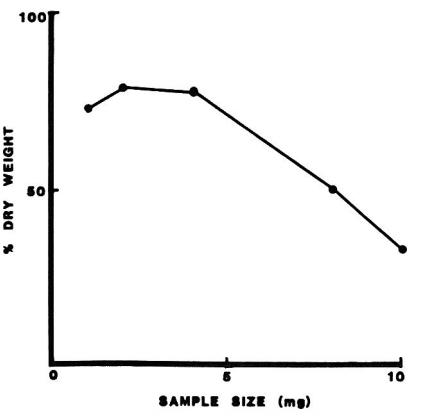


Fig. 5—The effect of increasingly larger sample (apple) sizes on sugar extraction efficiency utilizing a constant volume (100μL) of STOX-pyridine solution. Values represent an average of duplicate samples.

Table 1—Sugar content of fruits as determined by the rapid direct extraction-derivatization method

Fruit	Fructose[a]	Glucose[a]	Sucrose[a]	Total[b]
Banana	64.2 ± 4.4	65.6 ± 3.1	410 ± 5	540 ± 29
Apple (Red Delicious)	461 ± 14	149 ± 6	107 ± 2	717 ± 21
Nectarine	176 ± 8	147 ± 5	379 ± 2.6	702 ± 12
Tomato (Green, Rutgers)	220 ± 6	227 ± 7	15.0 ± 2.7	462 ± 12
Tomato (Ripe Rutgers)	299 ± 9	286 ± 10	7.4 ± 1.2	592 ± 15

[a] Mean for five replicates, μg/mg dry weight ± Standard Error
[b] Σ(Fructose + Glucose + Sucrose)

OX fructose and glucose as well as TMS sucrose from banana and Red Delicious apple. Gas chromatograms of TMS/OX and TMS sugars from nectarine and tomato were similar to banana and Red Delicious apple showing no interfering peaks. The identity and purity of peaks were confirmed via mass spectrometry, by comparison to authentic standards, which had been derivatized using identical conditions (Fig. 2A,B,C,). The mass spectra of TMS/OX fructose and glucose as well as TMS sucrose from all samples were identical to those of authentic standards. The identity of minor peaks observed have not been determined.

Gas chromatograms of sugar standards (Fig. 3) demonstrate complete conversion of reducing sugars to oximes as evidenced by the lack of additional peaks which would be present due to TMS derivatives of tautomeric reducing sugars. Clearly, the process was not hindered by matrix effects. In addition, previous work by Schaffler and DuBoil (1981) suggested hydrolysis of sucrose (<0.15%) may occur during derivatization due to the acidic nature of the reagent. Hydrolysis of sucrose would decrease sucrose values and elevate glucose and fructose values. The 1:1 dilution of STOX with silylation grade pyridine, as used in this procedure, minimized the hydrolysis of sucrose (Fig. 4). Peaks that correspond to TMS/OX fructose and glucose (Fig. 4) represent less that 0.5% of the total sucrose derivatized when standard sucrose was subjected to the oxime derivatizing procedure. The method outlined results in complete conversion of reducing sugars to their respective oximes while minimizing sucrose hydrolysis.

The efficiency of extraction of free sugars from increasingly larger sample sizes of apple, while keeping the volume of the STOX-pyridine solution constant, showed (Fig. 5) that large sample sizes could exceed the capacity of a given volume of STOX-pyridine solution to completely extract the free sugars.

A 100 μL volume of STOX-pyridine solution began to lose its ability to extract sugar from samples larger than 4 mg. To determine if increasing volume in proportion to an increase in sample size would eliminate the problem and allow for the use of larger sample sizes, another experiment was performed. Results showed that by proportionally increasing the volume of STOX-pyridine solution, samples as large as 10 mg could be used to give representative results. Maintaining a ratio of 50:1 of STOX-pyridine solution to sample size resulted in consistent extraction of free sugars (Fig. 6).

SUMMARY

THE METHOD developed for the direct extraction and derivatization of free sugars from freeze-dried fruit tissues is simple, reproducible and quantitative. The method allows for the isolation and quantitation of extractable free sugars such as fructose, glucose and sucrose, from a solid matrix, thereby, eliminating the need for large sample sizes and tedious extraction steps. Interferences due to contaminating compounds were not experienced and hydrolysis of sucrose was minimized. Strict attention to analytical detail is required to give consistent results.

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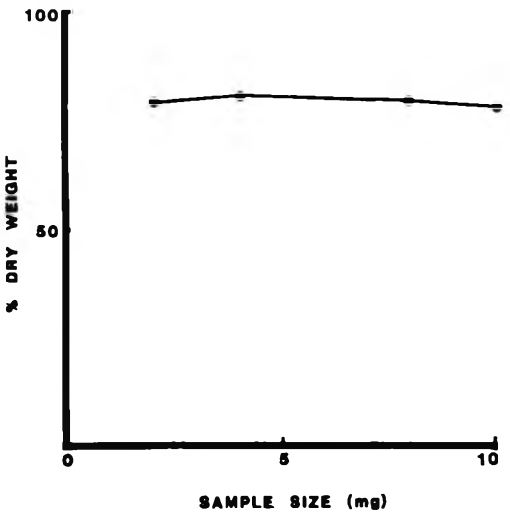

Fig. 6—The effect of utilizing a constant 50:1 ratio of STOX-pyridine solution (μL) per mg of dry Red Delicious apple tissue on sugar extraction efficiency. Values represent an average of duplicate samples.

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Ms received 5/19/86; revised 8/15/86; accepted 8/16/86.

Salaries and research support provided by State and Federal funds appropriated by The Ohio Agricultural Research and Development Center, The Ohio State University. Journal Article No. 63-86.
Mass spectra were generated by David Chang of the Campus Chemical Instrumentation Center, The Ohio State Univ., Columbus, OH.

ered along with the potential nutritional contribution of the free amino acids in a mushroom. As shown in Table 3, the sum of the residual weights of the whole protein amino acids in the mushrooms, which must be considered as available protein, is very close to that directly estimated using an average factor of 4, except that *A. bisporus* contains large amounts of nonprotein amino acids in the NPN fraction (Oka et al., 1981). Therefore, protein of the cultivated mushrooms would be lower than that cited in *Standard Table of Food Composition in Japan* (1982) and in *Amino Acid Content of Foods and Biological Data on Proteins* (FAO, 1970), in which a factor of 6.25 is tentatively adopted for the calculation.

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Detection of Phenylalanine Ammonia-Lyase in the Skin of Blueberry and Cranberry Fruits

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ABSTRACT

Phenylalanine ammonia-lyase (PAL) was extracted from the skin of individual berries and assayed spectrophotometrically under conditions minimizing interference from co-extracted constituents. Analyses for total anthocyanin (TAcy), soluble solids (SS), and titratable acidity (A) were performed on the same berries. In blueberry fruits, PAL activity was present at all maturity levels examined. Cultivar differences in anthocyanin accumulation were unrelated to PAL activity. PAL activity in cranberry fruit was unrelated to harvest date, cultivar differences in anthocyanin accumulation, or values of TAcy and SS/A in individual berries. Activity was retained in cranberries during 4 wk of refrigerated storage.

INTRODUCTION

PREVIOUSLY, we have investigated the relationship between the fruit color and anthocyanin content of highbush blueberry (Sapers et al., 1984) and cranberry (Sapers et al., 1983, 1986a). The biosynthesis of anthocyanins in the skin of these fruits during ripening and post-harvest storage is of particular interest because of the possibility that coloration might be improved by stimulation of pigmentation through exposure to such environmental factors as ethylene and light (Cracker, 1971; Proctor and Creasy, 1971; Watanabe and Arakawa, 1983). The enzyme phenylalanine ammonia-lyase (PAL) plays a key role in anthocyanin biosynthesis, possibly controlling the extent of anthocyanin accumulation (Hahlbrock, 1981; Rhodes, 1983). PAL has been detected in various plant tissues (Hanson and Havir, 1981) including fruit parenchyma cell suspension cultures (Macheix et al., 1981; Sakamoto et al., 1980) but, to our knowledge, not in the skin of blueberry or cranberry fruit. Riov et al. (1969) investigated the ethylene-controlled induction of PAL in citrus fruit peel, extracting the enzyme from an acetone powder of the peel tissue with borate buffer and partially purifying the enzyme by ammonium sulfate precipitation. Tan (1979) investigated the relationship between anthocyanin, PAL, and a PAL-inactivating system in apple skin, extracting the enzyme from a frozen powder, prepared by pulverizing the skin in liquid nitrogen, with borate buffer in the presence of polyvinylpolypyrrolidone (PVPP). The detection of PAL in pigmented fruit skins is likely to be complicated by the presence of phenolic compounds, quinones (generated from the former by polyphenol oxidase), and high concentrations of flavonoids which can complex or condense with proteins (Loomis, 1974) as well as interfere with the assay because of high background absorbance at 280 nm. Loomis (1974) has described the use of buffers, polymeric absorbents, antioxidants, and low temperature extraction to overcome these problems. Our objectives in the present study were to develop a PAL assay for the skins of individual small fruits that could be used in conjunction with other measurements to determine the relationship between PAL activity, the anthocyanin content, and fruit ripeness, and to apply this procedure to ripening blueberry and cranberry fruits.

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MATERIALS & METHODS

Experimental materials

Blueberry and cranberry samples, harvested at the Rutgers University Blueberry and Cranberry Research Center (Chatsworth, NJ) in 1984, were stored at −29°C until assayed. Blueberry fruits of several cultivars, representing different stages of color development, were harvested at Chatsworth in July, 1985, and stored briefly at 3°C until assayed. Cranberry fruits that had been tagged at the pink-white stage of color development were harvested at intervals and then assayed immediately and again after 4 weeks storage at 3°C. Samples of other fresh fruits were obtained at local supermarkets. PAL (EC 4.3.1.5; purified from *Rhodotorula glutinis*) was obtained from P.L. Biochemicals, Inc. (Milwaukee, WI) or from Sigma Chemical Co. (St. Louis, MO) and stored at −18°C. PVPP (Sigma Chemical Co.) was purified by the method of Loomis (1974). Amberlite XAD-7 polymeric adsorbent (Rohm and Haas Company, Philadelphia, PA) was purified by a water-washing procedure, as recommended by the manufacturer (Anon., 1969).

Extraction and purification procedure

The skin of individual weighed fruits (fresh or frozen) was completely removed with a razor blade and tweezers and immediately frozen, together with the peeled fruit, in liquid nitrogen. The frozen skin fragments were ground in liquid N_2 to a fine powder with a precooled mortar and pestle. To extract PAL and the anthocyanins, the frozen powder was ground with 5 mL 20 mM 2-mercaptoethanol in 0.1M sodium borate buffer, pH 8.8, and 0.4g purified PVPP. The resulting suspension was then quantitatively transferred to a 1 cm diam PVPP column, prepared with 2.5g moist, purified resin (1g dry PVPP = 3.5g moist PVPP), and held at 4°C. PAL was recovered from the column by elution with 15 mL distilled H_2O. Anthocyanins were recovered from the PVPP column following PAL recovery by elution with 20–60 mL 95% ethanol:1.5M HCl (85:15).

PAL assay

Aliquots of dilute PAL solutions, fruit skin extracts, and column eluates not exceeding 5 mL in volume were diluted to 10 mL with 0.1M sodium borate buffer, pH 8.8, in 16 × 125 mm screw cap test tubes. Five milliliters 0.06M L-phenylalanine were added to each tube at zero time. A reagent blank containing borate buffer in place of the PAL solution and an enzyme blank containing distilled H_2O in place of L-phenylalanine solution also were prepared. The tubes were capped, gently mixed by inversion, and incubated for 90 min in a 37°C water bath. The absorbance of the reaction mixtures and enzyme blank was determined against the reagent blank at 280 nm at 10 min intervals with a Perkin-Elmer Model 552 UV-visible spectrophotometer, equipped with a Perkin-Elmer Super Sipper having a temperature-controlled micro-flow cell set to 37°C. PAL activity was calculated from the slopes of absorbance vs time curves, obtained by linear regression, a unit of activity being defined as the production of 1 μmole cinnamate per min at 30°C (Havir and Hanson, 1970). A molecular extinction coefficient for cinnamate of 16,100, which is equivalent to 0.932 μmolcs per absorbance unit under the conditions of the assay, was determined experimentally. Since enzyme blanks underwent little or no change in absorbance during the assay, no enzyme blank correction was required. A temperature correction factor of 0.598, corresponding to an Arrhenius activation energy of 13.7 kcal/mole, was used to convert the activity values from 37° to 30°C, as described by Havir and Hanson (1970). Thus, the activity was calculated as:

$$\text{Activity (units)} = \text{Slope} \times \text{Dilution factor} \times 0.557$$

where 0.557 is the product of 0.932 and 0.598.

Determination of total and individual anthocyanins

The total anthocyanin content of fruits assayed for PAL was determined by spectrophotometric analysis of the alcoholic column eluates at 543 nm for blueberry and at 535 nm for cranberry. Concentrations were expressed as mg anthocyanin per 100 ml for cranberry, based on the extinction coefficients determined by Fuleki and Francis (1968 a); concentrations were expressed as absorbance units (A.U.) per 100 mL for blueberry, since extinction coefficients were not available. Because the anthocyanins were derived entirely from the skin, the anthocyanin contents of these berries was expressed as mg or A.U. per cm^2 of surface area, calculated from the quantity of anthocyanin eluted from the column and an estimate of the surface area, obtained from the berry weight, W, based on the assumption that the berries were spherical and had a density of 1.04 (Sapers and Phillips, 1985).

$$\text{Surface area } (cm^2) = 4.71\ W^{2/3}$$

The proportions of individual anthocyanins in cranberry juice and alcoholic eluates was determined by HPLC, as described by Sapers et al. (1986b).

Determination of fruit soluble solids-acidity ratio (SS/A)

Each frozen peeled berry was weighed, shattered into fragments with a mortar and pestle, and transferred to a stainless steel microblender jar with an equal weight of distilled H_2O. After about 1 min to allow for partial thawing, the berry was homogenized at high speed for 2 min. A drop of homogenate was used to obtain the soluble solids content by refractometry with a B & L Abbe −3L Refractometer (Bausch & Lomb, Inc., Rochester, NY). Weighed quantities of homogenate were diluted with 50 mL distilled H_2O and titrated with 0.1M NaOH to a pH 8.1 endpoint. Values of SS/A were calculated as the ratio of the percent soluble solids (corrected to 20°C) to the titratable acidity (expressed as percent citric acid).

Evaluation of PVPP column technique

The PVPP column technique was evaluated with buffered cranberry juice containing added PAL rather than with extracts of individual berry skins which would be highly variable in composition and sufficient only for a few trials. The juice was pressed from thawed cranberry samples (Sapers et al., 1983), adjusted to pH 4, 5 or 7 with borate buffer, and spiked with varying amounts of PAL (in borate buffer), added after pH adjustment. Juice samples (which contained no PAL activity), were analyzed spectrophotometrically for total anthocyanin by the pH differential method of Fuleki and Francis (1968b). PAL assays were performed daily on enzyme solutions used in each experiment. PVPP columns equilibrated at 20° or 4°C, were charged with varying amounts of juice at different pH values and added PAL levels. PAL was eluted, and the percent recovery of enzyme activity and percent removal of substances absorbing at 280 nm were calculated. The anthocyanins were then eluted, and the percent recovery determined. Additional recovery experiments more closely resembling berry skin assays were performed with simulated skin extracts comprising 0.5 mL cranberry juice, diluted with 0.6, 1.0, 1.7 or 4.0 mL borate buffer containing 20 mM 2-mercaptoethanol and sufficient water to bring the final volume to 5 mL, giving pH values of 4.2, 5.1, 7.4, or 8.0, respectively. Each simulated extract was combined with 0.4g PVPP and 0.005 units of PAL and then added to a 2.5g PVPP column, equilibrated at 4°C, for PAL and anthocyanin elution.

RESULTS & DISCUSSION

Preliminary experiments with purified PAL

Assays performed on dilutions of *Rhodotorula glutinis* PAL in pH 8.8 borate buffer, to determine the lower limits of PAL detection, gave a constant activity per milliliter of enzyme concentrate for slopes between 0.5×10^{-3} and 14.5×10^{-3} absorbance units per min. A highly variable response was observed with more dilute enzyme solutions, i.e., those giving slope values smaller than 0.5×10^{-3} absorbance units/min, which is equivalent to a PAL concentration of about 2×10^{-5} activity units/mL. Ideally, fruit skin samples to be assayed for PAL should be large enough to contain 3×10^{-4} activity units for the assay to be in the useable slope range, assuming that the fruit skin PAL is similar in behavior to PAL from *R. glutinis*.

Attempts to demonstrate PAL activity in extracts of cranberry, blueberry and blackberry fruits prepared by blending the berries and borate buffer for 2 min at high speed in a stainless steel semi-micro blending jar on a Waring base, adding 5% Celite Analytical Filter Aid to the homogenate and then filtering through Whatman No. 5 paper under suction, were unsuccessful, even when the berries were "spiked" with PAL prior to blending. Blending PAL in buffer (no added berries) without any further treatment was sufficient to cause the loss of all PAL activity. PAL activity also was completely lost when 0.5–5.0% Celite was added to borate buffer solutions of the purified enzyme or to spiked cranberry homogenates diluted with buffer and then removed by filtration through Whatman No. 5 paper under suction prior to the assay. About half of the added PAL activity was lost when solutions of the enzyme in borate buffer or in previously clarified buffer extracts of cranberry, blueberry or blackberry fruits were filtered through Whatman No. 5 paper without added Celite. These berry extracts contained no endogenous PAL activity (as a consequence of blending and Celite addition) and, when spiked with PAL, were equivalent in enzyme activity to spiked borate buffer.

Finally, PAL activity was completely lost when cranberry juice was spiked prior to dilution with borate buffer. The loss in activity could be avoided by buffering the highly acidic juice (pH 2.5) to pH 5 (juice:buffer = 1:2) before the addition of PAL.

Thus, extraction procedures that entail high speed blending, the use of Celite, filtration, or prolonged exposure of the extracted PAL to a low pH due to co-extracted fruit acids should be avoided.

PVPP column for sample clean-up

To perform assays for PAL in intensely colored fruit skin extracts, interfering flavonoids and other phenolic compounds must first be removed. We investigated the use of PVPP columns to remove these compounds from cranberry juice spiked with PAL and also examined the recovery of adsorbed PAL and anthocyanins from the spent PVPP for subsequent quantitation (Table 1).

All treatments reduced the absorbance of extracts at 280 nm by 70–90%, presumably by the adsorption of flavonoids (including the anthocyanins), polyphenols, and other aromatic compounds on the column. The pH had a marked effect on PAL recovery, the enzyme being inactivated at pH 4; anthocyanin recovery was greater at the lower pH values than at pH 7 (Expt. 1). PAL recovery increased and anthocyanin recovery decreased as the quantity of cranberry juice loaded on the column decreased (Expt. 2). We estimate from the mean berry weights and anthocyanin contents of representative cranberry cultivars that the 1 mL juice level was equivalent in pigment content to the skin of 1 large berry (Sapers et al., 1986c). Column size (Expt. 3) had little if any effect on PAL or anthocyanin recovery. PAL recovery was improved when the column treatment was carried out at 4°C instead of at room temperature (Expt. 4). PVPP columns were compared with columns containing Amberlite XAD-7, alone or mixed with PVPP. Higher PAL recoveries were obtained with PVPP columns than with the mixed resins (Expt. 5) or XAD-7 alone (data not shown). Consequently, PVPP columns were used for extract clean-up in all subsequent PAL assays. PAL recovery decreased when the level of enzyme added to the column was reduced from 0.008 to 0.002 units (Expt. 6). This may be due in part to the variability of the assay at low PAL concentrations as well as to losses in activity during extraction and clean-up on the PVPP column.

Under conditions more closely simulating the assay of berry skins (Expt. 7), the PVPP column reduced the absorbance of simulated extracts at 280 nm by 70–78% and gave PAL recoveries of 78–87% as well as anthocyanin recoveries of 88–

Table 1—Performance of PVPP column in clean-up of cranberry juice for PAL assay.

Expt.	Treatment: Column size (g PVPP)[a]	Added PAL (units)	Added cranberry juice (mL)	pH	Column temp (°C)	Performance: A280 removed (%)	PAL recovery (%)	Anthocyanin recovery (%)
1	1.8	0.011	4	4	20	78	0	95
	1.8	0.011	4	5	20	89	50	91
	1.8	0.011	4	7	20	85	65	31
2	1.8	0.011	2	5	20	85	68	78
	1.8	0.012	1	5	20	85	88	36
3	1.8	0.010	1	5	20	91	74	56
	2.5	0.010	1	5	20	91	76	52
4	1.5[b]	0.009	1	5	20	90	57	—
	1.5[b]	0.009	1	5	4	90	69	—
5	1.5[b]	0.006	1	5	4	92	59	—
	1.5	0.006	1	5	4	89	77	—
	2.5	0.006	1	5	4	90	87	—
6	2.5	0.008	1	5	4	89	109	110
	2.5	0.004	1	5	4	92	70	113
	2.5	0.002	1	5	4	93	24	111
7[c]	2.5	0.005	0.5	4	4	82 ± 3	35 ± 15	91 ± 8
	2.5	0.005	0.5	5	4	78 ± 2	78 ± 10	98 ± 8
	2.5	0.005	0.5	7	4	76 ± 5	87 ± 9	95 ± 8
	2.5	0.005	0.5	8	4	70 ± 3	83 ± 4	88 ± 4

[a] Moist, purified PVPP packed in 1 cm diam column.
[b] 1:500 dilution of PAL standard.
[c] 1.5g PVPP + 0.75g XAD-7.
[d] Determined in triplicate; mean values ± standard deviation.

Table 2—Recovery of individual anthocyanins from PVPP column used in PAL assay of cranberries

Trial	Peak no.[b]	Peak area (%)[a]: Juice added[c] to column	Alcoholic eluate[d] from column
1	1	27.4	23.5
	3	13.0	12.1
	4	39.5	41.6
	5	4.2	5.2
	6	12.2	13.6
2	1	27.2	26.2
	3	13.0	13.1
	4	39.6	38.8
	5	4.1	4.8
	6	12.3	12.7

[a] Mean of duplicate determinations for identical columns (1 and 2) prepared on successive days.
[b] Peaks 1, 3, 4 and 6 tentatively identified as cyanidin-3-galactoside, cyanidin-3-arabinoside, peonidin-3-galactoside, and peonidin-3-arabinoside, respectively. Peak 5 not identified. (Sapers and Hargrave, 1987).
[c] 1 mL Ben Lear cranberry juice, pH 5, added to 2.5g PVPP column at 4°C.
[d] Eluted with 40 mL 95% ethanol:1.5M HCl (85:15).

Table 3—Effect of PVPP and 2-mercaptoethanol addition during extraction on the level of PAL detected in the skin of Santa Rosa plum

Expt.	Treatment	PAL activity in skin (units/g × 10^{-3})
I	Control	0.87
	10 mM 2-mercaptoethanol	3.50
	20 mM 2-mercaptoethanol	6.72
	40 mM 2-mercaptoethanol	3.76
II	Control	0.97
	0.1g PVPP	1.08
	20 mM 2-mercaptoethanol	1.84
	0.1g PVPP + 20 mM 2-mercaptoethanol	3.64
III	Control	1.58
	0.4g PVPP	1.18
	20 mM 2-mercaptoethanel	2.56
	0.4g PVPP + 20 mM 2-mercaptoethanol	2.85

98% at pH values of 5–8. As expected, PAL recovery was much lower at pH 4.

To determine whether the column treatment had any effect on individual anthocyanins adsorbed from berry skin extracts, we compared the proportions of individual anthocyanins, determined by HPLC, in cranberry juice and in alcoholic eluates from a PVPP column loaded with 1 mL of the same juice (Table 2). The proportions of individual anthocyanins were very similar in the original juice samples and column eluates. Consequently, the anthocyanin composition of a berry being assayed for PAL activity could be determined by means of HPLC analysis of the alcoholic eluate.

Detection of PAL in fruit skin

Initial efforts to demonstrate PAL activity in fruit skins ground in liquid N_2, extracted with cold borate buffer, and assayed following PVPP column pretreatment, were successful with Santa Rosa plum and sweet cherry but not cranberry (frozen) or apple. When the borate buffer used to extract frozen cranberry skin powder was spiked with 0.0027 units PAL, about 50% of the added activity could be recovered in the column eluate following PVPP clean-up.

To obtain further improvements in PAL recovery from fruit skin, 2-mercaptoethanol and PVPP were added to the borate buffer used to extract the frozen, powdered skin (Table 3). These experiments were performed with skin from Santa Rosa plums instead of blueberries or cranberries (which have only about 5-10% of the surface area of a plum) so that sufficient material of similar composition would be available for the evaluation of multilevel treatments. The recovered PAL activity was greatest when the buffer contained 20 mM 2-mercaptoethanol (Expt. I). PVPP was ineffective by itself but consistently increased the level of detected PAL activity when used in combination with 2-mercaptoethanol (Expts. II and III). The combination of 20 mM 2-mercaptoethanol and 0.4g PVPP was employed in subsequent assays for PAL in fruit skin, the amount of PVPP conforming to the use level recommended by Loomis (1968). Extracts obtained with this combination had pH values between 7.7 and 8.6, depending on the acidity and mass of the berry skins. With the modified extraction procedure, we were able to detect PAL activity in the skins of fresh blueberries (2.3×10^{-3} units/g) and frozen cranberries ($0 - 3.5 \times 10^{-3}$ units/g) but not in nectarine or peach skins. The recovery of PAL from samples of blueberry and cranberry skin spiked with 0.0040 enzyme units exceeded 80% (with correction for endogenous PAL).

Table 4—PAL activity in the skins of individual blueberry fruits of different coloration

Cultivar	Berry color	No. berries assayed	Mean ± Standard deviation: PAL activity (units/cm² × 10⁻⁴)[a]	Total anthocyanin (A.U./cm²)[a,b]	SS/A
Bluecrop	Pink-green	2	0	0.3[c]	0.7[c]
	Red-purple	3	1.1 ± 0.1	1.5[c]	4.5[c]
	Purple-blue	3	1.9 ± 0.5	3.0[c]	6.5[c]
	Blue	2	0.8 ± 0.1	18.3[c]	10.0[c]
	Black	4	1.6 ± 0.8	12.9[c]	42.6[c]
Coville	Pink-green	2	0.5 ± 0.6	0.4 ± 0.0	2.4 ± 0.2
	Red-purple	2	1.5 ± 0.0	2.8 ± 0.4	5.0 ± 1.1
	Purple-blue	2	0.8 ± 0.1	6.2 ± 2.3	5.4 ± 0.4
	Blue	2	0.6 ± 0.2	27.0 ± 12.3	13.6 ± 1.2
	Black	2	0.6 ± 0.2	25.9 ± 9.8	20.6 ± 3.7

[a] Calculated from estimated surface area of each berry.
[b] A.U. (absorbance unit) = absorbance at 543 nm × volume × dilution factor.
[c] Single berry only.

Table 5—PAL activity vs total anthocyanin and SS/A in ripening cranberry fruit after harvest and following 4 wk storage at 3°C

Cultivar	Days after tagging[b]	Mean ± standard deviation[a]: After harvest: PAL (units/cm² × 10⁻⁴)[c]	Total anthocyanin (mg/cm²)[c]	SS/A	After storage: PAL (units/cm² × 10⁻⁴)[c]	Total anthocyanin (mg/cm²)[c]	SS/A
Franklin	0	1.5 ± 0.7	0.009 ± 0.006	3.6 ± 0.7	2.8 ± 0.5	0.044 ± 0.045	3.1 ± 0.5
	7	3.1 ± 1.6	0.089 ± 0.066	4.2 ± 0.9	2.1 ± 0.6	0.046 ± 0.022	3.2 ± 0.3
	15	1.6 ± 0.6	0.044 ± 0.030	4.1 ± 1.7	2.4 ± 0.6	0.053 ± 0.031	3.6 ± 0.6
	21	2.2 ± 0.3	0.057 ± 0.035	3.6 ± 0.1	3.0 ± 0.9	0.089 ± 0.030	4.2 ± 0.6
Howes	0	3.4 ± 0.5	0.006 ± 0.001	2.9 ± 0.4	2.3 ± 1.3	0.014 ± 0.012	2.9 ± 0.1
	7	3.8 ± 1.2	0.030 ± 0.019	3.9 ± 0.2	2.6 ± 0.4	0.023 ± 0.016	3.6 ± 0.3
	15	2.8 ± 0.8	0.054 ± 0.033	3.7 ± 0.6	3.2 ± 1.1	0.079 ± 0.019	3.9 ± 0.7
	21	2.5 ± 0.4	0.071 ± 0.064	4.5 ± 0.8	2.4 ± 0.4	0.087 ± 0.046	3.5 ± 0.4

[a] Mean of four berries per treatment.
[b] Tagged at pink-white stage of color development.
[c] Calculated from estimated surface area of each berry.

Quantitative studies with blueberry and cranberry

To determine whether PAL activity in blueberry was related to anthocyanin content or ripeness, we carried out a quantitative study of blueberry fruits representing 2 cultivars, harvested at different stages of color development (Table 4). Our data indicated somewhat variable PAL activity in fruits of different maturity, as indicated by coloration, total anthocyanin, and SS/A values. Correlations between PAL activity and total anthocyanin or SS/A were not significant for either cultivar. The presence of PAL in fully ripe and over-ripe blueberries is probably of marginal importance with respect to fruit color, even if anthocyanin biosynthesis continues in the skin, since the color of such fruit is greatly influenced by the state of their epicuticular wax, i.e., the presence of a rodlet wax structure which produces the glaucous effect responsible for the typical light blue color of blueberries (Albrigo et al., 1980).

Although the presence of PAL in the skin of ripening blueberries may be related in some way to their capacity to accumulate anthocyanin, there was no relationship between PAL activity in Coville berries which are high in total anthocyanin content (150 A.U./g in fully ripe berries) and in Bluecrop which are low (63 A.U./g). It would be of interest to extend the methodology described herein to other enzymes involved in anthocyanin biosynthesis as well as to potential substrates and inhibitors so that the basis for genetic differences in total anthocyanin accumulation and in the pattern of individual anthocyanins observed in previous studies (Sapers et al., 1984) could be elucidated.

The level of PAL activity found in frozen cranberry samples (data not shown) varied greatly between berries but did not appear to be related to berry coloration or cultivar. Due to the possibility that these results may have been influenced by the frozen storage of the fruit (8 months at −29°C), we repeated the study with fresh cranberries that were tagged in the pink-white stage of color development and then harvested at 1 wk intervals over 3 wk. Analytical data obtained for two cultivars at the time of harvest and after 4 wk at 3°C (Table 5) indicated no relationship between PAL activity and harvest date or post-harvest storage. Similarly, we observed no correlations between the PAL activities of individual berries and their total anthocyanin contents ($r = 0.46$ and -0.16 for Franklin and Howes cranberries, respectively) or SS/A values ($r = -0.19$ and -0.04 for Franklin and Howes respectively). Values of the latter parameter are indicative of cranberry fruit ripeness (Sapers et al., 1986a). Our data did not show the extent of post-harvest color development since we intentionally selected berries representing a range of colorations at each sampling time to permit the comparison of PAL activity with total anthocyanin content. However, the presence of PAL activity in fruits of different ripeness and anthocyanin content after 4 weeks of post-harvest storage is consistent with the well known ability of cranberries to increase in pigment content during storage (Zukerman et al., 1966).

In a previous study (Sapers et al., 1986c), we reported large differences in the capacity of Franklin and Howes cranberries to accumulate anthocyanin, fully colored fruits of the former cultivar containing twice as much pigment as the latter. This characteristic does not appear to be related to PAL activity in the fruit skin. The extent to which PAL or other enzymes, substrates, or inhibitors limit anthocyanin accumulation in ripening cranberries and during post-harvest storage requires further investigation. Such information may shed light on genetic differences in anthocyanin accumulation and suggest new approaches to the enhancement of cranberry fruit color.

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Quality of Freeze Concentrated Orange Juice

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ABSTRACT

Pasteurized Valencia and Temple orange juices concentrated to 45°Brix by freeze concentration retained their fresh juice flavor. Direct steam infusion heating to inactivate enzymes allowed more rapid heating than indirect heating and successfully lowered juice peel oil during vacuum cooling. Except for considerable pulp reduction of feedstream juices, there were few differences from normal citrus juice recovery procedures for freeze concentration. Since the product retained most of the aroma constituents of fresh juice, careful handling and high quality feed juice prior to freeze concentration was much more important than for evaporation. Fresh juice freeze concentrated to 45°B, then pasteurized at temperatures of 80°, 97° and 111°C had reduced sucrose (up to 25%) as the temperature increased to 111°C.

INTRODUCTION

USE OF FREEZE CONCENTRATION instead of evaporation to concentrate orange juice could reduce loss of volatile aromas and thermal degradation of product. Stahl (1944) reported that freeze concentrated citrus juice possessed "a richer fruit flavor than that previously obtained by any other known process, because no volatile aromas or flavors are lost and the chemical changes liable to occur during concentration are reduced to a minimum." Smith (1965) showed that quality improvement (measured by trained and consumer taste panels) of orange concentrates occurred when blending with freeze concentrate compared with single strength juice cutback. Muller (1967) and Deshpande et al. (1982, 1984) reviewed freeze concentration processes for fruit juices and concluded that the aroma, flavor and nutrient content of products were of superior quality.

While the earlier processes yielded high quality products, many had considerable losses of soluble solids during product recovery. A multistage commercial process capable of high aroma retention and minimal loss of juice solids has been developed (van Pelt, 1984). Comparison of current orange juice evaporation processes with freeze concentration resulted in the conclusion that freeze concentrated product had higher quality (van Pelt and Swinkels, 1984). Other descriptions of citrus juice concentration by freeze concentration have claimed that product with superior aroma and flavor qualities is produced with this technique (Strobel, 1983, 1984).

The purpose of this study, based on results of pilot scale experiments, was to determine the effects of some juice processing variables, such as heat inactivation of enzymes and pulp content on quality of freeze concentrated orange juice. Some advantages and limitations of the process compared with commercial evaporation will be discussed.

MATERIALS & METHODS

Juice recovery

Table 1 contains a general description of extracted juices and conditions used during this study. Orange juice extraction involved the use of two common commercial extractors during the times experiments were conducted. One type reamed the juice from fruit (Model 700, Brown International, Inc., Covina, CA), while the other squeezed the fruit (Model 291B, FMC Corp., Citrus Machinery Div., Lakeland, FL). Different extractors were used because of availability at the time of fruit maturity, not because of plans to compare product from each extractor. Juice sample size was from 500-800 L. After extraction, juices were screened (0.5 mm) in a screw finisher to reduce pulp content. Additional pulp reduction to 2-3% was performed on some samples. This was accomplished by continuous centrifugation (Westfalia Model SA 7-06-076, Centrico, Northvale, NJ) of fresh or heat stabilized juices.

Table 1—Descriptions and abbreviations of samples

Description	Code
Valencia juice	VAL
Temple juice	TPL
Early maturity	EM
Prime maturity	PM
Late maturity	LM
Freeze concentrated juice	FC
Evaporator concentrated juice	EC
Nonpasteurized juice	NP
Pasteurized at 80°C	P80
Pasteurized at 90°C	P90
Pasteurized at 97°C	P97
Pasteurized at 111°C	P111
Single strength juice	SS
Hot-pack juice	HP
Steam infusion 90°C pasteurized juice	SI

Heat stabilization

Microbiological stability and cloud stability of juices were achieved by heating to temperatures greater than 80°C using pilot scale heat exchangers. Temple orange juice was heat stabilized at 88°C in either of two heat exchangers, a very rapid direct heating steam infusion process (APV Crepaco, Lake Mills, WI) or an indirect contact plate system (Junior Paraflow, APV). One sample of finished Temple juice (380 L) was heat stabilized at 88°C for 12 sec by steam infusion, centrifuged to 2–3% pulp and chilled to near 0°C to feed the freeze concentrator. Another sample (190 L) was heated in the plate exchanger to 88°C. One-half was hot-filled and one-half was held 12 sec then cooled to 25°C before sealing into 176 mL enamel lined metal cans. Samples were cased after canning and held at −20°C prior to evaluation.

Valencia orange juice was extracted from fruit of different maturities during a period extending over two processing seasons. Juice was recovered from oranges picked at late maturity (June), early maturity (March) and prime maturity (May). Some finished juice was heat stabilized by steam infusion or plate exchanger. Other juice was freeze concentrated without any heat treatment and samples were heat-stabilized 6 sec at 80°, 97° or 111°C in a tubular heat exchanger (Unitherm IV, Cherry-Burrell Corp., Cedar Rapids, IA). Samples were canned as before. All quality tests and evaluations were completed within one month of the time the juice was processed.

Freeze concentration

Juices were concentrated at temperatures below −2°C using pilot equipment (Model W-6 or W-8, Grenco Process Technology B.V., 's-Hertogenbosch, The Netherlands) operating at approximately 10 kg/hr water removal from single strength juices. Concentration continued about 30 hr until approximately 50 kg of 45°Brix concentrates were obtained. Samples of the feed, concentrate and ice melt water were canned and stored at −20°C for evaluation. Other details of operation of the freeze concentrators may be obtained from Grenco (van Pelt, 1984; van Pelt and Swinkels, 1984). Juice from late maturity Valencia

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fruit was also concentrated by evaporation in a pilot scale 225 kg/hr (water removal), 3-effect, tubular falling-film evaporator (Gulf Machinery, Inc., Safety Harbor, FL).

Quality tests

Diluted concentrate and single strength juices were analyzed for total soluble solids (°Brix) by refractometer, titratable acidity (%, as citrate), pulp (vol. %), peel oil (vol. %), color, enzyme (PEU) and viscosity (Matthews and Gray, 1973). Malic and citric acids were also measured quantitatively by comparison with standards, using a liquid chromatography technique. The mobile phase was 1.0% potassium phosphate buffer, pH 2.5 using a Zorbax C18 column. Total hexose was measured using an anthrone reagent (Stewart, 1975); while glucose, fructose and sucrose were determined by gas-liquid chromatography (GLC) (Braddock and Marcy, 1985). Ascorbic acid was determined using an indophenol dye titration (AOAC, 1980). Degree of browning (browning index) of the samples was also estimated (Meydav et al., 1977).

Aromas of some samples were estimated by GLC peak area percents of volatiles stripped with helium from juice and recovered in a liquid nitrogen trap. The stripping/trapping procedure was used for comparison of juices from various proces streams or from different thermal treatments. A modified procedure (Strobel, 1984) involved heating 300 mL of juice to 55°C, purging with helium at a flow rate of 100 mL/min for 15 min, with the vapor passing through an ice water reflux column into a 2 mm i.d. glass u-tube immersed in liquid nitrogen. A sample taken directly from the amount collected in the u-tube was injected into the GLC (Perkin Elmer Sigma 3B, Norwalk, CT). Conditions were as follows: 1.0 μL injection; 0.25 mm × 60M fused silica column with SE 30 (J & W Scientific, Inc., Rancho Cordova, CA); hydrogen carrier gas; injector and detector at 275°C; oven conditions, 50°C for 5 min, 8°/min to 200°C, hold 15 min. Peaks of interest were identified using a GLC-mass spectral system (MS-25, Kratos Instruments, Ramsey, NJ) and an on-column injection technique (Klim et al., 1984).

Taste evaluations

Taste panel evaluations were performed by a 15 member panel experienced in tasting citrus juices. Concentrates were diluted to single strength with deionized water for analyses. To become familiar with juice properties important to this study, panelists were presented with samples identified as being either fresh or heat processed juice. The heat processed standard was heated to 90°C for 15 min in a covered stainless steel cooking pot. After two training sessions, the panel ranked juices from various process treatments according to specified attributes. To minimize fatigue, only three samples were ranked (most = 1, least = 3) per session. Panelists were asked to rank juices from most to least fresh juice flavor in one session. At the next session, they were asked to rank the same samples (different codes) from most to least processed flavor. Statistical analyses of the data were performed using a computer program (Ryan et al., 1982).

RESULTS & DISCUSSION

Processing

Commercialization of freeze concentration of citrus juices is now being implemented. Probably, the most significant advantage of this technique is the retention of fresh juice aroma and character through the process into the concentrated product. However, citrus juice recovery and processing involves coordination of sophisticated techniques and operations to produce high quality products with other properties besides flavor. In this study, juice yield was not a factor which was studied; however, the extractors were set to recover juice of good quality without over-extraction. Basically, we recovered and freeze concentrated juices from Valencia oranges at three stages of horticultural maturity (EM, PM, LM; see Table 1 for abbreviations) and mature Temple oranges. Processing the EM and LM oranges, we found that freeze concentrated juice retained both immature or overripe flavor characteristics. Many undesirable odors are lost during evaporation but remain in the product during freeze concentration.

Other properties of citrus juices important to quality are listed in Table 2. Of these, some are determined by processing conditions. For example, the amounts of peel oil and pulp in the juices are adjustable and can significantly alter quality, depending on the amount. High pulp can be a major contributor to increased juice viscosity; while, too much peel oil results in a burning sensation of the lips and tongue. Results of this study indicated that the freeze concentration process operated satisfactorily without significant loss of soluble solids in the melted ice, whether the pulp in the feed stream was 1% or 10%. However, as we found in previous studies with pineapple and grapefruit juices, there was a considerable reduction of juice pulp during the freeze concentration process (Braddock and Marcy, 1985; Braddock, 1986). This would necessitate some form of juice pulp reduction by finishing and/or centrifugation prior to the concentration process. It should be noted that pulp reduction prior to evaporation is common industry practice, although the reduction is not of the magnitude (to a value of 1–2%) as done for this study (Table 2). In practice, pulp reduction results in loss of juice yield. For freeze concentration, this could increase processing cost, unless juice solids in the pulp were recovered.

Raw orange juice recovered using commercial extractors set for optimum juice yield conditions commonly will have peel oil higher than 0.025%. For example, the SSNP sample (Table 2) contained 0.037% oil immediately following extraction and finishing. Optimum oil standards for frozen concentrated orange juice are in the range, 0.012–0.015%. Some reduction to about 0.02% oil can be made by changing juice extractor variables; however, changes giving lower oil levels resulted in reduced juice yield in our pilot study. Lower yield would need to be justified under commercial conditions.

Processing conditions also affected other juice properties listed in Table 2. Most notably, centrifugation to reduce juice pulp concentration reduced the color (N) and viscosity of all juices processed, including EMVAL and LMVAL (not listed in Table 2). While a viscosity reduction would be beneficial to the concentration process, loss of juice color is considered detrimental to juice quality. During commercial manufacture, pulp is commonly added to adjust the amount in the finished juice to a level considered desirable for consumers. In such cases, one might expect some color improvement, depending on the pulp concentration in the juice.

Heat stabilization

For evaluation purposes, freeze concentration of fresh, unpasteurized (NP) juice was performed during this study. However, microbiological and cloud stability problems could occur before and after the concentration process during handling or storage of the fresh juice feed or the finished product. This might be illustrated by examination of the pectinesterase activity data listed in Table 2. Prior to pulp reduction, the fresh juice sample (SSNP) had enzyme activity of 0.7 PEU. The same juice with the pulp reduced by centrifugation to 1%, reconstituted following freeze concentration, had enzyme activity of 0.5 PEU. Experience will show that under refrigerated, and especially ambient temperature conditions, at 0.5 PEU, the serum and cloud in orange juice will separate, an undesirable quality attribute.

Heat stabilization by direct steam infusion heating followed by vacuum flash cooling of juice was a technique evaluated during this study. Advantages of this process are rapid heating times (0.5 sec) allowing quality preservation through reduced times, minimal contact with heated surfaces and the potential to lower the high level of peel oil in the freshly extracted juice during steam condensate removal by vacuum cooling. The oil reduction was easily accomplished for the Valencia orange juice. Compare SSNP with SI in Table 2, which shows reduction from 0.037 to 0.015% following steam infusion and centrifugation to lower the pulp content. However, during the Temple orange run, water pressure variation caused product dilution with steam condensate and higher juice discharge tem-

Table 2—Summary of common juice quality properties from various processing treatments

	PMVAL[a]						TPL		
	SSNP	SI	NPFC	FCP80	FCP97	FCP111	SSP90	SI	SIFC
SS basis:									
°Brix	13.5	13.3	13.3	13.3	13.3	13.3	15.0	9.86	15.0
Acid (%)	0.9	0.85	0.85	0.85	0.85	0.85	1.33	0.84	1.28
Ratio (°B/acid)	15	15.6	15.7	15.7	15.7	15.7	11.2	11.7	11.7
Oil (%)	0.037	0.015	0.020	0.018	0.017	0.016	0.02	0.003	0.003
Hexose (g/100 g)	13.3	13.0	12.7	13.0	12.0	12.0	14.4	9.6	14.7
Vitamin C (mg/100 ml)	46	42	43	44	43	41	57	35	54
Browning index	0.18	0.12	0.13	0.12	0.13	0.13	0.20	0.12	0.17
Color (C_R)	33.8	21.4	21.7	21.2	20.8	22.5	36.7	27.7	31.8
(C_Y)	83.0	64.2	64.6	64.2	64.6	65.3	85.4	77.3	80.5
(N)	37.2	33.1	33.2	33.1	33.1	33.4	38.0	35.6	36.6
Pulp (%)	10.0	2	1	1	1	1	14	5	2
Enzyme (PEU)	0.7	0	0.5	0.1	0	0	0	0	0
Viscosity (mPa·s)	5	3	3	3	4	4	7	3	4
Concentrate:									
°Brix	—	—	45.6	45.6	45.7	45.6	—	—	45
Acid (%)	—	—	2.9	2.9	2.9	2.9	—	—	3.8
Viscosity (mPa·s)	—	—	12	12	13	13	—	—	20

[a] Abbreviations are defined in Table 1.

perature from the equipment. This juice (TPLSI) had very low oil concentration, 0.003%, compared with 0.02% for the feed juice. This dilution meant that more water had to be removed during freeze concentration; otherwise, the pulp reduction from 5 to 2% was the major difference noted when the SI juice was concentrated (Table 2). There were few other differences between treatments which could be attributed to the steam infusion heating-cooling process for either juice listed in Table 2.

Pasteurization following concentration of orange juice (PMVAL) was also performed (Table 2). Here, the rationale was the considerable reduction in the mass of water which would need to be heated to stabilize the juice. The system used was capable of pumping the viscous concentrate at high velocity to get good heat transfer, resulting in inactivation of the pectinesterase except for the lowest temperature studied (80°C). Juice browning would be expected to increase with increased process temperature up to 111°C; however, no increase or color change in the heated concentrate was found (Table 2). There was a significant change in the sugar profile (gas chromatograph peak areas) with increased heating. Results in Table 3 indicate sucrose decreased from 46 to 34% of the area for the three sugars, while fructose increased from 25 to 35%, when the concentrate was heated at temperatures of 80°, 97° and 111°C. We were unable to explain why glucose content only showed a corresponding small increase, except that it may be less stable to the heat treatment.

Samples of single strength Temple juice were also compared to reconstituted freeze concentrate. The comparison of significance showed a reduced level of sucrose and increased fructose in samples which were hot-packed (Table 3). Again, the glucose was apparently less heat stable than the fructose, not increasing as the sucrose decreased. Other results in Table 3 show very little differences between organic acids at the various treatments or between sugars comparing steam infusion with plate pasteurization of the single strength Temple juice. Plate pasteurization of the single strength VAL juice (SSP90) was also comparable to the tubular pasteurization of the freeze concentrate at 80°C.

Table 3—Sugars and acids in processed Valencia (VAL) and Temple (TPL) orange juice[a]

	(% GLC area[b])			Acid (mg/ml SS juice)	
Sample	Fructose	Glucose	Sucrose	Malic	Citric
PMVAL:					
FCP80	25	29	46	3.1	11.1
FCP97	28	31	41	3.1	11.3
FCP111	35	31	34	2.8	11.5
SSP90	26	29	45	2.9	10.9
TPL:					
SSP90	26	29	45	5.9	13.9
SIFC	25	30	45	5.7	12.5
SSHP	32	30	39	5.5	12.8

[a] Abbreviations are defined in Table 1.
[b] Values were obtained by summation of the areas for the three sugars and calculating the % contribution of each.

Volatile aroma analyses

It has been established that freeze concentration processes have the potential for preserving the volatile aromas present in fresh fruit juice (Deshpande et al., 1984). For orange juices, there have been data published that illustrated the superior aroma quality of freeze concentrate compared with evaporator concentrate. Some studies compared GLC analyses of volatiles stripped from juices under specific conditions, showing retention of compounds with low and high boiling points (Strobel, 1983, 1984, 1985). We agree with these reports, but would like to discuss some factors which are important to interpretation of analytical data from GLC aroma analyses. For example, Strobel (1984) reported that an orange juice with a ratio of ethyl butyrate:limonene in the range 0.004–0.4 would have a preferred aroma and flavor. This broad range could encompass many factors relating to fruit maturity and processing variables or the stripping and recovery/analysis technique used.

The data in Table 4 can be used to illustrate some effects of certain processing conditions on volatile aroma compounds in a sample of very late maturity Valencia orange juice. First, the very high ethanol content (77.8% area) in the aroma stripped from unpasteurized single strength juice (SSNP) was indicative of the late season, over-mature quality of the fruit as well as

Table 4—GLC peak area percent of aroma compounds recovered from fresh, pasteurized and freeze concentrated juice from late mature Valencia oranges

Compound	SSNP[a,b]	SSP90	NPFC	P90FC
MeOH/acetaldehyde	2.80	1.10	1.20	0.30
Ethanol	77.80	19.0	26.30	6.40
Ethyl acetate	0.06	0.03	0.02	0.01
Acetal	0.03	0.03	0.03	0.02
Hexanal	0.02	0.14	0.11	0.06
Ethyl butyrate	0.06	0.19	0.18	0.09
Pinene	0.07	0.41	0.38	0.44
Octanal	0.05	0.32	0.26	0.22
Myrcene	0.34	1.97	1.45	1.90
Limonene	17.70	74.70	68.70	89.10
Linalool	0.02	0.20	0.13	0.24
Decanal	0.19	0.60	0.18	0.02
Neral	0.05	0.05	0.07	0.02
Geranial	0.12	0.04	0.08	0.03
Valencene	0.43	0.20	0.25	0.19

[a] Average of triplicate samples, two injections per sample, pooled standard deviation 15% of value.
[b] Abbreviations are defined in Table 1.

the handling of the raw juice following extraction. Those familiar with the flavor of fresh juice from over mature fruit would probably not consider this juice to be of best quality. If one examines all the samples listed in Table 4 according to the proposal of Strobel (1983, 1984), then none of these juices would meet the preferred flavor criteria for the above-mentioned ethyl butyrate and limonene contents.

Data in Table 4 also indicate that the most highly volatile constituents, methanol, acetaldehyde and ethanol are subject to considerable losses during pasteurization and cooling. These reductions affect the composition of the GLC aroma profile, most notably increasing the proportions of the less volatile substances, particularly limonene. Important interpretations of this data, considering Strobel's conclusions and those of Carpenter et al. (1983), are that changes in the highly volatile aroma fractions can be indicative of fruit condition and processing variables. Certainly, the feedstock juice for a freeze concentration process would need to have initial good volatile aroma quality because the final concentrate will be similar.

Taste evaluation

Concentration of fresh juice followed by a heating step to inactivate enzymes and microorganisms was studied. One would expect that heating effects on more concentrated constituents would be greater than when heating the dilute materials. Some taste test ranking studies confirm that panelists can detect increased heating temperatures in concentrated fresh orange juice which was subsequently pasteurized (Table 5). As indicated, panelists ranked the concentrate heated to the highest temperature as having the least fresh juice flavor and the most processed flavor. Panelists were unable to detect significant flavor differences between concentrates heated to 80° and 97°C.

In a study involving different heating methods applied to Temple orange juice (a citrus juice very sensitive to heat), taste test ranking studies compared plate and steam infusion heat exchangers and hot-packing juices. Results of these tests showed that hot-packed juice had the least fresh and most processed flavor (Table 6). These results were valid even though the steam infusion process reduced the volatile essential oils in the TPL juice to very low levels (Table 2). Apparently, some thermally induced off-flavors were nonvolatile and detectable by the taste panel. Since all three samples were heated to the same temperature (90°C), the time of exposure was the apparent reason the hot-packed juice had the least fresh flavor. During the experiment, cooling time to ambient temperature for this juice was not measured but would have been at least several hours.

Table 5—Mean and standard deviations for ranking prime maturity Valencia (PMVAL) orange juices according to fresh and processed flavor

Ranking	Sample	N	Mean	Std. dev.
Fresh	FCP80[a]	15	1.5 c	0.64
	FCP97	15	1.7 c	0.70
	FCP111	15	2.8 d	0.41
Processed	FCP111	15	1.3 e	0.72
	FCP97	15	2.3 f	0.62
	FCP80	15	2.3 f	0.71

[a] Abbreviations are defined in Table 1. Means with similar letters are not significantly different. N is the number of evaluations.

Table 6—Mean and standard deviations for ranking various processed Temple (TPL) orange juices according to fresh and processed flavor[a]

Ranking	Sample	N	Mean	Std. dev.
Fresh	SSP90	36	1.9 c	0.85
	SIFC	36	1.9 c	0.80
	SSHP	36	2.3 d	0.81
Processed	SSHP	36	1.4 e	0.68
	SIFC	36	2.1 f	0.61
	SSP90	36	2.5 f	0.78

[a] Abbreviations are defined in Table 1. Means with similar letters are not significantly different. N is the number of evaluations.

These results as well as our experience with citrus processing emphasize the importance of the enzyme stabilization/pasteurization process prior to freeze concentration. High amounts of ethanol in the volatile fraction of raw juice from over-mature fruit could also be a factor for juice handled improperly or held for long periods allowing yeast growth before heat stabilization. Too much heat may also adversely affect the quality as shown in the results in Tables 5 and 6. Although concepts of chemical kinetics do not support improved product quality when concentration is followed by heating, the potential exists to do so, if raw juice handling could be carefully controlled. Plant and equipment sanitation and rapid chilling to low temperatures of freeze concentrator feed streams would have to be stringently monitored. The pilot freeze concentration process described has recently become commercial for orange, grapefruit and mandarin juices. As interest and applications expand, the industry will solve such problems as mentioned above. Our main conclusion is that flavor and quality parameters of orange juices studied were mostly determined by horticultural factors, handling and thermal processes and not a result of freeze concentration.

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Ms received 6/23/86; revised 9/12/86; accepted 9/12/86.
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High Performance Liquid Chromatography of 2,5-Dimethyl-4-Hydroxy-3 (2H)-Furanone in Pineapple and Grapefruit Juices

HYOUNG S. LEE and STEVEN NAGY

ABSTRACT

A reversed-phase HPLC method was developed for rapid analysis of 2,5-dimethyl-4-hydroxy-3(2H)-furanone (DMHF), the compound responsible for the "burnt-pineapple" flavor in pineapple juices. A Zorbax ODS column was used with a mobile phase of 0.05M sodium acetate (pH 4.0)/methanol (70:30); detection was by UV at 290 nm. Sample clean up was accomplished by solid-phase extraction with C-18 Sep-Pak cartridges. DMHF contents ranged from 1.6 to 27.3 ppm from 10 fresh pineapple juices. Using this system, the production of DMHF was monitored in canned grapefruit juices during storage at varying temperatures.

INTRODUCTION

2,5-DIMETHYL-4-HYDROXY-3(2H)-FURANONE (DMHF) is a very important compound due to its pleasant organoleptic character, and is used extensively for flavoring beverages and foods (Hirvi et al., 1980). Rodin et al. (1965) stated that it is the character impact compound of pineapple flavor, and has been identified in many fruits such as strawberries (Ohloff, 1969; Pickenhagen et al., 1981), raspberries (Honkanen et al., 1980), mango (Pickenhagen et al., 1981), and arctic bramble (Kallio, 1976). This compound has been found as one of the degradation products of sugars (Shaw et al., 1968). Also, it was responsible for the characteristic pineapple-like note of aged canned orange juice (Tatum et al., 1975).

DMHF is somewhat unstable in air and in an aqueous solution; its stability has been studied by many investigators (Hodge et al., 1963; Hirvi et al., 1980; Shu et al., 1985). However, there are limited reports on quantitation and analytical procedures because it does not survive injection into a stainless steel, open-tubular GC column (Flath and Forrey, 1970). Recently, Pickenhagen et al. (1981) improved the GC method by using a fused silica column. DMHF is highly oxygenated and does not steam distill. Yield is poor and it easily disappears from the distillate (Kallio, 1976). Thus, time consuming liquid-liquid extraction has been the only possible approach in sample preparation.

Application of liquid chromatographic methodology to pineapple flavor characterization had not been reported in the literature to our knowledge. The purpose of the present investigation is to develop a simple and rapid liquid chromatographic procedure to quantitate DMHF in pineapple juice and, additionally, apply that methodology to a study of DMHF changes in grapefruit juice during storage.

MATERIALS & METHODS

Samples

Fresh pineapples [*Ananas comosus* (L.) Merr.] grown in Hawaii and Costa Rica were purchased from a local market. The fresh juice was prepared from skinned, cut and cored whole fruit. Four different brands of commercially canned, single-strength pineapple juice were purchased from local outlets. For storage tests, commercially canned grapefruit juices were obtained from Citrus World, Inc., Lake Wales, FL. Samples were placed in laboratory lockers at 10°, 20°, 30°, 40°, and 50°C for 15-wk periods.

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Reagents and standards

The 2,5-dimethyl-4-hydroxy-3(2H)-furanone (DMHF) was kindly provided by B.D. Mookherjee (International Flavors and Fragrances, Union Beach, NJ). Furfural and 5-hydroxymethylfurfural were purchased from Fisher Scientific Co. (Fair Lawn, NJ) and Sigma Co. (St. Louis, MO). All solvents were HPLC grade from J. T. Baker Chemical Co. (Phillipsburg, NJ).

Isolation and purification of DMHF

For the isolation of DMHF, our previous work (Lee et al., 1986) was applied with modification. Briefly, to 10 mL juice, 0.5 mL Carrez solutions (30% zinc acetate and 15% potassium ferrocyanide) were added. After standing for 5 min, the mixture was centrifuged at 2000 g for 5 min. One ml of supernatant was passed through a C-18 Sep-Pak cartridge (Waters Assoc., Milford, MA). After washing the cartridge with 0.5 mL hexane, the DMHF was eluted three times with 3 mL of ethyl acetate, dried over anhydrous sodium sulfate, and concentrated by nitrogen gas to 6 ml (pineapple juices) and to 1 mL (grapefruit juices). Each sample was filtered through a 0.45 μm Durapore filter (Millipore Co., Bedford MA) before injection.

Recovery studies

The percent recoveries were determined by comparing chromatographic peak areas from fresh grapefruit juice with spiked known amount of DMHF (1, 10 and 50 ppm) against peak areas generated by direct injection of standard solutions of DMHF. Analysis was carried out in duplicate and each sample was prepared as described above.

High performance liquid chromatography

Quantitative liquid chromatographic determinations were conducted with Waters Model 6000A pump, Model U6K injector (Milford, MA), LDC/Milton Roy spectrophotometer D (Riviera Beach, FL) and SP 4270 computing integrator (Spectra Physics, San Jose, CA). A Zorbax ODS column (250 × 4.6 mm, i.d., E.I. du Pont de Nemours Co., Wilmington, DE) was coupled to a RP-18 guard column (30 × 4.6 mm, i.d., Rainin Instrument Co., Woburn, MA). Column temperature was kept at 30° ± 1°C using a water jacket, and the mobile phase was 0.05 M sodium acetate (pH 4.0)/methanol (70:30). Operating conditions were: flow rate, 0.5 mL/min; UV, 290 nm (0.02 AUFS); chart speed, 0.25 cm/min; and injection volume, 10 μL.

RESULTS & DISCUSSION

High performance liquid chromatography (HPLC)

A reversed-phase HPLC procedure was developed to resolve and quantify DMHF in pineapple and grapefruit juices. The chromatograms of samples obtained from fresh and canned pineapple juices are shown in Fig. 1. The total analysis time was less than 30 min. The DMHF was eluted around 12.6 ± 0.02 min, and identified by comparison of its retention time with that for the standard, and by spiking the sample with DMHF. The coefficient of variation (CV) of retention time and response factor for the six runs of DMHF standard was less than 3.1%.

In initial efforts, mobile phases with aqueous methanol (30%) or a phosphate/phosphoric acid buffer with 30% methanol system was unable to elute this compound from the C-18 column

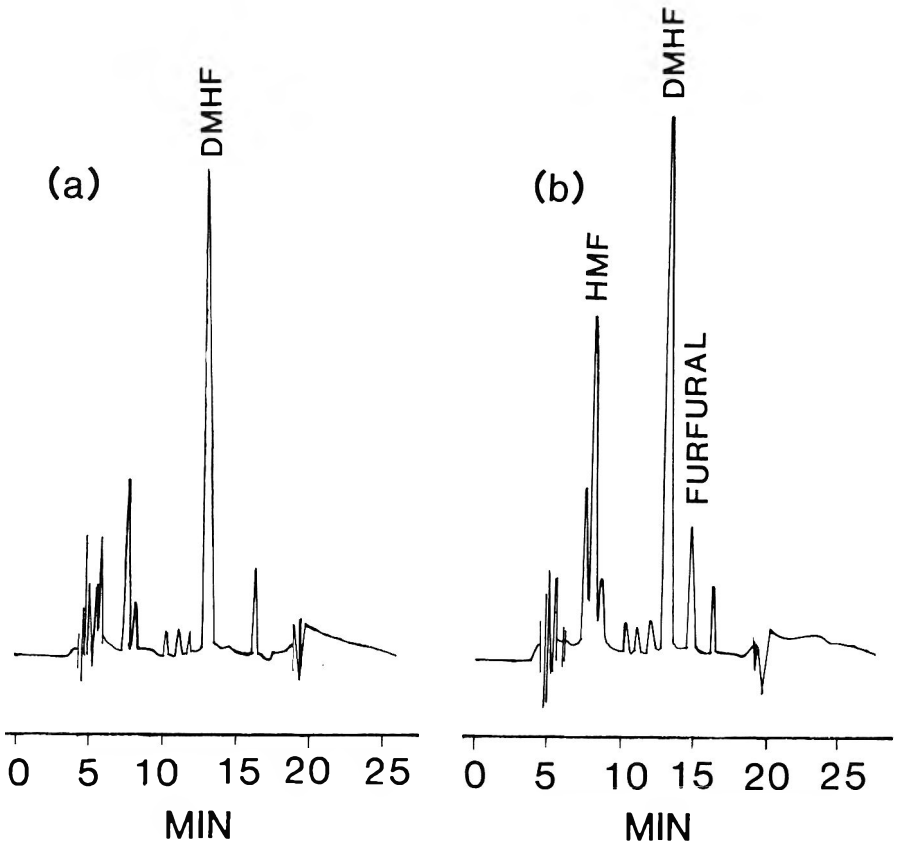

Fig. 1—HPLC chromatogram of pineapple juices: (a) fresh; (b) canned.

or tended to elute as a severe tailing peak. Finally, an acetate/acetic acid buffer with the 30% methanol mixture provided a satisfactory symmetrical peak at the same pH (4.0) and the same molarity (0.05M). No interfering peaks were observed for DMHF in pineapple juice samples. However, there were numerous peaks in grapefruit juices and an unidentified peak that could not be resolved from DMHF initially. A slight column temperature increase from ambient to 30 ± 1°C provided clear separation of DMHF from the unknown interfering peak. Typical separation of DMHF from grapefruit juice stored 15 wk at 50°C is noted in Fig. 2. As time and temperature changed during storage of the grapefruit juices, the chromatogram became complex; some peaks disappeared while other peaks formed. However, the resolution of DMHF peak was not affected.

Cilliers and Van Niekerk (1984) described difficulties working with viscous tropical juices, such as pineapple, for HPLC analysis. Preliminary sample purification with the clarification agents (Carrez reagents) could remove the pulp, fat, protein and carotenoids (Lee et al., 1986; Wallrauch, 1984; White, 1979). The proposed sample preparation method using solid-phase extraction with C-18 cartridge required significantly less time in comparison to liquid-liquid extraction (18 hr, Pickenhagen et al., 1981). With 0.05M sodium acetate buffer (pH 4.0)/methanol (70:30) eluates, linear responses were obtained between peak areas and injected amounts. Two replicated injections of standard amounts of DMHF in the range 50–5000 ppb yielded r = 0.9999; slope = 48.8; intercept = 4167. Recovery of DMHF by the analytical procedure was determined by spiking the three levels (1, 10 and 50 ppm) of DMHF to the fresh grapefruit juice. The mean recovery was 92.6% with 2.5% CV. Hexane was used to flush the residual water from the cartridge prior to sample elution (Anonymous, 1984). There was a loss of less than 0.5% of DMHF as a result of prewashing the cartridge with 0.5 mL hexane. Alternatively, the cartridge can be dried under vacuum (Anonymous, 1984). The limit of detection of DMHF was 50 ppb.

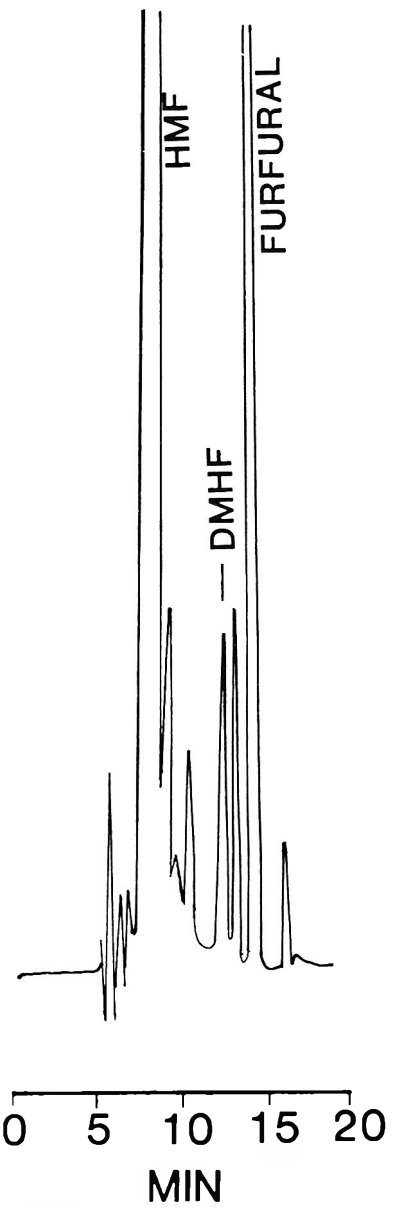

Fig. 2—HPLC chromatogram of grapefruit juice stored for 15 wks at 50°C.

DMHF contents in pineapple juices

Quantitative data were normalized to an 13.5° Brix single-strength standard as listed in Table 1. The DMHF contents varied between 1.6 and 27.3 ppm with a mean of 15.0 ppm for 10 fresh pineapple juices. Average DMHF content for Hawaiian pineapple juices was 25.1 ppm, whereas it was 4.9 ppm for the Costa Rican juice. Although maturity information on these samples is lacking, out preliminary experiment indicated that there are significant differences ($P < 0.05$) in DMHF values with regard to Hawaiian and Costa Rican pineapples. In a study conducted by Pickenhagen and co-workers (1981), wide natural variations were noted between cultivated and wild species of strawberries in DMHF contents. Post-harvest storage effects also influenced DMHF contents.

Hirvi et al. (1980) noted that DMHF can be totally destroyed during the processing or canning of fruits such as pineapples, but this compound seems to be somewhat stable during canning. Our canned pineapple samples showed the presence of relatively high amounts of DMHF. In the 24 canned pineapple juices from four different brands, the mean value was 24.1 ppm and varied from 11.2 to 28.6 ppm, (Table 1). Also, an average of 0.8 ppm of furfural and 1.9 ppm of 5-hydroxy

Table 1—2,5-Dimethyl-4-hydroxy-3(2H)-furanone (DMHF) contents in pineapple juices[a]

	No. of samples	Brix (°Bx)	DMHF (ppm)[b] Range	Mean
Fresh				
Hawaiian	5	13.7	22.7–27.3	25.1
Costa Rican	5	12.0	1.6– 7.2	4.9
Total	10		1.6–27.3	15.0
Canned (Brand)				
A	7	13.1	23.7–27.7	26.4
B	5	13.3	11.2–20.3	16.0
C	6	13.3	25.8–28.6	26.9
D	6	13.0	25.5–28.0	27.0
Total	24		11.2–28.6	24.1

[a] Means of duplicate analysis for each sample.
[b] Results normalized to 13.5° Brix.

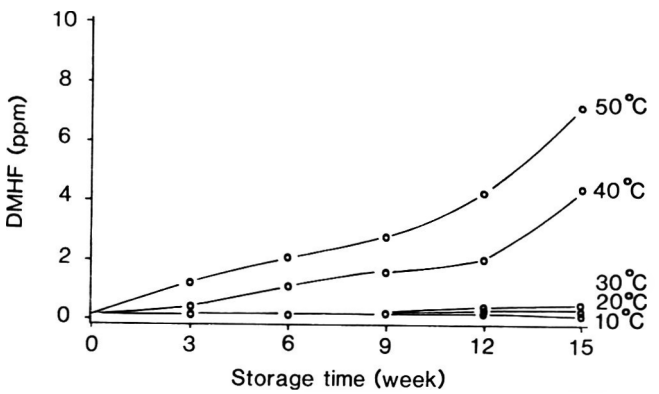

Fig. 3—Build-up of DMHF contents in canned grapefruit juices stored for different periods and at different temperatures.

methylfurfural (HMF) were found in canned juices. They are known to be produced from the degradation of sugars and ascorbic acid and it is probable that these compounds were formed in the juices either during processing or subsequently during storage (Gawler, 1962).

DMHF content in grapefruit juices

Tatum et al. (1975) identified DMHF in temperature-abused, canned orange juice and judged it to be one of the compounds primarily responsible for the malodorous property of temperature-abused juice. Since one of the goals of this study was to monitor the DMHF content during the storage of grapefruit juices, a long term storage study was conducted. The build-up of DMHF contents in canned grapefruit juices stored for different periods and at different temperatures is shown in Fig. 3. Samples stored at − 10°C were used as a reference and 0.19 ppm of DMHF was detected.

At a storage temperature of 10°C, the DMHF content did not increase, and at 30°C, the increase was relatively small. After 15 wk storage, the DMHF levels in the juices were 0.26 ppm (20°C), 0.45 ppm (30°C), 4.38 ppm (40°C) and 7.15 ppm (50°C). Thus, juice stored at 50°C for 15 weeks contained about 28 times more DMHF than the 20°C stored juice. Underwood (1971) also observed its formation with heating time in maple syrup. Based on the results obtained in these storage experiments, increasing the temperature and storage period cause more DMHF to form and accumulate.

The increase in DMHF level is highly important to juice quality because it is reportedly a powerful flavoring agent; its odor threshold value in water has been reported at 0.04 ppb (Fors, 1983) and 0.1–0.2 ppm (Pittet et al., 1970). Also masking or depressing the full orange-like aroma has been observed at 0.05 ppm level in orange juice (Tatum et al., 1975). Although our study was not intended to correlate sensory deterioration with DMHF content, we could easily sniff the strong pineapple-like aged flavor in the 40°C samples.

The formation mechanism of DMHF was not the objective of this storage study, but it is likely that DMHF may be formed through a series of enolisation and dehydration reactions of Amadori compounds (Baltes, 1982; Hodge et al., 1963, 1972), as well as other furan derivatives such as furfural and HMF. In stored juice, it appears that DMHF may serve as a quality deterioration index, as well as furfural and HMF (Berry and Tatum, 1965; Meydav and Berk, 1978; Nagy and Randall, 1973; Tatum et al., 1975) in temperature-abused grapefruit juices.

In conclusion, a simplified liquid chromatographic procedure was developed to quantitate DMHF, a high boiling unstable compound, without deterioration. By using solid-phase extraction with a disposable C-18 catridge, the need for liquid-liquid extraction is eliminated, and more reproducible results are attained. This method could be applicable to monitor the DMHF contents of grapefruit juices during storage.

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Ms received 6/6/86; revised 9/8/86; accepted 10/8/86.

Shelf Life Study of Oil/Water Emulsions using Various Commercial Hydrocolloids

K. A. COIA and K. R. STAUFFER

ABSTRACT

This study compares and evaluates commercial hydrocolloids for shelf life stability in an oil/water emulsion system stored at 25°C. Observations of surface or interfacial changes and rheological parameters took place over 90 days. Parameters; n, pseudoplastic index; K, consistency index; and τ_0, yield stress were reported. Apparent viscosities η_{20} (quality control index) and η_{50} (oral flow index) were monitored throughout the study and correlated well with stability changes. The study produced the following results: propylene glycol alginate promotes creaminess without significant rheological contribution (η_a, η_{20}, η_{50} and τ_0). Xanthan gum contributes to body; however, oiling off was observed. Gum tragacanth and combination(xanthan, tragacanth and PGA, 1:1:1) act as bifunctional stabilizers, controlling both oiling off and body.

INTRODUCTION

AN EMULSION is a thermodynamically unstable system consisting of at least one immiscible liquid intimately dispersed in another in the form of droplets whose diameter in general exceeds 0.1μ. Such systems possess a minimal stability, which may be improved by surface-active agents such as finely divided solids, lipoproteins, mono- and diglycerides, etc. (Becher, 1965). Surface-active agents which are added to an emulsion to increase its stability by interfacial action are known as emulsifying agents. Several gums historically have been considered as auxiliary emulsifiers and were thought to promote emulsion stability by increasing the viscosity of the aqueous phase and slowing down coalescence (Powrie and Tung, 1976). Recent studies (Stauffer, 1980; Anonymous, 1977) have shown that certain gums (gum tragacanth and propylene glycol alginate (PGA)) were surface-active and contributed to emulsion stability by a combination of viscosity and surface activity. Xanthan gum (1%) did not lower the surface tension of water (Anonymous, 1975).

Oil-in-water (O/W) emulsions containing vegetable oil, salt, acid (vinegar), plus sugar and seasoning-collectively referred to as salad dressing-were originally stabilized by gum tragacanth and, later, by propylene glycol alginate. Xanthan gum, a microbial exocellular polysaccharide, was developed at Northern Regional Research Laboratory in Peoria, Illinois and approved by the FDA for use in food, and it is now used to stabilize most salad dressings. Currently, most manufactured salad dressings are stabilized by xanthan gum and PGA. Xanthan gum and gum tragacanth combinations are also used on occasion.

The purpose of this investigation was first to establish universal rheological parameters-such as pseudoplastic index, n; consistency index, K; and yield stress, τ_0; plus surface and interfacial activities-of the three major acid-stable and salt-compatable gums-xanthan, tragacanth and propylene glycol alginate (PGA)-which are used predominately for oil-in-water dressing emulsions. The second objective was to study these gum systems over a 90-day storage period to identify the physical (rheological parameters and surface activity) and functional properties associated with storage stability. Such properties include; η, K and τ_0, along with surface active changes in both surface tension of the continuous phase and interfacial tension between the aqueous and lipid phase.

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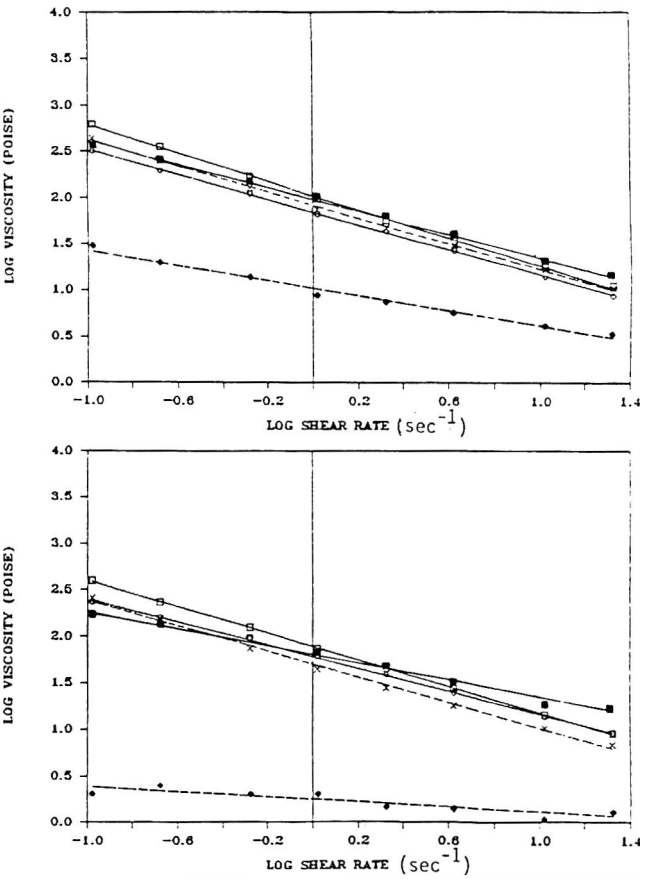

Fig. 1—Changes in apparent viscosity (η) of the emulsion from initial (top) to after 90 days (bottom) of storage at 25°C. □—Xanthan (Keltrol F), ◇—Xanthan (Rhodigel 23), X- - -Combination, ■—Tragacanth (Flake #27), ◆- - -Propylene Glycol Alginate.

Table 1—Calabrated Brookfield (RVT) viscometer and its generated shear rates (sec^{-1}) that were used to calculate the flow behavior constants from the power law equation

rpm	Shear rate (sec^{-1})
0.5	0.1051
1.0	0.2102
2.5	0.5254
5.0	1.0508
10.0	2.1016
20.0	4.2032
50.0	10.5080
100.0	21.0160

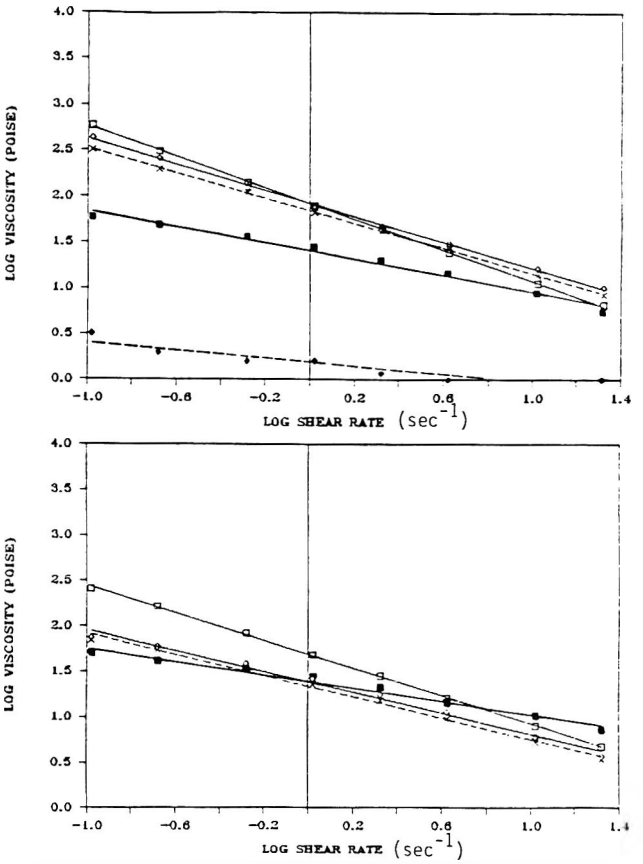

Fig. 2—Changes in apparent viscosity (η) of the continuous phase from initial (top) to after 90 days (bottom) of storage at 25°C. □—Xanthan (Keltrol F), ◇—Xanthan (Rhodigel 23), X- - -Combination, ■—Tragacanth (Flake #27), ♦- - -Propylene Glycol Alginate.

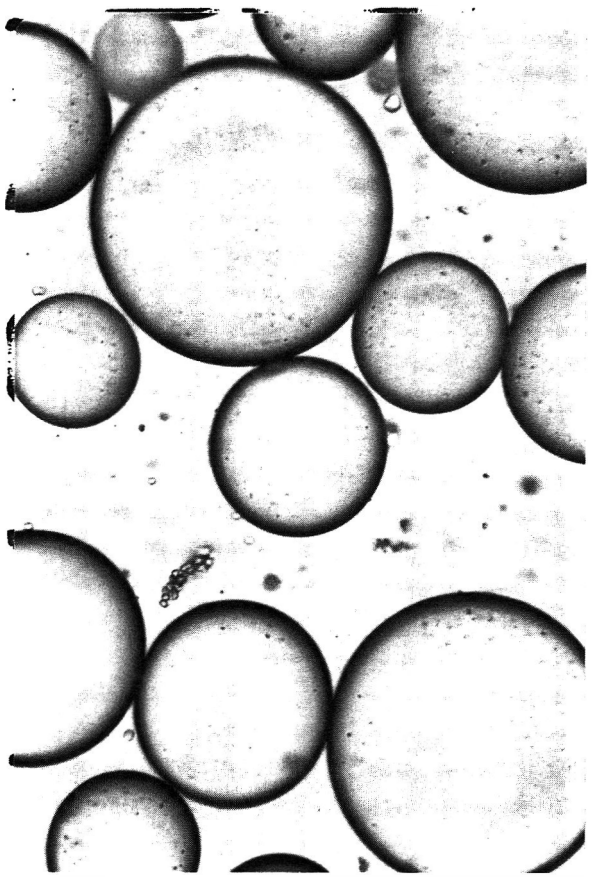

Fig. 3—Photomicrograph of 40% oil-in-water model dressing system where gum tragacanth was the stabilizer. Magnification was 200X of the emulsion that was approximately 1 mo. old.

MATERIAL & METHODS

Sample materials

Food grade xanthan gums, Keltrol-F and Rhodigel 23, were obtained from Kelco (Clark, NJ) and Rhone-Poulenc (Paris, France), respectively. Gum tragacanth (Flake #27) and propylene glycol alginate (PGA), type BH, were donated by Colony Import & Export Corp., NY, and a combination (XTP) of equal proportion of gums, xanthan, tragacanth and PGA, was obtained from Shangum, Inc. (Bradford, RI). Analysis of the tap water by the Rhode Island Department of Health indicated a pH of 5.7, total solids content 103.0 ppm, total hardness ($CaCO_3$) 46.0 ppm, chlorides <2.0 ppm, sulphates 20.0 ppm and calcium 15.6 ppm. Commercial white grain vinegar (45 grain) had a total acidity of 4.5. Noniodized table salt was used in all 40% O/W emulsion systems. A winterized vegetable oil (corn), which was obtained from Procter & Gamble (Cincinnati, OH), was used in all emulsions.

Emulsification

The following methods were developed to test the emulsifying properties of gums and/or combination. Low-fiscosity PGA and moderate-to-high viscosity gum tragacanth were used at a weight level of 1.25g/100 of the aqueous phase. Xanthan gums, Keltrol-F and Rhodigel 23, and the XTP were used at levels of 0.67/100 of the aqueous phase to bring all systems to an initial apparent viscosity (η_{20}) at 20 Brookfield rpm. In the case of PGA, however, this was difficult since gum concentrations should not be greater than 0.75% of the total emulsion to meet standards of identity requirements.

The gums, or combination of them, were first dispersed in water (573.0 mL) at low speed for 60 sec in a Waring Blendor. The vinegar (27 mL) was then added and mixed for an additional 60 sec. This slurry is the continuous phase. The continuous phase was subdivided into two 300 mL aliquots; one sample was set aside for the continuous phase studies, and the remainder was used to prepare the emulsion.

The emulsions were prepared by adding 200 mL vegetable oil to the continuous phase in the Waring Blendor for 120 sec at high speed. They were then deairated by vaccum and used for shelf life studies.

Surface and interfacial tension

The 300 mL continuous phase aliquots remained at 21.5°C ± 0.5 for a minimum of 24 hr before the surface tensions were recorded. The surface skin (concentrated gum near the surface) that formed on the gum solution on aging was disturbed prior to measurement and the solution was allowed to stand an additional 90 min to equilibrate. Al-Jassir and Stauffer (1983) have shown that after 90 min the surface tension leveled off, permitting good indication of surface activity. The same procedure was followed for interfacial tension. However, after the patinum-iridium ring was wetted in the gum solution, 40 mL vegetable oil was floated on the surface of the aqueous solutions. The oil and water interface was allowed to age for 60 sec before readings were taken with the Fisher model 215, Autotensiomat Surface Tension Analyzer (du Nouy type). Shotton and White (1963) have shown that films can develop in 15-20 sec for vegetable gums.

Determination of mean globule size

The particle size distribution and mean globule size of all emulsions were determined just before their flow properties were examined. The "mean volume diameter" (D_m) is considered most suitable for correlation with viscosity (Shotton and White, 1963). This is defined as:

$$D_m = \sqrt{\frac{\Sigma_n D_3}{\Sigma_n}}$$

where n is the number of globules with diameter D.

Each determination of D_m was based on the examination of not less than 600 globules. Photomicrographs were taken at 200X magnification to determine the emulsion droplet size.

Table 2—Viscometric constant (flow behavior index and consistency index) and apparent viscosity (poise) at various Brookfield RPM initially and after 90 days of storage at 25°C

	Continuous phase										Emulsion									
	Initial					After 90 days					Initial					After 90 days				
	K	η^a_{20}	η^b_{50}	n	r^c	K	$\eta_{2c}{}^a$	η^b_{50}	n	r^c	K	η^a_{20}	η^b_{50}	n	r^c	K	η^a_{20}	η^b_{50}	n	r^c
Xanthan (Keltrol F)	84.7	25.2	11.6	0.15	0.999	49.3	16.5	8.2	0.24	0.999	106.7	35.6	17.7	0.24	0.999	78.1	28.1	14.6	0.20	0.999
Xanthan (Rhodigel 23)	84.8	31.9	16.3	0.30	0.999	24.5	10.7	6.3	0.42	0.995	70.6	26.6	14.3	0.32	0.999	61.0	25.1	14.2	0.38	0.999
XTP[d]	70.6	26.6	14.3	0.32	0.999	21.9	9.4	5.5	0.42	0.997	84.8	31.0	16.3	0.30	0.999	61.0	18.9	10.1	0.38	0.999
Tragacanth	25.8	13.7	9.1	0.56	0.992	24.5	14.5	10.5	0.64	0.989	96.7	38.9	21.8	0.37	0.998	64.8	33.9	22.4	0.55	0.994
Propylene glycol alginate	1.6	1.2	0.9	0.79	0.933	0.4	0.3	0.0	0.96	0.924	10.7	6.0	4.1	0.59	0.993	1.8	1.5	1.3	0.87	0.892

[a] Apparent viscosity (poise) at 20 Brookfield.
[b] Apparent viscosity (poise) at 50 Brookfield.
[c] Correlation coefficient.
[d] Combination of xanthan, tragacanth, and PGA gums at a (1:1:1) ratio.

Table 3—Mean particle size of the noncontinuous oil phase initially and after 90 days of storage at 25°C

	Initial D_m (μ)	After 90 days D_m (μ)	Particle size distribution[b]
Xanthan (Keltrol F)	10	14	bell shaped around mean
Xanthan (Rhodigel 23)	8	12	bell shaped around mean
XTP[c]	12	12	bell shaped around mean
Tragacanth (Flake #27)	30	34	skew to left with a mode (13μ)
Propylene glycol alginate	5	8	skew to left with a mode (2μ)

[a] the mean volume diameter.
[b] oil layers were not considered for D_m or distribution.
[c] combination of xanthan, tragacanth, and PGA gums at a (1:1:1) ratio.

Table 4—Particle size distribution of gum tragacanth of the non-continuous phase after 90 days of storage

Gum tragacanth	
Particle size (μ)	Percentage of distribution
2.0	23.0
4.0	24.0
13.0	9.0
18.0	2.0
23.0	7.0
28.0	2.0
33.0	5.0
43.0	4.0
58.0	2.0
68.0	2.0
75.0	4.0
85.0	3.0
95.0	2.0
110.0	5.0
130.0	3.0
170.0	3.0

Rheology

Calibration. All continuous phases and emulsions were tested with the Wells-Brookfield Synchrolectric RVT model (7, 187 dyne/cm spring torque for maximum reading on scale; 8 speeds). The Brookfield was calibrated against the Rheometrics Fluids Rheometer (RFR) using several Brookfield Newtonian standards in the range of 40 to 0.1 poise. Also, a pseudoplastic standard of 0.2% Carbopol 941 was used to calibrate the shear rate (sec^{-1}) of the Rheometrics Fluids Rheometer (RFR) to the rpm of the Brookfield RVT viscometer. As a result of the above calibration, and the rpm generated from the Brookfield RVT model correlated to the shear rate (sec^{-1}) as shown in Table 1.

Curve fitting. The rheograms generated from the viscometers above were curve-fitted by the use of the power law:

$$\eta^a = K \dot{\gamma}^{n-1}$$

where η^a = apparent viscosity; K = consistency index; $\dot{\gamma}$ = shear rate; and n = flow behavior index. The consistency index (K) is located at 10° sec^{-1} which relates to Brookfield RVT at 5 rpm (Fig. 1 and 2).

Yield stress. Casson (1959) proposed a plot of the square roots of shear stress and shear rate using the following equation:

$$\tau^{0.5} \tau_0{}^{0.5} = K \dot{\gamma}^{0.5}$$

where τ is the shear stress; τ_0 is the yield stress; $\dot{\gamma}$ is the shear rate; and K is known as the consistency index. The Casson flow model (Casson, 1959) was used to estimate yield stress.

The data were treated by correlation coefficients according to standard statistical methods (Khazanie, 1979).

RESULTS & DISCUSSION

Sensory stimuli (η_{50})

Shama and Sherman (1973a, b) have shown that with high viscosity products (η_a) > 70 cps) the oral stimulus was found to be the shear stress approximately at a constant shear rate of 10 sec^{-1}, which relates to the Brookfield RVT at 50 rpm (Table 1). Therefore, the oral flow index, measured at 50 rpm, is designated as apparent viscosity η_{50}.

There was a 10–30% decrease for the emulsions and continuous phase (Table 2), except for gum tragacanth which increased. Initial emulsions prepared with xanthan gums showed η_{50} equal to 16.0 ± 1.7 poise, the XTP equaled 16.3 poise,

Table 5—Changes in surface tension (dyne/cm) of the various hydrocolloids initially and after 90 days of storage at 25°C

	Continuous phase		Emulsion	
	Initial	After 90 days	Initial	After 90 days
Xanthan (Keltrol F)	63.0	64.0	57.7	60.5
Xanthan (Rhodigel 23)	62.9	66.0	57.4	60.5
XTP[a]	56.4	58.4	58.8	63.0
Tragacanth (Flake #27)	45.7	53.1	45.3	47.5
Propylene glycol alginate	49.7	44.2	54.6	48.5
Continuous phase (minus gum)[b]	67.5			

[a]Combination of xanthan, tragacanth, and PGA gums at a (1:1:1) ratio.
[b] 4.5% vinegar, 2% salt.

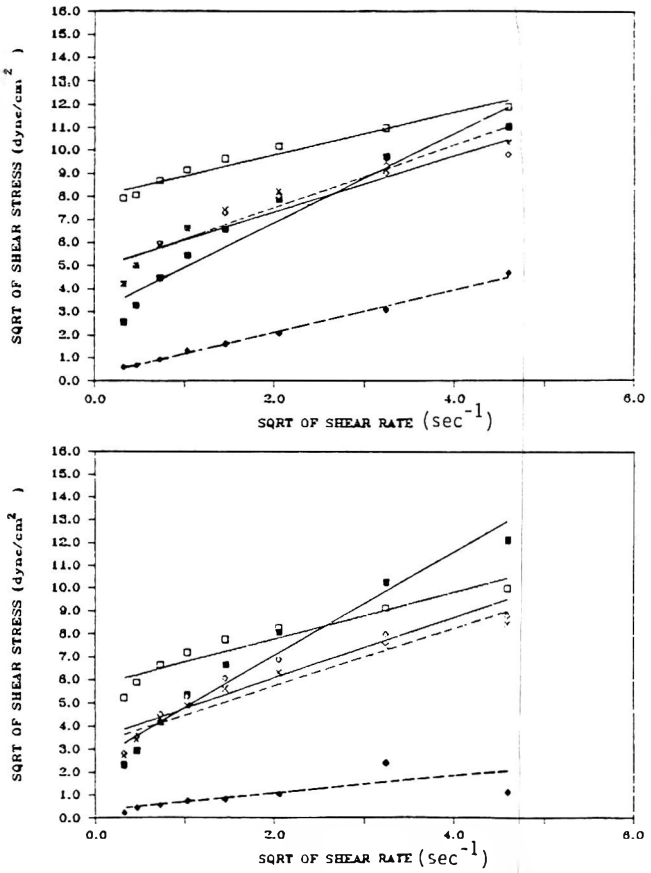

Fig. 4—Changes in yield value of the continuous phase from initial (top) to after 90 days (bottom) of storage at 25°C. □—Xanthan (Keltrol F), ◇—Xanthan (Rhodigel 23), X- - -Combination, ■—Tragacanth (Flake #27), ♦- - -Propylene Glycol Alginate.

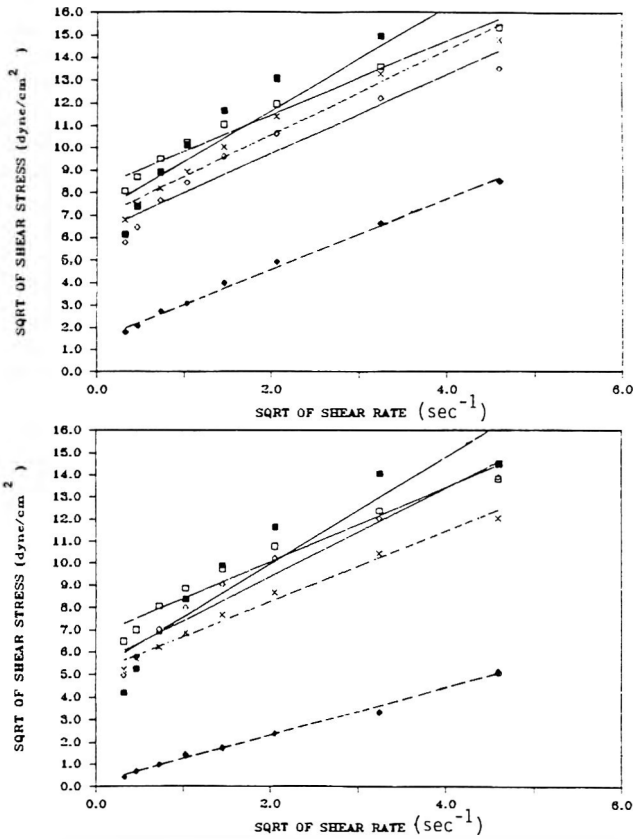

Fig. 5—Changes in yield value of the emulsion from initial (top) to after 90 days (bottom) of storage at 25°C. □—Xanthan (Keltrol F), ◇—Xanthan (Rhodigel 23), X- - -Combination, ■—Tragacanth (Flake #27), ♦- - -Propylene Glycol Alginate.

gum tragacanth equaled 21.8 poise, and PGA was 4.1 poise. Therefore, from an oral or textural point of view, gum tragacanth emulsion remained the most stable, followed by xanthan gums, XTP and PGA.

Apparent viscosity quality control index (η_{20})

Although η_{50} related to the oral flow index, commercial specifications for viscosities of gum dispersions or emulsions are usually reported at Brookfield 20 rpm. Therefore, to compare our data with manufacturer's apparent viscosities, we have analyzed our results at 20 rpm or η_{20}.

η_{20}, or quality control index, follows the same trend as the apparent viscosity at 50 Brookfield rpm. Any differences can be accounted for by the differences in pseudoplasticity (n) of the various systems. Table 2 show the continuous phases and emulsions before and after 90 days of storage.

Flow behavior index (FBI) (n)

Figures 1 and 2 show the pseudoplasticity, or shear thinning, of all emulsions or their continuous phases. The slope, or flow behavior index (n), is given in Table 3.

Continuous phase. Keltrol-F was the most pseudoplastic before and after 90 days of storage. On the other hand, PGA, the least pseudoplastic initially, showed near Newtonian flow after 90 days. The correlation coefficient r for the applicability of the power law model ranged between 0.999 and 0.924 for the continuous phases throughout 90 days of storage.

The FBI (n) values of xanthan gum Keltrol-F versus Rhodigel 23 can be related to their molecular weights. Low molecular weight materials conform to flow alignment easier than higher molecular weight polymers. Thus, Keltrol-F had a higher molecular weight than Rhodigel 23, since the FBI (n) was lower at 10^{-1} sec^{-1} (Table 3 and Fig. 2). Gum tragacanth is more branched than other gums studied and has a lower molecular weight than xanthan gum (Glicksman, 1969), but it has a higher molecular weight than PGA. The indicated molecular weight order of Keltrol-F > Rhodigel 23 > tragacanth (Flake #27) > PGA (type BH), agrees with previous studies (Glicksman, 1969).

Emulsion. The initial FBI for Keltrol-F was the most pseudoplastic of all systems studied, followed by XTP, Rhodigel 23, gum tragacanth, and PGA. However, after 90 days, PGA was the least pseudoplastic. The correlation coefficient r for the applicability of the power law model ranged between 0.999 and 0.892 for the emulsions after 90 days of storage. In general, all emulsions were pseudoplastic with PGA showing the lowest pseudoplasticity (Table 3).

The flow behavior index for emulsions should relate to the following factors: (a) the mean particle size (oil droplets); (b) particle size distribution; (c) the colloidal nature of the continuous phase.

The mean particle size for gum tragacanth was 30-34μ (Table 4) initially and after 90 days of shelf life study; however, the mode was 13μ. Most other emulsions showed bell-shaped distribution around the mean. For gum tragacanth-prepared emulsions the oil droplet size was polydispersed (i.e. 47% were 4μ or less, and 10% were 100μ or more) (Table 5). Therefore, the particle size distribution, b, played a major role in the flow behavior (Table 3), since FBI (n) decreased from 0.56 (continuous phase) to 0.37 (emulsion). This trend was also observed after 90 days of storage. Emulsions prepared from PGA showed flow behavior similar to gum tragacanth.

The initial emulsions prepared with Rhodigel 23 and XTP relate best to c, the colloidal nature of the continuous phase, since the initial emulsion FBI (n) essentially followed that of

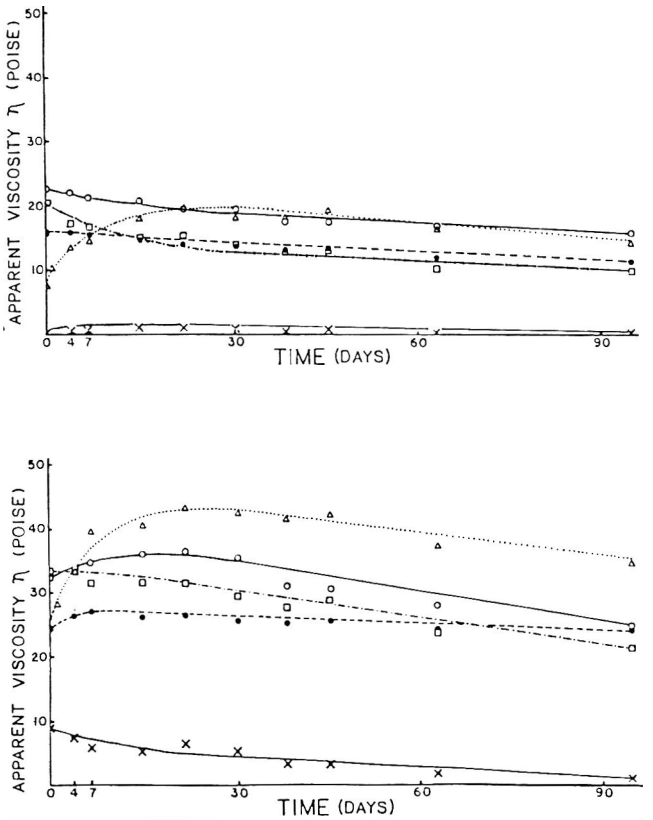

Fig. 6—Changes in apparent viscosity (η_{20}) at 20 rpm Brookfield RVT) of the continuous phase (top) and emulsion (bottom) during 90 days of storage at 25°C. ○—Xanthan (Keltrol F), ●- - - Xanthan (Rhodigel 23), □· - · - ·Combination, △· · · · ·Tragacanth (Flake #27), X—Propylene Glycol Alginate.

Table 6—Changes in interfacial tension (dyne/cm) of various hydrocolloids initially and after 90 days of storage at 25°C

	Continuous phase	
	Initial	After 90 days
Xanthan (Keltrol F)	27.6	21.4
Xanthan (Rhodigel 23)	25.6	20.0
XTP[a]	- - - -	18.2
Tragacanth (Flake #27)	12.5	13.2
Propylene glycol alginate	27.5	13.8
Water and oil (control)	22.2	

[a] Combination of xanthan, tragacanth, and PGA gums at a (1:1:1) ratio.

the initial continuous phase flow behavior index. The emulsion prepared with PGA related best to a, the mean particle size (Table 4), in that the oil droplets were very small and uniform (Becher, 1965), and thus the least pseudoplastic of all emulsions studied (Table 3). Emulsion prepared with gum tragacanth related to a and b. The change in FBI (n) of Keltrol-F for initial studies shows that the emulsion was less pseudoplastic than the continuous phase. This relates to a, the mean particle size of the oil droplets (Table 4).

Surface phenomenon

Surface tensions and interfacial tensions of both the continuous phase and emulsion system, initially and after 90 days storage, are shown in Tables 6 and 7.

Continuous phase. Colloidal dispersions of both xanthan gums showed the least surface tension. Gum tragacanth and PGA were the most surface active. Aging of the continuous phase had a slight negative effect on all systems except PGA, which continued to decrease. The continuous phase of gum tragacanth essentially showed no change in interfacial tension after 90 days of storage (Table 7).

Emulsion. The surface tension of the emulsions was measured initially and after 90 days. All surface tensions increased after 90 days, except for PGA, which decreased.

Emulsion prepared with gum tragacanth was unique in that an obvious interfacial surface skin was formed around each oil droplet (Fig. 3). PGA also showed a slight surface film. Emulsions prepared with xanthan gums did not show surface films.

Yield stress

Our research is based upon the Casson plot analysis and is used only to compare one system to another, not to substantiate any actual values. These differences relate well to emulsion stability (Charm, 1965) and, therefore, systems with higher yield stress values relate to increased emulsion stability. Figures 4, 5 and Table 8 show the Casson yield stress values (if any) of the emulsions and continuous phases.

Continuous phase. A study of the changes in Casson yield stress values of the continuous phases after 90 days of storage (Table 8) indicate that xanthan gum (Keltrol-F) had the highest value, whereas PGA had no yield stress value. XTP and gum tragacanth were between Keltrol-F and PGA. The yield stress value of gum tragacanth did not change much during storage, whereas that of all other continuous phases decreased. Keltrol-F was notable for its exceptionally high yield stress value, after 90 days of storage.

Emulsion. Initially, xanthan gum (Keltrol-F) had the highest, and PGA had the lowest yield stress values. XTP and gum tragacanth initially were between Keltrol-F and PGA. The yield stress of all emulsions decreased during the 90 days of storage. Keltrol-F was again notable for its exceptionally high value, whereas PGA had no yield stress value after 90 days of storage. The magnitude of the correlation coefficient r for the applicability of the Casson model ranged between 0.997 and 0.949 for the initial emulsions and between 0.997 and 0.938 for the emulsions after 90 days of storage.

Yield stress of a fluid relates to the force required to initiate flow (dyne/cm^2). Actual yield stress values for fluids are at best controversial (Rha, 1978). Some investigators (Sherman, 1970) do not recognize yield stress as an exact physical parameter, but merely as an empirical parameter relating to the manner in which the material was subjected. Yield stress have been criticized by many rheologists (Sherman, 1970; Blanshard and Mitchell, 1978). Charm (1965) related yield stress to the particle diameter, density difference of phases and gravity by the following equation:

$$\tau_0 = \frac{D_p\,(\Delta d)\,g}{3}$$

Table 7—Changes in yield value (dyne/cm^2) of various hydrocolloids initially and after 90 days of storage at 25°C

	Continuous phase				Emulsion			
	Initial	r[a]	After 90 days	r[a]	Initial	r[a]	After 90 days	r[a]
Xanthan (Keltrol F)	63.9	0.978	32.9	0.954	67.7	0.986	45.5	0.975
Xanthan (Rhodigel 23)	24.1	0.943	11.8	0.947	39.3	0.965	29.4	0.974
XTP[b]	23.2	0.954	10.4	0.960	47.1	0.983	26.2	0.993
Tragacanth (Flake #27)	8.7	0.967	6.3	0.977	51.1	0.949	27.0	0.938
Propylene glycol alginate	0.0	0.997	0.0	0.658	2.2	0.997	0.0	0.997

[a] Correlation coefficient.
[b] Combination of xanthan, tragacanth, and PGA gums at a (1:1:1) ratio.

Table 8—Number of days for separation to occur in an emulsion system using various hydrocolloids during 90 days of storage at 25°C

	Serum layer[a]	Oil layer[b]
Xanthan (Keltrol F)	none	46
Xanthan (Rhodigel 23)	none	46
XTP	none	none
Tragacanth (Flake #27)	75	none
Propylene glycol alginate	4	none

[a] Water layer.
[b] Oil layer.
[c] Combination of xanthan, tragacanth, and PGA gums at a (1:1:1) ratio.

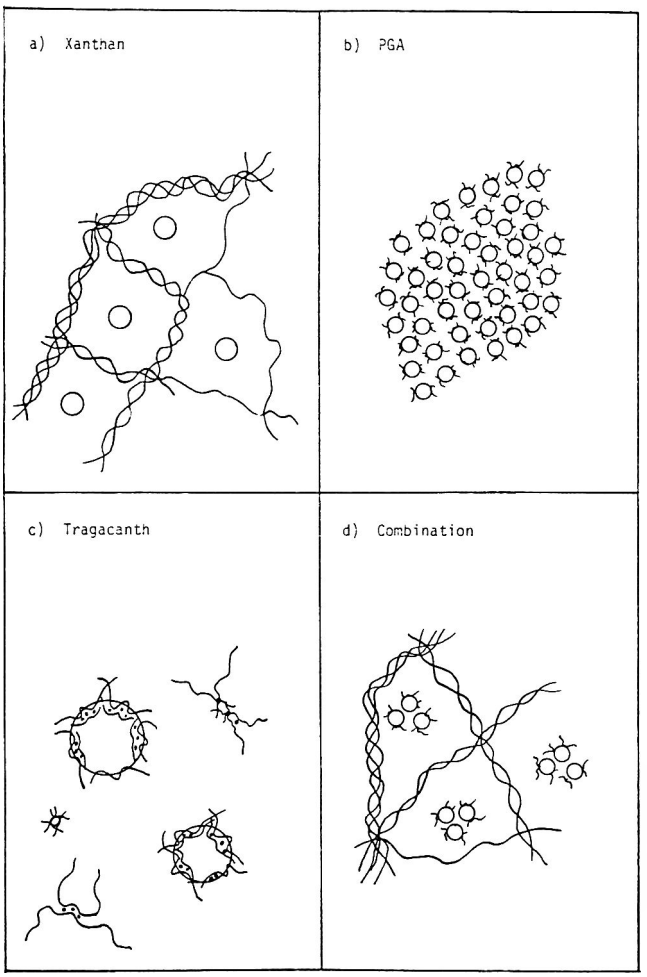

Fig. 7—Proposed models for oil-in-water stability.
○ = noncontinuous oil phase;
= single, double or triple stranded xanthan gum;
= crystalline region of gum tragacanth;
= PGA-oil interface.

where D_p is the diameter of the particle; Δd is the difference in density of two phases; and g is the gravitational constant. Charm's equation shows that the larger the yield stress value (or the smaller the non-continuous phase particle diameter) the more permanent the emulsion or suspension. However, this equation is impractical because particle diameters of noncontinuous phase are nonuniform.

Yield stress indicates an inter-molecular association of colloidal polymers which produce a three-dimensional network structure throughout the continuous phase. Inter-molecular hydrogen bonds and molecular entanglement are considered for major factors contributing to yield stress in both suspensions and emulsions (Glicksman, 1969; Heckman, 1977; Hodge and Osman, 1976).

90-day study

The changes in apparent viscosity (η_{20}) for both the emulsions and continuous phases throughout the 90 days of storage are shown in Fig. 6.

Apparent viscosity. Gum tragacanth was unique in that both the continuous phase and emulsion viscosity increased for the first 20–30 days before any loss occurred. The actual initial viscosity of the gum tragacanth emulsion was lower than the emulsion prepared with xanthan gums or XTP, but after 90 days the viscosity was higher.

It is possible that gum tragacanth was amorphous and crystalline in its solid state. The initial viscosity (η_{20}) resulted from the hydration of the amorphous region. The crystalline bodies were soluble and, therefore, more hydrophobic. Thus, they could migrate to the interfacial area producing films around the skins. The remaining crystalline bodies were unable to-migrate to the continuous phase and, upon standing (90 day storage), slowly hydrate, thus producing additional viscosity.

PGA did not show a high viscosity throughout the 90 day study. Xanthan gum (Keltrol-F) also showed a slight increase in viscosity up to 20 days in the emulsion before any decrease in viscosity occurred. XTP showed an apparent viscosity profile between PGA and the other emulsions. This relates to the combined effect of its constituent xanthan, tragacanth and PGA gums.

Phase changes of the emulsions. The changes in apparent viscosity, yield stress value and surface phenomenon relate to the physical changes that can occur in an emulsion. The two most significant phase changes in an emulsion are:

Oiling off. The noncontinuous oil droplets coalesece to larger oil droplets, and finally an oil layer is produced at the top of an emulsion.

Serum layer. The encapsulated oil droplets flocculate, producing a large particle diameter which causes a migration of the flocculate upwards. This produces an aqueous serum layer at the bottom of the emulsion. The number of days for separation to occur in an emulsion system during 90 days of storage are shown in Table 9.

Xanthan gums (Keltrol-F and Rhodigel 23) showed a strong resistance to serum separation but were susceptible to oiling off during storage. They were not surface active and, therefore, oiling off occurred more readily than with other surface active gum systems. Since xanthan gums function by having high yield stress values and high apparent viscosities, they should be used in O/W emulsions to produce viscosity and body, but not necessarily to inhibit oil separation (Fig. 7a).

PGA showed serum separation, readily apparent after four days, but the oil layer formation was impeded. PGA also had low viscosity and insignificant yield stress value but was very surface active and, therefore, should be used in an emulsion to inhibit oil separation (Fig. 7b).

Gum tragacanth showed no oiling off, but a very small serum layer appeared at the bottom of the emulsion after 75 days. This probably was due to the oil droplets, with skin intact, migrating to the top, according to Stokes Law. Gum tragacanth was unique in that it had a moderate yield stress value and viscosity that either increased or stabilized throughout the 90-day study. It was also surface active and helped oil phase changes.

Gum tragacanth produced polydispersed droplets which eventually resulted in a slight serum layer. Interfacial skins were kept intact throughout the study. Proposed crystalline bodies were responsible for both surface activity and delayed hydration (Fig. 7c). Tragacanthic acid is most likely to possess the unhydrated crystalline region, since it is considered to have a swolling metrix as opposed to being fully hydrated when in a colloidal dispersion. The arabinogalactan is a water soluble fraction much like gum arabic and gum ghatti, both of which produce interfacial films in oil-in-water emulsions. Interfacial films may be formed by either the arabinogalactan polymer

and/or unhydrated crystalline hydrophobic bodies from tragacanthic acid.

The gum XTP showed no serum separation or oil separation throughout the study. Combinations are typical in O/W pourable condiments such as French and Russian dressing. The combination (XTP) studied here showed that rheological parameters of xanthan, tragacanth, and PGA gums were incorporated to combat the oil and serum changes. This lack of oiling off relates to the gum tragacanth and/or PGA, whereas the yield stress and apparent viscosity relates to additive values of the respective gum components (Fig. 7d). No observed synergism was detected.

CONCLUSION

THE ABOVE RESULTS show that the gums perform various functions. Each gum studied had at least one unique stabilizing property. No single gum produced a complete stabilizing system for 90 days, whereas the combination (XTP) of xanthan, tragacanth, and propylene glycol alginate (1:1:1) demonstrated stabilizing forces related to the individual gum component functions.

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The authors acknowledge the assistance of Dr. Nicholi Vorsa, Rutgers University Blueberry and Cranberry Research Center in Chatsworth, NJ, who provided us with fruit samples, and Donna L. Hargrave, an employee of the Eastern Regional Research Center, who performed the HPLC analyses.

Changes in Phytase Activity and Phytate During the Germination of Six Canola Cultivars

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ABSTRACT

Six canola cultivars were germinated for 8-days and the levels of phytate and phytase activity monitored. Of these, the cultivar Regent exhibited the lowest level of phytate as well as the highest phytase activity. Germination of Regent for up to 2-days resulted in the reduction of phytate from 1.26% to 0.85% without any deleterious changes in the quality or quantity of the oil, based on the negligible levels of free fatty acids produced.

INTRODUCTION

CANOLA or rapeseed, the major oilseed crop in Canada, is used extensively for salad oil and in the manufacture of margarine and shortenings (Vaisey-Genser and Eskin, 1982). The presence of phytic acid in canola protein, however, is responsible, in part, for its limited use in human food. Phytic acid is an antinutritional factor due to its ability to chelate divalent metal ions rendering them unavailable biologically (Cheryan, 1980; Erdman, 1979). The ability of phytic acid to bind metal ions has been demonstrated for a number of metal ions including Ca, Mg, Zn, and Fe (Oberleas, 1973), and Cu and Zn (Davies and Nightingale, 1975). Atwal et al. (1980) reported a decrease in Zn availability in experimental rats fed rapeseed diets containing increasing levels of phytic acid.

A number of chemical methods has been reported in the literature for decreasing phytate. Germination of the seeds, however, appears to be a relatively simple nonchemical approach for increasing phytase levels to effect a decrease in phytic acid. The latter has been demonstrated in fababeans. (Eskin and Wiebe, 1983), horse grams and moth bean (Borade et al., 1984). Phytase (myoinositol hexaphosphate phosphohydrolase EC.3.1.3.8), catalyzes the release of free orthophosphate from phytate necessary for the production of adenosine triphosphate (ATP) and related high energy compounds essential for biosynthetic processes. The high level of phytic acid in canola relative to other legumes suggests the possibility of using the endogenous phytase for its reduction (Latta and Eskin, 1980). Reduction of phytate in the canola cultivar (*Brassica napus* var Tower) was reported by Thompson and Serraino (1985) during 7-days germination. This was accompanied by a decrease in oil as well as the deterioration of the oil. There have been no reported studies related to the presence of phytase in canola.

This study was conducted to determine the levels of phytase activity and phytate in four varieties of summer rape species (*Brassica napus*) and two varieties of summer turnip rape species (*Brassica campestris*) germinated over an 8-day period.

MATERIALS AND METHODS

Sample preparation

Seed samples of canola cultivars from summer rape species (*Brassica napus*), Tower and Regent, were provided by Dr. B. Stefannson, Dept. of Plant Science, Univ of Manitoba, and Altex and Andor, provided by Dr. J. Kondra, Dept. of Crop Science, Univ. of Alberta. Seed samples of canola cultivars from summer turnip rape species (*Brassica campestris*) included Candle and Tobin and were provided by Dr. K. Downey, Agriculture Canada Research Station, Saskatchewan. The dry seeds were geminated over an 8-day period and samples removed at 0, 2, 5, and 8 days following germination as described previously (Eskin and Wiebe (1983). Individual samples were freeze-dried and ground in a Wiley Mill through a 30-mesh screen. All experiments were replicated twice.

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Enzyme extraction

Crude phytase extracts were obtained from the different canola seed cultivers using the procedure described by Goel and Sharma (1979). The crude phytase extract was treated with ammonium sulfate and the fraction obtained between 40–70% collected. This fraction was found to contain all the phytase activity.

Enzyme assay

Phytase activity was monitored using the procedure of Lolas and Markarkis (1977). The reaction mixture consisted of 0.2 mL of 0.6M sodium acetate buffer pH 5.3; 0.15 mL of 2 mM sodium phytate (Sigma Chemical Co. St. Louis, MO) previously adjusted to pH 5.3 with IN HCl; 0.2 mL enzyme solution and 0.65 mL distilled water. The final volume was 1.2 mL with final concentrations of buffer and phytate of 0.1M and 0.25 mM, respectively. The reaction mixture was held at 50 + 1°C for 30 min in a water bath and the reaction terminated with 1.0 mL of cold 1.54M trichloroacetic acid. The mixture was then chilled in an ice bath for 15 min and centrifuged at $30,000 \times g$ for 10 min. The supernatant was subsequently filtered through Whatman No. 1 filter paper and aliquots removed for determination of inorganic phosphorus (Pi) by the method of Chen et al. (1956). Phytase activity was expressed as μg Pi/mg protein /30 min.

Chemical methods

Protein was determined by the method of Lowry et al. (1951) using crystalline bovine serum albumin as standard. Phytate was determined using the colorimetric method of Latta and Eskin (1980). This method is based on the interference by phytate on the formation of a colored complex between sulfosalicylic acid and ferric chloride. This method does not distinguish between phytate (inositol hexaphosphate) and other inositol phosphates (tri-, tetra-, and penta-) that may be formed during germination. Phytate levels are therefore expressed as 'apparent phytate' to indicate the possible presence of these inositol phosphates. The lower inositol phosphate esters are eluted with the inorganic phosphorus fraction.

Total fat was determined by extraction with hexane using the Soxhlet method (AOCS, 1979). Free fatty acids were measured following the procedure of Lowry and Tinsley (1976).

Table 1—Phytate content of six canola seed cultivars

Cultivars	Phytate[a] (%)[b]	S.D.[c]
Altex	1.80	±0.02
Andor	2.30	±0.04
Regent	1.26	±0.02
Tower	2.10	±0.04
Tower	2.10	±0.04
Candle	2.20	±0.07
Tobin	1.83	±0.02

[a] Dry weight basis
[b] Mean of three replicates
[c] Standard deviation

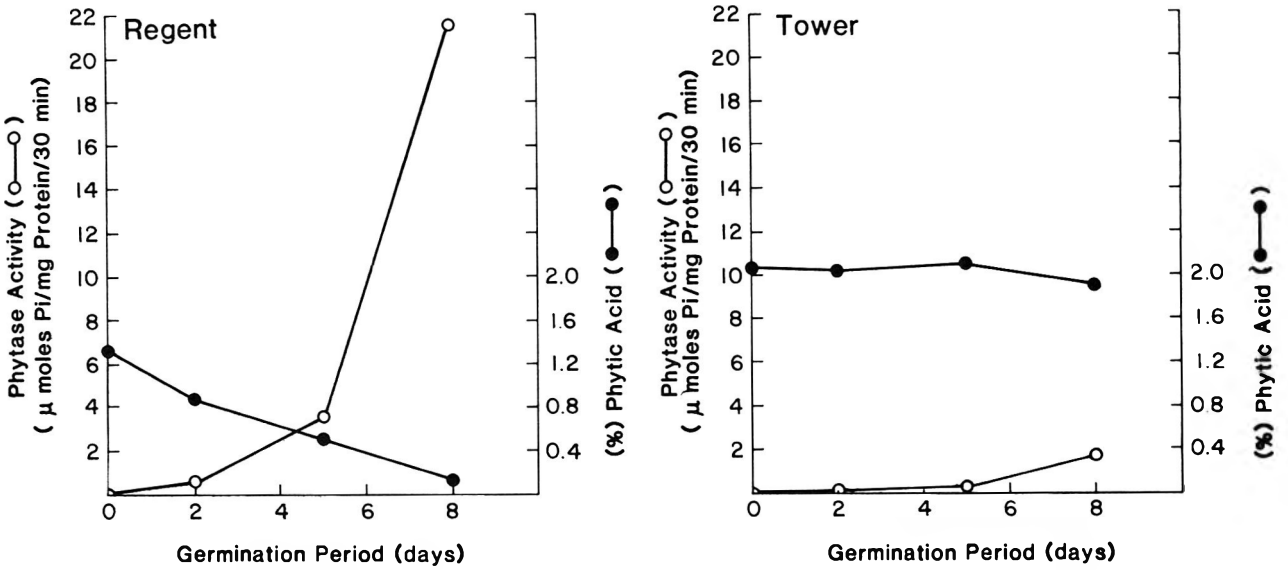

Fig. 1—Changes in phytase activity and phytate during germination of Regent and Tower (Brassica napus).

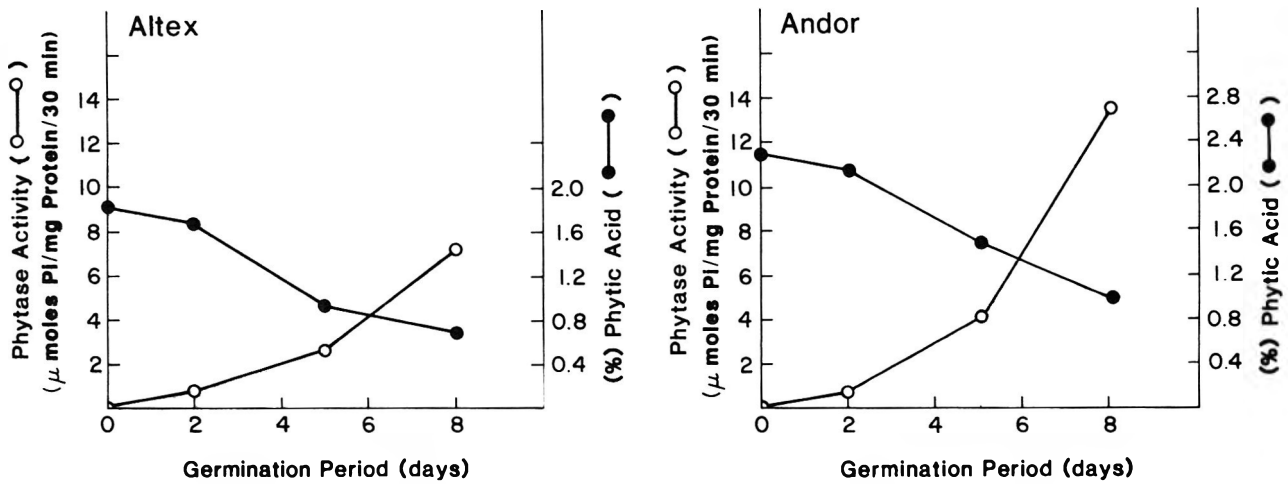

Fig. 2—Changes in phytase activity and phytate during germination of Altex and Andor (Brassica napus).

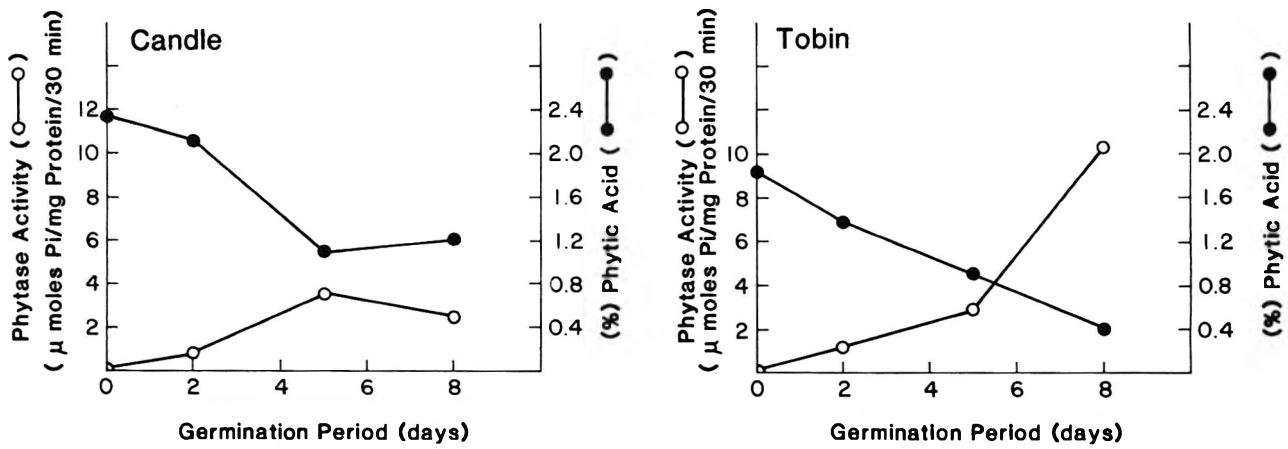

Fig. 3—Changes in phytase activity and phytate during germination of Candle and Tobin (Brassica campestris).

RESULTS & DISCUSSION

THE INITIAL LEVELS of phytate in the canola cultivars prior to germination are shown in Table 1. With the exception of Regent the level of phytate ranged from 1.8% to 2.3%. The canola cultivar Regent had a phytate level of 1.26% (Table 1), which was 30–45% less than the other canola varieties. The results of the germination studies are shown in Fig. 1, 2, and 3 in which an increase in phytase activity is evident for all cultivars studied except for Tower. Considerable variation in the levels of phytase activity was evident among those cultivars

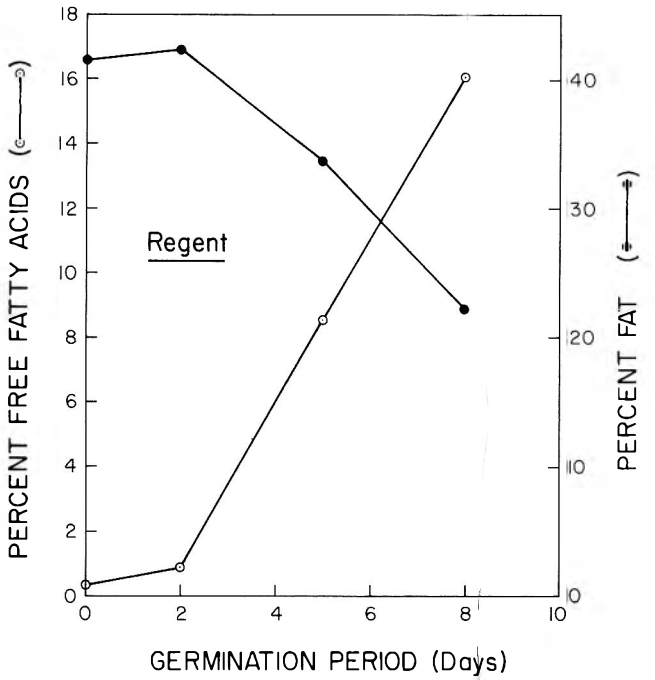

Fig. 4—Changes in oil content and free fatty acids during germination of Regent (Brassica napus).

mination. Of the cultivars germinated, Regent showed the most rapid rise in phytase activity increasing markedly after 8 days germination (Fig. 1). This was accompanied by a decrease in apparent phytate from 1.26% to less than 0.1%. In the case of Tower only slight phytase activity was evident following 8 days of germination which was accompanied by a very slight decrease in phytate levels. This differed from that reported for Tower by Thompson and Serraino (1985) where a marked reduction in phytate was observed. With respect to the cultivars Altex, Tobin, and Andor a definite rise in phytase activity was evident after 5-days germination which was accompanied by the reduction in phytate ranging from 1.80–2.3% to 0.39–1.0%, respectively (Fig. 2 and 3). In the case of the cultivar Candle, an increase in phytase activity was observed after 5-days germination, causing a 50% decrease in apparent phytate from 2.2% to 1.1%. Between 5 and 8-days of germination, there was a slight drop in phytase activity which was reflected by little change in the apparent phytate level during this period.

Thompson and Serraino (1985) reported that germination of canola beyond 2 days to reduce phytate was accompanied by deterioration of the oil due to lipase activity. Samples of Regent were examined for fat and free fatty acids over the 8-day storage period. The results (Fig. 4) confirmed that little change in free fatty acids occurred during the first 2 days of germination as reported previously (Thompson and Serraino, 1985). During the subsequent germination period, a 50% reduction in fat was evident which was accompanied by a rapid rise in free fatty acids. Nevertheless, after 2-days germination the level of apparent phytate in Regent was reduced by 33% to 0.85% without either the quantity (% lat) or quality (free fatty acids) of the oil affected. This compared to the results of Thompson and Serraino (1985) where the level of phytate after 2-days germination was still over 1.5% and took 7-days germination to decrease to around 0.8%. Based on these results it was evident that in the case of the canola cultivar Regent, a short germination period could be used to reduce phytate without adversely affecting the quantity or quality of the oil in this seed.

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Ms received 10/23/85; revised 9/10/86; accepted 9/12/86.

This work was supported by a National Science and Engineering Research Council Grant, Canada.

Laboratory Sprout Damage and Effect of Heat Treatment on Milling and Baking Properties of Indian Wheats

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ABSTRACT

Effect of sprouting and heat treatment of wheat on physicochemical, milling, rheological and baking properties of three high yielding Indian wheat varieties were investigated. Flour recovery, diastatic activity and total sugars increased significantly in sprouted samples whereas damaged starch and gluten decreased with concomitant increase in free amino nitrogen. Poor rheological properties were exhibited by sprouted wheats which considerably improved with heat treatment. The bread baked from sprouted wheat flours had smaller loaf volume with poor crumb characteristics but cookies prepared from the same flour exhibited better spread. Heat treatment brought about considerable improvement in baked volume and crumb characteristics of breads.

INTRODUCTION

UNTIMELY RAINS during the harvesting and threshing period of wheat lead to field sprouting of grains and bring about increase in the enzymatic activity along with several other chemical changes in the grain. Shorina et al. (1967) reported induced synthesis of alpha-amylase and 320-fold increase in its activity with sprouting of 120 hr. They also reported manifold increase in proteolytic activity resulting in softening of gluten together with decrease in disulfide crosslinks by disulfide reductases. William (1977) reported reduction in the damage starch content of the sprouted wheats while other workers (Lemar and Swanson, 1976; Lorenz and Valvono, 1981) reported an increase in the ash of flours obtained from sprouted wheats. Degree of sprouting had an important bearing on the effective utilization of wheat in bread making. Farinographic water absorption and dough development time decreased with sprouting of grains (Hwang and Bushuk, 1973) and application of heat to the grains before milling was found to have a favorable effect on these properties (Gawda, 1973; Ranhotra et al., 1977). The bread baked from sprouted wheat flour having excessive degradation of starch produced inferior and defective crumb (Gawda, 1973). Due to the high frequency of untimely rains and inadequate storage facilities, the incidence of sprouting of wheat has become a common problem for bakers in producing good quality bread. The present study was undertaken to get comprehensive information on the effect of the degree of sprouting and heat treatment on the bread making properties of high yielding Indian cultivars of wheat.

MATERIALS AND METHODS

REPRESENTATIVE SAMPLES of three high yielding cultivars of wheat namely, WL-1562, WL-711 and S-308 grown in Rabi 1982-83, were obtained from the wheat section of Punjab Agricultural Univ., Ludhiana.

Soaking and sprouting of wheat

The cleaned samples of wheat were soaked for 8 hr at 32°C. The excess water was drained off and superficial water present on the grains was removed with filter paper. The soaked sample was divided into four lots and incubated at 32°C and 75% relative humidity in a Yorco humidity temperature controlled incubator for 24 hr and 48 hr.

Heat treatment of grains

One lot of each of the samples after 24 hr and 48 hr incubation was subjected to heat treatment at 80°C for 15 min to partially inactivate the enzymes. In all, five samples were obtained for each variety: (1) control; (2) 24 hr sprouted; (3) 48 hr sprouted; (4) 24 hr sprouted and heat treated; (5) 48 hr sprouted and heat treated. All the samples were dried in a hot air drier to 12% moisture and kept in air-tight containers for further use.

Conditioning and milling of wheat

The calculated amount of water was intimately mixed with the grain to desired moisture level of 14%. The samples were shaken after each small addition of water and allowed to stand for 48 hr to ensure uniform penetration of moisture in the grain. The samples of conditioned wheat were milled in the Buhler Pneumatic Laboratory Mill (MLU-202 Buhler Brothers, Uzwil, Switzerland). Several pilot millings were conducted to determine the appropriate conditions of feed rate and roll settings for the mill. The yields of straight grade flour, bran and shorts were calculated on recovered product basis.

Physicochemical and rheological properties of flour

Analytical methods for ash (08-01), protein (46-13), gluten (38-10), total sugars (80-60), diastatic activity (22-15), and damaged starch (76-30A) were followed (AACC, 1976). Free amino nitrogen was determined by the method of Lie (1973). The constant flour weight method (AACC, 1976) was used for farinographic characteristics of flours.

Baking studies

The straight dough method with remix procedure of Irvine and McMullan (1960) was used for test baking and breads were evaluated after recording volume by the rapeseed displacement method, for crumb color and texture by the objective method given in AACC (1976). Cookies were prepared according to the method of AACC (1976). Spread factor of cookies was calculated using W/T as indicated in the AACC (1976) method.

All results were expressed on a 14% moisture basis unless otherwise stated. The data were subjected to analysis of variance; Duncan's multiple range test according to the method of Pence and Sukhatme (1967) was used.

RESULTS AND DISCUSSION

THE MILLING YIELD and ash of the flour as affected by sprouting and heat treatment are shown in Table 1. Varieties and treatments differed significantly with respect to flour yield and ash. Sprouted wheat recorded significantly higher flour yield compared to unsprouted wheat. However, no significant differences in flour yields were observed for wheat sprouted for 24 and 48 hr.

Heat treatment did not bring any change in flour yields of samples sprouted for 24 hr, but brought about a significant increase in flour yield in 48 hr sprouted wheat. This increase could be due to decreased amounts of shorts produced during milling in sprouted samples and higher retention of bran in

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Table 1—Effect of sprouting and heat treatment on flour yield and ash

Cultivar	Characteristics	Hr of sprouting: No treatment 0	No treatment 24	No treatment 48	Heat treatment (80°C for 15 min) 24	Heat treatment (80°C for 15 min) 48
WL-1562	Flour yield (%)	72.6	75.0	74.4	75.0	76.2
	Ash (%)	0.53	0.54	0.58	0.55	0.59
WL-711	Flour yield (%)	70.3	73.0	72.5	73.0	74.2
	Ash (%)	0.49	0.51	0.53	0.52	0.55
S-308	Flour yield (%)	64.9	70.0	70.2	70.1	72.5
	Ash (%)	0.48	0.50	0.51	0.53	0.53

	L.S.D. (0.05) varieties	L.S.D. (0.05) treatments
Flour yield	1.26	1.63
Ash	0.009	0.019

Table 2—Effect of sprouting and heat treatment on chemical properties of wheat flours

Cultivar	Characteristics	Hr of sprouting: No heat treatment 0	No heat treatment 24	No heat treatment 48	Heat treatment (80°C for 15 min) 24	Heat treatment (80°C for 15 min) 48
WL-1562	Protein (%, N × 5.7)	8.6	9.1	9.3	9.0	9.2
	Gluten (%)	9.2	8.8	—	8.8	—
	Free amino nitrogen (mg/g)	3.95	4.65	4.75	4.65	4.80
	Total sugars (% maltose)	1.85	2.25	3.20	2.20	2.89
	Diastatic activity (mg maltose/10g)	176	225	328	218	256
	Damaged starch (%)	8.2	5.9	5.9	6.1	6.7
WL-711	Protein (%, N × 5.7)	9.2	9.7	9.9	9.7	9.8
	Gluten (%)	9.7	8.0	—	8.3	—
	Free amino nitrogen (mg/g)	2.96	4.60	4.98	4.55	5.00
	Total sugars (% maltose)	1.64	2.76	3.94	2.62	3.50
	Diastatic activity (mg maltose/10g)	116	234	322	226	250
	Damaged starch(%)	8.5	6.0	6.0	6.2	7.0
S-308	Protein (% N × 5.7)	8.4	9.0	9.1	8.9	9.1
	Gluten (%)	9.4	8.6	—	9.1	—
	Fee amino nitrogen (mg/g)	3.25	3.53	3.71	3.50	3.75
	Total sugars (% maltose)	1.69	2.30	2.97	1.96	2.62
	Diastatic activity (mg maltose/10g)	207	251	315	192	251
	Damaged starch (%)	6.0	4.2	4.4	4.3	5.0

	L.S.D. (0.05) varieties	L.S.D. (0.05) treatments
Protein	0.4	0.5
Gluten	—	—
Free amino acids	0.48	0.62
Total sugars	0.43	0.33
Diastatic activity	N.S.	46.41
Damaged starch	0.26	0.34

flour in the heat-treated samples. The sprouted as well as heat treated samples recorded significantly higher ash with respect to control except for 24 hr sprouted samples from variety WL-1562. This might be due to the higher extraction rates of flour in which bran contamination could be higher.

Protein increased significantly with sprouting. However, the differences were nonsignificant between samples sprouted for 24 and 48 hr. The heat treatment given to the 24 and 48 hr sprouted samples did not show any difference in protein but interestingly the gluten of sprouted wheat decreased markedly compared to the unsprouted samples (Table 2). There was extensive softening of gluten after 48 hr of incubation accompanied by increased solubility, since it had not been possible to wash out gluten from these samples. The decreased gluten in these samples had been attributed to the increased activity of proteolytic enzymes during sprouting (Beresh, 1969; Handford, 1967). This observation was further supported by increased free amino nitrogen in sprouted samples. The heat treatment of 48 hr sprouted samples did not improve the gluten characteristics.

Total sugars and diastatic activity increased with sprouting (Table 2). Significantly higher values were recorded for these characteristics for wheat after 48 hr sprouting compared to those sprouted for 24 hr. Heat treatment decreased total sugar in all the sprouted samples. The increased sugar in sprouted samples was also reported by Dronzek et al. (1972) and may be explained by enzymatic degradation of starch.

The increase in diastatic activity in cultivar WL-711 was much higher compared to that of WL-1562 and S-308 cultivars. Heat treatment of sprouted samples considerably reduced the enzymatic activity but in no case did it match that of the unsprouted samples. Several workers (Hwang and Bushuk, 1973; Bhatt et al., 1981) also reported increased diastatic activity with sprouted grains. The damaged starch content decreased significantly with sprouting. Heat treatment resulted in higher damaged starch particularly in samples with 48 hr sprouting. The reduction in damaged starch from sprouted grains has been reported by William (1977).

Rheological properties

Farinograms obtained from the flours of sound and sprouted wheat are shown in Fig. 1; results are tabulated in Table 3. Farinograph water absorption decreased significantly for samples sprouted for 24 hr and 48 hr. The lower water absorption by these samples may be due to the lesser damage in starch in sprouted samples. The capacity of flours to absorb water increased when sprouted samples were given the heat treatment. The dough development time and dough stability decreased with the increase in sprouting period. Tremendous softening of dough was observed due to sprouting, and heat treatment was found to be beneficial in reducing the softening and increasing the dough development time. The softening of dough was considerably higher for doughs prepared from cultivars

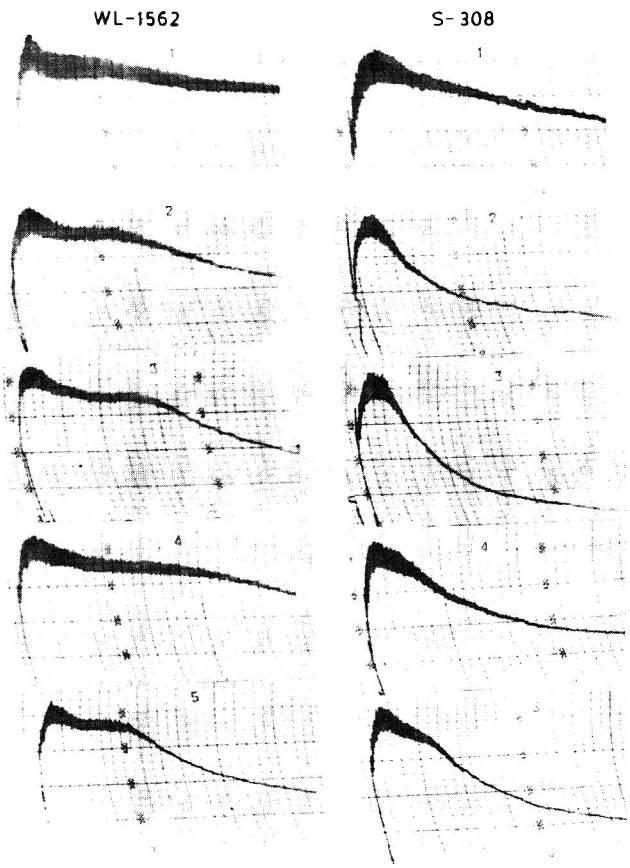

Fig. 1—Farinogram showing effect of sprouting and heat treatment on the rheological properties of WL-1562 and S-308 wheat flours. (1) Control; (2) 24 hr sprouted; (3) 48 hr sprouted; (4) 24 hr sprouted and heat treated, 80°C for 15 min; (5) 48 hr sprouted and heat treated, 80°C for 15 min.

WL-711 and S-308 than that from WL-1562. The differences were significant. Similar effects of sprouting on dough properties have been reported by Hwang and Bushuk (1973), Bean et al. (1976) and Ciacco and D'Appolonia (1982). The decreased strength of dough was attributed to the increased activity of the proteolytic enzymes which hydrolyzed the gluten and protein disulfide reductase developed during sprouting.

Baking characteristics of flour

Photographs of breads prepared from sprouted and unsprouted wheat flour are shown in Fig. 2 and 3. Bread baking qualities of flours as affected by sprouting and heat treatment were studied taking into consideration loaf volume, specific volume, crumb color and texture (Table 4). The increased sprouting period brought about significant reduction in the loaf volume. The heat treatment of flours rectified the ill effects of sprouting on the loaf volume of bread to a great extent. The flours from the sprouted wheats were found to produce breads with poorer crumb characteristics than those produced from sound wheat flours. The crumb of the bread baked from sprouted wheat was gummy having larger holes. The heat treatment brought about considerable improvement in bread quality. Huang and Bushuk (1973); Ranhotra et al. (1977), and Finney et al. (1981) reported that reduction in loaf volume of bread was due to the poor mechanics of doughs from sprouted wheats. Inferior crumb characteristics could be the result of excessive degradation of starch to sugars and dextrins which imparted undesirable stickiness and gumminess to the crumb (Ibrahim and D'Appolonia, 1979; Lorenz and Valvono, 1981; Finney et al., 1981). The spread factor of cookies was higher for sprouted samples than for unsprouted wheat (Fig. 4). The heat treatment brought about significant increase in spread factor over the control especially in samples sprouted for 48 hr.

The studies showed that sprouting of wheat had an adverse effect on the rheological and bread-making properties of wheat.

Table 3—Effect of sprouting and heat treatment on farinographic characteristics of flour

		Hr of sprouting				
		No treatment			Heat treatment (80°C for 15 min)	
Cultivar	Characteristics	0	24	48	24	48
WL-1562	Farinograph water absorption (%)	61.6	59.2	57.0	60.6	58.7
	Dough development time (min)	2.0	1.5	0.8	1.5	1.0
	Degree of softening (B.U.)	70	160	230	110	200
	Dough stability (min)	5.8	2.0	1.5	2.5	1.5
WL-711	Farinograph water absorption (%)	61.2	57.6	55.2	59.0	57.0
	Dough development time (min)	1.3	0.8	0.5	0.8	0.5
	Degree of softening (B.U.)	170	285	365	245	300
	Dough stability (min)	2.5	1.5	0.5	1.8	0.8
S-308	Farinograph water absorption (%)	57.2	56.0	55.6	56.8	56.2
	Dough development time (min)	2.5	1.0	0.8	1.5	1.0
	Degree of softening (B.U.)	160	250	320	220	260
	Dough stability (min)	2.5	1.8	1.3	2.0	1.5
		L.S.D. (0.05) varieties			L.S.D. (0.05) treatments	
Farinograph water absorption		1.26			1.63	
Dough development time		0.36			0.47	
Degree of softening		17.18			22.18	
Dough stability		NS			1.41	

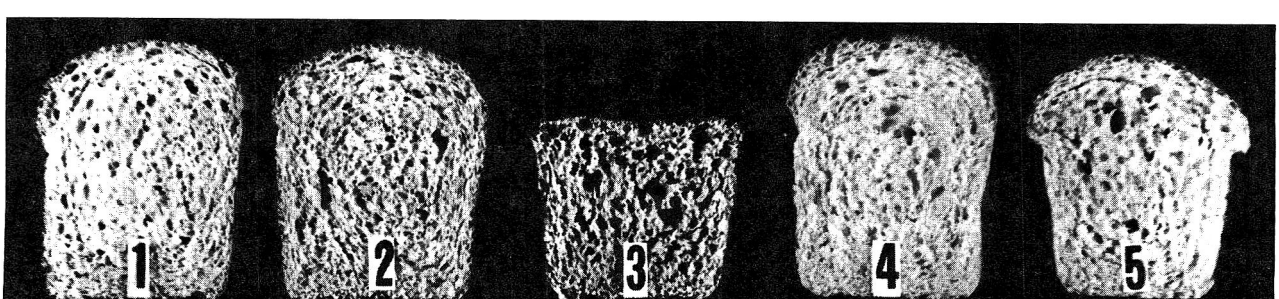

Fig 2—Effect of sprouting and heat treatment on loaf volume and crumb characteristics of bread from WL-711 wheat. (1) Control; (2) 24 hr sprouted; (3) 48 hr sprouted; (4) 24 hr sprouted and heat treated, 80°C for 15 min; (5) 48 hr sprouted and heat treated, 80°C for 15 min.

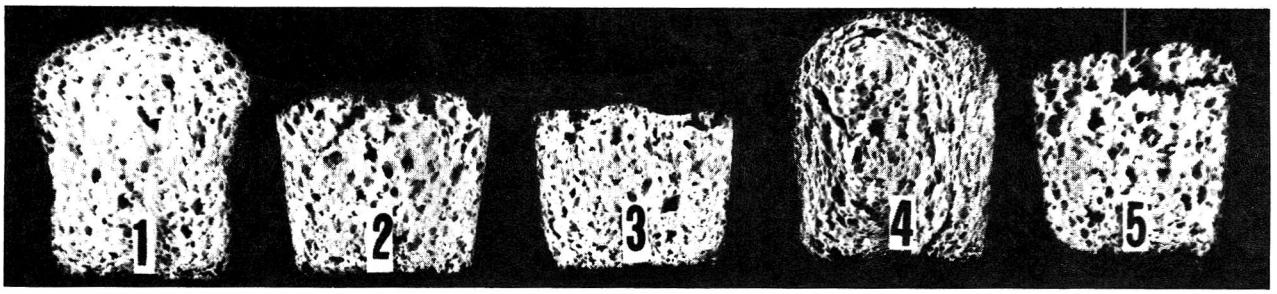

Fig 3—Effect of sprouting and heat treatment on loaf volume and crumb characteristics of bread from S-308 wheat. (1) Control; (2) 24 hr sprouted; (3) 48 hr sprouted; (4) 24 hr sprouted and heat treated, 80°C for 15 min; (5) 48 hr sprouted and heat treated, 80°C for 15 min.

Heat treatment of sprout-damaged grains effectively but not completely checked the adverse effect of sprout damage.

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Table 4—Effect of sprouting and heat treatment on baking properties of wheat flour

		Hr of sprouting				
		No treatment			Heat treatment (80°C for 15 min)	
Cultivar	Characteristics	0	24	48	24	48
WL-1562	Loaf vol. (cc)	695	600	500	645	560
	Specific vol. (cc/g)	5.03	4.41	3.63	4.74	4.29
	Crumb color	Yellowish creamy	Creamy	Creamy	Yellowish creamy	Creamy
	Crumb texture	Good	Poor	Poor	Good	Poor
	Cookies spread factor (W/T)	4.52	4.95	5.07	4.90	5.15
WL-711	Loaf vol. (cc)	560	400	360	520	465
	Specific vol. (cc/g)	4.16	2.96	2.69	3.72	3.0
	Crumb color	Creamy	Creamy	Creamy	Creamy	Creamy
	Crumb texture	Good	Fair	Poor	Good	Poor
	Cookies spread factor (W/T)	4.56	4.63	4.95	6.30	6.20
S-308	Loaf vol. (cc)	575	530	390	550	515
	Specific vol. (cc/g)	4.13	3.74	2.78	3.88	3.42
	Crum color	Yellowish Creamy	Creamy	Creamy	Yellowish Creamy	Creamy
	Crumb texture	Good	Good	Poor	Good	Good
	Cookies spread factor (W/T)	5.22	5.38	5.18	5.10	5.73
		L.S.D. (0.05) varieties			L.S.D. (0.05) treatments	
Loaf volume		40.03			51.68	
Specific volume		0.24			0.30	
Cookie spread factor		NS			0.50	

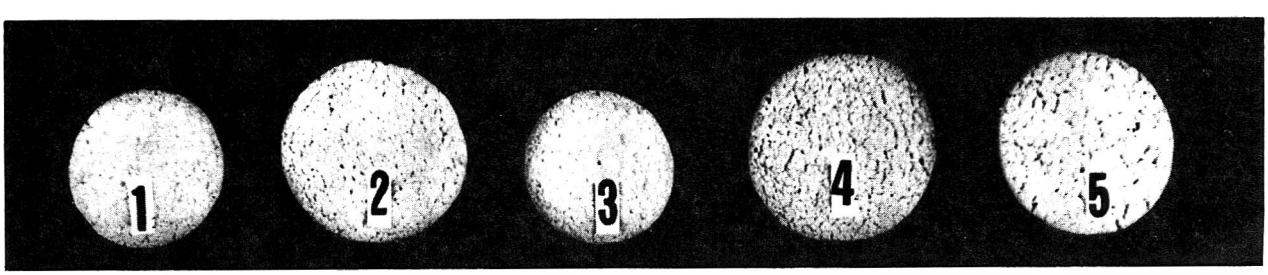

Fig 4—Effect of sprouting and heat treatment on appearance and spread factor of cookies from WL-1562 wheat flour. (1) Control; (2) 24 hr sprouted; (3) 48 hr sprouted; (4) 24 hr sprouted and heat treated, 80°C for 15 min; (5) 48 hr sprouted and heat treated, 80°C for 15 min.

Squeezing Flow Viscosimetry of Peanut Butter

O. H. CAMPANELLA and M. PELEG

ABSTRACT

The rheological characteristics of peanut butter were determined in two types of lubricated squeezing flow tests, one based on constant deformation rate and the other on deformation under constant stress (creep). Elongational flow was observed in both types of tests. When the flow regime was not governed by viscoelastic effects, peanut butter could be described as a power law fluid with consistency on the order of 70–200 KPa and flow index of 0.5–0.7.

INTRODUCTION

THE RHEOLOGICAL CHARACTERISTICS of viscous semi-liquid foods are usually determined in shear flow using a coaxial viscosimeter and less frequently by a cone and plate viscosimeter. One of the prerequisites for such determinations is that the sample is indeed sheared and that there is no slip between the instrument surfaces and the tested fluid. This condition is satisfied in most semi-liquid foods but there is a notable group of foods for which the "no slip" assumption cannot be taken for granted. This group consists of highly viscous materials which contain a considerable amount of fat (e.g., melted cheeses, peanut butter). These materials can be considered as self-lubricating and, therefore, their flow pattern in a coaxial (or capillary) rheometer is either a distorted shear flow or plug flow. A way to avoid the problems that are associated with self-lubrication is to use squeezing flow viscosimetry with lubricated plates. In such tests the existence of lubrication and slip is not only acknowledged but also incorporated in the calculation of the results.

The objectives of this work were to test the applicability of lubricated squeezing flow viscosimetry to peanut butter and to determine its rheological properties using this method.

THEORY

Squeezing flow viscosimetry

Squeezing flow viscosimetry is based on compression of a fluid specimen between two parallel plates. There are four types of test arrays which offer both technical simplicity and a convenient rheological interpretation of the results. The four arrays are schematically described in Fig. 1. They can be classified as those based on constant area or constant volume, or alternatively, those based on constant load or constant rate of deformation. The mathematical aspects of squeezing flow, with and without lubrication, have been discussed extensively in the rheological and polymer literature (e.g., Oka, 1960; Leider, 1974; Leider and Bird, 1974; McClelland and Finlayson, 1983; Chatraei et al., 1981).

Consequently, there are a few published equations relating force-time or deformation-time relationships to the flow characteristics of different types of fluids, i.e., Newtonian, power law. These expressions can be used to calculate the rheological constants of such fluids from experimentally determined relationships. The existence of theoretical solutions to squeezing flow problems by themselves, however, is not a guarantee for meaningful interpretation of such tests. The reason is that instrumental or procedural artifacts can, at least theoretically, affect the magnitude of the calculated rheological constants, while the general character of the underlying mathematical relationships remains unchanged. Methodologically, therefore, it is imperative to determine the rheological constants either by two different kinds of tests and/or by the same kind of test performed under different conditions (e.g., different specimen height, load range and deformation rate). This approach was followed in this work, and the tests selected were the constant area array with constant load (stress) and constant deformation (displacement) rate (Fig. 1).

Mathematical relationships in squeezing flow

Lubricated squeezing flow of a specimen with a constant area is governed by the equation (Leider and Bird, 1974; Chatraei et al., 1981)

$$F(t) = 2\pi \int_o^R (T_{zz} - P_o) r dr \qquad (1)$$

where F(t) is the force, T_{zz} is the normal component of the stress tensor, P_o the atmospheric pressure, R is the specimen's radius and r is the distance from the center. For Newtonian fluid, Eq. (1) results in the relationship (Bird et al., 1977; Chatraei et al., 1981)

$$F(t) = \frac{3\pi R^2 \mu}{H(t)} \left(- \frac{dH(t)}{dt} \right) \qquad (2)$$

where μ is the Newtonian viscosity and H(t) is the momentary height of the specimen. For a power law fluid, i.e., obeying the equation (Showalter, 1978)

$$\tau = -K \left(\frac{1}{2} \dot{\gamma} : \dot{\gamma} \right)^{\frac{n-1}{2}} \dot{\gamma} \qquad (3)$$

where τ is the stress tensor, $\dot{\gamma}$ the strain rate tensor, $\dot{\gamma} : \dot{\gamma}$ is the scalar (double) product of the shear rate tensor, and K and n constants (also known as the consistency and flow index, respectively). Eq. (1) yields, upon integration, the following relationship between the normal component of the stress tensor (T_{zz}) and the true strain rate (dH/Hdt)

$$T_{zz} - P_o = 3^{\frac{n+1}{2}} K \left(- \frac{dH(t)}{H(t)dt} \right)^n \qquad (4)$$

which in turn gives the following:

For flow under constant deformation rate (i.e., $- \frac{dH(t)}{dt} = \text{const} = V$)

$$F(t) = \frac{3^{\frac{n+1}{2}} \pi R^2 K V^n}{[H(t)]^n} \qquad (5)$$

or

$$\ln F(t) = C + S_1 \ln \left(\frac{1}{H(t)} \right) \qquad (6)$$

where

$$C = \ln \left[3^{\frac{n+1}{2}} \pi R^2 K V^n \right] \qquad (7)$$

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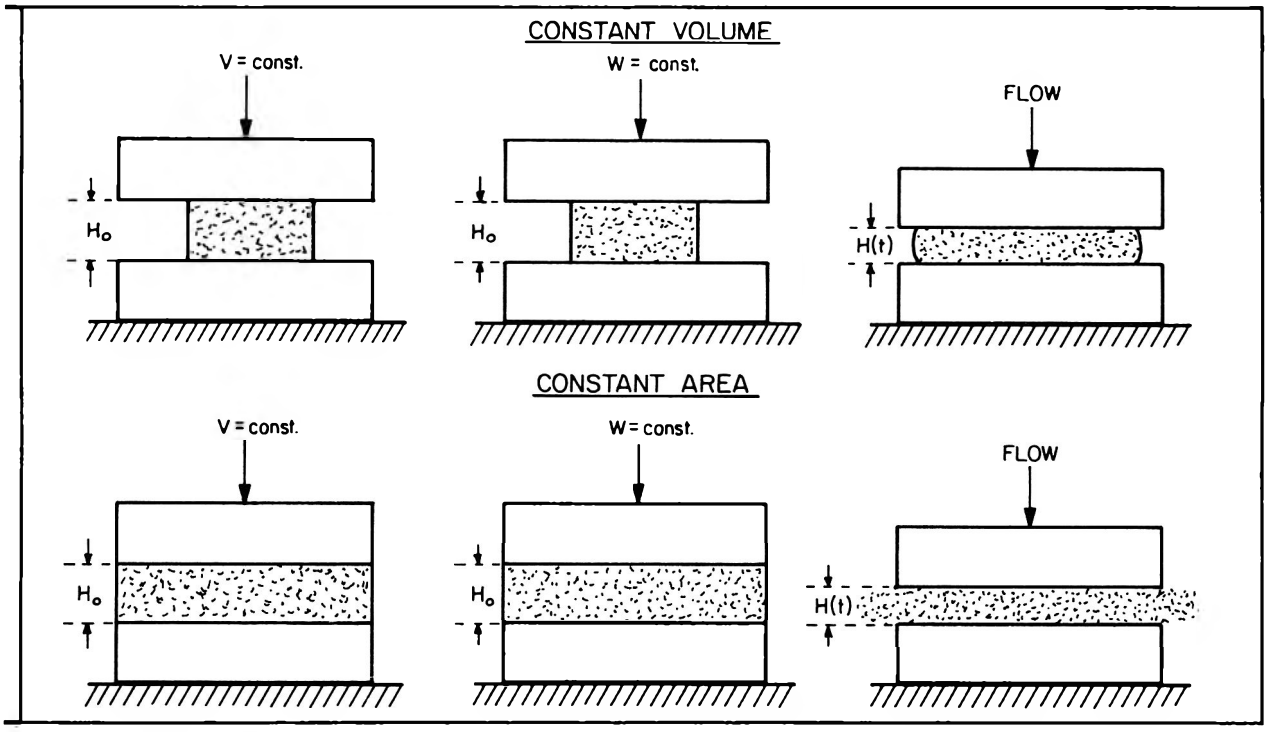


Fig. 1—Schematic view of the different arrays of squeezing flow rheometry. V is a constant deformation (displacement) rate and W, a constant load (weight). H_o, the initial specimen height, H(t), the specimen height after time t.

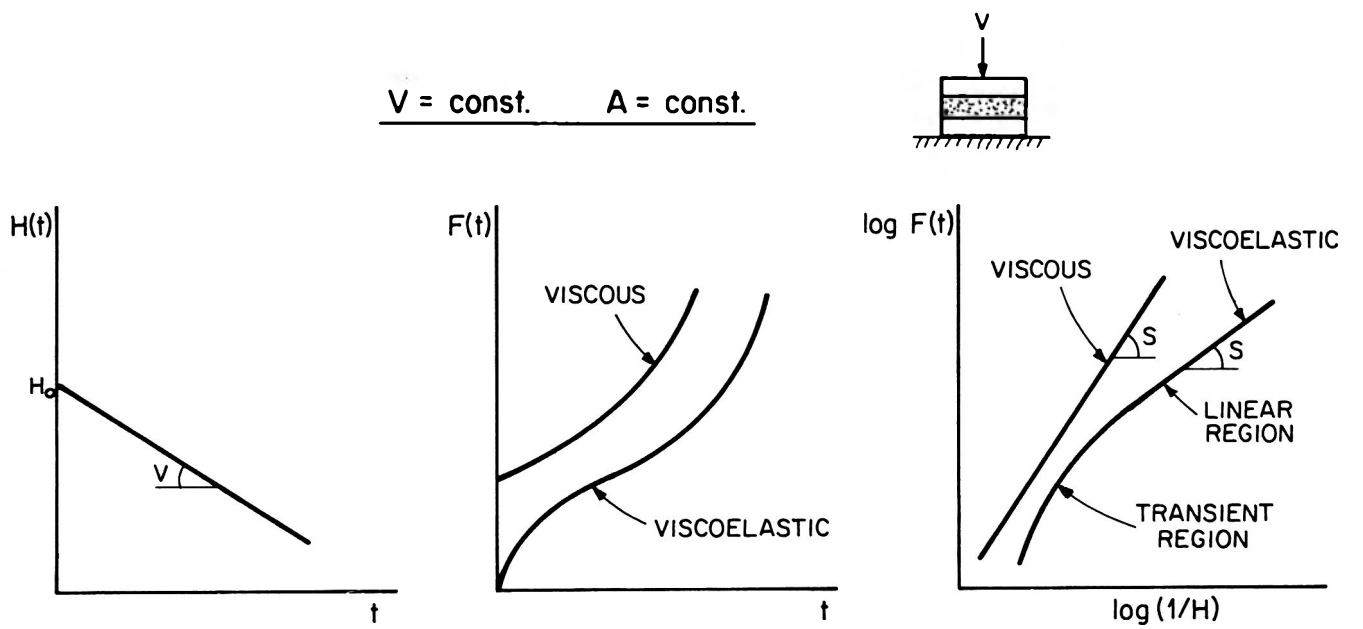


Fig. 2—Schematic view of the data retrieval procedure from a squeezing flow experiment performed with constant deformation rate (V) and constant specimen area (A). (H is the specimen momentary height, H_o the initial height, F the momentary force, t the time, and S the slope of the lower region).

and $S_1 = n$ if the relationship is indeed linear (see Fig. 2). The values of K and n, therefore, can be calculated either by linear regression of the data presented in the form of Eq. (6) or by nonlinear regression of the data presented in the form of Eq. (5).

For flow under constant load (constant stress), i.e., F(t) = const = W (see Fig. 3).

$$\ln \frac{H(t)}{H_o} = -S_2 t \tag{8}$$

where

$$S_2 = \left(\frac{W}{3^{\frac{n+1}{2}} \pi R^2 K} \right)^{1/n} \tag{9}$$

and H_o the specimen's initial height.

Recording force time relationships under constant deformation rate or height decreases under constant force enables the calculation of the constants K and n from the fit of Eq. (6) and (8) to experimental data in regions where viscoelastic effects vanish or can be neglected. It should be noted that since Eq. (9) contains both constants as part of the slope, their magnitude can only be calculated from repeated experiments with at least two different loads (W).

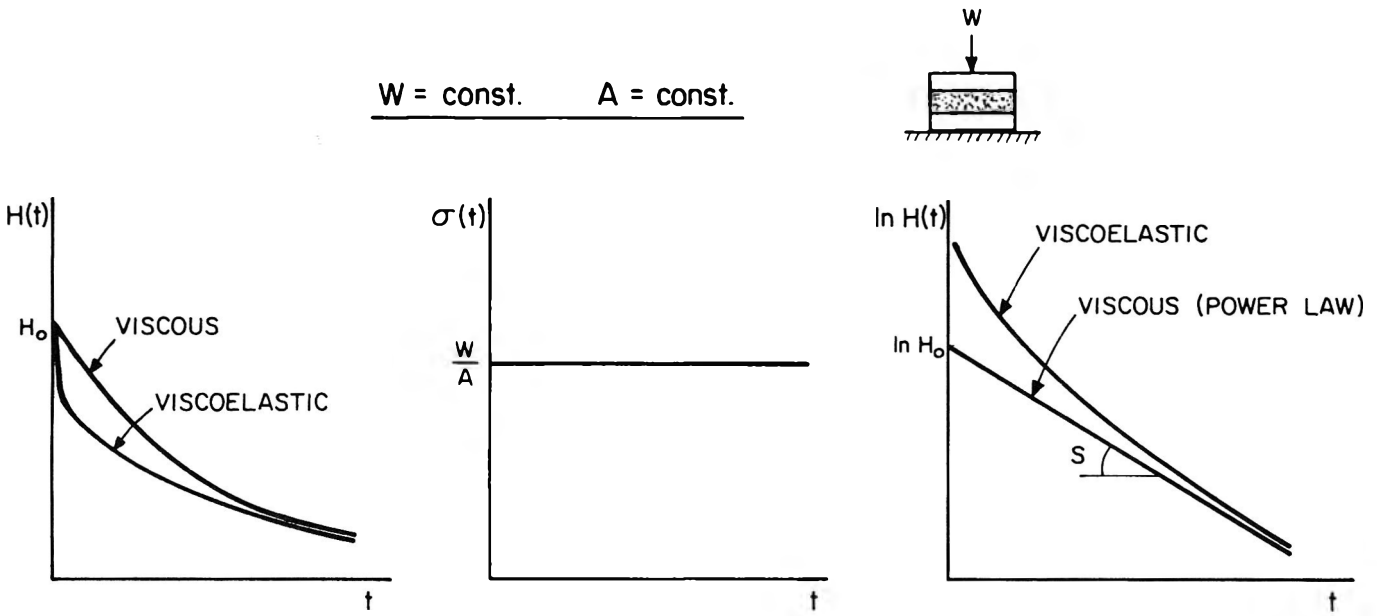


Fig. 3—Schematic view of the data retrieval procedure from a squeezing flow experiment (creep) performed with a constant load (W) and constant specimen area (A). (H is the specimen momentary height, H_o the initial height, σ(t) is the stress, t is the time, and S the slope of the linear region).

MATERIALS & METHODS

JARS OF PEANUT BUTTER of a national brand and of a supermarket chain were purchased at a local supermarket. They were left to equilibrate at laboratory ambient temperature and were tested at about 22–24°C. Samples of the peanut butter were placed between two parallel lubricated plastic platens with known diameter in the form shown schematically in Fig. 1 (constant area). The initial thickness (height) of each sample was monitored. Prior to testing the samples were allowed to rest for a few minutes to relieve the shear effects produced during the sample preparation.

Part of the samples were subjected to uniaxial deformation at various constant deformation (displacement rates using an Instron Testing Machine model TM.) Another part was subjected to uniaxial creep deformation under various constant loads using the creep tester recently described in detail by Purkayastha et al. (1985). The recorded force-time and thickness-time relationships in the two tests were digitized, replotted and fitted by Eq. (5) or (6) and (8) (Fig. 2 and 3). The constants of these equations were used to calculate the rheological constants K and n using Eq. (7) and (9).

RESULTS & DISCUSSION

THE APPEARANCE of peanut butter specimens in lubricated squeezing flow is demonstrated in Fig. 4. The figure clearly demonstrates that the flow pattern is that of elongational flow. This is evident from the flat boundary of the expelled material and the fact that the specimen retained its cylindrical shape. Shear flow would have resulted in bulging of the edge and "barreling" of the specimen shape. It should be mentioned that a similar pattern was also observed when the platens were not lubricated indicating that, at least with the platens used, peanut butter can provide enough self-lubrication to induce elongational flow. The same behavior was observed in the creep experiments, again demonstrating that self-lubrication governed the flow regime.

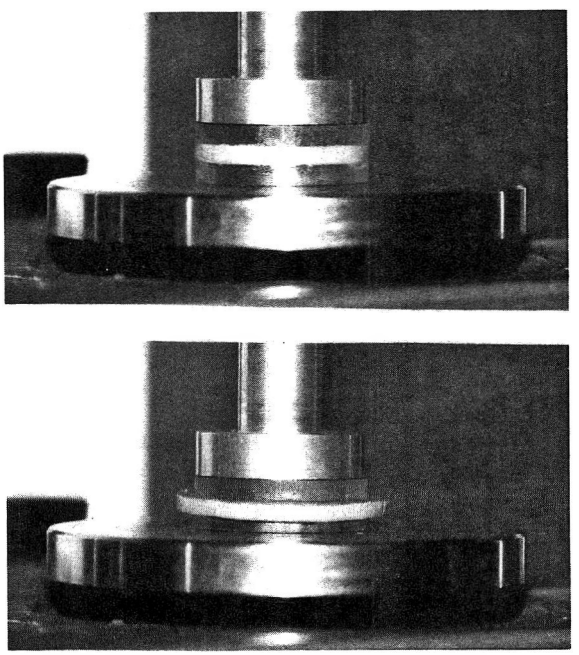
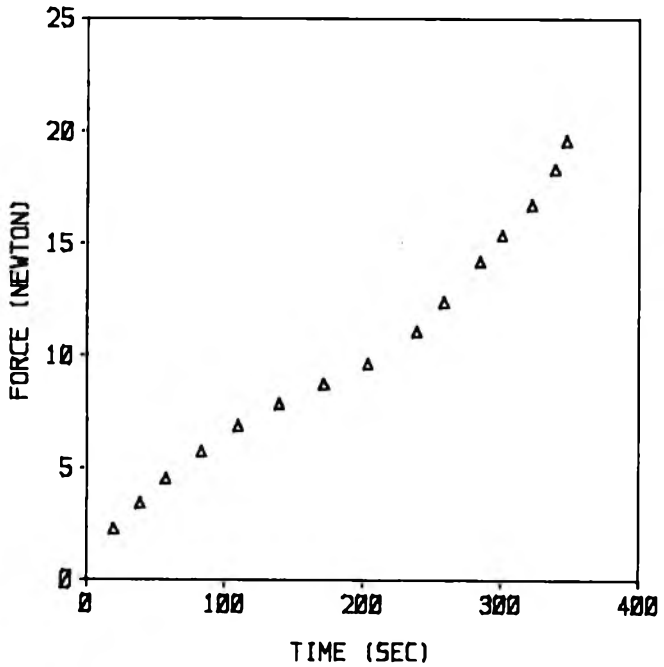


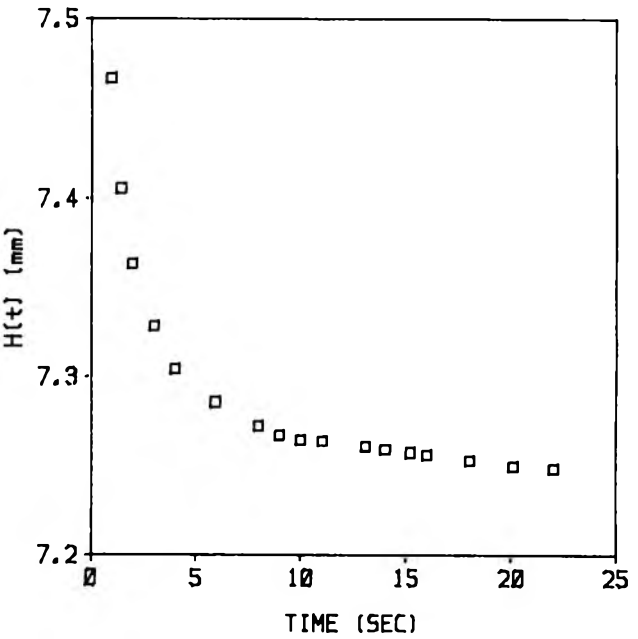
Fig. 4—The appearance of a peanut butter specimen before (top) and during lubricated squeezing flow experiments (bottom). Note the flat appearance of the deformed specimen boundary indicating elongational flow pattern.

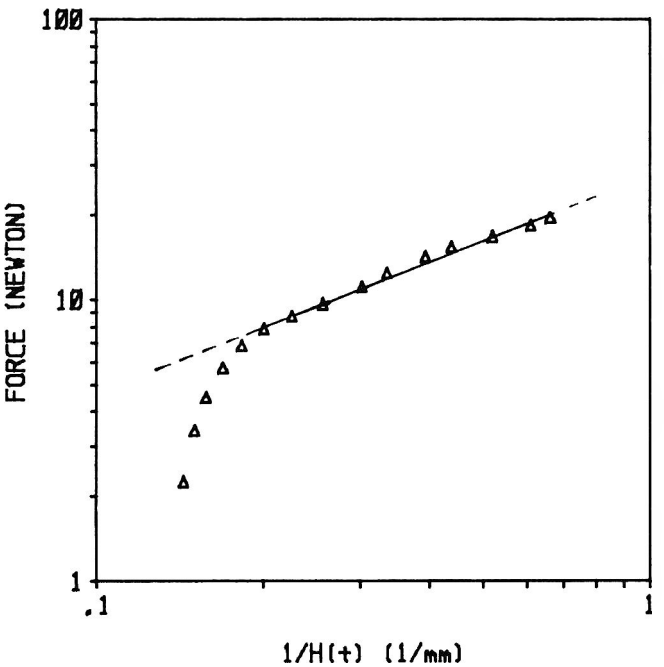
Calculation of the rheological constants

The experimental force-time (Fig. 5) and height-time (Fig. 6) relationships showed transient regions that were most probably produced by viscoelastic effects. Upon transformation to logarithmic relationships, however, the curves, as could be expected, had considerable linear portions that enabled calculation of the power law model constants. It is perhaps worth metioning that in the deformation tests, viscoelastic effects are evident from the start (i.e., the force deformation (time) curve starts from the original and not from a non-zero value as in the case of true liquids. Their role becomes dominant as the specimen becomes shorter as the result of the progressively increasing true strain rate). In creep, in contrast, viscoelastic effects are only manifested in the initial stage (i.e., exhibiting an "instantaneous" like compliance) and they rapidly dissipate subsequently, as the flow proceeds at an ever decreasing rate.

Calculated values of the rheological characteristics of two commercial peanut butter brands are presented in Table 1. As could be expected, the table shows that peanut butter can be described as a power law fluid with a flow index on the order 0.5–0.7. It is about ten times higher than previously published







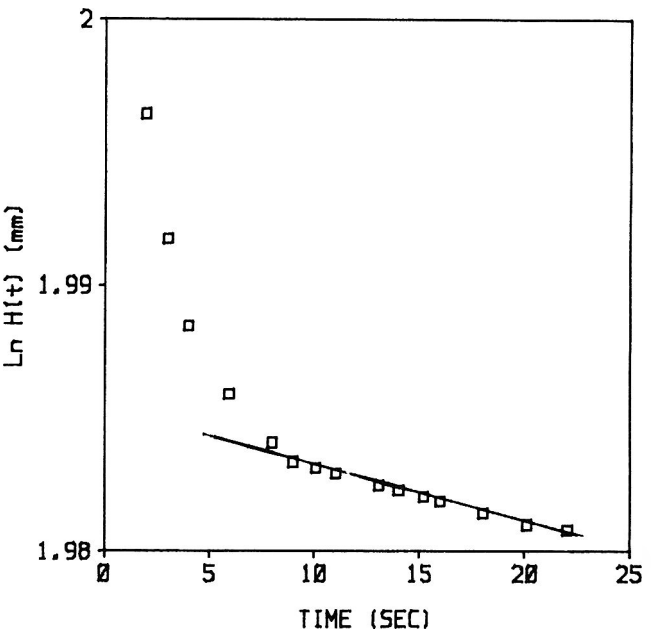


Fig. 5—Typical force-time and log F vs log 1/H(t) curves of peanut butter deformed at a constant deformation rate. Compare to Fig. 2. H(t) is the specimen height after time (t).

Fig. 6—Typical height-time and log H(t) vs time of peanut butter deformed under constant load (stress). Compare to Fig. 3. H(t) is the specimen height after time t.

values based on shear flow under much higher deformation rates (Dickie and Kokini, 1982). The highly viscous character of peanut butter is expressed by the magnitude of the "consistency" parameter K which had values on the order of 70–90 KPa for one brand and 140-200 KPa for another. These levels are in general agreement with the values published by Sharma and Sherman (1973). The scatter of the results was in the range of less than 10% for the values of n and on the order of up to 30% for the values of K. This scatter was probably the result of the crude manner in which the specimens were prepared and also some history related effects. Since peanut butter did show viscoelastic behavior (Fig. 5 and 6) and since the existence of rheological memory cannot be ruled out, at least part of the scatter can be related to differences in the stress history of the specimens before and during the tests. These effects were probably also responsible for the slight discrepancy between the results obtained in constant deformation rate and in tests performed with a constant load. Despite these, however, the difference between the rheological behavior of the two peanut butter brands was clearly evident, irrespective of the test employed or the conditions under which the test had been performed.

Role of yield stress

Although the reality of the yield stress has recently been challenged (Barnes and Walters, 1985), the concept itself is still useful in many practical applications. Under the conditions of the reported tests, however, the yield stress does not come into play. This is because elongational flow is either induced

Table 1—Rheological constants of peanut butter determined under different conditions in lubricated squeezing flow tests[a]

	Constant deformation rate				Constant load (Stress)				
Product	V (cm min^{-1})	H_o (mm)	K (kPa·S^n)	n (–)	$W/\pi R^2$ (kPa)	H_o (mm)	K (kPa·S^n)	n (–)	Strain rate[b] $\times 10^{-4}$ (sec^{-1})
National brand	0.05	6.9	57	0.53	2.9	14.6	99	0.49	1.4
	0.05	6.4	82	0.64	3.8	12.2			2.4
	0.10	6.5	82	0.72	6.1	11.3	85	0.47	6.5
	0.10	7.5	76	0.67	8.2	12.0			12.4
	mean:		74	0.64	mean:		92	0.48	
Supermarket chainbrand	0.05	6.4	132	0.64	1.8	9.9	205	0.58	0.63
	0.05	6.0	190	0.69	2.8	9.5			1.36
	0.1	7.1	130	0.70	4	10.9	202	0.58	2.5
	0.1	8.1	124	0.69	5	8.9			3.8
	mean:		144	0.68	mean:		203	0.58	

[a] V is the deformation rate, H_o the initial specimen height, W the imposed load and R the specimen radius. The consistency K and the flow index n were calculated using Eq. (5) to (9) employing the procedure described in the text.

[b] The strain rate where log H vs t was a straight line (see Fig. 6).

irrespective of its hypothetical existence (in constant deformation rate experiments) or because the measurements are taken under stresses that exceed its magnitude (in the constant stress experiments). Yield stress, however, can be determined in unlubricated squeezing flow experiments and a procedure for such determination is described elsewhere (Campanella and Peleg, 1987).

Comparison between constant deformation rate and constant stress experiment

The amount of data collected in this study, including additional experiments whose results are not reported in Table 1, and the strong possibility that the specimen history can influence the results, does not allow for a clear cut preference of either test as more meaningful rheologically. Both seemed to be on equal footing and provided a consistent picture with respect to differences between the peanut butter brands. As far as convenience was concerned, the constant deformation tests seemed to be by far the superior. The main reason was that only one curve was required for the determination of the constants K and n. The trial and error needed to select the appropriate load for the constant stress experiments were also avoided and the tests themselves were of much shorter duration. The snag is, of course, the equipment cost. While Universal testing machines are rather expensive, the "creep" experiments can be performed with self-assembled equipment at about a tenth of the cost. Since, however, Universal testing machines are becoming almost a standard item in food research laboratories, the cost becomes a lesser issue. In any case, the cost of such machines is by far less than that of sophisticated rheometers which would otherwise be required for the analysis of materials such as peanut butter.

Other implications

As previously proposed by Sharma and Sherman (1973), the sensation of peanut butter consistency in the mouth is not necessarily based on shear flow. If this is indeed the case, and we believe it is, then rheological parameters obtained by lubricated squeezing flow experiments are more pertinent to those obtained in shear. This may also be the case with respect to peanut butter "spreadability," a factor of prime concern to its manufacturer. Although the force required to spread peanut butter does involve shear, it has a significant component that results from squeezing. The reported experiments were certainly not intended to simulate spreading nor the events that occur in the mouth. Therefore, the reported values of K and n need not be directly related to the textural perception of peanut butter. However, if the stimulus for assessing the consistency of peanut butter sensorily is its resistence to deformation and flow, then it seems that the magnitude of this resistance is far greater than what could be assumed on the basis of shear flow data obtained by a more conventional type of rheometer.

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Ms received 1/16/86; revised 7/30/86; accepted 8/18/86.

Contribution of the Massachusetts Agricultural Experiment Station at Amherst. The financial support of the principal author by the Consejo Nacional de Investigaciones Cientificas y Tecnicas de la Republica Argentina (Programa BID-CONICET) is gratefully acknowledged.

Apparent Heat Transfer in a Forced Convection Oven and Properties of Baked Food

HIDEMI SATO, TSUNEO MATSUMURA, and SHOKO SHIBUKAWA

ABSTRACT

Parameters for expressing the heating performance and baking results of sponge cakes dependent on heating performance in a forced convection oven were studied. The heating performance of a forced convection oven may be expressed by the apparent heat transfer coefficient which was measured at various air temperatures and velocities. Both the air velocity and temperature of the circulating air affected the apparent heat transfer coefficient in a forced convection oven and determined the final properties of the baked food. The effects of these parameters on sponge cakes baked in the forced convection oven were observed.

INTRODUCTION

FORCED CONVECTION OVENS are gaining popularity both in the home and in the food industry. Currently, various forced convection ovens are available. In these ovens, food is exposed to circulating hot air and heated by convective heat transfer. However, the time required to heat food in different ovens, even at the same air temperature, may differ, because heating performance levels vary in forced convection ovens, due to the differences of heat transfer characteristics between food surface and hot air or hot wall.

Several studies have been done on drying techniques and on color formation in forced convection ovens (Skjöldebrand, 1980; Skjöldebrand and Hallstrom, 1980; Skjöldebrand and Öste, 1980). In other studies, the results of foods cooked in different ovens have been compared (Dagerskog and Sorenfors, 1978; Colleson and West, 1980). These studies have contributed much useful information on forced convection ovens. However, many problems still remain. One is the relation between the heating performance of the oven, a dominant characteristic which determines the heating rate or drying process of food, and food properties after baking in a forced convection oven. Further, no suitable indicator for expressing the heating performance has been reported.

The objectives of this study were to determine whether the heating performance of the forced convection oven could be expressed by the apparent heat transfer coefficient, defined by the air temperature and surface temperature of foods and to analyze the relationships of the baked sponge cake properties with both air velocity and temperature.

MATERIALS & METHODS

Apparatus

The equipment used in this study is shown in Fig. 1. Air heated by an electrical heater is circulated in the oven by a fan. Hot air flows into the cavity through the perforated wall which directs the air flow. The oven is designed to permit air velocity from 0 m/sec to 1.5 m/sec and air temperature from 20°C to 400°C. Air velocity near the sample was measured with a hotwire anemometer.

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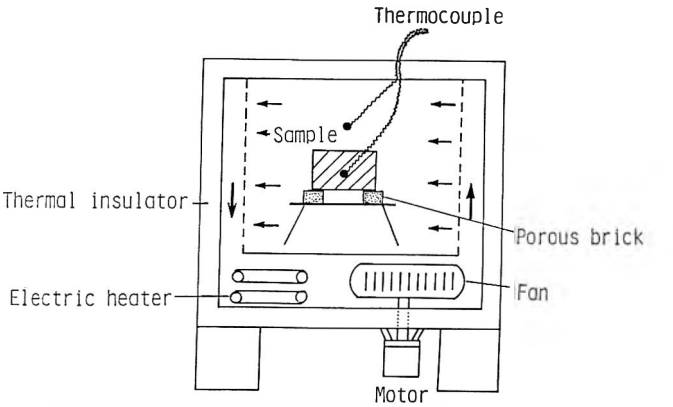

Fig. 1—Diagram of equipment used in experiments.

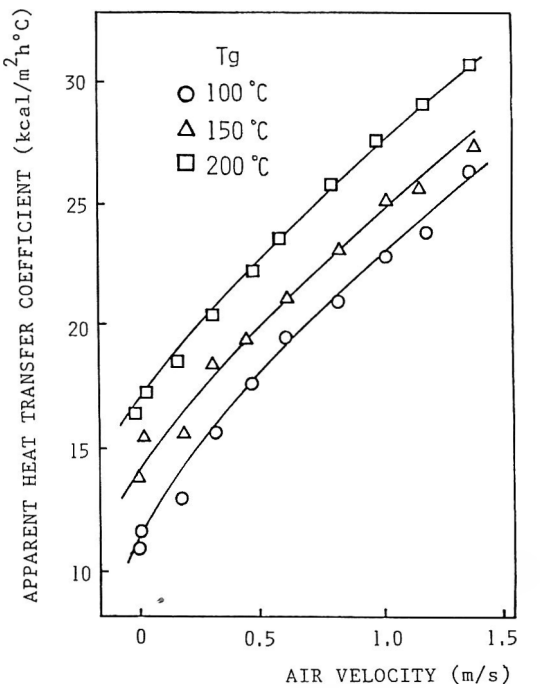

Fig. 2—Effect of changes in air velocity on the apparent heat transfer coefficient. Tg–air temperature.

The sample was placed on small porous bricks in the center of the cavity. The bricks were set at four points which would not interfere with normal heat transfer by convection and radiation in the oven. Their thermal conductivity was so low that any conductive heat transfer was negligible.

Measurement of the apparent heat transfer coefficient

The test sample was heated by convective heat transfer from the hot air and radiative heat transfer from the oven interior walls. Total heat transfer rate, Qt, from both radiation and convection in a forced convection oven is calculated by Eq. (1).

$$Qt = Qr + Qc = (h_r + h_c)\, A\, (Tg - T') \qquad (1)$$

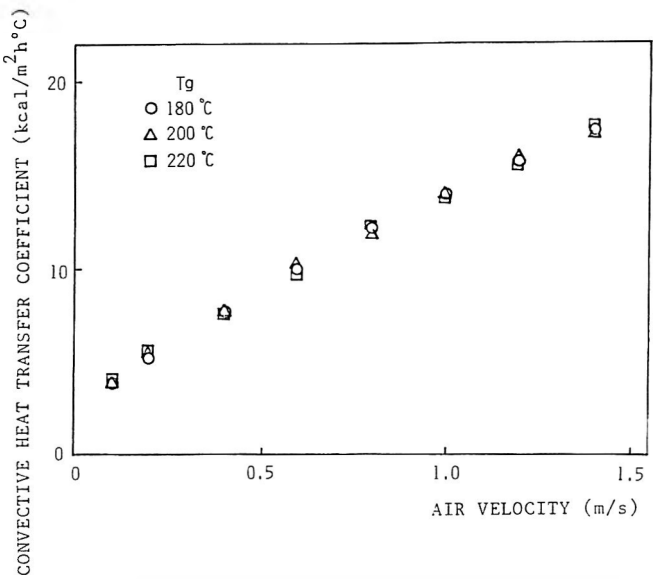

Fig. 3—Effect of changes in air velocity on the convective heat transfer coefficient. Tg–air temperature.

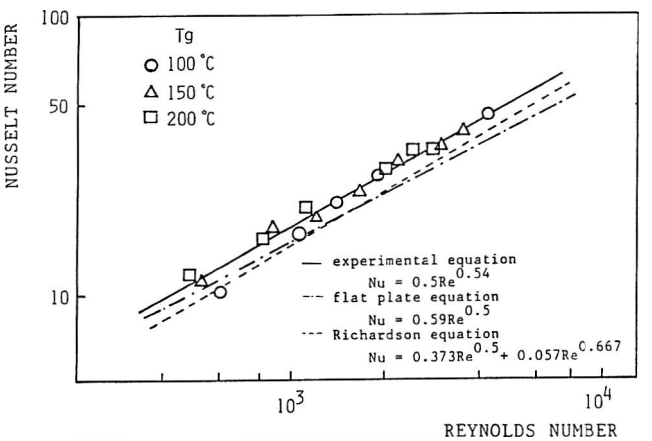

Fig. 4—Heat transfer by forced convection in the oven. Tg–air temperature; Re–Reynolds number and Nu–Nusselt number.

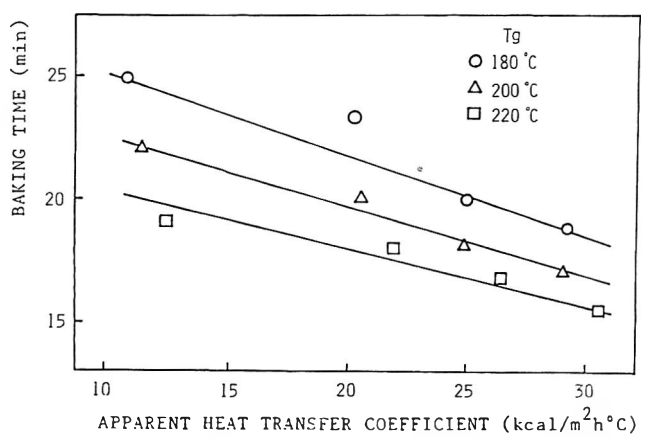

Fig. 5—Effect of apparent heat transfer coefficient on baking time. Tg–air temperature.

where Qr is the radiative heat transfer rate; Qc, the convective heat transfer rate; h_r, the radiative heat transfer coefficient; h_c, the convective heat transfer coefficient; A, the surface area of the sample; Tg, the air temperature; and T′, the surface temperature of the sample. $(h_r + h_c)$ in Eq. (1) represents the apparent heat transfer coefficient, h, in this study [Eq. (2)].

$$h = h_r + h_c \tag{2}$$

Radiative heat transfer coefficient can be expressed by the following equation in the case wherein a small sample is enclosed with a sufficiently large heating surface (Eckert and Drake, 1959).

$$h_r = \sigma \epsilon (\dot{T}' + \dot{T}g)(\dot{T}'^2 + \dot{T}g^2) \tag{3}$$

where σ is the Stefan-Boltzman constant, 4.88×10^{-8} (kcal/m²hr°K); ϵ, the emissivity of the sample surface; and the temperature with *, the absolute temperature, °K. Wall temperatures were nearly equal to air temperature, Tg, in this type of forced convection oven, because there was no heat source in the walls and walls were exposed to the flowing air.

The apparent heat transfer coefficient, h, was experimentally determined using the temperature-time curve of the aluminum cylinder which was 66 mm in diameter and 50 mm in height and was placed at the sample position shown in Fig. 1. The surface of the cylinder was coated with a silicon resin which made the emissivity of the coated surface nearly 1.0. The thermal conductivity of aluminum is so high that the temperature of the entire cylinder is uniform. The mean temperature, T, of the cylinder is assumed to be equal to the surface temperature, T′. Thus, Eq. (4) is derived from the heat balance assuming that temperature changes by dT over a short period of time dt

$$h A (Tg - T) dt = Cp W dT \tag{4}$$

where W is the weight (g); Cp, the specific heat (kcal/kg°C) and A, the cylinder surface area (m²).

Integration of Eq. (4) leads to Eq. (5) if $T = T_1$ at $t = t_1$, and $T = T_2$ at $t = t_2$.

$$h = \frac{Cp\ W}{A(t_1 - t_2)} \log \frac{Tg - T_2}{Tg - T_1} \tag{5}$$

Temperatures were measured using the alumel-chromel thermocouple inserted into the cylinder. From the measured temperature-time curve, the apparent heat transfer coefficient, h, can be determined with Eq. (5). The value of h_c is determined with Eq. (2) after calculating h_r using Eq. (3).

Food tests

Sponge cake was chosen to observe the effect of heating performance in the forced convection oven. The sponge cake was made from a batter containing 29.2% sugar, 29.2% flour, 29.2% egg, 11.7% water and 0.7% emulsifier (main components were sugar ester and glycerin monoester). Emulsifier was used in the batter to maintain equilibrium of the whipped eggs before baking. One hundred fifty grams were poured into an aluminium mold (ϕ 120 mm) which was placed in the oven set at a predetermined air temperature and velocity. The batter was baked until the temperature at the center reached 90°C and then heated 5 more min. The temperature at the center of the sample was measured by an alumel-chromel thermocouple. Pre-set air temperatures and velocities were determined by preliminary tests. Four different air velocities between 0 m/sec and 1.5 m/sec and temperatures of 180°C, 200°C, and 220°C were used.

The total weight loss of the cake was measured after baking and was expressed as a percentage of the initial weight. The shape of the cake was expressed as the difference in the height between the center and the side. Pictures of the longitudinal sections were taken to compare samples baked at different air temperatures and velocities. Texture refers to the firmness and cohesiveness of sponge cakes. Sponge cake textures were analyzed with a Rheolometer (Iio Electric Co. Ltd., Tokyo, Japan). A piece, 30 mm long, 30 mm wide and 20 mm high, was cut from each cake and placed in the Rheolometer, where the piece was crushed with a plunger. The curve of the force necessary to crush each piece was recorded. Firmness and cohesiveness were calculated from this curve. The formation of color on cake surfaces during baking was measured using a Color Measurement Unit (Koito Co. Ltd., Tokyo, Japan) Volume of the sample after baking was measured by the rapeseed displacement method (Grisworld, 1962). Experiments were repeated five times at each predetermined air temperature and velocity. Analysis of variance of the data was determined according to Bryant (1960).

Table 1—Cohesiveness and volume of baked sponge cakes[a,b]

Menu item		Air temperature (°C) 180	200	220
Cohesiveness (R.U.)[c]				
Air velocity (m/sec)	0	0.637 ± 0.011a	0.657 ± 0.018a	0.671 ± 0.013a
	0.5	0.637 ± 0.004a	0.669 ± 0.011a	0.674 ± 0.004a
	1.0	0.658 ± 0.008a	0.674 ± 0.013a	0.688 ± 0.005a
	1.5	0.664 ± 0.013a	0.688 ± 0.011a	0.686 ± 0.010a
Volume (cm^3)				
Air velocity (m/sec)	0	903.7 ± 22.3a	1029.1 ± 33.2b	1116.7 ± 30.9c
	0.5	932.9 ± 6.8a	1028.2 ± 39.9b	1140.0 ± 37.9c
	1.0	930.1 ± 5.3a	1025.4 ± 37.9b	1206.9 ± 37.0c
	1.5	946.3 ± 29.9a	1089.0 ± 35.8b	1184.3 ± 30.9c

[a] N = 5

[b] Where letters differ within a row, mean values differ significantly ($P<0.05$) from each other.

[c] (R.U.) means Rheolometer unit.

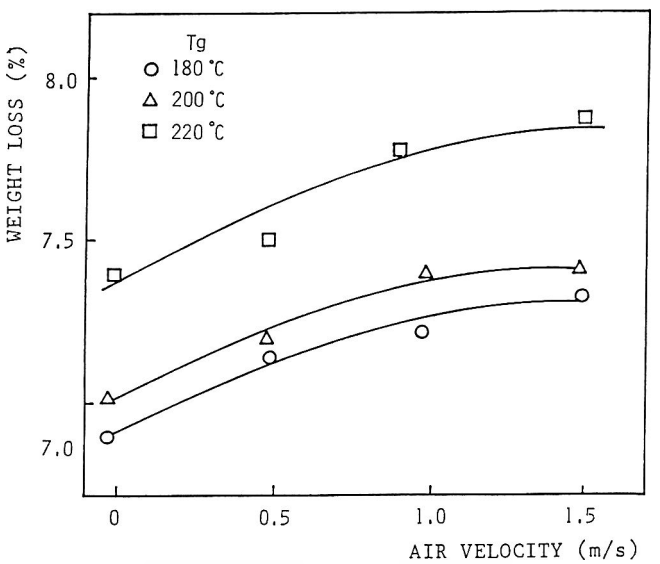

Fig. 6—Effect of changes in air velocity and temperature on weight loss. Tg–air temperature.

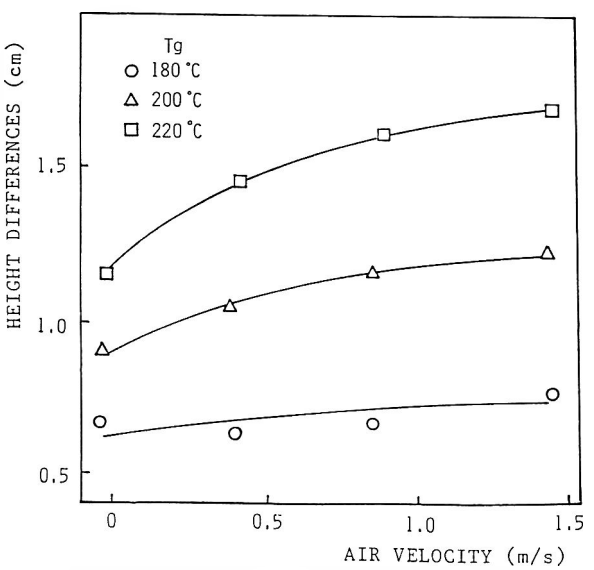

Fig. 7—Effect of changes in air velocity and temperature on height. Tg–air temperature.

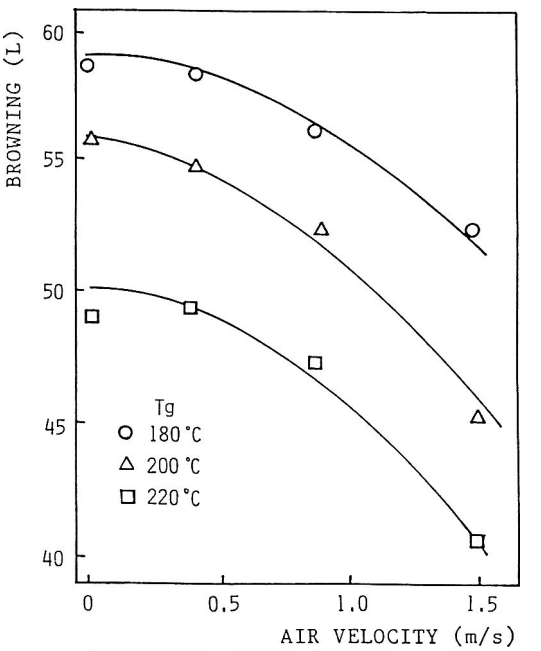

Fig. 8—Effect of changes in air velocity and temperature on surface color. L–brightness; Tg–air temperature.

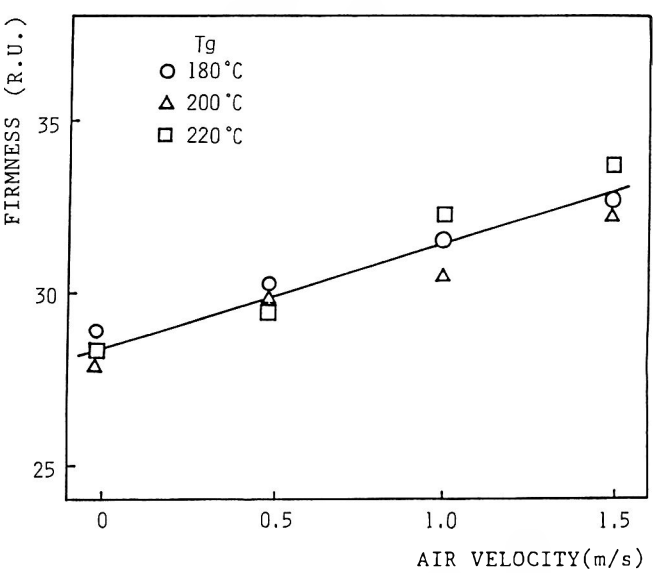

Fig. 9—Effect of changes in air velocity on firmness. R.U.–Rheolometer unit; Tg–air temperature.

RESULTS & DISCUSSION

Apparent heat transfer in the forced convection oven

Figure 2 shows the apparent heat transfer coefficient, h, measured with the aluminium cylinder. Increase of the air velocity and air temperature caused an increase in h. As shown in Eq. (2), h is given by the sum of h_c and h_r. In other words, heat is transferred by convection and radiation. Figure 3 shows

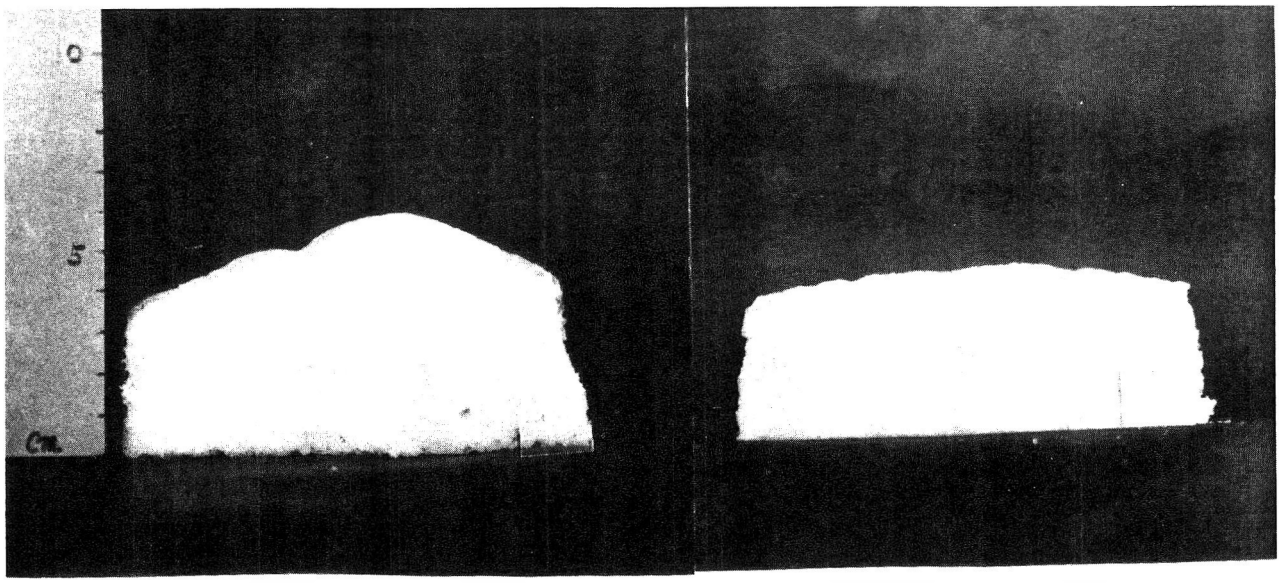

Fig. 10—Profile of sponge cakes baked at different air temperature (Tg) and velocities (v). A—Tg = 200°C, v = 0 m/sec; B—Tg = 160°C, v = 1.5 m/sec.

the convective heat transfer coefficient, h_c, which was obtained by experimental data of h and calculation for h_r using Eq. (2) and (3). The figure showed that the value of h_c was independent of Tg but that it was affected by air velocity, v. It is well known (McAdams, 1954) that the forced convective heat transfer coefficient is correlated by nondimensional groups: $Nu = d\,h_c/\lambda$, $Re = v\,d/\nu$ and Prandtl number, where d is the representative length; λ, the thermal conductivity and ν, the kinematic viscosity of circulating air. In the case of this experiment, however, the Prandtl number was nearly constant. Nusselt and Reynolds numbers are linearly correlated from the data as expressed in Eq. (6).

$$Nu = 0.5\,Re^{0.54} \quad (6)$$

In Fig. 4, the referenced correlations for a cylinder and for an isothermal flat board are also shown for comparison (Richardson, 1960; Pohlhausen, 1921). Thus, in a forced convection oven, the heat transferred to food in the shape of a finite cylinder is calculated from Eq. (1), (2), (3), and (6). An approximate value of the apparent heat transfer coefficient, h, can be determined with Eq. (7).

$$h = 14.8v^{0.54} + 5.0\times10^{-2}Tg + 1.9 \quad (7)$$

where the units are: (kcal/m^{2}hr°C) for h, (m/sec) for v and (°C) for Tg. As shown above, the apparent heat transfer coefficient as a representation of the heating performance of the forced convection oven can be easily measured with a metal block.

Baking food

The relation between baking time and the apparent heat transfer coefficient at different air temperatures is shown in Fig. 5. It was confirmed that the apparent heat transfer coefficient can be used to represent heating performance, because any increase in the apparent heat transfer coefficient shortened the baking time. An increase in air velocity causes an increase in the apparent heat transfer coefficient (Fig. 2). Baking time was affected by temperature and velocity of the circulating air (Fig. 5). The equation to predict the baking time, t, using the apparent heat transfer coefficient, h, and air temperature, Tg, is shown in Eq. (8).

$$t = (4.7\times10^{-3}Tg - 1.2)h - 0.17Tg + 59 \quad (8)$$

where t is the baking time (min) and h, the apparent heat transfer coefficient (kcal/m^{2}hr°C).

From Eq. (7) and (8), baking time, t, can be predicted using air velocity and air temperature with the following Eq. (9).

$$t = (0.071Tg - 18)v^{0.54} + 2.4\times10^{-4}Tg^2 - 0.22Tg + 57 \quad (9)$$

where v is the air velocity (m/sec). The above showed that the time needed to bake sponge cakes could be shortened by increasing air velocity and/or temperature.

Figures 6, 7, and 8, respectively, show the effect of air temperature and velocity on weight loss, height differences, and the degree of browning. Air velocity and temperature had significantly different effects upon the weight loss, height differences and browning of baked sponge cake ($P < 0.05$). Increases in air velocity and/or temperature resulted in a dry sponge cake shaped like a cone with dark surface color. Concerning the firmness of cake (Fig. 9), the only significant difference occurred when air velocity changed ($P<0.05$). Increases in air velocity produced a firmer sponge cake, independent of air temperature. Cohesiveness was not affected by air temperature or velocity (Table 1). Changes in air temperature tended to affect volume more than air velocity (Table 1). Increases in air temperature resulted in a higher cake.

There was no correlation between weight loss and firmness, because firmness was unaffected by changes in air temperature. However, air velocity affected sponge cake weight significantly. A correlation between volume and shape related to height differences was found. Increases in air temperature resulted in a cone shaped cake with great volume, unaffected by air velocity changes.

Sponge cake baking can be controlled by adjustments in air velocity and/or temperature. As for brightness, uniform surface color can be obtained with Tg = 200°C and v = 1.0 m/sec and with Tg = 180°C and v = 1.5 m/sec. The properties of a sponge cake can be further controlled. When a cake with good

—Continued on page 193

Characterization of the Oxygen-17 Nuclear Magnetic Resonance Water Mobility Response Surface

S. J. RICHARDSON, M. P. STEINBERG, R. E. DE VOR and J. W. SUTHERLAND

ABSTRACT

This study uses Factorial design techniques to investigate the Oxygen-17 (^{17}O) Nuclear Magnetic Resonance (NMR) relaxation rate response surface of a corn starch-water system as affected by three experimental conditions: concentration, time between sample preparation (hydration) and measurement and storage temperature. It was found that neither time (between 0.1 and 20 hours) nor the storage temperature (room temperature and low temperature) had a significant effect on the ^{17}O NMR relaxation rate response. The only variable which affected the ^{17}O NMR relaxation rate was the concentration of corn starch in water (10–70%, w/w). Mathematical modeling of the dependence of the ^{17}O NMR response on concentration is discussed in detail.

INTRODUCTION

CHARACTERIZATION of sorbed water and its mobility in food components and systems has been a subject of longstanding investigation (Vail and Bailey, 1940; Bull, 1944; Nemethy and Scheraga, 1962; Eisenberg and Kauzmann, 1969; Labuza, 1975; Leung and Steinberg, 1979; Lang and Steinberg, 1983). One of the most successful and relatively recent techniques for such characterization is Nuclear Magnetic Resonance (NMR) spectrosopy, specifically Oxygen-17 (^{17}O) NMR (Koening et al., 1975; Halle and Wennerstrom, 1981; Halle et al., 1981; Lioutas, 1984; Richardson et al., 1986). ^{17}O NMR transverse relaxation rate (R_2) measurements directly and noninvasively monitor the molecular motions of the water molecule (Richardson et al., 1986), and can thus be used to study the mobility of the states of water in food systems.

As with any analytical technique, the effects of the experimental conditions on the measured response are of extreme importance. The major question of interest here is how do the major experimental variables influence the ^{17}O NMR response in both magnitude and direction. The answer to this question is of utmost importance to the design of future experiments, the control of conditions required by the experiment, the reliability of the data obtained and the scientific interpretation of those data.

A Factorial design is a testing structure which is particularly suited to answer the above question (Lah et al., 1980; DeVor, 1985). Factorial designs are frequently performed and statistically analyzed to measure the effects of one or more independent variables on a response (Box et al., 1978). They allow for the identification of both the magnitude and direction of the effects of the experimental conditions on the desired response in the form of a mathematical model. These modeling techniques allow the investigator to determine, with very few trials, detailed information concerning the nature of the response surface under the experimental conditions of interest (Box et al., 1978). Examples of this are the application of Factorial designs to the development of complicated food formulations (Thalheimer and Rusch, 1970; Chow et al., 1983), as well as, the use of Factorial designs to elucidate the nature of the response surface in Process Optimization studies (Cochran and Cox, 1957; Montgomery, 1976; Lah et al., 1980; Motycka et al., 1984; Oh et al., 1985).

The purpose of this work was to employ Factorial design techniques to characterize the unknown ^{17}O NMR R_2 response surface as affected by three independent experimental variables; concentration, time between sample preparation (hydration) and measurement and temperature of storage.

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MATERIALS & METHODS

Sample analysis and preparation

The corn starch was commercial grade, Argo brand (Best Foods, Inc., Englewood Cliffs, NJ). Moisture content was 8.5% as determined by vacuum oven method (AOAC, 1980) using 60°C and 29.8 in Hg vacuum for 24 hr. Determinations were made in triplicate. Protein content (N × 6.25) was determined by the macro-Kjeldahl method (AOAC, 1980) to be 0.31%.

Thirteen corn starch samples ranging in concentration from 10 to 70%, weight/weight, were prepared by adding the calculated amount of distilled water and hand mixing for 2.5 min. The specific moisture contents studied were statistically selected based on the requirements of the Factorial design (Table 1). The calculated moisture contents were verified by the vacuum oven method.

NMR measurements

A laboratory assembled NSF-250 Multinuclear NMR Spectrometer of the Oldfield and Meadows (1978) design operating at 34 MHz ^{17}O NMR resonance frequency was used. Single pulse experiments were done in triplicate at 20°C upon sample equilibration from storage temperature, which required about 5 min. Pulse width was 45 microseconds, beta delay was 55 microseconds, and recycle time was 0.21 sec. Spectra were stored in a 16K point array which provided adequate resolution.

The ^{17}O NMR transverse relaxation rate (R_2) reponse was determined by measuring the line widths at half-height (υ_{obs}) of each spectrum at each concentration and design condition. To correct for any residual magnetic field inhomogeneity, the net line broadening ($\Delta\upsilon B$) was calculated by subtracting the line width of liquid water (υ_{free})

Table 1—Definition, levels and ^{17}O NMR differential relaxation rate responses for the Central Composite Design in Fig. 1

Natural variables		Coded variables		^{17}O ΔR_2 NMR response	
Conc *g starch* / mole water	Storage time, (hr)	Conc'n	Time	Room Temp[a] (Y_{RT}) storage, sec^{-1}	Low Temp[a] (Y_{LT}) storage, sec^{-1}
4.14	3	−1	−1	59.29	53.74
4.14	17	−1	1	57.18	57.64
23.02	3	1	−1	459.13	466.51
23.02	17	1	1	487.37	463.82
10.76	10	0	0	129.59	130.51
10.76	10	0	0	130.51	133.29
10.76	10	0	0	135.14	128.66
10.76	10	0	0	133.29	131.43
10.76	10	0	0	139.76	135.14
31.81	10	1.414	0	634.43	649.19
2.22	10	−1.414	0	31.08	34.32
10.76	19.9	0	1.414	124.19	127.64
10.76	.1	0	−1.414	130.79	135.77

[a] Y_{RT} = 25 ± 1°C and Y_{LT} = 2.5 ± 1°C

from that of the sample (υ_{obs}). The net or differential relaxation rates (ΔR_2, sec^{-1}) were calculated (± 0.5%) from the following standard net line broadening equation (Dwek, 1973):

$$\Delta R_2\ (sec^{-1}) = \pi \Delta \upsilon_B\ (sec^{-1}) \quad (1)$$

Experimental design

The critical nature of sample preparation and experimental conditions in water binding studies are discussed by D'Arcy and Watt (1981). The three conditions of interest in this study were: (1) concentration of solids, (2) time between sample preparation and ^{17}O NMR measurements, and (3) temperature of storage. The structure of the experimental design was intended to elucidate the effect of measurement time (0.1–20 hr) and temperature of storage (room and low temperature) on the ^{17}O NMR relaxation rate response at various corn starch-water concentrations (10–70%, weight/weight). In order to characterize the surface of the ^{17}O NMR response, a model of degree two or higher is required because of the possible curvature in the true response surface due to time and concentration. In most cases, the second order model:

$$\hat{y} = B_o + \sum_{i=1}^{k} B_i x_i + \sum_{i=1}^{k} B_{ii} x^2_i + \sum\sum_{i<j} B_{ij} x_i x_j \quad (2)$$

provides an adequate approximation (Montgomery, 1976). A preferred Factorial design for fitting such a second-order model is the Central Composite Design (CCD). These designs consist of a 2^k factorial (or fractional factorial) block augmented by 2k axial points and n_o center points. The CCD can be made rotatable by the choice of the axial point, α, where $\alpha = (F)^{1/4}$, (F is the number of points used in the factorial portion of the design); it can be made orthogonal or uniform precision by the choice of the number of n_o (Montgomery, 1976).

In this study k = 2; concentration (x_1) and time (x_2), F = 4, α = 1.414 and n_o = 5 (for uniform precision). The CCD for k = 2 is shown in Fig. 1, and the design in Table 1. The CCD was performed for both room temperature (Y_{RT}) (25 ± 1°C) and low temperature (Y_{LT}) (2.5 ± 1.0°C) conditions. The order in which each trial was performed was randomized (Steel and Torrie, 1980). CCD analysis of the quadratic regression was done by a computer (Sutherland, 1985).

RESULTS & DISCUSSION

TWO STATISTICAL ANALYSES were employed to evaluate the Factorial design results: (1) fit analysis of the second order CCD model for the concentration and time variables under each storage condition, separately, and (2) paired t-test analysis for the two storage conditions.

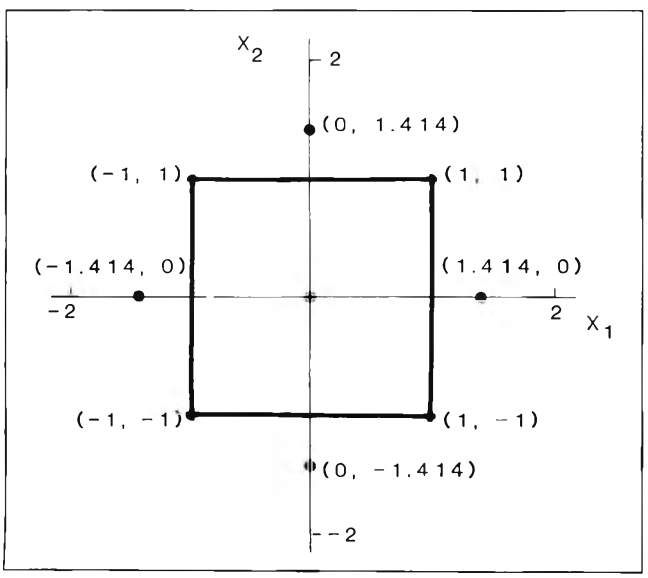

Fig. 1—Central composite design (CCD) display for two variables (k = 2): concentration (x_1) and time (x_2). CCD coordinates correspond to the coded variables defined in Table 1.

The second order model [Eq. (2)] was employed with the data from the CCD to estimate the true response surface of the ^{17}O NMR differential relaxation rate results for each temperature condition. For k = 2, Eq. (2) becomes:

$$\hat{y} = B_0 + B_1x_1 + B_2x_2 + B_3x_1x_2 + B_4x_1^2 + B_5 \quad (3)$$

where x_1 is concentration, x_2 is time between sample preparation and NMR measurement and B_0, B_1, B_2, B_3, B_4, and B_5 are the regression coefficients. This model allows evaluation of the effect of concentration and of time between sample preparation and ^{17}O NMR measurement under each temperature condition.

The results of the two trials to be fitted (room temperature and low temperature) are shown in Table 1. The regression coefficients (Table 2) were tested for significance with a 95% confidence interval (Steel and Torrie, 1980). For both temperature conditions only B_0, B_1, and B_4 regression coefficients were significant. This indicates that the only statistically significant experimental variable was concentration and that time from 0.1–20 hr had no effect on the ^{17}O NMR response.

The second order polynomial approximation of the true but unknown response surface employing all six (B_0 through B_5) of the regression coefficients (Table 2) was used to develop contour plots of the predicted response surface over the range of variables tested. The contour plots for the room temperature and low temperature conditions are shown in Fig. 2 and 3, respectively. Each plot shows the ^{17}O NMR differential relaxation rate response as a function of concentration and time. These full second order model plots visually reflect the trends seen in the regression coefficients that the ^{17}O NMR response is affected only by the concentration and not by the time between sample preparation and instrument measurement.

The Analysis of Variance (ANOVA) for each temperature condition (Table 3 for room-and Table 4 for low-temperature) show that although the full second order model [Eq. (3)] was

Table 2—Regression coefficients and 95% confidence intervals (CI) for room and low temperature storage conditions

	Room temperature		Low temperature	
Parameter	Estimated coefficient	± CI	Estimated coefficient	± CI
B_0	133.65	±18.00	131.80	±13.04
B_1	210.46	±14.23	211.07	±10.31
B_2	2.13	±14.23	−1.29	±10.31
B_3	7.66	± 7.54	−1.65	±14.58
B_4	108.50	±15.26	110.93	±11.06
B_5	5.84	±15.26	5.87	±11.06

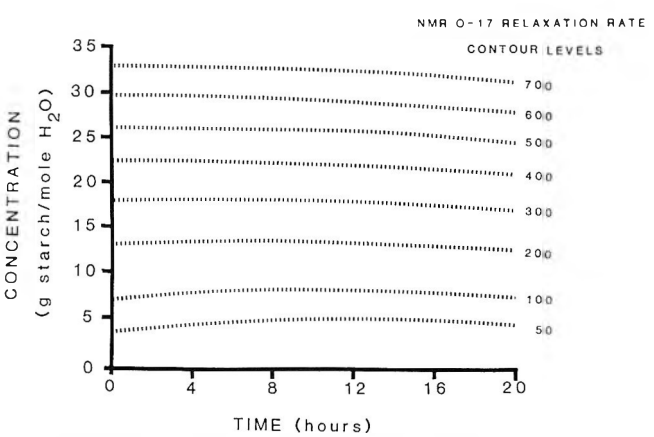

Fig. 2—Response surface contours for ^{17}O NMR relaxation rate response for room temperature conditions in the regions tested as a function of concentration and time. Levels of variables correspond to the natural variables given in Table 1.

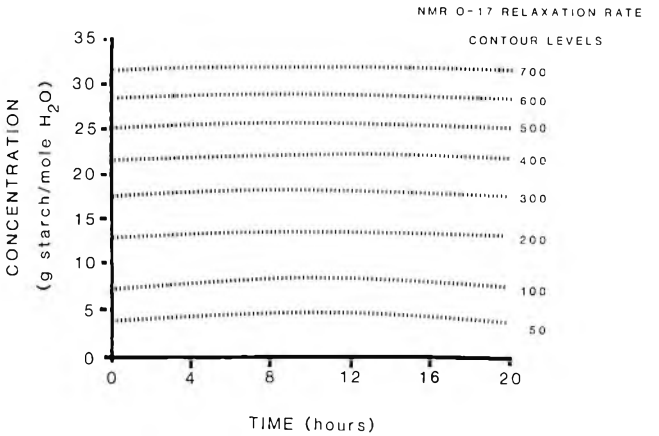

Fig. 3—Response surface contours for ^{17}O NMR relaxation rate response for low temperature conditions in the regions tested as a function of concentration and time. Levels of variables correspond to the natural variables given in Table 1.

Table 3—Analysis of variance for the room temperature condition under the full second order model [Eq. (3)]

Source	df	Sums of squares	Mean squares	F
Model	6	977,920.49	162,986.75	402.51[a]
Residual	7	2,834.51	404.93	
• Lack of fit	3	2,768.49	922.83	55.90[a]
• Pure error	4	66.02	16.51	
Total	13	980,755.00		

[a] Significant at the 1% level. R^2 = 0.99711; R^2corr = 0.99355.

Table 4—Analysis of variance for the low temperature storage condition under the full second order model [Eq. (3)]

Source	df	Sums of squares	Mean squares	F
Model	6	981,727.99	163,621.33	770.24[a]
Residual	7	1,487.01	212.43	
• Lack of fit	3	1,461.98	487.33	77.85[a]
• Pure error	4	25.03	6.26	
Total	13	983,215.00		

[a] Significant at the 1% level. R^2 = 0.99849; R^2corr = 0.99665.

highly significant (F = 402.51), there was a large lack of fit (F = 55.90) for the model under both storage temperature conditions.

Thus, the key result of this first statistical analysis was that the ^{17}O NMR transverse relaxation rate response was independent of the time variable under the range tested in this experiment, under each storage condition. Therefore, the true response surface data can be used to determine whether the effect of concentration on the ^{17}O NMR response can be modeled.

Since only regression coefficients B_0, B_1 and B_4 were found to be significant (Table 2) the full second-order model [Eq. (3)] reduces to:

$$\hat{y} = B_0 + B_1x_1 + B_4x_1^2 \quad (4)$$

for each temperature condition. In order to determine the suitability of this model to describe the behavior of the ^{17}O NMR relaxation response against concentration, a least squares fit was done on a computer (Sutherland, 1985). The ANOVA for each temperature condition is given in Tables 5 and 6. The model for room temperature ^{17}O NMR response accounted for 0.99659 of the total variation (Table 5). The R^2 corrected value, which is calculated as ((Model SS - Mean SS)/(Total SS - Mean SS)) and describes the actual variation accounted for by the model when corrected for the mean, was 0.99240 (Table 5). The model for low temperature ^{17}O NMR response accounted for 0.99822 of the total variation and had an R^2 corrected value of 0.99606 (Table 6). The ANOVA (Tables 5 and 6) indicated that although the second order model [Eq. (4)] was highly significant, a large lack of fit was evident for the model under each temperature condition.

Table 5—Analysis of variance for the room temperature storage condition under the second order model [Eq. (4)]

Source	df	Sums of squares	Mean squares	F
Model	3	977,417.80	325,805.93	974.82[a]
Residual	10	3,342.20	334.22	
• Lack of fit	4	3,200.05	800.01	33.77[a]
• Pure error	6	142.15	23.69	
Total	13	980,076.00		

[a] Significant at the 1% level. R^2 = 0.99659; R^2corr = 0.99240.

Table 6—Analysis of variance for the low temperature storage condition under the second order model [Eq. (4)]

Source	df	Sums of squares	Mean squares	F
Model	3	981,469.30	327,156.43	1,868.72[a]
Residual	10	1,750.70	175.07	
• Lack of fit	4	1,693.40	423.35	44.33[a]
• Pure error	6	57.30	9.55	
Total	13	983,220.00		

[a] Significant at the 1% level. R^2 = 0.99822; R^2corr = 0.99606.

Table 7—Analysis of variance for the room temperature storage condition under the log transformation model [Eq. (5)]

Source	df	Sums of squares	Mean squares	F
Model	3	329.06267	109.68756	10,222.513[a]
Residual	10	0.10733	0.01073	
• Lack of fit	4	0.09909	0.02477	18.080[a]
• Pure error	6	0.00824	0.00137	
Total	13	329.17000		

[a] Significant at the 1% level. R^2 = 0.99967; R^2corr = 0.98819.

Table 8—Analysis of variance for the low temperature storage condition under the log transformation model [Eq. (5)]

Source	df	Sums of squares	Mean squares	F
Model	3	328.90759	109.63586	20,883.021[a]
Residual	10	0.05253	0.00525	
• Lack of fit	4	0.04916	0.01229	21.946[a]
• Pure error	6	0.00337	0.00056	
Total	13	328.96012		

[a] Significant at the 1% level. R^2 = 0.99984; R^2corr = 0.99413.

In order to investigate the cause of this large lack of fit, analysis of the residuals (observed response − predicted response) associated with the model [Eq. (4)] was done. The assumptions underlying the statistical model expressed by Eq. (4) are that the errors associated with the true response (Y) are normally and independently distributed with constant variance (Box et al., 1978). However, when the residuals were plotted against the predicted response (not shown) it was revealed that the experimental errors associated with the ^{17}O NMR R_2 response were larger at both the lower and higher concentrations than at the intermediate concentrations. In an effort to stabilize the variance of this model, a log transformation was applied to the model [Eq. (4)] (Box et al., 1978):

$$\ln\hat{y} = B_0 + B_1x_1 + B_4x_1^2 \quad (5)$$

The log transformation did decrease the lack of fit (Tables 7 and 8); however, the F ratios were still significant at the 1% level. Analysis of the residuals still revealed that the experimental errors associated with the ^{17}O NMR R_2 response were larger at the low and high concentrations than at the intermediate concentrations.

The experimentally observed ^{17}O NMR differential relaxation rate response and the second order and log transformation predicted responses plotted against starch concentration for the room and low temperature storage conditions are shown in Fig.

4 and 5, respectively. One can visually observe the location of the lack of fit in the second order (Equation 4) and log tranformation [Eq. (5)] models. The lack of fit in the second order model is most apparent at the lower starch concentrations, whereas the log transformation model fits better at lower concentrations but over estimates the response at higher concentrations.

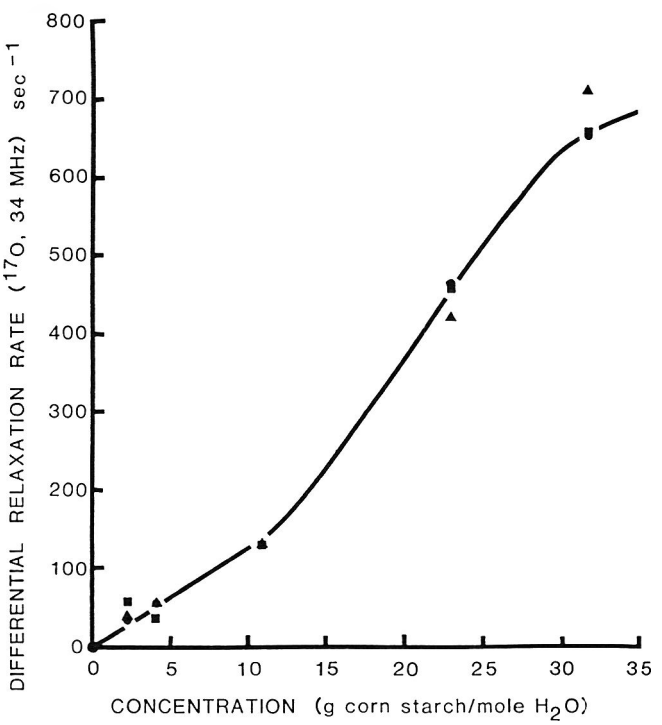

Fig. 4—Comparison of the experimentally observed ^{17}O NMR differential relaxation rate response (●) and the second order model (■) and log transformation model (▲) predicted responses plotted against starch concentration for the room temperature storage condition.

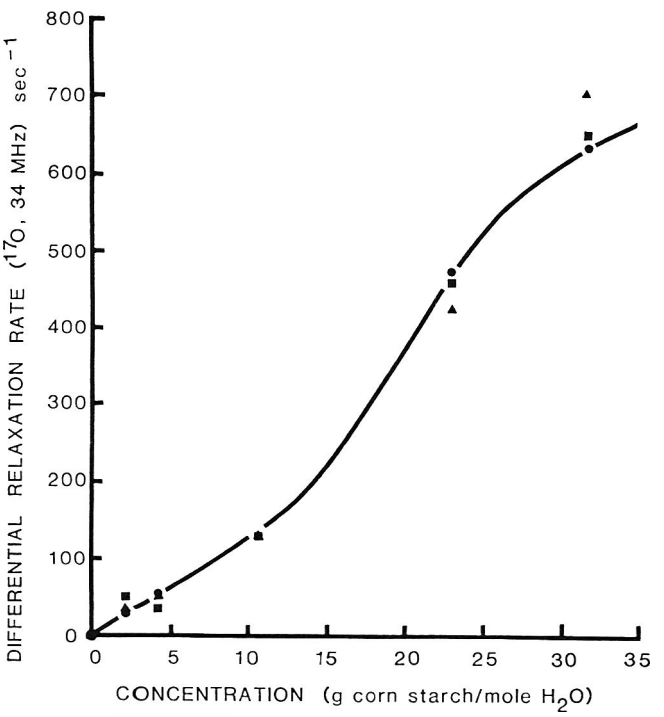

Fig. 5—Comparison of the experimentally observed ^{17}O NMR differential relaxation rate response (●) and the second order model (■) and log transformation model (▲) predicted responses plotted against starch concentration for the low temperature storage condition.

It must be noted that a large portion of the residual lack of fit of the two models may be attributable to the estimate of pure error used in the models. The pure error in the ANOVA Tables (Tables 3, 4, 5, 6, 7 and 8) reflect the pure error associated with the replicated center point of the CCD design, n_0. As discussed, the experimental errors associated with the ^{17}O NMR relaxation rate response are larger at both the lower and higher concentrations than at the intermediate concentrations (at n_0). Thus, if the pure error would be estimated from the sum of squares of the difference between the individual response and the average over the entire concentration range, the lack of fit would decrease significantly for both the second order and log transformation models.

This lack of fit in the second order and log transformation models indicates that, even though these mathematical models were able to account for a significant portion of the variability in the data, further refinement of the model should be possible, either by taking into account additional unidentified variables and/or by obtaining a better estimate of the pure error, as previously discussed.

Recently a mechanistic model for the interpretation of the dependence of the NMR relaxation rates on concentration has been investigated (Kumosinski and Pessen, 1982; Richardson et al. 1986). The Kumosinski model based on charge repulsion or charge fluctuation effects attempts to physically account for the non-linearity of the concentration dependence of the NMR relaxation rates (Kumosinski and Pessen, 1982). The theory and application of this mechanistic model to the corn starch-water system is currently being investigated.

Paired t-test

The paired t-test was employed to determine whether there was a statistically significant difference at the 95% confidence level between the ^{17}O NMR differential transverse relaxation rate (ΔR_2) response under the two storage temperature conditions. The paired t-test analysis, done with a computer (Sutherland, 1985), yielded a calculated t-value equal to -0.147 (Null hypothesis: $\mu_{LT} - \mu_R = 0$; alternative hypothesis $\mu_{LT} - \mu_{RT} \neq 0$); with a confidence interval of -5.815 to 5.079 for the true mean. From the standard t-tables (Steel and Torrie, 1980) $t_{0.05,_{12}}$ equals 0.443; therefore, we fail to reject the null hypothesis at the 5% significance level and conclude that there is no strong evidence to suggest that the true means of the room and low temperature storage conditions differ. Therefore, we concluded that there is no significant difference between the room and low storage temperature ^{17}O NMR relaxation responses for the 0.1–20 hr interval tested.

CONCLUSIONS

UNDERSTANDING what conditions affect the ^{17}O NMR relaxation rate both in magnitude and direction is critical to the design and interpretation of future ^{17}O NMR experiments. The results of this study showed that the ^{17}O NMR relaxation rate response was not affected by the experimental conditions: (1) time between sample hydration and measurement, and (2) storage temperature. However, it was affected by corn starch concentration, i.e., moisture content.

Further studies are required to determine whether the other food constituents, such as proteins and sugars, would be affected in the same way by these variables.

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Ms. received 4/7/86; revised 7/28/86; accepted 8/12/86.

Contribution of Project No. 50-0340 of the Agricultural Experiment Station, Univ. of Illinois at Urbana-Champaign. S.J.R. was the recipient of a Hunter-Hacket-Wright Graduate Scholarship and a Lipton IFT Graduate Scholarship.

APPARENT HEAT TRANSFER IN THE OVEN. . .From page 188

texture is desired and shape is not important, the cake should be baked with a low air velocity and a high temperature (Fig. 10A). When a flat cake suitable for decoration as shown in Fig. 10B is desired, baking should be done at a high air velocity and a low temperature.

CONCLUSION

THE HEATING PERFORMANCE of the forced convection oven could be expressed by the apparent heat transfer coefficient.

The time needed to bake sponge cakes could be reduced by increasing air velocity and/or air temperature; both factors affect the apparent heat transfer coefficient.

There was a strong relation between baked sponge cake characteristics and changes in air velocity or air temperature. Sponge cake with good texture but of variable shape can be baked with a low air velocity and a high temperature. Flat cakes suitable for decoration should be baked with a high air velocity and a low temperature.

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Ms received 10/21/85; revised 9/23/86; accepted 9/23/86.

The authors are grateful to Professor Y. Iida of Yokohama National Univ. for his discussion on the heat transfer.

Isolation of a Caffeine-Resistant Mutant of *Aspergillus parasiticus*

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ABSTRACT

A caffeine-resistant mutant of *Aspergillus parasiticus* NRRL 2999 was isolated and subsequently designated strain BCR1. The mutant strain grew in the presence of > 8 mg/mL caffeine, while growth of the parent strain was delayed by 1 mg/mL and inhibited by 2 mg/mL. Strain BCR1 produced abundant amounts of aflatoxin only when cultured in media containing caffeine. Residual caffeine analyses indicated that caffeine-resistance in BCR1 was not due to the metabolic elimination of caffeine.

INTRODUCTION

PREVIOUS INVESTIGATIONS have demonstrated that caffeine (1,3,7-trimethylxanthine) inhibits growth and polyketide mycotoxin production by a variety *Aspergillus* and *Penicillium* species (Buchanan and Fletcher, 1978; Nartowicz et al., 1979; Lenovich, 1981; Buchanan et al., 1982, 1983a, 1983b; Buchanan and Lewis, 1984a). Buchanan et al. (1983b) suggested that the inhibition of growth and aflatoxin production may involve two separate mechanisms. Subsequent investigations (Buchanan and Lewis, 1984a) suggested that the inhibition of aflatoxin production may involve an inhibition of carbohydrate uptake and/or utilization, though the specific loci affected by caffeine were not identified. The objective of the current study was to develop a caffeine-resistant mutant of *Aspergillus parasiticus* that could be used to further elucidate how this naturally occurring compound prevented the formation of aflatoxin. Further impetus for this work was provided by the recent study with green coffee beans by Tsubouchi et al. (1985) who reported the isolation of *Aspergillus ochraceus* strains that grew and produced ochratoxin A in the presence of elevated levels of caffeine.

MATERIALS & METHODS

Microorganism

Aspergillus parasiticus NRRL 2999 was employed as the parent strain. The mold was maintained on Potato Dextrose Agar (PDA) (Difco) slants stored at 4°C. Spore suspensions were prepared as described previously (Tice and Buchanan, 1982) and diluted to contain 10^6 conidia/mL.

Media

Yeast Extract-Sucrose (YES) (Davis et al., 1966), Peptone-Mineral Salts (PMS) (Buchanan and Lewis, 1984b), and Yeast Extract-Glucose (YEG) (Buchanan and Lewis, 1984a) media were prepared as described previously. Plating media were prepared by adding agar at 20 g/L to the respective liquid media. Caffeine was added to the media prior to sterilization by autoclaving (15 min at 15 psi).

Isolation of mutants

Caffeine-resistant mutants were isolated using YES agar containing 4 mg/mL caffeine. Plates were inoculated with 0.5 mL (5×10^5 conidia) of a spore suspension of *A. parasiticus* NRRL 2999 and incubated at 37°C for up to 10 days. The plates were examined daily for rapidly growing colonies, which were subsequently subcultured and maintained on PDA + 4 mg/mL caffeine slants.

Assessment of caffeine resistance

The effects of caffeine on growth and aflatoxin production by the parent and caffeine-resistant strains were assessed using 125-mL flasks containing 25 mL of YES with 0, 1, 2, 4, or 8 mg/mL caffiene. The cultures were inoculated with 0.5 mL of spore suspension to achieve an inoculum of approximately 2×10^4 conidia/mL. Cultures were incubated at 28°C with or without agitation (0 vs 150 rpm), and analyzed for pH, aflatoxin production, residual caffeine and mycelium dry weight after 3, 6, and 9 days.

The effect of caffeine on aflatoxin production was also evaluated using a modification of the replacement culture technique of Buchanan and Lewis (1984b). YES medium (300 mL in 1000-mL flasks) was inoculated with 2 mL of spore suspension to achieve an inoculum of 6.7×10^3 conidia/mL. Cultures were incubated for 72 hr at 28°C on a rotary shaker (150 rpm). The mycelial pellets were harvested on cheesecloth and subsequently disrupted in a blender. The mycelia were then transferred in 10g portions (wet weight) to 1000 mL flasks containing 300 mL of PMS and incubated for 24 hr at 28°C and 150 rpm. The mycelia were then reharvested on cheesecloth and transferred in 2.0g portions (wet weight) to 50-mL flasks containing 20 mL of YEG with 0, 1, 2, 4 and 8 mg/mL caffeine. After initial mixing, these cultures were incubated at 28°C without agitation for 72 hr and then analyzed for pH, aflatoxin production, residual caffeine concentration and mycelial dry weight.

Analyses

Aflatoxins were extracted, separated by thin-layer chromatography, and quantitated by fluorodensitometry as described previously (Buchanan et al., 1985). Mycelium dry weights were determined gravimetrically after drying the extracted mycelia for 18 hr at 85°C.

Residual caffeine concentrations were determined by high performance liquid chromatographic analysis of medium samples. After clarifying the samples with 0.45 μm filter and diluting with water to achieve an approximate caffeine concentration of 0.02 mg/mL, caffeine was separated on a reversed phase column (Altex Ultrasphere ODS-C_{18}, 4.6 × 250 mm). Elution was carried out isocratically with 1% acetic acid + 12% acetonitrile in water at a flow rate of 1 mL/min. Caffeine was detected by monitoring UV absorbance at 254 nm and quantitated by comparison of peak heights against external caffeine standards. Preliminary studies were performed to assure that there were no interfering fungal metabolites or medium components.

Estimation of sporulation

The quantitative effect of caffeine on sporulation was estimated by inoculating YES agar plates containing various levels of caffeine with 2.5×10^4 conidia and incubating the plates at 28°C. After 3, 7, 10, and 14 days, triplicate plates of each caffeine concentration were flooded three times with 3 ml water + Tween 80 (20 μl/mL), with the conidia being gently dislodged with an inoculating loop. The spore suspensions were brought up to 15 mL, and the conidia enumerated by direct microscopic examination using a counting chamber.

RESULTS

INCUBATION of 172 YES + 4 mg/mL caffeine agar plates (each inoculated with 5×10^5 conidia) yielded six rapidly growing colonies. Subsequent passage on YES agar with and without caffeine confirmed that these rapidly growing isolates had stable caffeine-resistance phenotypes. One isolate (designated strain BCR1) was selected for further characterization.

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BCR1 sporulated poorly ($< 2 \times 10^5$ conidia/plate) on YES agar, but there was a dose-related increase in sporulation to moderate levels ($> 10^7$ conidia/plate) on YES agar supplemented with up to 4 mg/ml caffeine. Further increases in the caffeine concentration of YES agar resulted in decreased levels of sporulation. Good sporulation ($>10^9$ condia/plate) occurred on PDA with or without caffeine supplementation. Stock cultures of BCR1 were subsequently grown and maintained on PDA + 4 mg/mL caffeine as a precaution against reversion.

The effects of caffeine on growth and aflatoxin production by non-agitated, conidia-initiated YES cultures of NRRL 2999 and BCR1 are presented in Fig 1. Caffeine affected the parent strain as described previously (Buchanan and Fletcher, 1978; Buchanan et al., 1983b) with 1 mg/mL delaying and 2 mg/mL inhibiting growth and aflatoxin formation. The mutant strain grew in the presence of 8 mg/mL caffeine, though the growth rate decreased with increasing caffeine concentration. The extent of growth was equivalent at caffeine levels $\leq$ 4 mg/mL, but was depressed at 8 mg/mL. Subsequent studies (not shown) indicated that BCR1 grew slowly in media saturated with caffeine. Aflatoxin production by BCR1 was caffeine-dependent. Little aflatoxin production occurred in the absence of caffeine, while the cultures supplemented with 4 mg/mL caffeine produced aflatoxins at levels roughly equivalent to those observed with the parent strain in the absence of caffeine. Similar studies performed with agitated cultures (data not shown) indicated that aeration did not influence the strains' responses to caffeine. BCR1 produced the greatest amount of aflatoxin when cultured in YES adjusted to an initial pH of 6.5 (Table 1); however, changes in initial pH did not greatly affect the overall caffeine-dependent nature of aflatoxin production by the isolate.

The effects of caffeine on aflatoxin production by replacement cultures of *A. parasiticus* NRRL 2999 and BCR1 were also studied to assess toxin formation more directly by minimizing differentials in the rate and extent of growth (Buchanan and Lewis, 1984a, b). After sequential culturing in YES and PMS, 2.0g portions of the pregrown, previously disrupted mycelia (equivalent to 150–200 mg dry weight) were transferred to YEG medium and evaluated at 72 hr for toxin production (Table 2). The parent strain (NRRL 2999) produced abundant amounts of aflatoxins when incubated in YEG without caffeine. Toxin formation was strongly depressed by 1 mg/mL caffeine, and only low levels of toxin production were observed in cultures containing $\geq$ 2 mg/mL caffeine. Caffeine also prevented the approximate doubling in dry weight observed in the replacement cultures without caffeine. The observed effects of caffeine on aflatoxin production by replacement cultures of NRRL 2999 were similar to those observed with conidia-initiated cultures (Fig. 1) and agree with previous work employing replacement cultures (Buchanan and Lewis, 1984a).

The replacement cultures of strain BCR1 displayed equivalent increases in dry weight with caffeine levels up to 4 mg/mL but were inhibited by 8 mg/mL. Aflatoxin production was dependent on caffeine, with maximal production occurring in the cultures containing 2–4 mg/mL. However, unlike the conidia-initiated cultures, the caffeine-supplemented replacement cultures of BCR1 did not achieve a level of aflatoxin production equivalent to that attained by the parent strain in the absence of caffeine. The reason for this difference is not known; however, since the replacement culture technique involves growing the mold initially in caffeine-free media prior to transferring the mycelia to caffeine-supplemented replacement medium, this suggests that caffeine may affect aflatoxin synthesis in BCR1 by altering the mold's primary metabolism. Further experimentation is currently underway to evaluate this hypothesis.

The residual caffeine content of conidia-initiated YES cultures containing various initial levels of caffeine was monitored to determine if caffeine-resistance in BCR1 was due to the

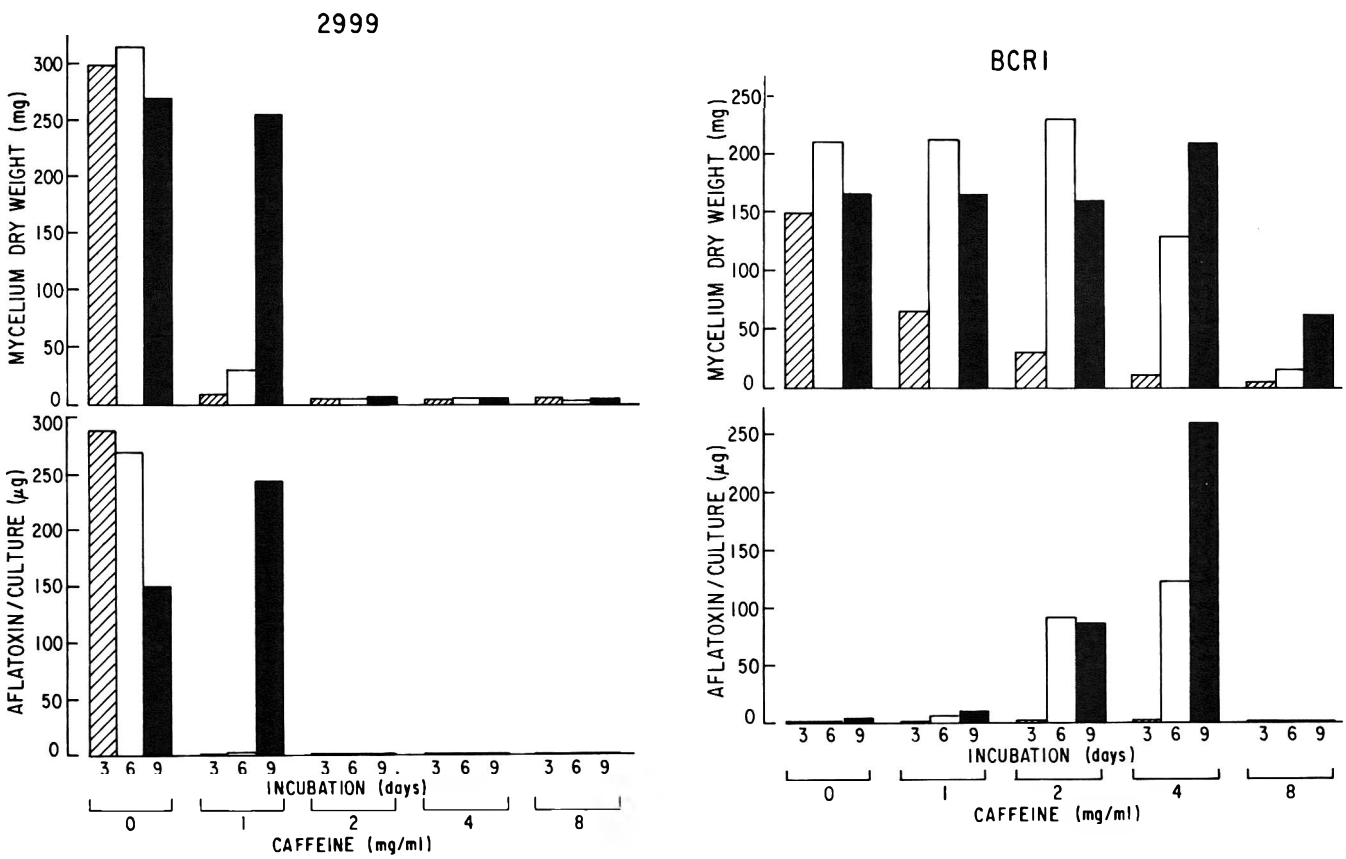

Fig. 1—Effect of caffeine on growth and aflatoxin production by Conidia-initiated YES cultures of Aspergillus parasiticus *NRRL 2999 (left) and BCR1 (right).*

Table 1—Effect of initial pH on growth and aflatoxin production by A. parasiticus *BCR1 in conidia-initial YES cultures containing 0 and 4 mg/mL caffeine*

Caffeine conc. (mg/mL)	Initial pH	Incubation time (days)	Mycelium dry wt (mg)[a]	pH[a]	Aflatoxin culture (μg)[a]	Aflatoxin mycelium (ng/mg)
0	4.5	5	199(30)	7.4	0.0	0
		11	139(1)	8.2	0.0	0
	5.5	5	211(5)	7.1	0.0	0
		11	134(2)	8.3	0.0	0
	6.5	5	217(3)	6.9	0.0	0
		11	135(3)	8.3	2.9(0.6)	21
	7.5	5	209(4)	6.7	0.3(0.3)	1
		11	132(3)	8.1	1.7(0.5)	13
4	4.5	5	48(5)	4.6	0.3(0.1)	63
		11	143(3)	8.1	41.0(5.6)	287
	5.5	5	70(4)	5.2	3.6(0.8)	51
		11	147(7)	8.3	62.2(13.5)	423
	6.5	5	71(8)	5.6	46.6(10.3)	656
		11	140(2)	8.2	90.3(1.3)	644
	7.5	5	33(4)	6.3	6.1(1.9)	185
		11	132(2)	8.1	71.7(6.3)	543

[a] $\bar{X}$(± SEM), n = 3 replicate cultures.

Table 2—Effect of caffeine on the production of aflatoxin by replacement cultures of A. parasiticus *NRRL 299 and BCR1*[a]

Strain	Caffeine conc (mg/mL)	Mycelium dry wt (mg[b])	pH[b]	Aflatoxin culture (μg)[b]	Aflatoxin mycelium (ng/mg)[b]
NRRL	0	428(11)	4.6	712.8(53.0)	1669(136)
2999	1	350(7)	5.0	15.9(0.6)	44(3)
	2	213(4)	6.0	2.1(0.1)	11(1)
	4	192(5)	6.1	2.6(0.3)	14(2)
	8	178(2)	6.2	1.9(0.1)	11(1)
BCR1	0	382(6)	4.9	6.4(1.3)	17(3)
	1	380(4)	5.2	5.9(1.6)	16(4)
	2	376(16)	5.2	27.4(1.8)	73(5)
	4	377(19)	5.3	21.2(3.1)	57(10)
	8	194(9)	6.0	0.0	0

[a] Sequentially cultured in YES and PMS and then transferred to YEG and analyzed after 72 hr of post-transfer incubation.
[b] $\bar{X}$(± SEM), n = 4 replicate cultures.

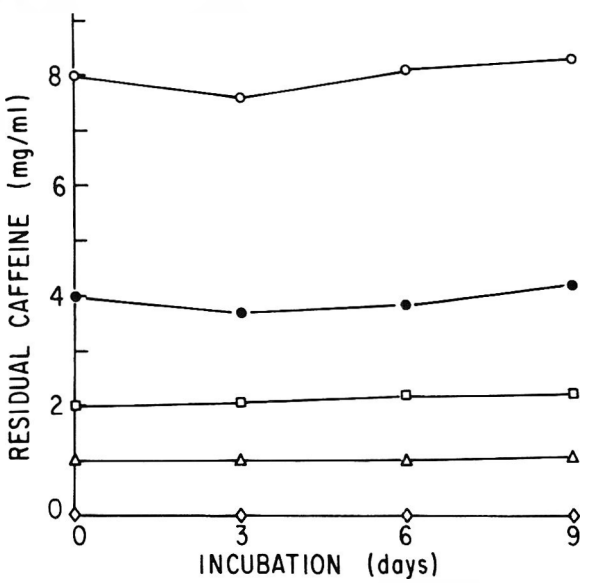

Fig. 2—Residual caffeine content of Conidia-initiated YES cultures of A. parasiticus *BCR1 having an initial caffeine content of 0 (♦), 1 (△), 2 (□), 4 (●), and 8 (○), mg/mL.*

strain having acquired the ability to metabolize caffeine (Fig. 2). Caffeine levels in the medium remained constant over the course of the nine day incubation, even though the mold actively grew at all caffeine concentrations. Similar results (not shown) were obtained with replacement cultures of BCR1 and NRRL 2999 having initial caffeine concentrations of 4 mg/mL, indicating that neither the parent nor mutant strain were capable of metabolizing caffeine. It appeared that caffeine-resistance in BCR1 was not due to detoxification of caffeine.

DISCUSSION

THE PRESENT STUDY demonstrated the possibility of isolating variants of *A. parasiticus* that were able to grow and produce aflatoxins at caffeine concentrations that normally inhibited the mold. In some ways, the caffeine-resistant strain isolated in the current study is similar to the caffeine–resistant strains of *A. ochraceus* reported by Tsubouchi et al. (1985). For example, neither species produced large amounts of their respective mycotoxins unless cultured with a caffeine supplement. However, unlike *A. ochraceus* which completely depleted added caffeine, caffeine-resistance in *A. parasiticus* BCR1 was not associated with metabolic elimination of the compound. The caffeine-dependent nature of both aflatoxin production and sporulation in BCR1 suggested that the mutation involved a bioregulatory locus that influenced multiple developmental processes within the mold.

The mutants isolated in the current study were obtained without employing induced mutagenesis at a mutation rate of 7×10^{-8}. This ready isolation of caffeine-resistant variants suggested that naturally-occurring resistant strains of *A. parasiticus* (and probably *Aspergillus flavus*) were likely to exist in conjunction with caffeine-containing commodities. Confirmation of this supposition will require examination of isolates from commodities such as coffee, tea, and cocoa beans to assess caffeine-resistance and mycotoxicogenesis. The results of the current study indicated further that in any assessment of mycotoxicogenesis of molds isolated from materials containing a naturally-occurring inhibitor, mycotoxin production should be determined both in the presence and absence of the inhibitory compound. Strain BCR1 would have been classified as only a low-level aflatoxin producer if it had been evaluated only in media without caffeine.

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Ms received 5/23/86; revised 9/8/86; accepted 9/12/86.

Characterization of Solution Properties of Four Iron Sources in Model Systems by Solubility Studies and IR/VIS Reflectance Spectrophotometry

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ABSTRACT

The hydrolysis of iron and the effect of aging on the resolubilization of iron hydroxides under simulated gastric pH conditions were measured and spectroscopy was evaluated as a predictor of these changes. Model systems of ferrous sulfate ($FeSO_4$), ferric sulfate ($Fe_2(SO_4)_3$), ferric chloride ($FeCl_3$) and hydrogen-reduced elemental iron (HRFe), with and without ascorbic acid, were analyzed at intervals over 10 wk storage for both soluble iron and iron resolubilized from insoluble iron hydroxide polymers. Irreversible hydrolysis, resulting in polymers with ordered structures, occurred in the $FeSO_4$ and HRFe systems. Spectra of air-dried precipitates correlated with solution behavior of iron providing a possible predictive technique. Added ascorbic acid increased the levels of soluble iron in all systems.

INTRODUCTION

THE HYDROLYSIS of iron has a profound effect on the solution properties and physicochemical behavior of iron in food systems (Spiro and Saltman, 1969). This hydrolysis has been well documented from the last century to the present (Bineau, 1879; Weiser, 1935; Arden, 1950, 1951; Biedermann and Schindler, 1957; Bernal et al, 1959). Hydroxides precipitate from solutions of Fe^{2+} above pH 5.5 (Mellor, 1935; Britton, 1925) and from Fe^{3+} above pH 2-3 (Arden, 1951; Hedstrom, 1952). Hydrolysis proceeds from free ions in solution to various iron oxides along the pathways shown in Fig. 1. As can be seen, alkalinization, oxidation and heating all drive the reactants toward dehydrated polymers with increasingly ordered structures. In solutions at room temperature iron hydroxide gels undergo hardening over time as a result of this increasingly ordered structure (Gheith, 1952; Weiser, 1935). This hardening corresponds to the emergence of various crystalline mineral forms which have been identified by such techniques as differential thermal analysis (Mackenzie, 1952, 1957; Gheith, 1952), electron microscopy (Joekes et al., 1981; Mackenzie and Meldau, 1959; Sato et al., 1969), X-ray diffraction (Mackenzie and Meldau, 1959; Biedermann and Schindler, 1957) and UV/Vis/IR spectrophotometry (Mackenzie and Meldau, 1959; Nobuoka, 1965; Sato et al., 1969; Morris et al., 1985). As well, the transformations and chemical nature of iron hydroxide polymers can be followed by these techniques.

The aged iron hydroxide gels are increasingly resistant to acid attack (Weiser, 1935; Biedermann and Schindler, 1957; Spiro et al, 1966; Sommer and Margerum, 1970; Sommer et al., 1973). This decrease in reactivity may explain the lower bioavailability of these compounds as compared to iron salts and endogenous iron in foods (Fritz et al., 1970; McNaughton et al., 1976; Derman et al., 1977). Relative solubilities of iron have been shown to correlate with relative biological value (RBV) of iron sources (Rao and Prabhavathti, 1978; Smith, 1983). Therefore, the RBV can be affected by resolubilization properties which may be amenable to monitoring.

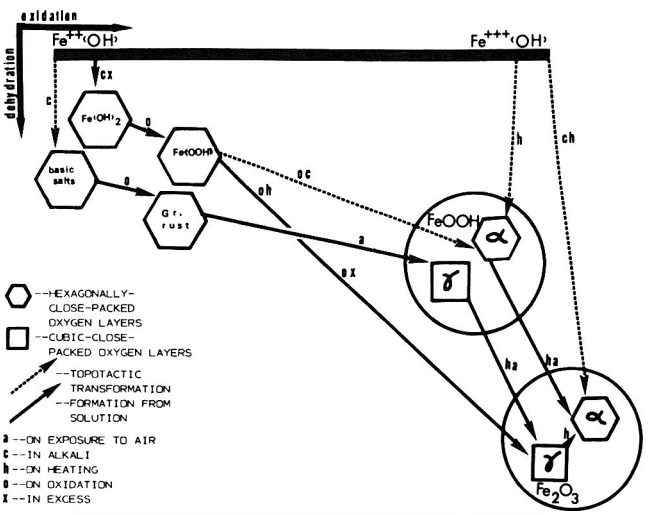

Fig. 1—Structural transformations in the iron oxide hydroxide system. Adapted from Bernal et al. (1959).

Some chelating agents may bind iron and prevent hydrolysis or minimize its effects (Chaberek and Martell, 1959). Much attention has been given to ascorbic acid (AA) due to the remarkable increase in iron absorption noted when it is consumed with iron-containing meals (Forth and Rummel, 1975; Hallberg, 1981; Clydesdale, 1982, 1983).

Because of its relationship to bioavailability, this study focused on the degree of hydrolysis of different iron sources in model systems, with and without ascorbic acid, and the reversibility of hydrolysis under simulated gastric pH conditions. Visible/infrared spectrophotometry was also evaluated as a possible predictor of both iron hydrolysis and the potential of the hydrolyzed iron to be resolubilized at gastric pH.

MATERIALS & METHODS

Iron sources

Ferrous sulfate ($FeSO_4.7H_2O$, certified ACS), ferric sulfate ($Fe_2(SO_4)_3.H_2O$), and ferric chloride ($FeCl_3$, certified ACS) were obtained from Fisher Scientific Co., NJ, and hydrogen-reduced elemental iron (HRFe–97% less than 44 microns particle size) from Glidden-Durkee Co., NY.

Since it was found that particles adhered to the glass surface of the container and could not be dissolved, when dry HRFe was added to the buffer-water system, the following procedure was developed. Approximately 1g HRFe was added to 50 mL 0.01N HCl, stirred, and allowed to stand for 30 min at 25° C. A 40 mL aliquot was decanted to a separate container and allowed to stand for 20–24 hr to insure dissolution of all particles. The solution was then analyzed by atomic absorption spectrophotometry (AAS) to determine the iron concentration.

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From previous studies by Lee and Clydesdale (1980) and Nojeim and Clydesdale (1981) it was known the HRFe is converted to soluble iron in the form of Fe^{+2}. Therefore, this portion of the study is in fact an investigation of the behaviour of acid solubilized HRFe as might occur in a fortified fruit beverage such as that studied by Lee and Clydesdale (1980).

Preparation of model systems

All glassware was acid-washed and well rinsed with double distilled, deionized water (DDD) to prevent contamination with iron.

One part DDD was added to two parts Prep Tyrode buffer to form the model systems. Prep Tyrode buffer consists of (g): NaCl (16.0), KCl (0.4), $NaH_2PO_4.H_2O$ (0.13), $NaHCO_3$ (2.0), glucose (2.0), $MgSO_4.7H_2O$ (0.52) and $CaCl_2$(0.4), NaN_3 (0.4), made up to 2L with DDD. Half of the systems were treated with ascorbic acid (AA) (L-ascorbic acid, Fisher Scientific Co., NJ) at a molar ratio of 1.25:1 ascorbic acid: iron as suggested by Clydesdale and Nadeau (1985). Each iron source was added to one AA-treated and one untreated model system. All iron sources were added at a concentration of 0.0014M (77.5 ppm) Fe, to simulate the U.S. RDA (18 mg) of iron delivered in an 8 fl oz (237 mL) serving of food.

Iron sources were added to the prepared model systems to a total volume of 900 mL and mixed vigorously for 15 sec. Twenty mL aliquots were volumetrically pipetted into 50 mL centrifuge tubes and stored at room temperature. Duplicate tubes were analyzed at 0, 20, and 40 min., 1, 2.5, and 5 hr, 1, 2, 6, and 12 days, 4 and 10 wk, for the various forms of iron described in the following section with a Perkin Elmer 372 double beam atomic absorption spectrophotometer (AAS) with an iron specific hollow cathode lamp. A 300 mL aliquot was also taken from which samples were removed under the same time schedule noted above for visible/infrared reflectance spectrophotometry as described in a subsequent section.

Total iron

Total iron was determined by digesting replicates, taken during stirring, of the iron model systems in HNO_3 for 20 min. and, after dilution, measuring iron concentration via AAS.

Soluble iron

Duplicate samples were centrifuged for 10 min at 2,335g in an International Equipment Co. Model K centrifuge. The supernatant was analyzed for iron soluble at the endogenous model system pH.

Resoluble iron

Resolubilization of the insoluble iron hydroxide polymers, obtained from the pellets after centrifugation (described in the last section under "soluble iron") was evaluated by adding 0.01N HCl and incubating for 90 min at 37°C in a shaking water bath at 110 oscillations per min (Blue "M" magni-whirl, Fisher Scientific Co., NJ) to simulate gastric conditions (Lock and Bender, 1980; Rao and Prabhavathi, 1978; Jacobs, 1975). After incubation, the samples were centrifuged and resolubilized iron in the supernatant was analyzed via AAS.

Total soluble iron

Total soluble iron was defined as soluble plus resoluble iron obtained under gastric pH conditions, which represents the amount of iron potentially available for absorption in the duodenum.

Insoluble iron

In this study, insoluble iron refers to the iron remaining insoluble after the stored samples were subjected to the resolubilization procedure described previously utilizing a simulated gastric pH obtained with 0.01N HCl. It was determined by difference: insoluble iron equals total iron minus total soluble iron (soluble plus resoluble iron). It does not refer to the insoluble hydroxides formed in the total system which were air dried at various storage times for Vis/IR reflectance spectral analysis.

Complexed iron

Soluble complexed iron is defined as any soluble iron not determined by the bathophenanthroline method for ionic iron.

Iron profile

The ionic profiles for the soluble and resoluble iron were determined using the bathophenanthroline (batho) procedure described in detail by Rizk and Clydesdale (1983). However, it should be noted that this is, in reality, bathophenanthroline reactive iron and as such provides a measure of ionic iron as discussed by Clydesdale and Nadeau (1985).

Visible/infrared reflectance spectrophotometry (Vis/IR)

Reflectance spectra of samples of precipitated iron-polymers, air-dried on glass cover slips, were obtained, using a Perkin Elmer 330 UV/VIS/IR spectrophotometer from 350 to 2600 nm. Precipitation occurred initially at various times depending on the source. $FeSo_4$ and

Table 1—Distribution of iron, expressed in ppm, in model systems containing four iron sources, with and without 1.25 mol ascorbic acid/mol Fe. S = ppm iron soluble after centrifugation. R = ppm iron resolubilized from the precipitate after incubation at pH 2. I = ppm iron remaining insoluble after incubation at pH2. Results shown are the mean of duplicate samples ± the standad error of the mean — indicates that iron was not detected by the analytical method used in this study.

Sample		Time 0	20 min	40 min	60 min	2½ hr	5 hr	1 day	2 days	6 days	12 days	4 wk	10 wk
	S	24.0±0.1	13.3±0.2	12.0±0.1	10.1±0.1	5.7±0.1	2.6±0.1	0.7±0.1	0.9±0.2	1.1±0.1	0.9±0.2	0.0±0.0	0.0±0.0
$FeSO_4$	R	37.9±0.1	50.2±0.3	53.8±0.3	55.8±0.2	58.9±0.1	60.7±0.2	59.0±0.2	55.8±1.4	53.6±0.4	57.8±0.5	52.9±0.4	47.7±0.0
	I	11.2±0.1	9.5±0.1	7.3±0.2	7.1±0.2	8.4±0.2	9.7±0.2	13.3±0.3	16.4±1.2	18.3±0.4	14.3±0.4	20.1±0.4	25.3±0.0
	S	54.0±0.5	54.3±0.2	54.2±0.2	54.3±0.2	5.1±0.0	49.9±0.3	51.9±0.3	54.4±0.0	56.4±0.0	58.5±0.5	59.1±1.5	59.1±1.5
$FeSO_4$ + AA	R	19.7±0.2	19.6±0.5	19.4±0.2	19.3±0.2	18.5±0.0	23.7±0.3	21.8±0.3	19.2±0.0	19.2±0.0	15.1±0.5	14.5±1.5	14.5±1.5
	I	—	—	—	—	—	—	—	—	—	—	—	—
	S	—	—	—	—	—	—	—	—	—	—	—	—
$Fe_2(SO_4)_3$	R	60.9±0.1	62.8±0.3	63.2±0.1	61.5±0.2	61.4±0.2	59.5±0.4	57.2±0.0	56.7±1.0	55.7±0.2	59.8±0.5	61.5±0.3	62.0±0.2
	I	2.1±0.2	0.2±0.3	0.0±0.2	2.5±0.3	2.6±0.3	3.5±0.4	5.8±0.2	6.3±1.0	7.3±0.5	3.2±0.5	1.5±0.4	1.0±0.2
	S	41.7±0.7	42.7±0.3	43.6±1.2	40.2±0.2	37.5±2.3	39.5±0.5	41.5±0.2	43.1±0.3	43.8±0.8	45.4±0.5	47.1±0.6	47.7±0.2
$Fe_2(SO_4)_3$	R	19.8±0.5	20.9±0.4	19.2±0.2	20.3±0.3	23.9±1.4	18.9±0.6	18.6±0.6	15.9±0.4	15.9±0.9	15.9±0.3	16.2±0.2	14.3±0.2
+ AA	I	0.3±0.0	0.0±1.0	0.0±1.1	1.5±0.5	1.6±3.7	3.6±1.1	2.5±1.2	2.3±0.1	1.5±1.0	0.7±0.2	0.0±0.4	0.0±0.3
	S	—	—	—	—	—	—	—	—	—	—	—	—
$FeCl_3$	R	74.3±1.0	72.5±0.8	70.9±0.9	73.1±0.3	73.8±0.5	71.5±1.7	71.4±1.7	76.2±0.5	73.3±0.0	73.4±0.1	67.6±0.9	74.2±0.9
	I	0.0±1.0	1.5±0.8	3.1±0.9	0.9±0.3	0.2±0.5	2.5±1.7	2.6±1.7	0.0±0.5	0.7±0.0	0.6±0.1	6.4±0.9	0.0±0.9
	S	48.5±0.3	59.2±4.2	51.2±0.0	49.9±0.1	56.2±0.6	52.0±0.0	55.3±0.3	58.6±0.6	53.4±0.4	53.1±0.3	59.7±0.7	65.9±0.5
$FeCl_3$ + AA	R	25.3±0.4	14.6±4.4	22.6±0.2	23.9±0.3	17.6±0.8	21.8±0.8	18.5±0.5	15.2±0.8	20.4±0.4	20.7±0.5	14.1±0.4	7.9±0.6
	I	—	—	—	—	—	—	—	—	—	—	—	—
	S	71.1±1.1	68.2±1.0	67.7±0.5	68.6±0.9	67.0±0.0	54.4±0.0	72.2±0.2	39.3±0.3	9.8±0.6	5.2±0.0	3.9±0.5	1.7±0.1
HRFe	R	2.1±1.3	4.8±1.0	5.3±0.6	4.4±0.7	6.0±0.1	13.1±0.4	0.8±0.3	15.8±0.2	21.1±0.9	10.1±0.4	11.1±0.3	32.6±2.8
	I	—	—	—	—	—	5.5±0.4	0.0±0.0	17.9±0.1	42.0±1.5	57.7±0.4	58.0±0.2	38.7±2.9
	S	77.8±0.0	78.0±0.0	77.8±0.2	78.0±0.0	77.5±0.1	71.7±0.9	72.0±0.0	70.1±0.7	71.3±0.3	65.6±0.4	69.4±0.2	58.6±0.4
HRFe + AA	R	0.2±0.1	0.0±0.1	0.2±0.2	0.0±0.1	0.5±0.2	6.3±0.8	6.0±0.2	7.9±0.8	6.9±0.3	12.4±0.5	8.6±0.2	14.1±0.4
	I	—	—	—	—	—	—	—	—	—	—	—	5.3±0.8

$FeSO_4$ plus AA showed some initial precipitation at 60 min. while the acid solubilized HRFe did not precipitate until after 2 days storage without AA and after 6 days with AA. Spectra were measured by the VIS detector from 350 to 898 nm with the detector switch set for 900 nm and by the IR detector from 852 to 2600 nm with the detector switch set for 850 nm. Triplicate spectra, both VIS and IR, were computer-averaged and then joined via multiplicative offset. Multiplicative offset involved lowering the IR spectra to match the reflectance levels of the VIS spectra in the overlap region (852 to 898 nm) by multiplying the data points in the IR spectra by reflectance from the VIS at a selected wavelength divided by % reflectance from the IR at the same wavelength.

Selected spectra were analyzed in a band detection program (Huguenin and Jones, 1985) to obtain the wavelengths where band centers occurred. The interpretations of the electronic interactions, and, thus, the changes in chemical structure of the iron compounds, causing shifts in absorption band positions, were based upon the work of Burns (1970).

Statistical analysis

Bivariate plots and multiple linear regressions were obtained for soluble, resoluble and total soluble iron data grouped according to iron source and ascorbic acid treatment (BMDP Statistical Software, Los Angeles, CA). Standard errors of the mean of all iron analyses were calculated according to Steel and Torrie (1980).

RESULTS & DISCUSSION

FIGURE 1 illustrates some of the classical qualitative structural changes thought to occur in the aging of iron compounds in the presence of various environmental factors (Bernal et al., 1959). The complexity of the system under study is evident from Fig. 1 and it helps to explain in part the cycling noted in the solution characteristics, with time, of some of the iron compounds in Table 1. Obviously, the degree of packing and the crystalline structure of the iron compound formed at any given time will make it more or less susceptible to both solubilization and resolubilization.

Iron hydrolysis occurred in six of the eight model systems to some extent, as evidenced by a decrease in soluble iron (Table 1) and the appearance of a precipitate. It should be noted that some precipitation (up to approximately 0.15 mM) might have occurred due to interaction with the phosphates in the buffer rather than as a result of hydrolysis.

$FeSO_4$

Soluble and total soluble iron (Table 1) were found to decrease in a linear manner with time for the untreated ferrous sulfate system ($p<0.05$ and $p<0.01$, respectively). The results are very similar to a decrease in bioavailability reported by Park et al. (1983) after 3 months' storage of $FeSO_4$.

The VIS/IR spectra of the air dried samples obtained at 60 min and 10 weeks are shown in Fig. 2. An increase in absorption of 425 nm can be seen as evidenced by a decrease in reflectance from 26.5 to approximately 22% at 10 wk, indicative of Fe^{+3} formation and oxidation of the polymer (Burns, 1970).

$Fe_2(SO_4)_3$ and $FeCl_3$

The concentration of soluble, resoluble and total soluble iron did not change significantly over time in the $Fe_2(SO_4)_3$ and $FeCl_3$ systems (Table 1). As well, the spectra of the air dried samples of $Fe_2(SO_4)i3$ and $FeCl_3$ taken for VIS/IR analysis indicated little change over 10 wk and therefore the spectra are not included.

HRFe

The acid solubilized samples of HRFe showed a highly significant ($p<0.01$) decrease in both soluble and total soluble iron and an increase ($p<0.01$) in resoluble iron over the 10 wk sampling period (Table 1). The soluble iron in this system (pH = 5.9), which represented only 2% at 10 wk, was in the form of Fe^{+2} as measured by bathophenanthroline analysis in this system. This may have been due to the acid used in the solubilization of this iron source and may be related to the initial ionic profile of the HRFe in HCl which indicated that 94% was Fe^{+2} and the remaining 6% soluble complexed iron. Interestingly, the Fe^{+2} produced in this system from HRFe acted differently than the Fe^{+2} produced from the other ferrous salts evaluated. Thus, HRFe in an acid beverage might provide a different bioavailability pattern from other iron sources due to its different solution behavior.

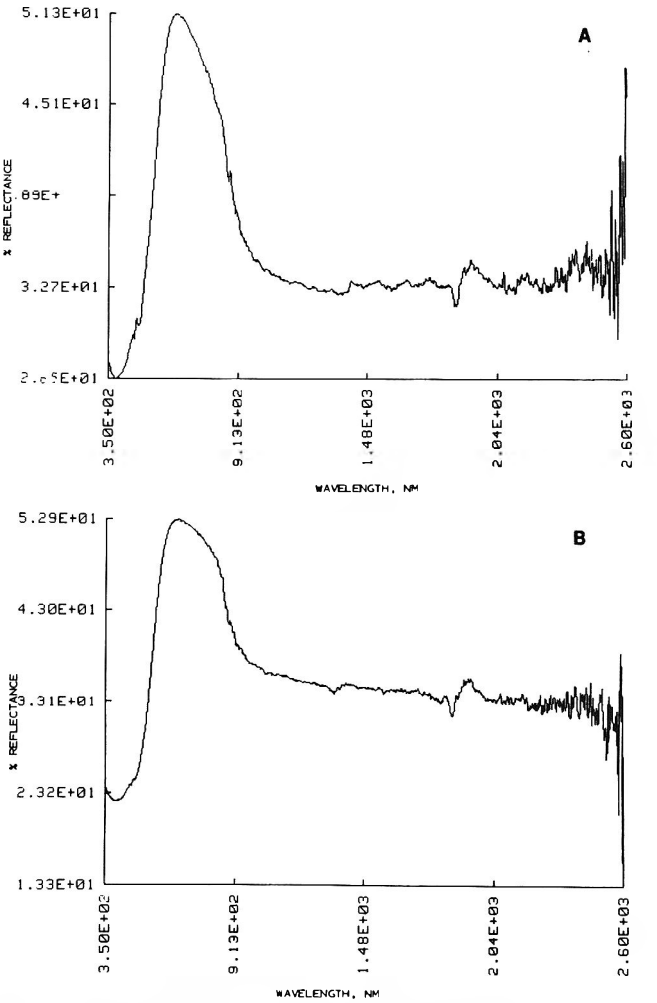

Fig. 2—Averaged spectra of air-dried precipitates from $FeSO_4$. (A) after 60 min storage; (B) after 10 wk storage.

The decrease in insoluble iron from 79% at 4 wk to 53% at 10 wk may be due to some particular characteristic of acid solubilized HRFe which allowed for structural changes in the iron polymer causing a change in the degree of susceptibility to solubilization (Weiser, 1935).

In contrast to the soluble iron, the air dried samples used for IR/VIS analysis contained a predominance of Fe^{+3} as indicated by the band at 425 nm rather than 350 nm (Fig. 4). This band showed an increased absorption from 2 days until 10 wk as evidenced by a decrease in reflectance from 38% to 23.4%. Further evidence of increasing Fe^{+3} is seen by the slight decrease in absorption and a narrowing of the shoulder at 700 nm. This spectral evidence of increasing Fe^{+3} correlates with the solubility studies shown in Table 1 indicating a decrease in soluble iron from 71.1 ppm to 1.7 ppm over the 10 wk period.

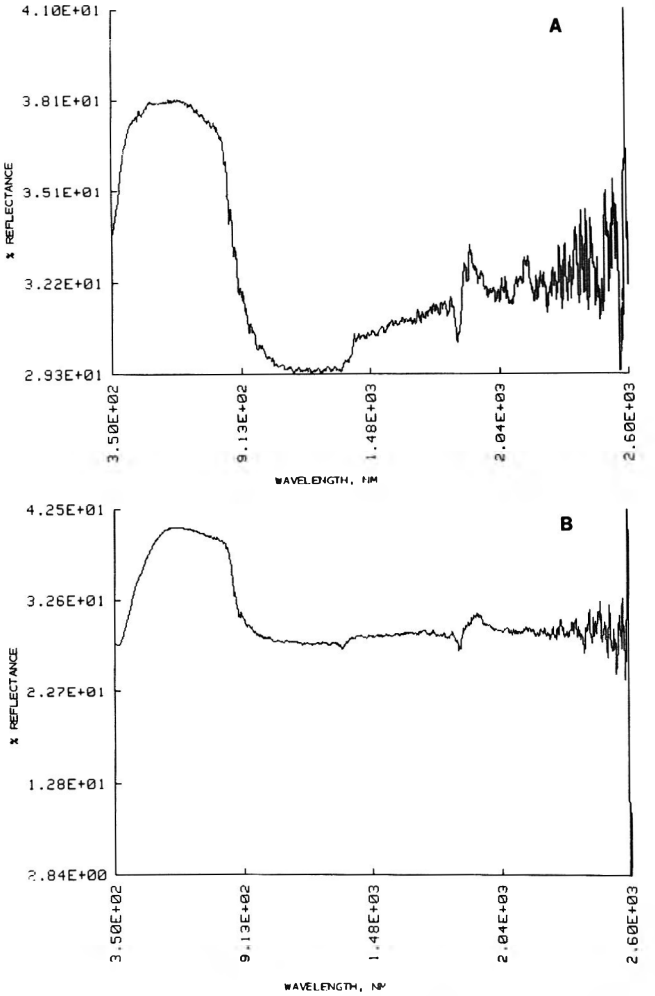

Fig. 3—Averaged spectra of air-dried precipitates from $FeSO_4$ plus AA. (A) after 60 min storage; (B) after 10 wk storage.

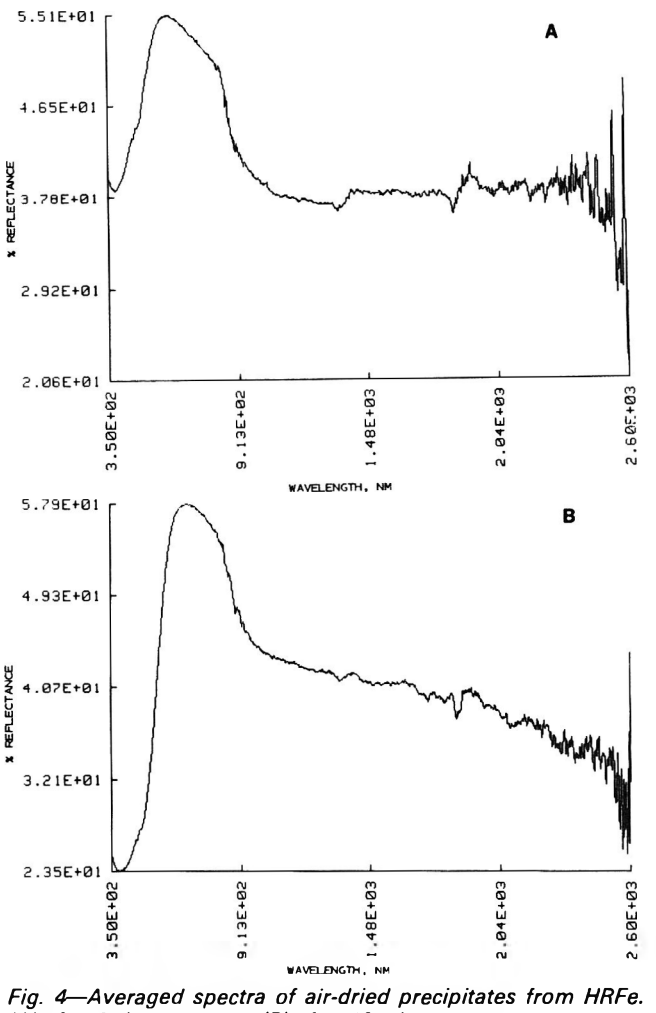

Fig. 4—Averaged spectra of air-dried precipitates from HRFe. (A) after 2 days storage; (B) after 10 wk storage.

Valence effect

Both $FeSO_4$ and $Fe_2(SO_4)_3$ were used in the study to provide a comparison of the solution characteristics between different valence states. The results shown in Table 1 indicate that Fe^{2+} and Fe^{3+} have distinct differences under the conditions of this study. Initially, 33% of the Fe^{2+} was soluble whereas Fe^{3+} was insoluble. However, these two cations reacted in a significantly different manner to resolubilization in 0.01 NHCl ($P<0.05$) with Fe^{+3} producing more resoluble iron than Fe^{+2} at every time period. This might be explained in part by reference to Fig. 1 where it can be seen that at room temperature, upon oxidation, and in the presence of alkali, Fe^{+2} can proceed to the insoluble and stable oxyhydroxide (FeOOH), while Fe^{3+} cannot proceed to the stable FeOOH or Fe_2O_3 without heat. Therefore, the Fe^{3+} may have formed a less stable intermediate which was more susceptible to resolubilization.

Ascorbic acid

Ascorbic acid treatment significantly increased ($p<0.05$) the amount of soluble iron in all systems when compared to those without AA. This occurred despite the face that these solutions ranged in pH from 4.5 to 7.5. Ascorbic acid may solubilize ferric iron both by complexation and/or by reduction to Fe^{2+} (Clydesdale, 1982, 1983). The reduction of Fe^{3+} to Fe^{2+} is well-known chemically and has previously been identified in food by Hodson (1970) and Rizk and Clydesdale (1984).

In all treated systems, soluble and resoluble iron concentrations changed in a highly significant manner over time ($p<0.01$). This change is reflected as a decrease in resoluble iron for the iron salts: $FeSO_4$, $Fe_2(SO_4)_3$, and $FeCl_3$ and an increase in the iron soluble at the model system pH for these sources. The net result was no significant change in total soluble iron over time. Opposite, but still highly significant changes occurred with HRFe. Total soluble iron decreased in a highly significant way ($p<0.01$) over time, leaving 7% of the iron remaining insoluble at 10 wk.

Proportionally more iron was resolubilized from the AA treated samples as Fe^{2+} than as Fe^{3+} or soluble complexed when compared to untreated samples.

This change in valence is supported by the IR/VIS spectra obtained from air dried samples with AA shown in Figs. 3 and 5. It is apparent that the Fe^{3+} band at 425 nm noted in the samples without AA (Fig. 2 and 4) has shifted to 350 nm, the band indicative of Fe^{2+}. The increased solubility of AA treated samples noted in Table 1, might also be predicted from the spectra in Fig. 3 and 5 where the peak at 700 nm noted in the samples not treated with AA (Fig. 2 and 4) has become diffuse over the range 600–800 nm. This indicates a less ordered polymer structure (Burns, 1970) which would be potentially more readily soluble than a more ordered structure.

An increased amount of the soluble iron found in the AA treated samples over time was complexed (23–84% respectively) while the untreated samples did not contain any soluble complexed iron. This resulted in a protective effect on the iron in the AA treated model systems from most time-dependent fluctuations in iron insolubilization due to hydrolysis. This solubilizing effect of ascorbic acid has been previously noted by Lee and Clydesdale (1979, 1980) and Clydesdale (1982, 1983).

These results offer further explanation for the increase in

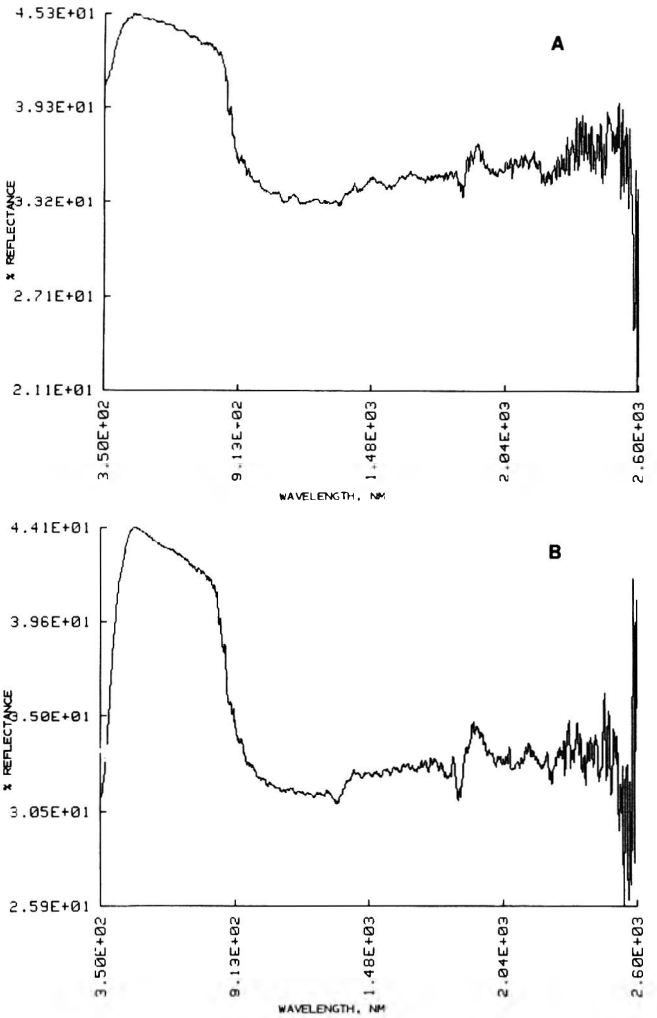

Fig. 5—Averaged spectra of air-dried precipitates from HRFe plus AA. (A) after 6 days storage; (B) after 10 wk storage.

RBV of iron sources fed with ascorbic acid observed by many workers (Brise and Hallberg, 1962; Conrad and Schade, 1968; Fritz et al., 1970; Cook and Monsen, 1977). Either a stable AA-Fe complex or reducing environment was maintained in these model systems until the hydrolysis potential became very strong, as in the HRFe + AA system, where some iron was given up to irreversible hydrolysis, but only after 10 wk storage.

This study indicated that iron hydrolysis occurred at a significant level for the HRFe and untreated $FeSO_4$ systems. The lack of irreversible hydrolysis of ferric iron sources to a stable iron oxide during aging at room temperature raises the question of how processing temperature might affect the structure and behavior of iron in fortified foods where iron is added before the heat treatment and ascorbic acid is present. However, ascorbic acid enhanced solubility under all conditions.

Most importantly, perhaps, IR/VIS reflectance spectroscopy of air dried parallel samples of the iron sources studied correlated with some of the solubility characteristics noted in the model system solubility study. As such, this spectroscopic technique provided a reasonably objective picture of the changes in the physicochemical state of the solution behaviour of the iron indicating that it might be considered for further development as a predictive tool for the measurement of iron hydrolysis, and thus potential iron bioavailability, in food systems.

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A Method for Thermal Process Evaluation of Conduction Heated Foods in Retortable Pouches

SANTI R. BHOWMIK and SHWETA TANDON

ABSTRACT

A mathematical model was developed to evaluate thermal processing of conduction heated foods in retortable pouches. This model uses any given finite value of surface thermal conductance (h) which is encountered in commercial sterilization of retortable pouches using hot water as the heating medium. The model is capable of determining process time, mass average sterilizing value and nutrient retention for a prefixed critical point sterilizing value using any retort temperature profile. Results of thiamin assay in pea puree processed in retortable pouches indicated good agreement between experimental and model predicted values of thiamin retention.

INTRODUCTION

SATURATED STEAM is the most commonly used heating medium for commercial sterilization of packaged foods due to several advantages. During the heating period, steam condenses on the surface of the package resulting in very large values of surface thermal conductance (h). The rate of heat transfer from the heating medium (steam) through the package wall into the outer layer of food is high. From this point, penetration of heat into the coldest region of the food is controlled by thermal properties of the food itself. Although steam is a highly desirable heating medium, its application in certain cases is limited.

Retortable pouches, because of their flexible nature and limited seal strength, are unable to support internal pressure developed by expansion of headspace gases at thermal processing temperatures. Therefore, the pouches are sterilized in an environment where the external pressure in the retort is equal to or greater than the internal pressure of the pouches during heating and cooling cycles of the process. In most cases, this is achieved by processing the pouches using hot water (heating medium) under overriding air pressure to equalize the internal and the external pressures of the pouches undergoing sterilization (Mermelstein, 1978). However, this reduces the value of surface thermal conductance (h) to a finite value as opposed to a very large value achieved by using steam.

Beverly et al. (1980) identified h as one of the critical operational parameters involved in safe thermal processing of retortable pouches. Pflug and Barrero (1967) observed an increase in the rate of heat penetration into flexible packages by increasing the rate of hot water circulation which was associated with a rise in h value. Terajima (1975) reported that the effect of h on heat transfer into retortable pouches was greater with conduction heated than with convection heated foods. Peterson and Adams (1983) determined the values of h for retortable pouches at various flowrates of hot water (heating medium) in an experimental retort. They showed that the assumption of a very large value of h was not valid for thermal processing of retortable pouches while hot water was used as the heating medium. They stated that large unknown variations in the h values due to differences in retort hook-up and flowrate of hot water could actually cause potential health hazard due to inadequate sterilization of the processed food.

To date, all published mathematical models for thermal process evaluation of retortable pouches are based upon a very large value of h assuming negligible resistance to heat transfer on the surface of the pouches. A review of the literature indicated no method available for evaluation of thermal processing of retortable pouches considering finite values of h while using hot water as the heating medium. This research was initiated to develop such a model using a finite difference technique for any time variable retort temperature profile, and to verify the model experimentally.

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THEORY

Development of mathematical model

Institutional size retortable pouches (28.6 × 40.6 × 4.5cm) were used for this study. The length of a pouch was considerably larger than its width and thickness. Hence, a two-dimensional equation was considered for heat transfer analysis in a retortable pouch. The transient, isotropic heat conduction in a pouch containing conduction heated foods can be expressed by the following equation: (All symbols are defined in the Nomenclature)

$$\rho C_p \frac{\partial T}{\partial t} = K \left(\frac{\partial^2 T}{\partial x^2} + \frac{\partial^2 T}{\partial y^2} \right) \quad (1)$$

where, T = temperature as a function of x, y, and t; t = time; c_p = specific heat of food; K = thermal conductivity of food; ρ = density of food.

When the temperatures at the boundary points are unknown and resistance to heat transfer on the surface of the pouch is high, the boundary condition effecting the heat transfer phenomenon is described by the equation shown below:

$$\frac{\partial T}{\partial n} = \frac{h}{K} (T_r - T_b) \quad (2)$$

Where, T_r = heating medium temperature; T_b = boundary node point temperature; $\frac{\partial T}{\partial n}$ = outward normal gradient of temperature; h = apparent surface thermal conductance (heat transfer coefficient).

The finite difference forms of the heat conduction equation (Eq. 1) with convective boundary condition (Eq. 2) were derived using energy balance principle (Croft and Lilley, 1977) as the following.

Considering two-dimensional heat transfer, the cross section of a food-filled pouch was divided into elements of volume Δx.Δy.1 superimposing a rectangular grid pattern. It may be noted that although two-dimensional heat transfer was considered in the pouch, a numerical technique that is based upon the concept of cell control volume of unit width in Z-direction, without any heat flow in this direction, was applied. The ir-

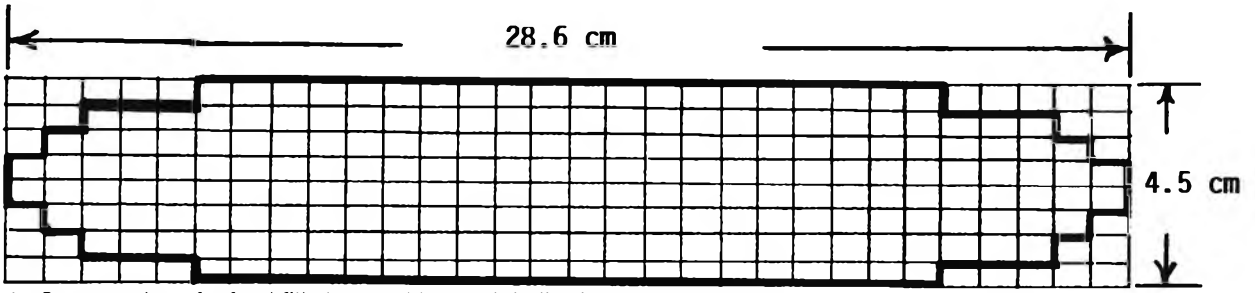

Fig. 1—Cross section of a food filled retortable pouch indicating rectangular grid pattern.

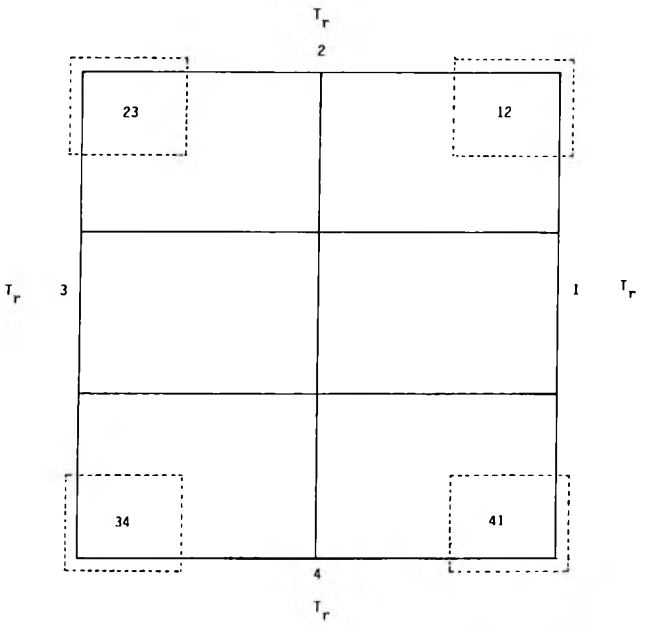

Fig. 2—Codes for external corners and side of the pouch.

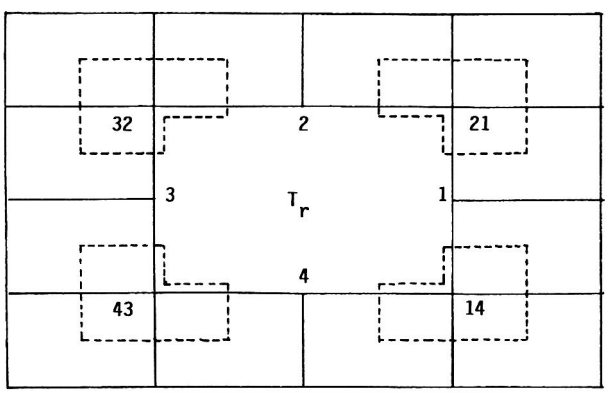

Fig. 3—Codes for internal corners and side of the pouch.

regular boundary was approximated as shown in Fig. 1, so that the boundary points would always coincide with the superimposed grid pattern's side nodes. Location of each node point with respect to the boundary was then determined by the computer program developed in this study. Each node point was taken as representative of a region around it, called a cell control volume as shown by dotted lines in Fig. 2 and Fig. 3. Heat flows across the surfaces of such a region from adjoining regions by convection and/or by conduction depending on the location of the node. Three types of boundary nodes occur as portrayed by Fig. 4 (side nodes), Fig. 5 (external corner nodes) and Fig. 6 (internal corner nodes). An interior node is represented by Fig. 7.

Considering Fig. 4, the pattern of heat flow (q) across the surfaces of a cell control volume can be represented as the

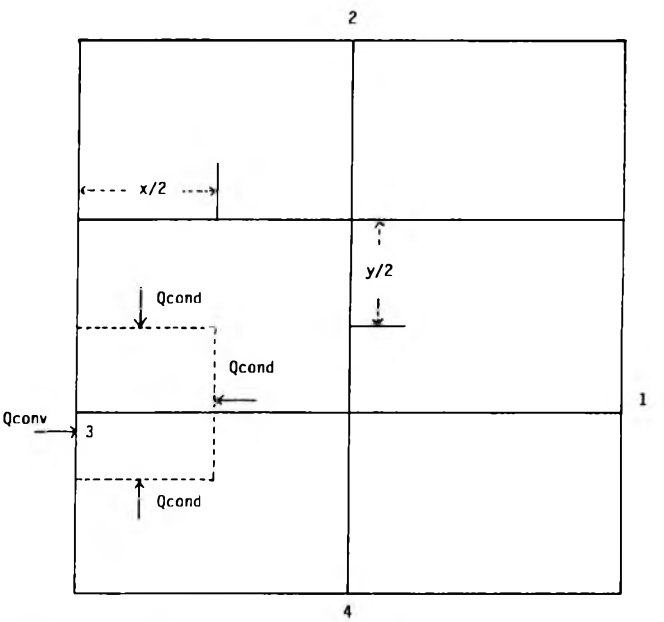

Fig. 4—Heat flow pattern at node point on side 3.

following:

$$q_{i}+1,j \rightarrow i,j = \frac{K\Delta y}{\Delta x}(T_{i+1,j} - T_{i,j}) \quad (3)$$

$$q_{i}-1,j \rightarrow i,j = h\Delta y(T_{r} - T_{i,j}) \quad (4)$$

$$q_{i,j}+1 \rightarrow i,j = \frac{K\Delta x}{2\Delta y}(T_{i,j-1} - T_{i,j}) \quad (5)$$

$$q_{i,j}-1 \rightarrow i,j = \frac{K\Delta x}{2\Delta y}(T_{i,j-1} - T_{i,j}) \quad (6)$$

$$\Sigma q_{i,j} = \rho C_{p} V \frac{\partial T}{\partial t} \text{ (where summation is over all the cell faces)} \quad (7)$$

where, i and j signify the location of the nodes; Δx is increment of lengtyh in x-direction; Δy is increment of length in y-direction; V is volume of a cell control volume.

Therefore,

$$\rho C_{p}\frac{\Delta x\Delta y}{2}\frac{\partial T}{\partial t} = \frac{K\Delta y}{\Delta x}(T_{i+1,j} - T_{i,j}) + h\Delta y(T_{r} - T_{i,j}) + \frac{K\Delta x}{2\Delta y}(T_{i,j-1} - 2T_{i,j} + T_{i,j+1}) \quad (8)$$

Replacing $\frac{K}{C_{p}\rho}$ with alpha, where alpha is thermal diffusivity of food, and applying the ADI (Alternating Direction Implicit)

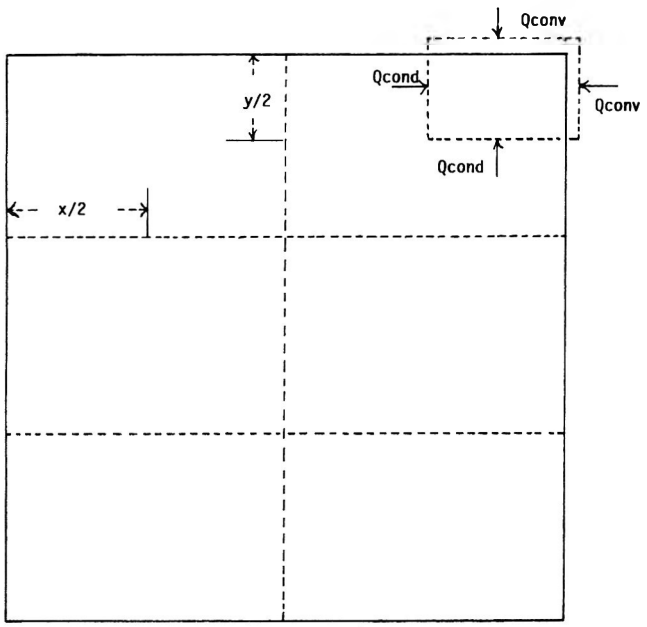

Fig. 5—Heat flow pattern at corner node point 12.

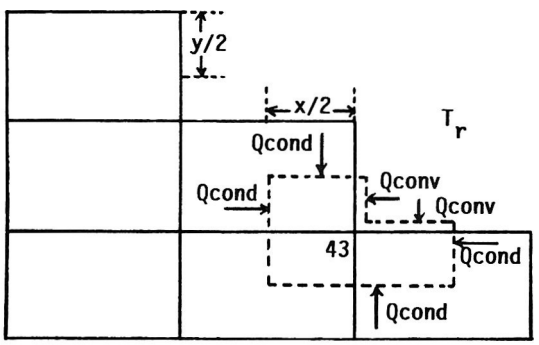

Fig. 6—Heat flow pattern at corner node point 43.

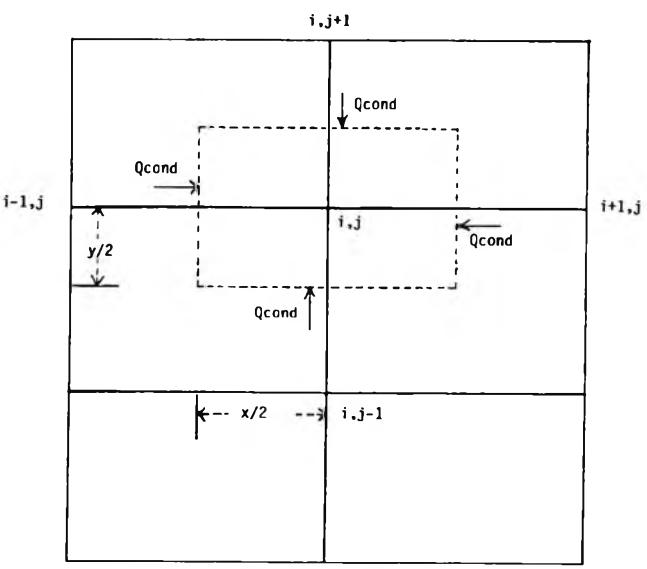

Fig. 7—Heat flow pattern at an interior node point.

method (Ames, 1977) to resolve problems of instability and minimize the number of iterations, we obtained the following finite difference equations. The equation for implicit in x-direction is:

$$T^*_{i,j} - T_{i,j,n} = \frac{\alpha\Delta t}{\Delta x^2}(T^*_{i+1,j} - T^*_{i,j}) + \frac{h\alpha\Delta t}{\Delta xK}(T_m - T^*_{i,j}) + \frac{\alpha\Delta t}{2\Delta y^2}(T_{i,j-1,n} - 2T_{i,j,n} + T_{i,j+1,n}) \quad (9)$$

The corresponding equation implicit in the y-direction is:

$$T_{i,j,n+1} - T^*_{i,j} = \frac{\alpha\Delta t}{\Delta x^2}(T^*_{i+1,j} - T^*_{i,j}) + \frac{h\alpha\Delta t}{\Delta xK}(T_{r\,n} - T^*_{i,j}) + \frac{\alpha\Delta t}{2\Delta y^2}(T_{i,j-1,n+1} - 2T_{i,j,n+1} + T_{i,j+1,n+1}) \quad (10)$$

where subscript n indicates time instant, and T* is the intermediate value of temperature at the end of the first half-time step.

Similarly, we obtained the following equation for surface corner node point 12 (Fig. 5). The equation for implicit in x-direction is:

$$T^*_{i,j} - T_{i,j,n} = \frac{\alpha\Delta t}{\Delta x^2}(T^*_{i-1,j} - T^*_{i,j}) + \frac{\alpha h\Delta t}{\Delta xK}(T_{r\,n} - T_{i,j,n}) + \frac{\alpha h\Delta t}{\Delta yK}(T_{r\,n} - T^*_{i,j,n},) + \frac{\alpha\Delta t}{\Delta y^2}(T_{i,j-1,n} - T_{i,j,n}) \quad (11)$$

while the corresponding equation implicit in y-direction is:

$$T_{i,j,n+1} - T^*_{i,j} = \frac{\alpha\Delta t}{\Delta x^2}(T^*_{i-1,j} - T^*_{i,j}) + \frac{\alpha h\Delta t}{\Delta xK}(T_{rn} - T^*_{i,j}) + \frac{\alpha h\Delta t}{\Delta yK}(T_{rn} - T^*_{i,j}) + \frac{\alpha\Delta t}{\Delta y^2}(T_{i,j-1,n+1} - T_{i,j,n+1}) \quad (12)$$

Equations for interior corner node point 43 (Fig. 6):

The equation for implicit in x-direction is:

$$T^*_{i,j} - T_{i,j,n} = \frac{\alpha\Delta t}{3\Delta x^2}(2T^*_{i-1,j} - 3T^*_{i,j} + T^*_{i+1,j}) + \frac{\alpha h\Delta t}{3\Delta xK}(T_m - T_{i,j,n}) + \frac{\alpha h\Delta t}{3\Delta yK}(T_m - T_{i,j,n}) + \frac{\alpha\Delta t}{3\Delta y^2}(2T_{i,j-1,n} - 3T_{i,j,n} - T_{i,j+1,n}) \quad (13)$$

While the corresponding equation implicit in y-direction is:

$$T_{i,j,n+1} - T^*_{i,j} = \frac{\alpha\Delta t}{3\Delta x^2}(2T^*_{i-1,j} - 3T^*_{i,j} + T^*_{i+1,j}) + \frac{\alpha h\Delta t}{3\Delta xK}(T_{rn} - T^*_{i,j}) + \frac{\alpha h\Delta t}{3\Delta yK}(T_{rn} - T^*_{i,j}) + \frac{\alpha\Delta t}{3\Delta y^2}(2T_{i,j-1,n+1} - 3T_{i,j,n+1} + T_{i,j+1,n+1}) \quad (14)$$

Equations for internal node point (Fig. 7):

The equation for implicit in x-direction is:

$$T^*_{i,j} - T_{i,j,n} = \frac{\alpha\Delta t}{2\Delta x^2}(T^*_{i+1,j} - 2T^*_{i,j} + T^*_{i+1,j}) + \frac{\alpha\Delta t}{2\Delta y^2}(T_{i,j-1,n} - 2T_{i,j,n} + T_{i,j+1,n}) \quad (15)$$

While the corresponding equation implicit in y-direction is:

$$T_{i,j,n+1} - T^*_{i,j} = \frac{\alpha\Delta t}{2\Delta x^2}(T^*_{i+1,j} - 2T^*_{i,j} + T^*_{i+1,j}) + \frac{\alpha\Delta t}{2\Delta y^2}(T_{i,j-1,n+1} - 2T_{i,j,n+1} + T_{i,j+1,n+1}) \quad (16)$$

An abridged flow diagram (Fig. 8) outlines the major steps involved in execution of the computer program using the above Eq. (3) through 16 and the relevant process parameters (Table 1). The temperature for each node point was estimated for a transient boundary condition. Each of these estimated temperatures was used to determine the concentration of survived target bacterial spore or nutrient by applying first-order reaction kinetics equation for inactivation of spores or destruction of nutrients, respectively, as shown below:

$$-\frac{dC}{dt} = KC \quad (17)$$

which integrates to:

$$K = \frac{2.303}{t}\log C_o/C \quad (18)$$

where, K = proportionality factor and

Table 1—Process parameters

C_o	= 100,000
C_{no}	= 100%
T_o	= 71.0°C
α	= 0.1903 × 10^{-6} m²/sec
z	= 10.0 C°
z_n	= 31.0 C°
D_r	= 1.0 min
D_{nr}	= 140.4 min
Δt	= 0.125 min
T_r	= 119°C

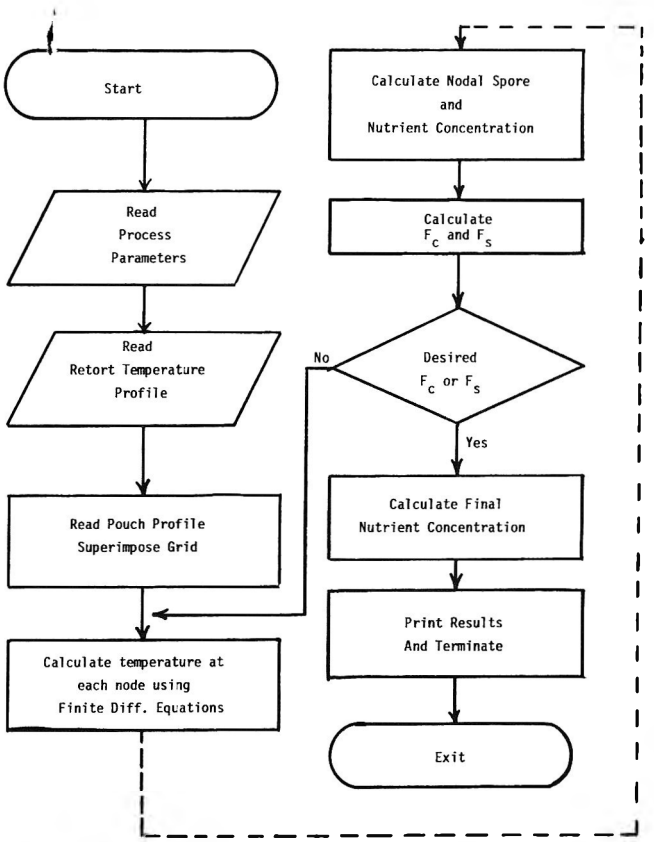

Fig. 8—Abridged flow diagram for thermal process evaluation using the developed computer model.

$$K = \frac{2.303}{D} \quad (19)$$

Rearranging:

$$C = C_o\,10^{-t/D} \quad (20)$$

The temperature dependence of D is expressed by:

$$D = D_r 10^{[(T_r - T)/z]} \quad (21)$$

The critical point sterilizing value F_c, at the end of each time step was estimated by:

$$F_c = \int_o^t 10^{[(T-T_r)/z]}\,dt \quad (22)$$

where T is the temperature at the critical point or slowest heating point in the pouched product. The sterilizing value at the end of the process was estimated by summing up the lethality at the critical point for each time step using Simpson's (Carnahan et al., 1969) Rule.

The mass average sterilizing value, F_s, was estimated using the following relationship:

$$F_s = D_r(\log C_o - \log C) \quad (23)$$

MATERIALS & METHODS

EXPERIMENTS to verify the mathematical model were performed in a vertical still retort (Modified RDTI-3 Dixi retort, Fig. 9). The temperatures in the retort and at different locations in the test retort pouch filled with 10% bentonite suspension or pea puree were measured with 36 guage copper-constantan thermocouples coated with Teflon-insulation (Omega Engineering, Stamford, CT.). Institutional size (28.6 × 40.6 × 4.5 cm) retort pouches with five internal thermocouples

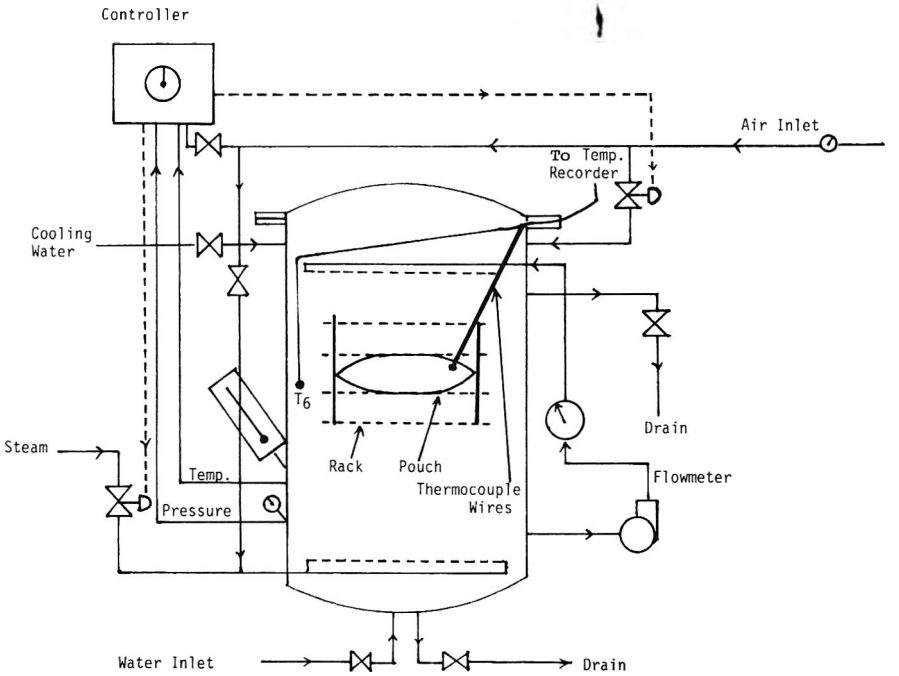

Fig. 9—Line diagram of the experimental set-up indicating position of pouch in the retort.

were used for this study. The pouches were supplied by American Can Co. (Neenah, WI).

Thermocouple junctions were made using Kup-L-weld (Burrell Corp., Pittsburgh, PA.) and were inserted into the pouch through a pin-hole in the pouch wall. A small circular area on the exterior surrounding the hole was roughened with sand paper to expose the aluminum layer. A high temperature silicone sealant was applied on the roughened area of the pouch to seal the thermocouple wires in place (Fig. 10). An Ecklund packing gland was not used to prevent errors due to heat sink effect as discussed by Kopetz et al., (1979).

A precut strip of pouch material (flexible support strip, Fig. 10) was used to hold the thermocouple junctions at fixed points in the pouch. Two strips holding three thermocouples in fixed positions, 22 mm from each end surface, were heat-sealed. Then the two end flaps of these strips were heat-tacked to the inside surface of the pouch to form the flexible support strip. The support strip was placed such that when the pouch was filled to its maximum thickness of 4.5 cm, the thermocouples 1, 2 and 3 shown in Fig. 10 would be positioned at the midplane of the pouch. Two more thermocouples, 4 and 5 shown in Fig. 10, were heat-sealed between the flexible support strip and the inner top and bottom pouch wall, to record internal surface temperatures. Five thermocouples were thus installed in the experimental pouches before filling with food. One thermocouple junction (Thermocouple 6 in Fig. 9) was placed close to the rack holding the pouch to record heating medium temperature.

All food-filled pouches were vacuum sealed with a heating bar (Multivac, Sepp Haggenmüller KG, W. Germany) to minimize the level of entrapped gases. The pouches were placed in racks made out of 1.6 mm thick perforated aluminum sheet with 40% open area (6.5 mm holes on 9.5 cm staggered centers) designed to fix maximum pouch thickness of 4.5 mm during processing.

The actual shapes of two filled pouches were determined with a gelatin-water mixture (Tandon and Bhowmik, 1986). The fill volume was the same as that used for 10% bentonite suspension and pea puree. The pouches were vacuum sealed and placed in racks to fix thickness to 4.5 cm and transferred into a refrigerator. After the jello had set, the pouch was cut open. The largest cross section of the pouch was cut-out and outlined on graph paper. The x, y coordinates noted for the boundary points of the pouch cross section were used as input data to the computer program to simulate the actual shape of a food-filled pouch. The program automatically superimposed one x-y grid patterns as shown in Fig. 1.

The following experimental procedure was followed to collect data. A single institutional size pouch with 5 internal thermocouples filled either with stabilized 10% bentonite (Townsend et al., 1949) or pea puree, was placed horizontally in the rack leaving the top and bottom surfaces of the pouch exposed to the heating medium to promote uniform heat transfer at both surfaces (Fig. 9). The position of the pouch was raised about 50 cm from the steam spreader to prevent direct contact of the pouch with steam and air-injected through the steam spreader at the bottom of the retort. The racked pouch was preheated to a uniform initial temperature. Subsequently, the temperature of the heating water was brought up to processing temperature of 121°C. The pouch was heat-processed under 124.1 kN/m^2 overriding air pressure until a desired value of F_c was reached. Steam was then cut-off and cooling water was circulated until the temperature of the center point in the pouch was reduced to 55°C.

To increase the rate of heat penetration into the pouch, a centrifugal pump was installed to provide recirculation of water during processing (Fig. 10). Hot water (heating medium) was pumped out from the bottom of the retort by the pump and introduced through the water spreader at the retort top to insure a uniform flowback through the retort. A flowmeter (Brown Morrison, Charlotte, N.C.) was used to record the flowrate of the recirculating water. A constant flowrate of water at 0.186 m^3/min was maintained during preheating, come-up, heating, and cooling periods in the retort of 0.254 m^3 total capacity. To maintain proper agitation and temperature distribution of the heating medium, steam and air were injected through the steam spreader at the bottom of the retort during the processing period. Temperatures were continuously recorded with a data logger (Digistrip II, Kaye Instruments, Bedford, MA.) at one-minute intervals at different locations within the pouch and retort.

Before the experimental runs with food-filled pouches, heat distri-

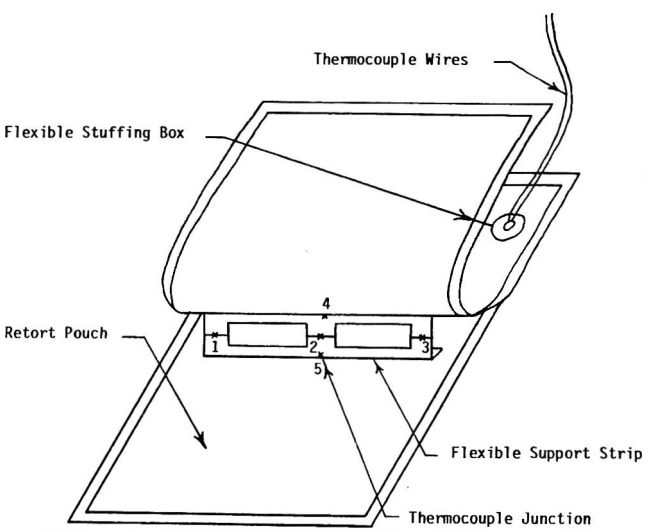

Fig. 10—Cut-out view to show locations of thermocouples in the experimental retortable pouch.

tests were performed inside the retort to insure uniformity of heating medium temperature throughout the retort during processing according to the procedure described in the *Federal Register*, March 16, 1979. These tests were carried out under similar conditions for experimental runs in terms of number of pouches per retort crate, position of pouches, quantity of air flow, and water circulation. Sixteen thermocouples located at various locations (*Federal Register*, 1979) recorded temperatures with a variation of 0.5 to 1°C within the retort.

A total of six runs was performed using the above procedure. Three runs (#1 through #3) were performed with a pouch-filled with stabilized suspensions of 10% bentonite. Temperatures collected from these experiments were used to calculate average values of f_h. Finally the value of h was determined following the procedure reported by Hanson and Adams (1983). This procedure was based upon the following equation that was obtained from Ball and Olson (1957) and Hayakawa (1973):

$$h = k \sqrt{2.303/(\alpha f_h)} \tan \sqrt{2.303\ a^2/(\alpha f_h)} \qquad (24)$$

where, a is the half-thickness of a retortable pouch; f_h is the slope value of the heating curve of food; α is the thermal diffusivity of food; k is the thermal conductivity of food and h is the surface thermal conductance of the food-filled pouch.

Three other runs (#4 through #6) wer performed with pouches filled with pea puree. Average values of f_h and h were calculated using the temperatures collected from these runs and the results are listed in Table 2.

After processing, the contents (pea puree) of each pouch were blended in a food processor (Model No. 14-11, Sunbeam Corp., Chicago, IL). Samples from blended pea puree were analyzed to determine thiamine retention after processing using the "Thiochrome Method" (AOAC, 1975) and the results are listed in Table 3.

Preliminary experiments were carried-out with pure solutions of thiamine hydrochloride at low concentrations that produced deflections covering the entire scale of the photofluorometer (Turner Associates., Palo Alto, CA.). Concentration ranging from 0.0 to 0.2 μg/ml were used to prepare a standard reference curve of photofluorometer reading vs thiamin concentration. This technique of obtaining standard curves was repeated several times to determine repeatability of the assay results under ideal conditions.

Thiamin retention analysis was performed on triplicate samples of pea puree from each processed pouch simultaneously with triplicate control sample of pea puree stored in the refrigerator. The percent thiamin retention for a given thermal process was determined directly by taking the difference between lowest reading from the replicate assays of the control samples and processed sample, over the lowest reading for the control sample after subtracting the appropriate blank readings and multiplying by 100 for each analyzed sample. The experimentally determined thiamin retention values were then compared with similar values predicted by the computer model for similar retort temperature profile using rate data for thiamine destruction in pea puree reported by Felliciotti and Esseler, (1956).

Table 2—*Average values of physical and thermal properties*

Physical or thermal property	Pea puree	10% Bentonite
(sec)	3429.00	3329.40
(m²/sec)	0.159 × 10⁻⁶	0.19 × 10⁻⁶
(kg/m³)	1023.54	1054.05
(J/kg°K)	3558.00	3926.00
(W/m°K)	0.58	0.79
(W/m²°K)	295.94[a]	202.74[a]

[a] values are different for pea puree and bentonite suspension, since these values were obtained using Eq. (24) which indicates that h is a function of k, α and f_h of the food.

Table 3—*Comparison of observed and model predicted % nutrient retention in pea puree*

	Predicted % nutrient	Experimental[a] % nutrient	Heating time (min)	F_o
	41.39	39.2 (2.1)	64.0	9.89
	43.44	41.1 (1.6)	61.0	8.52
	43.61	43.1 (1.2)	60.0	8.39

[a] Tabulated values are averages of three experimental data. A value in parenthesis is maximum deviation (%).

RESULTS & DISCUSSION

THE PROCESS PARAMETERS shown in Table 1 were used to run the computer program developed in this study. The values of thermal diffusivity (alpha) for 10% bentonite suspension and pea puree were determined experimentally using the method of Bhowmik and Hayakawa (1979). The corresponding values of thermal conductivities for 10% bentonite suspension and pea puree were computed using the relationship $K = \alpha C_p \rho$. The values of densities for 10% bentonite suspension and pea puree were determined experimentally and their specific heat (C_p) values were obtained from literature (Niekamp et al., 1984; Dickerson, 1968).

The temperatures recorded at the inside surface of the pouch by the thermocouples 4 and 5 (Fig. 10) showed an appreciable lag between internal surface and heating medium (retort) temperatures (Fig. 11). This indicated that the assumption of very large surface heat transfer coefficient using hot water as the heating medium was not applicable to the processing conditions of retortable pouches. Consequently, no reliable comparison could be made between experimental and predicted temperatures by the computer model that did not include finite values of surface heat transfer coefficient (Tandon and Bhowmik, 1986).

For experimental verification of the developed model, heating medium temperatures recorded by Thermocouple No. 6 (Fig. 9) inside the retort for Run No. 1 were used as input to the model. Temperature distributions at the center point in a retortable pouch filled with 10% bentonite suspension as predicted by the model and measured experimentally for Run #1, are shown in Fig. 12.

The maximum difference between computer predicted and experimentally measured temperature at the center point (Fig. 12) was 1.5°C and absolute mean difference was 0.7°C. Various factors could be responsible for these differences. Firstly, a lag of 0.5 to 1.0°C was inherent in thermocouple response. Secondly, there was an unmeasurable magnitude of experimental error in placement of thermocouples inside the pouch, including accuracy of the recorder.

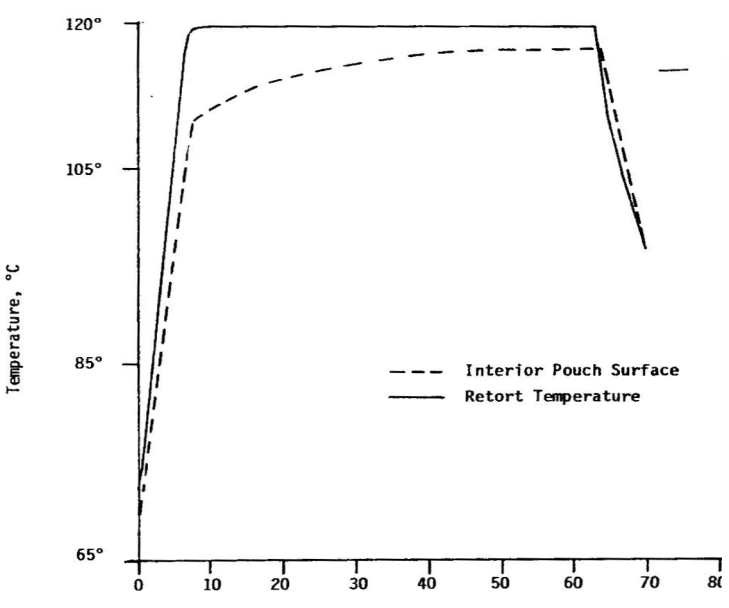

Fig. 11—Experimentally determined temperatures at the internal surface of a retortable pouch filled with 10% bentonite suspension.

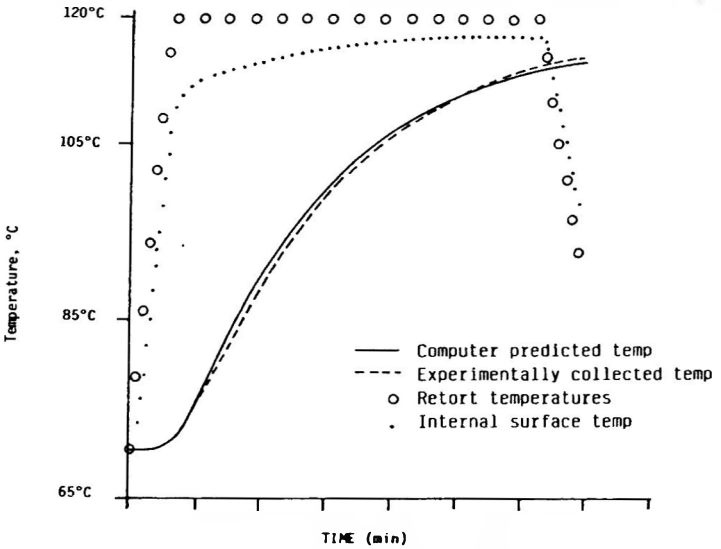

Fig. 12—Temperature history at coldest point as predicted by the computer model and as measured experimentally for a 10% bentonite suspension filled retortable pouch with convective boundary condition.

Effect of heat transfer coefficient on heating time and nutrient retention

Various critical processing factors influence the rate of heat penetration into the pouch. Pflug and Barrero (1967) observed that heat transfer coefficient was one of the critical processing factors. The rate of heat transfer on the surface of the pouch depends on the circulation rate of heating medium across pouch surface (Milleville and Badenhop, 1980; Peterson and Adams, 1983). Computer simulations were performed to estimate the effect of heat transfer coefficient on process time and % thiamin retention for processes to achieve the same level of lethality in an institutional-size pouch. Values of h reported by Peterson and Adams (1983) for different heating water flowrates with constant values of alpha, K and other processing parameters were used in this simulation and the results are shown in Table 4.

A 1% increase in thiamine retention was observed for a 4 min shorter heating time when the heating water flowrate was increased ninefold to provide a 37% increase in the heat transfer coefficient as indicated in Table 4. This shows that the value of the heat transfer coefficient on the surface of the pouch is influenced by the resistance to heat transfer of the pouch material, rack and the thermal properties of the food including the flowrate of the heating medium.

Thiamin assay results

In spite of slight random variations observed among the replicate assays in the standard curve, a linear relationship between fluorometer readings and thiamin concentration was obtained for all observations as reported in the literature. Therefore, the difference in fluorometer readings between the analyzed pea puree sample was found to be directly proportional to the difference in their thiamin concentration.

Thiamin retention in pea puree was experimentally determined. The results, including the model predicted thiamin retention for three experimental runs (Run #4 through 6), are shown in Table 3. Actual thiamin retention would agree closely with model predicted values provided the physical parameters, rate constant, and processing conditions were precisely supplied as input data to the computer model. In biological systems, it is unlikely to describe parameters precisely. Based on an error analysis study, Teixeira et al. (1969), concluded that an error of plus or minus 6% between the actual experimental values and computer predicted values of percent thiamin retention, should establish the validity of a computer model.

In this study the differences between predicted and experimentally assayed values of percent thiamin retention were analyzed and were found to be not significant at 95% confidence level. Thus, in spite of differences in predicting temperatures at specific node points (Fig. 12) there was no significant difference in the values of percent thiamin retention estimated by using the predicted and experimentally collected temperature distribution within the entire mass of a food-filled pouch. Therefore, the validity of the model developed in this study when using actual cross section of the pouch and finite values of heat transfer coefficient to evaluate a process schedule was clearly demonstrated.

CONCLUSION

THE DEVELOPED computer model closely emulates the effect of thermal processing on retortable pouches containing conduction heated foods using hot water as the heating medium with finite values of surface thermal conductance. This model has the capability of predicting temperature distribution at any location at the cross section of the food-filled pouch including estimation of integrated lethal effect on target bacterial spores and degradation of any thermally vulnerable factors (nutrient) for a constant or time-varying retort temperature profile. The temperatures predicted by the model compared well with experimentally determined temperatures at the center of a retortable pouch. Also, the estimation of nutrient retention by the model showed close agreement with experimental determination. Computer simulation using the developed model showed that flowrate of water had an appreciable effect on apparent surface thermal conductance (h). However, the values of h play a definite role in determination of process time and quality (nutrient) retention in the processed food.

NOMENCLATURE

C	Concentration of spores (numbers)
C_o	Initial concentration of spores (numbers)
C_n	Concentration of nutrients (thiamin) (%)
C_{no}	Initial concentration of nutrient (thiamin) (%)
C_p	Specific heat (J/kg C°)
D	Decimal reduction time of spores (min.)
D_r	Decimal reduction time at a reference temp. (min.)
D_{nr}	Decimal reduction time for target nutrient (min.)
F_c	Critical point sterilizing value (min.)
F_s	Mass average sterilizing value (min.)
f_h	Slope index of heating curve of food (min.)

Table 4—Effect of heat transfer coefficient (h) on process time and thiamin retention in 10% bentonite filled institutional size retort pouch based computer simulation

Water flowrate[a] (m^3/min)	Heat transfer coefficient[a] (h) W/$m^{2.°}$K	F_s (min)	% Thiamin retention (C_n)	Spore survivors (C)	Proc ti (mi
0.038	202.74	7.02	56.10	0.96×10^{-2}	54.
0.064	210.02	7.01	56.52	0.98×10^{-2}	52.
0.109	249.75	7.02	57.01	0.96×10^{-2}	51.
0.265	255.47	7.00	57.11	0.99×10^{-2}	50.
0.341	278.20	7.02	57.30	0.96×10^{-2}	50.

[a] Values obtained from Peterson and Adams (1983).

Surface thermal conductance (heat transfer coefficient due to convection) (Watt/m^2 C°)
Thermal conductivity of food (Watt/m C°)
Temperature (°C)
Intermediate temp (°C)
Initial temp of food (°C)
Retort temp (heating medium temp) (°C)
Time increment, min
Reciprocal of slope of thermal death-time curve of target microorganism (C°)
Thermal diffusivity of food (m^2/sec)
Density of food (kg/m^3)

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Ms received 4/18/86; revised 9/15/86; accepted 9/27/86.

Technical Contribution No. 2565 of the South Carolina Agricultural Experiment Station, Clemson Univ., Clemson, SC 29634-0371.

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Ms received 7/14/86; accepted 7/31/86.

Paper No. 2811, Massachusetts Agricultural Experiment Station, Univ. of Massachusetts at Amherst. This research supported in part from Experiment Station Project No. NE-116).

Calculation of Bound Water in Frozen Food

Q. T. PHAM

ABSTRACT

Using the widely accepted model of Schwartberg in which water is divided into free (freezable) and bound (unfreezable) fractions, a multiple linear regression enabled the bound water fraction to be calculated from enthalpy-temperature data. For each type of material (meat and fish, egg, bread or Tylose), the ratio of bound water to solids content was found to fall within a relatively narrow range.

INTRODUCTION

THE FREEZING and thawing of foods are important industrial processes. By using numerical methods, it is possible to acurately predict temperature changes in any situation provided the thermal properties are known. Although the heat conduction equation is usually written with a specific heat term, the most successful numerical methods tend to do away with specific heats and rely on enthalpy-temperature curves (Eyres et al., 1946; Furzeland, 1980; Pham, 1985). This is just as well, since enthalpies can be determined much more accurately than specific heats, which require a numerical differentiation to be calculated.

The enthalpy of various foods in the freezing range has been measured by Staph and Woolrich (1951), Riedel (1951, 1956, 1957a, b) and Fleming (1969). Theoretical studies have been carried out by Bartlett (1944), Heldman (1974, 1982), Schwartzberg (1976) and Chen (1985), and empirical correlations have been proposed by Riedel (1978), Chang and Tao (1981) and Succar and Hayakawa (1983). Most studies have followed Bartlett's approach, with the major modification being a correction for bound water. The existence of bound water, which cannot be frozen at any temperature, is a necessary postulate to correlate experimental enthalpy data (Heldman, 1974; Schwartzberg, 1976) and has been observed experimentally (Duckworth, 1971). The objective of this study was to show how the bound water fraction could be accurately determined from the enthalpy curve.

THEORY

FOOD can be considered a mixture of water, dissolved solids and undissolved solids. During freezing, the remaining liquid becomes more and more concentrated, and obeys the freezing point depression equation (Bartlett, 1944):

$$\frac{d(\ln a_w)}{dT} = \frac{M_w L}{RT^2} \quad (1)$$

Assuming that Raoult's law holds, the water activity is given by:

$$a_w = \frac{X_w/M_w}{X_w/M_w + X_s/M_s} \quad (2)$$

$$= \frac{X_w}{X_w + EX_s} \quad (3)$$

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Schwartzberg (1976) extends Eq. (3) to take into account the water that is bound to the solids and is unavailable for freezing:

$$a_w = \frac{X_w - X_b}{X_w - X_b + EX_s}$$

Substituting for a_w from Eq. (4) into Eq. (1) leads to an equation for the rate of ice formation. Making the further assumption that heat of solution effects are negligible, an expression for the apparent specific heat (i.e., including latent heat effects) is obtained:

$$c_a = c_w + (X_b - X_{wo})\,\Delta c + \frac{EX_s\, a_w}{1 - a_w}\left[\Delta c + \frac{M_w L^2}{(1 - a_w)RT^2}\right]$$

Schwartzberg (1976) showed that this equation can be approximated to within 0.03% by the simpler equation:

$$c_a = c_u + (X_b - X_{wo})\,\Delta c + EX_s\left[\frac{RT_o^2}{M_w t^2} - 0.8\,\Delta c\right]$$

Integration of this equation leads to an equation of the form

$$H = A + c_f t - B/t$$

where $c_f t$ is the sensible heat component, $-B/t$ the latent heat component, A an integration constant depending on the reference temperature, and

$$B = EX_s\, RT_o^2/M_w$$

The coefficients A, c_f and B can easily be found by regression. At the initial freezing point t_1, application of Eq. (1) and (4) leads to (Schwartzberg, 1977):

$$EX_s \simeq -M_w (X_{wo} - X_b)L_o t_1/(RT_o^2)$$

so that:

$$B = -(X_{wo} - X_b)L_o\, t_1$$

Thus, it appears that if the enthalpy curve is known, the free and bound components of water can be calculated from Eq. (9) and (10). To do this, however, t_1 must be accurately known, which is usually not the case. Since $/t_1/$ may be only about 1 or less, large errors are involved in using Eq. directly.

Charm and Moody (1966) calculated the bound water fraction by comparing the enthalpies of frozen whole fish and freeze-dried fish. Schwartzberg (1976) proposed finding it by comparing the freezing point at two different water con-

Table 1—Parameters values and correlation coefficient of equation $H = A + c_f t - B/t$ for various materials

Material	% water	Correlation coefficient	A kJ/kg	c_f kJ/kgK	B kJK/kg	t_1 °C
Lean beef	80.0	1.000	67.2	1.812	186.7	−0.733
Lean beef	74.0	1.000	68.8	1.904	224.9	−0.987
Lean beef	50.0	0.999	59.8	1.745	412.6	−3.628
Lean beef	26.1	1.000	50.8	1.542	432.3	−13.458
Lamb loin	64.9	0.996	64.6	1.880	172.9	−0.896
Lamb loin	52.5	0.998	62.4	1.754	125.0	−0.842
Lamb loin	44.4	0.998	57.5	1.579	102.1	−0.841
Calf veal	77.5	0.999	67.6	1.847	161.4	−0.682
Lamb kidney	79.8	0.996	78.7	2.313	224.8	−0.957
Cod muscle	82.0	1.000	66.8	1.804	234.9	−0.897
Cod muscle	50.0	0.999	68.2	1.990	418.5	−3.566
Haddock	83.6	1.000	64.9	1.739	240.6	−0.888
Cod	80.3	1.000	68.7	1.870	229.1	−0.907
Perch	79.1	0.999	66.7	1.787	214.8	−0.861
Egg white	86.5	1.000	67.4	1.780	143.5	−0.506
Egg yolk	50.0	0.999	77.5	2.053	80.3	−0.536
Egg yolk	40.0	1.000	67.3	1.781	109.2	−0.975
White bread	37.3	0.999	33.3	1.100	456.6	−4.833
Wholewheat bread	42.4	0.999	34.9	1.233	545.6	−4.545
Tylose	75.0	0.998	71.3	1.988	164.5	−0.768
Tylose	77.0	0.998	71.9	1.988	152.6	−0.680
Tylose salted	77.0	0.999	65.3	1.888	273.3	−1.189

Table 2—Percentage total and bound water, bound water/solids ratio and unfrozen water at −40°C

Material	Total water, %	Bound water, %	Bound water/ solids	% water unfrozen at −40°C
Lean beef	74.0	5.7	0.219	10.2
Lean beef	80.0	3.7	0.183	6.4
Lean beef	50.0	15.9	0.318	40.9
Lean beef	26.1	16.5	0.223	96.7
Lamb loin	64.9	7.1	0.201	13.1
Lamb loin	52.5	8.0	0.169	17.4
Lamb loin	44.4	8.0	0.144	20.1
Calf veal	77.5	6.5	0.290	10.1
Lamb kidney	79.8	9.4	0.466	14.2
Cod muscle	82.0	3.5	0.195	6.5
Cod muscle	50.0	14.8	0.296	38.6
Haddock	83.6	2.3	0.143	5.0
Cod	80.3	4.6	0.231	7.9
Perch	79.1	4.3	0.205	7.6
Egg white	86.5	1.5	0.109	3.0
Egg yolk	50.0	5.1	0.103	11.6
Egg yolk	40.0	6.4	0.107	18.5
White bread	37.3	9.0	0.143	36.2
Wholewheat bread	42.4	6.4	0.111	26.5
Tylose	75.0	10.8	0.430	16.3
Tylose	77.0	9.7	0.423	14.3
Tylose salted	77.0	8.1	0.352	13.5

Heldman (1974) suggested taking the unfrozen water at −40°C as being the bound water.

A better method is available by noting that at $t > t_1$, the enthalpy is to a good approximation a linear function of temperature:

$$H = H_o + c_u t \qquad (11)$$

Thus, at the initial freezing point both the following equations must be satisfied:

$$H_1 = H_o + c_u t_1 \qquad (12)$$

$$H_1 = A + c_f t_1 - B/t_1 \qquad (13)$$

Combination of these two equations gives:

$$B/t_1 = A - H_o - (c_u - c_f)t_1 \qquad (14)$$

Hence from Eq. (10):

$$X_b = X_{wo} - [H_o + (c_u - c_f)t_1 - A]/L_o \qquad (15)$$

Examination of Eq. (15) shows that the term $(c_u - c_f)t_1/L_o$ is quite small, while all the other terms on the right-hand side can be determined quite accurately by regression (provided enthalpy values over a range of temperatures are available). For example, using a reference temperature of −40°C, for lean beef $A/L_o \simeq 0.2$ (±2%), $H_o/L_o \simeq 0.9$ and $(c_u - c_f)t_1/L_o \simeq -0.005$. Thus, Eq. (15) allows X_b to be accurately determined. The initial freezing point t_1 may be found from direct measurement, by inspection of the enthalpy curve, or from solving simultaneously Eq. (12) and (13). In fact, for most *fresh* nondehydrated foods, t_1 is between 0 and −2°C, and the term containing t_1 can be dropped from Eq. (15) with negligible error:

$$X_b \simeq X_{wo} - (A - H_o)/L_o \qquad (16)$$

RESULTS

EQUATIONS (7) and (15) were applied to a range of foods for which enthalpy-temperature data have been measured (Riedel 1951, 1956, 1957a, b, 1960; Fleming, 1969). The coefficients A, B, H_o, c_u and c_f were all obtained by regression, after dividing the enthalpy curve into a frozen and an unfrozen range. To get reliable results, enthalpy data must be available

Table 3—Ratios of bound water to solids for various types of materials.

Type of materials	Range of solid mass fraction	No. of data	Bound water to solids ratio: Mean	S.d.	Min.	Max.
Meat & fish	0.124-0.739	13	0.217	0.056	0.143	0.318
Egg	0.135-0.600	3	0.106	0.003	0.103	0.109
Bread	0.576-0.627	2	0.127	0.023	0.111	0.143
Tylose	0.230-0.250	3	0.402	0.043	0.352	0.430

at fairly close intervals (about 1°C) just below the freezing point, and only datasets that satisfy this criterion were considered. This ruled out all the fruit and vegetable data of Riedel (1951), in which only two or three datapoints were given in the range between the freezing point and −10°C.

Table 1 shows the results. With two exceptions the correlation coefficients for Eq. (7) were 0.998 or better, and maximum errors in enthalpy were about 6kJ/kg or less, indicating very good fits. The exceptions were lamb kidney and lamb loin (65% water).

The foods were divided into four classes: meat and fish, egg, bread, and Tylose [a mixture of methylcellulose and water proposed by Riedel (1960) as a food analogue]. For each class, the ratio b of bound water to total solids was calculated (Table 2). In the meat and fish class, lamb kidney was an out-lier with an unusually high value of b (more than three standard deviations outside the mean) and also had a poor fit for Eq. (7). These differences may be due to the fact that kidney is the only non-muscle tissue in that class. When this material is excluded, the values of b for foods within each class are reasonably close together (Table 3), with means of 0.22 for meat and fish, 0.11 for egg components, 0.13 for bread and 0.40 for Tylose. The relatively small range for b within each class is remarkable when one considers that, for example, the solids mass fraction varies from 12.4% to 73.9% for the meats and fish and from 13.5% to 60% for the egg components.

For the three samples of lamb loin it is possible that the variability in the bound water/solids ratio is due to variations in the fat content, since fat binds very little water. The ratio of bound water to non-fat solids (i.e. protein) was therefore calculated. It was found to vary from 0.303 to 0.494, which means the variability is still quite large. Obviously the nature of the protein (muscle fibres or connective tissue) affects its water-binding properties.

Also shown in Table 2 are the values of the unfrozen water fraction at −40°C, F_u (which includes both bound water and unfrozen free water). The unfrozen free water mass fraction is approximately t_1/t. These values agree well with previous data. For example, for lean beef with 74% water, F_u was calculated to be 0.102, compared with a measured value of 0.12 (Riedel, 1957a).

DISCUSSION

THE MAIN LIMITATION of the method of this paper lies in the form of Schwartzberg's (1976) equation, Eq. (6). Succar and Hayakawa (1983) found empirically that for most materials the exponent of t in Eq. (6) is not exactly −2 but somewhat smaller. However, since no theoretical explanation has been found for this discrepancy, we have adhered to Schwartzberg's (1976) basic form. The generally high correlation (correlation coefficient = 0.998 to 1.000) found for Eq. (7) with most materials justifies this approach.

The mass fraction of bound water in foods can be accurately calculated from enthalpy data, provided the data cover both the frozen and unfrozen range, and provided a reasonable number of datapoints are available for temperatures not far below the initial freezing point to enable accurate regression. Precise measurement of the initial freezing point is not necessary.

Data for several materials with a wide range of solids show that although the ratio of bound water to solids is not constant, as assumed by Schwartzberg (1976), it falls within a relatively narrow range for each type of material: 0.143 to 0.318 for meat and fish, 0.103 to 0.109 for egg components, 0.111 and 0.143 for bread, and 0.352 to 0.430 for Tylose gels. That these ratios are not constant probably reflects the difference in molecular structure of the solids in each materials.

SYMBOLS

A	Constant [Eq. (7)], J/kg
a_w	Water activity
B	Constant [Eq. (7)], JK/kg
b	Bound water to total solids ratio
c	Specific heat, J/kg K
c_a	Apparent specific heat, J/kg K
Δc	Difference in specific heat between water and ice, J/kg K
E	M_w/M_s
F_u	Unfrozen water fraction at −40°C
H	Enthalpy, J/kg
L	Latent heat of freezing, J/kg
M	Molecular weight, kg/kmol
R	Gas constant, J/kmol K
T	Absolute temperature, K
t	Temperature in °C
X	Mass fraction

Subscripts

b	bound water
f	frozen food
s	soluble solids
u	unfrozen food
w	(unfrozen) water
o	at 0°C
1	at the onset of freezing

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Ms received 4/16/86; revised 9/22/86; accepted 9/22/86.

A Research Note

Is Chlorogenic Acid Bitter?

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ABSTRACT

Chlorogenic acid at 5× published threshold concentration (100 mg/L) in aqueous 0.2% potassium acid tartrate was not significantly more bitter than aqueous 0.2% potassium tartrate alone when evaluated by taste panel.

INTRODUCTION

CHLOROGENIC ACID and related hydroxycinnamates have most often been described as being bitter or astringent (Singleton and Nobel, 1976). Dadic and Belleau (1973) gave bitterness threshold values for chlorogenic acid of 10 mg/L in 5% ethanol and 20 mg/L in beer. Similar values were reported for the related compounds, caffeic and p-coumaric acids. Maga and Lorentz (1973) reported threshold values of 90 and 40 mg/L, respectively, for these compounds in water. Conversely, Meilgaard (1975) reported threshold values of 690 and 520 mg/L for the same compounds in beer and stated that they were predominantly astringent. A related compound, caffeoyl tartrate, which is found in grapes was reported to have a flavor threshold of 50 mg/L in water (Okamura and Watanabe, 1978).

Since these compounds are acids, the question arises whether the differences perceived are truly those of bitterness or acidity. The purpose of this investigation was to determine whether chlorogenic acid is bitter.

MATERIALS AND METHODS

A PAIRED COMPARISON test was used where the panelists (untrained) were presented a control and treated sample in random order. They were asked to choose the sample which was more bitter. All tasting sessions were conducted in partitioned booths with white fluorescent lighting. The panelists were served 20 ml portions of sample in 50 ml clear glass beakers or clear plastic containers. Unsalted crackers and room temperature distilled water were made available to the panelists. Samples were labelled with three digit random numbers.

The solutions for tasting were prepared with distilled water containing 0.2% potassium bi-tartrate (Baker Chemical Company, Philadelphia, PA). The treatments consisted of the 0.2% potassium bitartrate solution (pH 3.57) containing either chlorogenic acid (100 mg/L, Sigma Chemical Company, St. Louis, MO) or quinine sulfate (15 mg/L, Baker Grade, J. T. Baker Chemical Company, Phillipsburg, NJ). In the first two panels, the panelists were presented with four pairs of samples while in the later two panels, only one pair of samples was served to each judge. Significance of the results was determined from Table 1 of Roessler et al. (1978).

RESULTS & DISCUSSION

THE SAMPLES were purposely prepared containing potassium bitartrate in order to mask any effect on acidity of the samples. The results of the four tasting sessions are summarized in Table 1. The first session with chlorogenic acid resulted in a selection ($p = 0.01$) of the control over the samples containing the chlorogenic acid. When the taste panel was repeated under identical conditions, the results were the opposite. The panelists selected ($p = 0.02$) the samples containing chlorogenic acid as being more bitter.

Table 1—Taste panel paired difference tests for bitterness of quinine and chlorogenic acid in 0.2% potassium bitartrate solutions

Additive	No. of judges	Judgements: Total	Judgements: Bitartrate	Judgements: Bitartrate + additive
Chlorogenic (100 ppm)	35	140	85[a]	55
Chlorogenic (100 ppm)	25	100	39	61[b]
Quinine (15 ppm)	42	42	7	35[c]
Chlorogenic (100 ppm)	32	32	14[d]	18[d]
Total (Chlorogenic)	92	272	138[e]	134[e]

[a] $p = 0.01$
[b] $p = 0.02$
[c] $p = 0.001$
[d,e] Not significantly different

Because of the conflicting results, it was decided to test the panel with a known bitter compound, quinine sulfate, at approximately the same concentration above its reported threshold value (3 mg/L, Amerine et al., 1965). In this case, the panelists chose ($p = 0.001$) the treated (quinine sulfate) sample. Both in this taste panel and the following panel with chlorogenic acid, the panelists were only presented one pair of samples for judgment. This was done in order to rule out any possibility of fatigue, although analysis of the data in the previous panels did not show any suggestion of such a problem.

When the taste panel for chlorogenic acid was repeated, the results were non-significant. Twenty-three of the 32 judges had participated in the quinine sulfate tasting and twenty-one had correctly selected the quinine sulfate solution as bitter. Only nine of the same judges correctly identified the sample containing chlorogenic acid. When all of the data for chlorogenic acid are combined, the results are not significant. Therefore, it was concluded that at the concentration level used (100 mg/L) chlorogenic acid in not bitter when its acid character is masked.

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Ms received 10/20/86; accepted 10/27/86.

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A Research Note

Determination of the Yield Stress of Semi-Liquid Foods from Squeezing Flow Data

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ABSTRACT

The yield stress of tomato paste, ketchup, mustard, and mayonnaise was determined from the thickness vs time relationships of squeezed samples having constant area under constant force. The results were virtually independent of the force imposed and the initial thickness of the samples. The readings were in agreement with values determined from shear flow using a coaxial viscometer.

INTRODUCTION

THE DEFINITION of yield stress, in the context of semi-liquid foods, is the applied stress required to initiate shear flow. Since direct measurement of the yield stress, particularly in coaxial viscometers, requires elaborate experimental procedures, most of the published yield stress values of foods were obtained through indirect methods. These were mainly based on mathematical manipulation of the flow curve data to fit models that include a yield stress term (Rao, 1977; Mills and Kokini, 1984; Kaletunc-Gencer and Peleg, 1984) or determination of the yield stress from the residual stress after shear and relaxation (Mizrahi and Berk, 1972; Balmaceda et al., 1973; Robinson-Lang and Rha, 1981; Barbosa-Canovas and Peleg, 1983). The magnitude of the yield stresses determined by the residual stress approach is considerably lower than that determined by the flow curve methods (Barbosa-Canovas and Peleg, 1983). This is due to the destructive effect of shear on the "fluid internal structure" which causes the yield stress. The concept of yield stress in fluids has recently been challenged by Barnes and Walters (1985). According to these authors, the results of sensitive measurements show fluids always have finite viscosity, therefore, there is flow even under very small stresses (although at an extremely low rate).

Whether the yield stress in semi-liquid foods has a physical reality or is only an idealization appears to have little practical significance. In situations where the criterion is whether the fluid will flow under its own weight, as is the case in spreads, for example, or in a droplet adhered to an evaporator wall, there is no practical importance to any processes whose effect is only manifested in experiments on a much longer time scale. For this reason the concept of yield stress is still useful in rheological characterization of semi-liquid foods, and it is worth investigating new methods for its determination. "Squeezing Flow," a term currently used in the rheological and polymer literature (Leider, 1974), describes lateral flow that results from uniaxial compression and makes it clear that unlike in shear flow the driving force is perpendicular to the fluid's motion direction.

The objective of this work was to study the possibility of yield stress determination by squeezing flow and to compare the results with those obtained in shear flow measured by coaxial viscometers.

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MATERIALS & METHODS

COMMERCIAL TOMATO PASTE, ketchup, mustard and mayonnaise were subjected to compression under constant force in an apparatus shown schematically in Fig. 1. In this arrangement the area of the compressed part of the specimen remains constant during the test, a factor that facilitates rheological calculations. Tests were performed with a creep tester described by Purkayastha et al. (1985). The test conditions were varied with respect to the applied force and the initial specimen thickness, as shown in the table 1. Samples of the same food materials were also tested in a Haake Rotovisco RV-3 coaxial viscosimeter (Haake, Inc., Saddlebrook, NJ) and the yield stress was determined from the apparent viscosity vs shear stress relationships (Kaletunc-Gencer and Peleg, 1984). Yield stress values were also determined from the residual stress after various shear treatments and relaxation (Van Wazer et al., 1963).

THEORY

PROVIDED THE IMPOSED FORCE is not excessive, fluids that exhibit a yield stress when subjected to constant compressive stress, flow and reach an equilibrium height as shown schematically in Fig. 1. It can be shown (e.g., Leider and Bird, 1974; Leider, 1974; Schowalter, 1978; Covey and Stanmore, 1981), that for a Herschel-Bulkley fluid (i.e. pseudoplastic fluid with a yield stress) the flow under the conditions shown in Fig. 1 is governed by the equation:

$$\frac{n+1}{n}\,SnX^2 - \frac{n+1}{2n+1}\,(-1-X)^{(2n+1)/n} - (-1-X)^{(n+1)/n} = 0 \quad (1)$$

where: n is the power in the Herschel-Bulkley model, i.e.

$$\tau = \tau_o + K\,\dot{\gamma}^n \quad (2)$$

(τ the shear stress, τ_o the yield stress, K and n constants)

$$X = \frac{H(t)\,\tau_o}{2}\,\frac{dP}{dr} \quad (3)$$

(H(t) the instantaneous specimen height at time t and dP/dr the pressure gradient), and

$$Sn = -\frac{RK^{1/n}}{[H(t)]^2\,\tau_o^{1/n}}\,\frac{dH(t)}{dt} \text{ (the plasticity number)} \quad (4)$$

(R is the specimen radius).

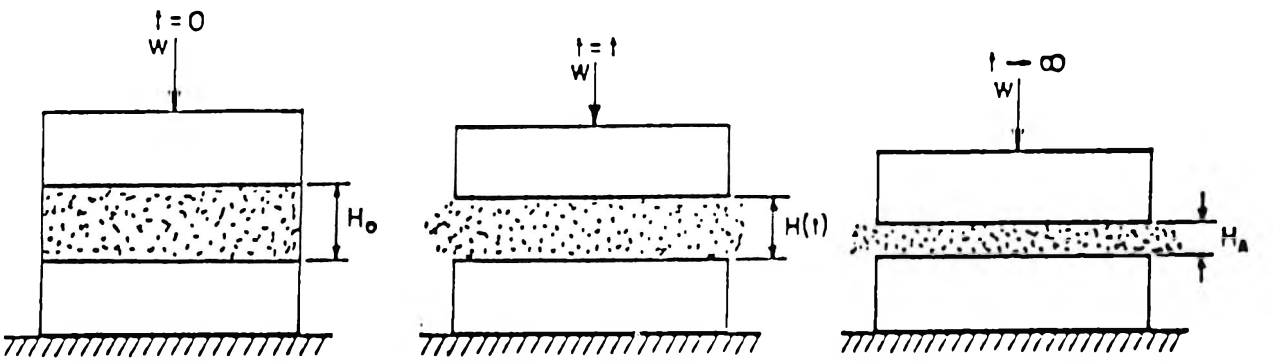
For fluids with low plasticity number (i.e. large τ_o) and small rates ($dH(t)/dt \rightarrow 0$) the relationship between X and Sn becomes linear with a small slope which, upon extrapolation yields the expression:

$$\tau_o = \frac{3WH_A}{2\pi R^3} \quad (5)$$

Table 1—Comparison between yield stress values of four semi-liquid foods determined in compression flow and a coaxial viscometer

Food	Total force (N)	Squeezing flow: Initial thickness (H_o) (mm)	Squeezing flow: Equilibrium thickness (H_A) (mm)	Squeezing flow: Yield stress (τ_o) (Pa)	Coaxial viscometer: Yield stress (Pa): From flow curve[a]	Coaxial viscometer: Yield stress (Pa): After relaxation[b]
Tomato paste	2.3	3.8	1.9	120	120-123	55-60
	2.3	5.8	1.75	110		
	2.3	4.8	1.9	120		
	2.3	7.8	1.9	120		
	2.3	11.1	1.7	107		
	2.6	5.8	1.85	133		
	4.8	8.0	1.0	130		
	7.1	10.0	0.7	135		
Ketchup	0.39	3.1	1.7	18	20-40	8-12
	0.57	4.8	1.7	26		
	0.39	4.3	2.5	26		
	0.73	4.8	1.5	30		
Mustard	0.75	6.2	3.8	78	70-80	13-18
	2.3	6.4	0.9	57		
	1.65	7.7	1.75	78		
	2.0	4.4	0.95	52		
	1.3	5.3	1.8	63		
Mayonnaise	1.3	6.2	2.6	91	70[c]	33-37
	1.65	7.3	1.8	81		
	2.0	7.0	1.5	81		

[a] Determined either from the discontinuity in the flow curve and by Mewises method (Kaletunc-Gencer and Peleg, 1984).
[b] Range of values obtained after shearing at 1, 130 and 250 sec^{-1} for mayonnaise and 1, 75, 470 sec^{-1} in the other foods.
[c] Calculated from the 2nd (down) flow curve obtained in the coaxial viscometer.



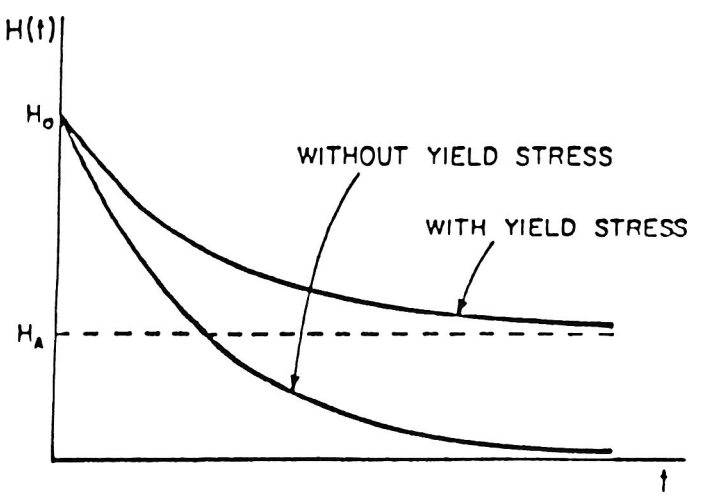


Fig. 1—Schematic view of the experimental apparatus and specimen thickness changes with time in a comprehensive constant area under constant force. (H_o, H(t) and H_A are the initial, momentary and equilibrium thickness respectively and W the total weight imposed on the specimen).

where W is the total weight imposed on the specimen and H_A the asymptotic (or residual) thickness of the specimen (Fig. 1).

RESULTS & DISCUSSION

YIELD STRESS VALUES of tomato paste, ketchup, mustard and mayonnaise, calculated by Eq. (5) are listed in Table 1. The calculated yield stress values showed little dependence on the test conditions. Thus the actual test conditions can be selected arbitrarily without significantly affecting the reading obtained.

Comparison of the yield stress values determined by the compression method and those obtained by coaxial viscometry

—Continued on page 217

A Research Note

Some Essential Elements of Two Species of Boletus Grown in Cordoba (Argentina)

ROLANDO P. PÉCORA, ROQUE A. SACCHETTA, and CARLOS A. GUZMÁN

ABSTRACT

Edible mushrooms (*Boletus tropicus* and *Boletus granulatus*) were comparatively analyzed for eight essential elements by atomic absorption spectrophotometry. Values obtained for Fe and Mn were three times higher in *B. tropicus* than in *B. granulatus*, whereas similar values were obtained with both species for K, Ca, Zn, Mn and Cu. No Co was detected in the samples analyzed.

INTRODUCTION

THE MINERAL COMPOSITION of edible mushrooms has been poorly investigated and results are contradictory and incomplete involving genera such as *Agaricus* (Stijve and Besson, 1976; Paul and Southgate, 1978; Turchetto et al., 1980; Staeva et al., 1981), *Boletus* (Mockus, 1970; Maruszewska and Gertig, 1979) or several wild edible mushrooms (Torley and Nedelkowits, 1961; Charlampowicz et al., 1973; Boettger, 1978; Schellman and Opitz, 1978; Byrne et al., 1979; Kikuchi et al., 1982). More information about the mineral composition of these food products are important from a nutritional point of view. Although knowledge of Mg, Fe, and Zn in food is important (NRC, 1980; Lindsay, 1981), no reports were found on the concentration of these elements in *Boletus*.

The worldwide *Boletus*, a wild edible mushroom present in Argentina as two species (*B. tropicus* and *B. granulatus*) is highly acceptable and more important commercially than the cultivated *Agaricus* but, due to their seasonal occurrence, they are utilized mainly in dried form rather than as a preserved product.

The objectives of this study were to determine, by atomic absorption spectrophotometry, the concentrations of Mg, Fe, and Zn, and to provide information on the amount of K, Ca, Cu, Mn, and Co of these edible mushrooms, analyzed in a form in which they reach the consumer.

MATERIALS & METHODS

DRIED MUSHROOMS were purchased in food stores from different places of Calamuchita and Punilla Valleys (Córdoba, República Argentina). As a control, a fresh *B. granulatus* mushroom sample (sample #7) was harvested in the woods (Bosque Alegre, Punilla), cleaned by hand and dried at 20–25°C, similar to commercial treatment. Species were identified by morphologic characters and microscopic observation of spores (Martinez, 1957). Samples were screened in a sieve to eliminate the adhered soil residue, ground in a stainless steel coffee grinder and oven dried at 80°C to constant weight.

Elemental assays by atomic absorption spectrophotometry were carried out by dry ashing according to Perkin Elmer specifications (1982) with minor modifications. Briefly, the dried samples (0.300g) were ashed in platinum crucibles in a muffle furnace for 2 hr at 600°C. Ashes were cooled, dissolved with hot 1M HCl and filtered through Whatman 41 (HCl and HF extracted) filter paper. Solutions obtained were collected in a 100 mL volumetric flask, then, HCl (conc) to a final concentration of 19% (v/v) and 5 mL of HNO_3 (conc) were added. These solutions were analyzed with a Perkin Elmer 372 Atomic Absorption Spectrophotometer using standards provided by the supplier (Perkin Elmer Corp., U.S.A.).

Chemical or ionization interferences were prevented by adding La, Na, or K to the samples and standards according to supplier specifications (Perkin Elmer Corp., 1982). Na was assayed in all samples but results were too erratic to be determined by this method. No Co was detected in these samples, hence it might be assumed that the values were below 3.0 mg/100g, the lowest limit detectable with the method. Samples were analyzed at least in triplicate and mean value reported.

RESULTS & DISCUSSION

GORSUCH (1970) reported that after the destruction of organic matter by dry oxidation techniques, recoveries of 94–100% were obtained for Ca, Cu, K, Mg, and Mn; 75–100% for Co; 81–100% for Fe; and 50–100% for Zn. Using this procedure with mushroom samples as test material, slightly higher recoveries than those listed above were obtained.

Table I shows the values found for K, Mg, Ca, Fe, Cu, Zn, and Mn in each sample of species analyzed including the control.

The most remarkable finding was the difference ($p < 0.05$) in Fe and Mn among the species, Fe and Mn being three times higher in *B. tropicus* than in *B. granulatus*. These dissimilarities could be explained by selective absorption and storage of these elements by the mushrooms (Laub et al., 1977; Turchetto et al., 1980) or by an influence of the physiology of the symbiotic tree partner on Boletus mineral composition. *Boletus granulatus* is mycorrhizal living in symbiotic association with *Pinus sp.* (Pinaceae), whereas *Boletus tropicus* is associated with *Fagara coco* (Rutaceae).

The Ca and Zn values obtained showed a great disparity among the samples studied which would be accounted by the influence of soil composition on mushroom mineral content as was described by Torley and Nedelkowitz (1962), Aichberger and Horak (1975), Laub et al. (1977), and Byrne et al. (1979) for several elements in wild and cultivated mushrooms.

In summary, this study provides useful information on the concentration of eight essential elements of two species of *Boletus* consumed in Argentina.

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Table 1—Mineral composition of two species of edible Boletus *grown in Córdoba (Argentina).*

Species	Sample no.	g/100g[b] (dry wt)	mg/100g (dry wt)[b]					
		K	Mg	Ca	Fe	Cu	Zn	Mn
Boletus tropicus	1 (3)[a]	3.9	160.2	11.2	41.7	3.7	2.9	1.1
	2 (3)	3.8	182.5	28.5	63.1	4.0	4.9	2.1
	3 (4)	3.9	164.5	27.0	47.2	3.9	5.0	1.3
	4 (3)	2.8	177.4	7.8	60.1	1.5	3.1	1.2
	5 (3)	3.6	223.5	15.6	45.2	2.6	7.7	1.1
	Average[c] ± S.D.	3.6 ± 0.4	181.6 ± 25.1	18.0 ± 9.3	51.5 ± 9.5*	3.1 ± 1.0	4.7 ± 1.9	1.4 ± 0.4*
Boletus granulatus	6 (5)	2.9	99.6	13.6	11.5	2.2	3.4	0.5
	7 (3)	3.8	158.9	11.1	18.1	3.6	3.1	0.6
	Average[c] ± S.D.	3.3 ± 0.6	129.2 ± 41.9	12.3 ± 1.7	14.8 ± 4.6*	2.9 ± 1.0	3.2 ± 0.2	0.5 ± 0.07*

[a] Figures in brackets indicate number of determinations.

[b] Data represents mean values.

[c] S.D. represents standard deviation. Average values marked by * are significantly different ($p < 0.05$). No Co was detected in the samples analyzed.

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Ms received 2/3/86; revised 8/9/86; accepted 9/12/86.

We thank Pedro Contreras for help with the atomic absorption spectrophotometry determinations.

YIELD STRESS IN SQUEEZING FLOW. . . From page 215

(Table 1) indicated that the readings were in general agreement. As was expected this was not the case when the yield stresses were determined after shear flow and relaxation as a result of the disruptive effect of shear on the structure of the test fluids. It was observed that the preparation of the specimen for testing in compression involve the imposition of considerably less shear compared with the filling and mounting of the coaxial viscometer cell. Therefore, the waiting period required to dissipate such shear effects for the compression test is much shorter than in coaxial viscometry (i.e. time scale of minutes rather than hours).

It was concluded that the squeezing flow method is a feasible and convenient method for yield stress determination in the foods tested and may be useful for testing other similar products.

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Contribution of the Massachusetts Agricultural Experiment Station in Amherst. The financial support of the principal author by the Consejo Nacional de Investigaciones Cientificas y Tecnicas de la Republica Argentina (programa BID-CONICET) is gratefully acknowledged.

A Research Note

Effect of Extrusion Cooking on *in Vitro* Protein Digestibility of Sorghum

O. O. FAPOJUWO, J. A. MAGA, and G. R. JANSEN

ABSTRACT

Extrusion variables including moisture (15 and 25%), temperature (50°, 125° and 200°C) and screw speed (50, 125 and 200 rpm) of two low tannin sorghum varieties extruded with 0, 2 and 4% calcium hydroxide were studied. Protein digestibility was determined by *in vitro* pepsin assay. Extrusion improved digestibility from 45.9 to 74.6% and 43.9 to 68.2% for the two varieties, respectively. Temperature was the key extrusion variable that influenced digestibility. Screw speed and moisture did not have significant effects. Alteration of pH before extrusion further improved digestibility.

INTRODUCTION

CONVENTIONAL COOKING does not improve the nutritional quality of sorghum (Price et al., 1980). However, digestibility studies in preschool children on extruded decorticated sorghum by MacLean et al. (1983) and *in vitro* digestibility measurement on the same extruded sorghum by Mertz et al. (1984) indicate that extrusion cooking of sorghum greatly increased digestibility of protein. However, the improved digestibility noted was based on a single extrusion of a single variety of sorghum run under a single set of conditions.

This research work was therefore designed to determine the effect of key extrusion variables, such as moisture, temperature and screw speed, on protein digestibility in two other varieties of sorghum. Another objective was to determine whether any changes in extrusion conditions, such as pH adjustment through the addition of calcium hydroxide might detoxify tannin during extrusion and post-extrusion treatments.

MATERIALS & METHODS

LOW TANNIN VARIETIES (CS 3541 and a typical U.S. yellow market class sorghum) grown in Brazos River bottom of College Station, TX were ground to pass a 0.2 mm screen.

A Brabender Plasticoder Extruder, Model PL-V500 was used. The barrel had a diameter of 19.05 mm with a 20:1 length to diameter ratio and was rifled with eight 0.79 mm × 3.18 mm longitudinal grooves. A 3:1 compression ratio screw was used along with a 1.90 mm diameter die.

In an earlier study the moisture content of the U.S. Market grade sorghum was adjusted to 15% and 25% in two separate batches before extrusion. Runs were performed at barrel temperatures of 50°, 125° and 200°C. At each of these barrel temperatures, runs were performed for screw speeds of 50, 125 and 200 rpm. Since the results of this experiment showed no significant effect of grain moisture and better digestibility value at a screw speed of 125 rpm, subsequent extrusion of the two grains without added calcium hydroxide and with 2 and 4% added calcium hydroxide were performed on grains with 25% moisture and only at screw speed of 125 rpm.

The *in vitro* pepsin method developed by Mertz et al. (1984) which was shown to give digestion values parallel to those in children was used.

To make the porridges, boiling water was added to the precooked samples extruded at 200°C at a ratio of 4:1 (H_2O/sorghum, W/W). Digestibility assays were performed on the thin porridges as well as on thick porridges prepared by adding boiling water to precooked sorghum samples that were extruded at 200°C (ratio - 3:1) and then heated with stirring for 5 min.

Statistical analyses included analysis of variance and least significant difference (LSD) at the 5% level of probability.

RESULTS & DISCUSSION

THE RESULTS of the initial study on the digestibility values of U.S. market class sorghum as influenced by extrusion temperature, screw speed, and grain moisture are shown in Fig. 1. There was no significant difference in digestibility values observed for grains containing 15 and 25% moisture. However, increased screw speeds and barrel temperature significantly improved sorghum digestibility.

Table 1 shows the results of the subsequent extrusion of the two sorghum varieties at a fixed grain moisture of 25% and a screw speed of 125 rpm. This table also shows the result of the effect of pH adjustment before extrusion. Digestibility values increased with increasing barrel temperature in all cases thus showing that extrusion temperature was a key factor in improving digestibility. The protein digestibility of raw sorghum variety CS3541 was 44.8% in the pepsin assay. This was increased to 74.6% by extrusion at 200°C. Likewise, the U.S. market class sorghum had a digestibility value of 43.3% and increased to 68.2% by exruding at 200°C. These results verify the conclusions of MacLean et al. (1983) and Mertz et al. (1984) on improved digestibility of sorghum.

The alteration of pH through the addition of $Ca(OH)_2$, before extrusion, also increased protein digestibility significantly as seen in Table 1. This table also shows the effect of added

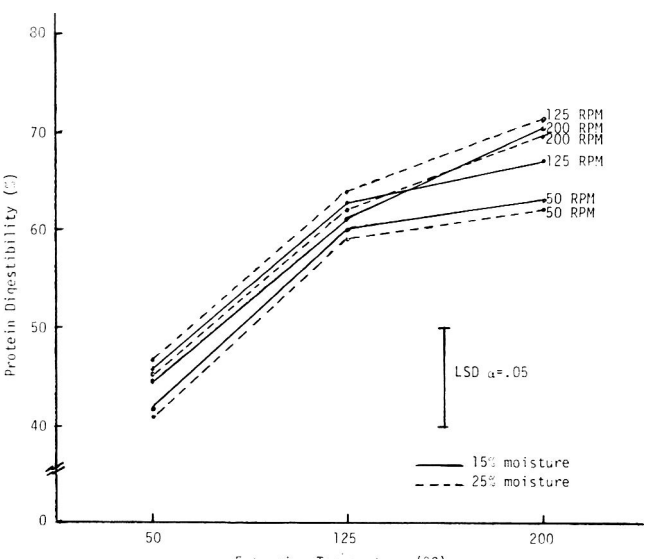

Fig. 1—Effect of extrusion temperature, screw speed, and moisture on the protein digestibility of U.S. market class sorghum.

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Table 1—Effect of extrusion cooking and added calcium hydroxide on the protein digestibility of sorghum[a]

Variety	Treatment before extrusion	Temp of extrusion (°C)	Protein digestibility (%)			
			Raw	Dry extruded product	Extruded product + Boiling H_2O No heating	Extruded product + H_2O, heated 5 Min.
CS 3541	Raw	—	44.8 ± 2.1[a]	—	—	—
	None	50	—	45.9 ± 1.0[d]	—	—
	None	125	—	66.3 ± 1.3[b]	—	—
	None	200	—	74.6 ± 3.0[c]	73.1 ± 0.9[c]	59.2 ± 1.7[d]
	2% $Ca(OH)_2$(pH7.8)	50	—	50.0 ± 2.2[d]	—	—
	2% $Ca(OH)_2$(pH7.8)	125	—	68.4 ± 1.3[b]	—	—
	2% $Ca(OH)_2$(pH7.8)	200	—	87.3 ± 2.1[c]	86.7 ± 1.2[c]	69.6 ± 2.2[b]
	4% $Ca(OH)_2$(pH11.0)	50	—	51.5 ± 2.2[d]	—	—
	4% $Ca(OH)_2$(pH11.0)	125	—	74.5 ± 1.9[c]	—	—
	4% $Ca(OH)_2$(pH11.0)	200	—	91.3 ± 2.8[c]	92.9 ± 2.8[c]	74.3 ± 2.9[b]
US market class	Raw	—	43.3 ± 1.2[a]	—	—	—
	None	50	—	43.9 ± 2.0[d]	—	—
	None	125	—	60.5 ± 1.3[b]	—	—
	None	200	—	68.2 ± 1.1[b]	66.8 ± 1.9[b]	50.6 ± 2.0[d]
	2% $Ca(OH)_2$(pH7.8)	50	—	45.4 ± 2.0[d]	—	—
	2% $Ca(OH)_2$(pH7.8)	125	—	60.0 ± 2.4[b]	—	—
	2% $Ca(OH)_2$(pH7.8)	200	—	75.6 ± 2.7[c]	73.2 ± 1.0[c]	59.1 ± 1.1[d]
	4% $Ca(OH)_2$(pH11.0)	50	—	57.4 ± 1.1[d]	—	—
	4% $Ca(OH)_2$(pH11.0)	125	—	73.9 ± 1.9[b]	—	—
	4% $Ca(OH)_2$(pH11.0)	200	—	83.5 ± 2.4[c]	83.6 ± 1.8[c]	60.3 ± 2.0[b]

[a] All extruded samples contained 25% moisture and were extruded at 125 rpm. Data represent mean ± SEM for four readings.
[b-d] Means within a group and column followed by different letters are significantly different at the 5% level.

boiling water and heating on the digestibility of sorghum extruded at 200°C. It is clear from the work of Anderson et al. (1969) on extrusion cooking of sorghum that the sorghum samples would have been well cooked at an extrusion temperature of 200°C.

Mere addition of boiling water to the extruded sorghum with mixing produced a smooth thin porridge. This post-extrusion treatment did not involve further heat treatment and the improved protein digestibility after extrusion was retained. This result demonstrated that extrusion of sorghums could replace the traditional cooking methods for making thin, unfermented, porridges for better nutritive quality.

However, when the precooked extruded product was mixed with boiling water and heated for 5 min to obtain a stiff porridge, protein digestibility was significantly reduced. The reduction in digestibility may be due to tannins complexing with proteins during heating. This observation suggested that the improvement of protein digestibility with extrusion was not due to tannin detoxification. Samples containing calcium hydroxide, which is known to detoxify tannins (Wah et al., 1977), showed better digestibility after the heat treatment. The mechanism by which extrusion cooking improves sorghum protein digestibility remains unknown. However, this study demonstrated that extrusion cooking can be used to produce a precooked sorghum that has improved protein digestibility.

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Ms received 4/7/86; revised 10/9/86; accepted 10/9/86.

A Research Note

Effect of Flavonoids and Related Compounds on Soybean Lipoxygenase-1 Activity

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ABSTRACT

Soybean lipoxygenase-1 activity was determined in the presence of the flavonoid compounds quercetin, rutin and kaempferol, and with caffeic acid and chlorogenic acid, which are cinnamic acids. All flavonoids and related compounds resulted in at least 10% inhibition of lipoxygenase activity on an equal weight basis. Inhibition of lipoxygenase-1 activity by flavonoids and cinnamic acids was compared to inhibition by butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). Kaempferol, caffeic acid and chlorogenic acid showed inhibition comparable to that of BHT. On an equimolar basis, rutin was the most effective inhibitor of lipoxygenase activity.

INTRODUCTION

LIPOXYGENASE (linoleate:oxygen oxidoreductase, EC 1.13.11.12) catalyzes the oxidation of unsaturated fatty acids containing a cis,cis-1,4-pentadiene system. Multiple forms of the enzyme are found in a variety of plant tissues, including leaves, stems, seeds and fruits (Pinsky et al., 1971). The best characterized isoenzyme is soybean lipoxygenase-1 (Chism, 1985).

Lipoxygenase has been associated with quality deterioration because of its involvement in off-flavor and odor production, loss of pigments such as carotene and chlorophyll, and destruction of essential fatty acids (Klein et al., 1985). Cis,trans-conjugated monohydroperoxides formed in the oxidation process probably do not contribute directly to flavor changes in foods because they are flavorless (Applewhite, 1985). However, decomposition of labile lipid hydroperoxides results in a complex mixture of compounds which contribute to off-flavors (Frankel et al., 1981; Frankel, 1984). The importance of lipoxygenase in the production of off-odors in vegetables has also been noted (Williams et al., 1985).

Flavonoids are a family of compounds that are widespread in plants and whose polyphenolic structure suggests that they might possess antioxidant properties. Anthocyanins, flavanols, and flavones are the most common plant flavonoids, and cinnamic acid and derivatives, which share a common biosynthetic pathway with one of the flavonoid rings, are frequently occurring plant phenolics (Harborne, 1984). Because of their widespread distribution in plants and close biosynthetic relationship to flavonoids, the role of cinnamic acids in the inhibition of enzymatic lipid oxidation was also considered. The objective of this study was to investigate the use of flavonoids and related compounds in the prevention of lipoxygenase-dependent lipid oxidation.

MATERIALS & METHODS

Lipoxygenase

Soybean lipoxygenase-1 (Sigma Type V, purified by affinity chromatography) had activity of 850,000 units/mg protein, where one unit is defined as an increase in absorbance at 234 nm of 0.001/min at pH 9 at 25°C and is equivalent to the oxidation of 0.12 μmol of linoleic acid. Dilutions of lipoxygenase were made with 0.2M sodium phosphate buffer, pH 6.5.

Antioxidants

Antioxidants tested included kaempferol and quercetin (flavonols), rutin (flavonol glycoside), chlorogenic acid and caffeic acid (cinnamic acids), and BHA and BHT (phenolics) (Sigma Chemical Co., St. Louis, MO). Ethanol or methanol was used to dissolve the compounds, with a final concentration of 2.5 mg/mL. Control reactions were run with ethanol or methanol instead of antioxidant solution for both types of assay.

Spectrophotometric assay

Lipoxygenase activity was measured under aerobic conditions at 25°C at pH 9, as the rate of conjugated diene formation at 234 nm during the linear phase of the reaction. The substrate was linoleic acid, prepared according to the method of Ben Aziz et al. (1970). The concentration of linoleic acid in the reaction mixture was 0.357 mM in 0.2M sodium borate buffer, pH 9.0; 2.0 mL substrate were added to the cuvette, followed by addition of 10 μL of antioxidant solution. The reaction was started by addition of 10 μL 0.04 mg/mL lipoxygenase solution.

Polarographic assay

Oxygen uptake was measured with a YSI Model 53 biological oxygen monitor with a Clark-type oxygen electrode, using linoleic acid, prepared as described above. The concentration of linoleic acid in the reaction mixture was 2.39 mM in sodium borate buffer, pH 9.0. Three milliliters substrate were added to the sample chamber and equilibrated at 25°C with oxygen at a flow rate of 28 cc/min. Fifty microliters of the various antioxidant solutions were injected into the sample chamber once the substrate had been equilibrated with oxygen for 3 min. The reaction was started by addition of 20 μL 0.3 mg/mL lipoxygenase solution.

RESULTS & DISCUSSION

INHIBITION of soybean lipoxygenase-1 activity by different flavonoids is given in Tables 1 and 2. Production of conjugated dienes was inhibited by all compounds tested (Table 1). Equal weights of the antioxidants resulted in lipoxygenase inhibition ranging from 10.9 to 28.5%. Kaempferol and caffeic acid

Table 1—Percent inhibition of soybean lipoxygenase-1 activity determined by spectrophotometric method; Equal weights of antioxidants were used

Compound	Class	Molarity (μM)	Percent inhibition[a] Equal wt basis	Equimolar basis
Quercetin	Flavonol	41.4	13.9	34
Kaempferol	Flavonol	43.7	19.4	44
Rutin	Flavonol glycoside	20.5	15.2	74
Chlorogenic acid	Cinnamic acid	35.3	10.9	31
Caffeic acid	Cinnamic acid	69.4	21.2	31
BHA	Phenolic	69.4	28.5	41
BHT	Phenolic	56.7	17.0	30

[a] Values for percent inhibition on an equimolar basis were calculated from equal weight data.

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Table 2—Percent inhibition of soybean lipoxygenase-1 activity determined by polarographic method; Equal weights of antioxidants were used

Compound	Molarity (μM)	Percent inhibition[a] Equal wt basis	Equimolar basis
Quercetin	137.9	18.9	14
Kaempferol	145.6	28.3	19
Rutin	68.2	13.4	20
Chlorogenic Acid	117.6	21.3	18
Caffeic Acid	231.3	14.9	6
BHA	231.5	30.6	13
BHT	189.1	29.2	15

[a] Values for percent inhibition on an equimolar basis were calculated from equal weight data.

showed inhibition of conjugated diene formation comparable to that of BHT. BHA showed the highest level of inhibition. On an equimolar basis, rutin was the most effective inhibitor of conjugated diene formation. Rutin is a naturally occurring glycoside of quercetin that is found in tobacco leaves, asparagus, apricots, and rhubarb (Herrmann, 1976).

When lipoxygenase activity was measured by oxygen uptake (Table 2), kaempferol showed inhibition comparable to that of BHA and BHT. Chlorogenic acid and quercetin had slightly lower levels of inhibition. On an equimolar basis, rutin again was the most effective inhibitor of lipoxygenase activity. Kaempferol and chlorogenic acid were comparable to rutin as inhibitors of oxygen uptake.

Differences between values in Tables 1 and 2 may be attributed to inherent differences in the two types of assays. While the spectrophotometric assay measures formation of the conjugated product, the polarographic assay is based on the measurement of oxygen uptake during the reaction, which may be less specific. Despite this difference, both the spectrophotometric and polarographic assays indicated that rutin (quercetin-3-rutinoside) has a higher level of inhibition of lipoxygenase activity than does quercetin on an equimolar basis. This was unexpected based on reports by Pratt and Watts (1964) that no differences were seen between the antioxidant activity of flavone glycosides extracted from plant tissues and the aglycones obtained by acid hydrolysis. More recently, Pratt (1976) reported that on an equimolar basis, the antioxidant activity of rutin is approximately half that of quercetin. The explanation of these different results may lie in the type of activity being assayed. Pratt and Watts (1964) and Pratt (1976) were studying antioxidant activity of flavonoids in a non-enzymatic system, measuring a decrease in the coupled oxidation of linoleic acid and β-carotene. In nonenzymatic systems, the flavonoids appear to have two modes of antioxidant action: primary (chain-breaking) and secondary (metal-complexing) (Letan, 1966a,b). In the enzymatic system containing lipoxygenase, flavonoids may act in a similar manner. Takahama (1985) has suggested that quercetin acts as a primary antioxidant in a soy lipoxygenase system. Because work in this area has been limited, however, the possibility of other interactions cannot be overlooked.

Although soybean lipoxygenase-1 is a well-studied enzyme, little work has addressed the possible effects of antioxidant compounds on lipoxygenase, and only a few studies have evaluated the effects of flavonoids. The results of this study shows that flavonoids and related compounds are effective inhibitors of lipoxygenase activity in a model system, reducing conjugated diene formation as well as oxygen uptake during lipoxygenase catalyzed linoleate oxidation.

Data from this study indicate that flavonoids and related compounds, which are widely distributed in the plant kingdom, may be as effective in the prevention of enzymatic lipid oxidation as the commonly used synthetic antioxidants BHA and BHT. Inactivation of lipoxygenase by means of a naturally occurring or a synthetic antioxidant could be of value in the food industry. Products that have enzyme activity that might be controlled by an antioxidant "dip" include fresh vegetables and fruit salads, and frozen unblanched or lightly blanched fruits and vegetables, including those frozen prior to further processing, such as those used in canned soups.

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Ms received 10/1/86; accepted 10/7/86.

Presented at the 46th Annual Meeting of the Institute of Food Technologists, Dallas, TX, June 15-18, 1986.

This study was a part of Project No. 60-323 of the Agricultural Experiment Station, College of Agriculture, Univ. of Illinois at Urbana-Champaign. It was supported in part by a grant from the Illinois Soybean Program Operating Board.

A Research Note

Effect of maturity and processing on the trypsin inhibitor and oligosaccharides of soybeans

KESHUN LIU and PERICLES MARKAKIS

ABSTRACT

Immature soybean seeds are used as human food, but they contain antinutritional factors. The trypsin inhibitory activity (TIA) increased slightly in soybean cv Beeson-80 but remained unchanged in cv Pella, as maturation advanced. Steaming or cooking for 20 min, as commonly practiced, fully eliminated TIA in immature seeds. Soaking (24 hr) plus cooking (20 min) was necessary for the complete disappearance of TIA in mature seeds. Sucrose, raffinose and stachyose concentrations increased significantly with maturation. Steamed or cooked immature seeds contained considerably smaller quantities of raffinose and stachyose, sugars associated with flatulence, than similarly treated mature seeds.

INTRODUCTION

WHILE MOST SOYBEANS are harvested at the mature stage and further processed to a variety of products for human or animal use, considerable quantities are also consumed as fresh, immature soybeans in China and elsewhere. Soybeans provide high quality plant protein and edible oil. But they also contain certain undesirable compounds. The trypsin inhibitor of mature soybeans and its inactivation have been studied extensively (Kunitz, 1946; Rackis et al., 1962; Birk et al., 1963; Rackis, 1972; Liener and Kakade, 1980). There is also considerable information on certain flatus-related α-galactosaccharides (mainly raffinose and stachyose) present in mature soybeans, and on the effect of processing on these sugars (Steggerda et al., 1966; Kim et al., 1973; Rackis, 1981; Bianchi et al., 1983; Savitri and Desikachar, 1985). Little, however, has been reported on the trypsin inhibitor of immature soybeans, and its inactivation (Bates et al., 1975; Collins and Sanders, 1976; Collins and Beaty, 1980; Sharma et al., 1975). Also, little is known about the appearance of α-galactosaccharides in the developing soybean while much has been reported on their disappearance during germination (Abdullah et al., 1984). The purpose of the present study was to provide additional information on the presence of these antinutritional factors in soybeans of varying maturity and on the effect of processing on these factors.

MATERIALS & METHODS

Sample preparation

Two soybean cultivars, 'Beeson-80' and 'Pella', were sown on 6/6/85 in an open field belonging to Michigan State University. The pods of 'Beeson-80' were harvested on 9/17, 9/26 and 10/18 and those of 'Pella' on 9/22, 9/30 and 10/30. At the first collection time, the pods were yellow-green and the seeds were green. At the second collection, the pods were yellow and the seeds were yellow-green. The third collection was the conventional harvest of mature soybeans. The immature and mature seeds were designated Immature I, Immature II and Mature, corresponding to the three collections. After shelling by hand, two samples from each cultivar and collection were subjected to each of the following treatments: (a) soaking in water at 22°C for 24 hr; (b) cooking in boiling water (100°C) for 20 min; (c) soaking and cooking as in (a) and (b); (d) steaming over boiling water for 20 min. Two samples were not treated and served as controls. All samples were freeze-dried, ground to pass through a No. 50 sieve and stored at room temperature for subsequent analysis.

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Proximate analysis

Moisture, crude protein (micro-Kjeldahl Nx6.25), crude fat and ash were determined by AOAC (1980) methods.

Trypsin inhibitory activity (TIA)

For measuring TIA, the Kakade et al. (1976) method was used and modified as follows. The extract was prepared from 0.5g 50-mesh powder by rotary shaking for 2 hrs with 50 mL 0.01N NaOH and then diluted 10-fold with water. (For raw samples, 0.8 mL of this dilution caused 40–60% trypsin inhibition.) From the diluted extract four aliquots (0.4, 0.8, 1.2 and 1.4 mL) were transferred to four test tubes and adjusted to a total volume of 2 mL with water. A fifth tube contained no extract. After this step the procedure of Kakade et al (1976) was followed, but the TIA was expressed as trypsin units inhibited (TUI) per mg dry soybean sample by *averaging* the results obtained with the four aliquots.

Oligosaccharides

Two grams freeze-dried sample were defatted by soaking the material in hexane twice and subsequently extracting with 40 mL 75% ethanol in an 80°C bath for 4 hr with occasional shaking. The extract was cleaned by adding, twice, 1 mL 10% lead acetate with mild heating and centrifuging down the impurities. After concentrating the purified extract by vacuum evaporation to a final volume of 25 mL, an aliquot was passed through a 0.22 μm membrane filter and a Sep-Pak C18 cartridge. A 20 μL sample was injected into an HPLC system (Waters Assoc., Milford, MA), composed of an M-45 pump, a guard-pak precolumn, an μ-Bondapak/carbohydrate column and an RI-401 refractometric detector. A Kontes Model-100 recorder was used. The solvent (acetonitrile/water, 80/20 v/v) passed with a flow rate of 3 mL/min. For standardization the following ranges of sugar concentrations were used: sucrose 0.1 – 0.6%; raffinose 0.02 – 0.10%; and stachyose 0.03 – 0.21%.

Statistics

The data were subjected to analysis of variance in a factorial design. When the F-test proved significant, the least-significant-differences procedure was applied to evaluate the differences among the means of different maturity stages or between the means of raw (control) and each individual treatment samples.

RESULTS & DISCUSSION

Proximate analysis

The differences in solids content among immature and mature seeds are large and statistically significant, whereas the differences in protein, oil and ash contents on a dry basis are small and in most cases insignificnt (Table 1). 'Beeson-80' is richer in protein than 'Pella'.

Trypsin inhibition

The procedure used for the determination of TIA is considered more reliable than both the Kakade et al. (1976) method

Table 1—Composition of developing 'Beeson-80' and 'Pella' soybeans

Stage of maturity	Total solids % wet wt		Protein % dry wt		Oil % dry wt		Ash % dry wt	
	'Beeson-80'	'Pella'	'Beeson-80'	'Pella'	'Beeson-80'	'Pella'	Beeson-80'	'Pella'
Immature I	35.3[a]	35.1[a]	40.4[a]	36.3[a]	19.0[a]	19.3[a]	4.9[a]	4.9[a]
Immature II	38.4[b]	38.6[a]	41.3[a]	36.3[a]	19.7[a]	20.8[ab]	4.8[a]	4.9[a]
Mature	92.2[c]	91.6[c]	42.9[a]	39.6[b]	20.2[a]	21.2[b]	5.0[a]	5.0[a]

[a] Column means bearing different superscripts differ statistically at p = 0.05. Row means connected by a line do not differ statistically at p = 0.05.

Table 2—Effect of maturity and processing on trypsin inhibition (TUI/mg dry wt) and oligosaccharide contents (% dry wt) of 'Beeson-80' & 'Pella' soybeans

	Maturity stage	Raw	Soaked	Steamed	Cooked	Soaked & Cooked
			'Beeson-80'			
	I	58.7[a]	57.1[a]	0.0[a]	0.0[a]	0.0[a]
Trypsin inhibition	II	62.0[b]	63.3[b]	0.0[a]	0.0[a]	0.0[a]
	III	64.3[b]	64.0[b]	13.1[b]	9.2[b]	0.0[a]
	I	4.17[a]	3.68[a]	4.49[a]	3.95[a]	3.75[a]
Sucrose	II	4.44[b]	3.95[b]	4.64[a]	3.75[a]	2.80[b]
	III	7.00[c]	6.20[c]	5.91[b]	5.82[b]	3.74[a]
	I	0.13[a]	0.00[a]	0.00[a]	0.00[a]	0.00[a]
Raffinose	II	0.35[b]	0.26[b]	0.20[b]	0.22[b]	0.15[b]
	III	0.52[c]	0.44[c]	0.37[c]	0.28[c]	0.30[c]
	I	0.00[a]	0.00[a]	0.00[a]	0.00[a]	0.00[a]
Stachyose	II	0.52[b]	0.46[b]	0.41[b]	0.34[b]	0.30[b]
	III	3.18[c]	2.60[c]	2.62[c]	2.73[c]	1.90[c]
			'Pella'			
	I	52.6[a]	53.8[a]	0.0[a]	0.0[a]	0.0[a]
Trypsin inhibition	II	53.4[a]	55.7[a]	0.0[a]	0.0[a]	0.0[a]
	III	56.6[a]	56.6[a]	6.3[b]	6.6[b]	0.0[a]
	I	4.43[a]	4.08[a]	5.76[a]	5.08[a]	4.03[a]
Sucrose	II	4.85[b]	4.29[a]	5.37[b]	4.10[b]	3.16[b]
	III	7.20[c]	5.72[b]	6.37[c]	5.28[a]	4.93[c]
	I	0.17[a]	0.00[a]	0.00[a]	0.00[a]	0.00[a]
Raffinose	II	0.27[b]	0.22[b]	0.20[b]	0.18[b]	0.15[b]
	III	0.66[c]	0.37[c]	0.38[c]	0.42[c]	0.31[c]
	I	0.00[a]	0.00[a]	0.00[a]	0.00[a]	0.00[a]
Stachyose	II	0.59[b]	0.43[b]	0.32[b]	0.41[b]	0.24[b]
	III	2.71[c]	2.20[c]	2.24[c]	2.06[c]	1.43[c]

[a-c] Column means bearing different superscripts differ statistically at p = 0.05. Row means underlined do not differ statistically at p = 0.05 from the raw sample means.

and the Hamerstrand et al. (1981) modification, which is based on that one portion of the extract which "inhibited at least 40 but not more than 60% of the trypsin". The Kakade et al. method presupposes a linear relationship (between volume of extract tested and TUI/mL) which was rare among the samples of this work. The Hamerstrand et al. modification gave very low values for TIA; among the four portions of extract used in this work, the one which inhibited at least 40 but not more than 60% of the trypsin produced the lowest TUI/mL value. The effect of maturity and processing on the TIA of soybeans is shown in Table 2. TIA was not affected by maturation in 'Pella', but TIA increased slightly in 'Beeson-80'. Collins and Sanders (1976) also found that maturation affected TIA differently in different cultivars. Soaking had no effect on the TIA value of either of the two cultivars. Steaming, or cooking, or soaking followed by cooking totally suppressed the trypsin inhibition of immature soybeans, but only soaking plus cooking eliminated this inhibition in mature soybeans. This finding is reassuring for the users of immature soybeans, as only cooking or steaming is applied in the preparation of this commodity.

Oligosaccharides

Sucrose, raffinose and stachyose are known to be present in mature soybeans (Hymowitz et al., 1972; Cegla and Bell, 1977). Quantitative analysis (Table 2) indicated that sucrose appeared early in the development of the seeds and remained the dominant sugar up to full maturity. Raffinose was present in small but measurable amounts already at maturity stage I. Stachyose was not detectable at maturity stage I, but subsequently increased quickly in concentration and surpassed that of raffinose. The fully mature seeds contained the greatest concentrations of all three oligosaccharides. Of particular interest is the much lower content in the flatus-related sugars, raffinose and stachyose, of the immature vs. the mature seeds. The treatments given the seeds generally resulted in decreasing the concentration of oligosaccharides. Soaking plus cooking removed the largest amount of all sugars. The sucrose content appeared slightly higher after certain treatments, perhaps as a result of differential extraction of water-solubles and/or enzymic activity. Again from a practical viewpoint, worth noting is the small quantity of raffinose and stachyose remaining in the cooked or steamed immature soybeans, a fact consistent with the common impression (undocumented) that flatulence is infrequent after ingestion of immature soybeans in contrast to ingestion of mature ones.

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A Research Note

Amino Acids in the American Groundnut (*Apios americana*)

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ABSTRACT

The amino acid composition of groundnut (*Apios americana* Medikus) was analyzed using ion-exchange column chromatography. Aspartic and glutamic acids were the predominant amino acids in both seeds and tubers. Other amino acids present in significant quantities were leucine, histidine and proline. The most abundant free amino acids, other than aspartic and glutamic acids, were arginine and leucine in the seeds and histidine and serine in the tubers.

INTRODUCTION

THE AMERICAN GROUNDNUT (*Apios americana* Medikus) is a bean-bearing, tuber-producing, nitrogen fixing plant which is widely distributed in and native to eastern North America where it ranges from Canada to southern Florida (Seabrook, 1973; Seabrook and Dionne, 1976). It was used as a food by the Indians, who apparently planted it at their campsites and introduced the early settlers to its sweet, starchy tubers (Beardsley, 1939). The potential of groundnut for domestication as a food crop has been discussed (Blackmon and Reynolds, 1986). Some analyses of its food value have been conducted, and a crude protein content of approximately 16.5% was reported for the tubers (Yanovsky and Kingsbury, 1938; Duke et al., 1984; Wilson et al., 1986). The objective of this study was to investigate the total and free amino acid concentrations of *A. americana* tubers and seeds to acquire a general idea of the plant's nutritional value.

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MATERIALS & METHODS

APPROXIMATELY 100g seeds and 300g tubers were collected for analyses from native populations of *A. americana* growing in Louisiana. Seeds were ground in a hammermill (10 mesh screen), mixed thoroughly on a ball mill apparatus and freeze-dried. Whole tubers were washed, coarsely chopped, freeze-dried, ground in the hammermill, and mixed thoroughly. Ground tuber and seed materials were each divided into triplicate portions. All samples were stored in a desiccator under vacuum for at least 48 hr prior to anlaysis. Residual moisture in freeze-dried seeds and tubers was determined (AOAC, 1984) and subsequently used to adjust results to a dry weight basis.

Lipids were extracted from the samples using the procedures previously described (Khor and Chan, 1985; Wilson et al., 1986). After lipid extraction, defatted samples were stored frozen in a desiccator.

Nitrogen content of the dry defatted samples was determined with a Technicon AutoAnalyzer II using standard instrument procedures (Technicon Industrial Systems, Tarrytown, NY). Samples of 0.5g were hydrolyzed for 45 min at 410°C in a Technicon BD-20 heating unit. The autoanalyzer was standardized using ammonium sulfate (J. T. Baker Chemical Co., Phillipsburg, NY) and checked with a casein standard (ICN Nutritional Biochemicals, Cleveland, OH).

Free and total amino acids were determined with an automated Beckman 116 Amino Acid Analyzer using standard instrument procedures (Spinco Division of Beckman Instruments Inc., Palo Alto, CA). Free amino acids were analyzed using 10g samples for both seeds and tubers. For total amino acids, samples were hydrolyzed in vacuum sealed tubes at 110°C for 24 hr prior to analysis. Sample sizes were 0.05 g for seeds and 0.1g for tubers. For each replicate, two injections were made onto the column with the results averaged. The analyzer was standardized with amino acids from Beckman Instruments Inc. (Palo Alto, CA).

RESULTS & DISCUSSION

GROUNDNUT SEEDS and tubers contained 4.10% and 1.78% nitrogen on a dry weight basis, respectively. Assuming a factor

Table 1—Total and free amino acid compositions of Apios americana *seeds and tubers (g amino acid per 100 g nitrogen)*[a]

	Seeds		Tubers	
Amino acid	Total	Free	Total	Free
Essential				
Cystine (half)	4.72 ± 0.09	0.08 ± 0.04	3.87 ± 0.92	0.07 ± 0.03
Isoleucine	17.54 ± 2.68	0.15 ± 0.03	25.42 ± 1.18	0.17 ± 0.03
Leucine	38.90 ± 0.95	1.10 ± 0.17	41.87 ± 1.47	0.21 ± 0.04
Lysine	10.92 ± 1.21	0.20 ± 0.06	12.66 ± 0.36	0.09 ± 0.03
Methionine	1.76 ± 0.34	----[b]	0.94 ± 0.03	----
Phenylalanine	25.76 ± 0.50	0.05 ± 0.01	28.31 ± 0.15	0.05 ± 0
Threonine	20.60 ± 0.33	0.77 ± 0.21	29.18 ± 2.60	0.41 ± 0.07
Tyrosine	12.22 ± 0.62	0.06 ± 0.02	7.50 ± 1.69	0.12 ± 0.03
Valine	25.26 ± 0.48	0.10 ± 0.05	30.41 ± 0.53	0.08 ± 0.03
Nonessential				
Alanine	26.71 ± 4.49	0.37 ± 0.14	29.80 ± 1.82	0.15 ± 0.03
Arginine	12.79 ± 0.26	1.70 ± 1.14	16.58 ± 0.21	0.12 ± 0.03
Aspartic acid	56.72 ± 1.57	2.13 ± 0.59	81.76 ± 1.83	3.45 ± 0.34
Glutamic acid	86.18 ± 2.54	2.29 ± 0.31	57.59 ± 4.32	1.71 ± 0.29
Glycine	25.33 ± 2.79	0.10 ± 0.04	19.32 ± 0.87	0.13 ± 0.03
Histidine	33.63 ± 0.62	0.36 ± 0.11	30.21 ± 1.05	2.07 ± 0.30
Proline	28.67 ± 1.65	0.49 ± 0.15	39.70 ± 1.65	0.41 ± 0.15
Serine	22.80 ± 0.15	0.71 ± 0.17	29.09 ± 0.46	0.98 ± 0.17
Ammonia	34.86 ± 0.21		16.93 ± 0.87	

[a] Mean ± s.e. of three replicates

[b] Not detected.

of 6.25, this corresponds to a protein content of 25.6% for seeds and 11.1% for the tubers. Levels of total and free amino acids in the tubers and seeds are given in Table 1. Aspartic acid was the most abundant amino acid in the tubers, while glutamic acid predominated in seeds. The ratios of these two amino acids in the seeds and tubers were almost exactly reversed. Other amino acids present in substantial amounts in tubers were leucine, proline, valine and histidine in descending order of abundance. In the seeds, the next most prominent were leucine, histidine, proline and alanine.

A recent study of the rare related species, *A. priceana* Robinson, by Walter et al. (1986) indicated that its tubers contained significantly less protein, 6.9%, with a different amino acid profile. Arginine was present in nearly twice the concentration of the next most prevalent amino acid, aspartic acid. In *A. americana* tubers, however, arginine concentration was insignificant compared with aspartic acid concentration. Glutamic acid appeared much less prevalent in *A. priceana* tubers than in those of *A. americana*.

Free amino acids in *A. americana* tubers and seeds were much less abundant than proteinaceous amino acids. The sum of the free amino acids represented about 2% of total amino acids. In seeds, free glutamic and aspartic acids were present in about equal amounts, followed by arginine and leucine. In tubers, aspartic acid was the most prevalent, with histidine, glutamic acid and serine following in descending order of abundance.

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Ms received 6/19/86; revised 9/26/86; accepted 10/17/86.

Acknowledgements:

Michigan Agric. Exper. Station Journal Article Number 12019.

The authors greatly appreciate the advice and cooperation of Dr. T.G. Isleib of this University and the partial support of this work by the Coca-Cola Co.

A Research Note

Microbial Production of Citric Acid by Solid State Fermentation of Kiwifruit Peel

Y.D. HANG, B.S. LUH, and E.E. WOODAMS

ABSTRACT

A solid-state fermentation method was developed for the production of citric acid from kiwifruit peel by *Aspergillus niger* NRRL 567. This method produced about 100g citric acid per kg of kiwifruit peel fermented in the presence of 2% methanol at 30°C in 4 days. The yield was more than 60% based on the amount of fermentable sugar consumed.

INTRODUCTION

KIWIFRUIT PEEL is a by-product resulting from the manufacture of kiwifruit into nectars or slices and represents nearly 10-16% of the weight of the original fruit, depending on the peeling method used. Current disposal of it poses considerable economic and environmental problems.

Citric acid is a commercially valuable product and has been produced primarily by submerged fungal fermentation of a sucrose or molasses medium (Kapoor et al. 1982). Recently Hang and Woodams (1984, 1985) reported that *Aspergillus niger* produced large amounts of citric acid when grown on apple and grape pomace under solid-state fermentation conditions. Solid-state fermentations refer to the cultivation of microorganisms on solid materials in the absence of free liquid and have been used for centuries in the Orient for the preparation of a variety of fermented food products (Hesseltine, 1972). The major advantages of using solid-state fermentation rather than submerged fermentation include (1) the yields are much higher than those in liquid media, (2) the space taken up by the fermentation vessel required is small relative to yield of product because less water is used and the substrate is concentrated and (3) the operating costs are much lower than those for liquid phase fermentation (Hesseltine, 1972).

The objective of this study was to determine the feasibility of citric acid production from kiwifruit peel by solid-state fermentation.

MATERIALS & METHODS

Substrate

The kiwifruit peel used throughout this study was prepared by the method of Wilman and Luh (1981) in a pilot-plant kiwi nectar processing unit. It was dried in a hot air dehydrator at 50°C for 60 hr to a moisture content of about 7% and stored at room temperature (22–25°C) until needed. Prior to its use, the dried peel was ground through the 0.125 cm screen of a Fitz Mill, Model D. (J. W. Fitzpatrick Co., Chicago, IL).

Cultures

Five citric acid-producing strains of *Aspergillus niger* were obtained from Dr. C.W. Hesseltine, Northern Regional Research Center, U.S. Dept. of Agriculture, Peoria, Illinois. Each culture was grown on a potato dextose agar slant at 33°C for 7 days. A spore inoculum was prepared by adding 3 ml sterile distilled water to the slant and shaking vigorously for 1 min.

Solid-state fermentation

Portions of 30g of dried kiwifruit peel were rehydrated with distilled water in 500 ml Erlenmeyer flasks to give a moisture of 65%. Each flask was inoculated with 0.2 mL of an appropriate spore inoculum and incubated at 30°C for 5 days. Methanol, 0–4%, was added to the flasks before fermentation. At the end of the fermentation, the fermented materials were extracted with distilled water, and the extracts were analyzed for residual sugar and citric acid.

Analytical methods

Moisture, protein (N × 6.25), fat, fiber and ash were determined by the AOAC (1960) methods. The sugar was analyzed as glucose by the phenolsulfuric acid method of Dubois et al. (1956) and the citric acid of the fermented pomace was determined by the colorimetric method of Marier and Boulet (1958).

RESULTS & DISCUSSION

THE KIWIFRUIT PEEL used throughout this study was found to contain the following: moisture, 6.6%; crude protein (N × 6.25), 6.4%; crude fat, 1.4%; crude fiber, 16.2%; crude ash, 5.2%; reducing sugar as glucose 44.2% and nitrogen-free extract (by difference), 20%.

Of the five citric acid-producing strains of *A. niger* (NRRL 328, NRRL 567, NRRL 599, NRRL 2001, and NRRL 2270) examined, strain NRRL 567 was found to produce the greatest amount of citric acid from kiwifruit peel. This culture has also been shown by Hang and Woodams (1984, 1985) to give a much higher yield of citric acid from apple and grape pomace than did other strains.

Methanol has an enhancing effect on fungal production of citric acid from kiwifruit peel (Fig. 1). Increasing the concen-

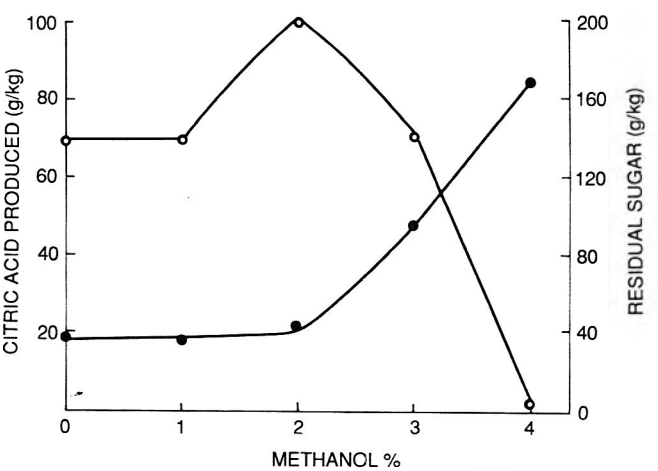

Fig. 1—Effect of methanol concentration on citric acid production from kiwifruit peel by A. niger *NRRL 567: ●——● sugar; ○——○ citric acid.*

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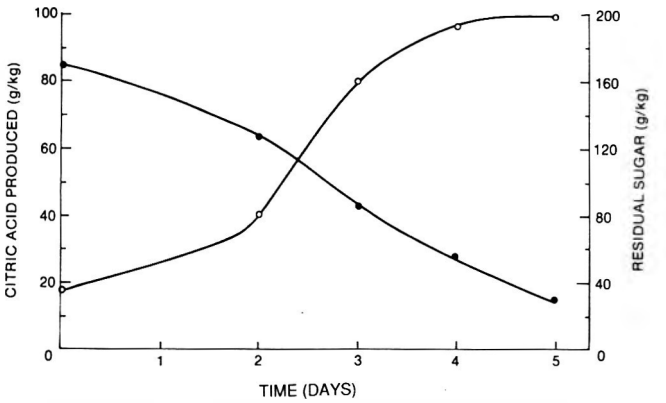

Fig. 2—Time course of citric acid production from kiwifruit peel by A. niger *NRRL 567; ●——● sugar; ○——○ citric acid.*

tration of methanol resulted in a marked increase in the production of citric acid from kiwifruit peel by *A. niger* NRRL 567. The mold produced the greatest amount of citric acid from kiwifruit peel in the presence of methanol at a concentration of 2%. Methanol at a concentration of 3% or higher, however, exerted an inhibitory effect on citric acid production. The influence of methanol in increasing citric acid yields appears to be a general phenomenon with strains of *A. niger* and the use of methanol has become a common practice (Kapoor et al. (1982). Methanol is not assimilated by *A. niger*, and its exact role in stimulating the production of citric acid is still not known. It is likely that methanol affects permeability properties of the mold and enables greater excretion of citric acid (Kapoor et al., 1982).

Figure 2 demonstrates the time course of citric acid production by *A. niger* NRRL 567 grown on kiwifruit peel in the presence of 2% methanol. The production of citric acid approximately paralleled the consumption of sugar. Citric acid production increased rapidly between 2–3 days and reached the maximum level on the fourth day. The sugar was reduced by about 80%. Kapoor et al. (1982) have reported that the time required for maximum citric acid production by submerged fermentation of a sucrose or molasses medium was usually 7–10 days.

When grown on kiwifruit peel in the presence of 2% methanol at different temperatures, the mold produced more citric acid at 30°C than at either 35°C or 25°C. The sporulation of *A. niger*, however, was more noticeable at 35°C than at lower temperatures.

Moisture content of kiwifruit peel had a profound influence on the production of citric acid by *A. niger* NRRL 567 in a solid-state fermentation system. The yields of citric acid based on the amount of sugar consumed varied widely with initial moisture of kiwifruit peel. In the absence of methanol, a sharp increase in citric acid production occurred as the substrate moisture decreased. Methanol markedly enhanced fungal production of citric acid from kiwifruit peel at all the moisture levels tested, but its effect in increasing citric acid yields was more pronounced at higher than at lower moisture.

Hang and Woodams (1984; 1985) reported that the yields of citric acid from apple and grape pomace based on the amount of sugar consumed were about 88% and 60%, respectively. In this work, the yield of citric acid from kiwifruit peel was found to be more than 60% under optimum solid-state fermentation conditions. From the results of this study and those of earlier work (Hang and Woodams, 1984, 1985), it was concluded that fruit processing solid residues can generally serve as a substrate for the production of citric acid by *A. niger* in a solid-state fermentation system.

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Ms received 6/19/86; revised 9/24/86; accepted 10/17/86.

Based on a paper presented at the 46th Annual Meeting of the Institute of Food Technologists, Dallas, TX, June 15-18, 1986.

A Research Note

Catalase, Lipoxygenase, and Peroxidase Activities in Cucumber Pickles as Affected by Fermentation, Processing, and Calcium Chloride

R. W. BUESCHER, C. McGUIRE, and B. SKULMAN

ABSTRACT

Catalase, lipoxygenase, and peroxidase activities were determined in tissues and brines of pickling cucumbers during fermentation, storage, and after processing. Lipoxygenase and catalase were inactivated within three days of exposure to fermentation brines. Peroxidase activity was relatively unaffected by the first three days of brining, but it declined during fermentation, storage, and after processing. $CaCl_2$ reduced the loss of peroxidase activity in tissue and brine during fermentation and storage. Alum in post-desalting brine reduced peroxidase activity in processed pickles although $CaCl_2$ ameliorated its efficacy. No activity of catalase, lipoxygenase or peroxidase was detected in cucumber juice extracts fermented by four different lactic acid bacteria.

INTRODUCTION

CATALASE (CAT), lipoxygenase (LOX) and peroxidase (POD) are present in cucumber (*Cucumis sativus*) fruits (Labee and Esselen, 1954; Wardale and Lambert, 1980). LOX activity is required for the production of aldehydes typical of fresh cucumber flavor (Fleming et al., 1968; Gaillard et al., 1976). In contrast, stale, off-flavors in pasteurized fresh-pack cucumber pickles occur from high levels of residual POD activity (Anderson et al., 1951; Labee and Esselen, 1954). Also, Labee and Esselen (1954) found that CAT supplemented in fresh pack pickle products could cause adverse flavors during storage.

Recently, POD activity was found in several commercial pickle products manufactured from fermented and brine-cured cucumbers (Buescher and McGuire, 1986). Thus, cucumber POD appears to be capable of surviving fermentation, brine storage and processing; however, the influence of these processes on its activity or on the activities of CAT and LOX are unknown. The effects of pasteurization on POD activity in fresh pack pickles have been reported (Anderson et al., 1951; Nebesky et al., 1950). The objectives of this study were to determine the activities of CAT, LOX and POD in cucumber tissues and brines as affected by fermentation, processing, storage, and $CaCl_2$ treatments.

MATERIALS & METHODS

Fermentation and processing

Freshly harvested pickling cucumbers (Carolina cultivar) were washed and sorted for defects and uniformity in diameter (3.2 ± 0.5 cm). Samples were weighed (8.5 kg), placed into 18L plastic buckets and held submerged in 8.5L of brine solution. The initial brine solution contained 10% NaCl, 0.1% potassium sorbate, 0.15% acetic acid, and no $CaCl_2$ (control) or 0.7% $CaCl_2$ adjusted to pH 5.0. Each treatment was replicated four times. *Pediococcus pentosaceus* (Aferm 772, Microlife Technics, Sarasota, FL) was added (0.5 mL) to each container to initiate fermentation. Fermentation was completed within 14 days at 22 ± 2°C. After 1 month additional NaCl was mixed into each container to provide an equilibrated concentration of 10% (wt/wt).

Samples were prepared for processing 75 days after initial brining by partially desalting the weighed pickles in an equal volume of water (1:1 wt/wt) at 50°C for 4.5 hr. Desalted samples from the two fermentation treatments (without and with $CaCl_2$) were each subdivided along with the desalting liquid into four equal portions and held (1) without treatment (control) or treated with (2) 0.3% alum [$ALK(SO_4)_2$], (3) 0.3% $CaCl_2$ or, (4) the combination of 0.3% alum and 0.3% $CaCl_2$ for 18 hr at 25°C. Then, the pickles were passed through a reel spray washer, packed into 710 mL glass jars (8 jars/treatment), covered with a 90°C solution containing 1.5% acetic acid, dill-flavored emulsion and tartrazine (FD&C yellow no. 5), capped, cooled to 30°C and stored at 22 ± 2°C until analyzed. The processed pickles had 1.01% acetic acid, 3.05% NaCl, and pH 3.10 after equilibration.

Fermentation microorganisms

To determine the contribution by fermentative microorganisms to CAT, LOX and POD activity during cucumber fermentation, activated pure cultures of *Lactobacillus brevis*, *Lactobacillus plantarum*, *Leuconostoc mesenteroides* and *Pediococcus pentosaceus* were grown in sterilized cucumber juice broth diluted with one part water and 5% NaCl similar to the procedure described by Fleming and Etchells (1967). In addition, CAT, LOX, and POD activities were determined when cucumbers were held in fermentation brines described earlier except that 0.2% sodium benzoate was included to prevent fermentation.

Assays

NaCl, pH, and titratable acidity were monitored (Buescher et al., 1981; Hudson and Buescher, 1985). Brine solutions and tissues were assayed periodically for enzyme activities during fermentation, storage, and after processing. Methods for extracting tissue and assaying POD activity have been reported (Buescher and McGuire, 1986).

CAT was extracted from cross-sectional slices of fruits by homogenizing in a cold solution of 0.1M sodium phosphate buffered to pH 7.5 with 0.05M citric acid (1g tissue/3 mL buffer). The homogenate was stirred for 2 hr at 4°C, filtered through Miracloth and centrifuged

Table 1—Peroxidase activity in cucumber pickles and brines as affected by fermentation, storage and $CaCl_2$

Days in brine	Peroxidase Activity[a]			
	Tissue		Brine	
	Control	$CaCl_2$	Control	$CaCl_2$
0	34.06	34.06	0.00	0.00
1	35.05	36.07	0.95	0.82
2	34.79	35.43	1.51	1.35
3	32.73	32.54	1.91	1.75
4	22.71	28.78*[b]	2.33	2.28
5	18.55	24.35*	2.36	2.44
6	9.49	18.87*	2.01	2.39
7	4.82	11.29*	1.80	2.41*[b]
10	2.46	5.41*	1.22	2.23*
17	1.70	4.93*	1.01	1.83*
24	1.22	4.53*	0.61	1.59*
31	0.74	4.21*	0.48	1.19*
38	0.47	3.76*	0.32	0.80*
45	0.18	3.60*	0.13	0.42*
59	0.05	2.68*	0.03	0.32*
80	0.03	1.19*	0.01	0.21*
130	0.02	0.19*	0.00	0.08*
180	0.01	0.13*	0.00	0.05*

[a] Peroxidase activity is expressed as mmoles of H_2O_2 decomposed/g tissue or ml of brine/min.

[b] * Indicates that activity of control and $CaCl_2$ treatments was significantly different ($P<0.05$).

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Table 2—Effect of fermentation, processing and storage on peroxidase activity in processed pickles[z]

	Peroxidase activity in processed pickles[y]							
	1 Month after processing				5 Months after processing			
	Tissue		Brine		Tissue		Brine	
Post-desalting treatment	Treatment of fermentation brines							
	Control	$CaCl_2$	Control	$CaCl_2$	Control	$CaCl_2$	Control	$CaCl_2$
Control	32ab[x]	1448a	5a	169a	0	406a	0	90a
Alum	11c	581c	3a	30c	0	45c	0	5c
$CaCl_2$	42a	1534a	4a	148a	0	518a	0	98a
Alum and $CaCl_2$	21b	845b	3a	84b	0	244b	0	42b

[z] Cucumbers were fermented and stored in brines containing no $CaCl_2$ (control) or 0.35% $CaCl_2$ for 75 days, then partially desalted and exposed to post-desalting treatments of no treatment (control), 0.3% alum, 0.3% $CaCl_2$, or the combination of alum and $CaCl_2$. The pickles were packed in jars, covered with fresh 90°C process brine, sealed, cooled, and stored at 22°C for 1 and 5 months.

[y] Peroxidase activity is expressed as micromoles of H_2O_2 decomposed/g tissue or ml brine/min.

[x] Means within columns with the same letter are not significantly different ($P<0.05$).

for 10 min at 10,000 × *g*. The supernatants from tissue extracts and brine samples were assayed for catalase activity by measuring at 240 nm, 25°C and pH 7.0 the linear rate of hydrogen peroxide degradation (Anonymous, 1979).

LOX was extracted from cross-sectional slices of fruits by homogenizing in cold 0.1M citrate-phosphate buffer, pH 6.5, containing 4 mM dithiothreitol and 0.2% Triton X-100 (1g tissue/3 mL buffer). The residue remaining after centrifugation at 10,000 × *g* for 10 min was re-suspended in buffer and centrifuged. The combined supernatant solution was assayed for LOX activity by measuring the rate of O_2 consumption (polarographic O_2 electrode) during linolenic acid oxidation at 30°C (Wardale and Lambert, 1980). LOX substrate solution contained 1.1 mM linolenic acid, 16 mM $CaCl_2$, and 0.03% Tween 20 in 0.05M MES buffer adjusted to pH 5.5.

RESULTS & DISCUSSION

CAT AND LOX ACTIVITY declined precipitously in cucumbers exposed to fermentation brine. CAT activity (mmoles H_2O_2 utilized/g tissue/min) decreased from 268.4 in fresh tissue to 20.8 in 24 hr, and after 48 hr in brine, no activity was detected. Concomitantly, LOX activity (micromoles O_2 consumed/g tissue/min) was 6.9, 2.5, 0.2 and 0 after cucumbers were in brine for 0, 24, 48 and 72 hr, respectively. $CaCl_2$ in brines did not alter ($P<0.05$) the loss of CAT and LOX activity. No activity of these enzymes was found in the brine solutions.

In contrast, POD activity in cucumber tissue was only slightly affected by three days exposure to fermentation brines; then, it declined steadily for 6 months (Table 1). POD activity was detected in the brine solutions one day after submerging the cucumbers. During the first week of fermentation, the POD activity in brines reached maximum levels and then declined. Compared to the control, $CaCl_2$ treatment of brines caused tissues and brines to have higher POD activity. $CaCl_2$ appeared to have assisted with stabilizing POD activity rather than stimulating its activity, since the differences between the control and $CaCl_2$ treatments became apparent only when activities began declining.

No CAT, LOX or POD activity was observed during fermentation of cucumber juice by *L. brevis, L. plantarum, Leuconostoc mesenteroides* and *Pediococcus pentosaceus*. Also, when cucumber fermentation was inhibited by benzoate the activities of CAT, LOX, and POD in tissues and brines were similar to the activities observed when fermentation was not inhibited (data not shown). Therefore, enzyme activities measured during cucumber fermentation were from cucumber tissue and were not from microbial sources. Previously, Whittenbury (1964) reported that certain strains of lactic acid bacteria produce CAT; however, Gregory and Fridovich (1974) were unable to detect CAT or POD activity in cultures of *L. plantarum*.

POD activity was present in brines and tissues of processed pickles (Table 2). Compared to untreated brines, $CaCl_2$ in fermentation and post-desalting brines caused POD activities to be higher in the processed pickles and their brines. Treatment with alum consistently reduced POD activity in tissues, although the reduction was attenuated when alum was used in combination with $CaCl_2$. Activity in all treatments declined during storage, resulting in the complete loss of detectable activity in pickles processed from fermentation brines untreated with $CaCl_2$.

In summary, CAT and LOX were rapidly inactivated in cucumbers exposed to fermentation brines. POD activity in tissues and after being released from tissues into brines gradually declined regardless of fermentation and processing treatments. $CaCl_2$ retarded POD inactivation, and inactivation was enhanced by alum.

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Ms received 6/19/86; revised 9/26/86; accepted 9/26/86.

This study was partially supported by research grants from Dow Chemical, USA and Pickle Packers International, Inc. The assistance of Green Bay Food Co. and Microlife Technics, Inc. in providing supplies for this research is gratefully acknowledged.

A Research Note

Effect of Various Cellulases and Pectinases on Viscosity Reduction of Mango Pulp

H.K. SREENATH, A.M. NANJUNDASWAMY, and K.R. SREEKANTIAH

ABSTRACT

Microbial cellulases and pectinases from various sources exhibited different degrees of activity when employed to reduce the viscosity of mango pulp. Ultrazym 100 rapidly reduced mango pulp viscosity up to 82% (unfiltered) and yielded pulp juices which could be filtered or centrifuged easily. Enzymatic liquefaction of mango pulp was influenced by temperature, enzyme concentration, and time of reaction.

INTRODUCTION

MANGO (*Mangifera indica* L.) is an important commercial fruit which serves as a raw material for several food processing industries in India. Recently, juice related products developed from this fruit (nectar, juice, jam, jelly, powder, fruit bars and flakes) have opened new avenues for economic utilization of pulp, a by-product of the canning industry and surplus fruit. To date, no means of improving juice recovery from pulps have been developed. Many modern processes for fruit and vegetable juice production employ enzymes as important processing aids to obtain higher juice yields and juices with higher soluble solids (Grampp, 1969; Rombouts and Pilnik, 1978, 1980). Although pectinases have been the principal enzymes used, mixtures of cellulolytic and pectinolytic enzymes are increasingly employed for partial or complete liquefaction of fruits and vegetable pulps (Dongowski and Bock, 1977; Kilara, 1982; Sreenath et al., 1984; Voragen et al., 1980). During liquefaction, cell walls are degraded leading to the release of cell contents which may be recovered in high yield (Sreenath et al., 1984). Exotic fruits and fruit pulps are obvious materials for further testing with selected enzyme preparations. An attempt was made in this investigation to optimize enzymatic treatment of mango pulp to obtain a product with low viscosity and low pulp content.

MATERIALS & METHODS

ENZYMES evaluated were: Rohament P and pectinol B_1 (Rohm GmbH., West Germany), cellulase and macerozyme (Kinki Yakult Co., Japan), ultrazym 100, pectinex ultra special and pectinex (Novo Industries, Denmark). Pectinase from *Aspergillus carbonarius* strain 1047 and cellulase from *A. oryzae* strain 55 were produced in our laboratory by solid state fermentation.

Filter paper cellulase {FPC} degrading activity (Mandels et al., 1976), pectin viscosity reducing activity {PVR} (Sreekantiah et al., 1971) and protein (Lowry et al., 1951) of the enzymes were determined. In a typical treatment, 0.25 kg of mango pulp (Totapuri variety, pressed, canned and stored for 6 months at +4°C) was stirred with 0.05% enzyme (w/v protein) and incubated at 40°C for 30 min. The treated pulp was then placed in a boiling water bath for 5–10 min to inactivate the enzyme. After cooling to room temperature (25°C), viscosity of the treated pulp was measured with a Brookfield viscometer (Brookfield Engineering Lab. Inc, Stoughton, MA). Later, the pulp was filtered through muslin cloth or centrifuged (after filtration) at 3.5×10^4 g for 10 min and viscosity was measured again. Factors affecting enzymic liquefaction of mango pulp, such as temperature, enzyme concentration and period of reaction were studied to optimize the viscosity reduction of pulp. On a pilot scale, batches of 30 kg mango pulp were treated with 0.05% enzyme (w/v protein) at 40°C for 30 min. The enzyme treated pulp was either filtered through a muslin cloth or centrifuged (after filtration) at 3.5×10^4 g for 10 min. The quantity of juice obtained and the viscosity, before and after filtration or centrifugation, were determined.

In each experiment, as a control, mango pulp was incubated under identical conditions without enzyme and viscosity measurements were made. Based on viscosity of the control mango pulp, the % reduction in viscosity of treated pulp due to added enzyme was calculated.

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RESULTS

ENZYMES employed in the present study differed in their FPC and PVR activities (Table 1). Ultrazym 100 was effective in reducing the viscosity of mango pulp much more rapidly than did other enzymes before filtration of the treated sample (Table 1). Pectinase produced by *A. carbonarius* and cellulase from *A. oryzae* failed to show the viscosity reduction that Ultrazym 100 did. Viscosity reduction of the pulp significantly increased with temperature between 25°C and 60°C as a function of Ultrazym treatment (Fig. 1). At 40°C, it was possible to obtain 82% reduction in viscosity with 0.05% enzyme and 30 min incubation. At 60°C, the decrease in viscosity was more rapid, but the 30 min viscosity was essentially the same as that obtained at 40°C.

The pH of the mango pulp (3.5) was not altered during enzyme treatment to maintain pulp quality. An enzyme concentration up to 0.05% (w/v) was found to be optimum for Ultrazym for maximum viscosity reduction of mango pulp (data not shown). A further increase in enzyme level had no significant affect on viscosity.

On pilot plant scale when batches of 30 kg of mango pulp

Table 1—Influence of filter paper degrading (FPC)[a] and pectin viscosity reducing (PVR)[b] activity of various enzymes on viscosity reduction of mango pulp

Enzyme	Specific activity (units/mg protein) FPC	PVR	Loss of viscosity before filtration (%)
Ultrazym 100	878	687	82
Pectinex	300	488	73
Pectinex ultra Special	209	213	74
Cellulase	1424	112	69
Macerozyme	530	317	71
Rohament P	530	1867	65
Pectinol B_1	925	806	69
Cellulase[c]	411	74	64
Pectinase[c]	330	217	41
Control[d]	—	—	0

[a] One unit of FPC activity is the amount of enzyme which catalyzed the formation of 1 μmol glucose/min under assay conditions (Mandels et al., 1976).
[b] One unit of PVR activity is the amount of enzyme which effected a 1% reduction in viscosity of a pectin solution under assay conditions (Sreekantiah, et al., 1971).
[c] Cellulase isolated from *A. Oryzae* and pectinase from *A. carbonarius*.
[d] Mango pulp was incubated without enzyme at 40°C for 30 min.

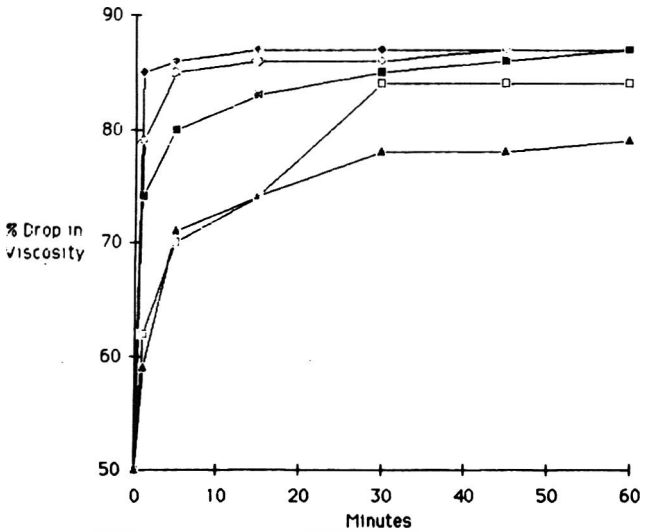

Fig. 1—Effect of temperature and period of reaction on viscosity reduction of mango pulp by Ultrazym 100: 60°C (—♦—), 50°C (—◇—), 40°C (—■—), 30°C (—□—) 25°C (—▲—).

were treated with Ultrazym 100 under optimum conditions (enzyme 0.05% (w/v) at 40°C for 30 min), 24 kg juice were obtained after filtration. The pulp of the filtered juice could be lowered by centrifugation to obtain a clear juice in a yield of 21 kg. On the contrary, the yield of juice without enzyme treatment was zero.

DISCUSSION

THE MANGO is relished throughout the world for its succulence, exotic flavor and delicious taste. Mango pulp, a base for the manufacture of several commercial products contains predominently carbohydrates rich in sugar, starch, celluloses and pectic substances (Subramanyam et al., 1975) and has a high viscosity. Technical preparations of pectinases and cellulases are mixture of many carbohydrates and it is impossible to assess the role of the separate activities. In other words, the reduction in pulp viscosity might not have been due solely to the enzymic activities assayed such as cellulase and pectinase (Table 1). The suitability of Ultrazym 100 in effecting rapid viscosity reduction of mango pulp, leading to maximum juice recovery under suitable conditions could be attributed to the presence of a well co-ordinated system of cell wall degrading enzymes and their synergistic activity in liquefaction (Sreenath et al., 1984).

Use of Ultrazym 100 also makes it possible to prepare nectar bases by first separating the pulp from the juice, then concentrating the juice and finally adding the pulp back to the concentrated juice which on reconstruction shows a stable cloud (Grampp, 1969). Commercially, mango juices and concentrates (low pulp with original aroma) are in high demand by the food and confectionary industry (Subramanyam et al., 1975), and this enzyme treatment can significantly improve juice yield and quality.

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Ms receivved 7/7/86; revised 9/8/86; accepted 9/12/86.

The authors thank Dr. B.L. Amla, Director, Central Food Technological Research Institute, Mysore, for his keen interest and helpful suggestions.

A Research Note

Prediction of Residual Peroxidase Activity in the Blanching-Cooling of Corn-on-the-Cob and Its Relation to Off-Flavor Development in Frozen Storage

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ABSTRACT

Through the use of a previously described thermo-kinetic model the average peroxidase activity retention in the kernel and outer cob during blanching-cooling of corn-on-the-cob is predicted. The residual peroxidase activity is correlated with sensory evaluation data, making it possible to predict the conditions under which there will be no off-flavor development after a 9 month storage at −18°C. The plots obtained allow for prediction of frozen storage life when processing variables are known. In addition, prediction of storage life can be made whether the product is water cooled at temperatures between 2 and 20°C or is air cooled at ambient air temperature.

INTRODUCTION

CORN-ON-THE-COB is one of the most difficult vegetables to effectively blanch because of its large dimension and the structural characteristics of its different parts. Corn-on-the-cob is normally blanched in steam; as heat is transferred by unsteady state conduction, long blanch times are necessary to inactivate the enzymes at all points within the cob. It is not yet known which enzymes are directly involved in the reactions producing off-flavor. Wagenknecht (1959) determined that off-flavor development of inadequately blanched corn-on-the-cob is due, at least in part, to the action of residual lipoxygenase. Bottcher (1975) established the optimal relationship between residual peroxidase activity and quality in frozen storage for several blanched vegetables. Lee and Hammes (1979) determined significant correlations between peroxidase residual activity in the outer cob and kernels of corn-on-the-cob blanched at 100°C for variable times, and off-flavor development detected by a panel of twelve experts after a 9 months storage at −18°C.

Garrote et al. (1985) performed a thorough study of the thermal inactivation of peroxidase and lipoxygenase enzymes at different locations in corn-on-the-cob, and Luna et al. (1986) developed a thermokinetic model describing peroxidase inactivation during blanching-cooling of corn on the cob.

The purpose of the present work is to connect the thermokinetic model predicting tool with experimental data obtained from the sensory evaluation of the frozen corn. Peroxidase residual activity is predicted for any blanching-cooling condition or corn-on-the-cob dimension. This information is linked to off-flavor development in frozen storage, as a method to predict product stability in relation to blanching-cooling variables employed.

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THEORY

PEROXIDASE thermal inactivation may be assumed as a first order reaction. Therefore:

$$\frac{dx}{dt} = -kx \qquad (1)$$

The specific constant of the rate of reaction, k, depends on temperature and varies at each point of the corn-on-the-cob, according to Arrhenius' equation (Garrote et al., 1985). Taking into account that the inactivation occurs during the heating and cooling steps, after integrating it yields:

$$x = \exp\left\{-k_{ref}\left\{\int_0^{t_h} \exp\left[-\frac{E_a}{R_g}\left(\frac{1}{T_h}-\frac{1}{T_{ref}}\right)\right]dt + \int_{t_h}^{t_h+t_c} \exp\left[-\frac{E_a}{R_g}\left(\frac{1}{T_c}-\frac{1}{T_{ref}}\right)\right]dt\right\}\right\} \qquad (2)$$

In Eq. (2), k_r and E_a as well as the temperature profiles during the heating-cooling steps, should be determined in order to calculate peroxidase retention. The model of Luna et al. (1986) has been applied in order to estimate peroxidase activity retention in the kernel and in the outer cob, having made the following assumptions: (a) corn-on-the-cob is considered as a finite cylinder made up of three layers (kernels, outer cob, and central cob); the three layers are assumed to have the same ρ, C_p, and K (Luna et al., 1986), making it possible to apply a homogeneous solution; (b) blanching is performed in steam at 80, 90, and 100°C; (c) Cooling is performed in stirred water at 2°C (Garrote et al., 1985); (d) Blanching and cooling times are equal (Lenz and Lund, 1977; Coffelt and Winter, 1973); (e) The thermal diffusivity in heating is equal to $\alpha_h = 1.40 \times 10^{-7}$ m²/sec and in cooling is equal to $\alpha_c = 1.10 \times 10^{-7}$ m²/sec (Luna et al., 1986); (f) The values of k_r and E_a employed are $k_{80°C} = 0.0057$ sec^{-1}, $k_{90°C} = 0.0134$ sec^{-1} and $k_{100°C} = 0.0221$ sec^{-1} and 19 Kcal/mol (Luna et al., 1986); (g) Each zone of the corn-on-the-cob constitutes the following percentages in weight: 60% (kernel), 27% (outer cob), and 13% (central cob); (h) The corn-on-the-cob diameters are: 40, 45, 50, and 55 mm, and 125 mm long.

MATERIALS & METHODS

The characteristics of the raw material employed (Jubilee variety), of the analytical determinations and of the blanching-cooling treatments are presented in Garrote et al., (1985) and Luna et al. (1986).

Table 1—Predicted average peroxidase activity (%) in kernel as a function of cooling water temperature and processing time.[a]

Cooling temp (°C)	Processing time ($t_h + t_c$), (min)			
	8	16	24	32
2	51.06	17.35	3.49	0.37
10	50.63	16.90	3.32	0.34
20	49.91	16.16	3.05	0.30
30	48.91	15.16	2.68	0.26

[a] (d = 45 mm., T_s = 100°C, T_i = 20°C, $t_h = t_c$).

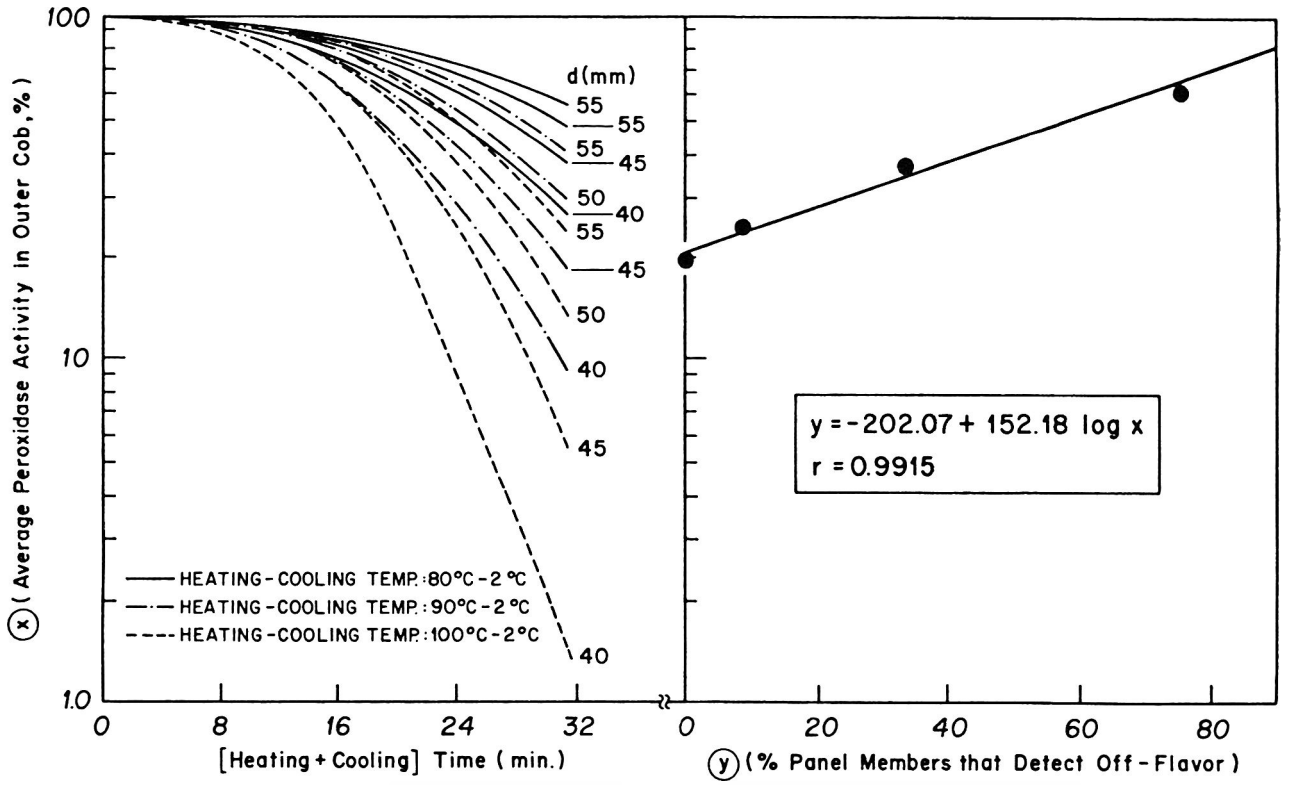

Fig. 1—Average peroxidase activity in outer cob (%), for several processing conditions, and its relation to off-flavor development in frozen corn-on-the-cob at −18°C after 9 month storage. (T_s = 80, 90 and 100°C; d = 40, 45, 50, and 55 mm.; T_w = 2°C and T_i = 20°C).

The sensory evaluation data were taken from Lee and Hammes (1979). Twelve expert (trained) panelists constituted a sensory evaluation panel to evaluate corn-on-the-cob samples stored for 9 months at −18°C. Product samples had been blanched for different times in 100°C steam and were water cooled.

The correlation obtained from Lee and Hammes data (1979) between residual peroxidase activity retention and sensory evaluation was verified for the Jubilee variety by quality control department personnel of the major corn-on-the-cob freezing plant of Argentina (Garrote, 1983).

RESULTS & DISCUSSION

THE MODEL of Luna et al. (1986) was verified for a stirred water cooling temperature of 2°C (Biot > 100). It is interesting to evaluate the influence that other cooling temperatures may have on peroxidase retention. Table 1 illustrates the predicted peroxidase retentions in the kernel at different cooling temperatures, the blanching temperature and the corn-on-the-cob diameter being kept constant. Similar results were obtained for other zones and diameters of corn-on-the-cob and for different blanching temperatures.

On the other hand, if the cooling step were performed in forced air at ambient temperature and 3 m/sec velocity, the cooling profile would be very similar to that obtained by cooling in water at the same temperature (Coffelt and Winter, 1973); this is due to the evaporation of superficial water which remarkably accelerates the cooling of the blanched vegetable. Even though there is a weight loss that may be significant, in the case of corn-on-the-cob it is not so important because it is sold by unit and not by weight (Bomben et al., 1975).

Coffelt and Winter (1973) and Carroad et al. (1980) have pointed out the possibilities of cooling in air. Therefore, if cooling in air follows a thermal pattern similar to cooling in water at 20°C, it is expected that the results generated by this work would also be applicable to corn-on-the-cob cooling in air.

The average peroxidase retention in the outer cob as a function of the processing conditions and how this residual activity correlates with the sensory evaluation of the frozen corn-on-the-cob after 9 months at −18°C is shown in Fig. 1. For kernels the linear regression equation obtained is $y = -4.51 + 66.66 \log \times$ ($r = 0.9887$). In order to achieve total acceptability (no off-flavor development), it is necessary to attain a residual activity not greater than about 20% in the outer cob (1% in kernels); it is easily seen that a temperature of 100°C and a processing time of 32 min will permit the attainment of stability in the storage of all sizes of corn-on-the-cob; for certain sizes (40 to 45 mm) a shorter time could even be used. For the other temperatures, except for 90°C, and for a corn-on-the-cob size of 40 to 45 mm. and processing time of 32 min, off-flavor development for any processing time is expected.

In conclusion, a rapid approximation of the stability possibilities of frozen corn-on-the-cob as a function of cob diameter and processing conditions (if the peroxidase initial activities are similar to those used in this research) can be obtained from Fig. 1.

NOMENCLATURE

Biot	Biot number
C	Enzyme concentration or activity at time "t".
C_o	Initial enzyme concentration or activity
C_p	Specific heat
d	Diameter
E_a	Activation energy
k	Rate constant at temperature T
k_{ref}	Rate constant at temperature T_{ref}
K	Thermal conductivity
R_g	Gas law constant
t	Time
t_c	Cooling time

—Continued on page 235

A Research Note

Effects of Chlorophyll and β-Carotene on the Oxidation Stability of Olive Oil

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ABSTRACT

Virgin olive oil was purified by silicic acid column chromatography to remove non-triglyceride components. The effects of chlorophyll and β-carotene on the oxidation stability of purified olive oil were studied by a combination of measuring peroxide value and oxygen disappearance in the headspace of sample bottles by gas chromatography. Chlorophyll in the purified oil acted as a photosensitizer for singlet oxygen formation under light. β-Carotene minimized lipid oxidation of purified oil under light storage by its light-filtering effect. Experiments clearly suggested that singlet oxygen was mainly responsible for the photooxidation of the oil containing chlorophyll.

INTRODUCTION

VIRGIN OLIVE OIL, which is obtained by pressing olive fruits, is one of the few oils that are consumed without any further refining process. It gives a characteristic rich flavor and yellow green color due to the presence of chlorophyll, carotenoid and many other minor components. Since chlorophyll and β-carotene have been reported to play important roles in generating or quenching singlet oxygen in model systems (Foote and Denny, 1968; Foote et al., 1970; Endo et al., 1984), the effects of chlorophyll and carotene on lipid oxidation have been a great concern to food scientists. The objective of this investigation was to study the effects of chlorophyll and β-carotene on the oxidation of olive oil in the presence of light during storage.

MATERIALS & METHODS

VIRGIN OLIVE OIL (VO) which was imported from Calamate, Greece was obtained from a local supermarket. The purified olive oil (PO) was obtained by passing 700 mL VO through a chromatographic column (600 mm × 40 mm i.d.) packed with 280g silicic acid (100 mesh, Mallinkrodt) which was activated by the method of Sahasrabudhe and Chapman (1961) and 70g of 2:1 mixture of activated charcoal (J.T. Baker Chemical Co.) and diatomaceous earth (John Manville Products). Peroxides, free fatty acids, phosphorus, chlorophyll and β-carotene in both oils were determined by AOCS (1980) methods. Chlorophyll and β-carotene were obtained from Sigma Chemical Co. (St. Louis, Mo.).

Air-tightly sealed 30 mL serum bottles containing 15g of PO and different levels of chlorophyll or β-carotene were placed on the wire netting which was 20cm above the Sylvania cool-white fluorescent light. The light intensity at the sample level was 4,000 lux and the temperature of samples was 25°C. All samples were analyzed in duplicate.

To study the mechanism of minimizing PO oxidation by β-carotene under light storage, the light-filtering effect of β-carotene was examined. The PO sample bottles were placed on the wire netting and the PO with or without 100 ppm β-carotene was placed between the wire netting and the fluorescent light.

Oxidation stability of sample was determined by a combination of measuring peroxide value of oil and the oxygen disappearance in the headspace of sample bottle by gas chromatography. Peroxide values of samples were determined by AOCS (1980) method. One milliliter of headspace vapor from the sample bottle was removed with a 2 mL gas tight syringe (Hamilton Co., Reno, NV) and injected into a Hewlett Packard 5880A Gas Chromatograph equipped with a thermal conductivity detector and an electronic integrator. A stainless steel column (1.83 m × 0.3 cm i.d.) packed with 80/100 mesh Molecular Sieve 13× (Alltech Associates, Inc., Deerfield, IL) was used and nitrogen gas flow rate was 20 mL/min. The temperatures of injection port, oven and detector were 200°, 35° and 250°C, respectively. The concentration (μmoles) of oxygen in the headspace was calculated by the linear regression analysis of standard curve obtained for the relationship between gas chromatographic peak area and oxygen volume injected.

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RESULTS & DISCUSSION

THE PO did not contain any peroxides, free fatty acids, phosphorus, chlorophyll or β-carotene. The VO contained 0.6% free fatty acids, 6 ppm phosphorus, 8 ppm chlorophyll and 9 ppm β-carotene. Coefficients of variation for PV and headspace oxygen analyses were 2% and 3%, respectively.

Table 1 shows that the addition of chlorophyll resulted in higher peroxides formation and greater oxygen disappearance in the headspace of PO stored under light. Chlorophyll added to the PO thus acted as prooxidant under light storage. The greater was the amount of chlorophyll in the PO, the less was the oxidation stability of oil. Even though results are not presented here, another experiment showed that different levels of chlorophyll added to the PO did not act as prooxidant under dark storage. This agrees with the reports that chlorophyll worked as a sensitizer to generate singlet oxygen in the photooxidation in the model system (Rawls and Van Santen, 1970; Clements et al., 1973; Carlsson et al., 1976).

Table 2 shows that the higher was the concentration of β-carotene in the PO, the lower was the peroxides formation in the PO and the higher was the residual oxygen in the headspace of the bottle under light. This suggested that β-carotene minimized the lipid oxidation of PO which did not contain sensitizers such as chlorophyll under light. The minimization of PO oxidation by added β-carotene under light must not be due to singlet oxygen quenching and/or free radical antioxidant effects because PO does not contain chlorophyll for singlet ox-

Table 1—Effects of chlorophyll on the peroxide formation and headspace oxygen of purified olive oil under light at 25°C

Storage time (hr)	P V (meq/kg Oil) chlorophyll (ppm) 0	2	4	Headspace oxygen (μmoles O_2/mL Headspace) chlorophyll (ppm) 0	2	4
0	0.00	0.00	0.00	9.51	9.51	9.51
3	0.60	3.95	4.72	9.47	9.10	8.93
6	0.70	4.68	6.65	9.44	8.79	8.33
10	1.15	5.65	7.10	9.34	8.48	8.14
16	1.95	7.10	8.90	9.33	7.98	7.58
28	2.10	8.90	11.60	9.03	7.10	6.52
40	3.15	11.30	13.80	8.67	6.43	5.92
52	3.72	13.75	15.60	8.61	5.92	5.34
70	4.50	14.75	17.20	8.35	5.23	4.55

Table 2—Effects of β-carotene on the peroxide formation and headspace oxygen of purified olive oil under light at 25°C

Storage time (hr)	P V (meq/Kg Oil) β-Carotene (ppm) 0	0[a]	5	10	20	Headspace Oxygen (μmoles O_2/mL Headspace) β-Carotene (ppm) 0	0*	5	10	20
0	0.00	0.00	0.00	0.00	0.00	9.43	9.43	9.43	9.43	9.43
12	1.73	0.65	1.09	0.98	0.76	9.13	9.23	9.26	9.33	9.29
24	3.22	0.90	1.70	1.80	1.15	8.41	9.19	8.68	8.83	8.91
36	4.40	1.09	2.70	2.50	2.09	7.99	9.08	8.33	8.37	8.53
48	5.15	—	3.28	2.60	2.42	7.75	—	8.31	8.34	8.49
60	6.50	1.60	4.31	3.46	2.75	7.22	8.92	7.98	8.07	8.39
72	6.56	—	4.52	3.64	3.75	7.00	—	7.76	7.93	7.99
84	7.80	3.60	5.20	4.95	4.02	6.80	7.74	7.62	7.50	7.92

[a] By placing PO with 100 ppm β-carotene between the wire netting and fluorescent light.

ygen production and β-carotene is not a free radical scavenger. The mechanism of minimizing lipid oxidation in the PO by β-carotene under light was further studied by placing the PO with or without 100 ppm β-carotene between the wire netting and fluorescent light. The PO sample bottles on the wire netting above the PO with 100 ppm β-carotene between the wire netting and light showed lower peroxide formation and higher residual oxygen in the headspace than the PO sample bottles on the wire netting above the PO without 100 ppm β-carotene between the wire netting and light as shown in Table 2. Therefore, the oxidation stability of the PO sample on the wire netting above the PO with 100 ppm β-carotene was higher than the PO sample placed on the wire netting above the PO without β-carotene. This suggested that β-carotene in the PO between the wire netting and light filtered out some of the light energy to minimize lipid oxidation of the PO on the wire netting. β-Carotene absorbs light between 400 and 500 nm (Goodwin, 1980) which corresponds to 21% of all energy emitted from light source (Anonymous, 1984). The PO containing β-carotene in the sample bottles will have less energy from light source and thus better oxidation stability than the PO containing no β-carotene.

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*RESIDUAL PEROXIDASE PREDICTION IN CORN-ON-THE-COB. . .*From page 233

t_h	Heating time
T	Temperature
T_c	Cooling temperature
T_h	Heating temperature
T_i	Initial temperature
T_{ref}	Reference temperature
T_S	Heating medium temperature
T_W	Cooling medium temperature
x	Relative retention = $[C/C_o]$
α	Thermal diffusivity
α_c	Thermal diffusivity for the cooling step
α_h	Thermal diffusivity for the heating step
ρ	Density

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The authors are grateful to Secretaria de Estado de Ciencia y Tecnologia de la Republica Argentina, to Consejo Nacional de Investigaciones Cientificas y Tecnicas (CONICET), and to Universidad Nacional del Litoral (UNL) for their financial aid. They are also grateful to Prof. Elsa Grimaldi for her valuable help in editing this manuscript.

A Research Note

Chemical Composition of Distillers' Dried Grains with Solubles (DDGS) from Soft White Wheat, Hard Red Wheat and Corn

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ABSTRACT

The chemical composition of distillers' dried grains with solubles (DDGS) produced from two varieties of soft white wheat, a blend of hard red wheats, and corn was determined. On the average, the concentration of protein increased 2.4–3.1 times, crude fiber 2.6–3.8 times, and lipid 1.4–2.4 times. The carbohydrate decreased by 30–50% in DDGS compared to the corresponding starting grain. The ash in DDGS was 3.8–7.8 times that of the original grain. Many of the differences ($p < 0.05$) in the concentration of lipid, protein, and crude fiber among the starting grains were also present in the DDGS products.

INTRODUCTION

DISTILLERS' DRIED GRAINS with solubles (DDGS) are the principal byproducts of the fermentation of dry milled whole grain to ethanol. DDGS are whole grain with a substantial portion of the starch removed by enzymic and microbial fermentation. The other components in the grain are concentrated by roughly a factor of three in DDGS (Waelti and Ebeling, 1982). The substantially higher protein and dietary fiber relative to the grain from which they are obtained make DDGS a desirable food component (Bookwalter et al., 1984; Tsen et al., 1982). In addition, the marketing of DDGS as a component in processed food would be economically advantageous for the ethanol industry which currently sells the material for animal feed. The compositions of DDGS produced in pilot scale operations from corn (Wu et al., 1981), wheat (Wu et al., 1984), barley (Wu, 1985) and sorghum (Wu and Sexson, 1984) have been reported. The composition of DDGS can vary widely depending on the manufacturing process (Tsen et al., 1983; Wall et al., 1984). Ash tends to be the most variable (Tsen et al., 1982).

The purpose of this study was to compare the proximate composition of DDGS manufactured from two varieties of soft white wheat of differing protein commercially important in the Pacific Northwest to DDGS from a blend of hard red wheats and corn. The change in proximate composition of each of the DDGS products with respect to the starting grains was determined.

MATERIALS & METHODS

DDGS were produced from four different grain products: Hill 81, a cultivar of soft white winter wheat grown in Southeastern Washington which contained a high level of protein (St. John Grain Growers, Inc., St. John, WA); corn (Grade #1, yellow dent); Tyee, a soft white winter wheat grown in Eastern Washington, and a red wheat blend (Grade #1 baker's blend containing 2/3 Weston, a cultivar of hard red winter wheat and 1/3 hard red spring wheat containing one or more of the varieties Fremont, Pilot or Bannock). All grains were from the 1985 crop and except for Hill 81 were purchased from Natural Foods Warehouse, Lynnwood, WA.

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The whole grains were ground through an alpine pin mill, with 100% passing through a U.S. standard No. 16 sieve. The guidelines for enzyme addition provided by the supplier were followed (Anon., 1980). The enzymes Takatherm L-340 and Diazyme L-200 were used (Miles Laboratories, Elkhart, IN) for liquefaction and saccharification. An atmospheric batch liquefaction was conducted. A yeast inoculum of approximately 5 million cells/mL (DADY, Universal Foods, Milwaukee, WI) was added. Fermentation was conducted at 27–30°C. After completion of fermentation, the ethanol was removed by evaporation or distillation and the whole stillage was concentrated to approximately 20% solids and drum-dried. Calculated ethanol yields were 0.37 L/Kg grain for the hard red wheat blend and Hill 81, 0.35 L/kg grain for corn and 0.30 L/kg grain for Tyee. This corresponded to 89%, 84%, and 63%, respectively, of the practical attainable yield of 0.60 L/kg starch for the different grain products.

The grains and the finished DDGS products were analyzed for the following components according to AOAC (1984) methods: moisture, 14.004; ash, 14.006; crude lipid, 14.018; crude fiber, 7.071; and nitrogen, 14.026. The conversion factor of 6.25 was used to determine the protein for corn; 5.70 was used for wheat. Carbohydrate was calculated by difference. Representative samples from each batch were anlayzed at least three times.

Data were analyzed by Student's t-test, one-way analysis of variance and Duncan's New Multiple Range Test (Steel and Torrie, 1960).

RESULTS & DISCUSSION

THE PROXIMATE COMPOSITION of DDGS is presented in Table 1. Compared to whole grain, the concentration of components in DDGS increased by the following amounts: ash, 3.8–7.8 times; lipid approximately 1.4–2.4 times, protein 2.4–3.1 times, crude fiber, 2.6–3.8 times. No significant changes were noted in the lipid of either red wheat or corn DDGS compared to the starting grain. Noncrude fiber carbohydrate decreased 30–50%. Based on neutral detergent fiber values (Dong and Rasco, 1986) and available process data, the following calculated starch conversions were obtained: 100% in the red wheat blend and Hill 81, 94% in corn and 73% in Tyee. Significant differences ($p < 0.05$) in the levels of lipid or protein among the starting grains were also apparent in the DDGS produced from them: in lipid, corn was greater than red wheat, Tyee, and Hill 81; in protein, Hill 81 and red wheat were greater than corn and Tyee. Even though higher levels of ash were found in Hill 81 and red wheat than in corn ($p < 0.05$) prior to fermentation, the levels of ash were similar for all types of DDGS produced in this study.

The levels of lipid, crude fiber and ash measured in corn DDGS were similar to those described by others; however, protein for the DDGS from corn produced in this study was somewhat lower (Wall et al., 1984; Wu et al., 1981; Waelti and Ebeling, 1982). Values for lipid, protein and crude fiber for DDGS from soft white wheat and hard red wheats were similar to the sum of the values reported by Wu et al. (1984) for distillers' grains, centrifuged solids and distillers' solubles fractions.

The calculated starch conversions for a red wheat blend, Hill 81 and corn were similar to those reported by Wu et al. (1981, 1984). However, Tyee which contains a higher level

Table 1—Proximate composition of soft white wheats, hard red wheat, and corn and DDGS products

	N[a]	H_2O (%)	Ash	Lipid	Protein	Crude fiber	Carbohydrate[b]
				% dry basis			
				$\bar{X}$ ± S.D.			
Soft white wheat (Tyee)	4	9.9 ± 0.4 (22)[c]	1.5 ± 0.1 (14)	1.7 ± 0.2 (14)	6.8 ± 0.6 (13)	2.9 ± 0.4 (13)	87.1 ± 1.2
DDGS	4	8.1 ± 1.5 (22)	8.4 ± 1.1 (20)	3.8 ± 1.5 (12)	19.6 ± 1.7 (12)	7.6 ± 1.0 (13)	60.7 ± 3.1
Soft white wheat (Hill 81)[d]	1	9.9 ± 0.5 (6)	1.8 ± 0.1 (6)	1.6 ± 0.1 (6)	14.3 ± 0.3 (3)	2.1 ± 0.2 (3)	80.2
DDGS	3	6.9 ± 3.0 (9)	7.1 ± 0.5 (9)	3.7 ± 0.3 (9)	38.4 ± 2.5 (11)	8.0 ± 0.4 (9)	42.7 ± 2.7
Hard red wheat	3	9.3 ± 0.7 (13)	1.8 ± 0.3 (12)	1.8 ± 0.1 (10)	14.1 ± 0.3 (11)	2.6 ± 0.6 (9)	79.7 ± 1.0
DDGS	3	9.4 ± 3.3 (14)	6.8 ± 0.7 (12)	2.5 ± 0.9 (10)	33.9 ± 4.5 (11)	6.8 ± 1.2 (15)	49.9 ± 4.9
Corn[e]	2	11.2 ± 0.9 (8)	1.3 ± 0.2 (6)	3.7 ± 0.3 (7)	7.4 ± 0.7 (6)	2.1 ± 0.3 (6)	85.5
Corn DDGS	2	10.3 ± 1.5 (6)	10.1 ± 2.2 (6)	9.0 ± 2.3 (6)	23.0 ± 2.0 (7)	6.3 ± 0.4 (7)	51.5

[a] N = number of lots (starting grain) or batches (DDGS). Samples from each lot or batch were analyzed a minimum of three numbers. The means and standard deviations shown were calculated from the mean value for each lot or batch.

[b] Noncrude fiber carbohydrate. Calculated by difference [100 − (% ash + % lipid + % protein + % crude fiber, dry weight basis) = % carbohydrate, dry weight basis]. Contains principally components of dietary fiber and for Tyee DDGS, also unconverted starch. (See text.)

[c] Total number of analyses.

[d] $\bar{X}$ ± S.D. calculated for the number of analyses in parentheses performed on one lot of Hill 81.

[e] $\bar{X}$ ± S.D. calculated for the number of analyses in parentheses performed on 2 lots/batches of corn/corn DDGS.

of starch than other wheat cultivars used in this study was found to be more difficult to enzymically convert using the methodology employed. Conditions of liquefaction would have to be modified to improve the degree of starch conversion for the higher starch substrates by either increasing pressure during liquefaction and/or increasing the length of enzyme treatment.

Ash in DDGS produced in this study was higher than that reported by other investigators for wheat (Wu et al., 1984) and corn (Waelti and Ebeling, 1982) but similar to the level for corn DDGS reported by Bookwalter *et al.* (1984). Variations in processing conditions, principally the reaction conditions for enzymic hydrolysis and amount of the soluble component incorporated into the DDGS, affect ash (Tsen et al., 1982; Bookwalter et al., 1984). The higher the proportion of solubles incorporated into the final DDGS product, the higher was the ash. With the exception of the study by Wu et al. (1984), most investigators do not specify the details of DDGS production. Many investigators received commercial samples from different sources and consequently did not have production information available. Therefore, it was not possible to readily determine what led to the variability in the ash concentration reported by others.

The higher protein and crude fiber of DDGS compared to that of the starting grain could make DDGS nutritionally advantageous as a flour supplement in baking mixes or other food products.

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Ms received 3/3/86; revised 8/25/86; accepted 8/25/86.

Presented at the 46th Annual Meeting of the Institute of Food Technologists, June 15-18, 1986, Dallas, TX.

This study was supported in part by the Washington, Oregon and Idaho Wheat Commissions. Sharon E. Downey was a recipient of the 1985 Miles Citric Acid Fellowship. The authors thank Mr. Orville J. Mayer of St. John Grain Growers, Inc. for the generous donation of Hill 81 soft white wheat.

A Research Note

Reduction in pH and Fermentation Time of Meat Mixtures Containing Dry Acid Whey

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ABSTRACT

The effect of various concentrations of dry acid whey on the pH of ground beef and pork and on fermentation activity of 11 commercial starter cultures added to a beef summer sausage formulation was determined. For every 1% (w/w) addition of dry acid (pH 4.0) whey, a reduction in meat pH of 0.11–0.13 resulted, independent of meat species, initial pH or fat content. Use of 3.5% (w/w) dry acid whey in conjunction with 1% dextrose resulted in a 1–2 hr reduction in the time required for the summer sausage mixture to reach pH 5.0 compared to a mixture without whey. Dry acid whey as a direct acidulant in fermented sausages could result in a savings of time and energy in manufacturing.

INTRODUCTION

TRADITIONALLY FERMENTED SAUSAGES are prepared from formulations containing ground meat, a fermentable carbohydrate, salt and a spice mix. Attempts have been made to eliminate the fermentation process by adding acidulants such as lactic acid or citric acid, glucono-delta-lactone or sodium acid pyrophosphate (Goodfellow, 1979; Everson, 1981). In 1984, the USDA approved the use of 3.5% (w/w) whey powder or whey products in standard-of-identity meat products, and no limits were placed on nonstandard-of-identity meat products.However, meat products containing greater than 3.5% (w/w) whey powder or whey products must be labeled imitation (CFR, 1985). The purpose of this research was to determine the effect of dry acid whey on the pH of ground meat and on the activity of added lactic acid bacterial cultures in a summer sausage formulation.

MATERIALS & METHODS

Effect of added dry acid whey on meat pH

Dry acid whey was obtained from a commercial source (High Acid Whey Powder, Extra Grade A, Product #174-A, Clofine Dairy Products, Inc., Linwood, NJ). The manufacturer specifies an average titratable acidity of 0.65% (w/w), expressed as lactic acid, and a pH of 4.0 for an aqueous solution of the whey Beef and pork having different initial pH values and various fat contents was cut into cubes of approximately 2 cm on a side, then passed through a food grinder equipped with a 9.5 mm plate. Dry acid whey was added to 50g meat samples and mixed by hand. The whey concentrations were 0.5, 1.0, 2.0, 3.0, 3.5, 5.0, 6.0, 7.0, 8.0, and 9.0% (w/w). These mixtures were then ground once through a 4.5 mm plate. Three 10g subsamples of the meat-dry acid whey mixture were homogenized 45 sec with 100 ml distilled water in a Willems Polytron Homogenizer (Model 45TE, Bronwill Scientific, Rochester, NY) at a speed setting of six and the pH determined. Fat content of the ground meat was estimated in triplicate using a Hobart Fat Percentage Indicator (Hobart Mfg. Co., Troy, OH).

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Effect of added whey on fermentation

The effect of adding 3.5% (w/w) dry acid whey on culture activity was determined using a summer sausage formulation containing either beef (pH 5.6 or 5.9) or turkey dark meat (pH 6.2). Eleven different frozen or lyophilized cultures from five different commercial sources were tested. These cultures were marketed for use in fermented sausage and included species from the genera *Pedicoccus*, *Lactobacillus* and *Micrococcus*. Cultures were used within the maximum storage time recommended by the supplier.

Beef (pH 5.6) and beef trim (pH 5.9) were purchased locally, ground through a 9.5 mm plate and then a 4.5 mm plate. Frozen turkey drumsticks were purchased locally, thawed overnight at 4°C, skinned and hand deboned, and ground as previously described. All ground meat was frozen and held at −30°C prior to use.

A summer sausage formulation was used for the two beef and the turkey meats to evaluate the following treatments: (1) nonfermented control containing no added carbohydrate; (2) nonfermented control containing added dry acid whey; (3) fermented control (culture plus 1% dextrose); (4) acid whey plus culture and no dextrose; and, (5) acid whey plus culture and 1% dextrose. Each formulation contained 1 kg meat, 9g spice mix, 25g salt, 35g whey powder (when added) and 10g dextrose (when added). The spice mix contained 130 ppm sodium nitrite and 550 ppm ascorbic acid, on a meat weight basis. No fat adjustment was made in the sausage formulation.

Ground beef or turkey dark meat, spice mix, whey powder and dextrose, when added, were mixed for 1 min at room temperature (20°C) with a harp attachment on an electric mixer. When cultures were added, the manufacturer's recommendations for thawing or reconstituting and for the amount to use were followed. The sausage formulation plus prepared culture were blended for 30 sec.

Ten 100g portions of sausage mix were packed in paper cups, covered with aluminum foil and incubated at the recommended optimum temperature for the culture being tested. Samples were removed hourly up to 10 hr and the pH determined as previously described. The uninoculated samples containing added dry acid whey were held at 10°C. "Fermentation time" was defined as the hours, from zero time, required for the sausage mixes to reach pH 5.0.

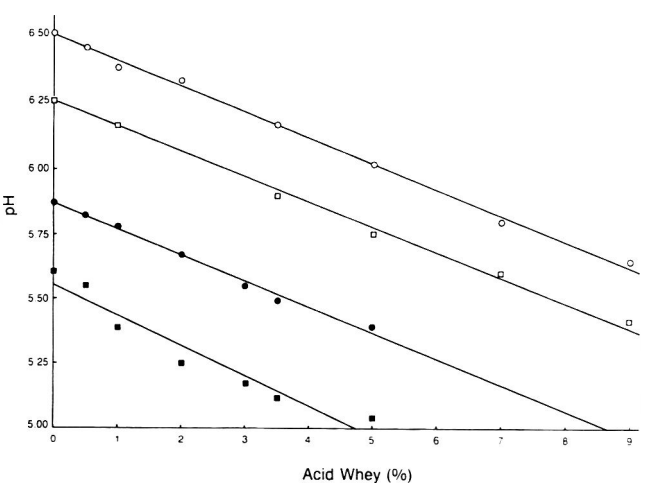

Fig. 1—Relationship of dry acid whey addition and meat pH for selected samples of beef and pork of various fat content and initial pH values. ○ = pork, 16.7% fat; □ = pork, 15.9% fat; ● = beef, 21.0% fat; ■ = beef, 6.3% fat.

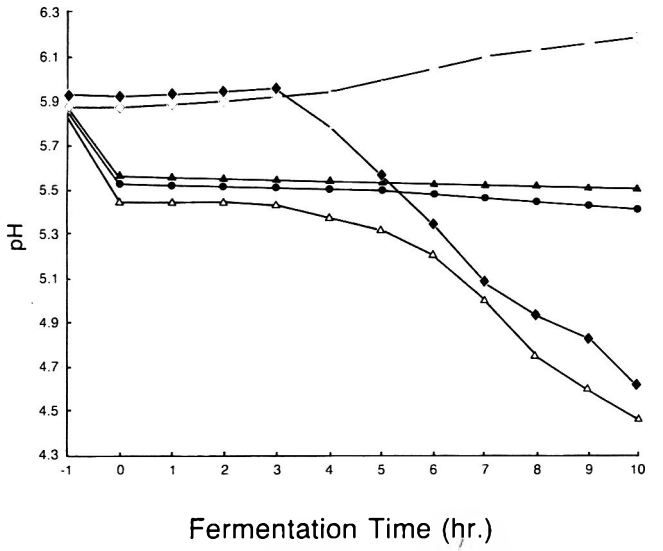

Fig. 2—Fermentation activity of a commercial starter culture in a summer sausage formulation prepared from pH 5.9 beef and containing: ◇ No acid whey, no culture, no dextrose, 43°C (non-fermented control); ▲ Acid whey, no culture, no dextrose, 10°C; ◆ No acid whey,, culture, 1% dextrose, 43°C (fermented control); ● Acid whey, culture, no dextrose, 43°C; △ Acid whey, culture, 1% dextrose, 43°C. Ingredients were added at −1 hr, and incubation of all samples was begun at 0 hr.

RESULTS & DISCUSSION

Effect of acid whey on meat pH

Initial meat pH ranged from 5.5 to 6.5, and fat ranged from 6 to 21%. The reduction in pH of ground beef and pork upon addition of various concentrations of dry acid whey for several of the meat samples tested is shown in Fig. 1. The relationship between meat pH and amount of whey added was linear, and visual examination showed the slopes of the regression lines to be equal. Thus, the effect of acid whey on meat pH was independent of meat species, fat content or initial pH. For each 1% (w/w) addition of dry acid whey, a reduction of 0.11 to 0.13 pH units occurred. Acid whey addition at the allowable maximum of 3.5% (w/w) resulted in a pH reduction of 0.40 to 0.43 units in the raw, ground meat. The linearity of the pH-whey added relationship permits addition of a predetermined amount of dry acid whey to achieve a desired pH based on initial pH of the meat.

Effect of acid whey on fermentation activity

Chemical acidulants, such as acid whey, used in fermented sausage reduce processing time (Jedlicka, 1984). Since commercial cultures are capable of completing sausage fermentation in 6 to 8 hr (Bacus, 1984; Haymon, 1984), 10 hr was chosen in this study as the maximum time allowed for sausage mixtures to reach pH 5.0.

Of the cultures used in this study, those able to ferment lactose and reduce the meat pH to 5.0 within 10 hr also showed improved activity when dextrose was added to the acid whey-meat mixture. As shown in Fig. 2, for one of the cultures tested, the addition of acid whey resulted in an immediate pH reduction in the meat mix of about 0.4 units. For a sample with added dextrose, an apparent lag phase of 3 hr was followed by a generally linear reduction in pH (Fig. 2). Once the organisms for each of the two dextrose-added samples were in the log growth phase, the rate of acid production was not influenced by the presence, or absence of whey. Because of the initial pH reduction by acid whey, the whey-added sample reached pH 5.0 in 1 to 2 hr less fermentation time with 8 of the 11 cultures studied. A similar reduction in fermentation time was obtained with pH 5.6 beef and pH 6.2 turkey meat. Thus, dry acid whey has the potential to reduce time and energy requirements for producing fermented sausages.

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Ms received 9/2/86; accepted 9/15/86.

College of Agriculture and Home Economics Research Center, Washington State Univ., Pullman, WA 99164-6240. Project No. 0297.

A Research Note

Protective Effect of Milk on Mineral Precipitation by Na Phytate

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ABSTRACT

Sodium phytate was added to milk with and without the addition of Fe or Zn and the solubility of the endogenous Ca and added Fe or Zn was not affected. However, when these experiments were repeated with a model system containing $CaCl_2$ at the same calcium concentration as milk, the addition of Na phytate caused a significant precipitation of each of these minerals. These results suggest that milk exerts a protective effect on the phytate induced precipitation of these minerals and thus their potential bioavailability.

INTRODUCTION

IT IS GENERALLY AGREED that osteoporosis is a major cause of disability in the United States among persons of middle and advanced age (Heaney et al., 1982; Seeman and Riggs, 1981; National Dairy Council, 1982). While the question of whether osteoporosis is a nutritional disorder, or not, remains unanswered, several dietary constituents includng Ca have been implicated as risk factors. Effective calcium intake depends not only on the dietary content, but also on the availability of the calcium once ingested (Heaney et al., 1982; National Dairy Council, 1982). Further, solubility is a major factor in bioavailability since all bioavailable forms are soluble under gastrointestinal conditions even though all soluble forms may not be bioavailable. Phytate is one of the dietary constituents that has been shown to decrease both the solubility and bioavailability of Ca (Allen, 1982; McCance, and Widdowsin; 1942a, b) and this effect appears to be more pronounced in the presence of other minerals (Wise, 1983; Subba Rao and Narasinga Rao; 1983; Oberleas et al., 1966).

The objective of the present study was to compare the effects of phytic acid on the solubility and, therefore, the potential bioavailability, of endogenous Ca in milk with its effect on inorganic Ca solubility, in a model system alone and in the presence of added Fe and Zn.

MATERIALS & METHODS

Milk

Skim milk was purchased from a local vendor.

Inorganic salts

Ferrous sulfate heptahydrate was obtained from Fisher Scientific Co. (Medford, MA); zinc sulfate heptahydrate and calcium chloride (anhydrous) were obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ).

Sodium phytate

Sodium phytate (corn crystalline) was purchased from Sigma Chemical Co. (St. Louis, MO).

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Procedure

Milk samples were analyzed for their Ca, Fe and Zn content by atomic absorption spectrophotometry (AAS). Model systems were designed according to the endogenous Ca content of the milk. Na phytate (1.44 mmol) was dissolved in 100 mL double distilled deionized water (DDW) and the pH adjusted to pH 6.5 with 1N HCl. One hundred milliliters of either (a) skim milk (containing 2.88 mmol endogenous Ca) or (b) $CaCl_2$ solution (2.88 mmol Ca), alone and in combination with either 5 mL ferrous sulfate (containing 0.48 mmol Fe) or 5 mL zinc sulfate (containing 0.48 mmol Zn) was added to the Na phytate systems. The systems were equilibrated at room temperature, with constant stirring, for 30 min. The pH was recorded and each system analyzed in duplicate for total and soluble mineral content. In the controls, the final pH was adjusted to those in the milk samples with 0.1N NaOH.

The ratios of Ca:phytate used in this research were based on the findings of Subba Rao and Narasinga Rao (1983) who showed that a molar ratio of 1:2 Na phytate:Ca ($CaCl_2$) caused a decrease in Ca solubility. As well, since it has been shown that Fe and Zn have a synergistic effect on Ca and phytic acid solubility (Subba Rao and Narasinga Rao, 1983; Oberleas et al., 1966) it was decided to add these at levels which created phytate:Ca:Fe and phytate:Ca:Zn ratios which might be found in food.

Analysis of minerals

Atomic absorption standards, reagents and apparatus used for the analysis of minerals have been described by Platt and Clydesdale (1985).

Sample preparation for total mineral analysis by AAS was as follows: A 10 mL aliquot of each sample was pipetted, in duplicate, into separate 100 mL digestion flasks containing 20 mL HNO_3, boiled for 10 min, cooled and made to volume with 0.5% aqueous lanthanum oxide solution in appropriate volumetric flasks.

Sample preparation for soluble mineral content was as follows: A 40 mL aliquot of each sample was centrifuged at 2,335 × *g* for 20 min. Immediately after centrifugation, the supernatant was decanted from the insoluble fraction and filtered through ashless #41 Whatman filter paper. A 10 mL aliquot of the filtered supernatant was then digested and analyzed for soluble minerals by AAS.

Statistics

One way analysis of variance was carried out according to Steel and Torrie (1980).

RESULTS & DISCUSSION

IN TABLE 1, it can be seen that when 1.44 mmol Na phytate was added to the milk sample (containing 2.88 mmol endogenous Ca), 99.0% of the Ca remained soluble. However, when Na phytate was added to the $CaCl_2$ system, containing an equivalent amount of Ca (2.88 mmol) only 33.9% of the Ca was soluble.

The addition of Na phytate to milk in the presence of either 0 .51 mmol Fe or 0.51 mmol of Zn had no significant effect on Ca solubility. However, when 0.50 mmol Fe or 0.51 mmol Zn was added to the $CaCl_2$ systems, in the presence of phytate, the Ca solubility was significantly reduced ($p<0.01$). Furthermore, approximately 100% of the Fe and Zn remained soluble in the milk systems, whereas only 55.4% and 50.2% of the Fe and Zn, respectively, was soluble in the inorganic salt systems.

Mineral solubility has been described as a primary prereq-

Table 1—Effects of Na phytate on mineral solubility in (I) milk and (II) $CaCl_2$ solutions, alone and in the presence of Fe or Zn

System	Final pH	Total (mmol)[b]			Phytate:Ca:Fe:Zn	Percent soluble[b]		
		Ca	Fe	Zn	Molar Ratios	Ca	Fe	Zn
1. Milk (control)	6.65	2.88	—	—		97.3	—	—
2. I) Milk + Na phytate[a]	6.35	2.83	—	—	3:6:0:0	99.0	—	—
II) $CaCl_2$ + Na phytate	6.40	2.77	—	—	3:6:0:0	33.9*	—	—
3. I) Milk + Fe + Na phytate	6.16	2.91	0.51	—	3:6:1:0	102.2	100.7	—
II) $CaCl_2$ + Fe + Na phytate	6.17	2.85	0.50	—	3:6:1:0	29.8*	55.4*	—
4. I) Milk + Zn + Na phytate	6.21	2.96	—	0.51	3:6:0:1	97.7	—	93.3
II) $CaCl_2$ + Zn + Na phytate	6.23	2.84	—	0.51	3:6:0:1	19.0*	—	50.2*

[a] 1.44 mmol of Na phytate was added to each of the systems.

[b] The symbol (—) means that the levels were below the detection level using AAS.

* Significantly different ($p<0.01$) between groups I and II within each system.

uisite of availability (Van Campen, 1983). The fact that milk prevented the precipitation of endogenous Ca and added minerals (Fe and Zn) in the presence of phytate suggests that the availability of these minerals may be enhanced by milk constituents. Knowledge regarding the ligands in milk that bind Ca is limited. However, several fractions may be involved including casein subunits, phosphate, and compounds with molecular weight <30,000 daltons (Lonnerdal and Glazier, 1985; Fransson and Lonnerdal, 1982).

Results, such as those found in this study, underscore the importance of the chemical interactions which occur in food and have major implications when evaluating the role of supplements in the diet.

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Ms received 9/26/86; accepted 10/2/86.

Paper No. 2815 Massachusetts Agricultural Experiment Station, University of Massachusetts at Amherst. This research supported in part from Experiment Station Project No. NE116.

A Research Note

Effects of Induced Low-Temperature Stress on Raw Peanuts

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ABSTRACT

Peanuts were exposed to low temperatures before they were dried to simulate temperature abuse. Samples were exposed to temperatures ranging from 0°C to −6°C. Visual examination of exposed samples revealed a glossy appearance around the edges and on the central surface of the cotyledons. A comparison of volatile profiles from exposed samples (0°C to −6°C) showed increased concentrations of acetaldehyde and ethanol with decreased temperatures. Conductivity was a measure of the electrolytes in the leachate from exposed samples and reflected the extent of membrane leakage. Concentrations of organic carbon and inorganic ions increased with decreased temperatures. Evidence of increased acetaldehyde and ethanol concentrations indicated anaerobic respiration was involved in quality deterioration of freeze damaged peanuts.

INTRODUCTION

LOW TEMPERATURE DAMAGE occurs in some fruit and vegetable crops when the external temperature falls below 10°C (Benedict and Ketring, 1972; Levitt, 1972; Lyons, 1973). Plants of subtropical origin such as peanut (*Arachis hypogaea* L.), cotton (*Gossypium hirsutum* L.), sweet potato (*Ipomoea batatas*) and tomato (*Lycopersicon esculentum*) are most susceptible to low temperatures. Low temperature damage may be subdivided into chilling or freeze damage depending on the cooling temperature, and the subsequent warming rate (Mazur, 1969). Similar structural changes in membranes and disruption of cellular function may occur under both types of injury (Benedict and Ketring, 1972; Lyons, 1973). Freeze damage, however, is characterized by intracellular ice formation which destroys cellular integrity (Benedict and Ketring, 1972; Murata, 1969).

Peanut harvesting practices and prevailing environmental conditions during the normal harvest period (late September and October) in the North Carolina-Virginia growing regions may subject the peanut crop to conditions favorable to chilling and freezing damage. At the time of digging, the peanut fruit has a moisture content of 35–55% (wet basis) (Woodroof, 1973). The freezing point of the peanut fruit has been observed to range from −2.2°C to −4.4°C at this moisture (Whiteman, 1957; Woodroof, 1973) with the average near −3.6°C. Visual damage to the seed from freeze damage includes discoloration, pitting, tissue deterioration and a glossy appearance on the external surface of the blanched seed (Ketring, 1979; Wampler, 1983). Peanuts that have been exposed to low temperatures prior to drying also develop an off-flavor. Aerobic respiration is inhibited in both chilling and freeze damage. Ketring (1979) observed increased ethylene and carbon dioxide production and changes in certain enzyme activities in freeze-damaged seeds. Murata (1969) observed an increase in acetaldehyde and ethanol production when banana fruit was chilled. These compounds are products of anaerobic respiration and contribute to off-flavors in food products. Whitaker and Dickens (1964) and Whitaker et al. (1974) studied the effects of curing temperatures on peanuts and found that anaerobic respiration is related to flavor deterioration in peanuts. Therefore, anaerobic respiration during freeze damage of peanuts may be responsible for off-flavor development.

This research was initiated to assess the effects of freeze damage on membrane permeability (as measured by leachate accumulation) and to characterize certain volatile components that contribute to flavor deterioration in peanuts.

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MATERIALS & METHODS

Seed material

Peanuts (cv. Florigiant) used in this study were growing in 1984 at the Peanut Research Station, Lewiston, NC using recommended cultural practices. The peanuts were combined immediately after digging, and dirt was removed from the pods by washing them with water. Surface water was removed from the pods with forced ambient air. The peanut kernels had moisture of about 40% (wet basis) immediately after digging.

Artificial freezing of peanuts

Each of five 14 kg samples of high moisture peanuts was exposed to one of the following temperatures: 25°C, 0°C, −2°C, −4°C, and −6°C. A shelf freeze dryer with shallow stainless steel pans was used to expose the peanuts to the low temperatures. Vacuum was not used. A thermocouple (copper-constantan) inserted into the peanut was used to monitor seed temperature. The peanuts remained in the freeze dryer for 6 hr and were dried to about 7% moisture using forced ambient air at 25°C. Following drying the treated peanuts were stored at 5°C and 60% relative humidity until analyzed. Analysis of samples began immediately after storage.

Isolation of volatiles

Volatiles from the peanuts were isolated by porous polymer trapping (Singleton and Pattee, 1980). A 100-g sample was homogenized in a Sorvall Omni-mixer with 300 mL deionized water for 1 min and placed in a 2L round bottom flask fitted with the porous polymer trap holder. The sample flask valve was closed and the sample allowed to incubate at 25°C for 15 min while stirred with a magnetic stirrer. Volatiles were removed from the sample and trapped on the porous polymer Chromosorb 102 (Johns-Manville) by attaching the collection apparatus to a water aspirator and applying a vacuum (ca. 760 mm Hg) for 5 min. Then the porous polymer trap was removed from the trap holder and transferred to a gas chromatograph for thermal desorption.

Gas chromatography

Isolated volatiles were thermally desorbed by placing the porous polymer trap into the injector port of a 3700 Varian gas chromatograph equilibrated at 200°C. The polymer trap remained in the injector port until the volatiles were separated. A packed Chromosorb 102 (80–100 mesh) analytical column (0.31 cm × 182.9 cm) programmed from 100°C to 200°C at 2°C/min was used to separate the volatile compounds. The chromatograph was equipped with dual FID detectors.

A standard solution (300 mL deionized water) containing 39.15 ppm acetaldehyde, 39.45 ppm ethanol, 39.25 ppm 2-propanol (IS), 31.30 ppm pentane, 40.45 ppm pentanal, and 40.70 ppm hexanal was used as the calibration standard. The amount of headspace volatiles collected on the porous polymer trap was controlled by equilibration time, flask size, amount of liquid, vacuum, collection time, and temperature of distillation flask (25°C). The internal standard (2-propanol) was added to the peanut sample prior to homogenization. Volatiles

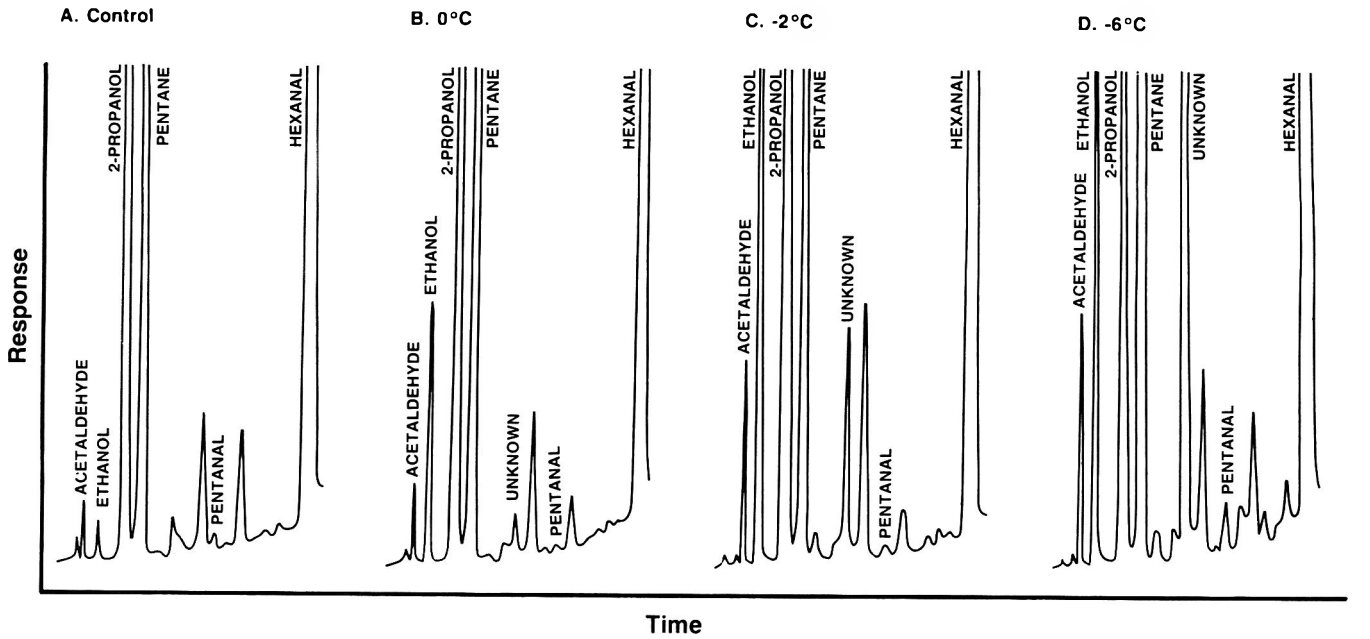

Fig. 1—Effects of different temperatures on the volatile profile of peanuts.

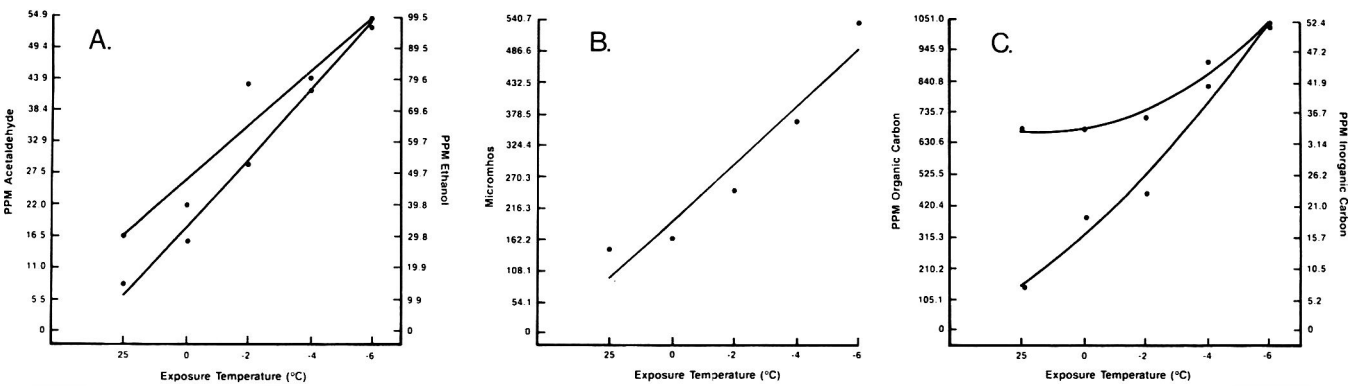

Fig. 2—Concentration of selected volatiles and analysis of leachates from peanuts exposed to different temperatures: Acetaldehyde and ethanol cocentrations (A); conductivity of leachates (B), and organic carbon and inorganic ion concentration in the leachate (C).

from the calibration mixture and samples were collected under identical conditions. Peaks were integrated using a Spectra Physics Integrator (Model 4200). Three replications were made on each sample. Identification of volatiles was accomplished by mass spectrometry and comparison of retention times to authentic standard compounds.

Conductivity measurements

Conductivity measurements were made on leachates from a portion of the kernels in each sample. Kernels that remained on a 6.35 mm mesh screen were used. A razor blade was used to cut through the testae of each unblanched kernel along the junction of the cotyledons opposite the hilum to permit an unimpeded flow of electrolytes (Wampler, 1983). A 50-g sample of kernels and 250 mL of deionized water were added to a 500 mL Erlenmeyer flask and shaken every 30 min for 2 hrs. After 2 hrs the leachate was decanted from the samples and the conductivity measured using a conductivity meter with a dip cell (Amber Sci., San Diego, CA). Conductivity measurements were replicated three times for each treatment.

Organic and inorganic carbon determinations

Total organic carbon was determined on the leachate by injecting a 40 μL sample into a Dorhmann Carbon Analyzer. Inorganic carbon was determined on the leachate by first acidifying the leachate and injecting a 40 μL sample of the leachate into the analyzer. Calibration was based on the external standard method using potassium hydrogen phthalate as the external standard.

RESULTS & DISCUSSION

Volatile profile analysis

Figure 1 shows the volatile profiles from selected low temperature treatments. Peaks of interest are identified on the chromatogram in Fig. 1. Acetaldehyde and ethanol increased with decreasing temperature. Pentane and hexanal were enzymatically produced in the homogenate and appeared not to be affected by low temperatures (Singleton et al., 1976). An unidentified peak that appeared in chromatogram D (Fig. 2) increased with a decrease in temperature. This component was not successfully identified.

A plot of acetaldehyde and ethanol concentrations versus exposure temperatures is shown in Fig. 2A. A linear relationship existed between concentration and decreasing temperature. Even at 0°C, increased production of acetaldehyde and ethanol occurred compared to the control sample (25°C). Accumulation of these components also contributes to off-flavors in peanuts and can affect other metabolic cellular functions (Pattee et al., 1965; Singleton et al., 1971).

Conductivity measurements of leachates

As temperature was lowered, conductivity of the leachates from exposed peanut samples increased (Fig. 2B). At −6°C the conductivity of the leachate was approximately 5 times higher than for the leachate of the control (25°C). Similar re-

sults have been shown to occur in sweet potatoes (Lieberman et al., 1958). Membrane lipids under normal temperature conditions have solid and liquid regions and as the temperature drops below 12°C solidification may occur at the liquid regions. This condition leads to a breakdown of cellular membrane integrity and a net loss of cellular solutes. Membrane lipids exist in a bilayer and are held together by various forces including hydrophobic bonding (Lyons and Breidenbach, 1979). Low temperature weakens hydrophobic bonding and disturbs the integrity of the cell membrane. The integrity of cell membranes in peanuts was disturbed as temperatures decreased leading to increased leakage as evidenced by inceased conductivity of the leachates.

Analysis of leachates for total organic and inorganic components from peanuts exposed to temperatures is shown in Fig. 2C. Decreases in exposure temperatures increased the amount of organic compounds and inorganic ions found in the leachate. Organic compounds such as organic acids, amino acids, and carbohydrates were primarily responsible for the total organic carbon found in the leachates; potassium was the most abundant ion found in the leachate from plat tissues exposed to cold treatment (Lieberman et al., 1958; Palta et al., 1977). Potassium is also the most abundant ion in peanuts (Bordini, 1975).

Results of this study showed that peanuts exposed to 0°C temperatures for 6 hr exhibited metabolic injury. Temperatures below −2°C caused considerable freeze damage in peanuts. Acetaldehyde and ethanol production increased in peanuts exposed to low temperatures. Membrane leakage also occured.

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Ms received 6/2/86; revised 9/8/86; accepted 10/2/86.

Paper No. 10312 of the Journal Series of the North Carolina Agricultural Research Service, Raleigh, NC. 27695-7601. The use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Research Service nor the United States Department of Agriculture of the products named, nor criticism of similar ones not mentioned.

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