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Dietary Effects of Frankfurters with Added Beef Fat and Peanut Oil

E. MARQUEZ, E. M. AHMED, R. B. SHIREMAN, J. A. CORNELL and R. L. WEST

ABSTRACT

Beef frankfurters containing 12% or 29% fat, either all beef fat or 40% beef fat plus 60% peanut oil, were fed to rabbits, resulting in a significant increase in serum cholesterol and triglyceride levels compared to rabbits fed rabbit chow. The absolute increase and rate of increase in triglyceride levels were affected by the amount of fat but not by the presence or absence of peanut oil. Cholesterol levels were not significantly different in animals fed frankfurters containing 12% or 29% beef fat except at 4 wk; the inclusion of peanut oil in the frankfurters significantly lowered serum cholesterol levels at 5 and 6 wk. Overall, frankfurters containing 12% final fat with 60% peanut oil substitution had the least hyperlipidemic effect.

INTRODUCTION

THE AMOUNT AND TYPE of fat in the food supply has emerged as a topic of increasing concern to consumers, producers, and government agencies. Interest in the proposed relationship between dietary fat, serum cholesterol concentration, and development of coronary heart disease exemplifies this concern. For example, the American Heart Association and other groups have recommended to the American public a reduction in the consumption of cholesterol and saturated fatty acids, and an increase in the consumption of polyunsaturated fatty acids (Winston, 1981) as a means of helping reduce serum cholesterol levels.

Because of the cholesterol and saturated fatty acid content of meat products, their inclusion in a healthful diet is controversial. Meats of all kinds contain cholesterol in the lean muscle and in adipose tissue. Higher concentrations of cholesterol have been found for separable fat than for separable lean tissue (Rhee and Smith, 1982). Saturated and monounsaturated fatty acids are predominant in meat fats (Dugan, 1971).

Fat is an important source of food energy and of fat-soluble vitamins; it is added to meat products for economic, flavor, and texture reasons (Rakosky, 1970). Presently, however, many meat processors are reducing the fat levels in their products because health-conscious consumers are avoiding food products that are high in fat. Law et al. (1971) reported that consumers preferred ground beef with a relatively low fat content of 5 to 20%, but data presently do not support the assumption that selecting meat lower in fat will actually lower fat and cholesterol consumption (Ono et al., 1985; Kregel et al., 1986).

This study was conducted to evaluate the effects of percent final fat and of partial substitution of peanut oil for beef fat in frankfurters on the development of hyperlipidemia using rabbits as the animal model.

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MATERIALS & METHODS

Analytical methods

Frankfurters were manufactured as described by Marquez et al. (1989). Total lipids were extracted from meat samples using the Folch et al. (1957) procedure. The extracted lipid was saponified (Rhee and Smith, 1982); the unsaponifiable material was extracted by hexane and cholesterol was determined by a colorimetric method based on the Liebermann-Burchard reaction (Ellefson and Caraway, 1976). Sterol content was calculated from the absorbances of the samples and of known standards. The colorimetric method did not discriminate between cholesterol and phytosterols and the final cholesterol content was determined by subtracting the phytosterol content provided by the amount of oil in each treatment. The phytosterol content in peanut oil was determined using the same saponification extraction and colorimetric procedures as described above (Folch et al., 1957; Rhee and Smith, 1982; Ellefson and Caraway, 1976).

For the fatty acid analysis, the extracted lipid was completely dried using a nitrogen stream and saponified as follows (Rhee and Smith 1982): 100 mg of the extracted fat was diluted to 10 mL chloroform. Duplicate 1 mL aliquots were taken and 0.2 mL Meth-Prep II (0.2N methanolic solution of m-trifluoruro-methylphenyltrimethylammonium hydroxide) was added to each and allowed to react for 30 min.

Fatty acids were determined by gas-liquid chromatography with a Perkin-Elmer Sigma 3B Dual FID Chromatograph using a 180 cm × 2 mm column packed with 10% SILAR-10 C on 80/100 mesh GAS-CROM Q. The oven temperature was 175°C, and injector and detector temperatures were 250°C. Nitrogen was used as the carrier gas at 30 mL/min. Retention time and area under the curve were obtained with a Perkin-Elmer LCI-1000 Laboratory Computing Integrator. Calibration curves were prepared for all fatty acids by injecting mixtures of the respective methyl esters (Supelco Co.).

Rabbit study

New Zealand white male rabbits, each weighing approximately 2.40 kg, were randomly divided into five groups of three animals each. The three animals in each group were assigned a specific diet. Each rabbit was housed in a stainless steel cage under controlled environmental conditions and maintain on the diet as indicated in Table 1 for six weeks. Based on preliminary experiments, animals were fed as follows: during the first and second weeks, all five groups were fed Purina commercial rabbit chow, and initial cholesterol and triglyceride plasma levels were determined at the end of each week. It was necessary to adapt the animals to frankfurters added to the chow, and during the third week each rabbit received, daily, 5g of the appropriate frankfurter preparation plus ad libitum chow. At week four, the ration was increased to 7.5g frankfurters, and during the last two weeks (fifth and sixth), 10 g/day were provided. The animals consumed all of the frankfurter rations each day and they ate the frankfurters in preference to their chow, so that all the meat rations were consumed

Table 1 — Composition of diets

Treatments	Diets
12-0	Chow + franks (12:0%)[a]
12-60	Chow + franks (12:60%)[b]
29-0	Chow + franks (29:0%)[c]
29-60	Chow + franks (29:60%)[d]
CONTROL	Chow[e]

[a] 12:0 = franks containing 12% final fat and no peanut oil substitution.
[b] 12:60 = franks containing 12% of final fat of which 60% is peanut oil.
[c] 29:0 = franks containing 29% of final fat and no peanut oil substitution.
[d] 29:60 = franks containing 29% final fat, of which 60% is peanut oil.
[e] Ralston Purina Chow.

within the first few hours of daily feeding. Commercial rabbit chow and water were provided ad libitum during the trial. Diet compositions are shown in Table 1.

Each week nonfasting blood samples were collected from the marginal ear vein before the daily feeding. Measurement of total serum cholesterol was made by the colorimetric method using the Fisher Diagnostic Total Cholesterol Kit procedure based on the Lieberman-Buchard reaction. Triglycerides were measured by an enzymatic assay (Fisher Diagnostics Triglyceride Assay, Fair Lawn, NJ).

Statistical analysis

The design consisted of a 2x2 factorial arrangement with the factors being final fat content at two concentrations (12 and 29%) and peanut oil substitution at two concentrations (0 and 60%). Data collected from this study were analyzed by analysis of variance using PROC GLM of SAS (1985). Pairwise comparisons of treatment means were performed using Duncan's Multiple Range Test (Duncan, 1955).

Simple linear regression was used to determine the relationship between length of diet feeding and the serum cholesterol or triglyceride response in rabbits. Comparisons of the slopes of the regression equations were performed using the procedure discussed by Draper and Smith (1981). Significance was determined by the F-test and differences at the 5% level of probability were considered statistically significant (Duncan, 1955).

RESULTS & DISCUSSION

MEAN VALUES for proximate composition expressed as percentage of moisture, fat and protein of the cooked frankfurter are reported by Marquez et al. (1989).

Fatty acid profiles

The concentrations of fat in the frankfurters had no effect (P > 0.05) on the individual fatty acid profile expressed as a percentage of the total fatty acids present (Table 2), since in both the 12 and 29% final fat groups, the substitution of peanut oil was fixed at the 0% or 60% level. Significant differences (P < 0.05) were observed in the fatty acid profiles as a result of peanut oil substitution. Products with 60% oil substitution were higher (P < 0.05) in oleic and linoleic acids and lower in myristic, palmitoleic, palmitic, and stearic acids, when compared to frankfurters without oil substitution. Overall, an increase in polyunsaturated fatty acids, a decrease in saturated fatty acids and, consequently, an increase in the ratio of the polyunsaturated to saturated (P/S) fatty acid (P < 0.05) were observed when peanut oil was substituted for 60% of the beef fat. There were no differences (P > 0.05) in the total monounsaturated fatty acid content due to the peanut oil.

Table 2 — Mean and standard error of the mean for fatty acid profiles of cooked frankfurters as influenced by peanut oil substitution

Fatty acids[b]	Treatment[a]		
	0	60	S.E.[c]
Myristic	4.4[x]	1.8[y]	0.17
Palmitic	24.2[x]	16.3[y]	0.29
Palmitoleic	6.5[x]	2.7[y]	0.15
Stearic	15.5[x]	8.9[y]	0.19
Oleic	40.7[x]	45.2[y]	0.44
Linoleic	3.2[x]	17.7[y]	0.23
Linolenic	2.1	1.7	0.09
Arachidic	0[x]	2.0[y]	0.15
Lignoceric	0[x]	0.8[y]	0.08
Total saturated	46.7[x]	29.1[y]	0.45
Total monounsaturated	47.3	47.9	0.67
Total polyunsaturated	5.1[x]	19.9[y]	0.36
P/S ratio[c]	0.1	0.7	0.02

[a] Oil treatments were as follows: 0 = No peanut oil; 60 = 60% of the final fat content was peanut oil.
[b] Fatty acids were expressed as percentages of the total amount of fatty acids present.
[c] Standard error of the mean (12 observations).
[d] Polyunsaturated/saturated fatty acid ratio.
[x,y] Means on a row bearing different superscripts differ significantly (P < 0.05).

Cholesterol

Cholesterol concentration of the beef frankfurters increased when the total fat content was increased from 12 to 29% (Table 3) due to the relatively high cholesterol concentration in beef fat. Our results agreed with those of Rhee and Smith (1982) who reported a decrease in cholesterol content in uncooked patties when beef fat was decreased.

Rabbit study

No differences in weight gain occurred (range 0.33–0.36 kg). The average daily dietary intake ranged from 0.12 to 0.17 kg/day, with highest consumption values for the control group. Mean values for serum cholesterol and triglyceride levels by dietary treatments and by week are presented in Fig. 1 and 2, respectively. Values reported for week two represent the base line or initial values for cholesterol or triglyceride levels in rabbit serum. The amounts of frankfurter were increased slowly

Table 3 — Mean values for cholesterol concentration (mg/100 g) of frankfurters

Oil[a], %	Final fat, %	
	12	29
0	62.0[y]	86.5[x]
60	51.5[z]	56.1[w]

[a] Oil treatments were as follow: 0 = No peanut oil; 60 = 60% of the final fat content was peanut oil.
[w,x,y,z] Means in the same row bearing different superscripts differ significantly (P < 0.05) as indicated by the LSD procedure. Means at 60% oil were significantly lower than the means at 0% oil.
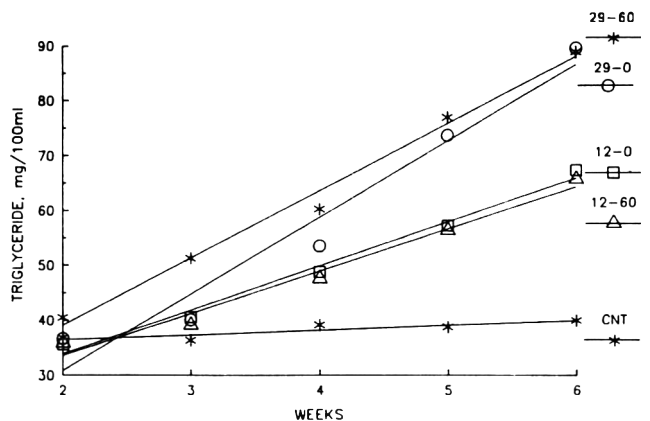
Fig. 1. — Simple regression equations of mean serum cholesterol levels vs time as influenced by dietary treatments. See Table 1 for treatment codes.
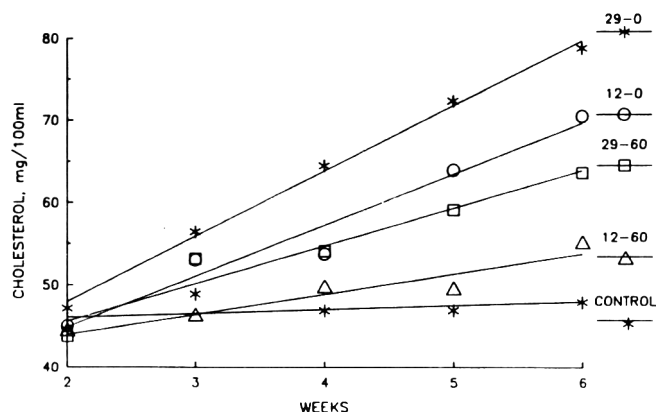
Fig. 2. — Simple regression equations of mean triglyceride levels vs time as influenced by dietary treatments. See Table 1 for treatment codes.

as previously indicated to ensure adaption and complete com-sumption. Values reported at weeks five and six correspond to the levels of cholesterol when rabbits were fed with 10 g/day of their respective treatments. The results indicated that most of the treatment combinations yielded a significant (P < 0.05) increase in the serum cholesterol and triglyceride levels of rabbits when compared to the controls after the six weeks of feeding, with the exception of treatment 12-60 (12% final fat content with 60% as peanut oil). Diets without peanut oil pro-duced significantly (P < 0.05) higher levels of serum choles-terol at five and six weeks compared to the control. Tests performed on the slopes of the fitted linear regression equations indicated that rabbits fed diets without dietary peanut oil had a significantly (P < 0.05) higher rate of increase in the serum cholesterol levels than rabbits receiving the dietary peanut oil treatment (Table 4).

Plots of serum triglyceride responses were made and tests of the slopes of the fitted equations indicated that the inclusion of frankfurters in rabbits' diets caused a significantly (P < 0.01) higher rate of increase in the serum triglyceride than in the control. Blood was always taken during the early morning before presenting the ration of frankfurters. Although the an-imals were not fasting at the time of blood drawing, the post-prandial effects of the frankfurters could be considered minimal since the animals readily consumed the frankfurters before eat-ing any of the chow. The final fat content had a significant (P < 0.01) effect on the rate of increase in serum triglyceride levels (Table 4).

Several studies have demonstrated that dietary polyunsatu-rated fatty acids from vegetable oils will reduce plasma cho-lesterol levels in humans and rabbits (Ahrens et al., 1957; Funch et al., 1962; Kritchevsky et al., 1971; Shepard et al., 1980; Mattson and Grundy, 1985). Recent studies in which polyunsaturated omega-3 fatty acids were used in diets resulted in a decrease in very low density lipoprotein formation and in plasma triglyceride levels in human subjects (Nestel et al., 1984; Phiillipson et al., 1985). Cook et al. (1970) and Ford et al. (1975) fed ruminant animals with a diet containing a polyunsaturated oil protected from rumimal bacterial action and as a result linoleic acid was increased in tissue nearly tenfold over the normal concentration. Meat with elevated lev-els of linoleic acid produced in this way has been studied in clinical trials in order to provide a diet with normal dietary components but containing higher concentration of polyunsat-urated fatty acids (Nestel et al., 1973). These researchers re-ported that increasing unsaturated fat in the diet of cattle lowered plasma cholesterol of subjects on diets containing food prod-ucts from such cattle, but the off-flavor development was re-ported to be a major drawback. The health conscious public still enjoys meat and meat-products but products with lower atherogenic potential seem to be desirable.

Many of the dietary studies to date have focused on purified or semi-purified diets. Kritchevsky et al. (1971, 1976) reported that peanut oil was atherogenic for rabbits fed a diet containing 2% cholesterol and also when compared with corn oil in semi-purified cholesterol-free diets. They attributed this atherogen-icity to the presence of long chain fatty acids such as arachidic (20:0) and behenic (22:0). Also, they concluded that the struc-ture of the triglyceride was very important because autoran-

domized peanut oil was less atherogenic than normal peanut oil. In the present study the substitution of peanut oil for some of the animal fat in a common meat product produced a very acceptable product; the peanut oil-modified product might also be considered significantly less hypercholesterolemic in rabbits than the product containing only animal fat.

A number of factors may be involved in the dietary effects in this study, i.e., the frankfurters with the higher fat content had a lower protein content and vice versa. Carroll and Ham-ilton (1975) reported that rabbits consuming animal proteins, including casein and beef, had higher plasma cholesterol levels than those on diets containing only plant proteins. They also reported that the addition of corn oil to the diet largely pre-vented the rise in plasma cholesterol. Consequently, it may be possible that the lack of differences observed in this study due to the final fat content was related to the differences in protein content. It is possible also that there is a response plateau in which 12% animal fat produces a response similar to that ob-tained with 29% animal fat. In this study, only the final fat content, and not the type of fat, determined the increase in triglyceride levels. Increased serum triglyceride levels have been reported in rabbits fed even less dietary fat (3% coconut oil) (Shireman et al., 1983).

Substitution of peanut oil for some of the animal fat in beef frankfurters resulted in a product with decreased cholesterol and saturated fatty acid contents and increased polyunsaturated fatty acids, as expected. Compared to the controls, all the meat-containing diets can be considered hyperlipidemic be-cause while those containing peanut oil were not hypercholes-terolemic, all affected the triglyceride response.

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Table 4—Estimates of regression slopes for cholesterol and serum tri-glyceride levels by different treatments

Treatment	Regression slopes	
	Cholesterol	Triglyceride
12-0	6.18[y]	7.98[y]
12-60	2.34[x]*	7.96[y]
29-0	7.90[y]	13.89[z]
29-60	4.57[x]	12.19[z]
CONTROL	0.55[x]	0.95[x]

[x,y,z] Slope estimates in the same column having different superscripts differ at the p < 0.05 level.

Properties of Low-Fat Frankfurters Containing Monounsaturated and Omega-3 Polyunsaturated Oils

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——————— ABSTRACT ———————

Properties of low-fat (<18%) frankfurters containing high-oleic acid sunflower oil (HOSO) and fish oil were studied. Replacement of animal fats by the oils had little effect on emulsion stability. Frankfurters with 5% fish oil had very low sensory scores due to undesirable fish flavor. Incorporation of maximum amounts of HOSO into low-fat beef/pork and all-beef frankfurters increased oleic acid by 34% and 62%, respectively, and the monounsaturated/saturated fatty acid ratio by 178% and 468%, respectively, compared to a regular (30% fat) product containing only animal fats. Sensory evaluation and instrumental texture profile analysis showed that the reduction in total fat caused texture problems, especially increased firmness and springiness and decreased juiciness.

INTRODUCTION

BECAUSE of an apparent relationship between the amount and type of fat consumed and the incidence of coronary heart disease (CHD)(Grundy, 1986), the composition of fat in the diet is becoming increasingly important. It is known that the ingestion of most saturated fatty acids increases the concentration of plasma low density lipoprotein (LDL)-cholesterol in humans, and elevated levels of plasma LDL-cholesterol are correlated with increased risk of CHD (Grundy, 1986). Substitution of monounsaturated fatty acids or polyunsaturated fatty acids (PUFA's) for saturated fatty acids decreases plasma LDL-cholesterol (Mattson and Grundy, 1985). However, incorporation of high levels of PUFA's in the diet has been reported to promote carcinogenesis in experimental animals (Gammal et al., 1967; Clinton et al., 1984). Furthermore, PUFA's reduce the level of plasma high density lipoprotein (HDL)-cholesterol which has been shown to have an inverse relationship with the incidence of CHD (Mattson and Grundy, 1985). In contrast, increasing monounsaturated fatty acids in the diet decreases the plasma LDL-cholesterol without reducing plasma HDL-cholesterol (Mattson and Grundy, 1985). Omega-3 PUFA's, which are found primarily in certain marine fish and fish oils, have received a great deal of interest because of their apparent beneficial effect relative to CHD (Jorgensen and Dyerberg, 1983; Carroll, 1986; Kinsella, 1986).

As the consumer's interest in reduced calorie foods is increasing, both the amount of total fat and the type of fat need to be altered for certain categories of meat products to make products that are highly desirable from a diet/health standpoint. Recently, the consumer's interest in reduced calorie foods has led the USDA Food Safety and Inspection Service to establish a standard for "light" labeled meat products which requires a minimum reduction of 25% in total fat (USDA, 1986). One of the meat products showing considerable potential for reduction of fat and inclusion of beneficial fatty acid sources is the frankfurter. There have been attempts to develop low-fat frankfurters by replacing the animal fat with soy proteins (Sofos and Allen, 1977) and water-gum suspensions (Foegeding and Ramsey, 1986) or by modification of formulations (Hand

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et al., 1987). St. John et al. (1986) increased the monounsaturated/saturated fatty acid ratio in low-fat frankfurters by using the lean and fat from pigs fed elevated levels of canola oil. Studies were conducted on frankfurters containing either 25% or 35% cottonseed oil to determine effects of the type and concentration of fat and the rate and temperature of comminution on characteristics and lipid dispersion of the products (Townsend et al., 1971; Ackerman et al., 1971). However, there has been no attempt to incorporate sources of monounsaturated fatty acids and/or ω-3 PUFA's into frankfurters.

The objectives of this study were to evaluate various quality characteristics of low-fat frankfurters as affected by incorporation of substantial amounts of monounsaturated fatty acids in the form of high-oleic acid sunflower oil (HOSO) and ω-3 PUFA's in the form of fish oil, and to examine limitations in the use of these in commercial frankfurter formulations.

MATERIALS & METHODS

Materials

Beef trimmings and kidney fat were obtained from a USDA Choice steer carcass, while Boston butt and pork backfat were used as sources of pork lean and fat. All meat materials were obtained from the Rosenthal Meat Science and Technology Center at Texas A&M University. Beef trimmings and Boston butts were trimmed of separable fat to obtain extra-lean meats. Each lean meat or fat source was ground separately through a 0.95 cm plate and subsamples were analyzed for fat and moisture using a CEM Automatic Volatility Computer (Model AVC-80) and a CEM Automatic Extraction System (CEM Corp., Indian Trail, NC). The ground lean and fat were vacuum-packaged and held at -20°C for 2 wk. All meats were tempered at 2°C for 20 hr prior to use.

HOSO was obtained from SVO Enterprises (Columbus, OH) and deodorized fish (menhaden) oil, stabilized with 0.02% tertiary butyl-hydroquinone and α-tocopherol, was secured from Zapata Haynie Corp. (Reedville, VA). The oils were stored at -20°C until used. Frankfurter seasoning was obtained from A.C. Legg Packing Co., Inc. (Birmingham, AL).

Experiment 1

In the manufacture of emulsion-type products such as frankfurters, the stability of the emulsion is largely determined by product constituents and processing conditions. In Experiment 1, emulsion stability tests, as described by Townsend et al. (1968), were conducted to investigate the effect of low-melting fats (HOSO and fish oil) on emulsion stability. Frankfurters were produced on a small scale basis, and each formulation was restricted to a 40/60 beef/pork blend having a final product composition of 15% total fat and 63–65% moisture. Water was added during formulation to compensate for smokehouse cooking losses (12%). Oils were added with a corresponding decrease in pork fat. Ground lean beef and pork were initially chopped with one-half ice, salt, cure and spices in a commercial food processor (Robot Coupe USA, Inc., Ridgeland, MA). Then, pork fat and the remaining ice were added and chopped so that the final temperature did not exceed 15°C. Oils were added during the final chopping phase of emulsification. Thirty-five gram portions of each emulsion were extruded, in triplicate, into tared polycarbonate centrifuge tubes (i.d. 2.5cm), stoppered, and placed in a 4°C waterbath. Immediately after placing the samples, the temperature setting of the waterbath was changed to 69°C, and emulsions were heated to an internal temperature of 69°C. The internal temperature was monitored with a thermocouple inserted into the geometric center of the sample. After samples were

removed from the waterbath, the free liquid was decanted into 10-mL graduated cylinders for measurement.

Experiment 2

Processing. Low-fat products containing no added oil (control), 7.5% HOSO (HOSO7.5), and 5% fish oil (FO5) were manufactured for comparison. The fish oil had a strong odor and, therefore, was not included at concentrations higher than 5%. The formulations presented in Table 1 were aimed at the same specifications as defined in Experiment 1 (15% total fat, 63–65% moisture) and at a cooking loss of 12%. Products were manufactured using standard industry practices. Emulsification of each batch was accomplished in a Hely Joly Silent Cutter (Model 339; Hely Joly Co., Tassin, France) beginning at an initial temperature of 4–9°C and ending when the final emulsion temperature was 10–15°C. Oils (HOSO and fish oil) were kept in a cold room (4°C) and added during final chopping sequence. Finished emulsions were stuffed into 2.6-cm diameter cellulose casings (Viskase Corp., Chicago, IL) with a Smith Stuffer (Smith Equipment Co., Clifton, NJ) and hand-linked (15cm length). The links were then heat-processed and smoked to a final internal temperature of 70°C in an Alkar (two truck) smokehouse (DEC International, Inc., Lodi, WI) followed by a cold shower to an internal temperature of 38°C. The products were chilled in a 4°C cold room overnight, peeled, and vacuum-packaged in high oxygen-barrier bags (30 - 40 cc $O_2/m^2/24$ hr), and held at 4°C.

Sensory evaluation. Sensory evaluations were conducted within a week after manufacture by a 19-member untrained, consumer-type panel. Links from each package were steeped in boiled water (95°C) for 7 min, sliced into 2.5cm-long pieces, and maintained at serving temperature by wrapping in aluminum foil and placing in an 80°C oven. Randomly selected samples were served to panelists situated in private booths. Distilled water was used between samples to cleanse the palate. The panelists evaluated products on a 6-point hedonic scale (1 = very undesirable, 6 = very desirable) for appearance, aroma, flavor, texture, juiciness, and overall desirability.

Experiment 3

Processing. To evaluate the effect of using maximum amounts of HOSO in combination with lean trimmings, 11.6% and 13.1% HOSO were added to a 40/60 beef/pork blend and all-beef trimmings, respectively, to produce low-fat frankfurters (15% fat target). Regular frankfurters containing 30% fat, but the same proportions of meats as the low-fat products, were produced for comparison. The products were designated as LF-HOSO-BP (low fat, maximum HOSO added, 40/60 beef/pork blend), HF-C-BP (high fat, no HOSO added, 40/60 beef/pork blend), LF-HOSO-B (low fat, maximum HOSO added, all-beef), and HF-C-B (high fat, no HOSO added, all-beef). The low-fat frankfurters were formulated on the basis of a 12% cooking loss and a final product composition of 15% fat and 64–65% moisture; regular frankfurters were formulated to contain 30% fat and 52 - 53% moisture (Table 2). Two replications per treatment were manufactured as described in Experiment 2. Cooking yields were determined by weighing products of individual treatments before and after cooking.

Total lipid determination. The total lipids were extracted using chloroform-methanol (2:1, v/v) according to the procedure of Folch

Table 1 – Formulations for frankfurters containing fats/oils from different sources (Experiment 2)

Ingredient	Product		
	Control	HOSO7.5[a]	FO5[b]
	< ----------- g/5000-g batch----------- >		
Lean beef trim (5.3% fat)	1700	1700	1700
Lean pork trim (7.6% fat)	829	1732	1431
Pork fat trim (34.7% fat)	1721	443	869
HOSO[c]	–	375	–
Fish oil	–	–	250
NaCl	112.5	112.5	112.5
Na nitrite	0.8	0.8	0.8
Na erythorbate	2.8	2.8	2.8
Seasoning[d]	34.4	34.4	34.4
Dextrose	100.0	100.0	100.0
Water	1100.0	1100.0	1100.0

[a] 7.5% high-oleic acid sunflower oil added.
[b] 5% fish oil added.
[c] High-oleic acid sunflower oil.
[d] Blend of white pepper, coriander, mustard, ginger, and mace.

Table 2 – Formulations, processing yields, and proximate compositions of low-fat (<18%) frankfurters containing HOSO[a] and regular-fat (30%) products (Experiment 3)

	Product			
	LF-HOSO-BP[b]	HF-C-BP[c]	LF-HOSO-B[d]	HF-C-B[e]
	< ----------------- g/6804-g batch----------------- >			
Ingredient				
Lean beef trim (2.7% fat)	2313	2313	4892	3572
Lean pork (6.2% fat)	2681	–	–	–
Pork regular (24.9% fat)	–	1109	–	–
Pork fat (72.1% fat)	–	2361	–	–
Beef fat (87.9% fat)	–	–	–	2211
HOSO	789	–	891	–
NaCl	153.1	153.1	153.1	153.1
Na nitrite	1.1	1.1	1.1	1.1
Na erythorbate	3.8	3.8	3.8	3.8
Dextrose	136.1	136.1	136.1	136.1
Seasoning[f]	46.8	46.8	46.8	46.8
Water	1497.0	1497.0	1497.0	1497.0
Finished product				
Processing yield (%)	82.0	86.5	81.3	87.1
Fat (%)	17.7	29.8	16.8	30.0
Moisture (%)	61.8	52.7	62.4	53.0
Protein (%)	17.5	14.5	17.0	14.0

[a] High-oleic acid sunflower oil.
[b] Low fat, maximum HOSO added, 40/60 beef/pork blend.
[c] High fat, no HOSO added, 40/60 beef/pork blend.
[d] Low fat, maximum HOSO added, all-beef.
[e] High fat, no HOSO added, all-beef.
[f] Blend of white pepper, coriander, mustard, ginger, mace.

et al. (1957). An aliquot of the total lipid extract (in triplicate) was freed of solvent and its lipid content was determined gravimetrically.

Moisture and protein determination. Moisture was determined according to the AOAC (1984) procedure. Protein was estimated, as described by St. John et al. (1986), by difference (100% - % total lipid - % moisture - % ash), with the assumption that the ash was 3% (USDA, 1979).

Sensory evaluation. A 6-member trained descriptive sensory panel evaluated products on an 8-point or 5-point scale for various attributes. Springiness (5 = extremely springy, 1 = not springy), firmness (8 = extremely firm, 1 = extremely soft), juiciness (8 = extremely juicy, 1 = extremely dry), oiliness (5 = extremely oily, 1 = not oily), off-flavor intensity (8 = extremely strong, 1 = extremely weak to none), flavor intensity (8 = extremely strong, 1 = extremely weak to none) and overall palatability (8 = extremely palatable, 1 = extremely unpalatable) were evaluated. The panelists were initially chosen on the basis of previous experience in evaluating frankfurters. Five training sessions were held in which a broad range of commercial frankfurters were compared using one of the products as a standard. Each attribute was discussed, and actual tests were initiated after the panelists agreed with the ballot scales. Samples were prepared and served as described for Experiment 2.

Texture profile analysis. Texture profile analysis (TPA) was performed as described by Bourne (1978) with an Instron Universal Testing Machine Model 1122 (Instron Corp., Canton, MA). Frankfurters from each treatment were steeped in 95°C water for 7 min, equilibrated to room temperature, and twelve 13 mm-long frankfurter sections were tested per replication. The frankfurter sections were compressed to 25% of their original height through a two-cycle compression. Force-time curves were obtained at a crosshead speed of 5 cm/min, chart speed of 50 cm/min, and full scale of 100 lb (45.4 kg). Texture variables of the force and area measurements were: FF = force required to fracture; F1 = maximum force required for the first compression; A1 = total energy required for the first compression; F2 = maximum force required for the second compression; A2 = total energy required for the second compression; springiness (S) = height that the sample recovered between the end of the first compression and start of the second; gumminess = F1 x A2/A1; chewiness = F1 x A2/A1 x S; and cohesiveness = A2/A1. Peak areas were determined by using the Ladd Graphic Data Analyzing System (Ladd Research Industries, Inc., Burlington, VT).

Determination of lipid oxidation. The 2-thiobarbituric acid (TBA) test was used to determine the extent of oxidative rancidity development during storage at 4°C for up to 12 wk. The TBA procedure of Zipser and Watts (1962) for cured meat was used with slight modifications. The amount of residual nitrite in each sample was measured by the AOAC (1984) method and the amounts of sulfanilamide to be added were determined according to the residual nitrite content, i.e.,

0.5% sulfanilamide (in the liquid before distillation) for 0 - 50 ppm residual nitrite, 1% for 50 - 100 ppm, 1.5% for 100 - 150 ppm and 2% for 150 - 200 ppm. These modifications were made to prevent excessive uses of sulfanilamide which would lead to underestimation of the extent of lipid oxidation (Shahidi et al., 1985).

Fatty acid profile determination. An aliquot of the total lipid extract was freed of solvent under nitrogen, and fatty acid methyl esters were prepared as described by Morrison and Smith (1964). Methyl esters were analyzed using a flame ionization gas chromatograph, with a fused silica capillary column, as described by Rhee et al. (1988).

Statistical analysis

In experiments 1 and 2, a single batch was processed per product (treatment). In Experiment 3, each product was processed in 2 replications (2 batches produced at different times). All sample analyses, except fatty acid analysis (in duplicate), and texture profile analysis (in 12 replicates) were performed in triplicate. Data were analyzed by analysis of variance, mean separation by the Student-Newman-Keuls test, and correlation analysis, where appropriate. Data analyses were performed using the SAS (1982) program.

RESULTS & DISCUSSION

Experiment 1

Weight losses after heating frankfurter emulsions containing different amounts of oils are shown in Table 3. The total fluids released during cooking were extremely small indicating that low fat (or high protein) concentrations resulted in very stable emulsions, in spite of the inclusion of substantial amounts of low-melting fats (HOSO and fish oil).

Experiment 2

Sensory scores of the three low-fat products (control, HOSO7.5 and FO5) are presented in Table 4. Frankfurters prepared with fish oil were considerably less desirable than the control or HOSO frankfurter. The undesirable flavor and aroma derived from fish oil were primarily responsible for the low

Table 3 — Emulsion stability of low-fat (15% as formulated) frankfurter batters as influenced by the addition of different types and amounts of oils (Experiment 1)

Oil added		Weight loss during heating (%)
Type	Amount (%)	
None (Control)	0	0.2
HOSO[a]	2.5	0.2
	5.0	0.8
	7.5	0.3
	9.2	0.5
	10.9	0.6
	12.8	0.5
Fish oil	2.5	0.7
	5.0	0.4
	7.5	0.5

[a] High-oleic acid sunflower oil.

Table 4 — Sensory scores of frankfurters containing fats/oils from different sources (Experiment 2)[d,e]

	Product[f]		
Attribute	Control	HOSO7.5	FO5
Appearance	4.4 (0.8)[b]	5.0 (0.9)[a]	4.6 (1.0)[b]
Aroma	4.3 (1.2)[a]	4.3 (1.1)[a]	3.2 (1.3)[b]
Flavor	4.5 (1.0)[a]	4.7 (1.1)[a]	2.1 (1.6)[b]
Texture	4.5 (1.0)[a]	4.2 (1.0)[a]	3.4 (1.6)[b]
Juiciness	4.4 (1.1)[a]	3.7 (1.0)[b]	3.0 (1.4)[c]
Overall desirability	4.4 (0.8)[a]	4.3 (0.9)[a]	2.4 (1.3)[b]

[a-c] Means within the same row with different superscript letters are significantly different (P<0.05).

[d] Data presented are means (standard deviations).

[e] Data based on a 6-point scale: 6 = very desirable, 1 = very undesirable.

[f] Products containing no oil (Control), 7.5% high-oleic acid sunflower oil (HOSO7.5) and 5% fish oil (FO5).

sensory scores of these products. The control (with no added oil) and samples with HOSO were not significantly different (P > 0.05) for most sensory parameters evaluated. Low texture and juiciness scores for frankfurters with fish oil cannot be explained. Because of extremely undesirable sensory properties (particularly flavor) of frankfurters with added fish oil, this product was not included in Experiment 3.

Experiment 3

Processing yields, fat, moisture and protein in the final products are shown in Table 2. Yields were 5–6% higher for the products with 30% fat (HF-C-BP and HF-C-B) compared to the low-fat (approximately 17%) products containing HOSO (LF-HOSO-BP and LF-HOSO-B). Total fat concentrations of the regular products were close to the targeted value of 30%, whereas total fat values of the low-fat products were slightly higher than the targeted value of 15%. Moisture values of HF-C-BP and HF-C-B frankfurters were as predicted, but those of LF-HOSO-BP and LF-HOSO-B products were lower than anticipated. Apparently, little fat loss occurred during processing of the low-fat frankfurters; moisture loss by evaporation seemed to be the main cause for the higher fat values. A higher moisture loss in low-fat frankfurters was probably due to a lower fat-to-protein (FP) ratio, as suggested by Mittal and Blaisdell (1983). They reported that the rate of moisture loss was inversely proportional to the FP ratio because fat was hydrophobic and offered resistance to the diffusion of moisture. Therefore, additional water may have to be added in processing frankfurters with a low FP ratio.

Sensory data (Table 5) indicated that the concentration of fat in the formulation had the greatest effect on sensory parameters. LF-HOSO-BP and LF-HOSO-B had higher scores for springiness and firmness but lower scores for juiciness and oiliness, compared to HF-C-BP and HF-C-B. These differences in texture apparently contributed to lower overall palatability scores for the low-fat products. No differences due to the type of meats (beef/pork blend vs all-beef) were detected. Rakosky (1970) reported that the firmness of a sausage product was dependent on the lean meat content and that fat softened the product while increasing tenderness.

Selected texture profile values are shown in Table 5. LF-HOSO-BP and LF-HOSO-B had much higher values for force to fracture (FF), first and second bite hardness (F1 and F2) and springiness (S) compared to HF-C-BP and HF-C-B. This was consistent with the sensory data for springiness and firmness. Between the two low-fat products, all-beef frankfurters (LF-HOSO-B) had slightly higher values (P < 0.05) in F1, F2, gumminess and chewiness, and a lower value (P < 0.05) in FF compared to the beef/pork blend (LF-HOSO-BP). Of the regular-fat products, all-beef frankfurters (HF-C-B) showed slightly higher values (P < 0.05) in FF, F1, F2, S, cohesiveness and gumminess than the beef/pork blend (HF-C-BP). These textural differences between the products with different proportions of meats were not detected by the trained sensory panel (Table 5). Cohesiveness, gumminess, and chewiness were higher (P < 0.05) for the low-fat products than for the regular products. Correlation coefficients for texture-associated sensory attributes (springiness, firmness, and juiciness) vs instrumental texture parameters and overall palatability (sensory attribute) are given in Table 6. All the texture-associated sensory attribute scores were highly correlated with the instrumental texture parameter values and sensory overall palatability scores with correlation coefficients ranging from 0.93 to 0.99 (P < 0.001).

It appears that the textural problems associated with the low-fat frankfurters in the current study may be reduced through incorporation of non-meat ingredients (e.g., oilseed ingredients and hydrocolloids) and/or special processing techniques. Sofos et al. (1977) reported that textured soy protein, when substituted for lean meat at concentrations greater than 25—30% (on an 1:2 hydrated basis), resulted in texture softening

Table 5—Sensory scores and instrumental texture profile values of low-fat (<18%) frankfurters containing maximum amounts of HOSO[e] and regular-fat (30%) products (Experiment 3)[f]

Parameter	LF-HOSO-BP[g]	HF-C-BP[h]	LF-HOSO-B[i]	HF-C-B[j]
Sensory attribute				
Springiness[k]	3.8 (0.7)[a]	2.3 (0.6)[b]	3.8 (0.7)[a]	2.3 (0.4)[b]
Firmness[l]	6.4 (0.7)[a]	3.8 (1.1)[b]	6.4 (0.8)[a]	4.0 (1.0)[b]
Juiciness[m]	3.4 (0.9)[b]	5.8 (0.5)[a]	3.3 (0.9)[b]	6.0 (0.6)[a]
Oiliness[n]	1.5 (0.5)[c]	3.1 (0.6)[a]	1.4 (0.4)[c]	3.4 (0.6)[a]
Off-flavor intensity[o]	2.9 (0.9)[a]	1.8 (1.0)[b]	2.1 (0.8)[b]	1.7 (1.0)[b]
Flavor intensity[p]	4.6 (0.9)[b]	5.1 (0.7)[a]	4.7 (1.0)[ab]	5.1 (0.8)[a]
Overall palatability[q]	4.0 (0.8)[b]	5.2 (1.1)[a]	4.0 (1.0)[b]	5.3 (1.1)[a]
Texture profile				
Fracturability (FF),N[r]	216.2 (35.1)[a]	58.5 (10.1)[d]	193.8 (24.6)[b]	79.0 (10.6)[c]
First bite hardness (F1),N	254.2 (39.7)[b]	80.4 (7.9)[d]	285.1 (34.4)[a]	108.5 (6.8)[c]
Second bite hardness (F2),N	204.9 (31.0)[b]	57.6 (6.0)[d]	226.8 (33.1)[a]	81.5 (5.6)[c]
Springiness (S), mm	7.8 (0.6)[a]	4.1 (0.5)[c]	7.5 (0.7)[a]	4.8 (0.6)[b]
Cohesiveness (A2/A1)	0.33 (0.04)[a]	0.18 (0.02)[c]	0.35 (0.04)[a]	0.22 (0.03)[b]
Gumminess (F1xA2/A1)	84.2 (18.6)[b]	14.6 (2.1)[d]	98.9 (13.8)[a]	24.1 (3.9)[c]
Chewiness (F1xA2/A1xS)	662.5 (160.8)[b]	59.4 (11.7)[c]	745.3 (124.2)[a]	116.5 (29.9)[c]

a-d Means within the same row with different superscript letters are significantly different (P<0.05).

q 8 = extremely palatable, 1 = extremely unpalatable.

a-d Means within the same row with different superscript letters are significantly different (P<0.05).

e High-oleic acid sunflower oil.

f Data presented are means (standard deviations)

g Low fat, maximum HOSO added, 40/60 beef/pork blend.

h High fat, no HOSO added, 40/60 beef/pork blend.

i Low fat, maximum HOSO added, all-beef.

j High fat, no HOSO added, all-beef.

k 5 = extremely springy, 1 = not springy.

l 8 = extremely firm, 1 = extremely soft.

m 8 = extremely juicy, 1 = extremely dry.

n 8 = extremely oily, 1 = not oily.

o 8 = extremely strong, 1 = extremely weak to none.

p 8 = extremely strong, 1 = extremely weak to none.

q 8 = extremely palatable, 1 = extremely unpalatable.

r N = Newtons.

Table 6—Correlation coefficients[a] between instrumental texture parameters and sensory attributes (Experiment 3)

Sensory attribute	Instrumental texture parameter							Sensory overall palatability
	FF[b]	F1[c]	F2[d]	S[e]	COH[f]	GUM[g]	CHEW[h]	
Springiness	0.98	0.93	0.93	0.98	0.97	0.96	0.98	−0.96
Firmness	0.99	0.95	0.95	0.99	0.98	0.97	0.99	−0.95
Juiciness	−0.98	−0.95	−0.95	−0.96	−0.95	−0.97	−0.99	0.96

a All correlation coefficients are significant at P<0.001.

b Fracturability.

c First bite hardness.

d Second bite hardness.

e Instrumental springiness.

f Cohesiveness.

g Gumminess.

h Chewiness.

of frankfurters formulated at 30% fat. Although the soft texture can be a limiting factor for the use of soy proteins in high-fat emulsion-type meat products, soy protein may be used advantageously as a fat substitute in the production low-fat, high-protein meat products (Rakosky, 1970). Conversely, the soft texture caused by elevated concentrations of a soy protein ingredient in a high-fat meat emulsion can be alleviated by reducing the total fat (Sofos and Allen, 1977). Foegeding and Ramsey (1986) produced low-fat frankfurters (11 - 12% fat) using water-gum suspension and reported that all low-fat products were as acceptable as the control (27% fat) product upon sensory evaluation using an effective method. An industry publication (FMC, 1987) showed that the low-fat (15%) frankfurters containing a carrageenan mixture had higher acceptability scores than the control (30% fat) when evaluated by a 25-member consumer-type panel. A decrease in firmness of a cooked meat emulsion due to an increase in added water was observed by Johnson et al. (1977). The recent allowance of added water for substituting the fat in meat products (USDA, 1988) appears promising for textural improvement of low-fat frankfurters.

TBA values of refrigerated, vacuum-packaged samples evaluated over a 12-wk period are shown in Table 7. Some differences (P<0.05) were observed among products; however, these differences were quite small numerically and of little significance from a practical standpoint. All products had very low TBA values (<0.54) even after 12 wk of storage.

Fatty acid profiles are presented in Table 8. Incorporation of HOSO into low-fat frankfurters significantly increased the percentage of 18:1. LF-HOSO-BP and LF-HOSO-B showed 34% and 62% (1.34- and 1.62-fold) increases, respectively, in 18:1 compared to their high-fat counterparts (HF-C-BP and

Table 7—Effect of storage time on TBA values (mg malonaldehyde/kg) of vacuum-packaged low-fat (<18%) frankfurters containing maximum amounts of HOSO[d] and regular-fat (30%) products (Experiment 3)

Product	Storage time at 4°C (wk)				
	0	2	4	6	12
LF-HOSO-BP[e]	0.48[a]	0.50[a]	0.52[a]	0.53[a]	0.53[a]
HF-C-BP[f]	0.47[a]	0.53[a]	0.46[ab]	0.44[b]	0.46[b]
LF-HOSO-B[g]	0.39[eb]	0.40[b]	0.43[b]	0.40[b]	0.44[b]
HF-C-B[h]	0.37[b]	0.34[c]	0.34[c]	0.41[b]	0.44[b]

a-c Means within the same column with different superscript letters are significantly different (P<0.05).

d High-oleic acid sunflower oil

e Low fat, maximum HOSO added, 40/60 beef/pork blend.

f High fat, no HOSO added, 40/60 beef/pork blend.

g Low fat, maximum HOSO added, all-beef.

h High fat, no HOSO added, all-beef.

HF-C-B). The monounsaturated/saturated fatty acid ratio was increased by 178% and 468%, respectively, and the total saturated fatty acids were decreased by 54% and 73%, respectively, when HOSO was added (LF-HOSO-BP vs HF-C-BP and LF-HOSO-B vs HF-C-B).

In conclusion, the low-fat frankfurters with a maximum concentration of HOSO (LF-HOSO-BP and LF-HOSO-B) would be highly desirable products from a diet/health standpoint because of high monounsaturated fatty acids and low saturated fatty acids in addition to low total fat. However, the texture problems associated with low-fat frankfurters must be solved to make the products more acceptable. Incorporation of a substantial amount of fish (menhaden) oil into frankfurter formulations would result in products unacceptable sensorially. Unless fish oil is extremely bland in flavor, its use for fatty acid modification of frankfurters may not be feasible.

Table 8—Fatty acid compositions for low-fat (<18%) frankfurters containing maximum amounts of HOSO[a] and regular-fat (30%) products (Experiment 3)

Product	14:0	16:0	16:1	18:0	18:1	18:2	20:4	Total sat.	Total unsat.	Total monounsat.	M/S ratio[c]
					Fatty acid (%)[b]						
LF-HOSO-BP[d]	0.3	8.5	0.6	5.7	72.3	11.3	1.2	14.5	85.4	72.9	5.03
HF-C-BP[e]	1.1	19.5	2.7	10.6	53.9	11.1	1.2	31.2	68.9	55.6	1.81
LF-HOSO-B[f]	0.3	6.5	0.3	5.3	75.3	11.1	1.3	12.1	88.0	75.6	6.25
HF-C-B[g]	3.2	25.6	2.4	15.5	46.4	5.5	1.4	44.3	55.7	43.8	1.10
HOSO[h]	–	2.8	–	4.1	87.5	5.6	–	6.9	93.1	87.5	12.68

[a] High-oleic acid sunflower oil.
[b] Percentage based on the toal peak area.
[c] The ratio of total monounsaturated fatty acids to total saturated fatty acids.
[d] Low fat, maximum HOSO added, 40/60 beef/pork blend.
[e] High fat, no HOSO added, 40/60 beef/pork blend.
[f] Low fat, maximum HOSO added, all-beef.
[g] High fat, no HOSO added, all-beef.
[h] High-oleic acid sunflower oil itself.

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Effects of Sodium Tripolyphosphate on the Physical, Chemical and Textural Properties of High-Collagen Frankfurters

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ABSTRACT

Two high-collagen meat emulsions were prepared by using two levels of sodium tripolyphosphate (STPP) with samples taken after one to four emulsification passes (EP) through an emulsion mill. Raw emulsions were evaluated for emulsion stability and comminuting temperature increase, whereas yield, color and texture tests were performed on cooked frankfurters. The addition of 0.25% STPP signicantly ($p < 0.05$) improved emulsion stability and consumer cook yield over the control. The number of EP signficantly ($p < 0.05$) increased emulsion temperature. As the number of EP increased, frankfurter color became lighter and less red. Frankfurters with STPP were signficantly ($p < 0.05$) lighter upon reheating. Skin texture scores decreased after three EP.

INTRODUCTION

FROM CURRENT RESEARCH, it is still widely accepted that high-collagen meat formulations produce meat emulsions with poor stability and bind (Comer, 1979; Sadowiski et al., 1980; Jones et al., 1982a,b; Gillett, 1987). Nevertheless, emulsion products are an excellent means of using this lower-functioning protein.

Previously, researchers have shown phosphates to be detrimental to solubility of collagen and stability of meat emulsions (Hamm, 1960; Ranganayaki et al., 1982). Kenney et al. (1986) observed that the addition of sodium tripolphosphate (STPP) to native wet-hide collagen suppressed the solubilization of collagen as a result of the initial high pH. Stabursvik and Martens (1980) also found similar results using intramuscular collagen. However, Ladwig et al. (1989), using skeletal muscle collagen, reported an increase in emulsion stability with the addition of sodium tripolphosphate (STPP). This decrease in cooking loss was observed in both high- and low-collagen emulsions.

The addition of collagen has been reported to increase the shear force value in various meat systems (Randall and Voisey, 1977; Rao and Henrickson, 1983). However, the addition of collagen to meat systems has also been found to lower viscosity and elasticity of homogenates (Sadowiski et al. 1980; Jones et al., 1982b).

Because collagen lacks myoglobin, its color is less red than myofibrillar proteins. Jones et al. (1982a) confirmed that meat emulsions with no added tripe were darker and more red than formulations that replaced myofibrillar proteins with tripe. In bologna extended with collagen, Rao and Henrickson (1983) reported that, as fat content decreased from 30 to 10%, bologna with collagen were signficantly less red than bologna without collagen.

If phosphates are used to stabilize high collagen formulations as suggested by Ladwig et al., (1989) then the effects of phosphates on other parameters must also be considered. For example, the color of cured meats has also been shown to be

adversely affected by phosphates (Swift and Ellis, 1957; Knipe et al., 1988). By increasing product pH, alkaline phosphates hinder the reduction of nitrosylmetmyoglobin to nitrosylmyoglobin (Fox and Thomson, 1963) and, ultimately, cured color development upon cooking.

Puolanne and Matikkala (1980) and Knipe et al. (1985) reported a decrease in firmness in cooked sausages when phosphate was added, whereas Swift and Ellis (1957) indicated that all phosphate mixtures tested increased the tensile strength of emulsified sausage products.

The objectives of this study were: (a) to determine the effects of sodium tripolyphosphate and the mechanical action of emulsion mills on emulsion stability and protein solubility of high collagen meat emulsions and on textural and color characteristics of high-collagen frankfurters.

MATERIALS & METHODS

Formulation

Formulations were prepared according to Ladwig et al. (1988).

Sample preparation

Meat emulsions were prepared according to Ladwig et al. (1988), with the following modifications. Chopping was continued until emulsions reached a temperature of 5°C, at which time the product was transferred to a Stephan Microcut (MCV 12B) emulsion mill for final emulsification. Samples were taken after 1, 2, 3 and 4 passes through the mill, with each treatment being replicated three times.

Production

Frankfurters were made with a portion of the emulsion following each pass through the emulsion mill. The meat emulsions were stuffed and linked using 22-mm-diameter, cellulose casings (type RP, Tee-pak, Inc., Chicago, IL) and a Vemag 500 suffer equipped with a linker attachment (Robert Reiser & Co., Inc., Boston, MA). The frankfurters were cooked to an internal temperature of 71°C in a Maurer and Sohne Allround System oven (H. Maurer & Sons, Riechenau, West Germany). After cooking, the franks were allowed to cool for 2 hrs before being peeled with a Ranger Apollo Peeler (Ranger Tool Co., Inc., Ellendale, TN). The next day, the franks were vacuum packaged (5 per pouch) in high-oxygen barrier pouches (nylon/surlyn/saran laminate, Curwood, Inc., New London, WI) using a Multivac chamber packaging machine (AG800, Sepp Haggenmulller KG, West Germany) with a vacuum of 33 cm mercury (vacuum setting of "2") and stored in a 1°C cooler until evaluation.

Cooking yield

Product cooking yield was determined by weighing the batches before and after cooking. Product yeild was calculated by:

$$\text{Percent product cooking yield} = \frac{\text{Cooked weight}}{\text{Raw weight}} \times 100$$

Consumer cook test

The consumer cook (reheating) test (Tauber and Lloyd, 1947) was performed 8 weeks after production of the frankfurters. This consists

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of adding the franks to boiling water and heating until boiling resumes. The container was removed from the heat and the water allowed to cool to 60°C. The franks were wiped free of external moisture and weighed to determine any weight change. This test was duplicated for each treatment per repetition.

Color

Objective color readings were obtained from a Hunter Lab Labscan Spectrocolorimeter (Hunter Associates Laboratories, Inc., Reston, VA). "L", "a", "b" values were measured, with illuminant F (light from a cool-white fluorescent source) as the light source, on the external surface of cooked and reheated frankfurters. The instrument was standardized with a white standard plate (x = 81.60, y = 86,68, z = 91.18). Six measurements on three frankfurters were taken after 8 weeks of storage.

Firmness

Skin and core firmness were measured objectively with the Instron Universal Testing Machine model 1122 with a 500-kg load cell. Warner-Bratzler shear peak heights were recorded with the crosshead speed set at 110 mm/min and the chart speed at 200 mm/min. Frankfurters were sliced into 25-mm sections, and core samples were taken by using a 15-mm core probe. Skin texture was determined with a 5-kg Full Scale Load setting, whereas core texture was measured with a 2-kg setting. Eight readings on five samples were taken on each pass per treatment on both skin and core texture.

Chemical analysis

Emulsion stability, soluble protein, hydroxyproline, proximate and statistical analysis were carried out according to Ladwig et al. (1988).

RESULTS

THE ADDITION of 0.25% STPP to high collagen emulsions significantly (p < 0.05) reduced gel-liquid and total cookout in emulsion stability test when averaged over all emulsion passes (Table 1). However, STPP did not decrease the temperature rise of the emulsion during comminuting or increase soluble-protein levels significantly (p > 0.05) over control groups.

The number of passes through the emulsion mill (EP) averaged over both treatments had no significant effect on gel-liquid and total cookout or protein solubility at the 0.05 confidence level (Table 1). Fat loss after one EP was significantly (p < 0.05) greater than fat loss after three EP. Temperature of the emulsions after subsequent passes increased significantly (p < 0.05) as expected.

Smokehouse and reheating yields were determined for high-collagen frankfurters and showed no significant (p > 0.05) dif-

ferences between treatments for the processed product (Table 2). However, STPP significantly reduced weight loss of reheated franks when compared with control franks.

As the number of EP increased, there were no significant (p > 0.05) differences in smokehouse yield. However, 2 and 3 EP significantly (p < 0.05) reduced reheating cook yields compared with only one pass (Table 2).

L, a and b values for surface color on both processed and reheated frankfurters are shown in Table 3. The addition of STPP showed no significant (p > 0.05) differences in L and a values over control groups of the processed product, but b values were significantly (p < 0.05) higher for the STPP-added product. After reheating, L (lightness) values became, and b (yellowness) values remained, significantly (p < 0.05) higher for the STPP product over the control. Differences in redness (a) values remained nonsignificant after heating (p > 0.05).

Frankfurters became significantly (p < 0.05) lighter (increased L values) after each subsequent EP for both processed and reheated products (Table 3). Before reheating, the franks passed once through the mill produced the reddest product (p < 0.05), but there was no significant difference (p > 0.05) in redness due to EP after reheating.

STPP increased skin and core shear values (Table 4), but these differences were not significant (p > 0.05). As the number of passes increased, the skin texture became significantly (p < 0.05) softer, although the core texture was not affected by the number of passes.

DISCUSSION

THE RESULTS indicate that the addition of 0.25% STPP to high-collagen emulsions improves overall stability. Gel-liquid and total cookout were significantly (p < 0.05) lower in the STPP-added emulsion in comparison with the control emulsion (Table 1). However, fat and solid cookout fractions were not significantly (p > 0.05) different in the two treatments. These findings are similar to those reported by Keeton et al. (1984) using 0.5% STPP. The ability of STPP to reduce water loss more effectively than fat loss can be explained partly by its solubility properties. STPP dissolves predominantly in polar solvents such as lean tissue, where water is concentrated, to exert its effect on ionic strength and pH (Trout and Schmidt, 1984).

On the other hand, gel-liquid and total cookout values were not significantly (p > 0.05) different after one to four passes through the emulsion mill (Table 1). This was not expected because emulsion temperature increased significantly (p < 0.05) after each subsequent EP. Fat cookout after three EP's was significantly less than after only one EP. The lowest fat cookout was observed after three EP and corresponded to an emulsion temperature of 19.0°C. This temperature optimum using an emulsifier was higher than previously reported by these authors when using a chopper (Ladwig et al., 1989). This implies that the physical action/duration may be more important to emulsion stability than final emulsion temperature as suggested by Schut (1976). The number of EP as well as STPP level had no effect on protein solubility.

The addition of STPP increased emulsion stability yields, but did not affect smokehouse processing yields (Table 2). This observation probably represents the difference in water and fat transmission through a cellulose casing. The yield in this study was determined by weighing the product encased in a cellulose casing before and after cooking. In effect, a large portion of fat cookout, located between the frankfurter and casing, would have been considered product yield and not product loss. In addition, the fat layer, being hydrophobic, could have resisted moisture diffusion from the product as well (Mittal and Blaisdell, 1983).

When evaluating frankfurter yield, the consumer cook (reheating) test may be considerably more accurate than the conventional processing yield determination. In this experiment, STPP-added treatments significantly (p < 0.05) increased yields

Table 1 – Effect of STPP[a] and number of EP[b] on emulsion stability, emulsion temperature increase[c], and protein solubility of high collagen meat emulsions

Treatments[d]	Fat (mL)[e]	Gel-Liquid (mL)[e]	Solid (mL)[e]	Total (mL)[e]	Emulsion temp. (°C)	Mg soluble protein per g emulsion
STPP Level						
0.00%	0.18[f,i]	4.05[f]	0.09[f]	4.32[f]	18.03[f]	46.82[f]
0.25%	0.14[f]	1.33[g]	0.03[f]	1.49[g]	16.50[f]	60.23[f]
S.E.	0.03	0.22	0.02	0.26	0.34	3.38
EP						
1	0.29[f,g]	3.05[f]	0.06[f]	3.40[f]	11.00[i]	50.93[f]
2	0.12[f,g]	2.41[f]	0.05[f]	2.58[f]	15.50[h]	55.97[f]
3	0.08[g]	2.59[f]	0.05[f]	2.73[f]	19.00[g]	53.27[f]
4	0.16[f,g]	2.70[f]	0.08[f]	2.93[f]	23.67[f]	53.93[f]
S.E.	0.06	0.25	0.01	0.26	0.53	2.03

[a] Sodium tripolyphosphate.
[b] Emulsification passes; sample of emulsion is taken after each pass through the emulsion mill in a continuous system averaged over both STPP levels.
[c] Average temperature increase in °C over four passes.
[d] Mean values for fat, gel-liquid, solid, total, emulsion temperature and soluble protein; N = 12 per treatment mean for STPP level; N = 6 per treatment mean for EP.
[e] mLs per 34g emulsion.
[f,i] Mean values in a column followed by different superscripts are significantly different (p < 0.05).

Table 2—Effects of STPP[a] level and number of EP[b] on smokehouse yield and consumer cook[c] yield of high collagen frankfurters

| Yield (%) | STPP Level | | | Treatments[d] | | | | |
	0.00%	0.25%	S.E.	EP 1	2	3	4	S.E.
Smokehouse	93.24[e,g]	93.26[e]	0.36	93.83[e]	93.07[e]	92.83[e]	93.27[e]	0.32
Consumer Cook	85.46[f]	96.08[e]	0.89	86.92[f]	92.52	93.17[e]	90.48[e,f]	1.04

[a] Sodium tripolyphosphate.

[b] Emulsification passes; sample of emulsion is taken after each pass through the emulsion mill in a continuous system averaged over both STPP levels.

[c] Consumer cook test (reheating test)

[d] Mean values for smokehouse and consumer cook yield; N = 12 per treatment mean for STPP level; N = 6 per treatment mean for EP.

[e-g] Mean values in a row followed by different letters (e, f) are significntly different ($p < 0.05$).

Table 3—Effects of STPP[a] level and number of EP[b] on Hunter Lab[c] color values in processed and reheated high collagen frankfurters

| Processed franks[e] | STPP Level | | | Treatments[d] | | | | |
	0.00%	0.25%	S.E.	EP 1	2	3	4	S.E.
L	55.92[f,i]	58.35[f]	0.79	55.03[h]	56.83[g]	58.67[f]	58.00[f]	0.23
a	8.93[f]	8.68[f]	0.13	8.97[f]	8.78[f,g]	8.62[g]	8.83[f,g]	0.07
b	11.29[g]	11.94[f]	0.04	11.33[g]	11.75[f]	11.67[f,g]	11.72[f]	0.10
Reheated Franks[e]	0.00%	0.25%	S.E.	1	2	3	4	S.E.
L	49.73[g,i]	51.21[f]	0.15	49.30[g]	50.50[f]	51.33[f]	50.73[f]	0.34
a	9.53[f]	9.31[f]	0.09	9.48[f]	9.18[f]	9.40[f]	9.60[f]	0.21
b	11.17[g]	12.07[f]	0.03	11.25[h]	11.65[g]	11.83[f]	11.73[f,g]	0.05

[a] Sodium tripolyphosphate.

[b] Emulsification passes; sample of emulsion is taken after each pass through the emulsion mill in a continuous system averaged over both STPP levels.

[c] Hunter Lab color: L = lightness, a = redness, b = yellowness.

[d] Mean values for L, a and b; N = 12 per treatment mean for STPP levels; N = 6 per treatment mean for EP.

[e] Processed franks measured for color scores unheated; reheated franks measured for color scores heated.

[f-i] Mean values in a row followed by different letters (f, g, h) are significantly different ($p < 0.05$).

Table 3—Effects of STPP[a] level and number of EP[b] on skin and core firmness scores[c] of high collagen frankfurters

| Texture | STPP Level | | | Treatments[d] | | | | |
	0.00%	0.25%	S.E.	EP 1	2	3	4	S.E.
Skin	1.43[e,g]	1.52[e]	0.02	1.58[e]	1.50[e,f]	1.42[f]	1.40[f]	0.04
Core	0.22[e]	0.23[e]	0.006	0.22[e]	0.23[e]	0.22[e]	0.24[e]	0.009

[a] Sodium tripolyphosphate.

[b] Emulsification passes; sample of emulsion is taken after each pass through the emulsion mill in a continuous system averaged over both STPP levels.

[c] Warner-Bratzler shear force in Kg

[d] Mean values for skin and core firmness; N = 12 per treatment mean for STPP level; N = 6 per treatment mean for EP.

[e-g] Mean values in a row followed by different letters (e, f) are significantly different ($p < 0.05$).

over control treatments when evaluated for reheating yield. The effect of EP also affected the reheating yield because one EP had a significantly ($p < 0.05$) lower yield than two or three EP. The consumer cook test results supported the emulsion stability results better than did smoke house yield measurements (Table 1). In the future, researchers should consider the consumer cook (reheating) test rather than or, at least in addition to, processing yields for assessing product stability.

The pH of a meat block is critical in meat color formation and stability. Just as a high pH in fresh meat may hinder brown color formation upon cooking, a high pH in cured meat may retard pink color development. The effects of STPP, an alkaline phosphate, concentration and EP on color scores are shown in Table 3. In the processed product, no significant ($p > 0.05$) differences in the STPP level were found for L and a values; however, upon reheating, L values decreased (became darker) in both treatments, and STPP-added franks became signficantly ($p < 0.05$) lighter than the control franks.

As EP increased, the processed franks became signficantly ($p < 0.05$) lighter (high L values) and less red (higher a values). This could be attributed to increased fat smearing and reduced particle definition as the number of EP increased. After heating, fewer differences were seen; however, the single-passed product remained significantly darker than product resulting from additional passes.

The addition of STPP did not significantly ($p > 0.05$) increase shear values for skin and core measurements (Table 4).

This is similar to the results of Knipe et al., (1985) but disagrees with Rongey and Bratzler (1966) and Keeton et al. (1984), who reported increased firmness in sausages with the addition of phosphates. These differences may be explained partly by the use of lower phosphate concentrations and greater connective tissue and watered In this experiment. Increased emulsification passes significantly ($p < 0.05$) softened the skin texture of franks.

The use of HC meats in fine-cut sausage products with the addition of STPP could prove beneficial by reducing costs as well as maintaining quality standards. From this research, it would be advisable to employ a double-pass emulsification system for fine-cut products in light of improved emulsion stability and reheating yield over the single-passed products assuming that temperature abuse with subsequent decrease in product stability does not occur.

In addition, this study indicates that specific attention should be given to cured color development as influenced by alkaline phosphates in the final reheated color of frankfurters.

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Thermal Conductivity of Fresh Lamb Meat, Offals and Fat in the Range −40 to +30°C: Measurements and Correlations

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ABSTRACT

The thermal conductivity of fresh lamb meat, offals and fat was measured over the temperature range −40°C to +30°C using a guarded hot plate apparatus. Simple empirical equations were presented for the conductivity of high-moisture (65 to 80%) meat and offals. With independently obtained values of physical parameters, several theoretical models were tested to see if thermal conductivity could be calculated from composition and temperature. Over a wide range of compositions and temperatures, best predictions (in terms of mean, standard deviation and range of errors) were obtained with Levy's modification to the Maxwell-Eucken equation. Its accuracy was not unduly sensitive to the uncertainties in the values of the physical parameters, the prediction errors remaining in the range ± 10% for all reasonable values of the latter.

INTRODUCTION

SIGNIFICANT PROGRESS has been made in recent years on calculation methods for food chilling, freezing and thawing processes. Analytical and numerical methods of increasing accuracy have been introduced. The availability of these methods has increased the need for more accurate thermophysical data, including thermal conductivity.

In the area of foodstuffs and more particularly of meat and other animal products, comprehensive reviews of existing data on thermal conductivity have been carried out by Lentz (1961), Morley (1966, 1972), Hill et al. (1967), Kostaropoulos (1971), Qashou et al. (1972), Sweat (1975), Mellor (1976), Miles et al. (1983) and Sanz et al. (1987). Data on beef, pork and poultry are plentiful, but only two previous studies, by Leitman (1967) and Bazan and Mascheroni (1984), deal with sheepmeat. These two sets of data show serious discrepancies, as will be seen later. No data on sheep offals or fat are available. Thus, there is a need for more data on sheep products.

Many measuring methods are available for thermal conductivity. Their use in the food area has been comprehensively reviewed by Kostaropoulos (1971), Mellor (1979), Nesvadba (1982) and Ohlsson (1983). There are two main classes of methods: steady-state methods, typified by the guarded hot plate apparatus; and unsteady-state methods, such as the line source method (Blackwell, 1954; Sweat, 1974). Our laboratory chose to use the guarded hot plate method, which, although slower than unsteady-state methods, can deal with nonisotropic materials in various configurations and can be used on frozen materials close to the freezing point. This method yields an average conductivity over a large area, an important consideration for non-homogeneous materials. It is also thought that the mathematics of this apparatus are better understood (De Ponte and Di Filippo, 1974; Pham and Smith, 1986; Pham, 1987a) and, therefore, sources of errors can be accurately taken into account. On the other hand, a COST 90 project has shown that line-source results obtained by different laboratories for the same material can differ by up to 30% (Kent et al., 1984) and results on the high side are often produced, although the

method can be quite accurate in the hands of experienced users (e.g., Sweat, 1974).

The objective of this study was to obtain more data on the thermal conductivity of lamb meat, offals and fat at temperatures ranging from −40°C to +30°C, which were of interest in common refrigeration processes. A second objective was to find out whether the data thus obtained agreed with any existing correlation.

MATERIALS & METHODS

Guarded hot plate design

The design of the guarded hot plate apparatus, shown in Fig. 1, follows the guidelines set out by the ASTM (1976). The measuring area was square, 200 mm × 200 mm, surrounded by a guard 100 mm wide. The gap between guard and measuring area was 3 mm wide. Hot and cold plates were made of 6 mm-thick black-anodized aluminum. Heating was by Pyrotenax sheathed electric elements sandwiched between the hot plates, one element for the measuring area and one for the guard. The free space between the hot plates was filled with a heat-conducting mixture of silicone grease and aluminum powder to ensure isothermality. A Redfern constant-current source supplied current to the heater with a stability better than 0.01%. The cold plate's temperature was controlled to within ±0.05°C by contact with a cooling plate, in which circulated ethyl alcohol pumped from a thermostatic bath. The whole apparatus is insulated from ambient. All thermocouples were inserted into holes drilled parallel to the plates' surfaces to avoid discontinuities on the surface.

The two-material specimen approach

Disregarding instrument errors, there are three basic sources of errors in a guarded hot plate apparatus: (1) thermal imbalance (i.e., temperature difference between hot plate and guard), (2) distortion of heat flow lines at the gap (or uncertainty in measuring area) and (3) edge effects (heat flow across the edge distorting the heat flow lines). Pham and Smith (1986) showed how effects (1) and (2) can be accurately calculated and presented a method to minimize errors from all three sources. This method consists of using an insulating material (expanded polystyrene) over the guard and gap area, thus having the test material over the central hot plate area only (Fig. 1). A further advantage of this technique is that only about one-quarter of the test material is needed compared to the conventional method. It is also much easier to prepare the specimen and ensure that it has a flat upper surface due to its small size.

Measurement and control

Eight copper-constantan thermocouples were embedded in each of the hot and cold plates for temperature measurement, making a total of 32. A 32-junction differential thermocouple measured the thermal imbalance between measuring area and guard and was used to control this imbalance to within ±1 μV (averaged value), equivalent to ±0.002 K. All thermocouple junctions were encased in epoxy-resin and tested to ensure an insulation resistance of at least 1 GΩ.

The power to the hot plate heater was calculated from the product of voltage drop and heater resistance. The latter was measured by interrupting the heating current for a fraction of a second every 10 to 30 min to send a standard 1 mA current through the element, while monitoring the voltage drop. A four-wire system was used, two wires for supplying the measuring current to the heater and two wires for measuring the voltage drop across the heater.

All measurements and control were done by a Hewlett-Packard HP
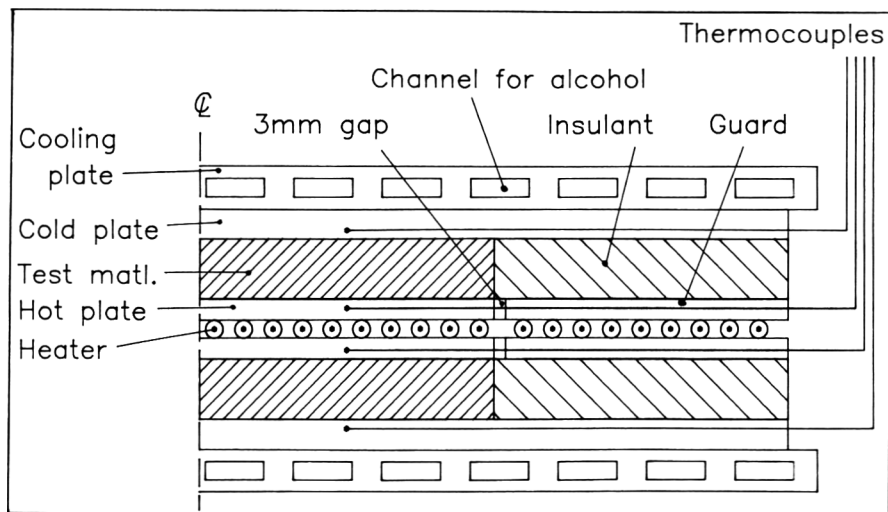
Fig. 1 — Thermal conductivity apparatus using two-material specimens. (Symmetry about centerline is assumed).

3054A data-acquisition unit driven by a HP 85B micro-computer. A computer program was written to carry out the measurements; average the data over several minutes; control the thermal imbalance (temperature difference between hot plate and guard); calculate and print out the results at regular intervals; and plot the thermal imbalance, mean temperature or thermal conductivity vs time on demand. In this way, we could ensure that steady-state was reached before changing the temperature.

Test procedure

Fresh material was obtained from a slaughterhouse. The material was sliced to a convenient size, or ground up and put on a cold or hot plate to a depth of about 20 mm, within an expanded polystyrene surrounding piece, which had been cut with a hot wire to precise dimensions. The specimens were prepared with great care. Small gaps were filled with a gel of similar water content, or, in the case of fat tissue, with soft rendered lamb fat, to ensure continuity of the thermal path and eliminate air bubbles. An oil-coated sheet of glass was put on the specimen to ensure that its surface was flat, free of bubbles and slightly protruding above the expanded polystyrene, then gently withdrawn. The whole apparatus was then assembled in the horizontal position, and a 50 kg weight put on top. The specimen thickness was determined by measuring the distance between cold plates at several points around the periphery with a Vernier-scale ruler reading to 0.1 mm.

At least two measurements were taken at above-freezing temperatures (one between 0 and 10°C and one between 25 and 30°C), after which the cold plates were cooled to −40°C to freeze the material. Measurements were then taken at several sub-freezing temperatures, at intervals of 10°C or less below −10°C, and 3°C or less above −10°C. An entire run took about 5 days.

After each run, the specimen was taken out (still hard frozen) and its dimensions remeasured, so that the k-values below freezing could be corrected for the specimen's expansion. Total volumetric expansion for the water-rich materials (i.e. all materials except fat) averaged 8%; however, because the specimens expanded in all directions, a smaller correction was applied to the k-value below freezing (4% on average). The specimen was then analyzed for water, protein, fat and ash content according to AOAC methods 24.003, 24.027, 24.005 and 24.009, respectively (Ellis, 1984).

Apparatus testing and calibration

The digital voltmeter in the data acquisition unit was checked against a reference cell and found to be accurate to 0.05%. The four-wire resistance-measuring method used for the hot plate heating element was tested against standard resistances and found accurate to 0.2%.

The zero error of the temperature difference between hot and cold plates was measured afterwards, by turning off the heating current. The long-term (one day) zero error was 0.00°C (Fig. 2), while there was a short-term error of up to 0.04°C, probably due to fluctuations in ambient conditions. Since the temperature difference used in the experiment was at least 2.0°C, the maximum error due to this source was 0.04/2.0 or 2%, but this error could be reduced by using long
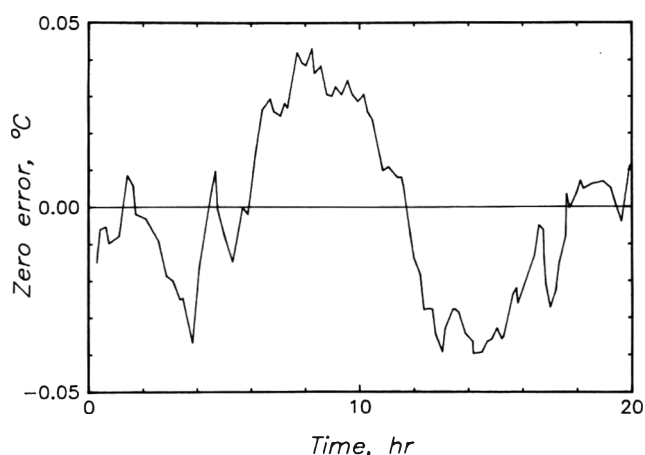
Fig. 2 — Zero-error of temperature difference between hot and cold plate.
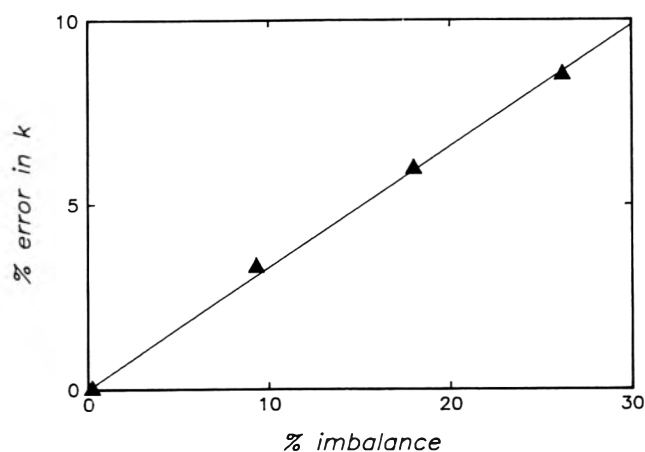
Fig. 3 — Effective thermal imbalance errors on measured k-value.

measuring times and large temperature differences. Errors over several data points should average out to zero.

The effect of thermal imbalance between hot plate and guard was tested using specimens of lamb thymus glands at −40°C. Non-negligible thermal imbalances were deliberately created using a non-zero set point on the thermal imbalance controller, and the thermal conductivity measured. Percentage errors in the measured k-values against percentage thermal imbalance (i.e., the ratio of the temperature difference between guard and hot plate to that between hot and cold plate, expressed as a percentage) are plotted in Fig. 3. A proportional relationship is obtained, as can be expected from theory (Pham and

Smith, 1986). The ratio, % error/% imbalance, is about 0.3. Normally the percentage imbalance is less than 0.1%, so the error due to this factor is negligible.

The other major error source is in the measurement of the specimen dimensions. The maximum error caused by this is estimated at +1.0% (+0.1 mm or +0.5% in the thickness, +0.5 mm or +0.25% in each of the two sides). Overall errors, therefore, range from 3.2% in the worst case to 1.2% if long measuring times or large cold-to-hot plate temperature differences are used.

Finally, the apparatus was tested on a gel of 1% agar in water. Thermal conductivity results were compared with four authoritative datasets quoted by Miles et al. (1983) for water, corrected for the presence of 1% agar by decreasing the water k-values by 0.5%. Very good agreement was obtained (Fig. 4).

RESULTS

RESULTS for 13 complete runs will be reported here, all done on fresh lamb products. These can be divided into three groups (Table 1). Group I consisted of all water-rich products except one. Group II consisted solely of leg muscle with fibers perpendicular to heat flow. This material was considered separately because, as is well known, the perpendicular configuration significantly reduced thermal conductivity. Group III consisted of fatty tissues. In addition there were some incomplete runs carried out above freezing, whose results will appear in Fig. 8 only.

Thermal conductivity results are shown in Fig. 5. Also plotted with each dataset is a regression equation of the form

$$k = a + bT + c/T \qquad (1)$$
$$\text{or } k = k_f + b(T - T_f) + c(1/T - 1/T_f) \qquad (1b)$$
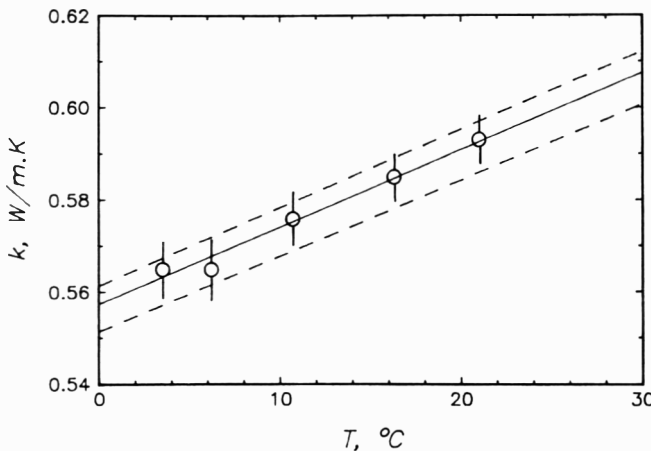
Fig. 4—Thermal conductivity of 1% agar gel. o this work: — Mean of four previous datasets for water (Miles et al., 1983) corrected for effect of agar; --- Upper and lower limits of previous datasets.

Table 1 — Composition of the materials tested

Materials	Water %	Protein %	Fat %	Ash %
Group I				
Leg muscle parallel	73.6	19.9	4.7	1.1
Leg muscle minced	73.9	18.6	4.5	1.0
Hearts	69.8	15.0	12.3	1.2
Hearts minced	68.8	14.9	13.9	1.1
Livers	68.9	19.4	6.2	1.5
Livers minced	67.7	20.8	3.7	2.0
Brains	79.0	10.3	7.6	1.4
Kidneys	79.9	14.5	3.1	1.3
Thymus	79.2	14.3	6.4	1.4
Thymus minced	75.9	13.5	9.2	1.7
Group II				
Leg muscle perpendicular	72.5	19.3	7.2	0.9
Group III				
Fat	13.3	3.2	85.4	0.1
Fat minced	11.1	2.9	86.3	0.2

for the sub-freezing range (Schwartzberg, 1977; Succar and Hayakawa, 1983), and

$$k = k_f + d(T - T_f) \qquad (2)$$

for the above-freezing range. Equation (1b) has a thermodynamical basis, since the frozen fraction of water varies as (1-T_f/T).

The initial freezing temperature T_i had been found to be $-0.9°C$ for several lamb tissues (Fleming, 1969; Pham, 1987b); this value was assumed to hold for all the materials tested here. "Initial freezing temperature" is a misnomer, since on cooling there are always supercooling effects and freezing starts at a temperature that depends on such factors as the rate of cooling and the presence of ice nuclei. T_f is defined as the temperature at which, on heating a frozen product, the ice fraction becomes 0 and a break occurs in the enthalpy-temperature curve. Pham (1987b) shows how this point can be found by intersecting the enthalpy-temperature regression curves above and below freezing.

The value of k_f was found by extrapolating the above-freezing results to -0.9°C. The parameters b and c were than found by linear regression. The values of k_f, b, c, and d are shown in Table 2 so that our experimental results can be regenerated. The term containing b accounted for the variation of the thermal conductivity of ice with temperature, while the term containing c accounted for the change in ice content with temperature. Although simpler than some of the more sophisticated equations that will be considered later, eqs. (1) or (1b) and (2) represent the data quite adequately, shown in Fig. 5.

The thermal conductivity data for the same materials in different forms (sliced and minced) agree with each other to within 5% (Fig. 5). Part of any difference will be experimental error and another part due to sample-to-sample variation. For example, sliced thymus gland had distinctly higher k-values than minced thymus, but the former had a moisture content of 79.2% compared with the latter's 75.9%. Sliced and minced fats were a similar case. Thus, it was unlikely that the actual form of the material affected its thermal conductivity (except for muscle with fibers perpendicular to heat flow).

For all Group I materials, the parameters k_f, b, c and d can be related to the water fraction x_w by the following empirical equations:

$$k_f = 0.060 + 0.52x_w \qquad (3)$$

$$b = -0.0057, \quad \text{s.d.} = 0.013 \qquad (4)$$

$$c = -0.446 + 1.62x_w, \quad R^2 = 0.678 \qquad (5)$$

$$d = 0.00090, \quad \text{s.d.} = 0.00029 \qquad (6)$$

Equations (3) to (6) when used in conjunction with eqs. (1b) and (2) agree with experimental data for Group I to ± 8%. No R^2-value is given for eq. (3), as this is simply Sweat's (1975) equation, eq. (7), adjusted to -0.9°C by a slight change in the constant term.

For Group II, the k-value of muscle with fibers perpendicular to heat flow was 8% lower than that of muscle with fibers parallel to heat flow, both above and below freezing.

Effect of previous history

It must be stressed that the values reported here are all for freshly obtained products, frozen in the guarded hot plate apparatus according to the procedure described under "Materials & Methods." Materials prefrozen in a different manner, or repeatedly frozen and thawed, may not have the same k-values due to the damage done to the tissue. As an example, Fig. 6 shows the k-T curves for freshly obtained brain (79.0% moisture) and brain (80.4% moisture) prefrozen in an uncontrolled manner (probably much more slowly), stored for an unknown period and thawed. The latter product (which will not be con-
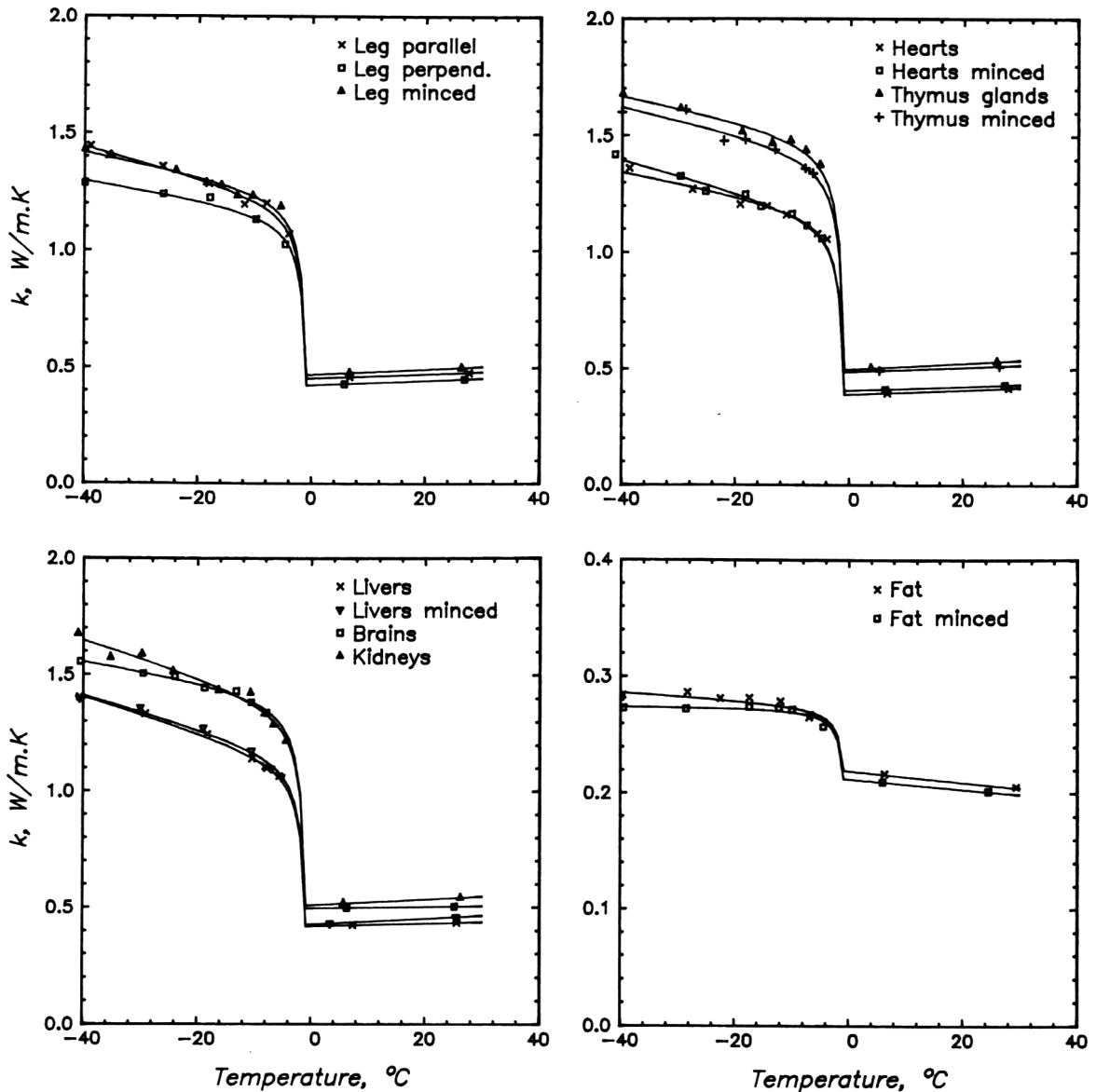
Fig. 5—Thermal conductivity data for lamb meat, offals, and fat.

Table 2—Empirical curve-fitting parameters for eqns (1b) and (2)

Material	k_f W/mK	b W/mK^2	c W/m	d W/mK^2
Group I				
Leg muscle parallel	0.450	−0.0063	0.69	0.0009
Leg muscle minced	0.466	−0.0043	0.71	0.0011
Hearts	0.390	−0.0046	0.71	0.0009
Hearts minced	0.407	−0.0065	0.68	0.0008
Liver	0.417	−0.0073	0.65	0.0006
Liver minced	0.425	−0.0067	0.67	0.0012
Brains	0.494	−0.0039	0.84	0.0003
Kidneys	0.507	−0.0075	0.78	0.0012
Thymus	0.497	−0.0047	0.91	0.0012
Thymus minced	0.487	−0.0053	0.85	0.0009
Group II				
Leg muscle perpendicular	0.421	−0.0037	0.67	0.0010
Group III				
Fat	0.219	−0.0003	0.05	−0.0005
Fat minced	0.212	−0.0000	0.06	−0.0004

sidered further) showed much higher k-values at low temperatures, suggesting that it had a higher freezable (unbound) water content.

Comparison with previous data

Whole lamb muscle. Only two sets of data have been published on the thermal conductivity of lamb or mutton muscle meat. Leitman's (1967) data, obtained over a more limited temperature range than in this work, are in reasonable agreement with the present data (maximum error 7% except for one point at -4°C, where the thermal conductivity changes very quickly with temperature) (Fig. 7) Leitman's data, however, indicate that thermal conductivity of unfrozen lamb is higher when fibers are perpendicular to heat flow. Bazan and Mascheroni's (1984) data for fibers PERPENDICULAR to heat flow seem to agree more with the present data (and Leitman's) for fibers PARALLEL to heat flow. Very little detail was published by Bazan and Mascheroni (1984) on the product's previous treatment or the experimental technique (these authors used a line-source method, but no calibration data against a known material were reported).

All Group I materials (nonfat, nonperpendicular). Sweat (1975) examined more than 200 previous datapoints on the thermal conductivity of meats with x_w in the range 0.6 to 0.8, and found that they can be best fitted with the following empirical equations:

$$k = 0.080 + 0.52x_w, \quad 0 < T < 60°C \qquad (7)$$

$$k = -0.28 + 1.9x_w - 0.0092T, \quad -40 < T < -5°C \qquad (8)$$

Equation (7) was originally obtained by Spells (1960). The standard deviation from eq. (7) was 8.6% for 110 datapoints

Fig. 6—Thermal conductivity of freshly obtained and prefrozen brains.

Fig. 7—Comparison of present and previous data for lamb meat. — Present data; --- Bazan and Mascheroni (1984), fibers perpendicular to heat flow; o Leitman (1967), fibers paralles to heat flow; x Leitman (1967), fibers perpendicular to heat flow.

Fig. 8—Comparison of present and previous data for all Group I materials. o Present data; — Sweat's (1975) regression equations; --- Range encompassing one standard deviation for previous data.

as the temperature dependency is described in a rather unsatisfactory manner, there being a gap in the range -5°C to T_f, which is important in, say, finite difference or finite element calculations. For such prediction the reader is referred to the section on comparison with predictive equations.

Group II materials (fibers perpendicular to heat flow). Sweat (1975) found from examining previous beef and pork data by Lentz (1961) and Hill et al. (1967) that "parallel" k-values below freezing are on average 8.7% lower than "perpendicular" values. This agrees with the 8% difference found in this study.

Group III materials (fats). No data have been previously reported for the thermal conductivity of mutton fat. However, that of beef fat with about 10% moisture had been reported as being 0.21 W/mK above freezing and 0.27 to 0.28 W/mK below freezing (Lentz, 1961; Morley, 1966), in excellent agreement ($\pm 5\%$) with this study.

Comparison with predictive equations

Description of models. Miles et al. (1983) list 31 equations for calculating thermal conductivity of foodstuffs, and this is by no means an exhaustive list. Most, however, are empirical equations derived to fit restricted datasets or a restricted range of parameters, similar to Eq. (1), (7) or (8), and cannot be used for predictive purposes. Some other equations, such as that of Choi and Okos (1986), are variations on one of those listed below. Still others, such as Van Beek's (1974) or Levy's (1982), require parameters that are not measurable and that would have to be found by curve fitting.

We shall, therefore, consider only six equations that rely on some kind of physical model to account for the contribution of individual components, and that are capable of general application. The models considered are:

and that from eq. (8) was 9.4% for 121 datapoints. These regression equations agree well with the present data for all Group I materials at $+20°C$ and $-30°C$ respectively (Fig. 8). (There are three extra datapoints at $+20°C$, which come from repeat runs on leg muscle parallel at 74.4% moisture, hearts at 77.3% moisture and minced hearts at 77.0% moisture, all unfrozen.) The maximum difference between the present data and Sweat's equations was within one standard deviation of all previous data examined by Sweat (1975). (The differences may be marginally higher if temperatures at the extremes of the ranges given by Sweat were used.) Thus, the present data are completely consistent with previous data for high-moisture meats and fish. However, the authors do not recommend using Eq. (7) and (8) for predictive purposes in precise calculations,

1. The parallel model: each component forms a parallel heat-conducting path.

$$k = \Sigma v_j k_j \qquad (9)$$

2. The series model: heat flows successively through each component so that their resistances are added.

$$1/k = \Sigma v_j/k_j \qquad (10)$$

3. The Maxwell-Eucken model (Eucken, 1940): one component (water or ice for water-rich materials, fat for fat-rich materials) forms a continuous phase, with the other components being widely dispersed as bubbles.

$$k/k_c = (1 - 2r.v_d)/(1 + r.v_d) \qquad (11)$$

where $r = (k_c - k_d)/(2k_c + k_d)$. In this case there are several dispersed components, so k_d will be calculated as the volume-averaged value of the minor components, using Eq. (7). Although not rigorous, this approach is acceptable since among the minor components one always predominates. Furthermore, the conductivities of the fat and protein components are almost equal.

4. Levy's (1981) model: a modification of the Maxwell-Eucken model (Eucken, 1940). To ensure symmetry between the continuous and dispersed phases (i.e. same numerical result with indices c and d reversed), Levy proposed to replace v_d in the Maxwell-Eucken equation by a function F, given by

$$2F = 2/s - 1 + 2v_d - [(2/s - 1 + 2v_d)^2 - 8vd/s]^{1/2} \qquad (12)$$

where $s = (k_d - k_c)^2/[(k_c + k_d)^2 + k_c.k_d/2]$ \qquad (12a)

5. Kopelman's (1966) model: particles or fibers of a dispersed phase in a continuous phase. Only Kopelman's perpendicular heat flow model will be considered here, as his parallel model gives worse results on this dataset. Thermal conductivity is given by

$$k/k_c = (1 - Q)/[1 - Q(1 - M)] \qquad (13)$$

where $M = v_d^{1/3}$ and $Q = (1 - k_d/k_c)M^2$

6. Hill et al. (1967) model: fibrous network in continuous phase with simultaneous parallel and series conduction:

$$k = (2t - t^2)k_d + (1 - 4t + 3t^2)k_c$$
$$+ 8(t - t^2)k_c k_d/[tk_c + (4 - t)k_d] \qquad (14)$$

where $t = 2 - \sqrt{4 - 2v_d}$ \qquad (14a)

To use the above models, the thermal conductivities and densities of the components must be known. Table 3 summarizes their values, taken from Miles et al. (1983). For water (Fig. 4) and ice (Choi and Okos, 1986):

$$k_w = 0.560 + 0.00165\ T \qquad (15)$$

$$k_i = 2.2196 - 0.0062489\ T + 0.00010154\ T^2 \qquad (16)$$

To calculate the ice fraction below freezing, the model of Schwartzberg (1976) is used, since it has been verified for meats by Pham (1987b) using calorimetric data. Schwartzberg divides the water into a bound fraction and a freezable fraction,

the latter behaving as an ideal solution so that its frozen fraction varies as $(1-T_f/T)$. Thus, the ice fraction is calculated from

$$x_i = (x_w - x_b)(1 - T_f/T) \qquad (17)$$

where the bound water fraction is related to the protein fraction by (Pham, 1987b)

$$x_b = 0.4x_p \qquad (18)$$

Pham (1987b) found that x_b can on average be expressed either as 0.3 (total solids) or 0.4 (protein fraction). However, using the former would lead to x_b values greater than the total water fraction x_w for fatty tissues. Since fat itself has very low water binding capacity, it was felt that relating the bound water fraction to the protein fraction was more justified.

The sum of mass percentages in Table 1 do not always add up to 100%, due to experimental errors. In the calculations it was assumed that the moisture contents were exactly as measured, since they were obtained by simple physical measurements, and the percentages of all other components were corrected by the same factor, to add up to 100% total.

Results of comparison

Thermal conductivity values at 0,-5 and -40°C were calculated for all Group I and III materials from the smoothed data [Eq. (1b) and (2) together with Table 2] as well as according to the five theoretical models above. For those models that require a continuous and a dispersed phase, the former was taken as the component with highest volume fraction. Thus, it can be liquid water, ice or fat, depending on composition and temperature. Percentage errors for each product are shown in Table 4 and their statistics summarized in Table 5.

The best predictions by far are obtained with Levy's model, which yields correct values (-6, +10%) over the whole range of compositions (11 to 80% water, 3 to 86% fat, 3 to 20% protein) and temperatures (both above and below freezing) considered. Both the mean error and the standard deviation are considerably smaller than the other models (Table 5). The drawback of Levy's equation is a certain lack of physical justification, since it was based on mathematical rather than physical arguments. It is also more complicated than the other equations, although this is not a serious drawback in this day of personal computers. Next in accuracy are the Maxwell-Eucken model and Kopelman's model. These two models produce similar results, as can be expected from their physical similarity. Next come Hill et al. (1967) model and the parallel model. The parallel model, which is favored by the COST 90 group (Meffert, 1983; Miles et al., 1983), gives high values for frozen meats, a result in agreement with the COST 90 group's for meat products. Hill et al. (1967) obtained good agreement between their model and data but only by using an unrealistic value for the dispersed (fibrous) phase conductivity. (They used 0.037W/mK, a bulk value measured for freeze-dried, nearly 90% porous fibers.) The series model gives very low predictions.

Note that as the k-values of all components are not too dissimilar above freezing, it is not surprizing that all models except the series model give similar, and reasonably good, results in that temperature range. If the anomalous low measured value of unfrozen hearts, which may have been due to air in the heart chambers, were ignored, mean errors were between -1% and +4%, standard deviations 4 to 5%, and errors limits -6 to +11% for unfrozen products, so with minor adjustments in the coefficients any model could be made to fit the data. The real test is at sub-freezing temperatures, when the thermal conductivity of one component (ice) is an order of magnitude higher than that of the others.

The accuracy of each model depends to a certain extent on the values of the physical parameters used. Of these, the most

Table 3 — Properties of meat components

j-th component	k_j W/mK	ρ_j kg/m³
Fat	0.18	930
Protein	0.20	1380
Water	Eq.(15)	1000
Ice	Eq.(16)	917
Mineral	0.26	2165

Table 4—Accuracy of theoretical models. Positive errors mean that theoretical values are higher than experimental values

Material	T °C	k W/mK	% Errors of theoretical models					
			Parallel	Series	Maxwell-Eucken	Levy	Kopelman	Hill et al.
Leg muscle parallel	0	0.45	6	-12	3	1	5	6
	-5	1.10	35	-46	21	7	25	24
	-40	1.44	33	-54	21	4	26	24
Leg muscle minced	0	0.47	3	-14	0	-2	1	2
	-5	1.13	32	-47	18	5	23	21
	-40	1.42	36	-53	23	6	29	27
Hearts	0	0.39	17	-6	13	10	15	16
	-5	1.06	34	-51	19	3	23	20
	-40	1.35	37	-58	22	1	28	23
Hearts minced	0	0.41	11	-12	7	4	9	10
	-5	1.05	33	-52	18	2	22	18
	-40	1.40	30	-61	16	-5	21	16
Livers	0	0.42	11	-10	7	4	9	10
	-5	1.04	34	-49	19	4	23	20
	-40	1.41	28	-59	14	-5	19	15
Livers minced	0	0.42	9	-11	5	3	7	8
	-5	1.06	29	-50	15	0	19	15
	-40	1.41	25	-59	11	-7	16	12
Brains	0	0.49	0	-16	-3	-5	-2	-1
	-5	1.27	25	-48	14	3	18	18
	-40	1.56	34	-51	24	9	29	28
Kidneys	0	0.51	-1	-15	-4	-5	-2	-2
	-5	1.25	29	-43	18	7	22	22
	-40	1.65	27	-50	18	5	23	22
Thymus glands	0	0.50	-1	-16	-3	-5	-2	-1
	-5	1.34	19	-50	8	-2	12	12
	-40	1.67	25	-53	15	2	20	19
Thumus glands minced	0	0.49	-1	-18	-4	-6	-3	-2
	-5	1.29	20	-53	8	-4	12	11
	-40	1.62	24	-57	13	-3	18	16
Fat sliced	0	0.22	4	-10	-3	-2	0	4
	-5	0.27	51	-24	-7	5	8	49
	-40	0.29	67	-29	-11	3	6	65
Fat minced	0	0.21	4	-8	-3	-1	0	4
	-5	0.26	39	-24	-10	0	4	37
	-40	0.27	57	-27	-12	1	5	55

[a] References for models: Eucken (1940), Levy (1981), Kopelman (1966), Hill et al. (1967)

Table 5—Error statistics of theoretical models, and their sensitivity to the value assumed for x_b/x_p

x_b/x_p used	Model	% error statistics			
		Mean	s.d.	Min.	Max.
0.4	Parallel	+24	16	-1	+67
	Series	-36	19	-60	-6
	Maxwell-Eucken[a]	+8	10	-11	+23
	Levy[a]	+1	4	-6	+10
	Kopelman[a]	+13	10	-2	+28
	Hill et al.[a]	+18	15	-2	+65
0.3	Parallel	+26	17	-1	+70
	Series	-36	18	-60	-6
	Maxwell-Eucken[a]	+10	12	-11	+27
	Levy[a]	+2	4	-6	+10
	Kopelman[a]	+15	11	-3	+32
	Hill et al.[a]	+20	16	-2	+68
0.5	Parallel	+22	16	-1	+64
	Series	-36	20	-61	-6
	Maxwell-Eucken[a]	+7	9	-11	+21
	Levy[a]	-0	4	-10	+10
	Kopelman[a]	+12	9	-2	+27
	Hill et al.[a]	+16	14	-2	+63

x_b: mass fraction of bound water; x_p: mass fraction of protein.

[a] References for models: Eucken (1940), Levy (1981), Kopelman (1966), Hill et al. (1967).

Fig. 9—Measured and predicted thermal conductivities of three materials. — Measured; --- Predicted from Levy's (1981) equation.

influential are the thermal conductivities of water and ice and the bound water to protein ratio, x_b/x_p. The first two parameters are well-established and unlikely to cause significant errors in the predictions. The ratio x_b/x_p would also have to be changed to quite unrealistic values before the accuracy was substantially affected. Pham (1987b) found that this ratio varied from 0.3 to 0.5. Using these extreme values instead of 0.4 leaves the accuracy of the models almost unchanged (Table 5).

For illustration purposes, the thermal conductitivites of three materials, as measured (smoothed data) and as predicted from Levy's equation are shown in Fig. 9. Although agreement was not perfect, these predicted curves would be quite acceptable in the absence of data.

Finally, it must be noted that in carrying out the above model checks, all parameters used were obtained from previous authoritative sources or independent calorimetric data, without any curve-fitting on the present dataset. It :s believed that this has never been successfully done before on food materials over such a wide range of compositions and temperatures. [Choi and Okos (1986) carried out a similar study, using the parallel

model, but used error minimization methods to find the component properties.]

CONCLUSIONS

THE TWO-MATERIAL guarded hot plate method proved particularly suitable for meat products. Thermal conductivity measured over a temperature range of -40°C to +30°C for a number of fresh lamb meat, fat and offals agreed very well with previous data.

Simple empirical equations were presented to correlate the results. The good agreement of the present data with previous data suggests that Eq. (1b) to (6) might be applicable to meats and offals $(0.6 < x_w < 0.8)$ in general. Above freezing, these equations are the same as Sweat's except for an extra temperature term. Below freezing, the present equations also agreed with Sweat's equations but had the advantage in that they provided a continuous k- T curve, with no gap in the freezing region (-5°C to T_f).

The previous thermal history of the product may be an important factor. Slow freezing, long storage times or repeated freezing and thawing may cause a breakdown in the tissue structure, with consequent changes in thermal conductivity.

Six theoretical models were tested to see if they could predict thermal conductivity from temperature and composition, using parameters values from independent sources. For unfrozen products, any model except the series model could be made to fit the data. Over the whole range of temperature, Levy's model was found to be very accurate, followed by the Maxwell-Eucken model and Kopelman's model. The applicability of Levy's equation extends over a wide range of compositions and temperatures (both above and below freezing), and was not unduly affected by uncertainties in the values of physical parameters.

NOMENCLATURE

a,b,c,d	Empirical parameters in Eq. (1) or (2)
k	Thermal conductivity, W/mK
T	Temperature, °C
v	Volume fraction
x	Mass fraction
ρ	Density, kg/m^3

Subscripts:

b	bound water
c	continuous phase
d	dispersed phase
f	at initial freezing temperature $(-0.9°C)$
i	ice
j	j-th component
p	protein
w	water

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Effect of Electrical Charge on Attachment of Salmonella typhimurium to Meat Surfaces

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───────── ABSTRACT ─────────

Treating tissue with electrical current was found to affect both the initial attachment and total numbers of attached Salmonella typhimurium to meat tissues. When lean tissue was attached to the positive terminal of the power supply operating at 50 volts/125 mA DC, the total number of attached cells increased. There was no effect when the samples were attached to the negative terminal. There was a significant (P<0.05) effect on the percentage of strongly attached bacteria with an increase in treating time. Electrical current was found to increase the percentage of strongly attached cells immediately after the current was applied.

INTRODUCTION

ELECTRICAL CURRENT (electrical stimulation) has been used to tenderize meat over the last several years (Kastner, 1982). It has been alternately reported that either electrically stimulated meat does not differ microbiologically from non-stimulated meat (Kotula and Emswiler-Rose, 1981) or that stimulated meat has significantly lower bacterial counts (Grimes et al., 1988). There does not appear to be a good explanation for these reported differences, although differences in redox potentials (Mrigadat et al., 1980), deleterious effects of electricity (Riley et al.; Lin et al., 1984), or accelerated glycolysis permitting rapid chilling of the carcass (Henrickson and Asghar, 1985) have been proposed.

Bacterial attachment to surfaces generally proceeds as a two step process: reversible and then irreversible attachment (Marshall et al., 1971). The initial, reversible attachment is thought to be influenced by a variety of cell surface factors, including extracellular polysaccharides (Fletcher and Floodgate, 1973), flagella (Notermans and Kampelmacher, 1974), and surface charge (Fletcher and Loeb, 1979). Of these factors, surface charge seems to be one of the major factors in attachment, since non-fimbriated (Meadows, 1971) and nonflagellated cells (Lillard, 1985) attach at similar rates to cells which possess these structures. Because of the importance of electrical charge in attachment, it would be reasonable to assume that bacterial attachment to surfaces could be modified by altering the electrical polarity on the substrate to which the bacteria are attaching.

Bacterial cells have a net negative charge on their cell walls (Corpe, 1970), although the magnitude of the charge varies from strain to strain. Ismaeel et al. (1987) has shown differences in the relative hydrophobicity between *Providencia stuartii*, *Escherichia coli*, and *Pseudomonas aeruginosa*. They were able to alter the cell hydrophobicity by pre-treating the cells with subinhibitory concentrations of chlorhexidine, which affects the surface of the cells. Hermansson et al. (1982) related cell surface structures to the relative hydrophobicity of *Salmonella typhimurium* and *Serratia marcescens*. The presence of fimbriae on *S. marcescens* resulted in a lower hydrophobicity than the non-fimbriated cells. The differences seen with

S. typhimurium were related to oligosaccharides in the cell surface structure.

Firstenberg-Eden et al. (1978) used an "S-value" to evaluate the relative strength of bacterial attachment to chicken and beef muscle surfaces. Farber and Idiziak (1984) also used S-values in measuring the attachment of psychrotrophic bacteria to beef muscle. The S-value determines the number of bacteria which are in each stage (reversible and irreversible) of the attachment process. An increase in S-value indicates an increase in the strength of bacterial attachment or conversely, a decrease in the relative ease of removal of the cells. Using the principles of the S-value determinations, an S_R value, was calculated which represents the percentage of the total population which is strongly attached to the muscle surface.

The objective of this experiment was to study the effects of electrical current and polarity on both the total numbers as well as the relative strength of bacterial attachment to meat surfaces.

MATERIALS & METHODS

Bacterium

S. typhimurium (ATCC 14028) was used in all of the trials. The bacterium was grown and maintained in tryptic soy broth (TSB, Difco) at 37°C. An 18 hr broth culture was harvested by centrifugation (3000 × g, 10 min 4°C) and the pellet resuspended in 9 mL Butterfield's phosphate buffer (FDA, 1984). Two mL of the resuspended cells were added to 18 mL of either buffer or attachment medium (Notermans and Kampelmacher, 1974; 8.7g NaCl, 1.66g Na_2HPO_4, 0.33g NaH_2PO_4, and 0.37g EDTA per liter distilled water) and mixed gently in a sterile beaker.

Preparation of meat tissue

Fresh beef trim (lean muscle and fat tissue) were obtained from the abattoir at the U.S. Meat Animal Research Center. The tissue was separated into two distinct groups (i.e. fat and lean), such that each tissue type was free of the other tissue type. Tissues were cut into 0.5 cm thick slices and frozen until use. Immediately prior to use, the tissue was thawed and aseptically cut into 1.0 × 3.0 × 0.5 cm (10 cm^2 surface) pieces using a sterile scalpel. These pieces typically contained less than 100 colony forming units (cfu) per 10 cm^2.

Enumeration of bacteria

The tissue samples were homogenized in sterile stomacher bags in a Tekmar Stomacher 400 (Tekmar Co., Cincinnati, OH) with sterile phosphate buffer for 2 min. The samples were serially diluted in phosphate buffer and then enumerated with tryptic soy agar (TSA, Difco) using the pour plate technique (Busta et al., 1984). The plates were incubated at 37°C for 24 hr, which resulted in good growth and clearly defined colonies.

S_R determination

Both loosely and strongly attached bacteria were enumerated for each sample for the S_R determinations. "Loosely attached bacteria" were defined as those bacteria which were removed when the tissue sample was placed in a sterile bottle containing 99 mL phosphate buffer and the bottle inverted gently 25 times over a period of approximately 15 sec. Samples of the buffer were removed for determination of the bacterial population and then the buffer was decanted. The tissue sample was transferred to a sterile stomacher bag and ho-

mogenized in 99 mL phosphate buffer as described above. The population of bacteria enumerated by the stomaching procedure were described as "strongly attached bacteria." Stomaching is assumed to release the majority of attached bacteria (Tootlebee, 1975) . The S_R value was calculated by dividing the strongly attached bacteria by the total population. "Total population" is defined as the sum of both the loosely and strongly attached bacteria. S_R essentially represents the percentage of the total population which is strongly attached.

Electrical charge experiments

Experiments using DC current were carried out using a Heath Regulated High Voltage Power Supply, Model SP-2717 (Heath Co., St. Paul, MN). The power supply was connected to the tissue samples using 18 gauge stranded wire and square ended alligator clips (Radio Shack, catalog number 270-345). Experiments using AC current were carried out using an isolation transformer (Model TR-110; Dyanscan Corp., Chicago, IL) connected to a variable autotransformer (type 3PN 1010, Staco, Inc., Dayton, OH), which was connected to a power transformer (115V, 50/60 Hz; Triad-Utrad Distributors, Huntington, IN). The power transformer was connected to the tissue samples using 18 gauge stranded wire and alligator clips. Voltage and current were determined with a digital multimeter (John Fluke Mfg. Co., Inc., Everett, WA).

To determine the effect on total numbers, tissue samples were connected to either the cathode or anode on the power supply, with the opposite electrode connected to a cylindrical metal probe which tapered to a sharp point (soldering iron tip, Radio Shack, catalog number 64-2065A). The sample and probe were inserted simultaneously into a 50 mL beaker containing 20 mL of the bacterium/buffer suspension. The power supply was energized and the current applied at either 25 or 50 volts DC (ca. 125 mA) or AC (ca. 20 mA). After 5 min (1 min for the 50 volt AC), the power supply was disconnected and the tissue sample was gently washed in 10 mL phosphate buffer for approximately 15 sec prior to enumerating the bacteria.

To determine the effect of charging time on S_R values, tissue samples were incubated in the bacterium/buffer mixture for 5 min, then connected to the power supply. The experiment was carried out as described above, except that the charge time was varied from 1 to 5 min and the solution in the charging beaker was changed to attachment medium. Loosely and strongly attached bacteria were enumerated after treatment.

To determine the S_R values after treatment, tissue samples were incubated in the bacterium/buffer mixture for 5 min. The tissue was then attached to the power supply lengthwise, such that one end of the sample was attached to each terminal of the power supply. The samples were charged for 1 min suspended in air by the alligator clips. The tissue samples were then suspended in sterile beakers by alligator clips so that the samples did not contact the sides of the beaker. Loosely and strongly attached bacteria were enumerated at specified time intervals. One set of charged samples was stored in a 5°C incubator for 24 hr prior to enumeration.

If the mechanism of bacterial attachment to the tissue surfaces was altered, the sensitivity to chemical sanitizers of the attached bacteria might also be altered. The sensitivity of attached bacteria to acetic acid was determined by incubating samples in the bacterium/buffer mixture for 5 min and connecting them to either the positive or negative terminal of the DC power supply. The opposite terminal was connected to the metal probe. The probe and sample were inserted into a beaker containing 20 mL sterile 2% acetic acid and treated with 25 or 50 volts for 10 sec, after which loosely and strongly attached bacteria were enumerated.

Alternately, the tissue samples were incubated in the bacterium/buffer solution for 5 min and connected lengthwise to both terminals of the power supply as described previously. The samples were treated with 50 volts DC for 1 min and suspended in sterile beakers as described in "S_R values after charging." After specified time intervals, the samples were washed in 20 mL filter-sterilized 2% acetic acid for 10 sec in 25 × 150 mm glass tubes on a Vortex Genie 2 (Scientific Industries, Bohemia, NY) set at 75% of the maximum speed setting. Loosely and strongly attached bacteria were enumerated after washing.

Statistical analysis

All experiments were carried out using a full factorial design with a minimum of two independent replications (Steel and Torrie, 1980).

RESULTS & DISCUSSION

Effect on total numbers

The initial experiments attempted to determine if electrical current could increase or decrease the number of bacteria which would attach to meat surfaces. Therefore, essentially sterile meat samples were placed in a bacterium/buffer solution and simultaneously exposed to electrical current. There was no significant difference in the numbers of attached bacteria between the control and DC (negative terminal) voltage treatments (Table 1). A 50 volt current caused an increase in attached bacteria when either fat or lean tissue was connected to the positive terminal. These results tend to support the report that bacterial cells have a net negative charge (Corpe, 1979) and therefore should migrate toward a positive charge. The effect of AC electrical current was different than that of DC current; there was a reduction in attached cells with an increase in voltage (Table 2). This effect is probably attributable to localized heating of the tissue samples, and is not an indication of the repulsive properties of AC current.

Effect of treatment time on S_R and total population

To assess the effects of electrical current on attached bacteria, tissue samples were inoculated prior to treatment. There was a significant ($P < 0.05$) increase in S_R values as treatment time increased (Fig. 1). The results were more consistent when the meat was connected to the positive terminal of the power supply. There was no significant ($P > 0.05$) difference between 25 and 50 volts when the tissue was connected to the positive terminal, although both treatments differed significantly ($P < 0.05$) from the controls at 0 and 120–300 sec. When the tissue was connected to the negative terminal, there was a significant ($P < 0.05$) difference between the two voltage settings after 240 sec of charging.

Treatment time also had an effect on the population of S. typhimurium (Fig. 2). There was a one half log (25 volts) to 1 log (50 volts) reduction in the count from 15 to 300 sec treatment time when the meat was connected to the positive terminal. The populations of the treated samples were significantly different ($P < 0.05$) from the control samples after 120 sec, and the different voltages were significantly ($P < 0.05$) different after 240 sec.

When the meat was connected to the negative terminal, the results were less consistent. At 15 sec, both the 25 and 50 volt samples were significantly ($P < 0.05$) lower than the controls. However, after 180 sec there was no significant difference

Table 1 — Effect of DC electrical current on numbers[c] of Salmonella typhimurium attached to meat tissues

| Tissue | Control | Volts DC[d] | | | |
		25 +	25 −	50 +	50 −
Lean	6.45[a]	6.32[a]	6.52[a]	6.91[b]	6.52[a]
Fat	6.24[a]	6.53[a]	6.29[a]	6.96[b]	6.44[a]

[a,b] Means within rows with different superscripts are significantly different ($P < 0.05$).
[c] Log_{10} colony forming units/10 cm^2; average of three to five experiments.
[d] Charge indicates terminal on power supply to which tissue was connected; treatment time = 5 min.

Table 2 — Effect of AC current on total numbers[c] of Salmonella typhimurium attached to meat tissues

| Tissue | Volts (AC) | | |
	0	25	50
Lean	7.03[a]	6.75[a,b]	6.48[b]
Fat	7.14[a]	7.26[a]	6.75[a,b]

[a,b] Means within rows and columns with different superscripts are significantly different ($P < 0.05$).
[c] Log_{10} colony forming units/10 cm^2; average of duplicate experiments.
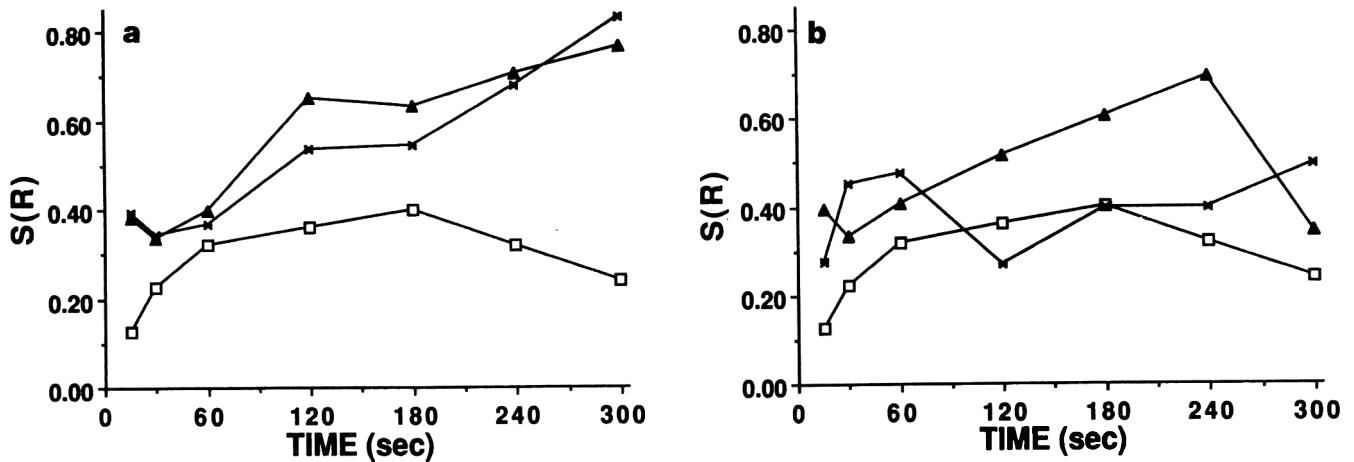
Fig. 1—Effect of charging time on S_R values of Salmonella typhimurium. (a) tissue connected to the positive terminal; (b) meat connected to the negative terminal. (□) = control; (X) = 25 volt DC; (▲) = 50 volt DC.
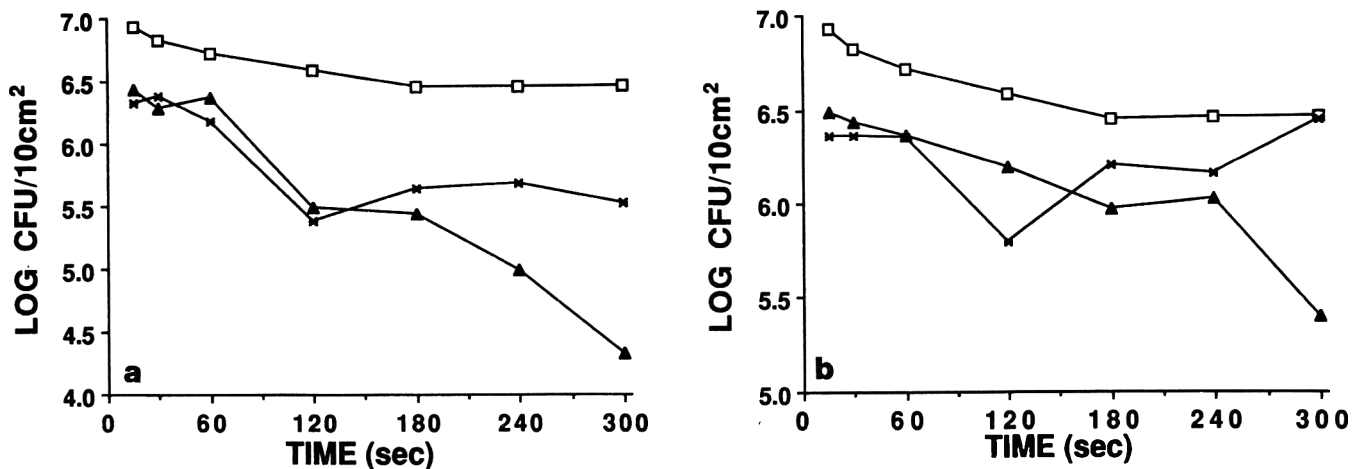
Fig. 2—Effect of treatment time on total of Salmonella typhimurium population. (a) tissue connected to the positive terminal; (b) meat connected to the negative terminal. (□) = control; (X) = 25 volt DC; (▲) = 50 volt DC.

between the control and the 25 volt sample. The 50 volt sample differed significantly (P < 0.05) from the control after 180 sec.

It is unlikely that the reduction in numbers of attached bacteria reflects a physical detachment from the meat surface. The reduction is probably attributable to either localized heating effects or other toxic effects of electrical current. There was some localized heating of the tissue samples, particularly in the areas immediately adjacent to the clips used to connect the tissue to the power supply, which may have accounted for this reduction in population. This heating was evident by the distinct "cooked" appearance of the sample in these areas. In addition, Lin et al. (1984) reported that electrical current (30 volt, 5 min) reduced the populations of several pure cultures of bacteria in normal saline by 4 to 5 log cycles, so that no viable bacteria could be recovered. The suspensions of bacteria were held in an ice bath to minimize the effects of heating. They speculated that this reduction could be attributed to either increased electrical flow or the production of chlorine by electrolysis.

Changes in S_R after treatment

The previous experiments measured the combined effects of electrical current and an aqueous charging system. Therefore, the experimental conditions were modified to eliminate this aqueous system, as well as minimize the effect of electrical polarity. The tissue was connected to the power source, so that one end was attached to each terminal. The tissue was then

suspended in air for a 1 min treatment time. These conditions also simulate more closely what happens to an electrically stimulated carcass.

There was a significant (P < 0.05) increase in the percentage of strongly attached bacteria from 0 to 30 min with the control samples, although there was no significant (P > 0.05) change from 30 to 60 min (Fig. 3). The percentage after 24 hr was significantly (P < 0.05) higher than after 60 min, although the greatest change in value occurred between 0 and 60 min. A 25 volt DC treatment for 1 min produced an S_R value at 0 min equivalent to the 10 min control sample. This value remained constant for 10 min, then increased. The 30, 60 and 24 hr samples did not differ from the control values. A 50 volt DC treatment resulted in an S_R value that was significantly (P < 0.05) greater than both the control and 25 volt DC sample at 0 and 10 min and was constant for the first 30 min. After 30 min, there was no difference between any of the samples, and the values for all of the samples increased with time.

There was no significant (P > 0.05) difference between the populations of either the control or the 25 volt sample at any time (Fig. 4). The 50 volt DC samples differed significantly (P < 0.05) from the control at 0 min and from the control and 25 volt DC samples at 30 min. For any treatment, There was no significant difference in population from 0 to 60 min. As expected, the populations decreased after 24 hr at 5°C for all treatments, although this decrease was not significant (P > 0.05) for the 50 volt DC samples.

By increasing the voltage, the percentage of strongly at-
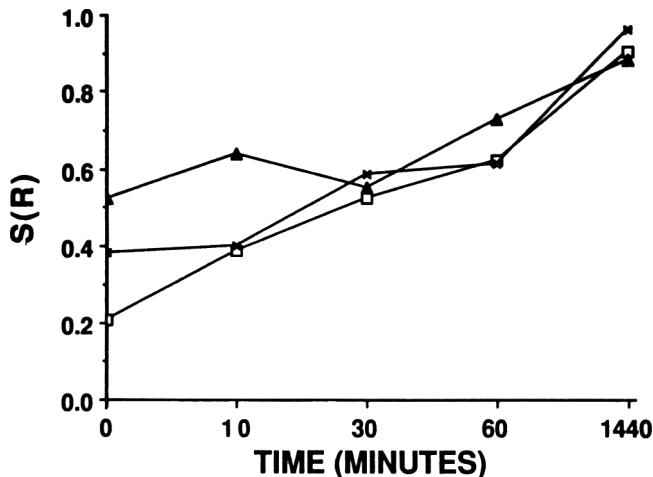
Fig. 3—Changes in S_R values of Salmonella typhimurium with time after treatment for 1 min. (□) = control; (X) = 25 volt; (▲) = 50 volt.
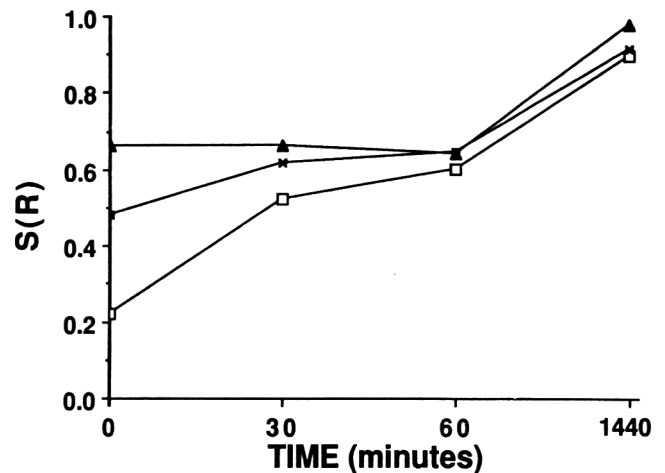
Fig. 5—Changes in S_R values of Salmonella typhimurium with time after treatment with AC electrical current for 1 min. (□) = control; (X) = 25 volt; (▲) = 50 volt.
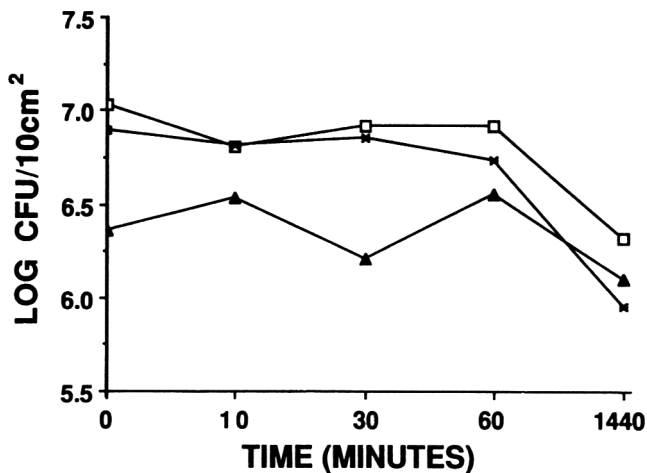
Fig. 4—Changes in total Salmonella typhimurium population with time after treatment with DC electrical current for 1 min. (□) = control; (x) = 25 volt; (▲) = 50 volt.

Table 3—Effect of AC current on total population[d] of Salmonella typhimurium attached to lean tissue

Time[a]	VOLTS (AC)		
	0	25	50
0	7.15[a]	6.93[a]	6.71[a]
30	6.99[a]	7.02[a]	6.72[a]
60	7.01[a]	6.94[a]	6.79[a]
1440[f]	6.46[b]	6.72[a]	5.57[c]

[a,b,c] Means within rows and columns with different superscripts are significantly different (P<0.05).
[d] Log$_{10}$ colony forming units/10 cm²; average of duplicate experiments.
[e] Time in minutes after treatment.
[f] Samples stored at 5°C.

Table 4—Simultaneous effects of electrical current and 2% acetic acid on Salmonella typhimurium and S_R values

Current	Total population[c]		S_R Value	
	+[d]	−	+[d]	−
0	6.68		0.412[a]	
25	6.62	6.57	0.526[a,b]	0.591[a,b]
50	6.50	6.61	0.684[b]	0.487[a,b]

[a,b] Means within rows and columns with different superscripts are significantly different (P<0.05); average S_R value of duplicate experiments.
[c] Log$_{10}$ colony forming units/10 cm²; average of duplicate experiments.
[d] Terminal of power supply to which tissue sample was connected.

Table 5—Effect of 2% acetic acid on Salmonella typhimurium and S_R values after treatment with electrical current

Voltage	Time (post treatment)		
	0 min	30 min	60 min
Total count			
0 (control)	5.77[d]	5.96	6.08
50 volt	6.08	5.93	5.82
S_R values			
0 (control)	0.752[3,a]	0.915[b]	0.837[b]
50 volt	0.845[a,b]	0.899[b]	0.948[b]

[a,b] Means within rows and columns with different superscripts are significantly (P<0.05) different.
[c] Log$_{10}$ colony forming units/10 cm²; average of duplicate experiments.
[d] No significant difference (P>0.05) between total counts.

tached bacteria was increased instantly from approximately 20% with the control to 52% with the 50 volt DC samples. There was a negative effect on population with a 50 volt DC treatment, although the net difference was less than 1 log cycle and not significant (P>0.05). AC current also influenced S_R values and total population. Overall, the S_R values were significantly lower (P<0.05) between the control and treated samples, and between the 25 volt (P<0.1) and 50 volt values (Fig. 5). After 24 hr, the total population on the 50 volt samples were significantly (P<0.05) lower than either the control or 25 volt samples (Table 3).

Sensitivity of attached bacteria to acetic acid

If electrical current altered the mechanism of bacterial attachment, the sensitivity of the attached bacteria to sanitizers might also be altered. However, DC electrical current did not alter the bacterium's sensitivity to 2% acetic acid, whether the acid was applied during (Table 4) or after (Table 5) treatment. There was no difference in the effects of acetic acid on total population with either the controls or the treated samples, irrespective of the time after inoculation and treatment. Acetic acid will generally result in approximately a one-half to 1 log cycle reduction in the population of bacteria (Quartey-Papafio et al., 1980), although the difference may not be statistically significant.

The mean S_R value of the 50 volt positive polarity samples was significantly (P<0.05) greater than that of the control when the current was applied with the sample in the sanitizer (Table 4), although there were no other differences in S_R means. The S_R value for the 0 min control was significantly (P<0.05) less than that of either the 60 min 50 volt treatment or either 30 min treatment (Table 5). Most of the effect of the acetic acid can be attributed to the effect on loosely attached bacteria,

since after washing most of the remaining bacteria were strongly attached (high S_R values). Time and electrical current were not factors in the effect of acetic acid on the control samples, although the increasing percentage of strongly attached bacteria (with time) should be kept in mind when designing carcass washing protocols (Anderson et al., 1987).

CONCLUSIONS

THE DATA strongly suggest that electrical charge is involved in the attachment process of bacteria to meat surfaces. The use of electrical current increased both the total numbers of attached bacteria to meat surfaces in aqueous solutions, as well as increasing the percentage of strongly attached bacteria when the current was passed directly through the tissue. The bacteriocidal effects of electrical charge were also noted, and, although the reductions attributed to electrical charge were not great, electrical charge may be responsible for some of the reported reductions in bacteria on electrically stimulated meat. Washing with acetic acid reduced the total numbers of bacteria on meat surfaces, but this reduction was largely attributable to the effect on loosely attached bacteria. Electrical current did not appear to alter the mechanism of attachment, since there was no difference in sensitivity to acetic acid by bacteria attached using electrical current.

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Influence of Phospholipid Content and Fatty Acid Composition of Individual Phospholipids in Muscle from Bison, Hereford and Brahman Steers on Flavor

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——————————— ABSTRACT ———————————

Neutral lipid (NL) and phospholipid (PL) fractions and their corresponding fatty acid profiles from the *L. dorsi* of Bison, Hereford, and Brahman steers of similar age finished on identical diets were determined. Compositional differences were related to sensory characteristics of the samples. Phosphatidylcholine + lysophosphatidylethanolamine (PC + LPE) existed in the highest concentration in all samples evaluated followed by lysophosphatidylcholine (LPC), phosphatidylethanolamine (PE), sphingomyelin (SPH), fraction one and phosphatidylserine (PS). Bison contained more total PL, particularly PC + LPE and LPC, than the other two bovine species. Polyunsaturated fatty acid (PUFA) composition of these PL was greater for Bison. Off-flavor and aftertaste increased with increasing levels of PC + LPE and LPC due to their greater composition of PUFA.

INTRODUCTION

VARIATIONS in bovine carcass composition have been attributed to genetic effects (Koch et al., 1976; Peacock et al., 1979), nutrition (Berg and Butterfield, 1968; Loveday and Dikeman, 1980) and physiological maturity (Johnson, 1974a,b). Different breeds of cattle, in particular, have been shown to differ in distribution of their carcass fat (Charles and Johnson, 1976; Kempster et al., 1976). Lunt and coworkers (1985) demonstrated that Brahman steers tended to deposit more of their total fat as subcutaneous fat early in their feeding period, whereas Angus steers have more seam fat as a percentage of carcass weight. Several studies (Joksimovic and Ognjanovic, 1977; Valin et al., 1984) have indicated the meat from Asian water buffalo *(Bubalus bubalis)* carcasses contains less intramuscular lipid than that from similarly fed beef cattle.

Fatty acid composition of bovine tissues has been widely studied. Results of these studies show that the fatty acid composition of bovine tissues can be influenced by diet (Brown et al., 1979; Melton et al., 1982; Skelly et al., 1978; Westerling and Hedrick, 1979). Animal age (Clemens et al., 1973; Hornstein et al., 1961; Link et al., 1970), sex characteristics (Eichhorn et al., 1985) and breed (Yoshimura and Namikawa, 1983) are other factors that can produce fatty acid compositional changes. Meat from the Asian water buffalo *(Bubalus bubalis)*, in particular, has been found to contain more polyunsaturated fatty acids compared with beef (Sinclair et al, 1982).

Fatty acids are important precursors of beef flavor since they are the primary source of carbonyl compounds upon heating (Selke et al., 1977, 1980). In general, the more unsaturated a fatty acid, the more susceptible it is to oxidation and a greater rate of autoxidation (Gokolp et al., 1983; Lea, 1957). The abundance of oxidatively unstable polyunsaturated fatty acids in animal tissues makes these tissues highly susceptible to off

flavors and in particular, warmed-over flavor (WOF), which is the rapid flavor deterioration in cooked meats (Pearson et al., 1977; Pearson and Gray, 1983; Younathan, 1985). Hornstein et al. (1961) concluded that upon exposure to the atmosphere, phospholipids (PL) extracted from pork and beef muscles developed rancid off-flavors much faster than neutral fats. This high susceptibility of phospholipids (PL) to oxidation is attributed to their high concentration of polyunsaturated fatty acids (Sato and Herring, 1973; Pearson et al., 1977). Wu and Sheldon (1988) recommended examining the polyunsaturated fatty acid (PUFA) profiles of individual PL fractions rather than the fatty acid profile of the total lipid fraction when evaluating the susceptibility of muscle foods to the development of off-flavors or WOF since it appears as though both the nitrogen moiety of the PL and the degree of unsaturation influence the level of lipid autoxidation.

The primary objective of this study was to evaluate the influence of genetic differences associated with three bovine species *(Bison bison, Bos taurus,* and *Bos indicus)* on the composition of lipid precursors of beef flavor. Specifically, the objectives were to: (1) establish the quantity and distribution of individual PL of Bison, Hereford, and Brahman cattle; (2) evaluate the fatty acid composition of the individual PL as influenced by animal and species differences; and (3) relate these findings to the sensory characteristics of the cooked meat.

MATERIALS & METHODS

Sample preparation

Animals used in this study included 10 Bison, 12 Hereford and 10 Brahman steers from an experiment in progress at the Roman L. Hruska U.S. Meat Animal Research Center, Clay Center, NE. All steers were finished on a diet consisting of corn silage (66%), corn (22%), and soybean-mineral supplement (12%) on a dry matter basis and slaughtered at approximately 18 months of age. After a 24 hr chill period, USDA quality and yield grades were determined (USDA, 1980) for each carcass. The right side of each carcass was stored at 2–3°C for seven days prior to processing. Seven 2.5 cm thick steaks from the anterior portion of the shortloin (Longissimus dorsi muscle) were removed from the right side of the carcass for sensory evaluation, objective tenderness and lipid analyses. Steaks one and three were used for modified descriptive analysis, five and six were used for Quantitative Descriptive Analysis, steak two for shear determination, steak four for myofibril fragmentation index, and steak seven for the lipid analyses described in this manuscript. The steak sample for lipid analysis was wrapped in laminated freezer paper, frozen and shipped overnight to North Carolina State University. Samples arrived in the frozen state and were immediately placed in the freezer and stored at −20°C until analyzed. Storage time prior to analysis ranged from four to six months with samples being randomly selected from the freezer to eliminate potential effects due to time in frozen storage.

Steaks were thawed, trimmed of external fat and connective tissue and the L. dorsi was diced into small pieces (5 mm square). Intramuscular lipids were extracted and washed from a 10 gm sample of lean by the procedure of Folch et al. (1957). All solvents used in this experiment were HPLC Grade (Fisher Scientific, Fair Lawn, NJ). Butylated hydroxy toluene (BHT) was added to the chloroform at 0.01% to increase the oxidative stability of the lipids during extraction. The lipid extracts were evaporated to dryness under vacuum at

30–35°C then redissolved in 20 mL chloroform:methanol (20:1, v/v). The entire sample (in 20 mL) was passed through a Sep-pak silica cartridge (Millipore, Waters Chromatography Division, Milford, MA). The unretained neutral lipid fraction was collected and the phospholipids were then eluted from the cartridge with 10 mL chloroform:methanol (1:1, v/v) and then 20 mL 100% methanol. The silica cartridge was recharged with 20 mL chloroform:methanol (20:1, v/v). The neutral lipid fraction was refiltered as described above and the two eluates of each lipid fraction were combined. The neutral lipid was diluted to 25 mL with chloroform:methanol (20:1, v/v) and the phospholipid fraction was evaporated to dryness at 30–35°C under vacuum and immediately redissolved in 2 mL chloroform:diethyl ether (1:2, v/v).

HPLC separation of phospholipids

Phospholipids were separated into six fractions by high performance liquid chromatography (HPLC) (Kaduce et al., 1983). A Waters (Millipore, Waters Chromatography Division, Milford, MA) liquid chromatograph system consisting of a Model 510 pump, UK6 injector and a 3.9 mm × 15 cm Resolve Column (Millipore, Waters Chromatography Division, Milford, MA) packed with 5μ spherical silica were used for separation. Lipids isocratically eluted from the column were monitored at 202 nm using a Model 490 programmable multiwavelength detector. Mobile phase composition, flow rate and sample size were optimized to acetonitrile:methanol:sulfuric acid (100:2.5:0.05, v/v/v), 0.8 mL/min and 20 μL, respectively. Phospholipid phosphorus content was quantitated by the method of Bartlett (1959) as modified by Dittmer and Wells (1969). Since phospholipids contain approximately 4% phosphorus, individual phospholipid concentrations were estimated by multiplying their phosphorus values by 25. Lysophospholipids contain only one fatty acid residue, therefore, the concentration of lysophosphatidylcholine (LPC) was estimated by multiplying the phosphorus concentration by 16. Since phosphatidylcholine (PC) and lysophosphatidylethanolamine (LPE) coelute, a factor of 25 was used. This may overestimate the contribution of LPE. The phospholipids were identified by comparing their retention times with authentic standards (Sigma Chemical Co., St. Louis, MO) and confirmed by thin-layer chromatography as reported earlier (Wu and Sheldon, 1988).

Capillary gas chromatography of fatty acids

Fatty acid composition of the neutral lipid and each phospholipid fraction was determined by preparing fatty acid methyl esters (FAME) (Morrison and Smith, 1964). Fatty acids were esterified from 0.5 mL of the neutral lipid fraction. Fatty acids in the phospholipid fractions were prepared immediately following HPLC by direct esterification of the HPLC column eluents.

FAME were analyzed using a Varian (Varian Corp., Palo Alto, CA) 3700 gas chromatograph equipped with an FID detector. FAME were separated on a 30 M SP2330 (Supelco Inc., Bellefonte, PA) capillary column with an internal diameter of 0.25 mm and a 0.2μ film thickness. The injector and detector were maintained at 230°C and 240°C, respectively. Column oven temperature was programmed from 150°C to 220°C at 4°C/min with an initial hold of 2 min and a final hold of 3 min. Helium carrier gas flow was 2 mL/min with a split ratio of 10:1 with the carrier gas at 20 psi. Sample size injected was 0.5 μL. Data were collected and detector signals integrated with a Waters 820 Chromatography Data Station (Millipore, Waters Chromatography Division, Milford, MA). Identification of the peaks was based on retention times of reference compounds (Nu Chek Prep, Inc., Elysian, MN). Peak areas of identified fatty acids were used to determine relative percent fatty acid composition of the total fatty acids which were used in the statistical analyses.

Sensory evaluation

Samples were prepared and presented to an eight member trained sensory panel at the Meat Animal Research Center, Clay Center, NE according to the "Guidelines for Cookery and Sensory Evaluation of Meat" (AMSA, 1978). Steaks were removed from the freezer, thawed (2°C) for 18–24 hr and broiled to an internal temperature of 70°C. Steaks were evaluated for juiciness, ease of fragmentation, amount of connective tissue, overall tenderness, off-flavor and aftertaste by a modified descriptive attribute panel (MDA). Steaks were rated on a scale of 1 through 8 for juiciness, ease of fragmentation, amount of connective tissue and overall tenderness, with 1 equal to extremely dry, difficult, abundant or tough and 8 equal to extremely juicy, easy,

none or tender. Off-flavor and aftertaste were scored on a scale of 1 to 4 with 1 equal to intense and 4 equal to none. The listing of off-flavor notes experienced was required for off-flavor scores of 3, 2, or 1. These listings were used to help determine the flavor notes to be evaluated by Quantitative Descriptive Analysis (QDA). Panelists selected for this panel received two weeks of specialized training in off-flavor recognition and QDA scoring (Cross et al., 1978). Each character note was scored by making a hash mark on a 12 cm line anchored at each end with the terms none and extreme. Quantitation of results was accomplished by measuring the distance from the left side (none) of which the panelists placed a mark.

Statistical analysis

Analysis of variance for the Randomized Complete Block Design was computed using the GLM regression procedure of the Statistical Analysis System (1985). When the F-test was significant, differences between means were determined by the Waller Duncan k-ratio t-test. Correlation coefficients were also computed for selected variables.

RESULTS & DISCUSSION

Neutral Lipid

The means for percentage composition of fatty acids (percent of total fatty acids) in the neutral lipid (NL) fraction of L. dorsi muscles of Bison, Hereford, and Brahman carcasses are presented in Table 1. The major fatty acids in the NL fraction of each were palmitic (16:0), stearic (18:0) and oleic (18:1) acids which together accounted for 88–90% of the total fatty acids. Neutral lipids were composed of about 48–53% saturated fatty acids (SFA), 47–52% unsaturated fatty acids (UFA) and 1–2% polyunsaturated fatty acids (PUFA). These data are similar to those reported in literature for intramuscular neutral lipid fractions of L. dorsi muscles from beef cattle (Eichhorn et al., 1985; Link et al., 1970; O'Keefe et al., 1968).

The NL fraction from Bison muscle contained lower levels of myristic (14:0) and myristoleic (14:1) acids than that from Brahman and lower levels of myristic, myristoleic and palmitic acids than that from Herefords. Bison contained higher levels of stearic, linoleic (18:2) and total PUFA (due mainly to 18:2) in the NL fraction than Brahman and higher levels of 18:2 and total PUFA than Herefords. Sinclair et al. (1982) determined the fatty acid composition of the total lipid fraction of lean beef and Asian water buffalo and found beef to contain more palmitic and oleic acids than water buffalo and water buffalo to be higher in total PUFA, in particular, linoleic, linolenic (18:3) and arachidonic (20:4) acids. When these same lean beef and Asian water buffalo samples were separated into triglycerides (TG) and PL fractions, beef contained 2.4% PUFA

Table 1 — Means for percentage composition of fatty acids in the neutral lipid fraction of L. dorsi muscles

Fatty acid component	Bison	Hereford	Brahman
14:0	2.80[b]	4.10[c]	4.10[c]
14:1	0.50[b]	1.10[c]	1.30[c]
16:0	30.10[b]	35.80[c]	31.60[b,c]
16:1	4.19[b]	4.21[b]	4.82[b]
18:0	17.00[c]	12.70[b,c]	12.30[b]
18:1	42.80[b]	40.60[b]	44.40[b]
18:2	2.20[c]	0.80[b]	1.10[b]
20:0	0.02[b]	0.30[b]	0.02[b]
18:3	0.09[b]	0.07[b]	0.08[b]
20:1	0.30[c]	0.20[b]	0.30[c]
20:2	0.04[b]	0.04[b]	0.04[b]
20:3	0.00[b]	0.01[b]	0.00[b]
22:1	0.02[b]	trace[b]	0.01[b]
24:1	0.00[b]	trace[b]	0.00[b]
Total SFA[a]	49.92[b]	52.90[b]	48.02[b]
Total UFA[a]	50.14[b]	47.03[b]	52.05[b]
Total PUFA[a]	2.33[c]	0.92[b]	1.22[b]

[a] SFA = saturated fatty acids; UFA = unsaturated fatty acids; PUFA = polyunsaturated fatty acids.

[b,c] Means bearing different superscripts on the same line differ significantly (P≤0.05).

Table 2—Composition of phospholipid of L. dorsi muscles (mg/100g lean tissue)

Phospholipid fraction[a]	Bison		Hereford		Brahman	
NL + PA + PG + CL + PI	35.06[c]	(6.30)[b]	37.48[c]	(7.94)	37.98[c]	(7.71)
PS	23.23[c]	(4.17)	21.50[c]	(4.55)	26.28[c]	(5.33)
PE	62.72[c]	(11.26)	44.31[c]	(9.39)	44.82[c]	(9.09)
PC + LPE	277.80[d]	(49.89)	249.94[c]	(52.95)	258.52[c]	(52.45)
LPC	110.50[d]	(19.84)	77.27[c]	(16.37)	79.88[c]	(16.21)
SPH	47.56[c]	(8.54)	41.56[c]	(8.80)	45.42[c]	(9.21)
Total	556.87[d]		472.06[c]		492.90[c,d]	

[a] NL – neutral lipid; PA phosphatidic acid; PG – phosphatidylglycerol; CL – cardiolipin; PI – phosphatidylinositol; PS – phosphatidylserine; PE – phosphatidylethanolamine; PC – phosphatidylcholine; LPE – lysophosphatidylethanolamine; LPC – lysophosphatidylcholine, SPH – sphingomyelin.

[b] Values in parentheses represent the percent of total phospholipid.

[c,d] Means bearing different superscripts on the same line differ significantly (P ≤ 0.05).

in the TG and 25.9% PUFA in the PL whereas water buffalo contained 3.0% PUFA in the TG and 34.1% PUFA in the PL fractions, thus indicating the PL fractions of water buffalo may actually be more unsaturated than beef (Sinclair et al., 1982).

Phospholipid composition

The polar lipids extracted from the L. dorsi muscles were resolved via HPLC into several fractions including: fraction one, phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine + lysophosphatidylethanolamine (PC + LPE), lysophosphatidylcholine (LPC) and sphingomyelin (SPH). Under these chromatographic conditions any non-separated NL, phosphatidic acid (PA), phosphatidylglycerol (PG), cardiolipin (CL), triacylglycerol (unretained by Sep-pak), cholesterol and cholesterol esters elute in the first fraction.

Based on phosphorus analysis, total PL ranged from 472-556 mg/100g lean tissue (Table 2). Bison contained more total PL than Hereford, due mainly to increased quantities of PE, PC + LPE and LPC. Hornstein et al. (1961) and O'Keefe et al. (1968) have reported values of 430–1004 mg phospholipid per 100g wet tissue in muscle from various sites in cattle. The % ether extractable fat (Koch et al., 1987) in the L. dorsi muscle was lowest for Bison carcasses. Brahman carcasses had more fat than Bison (3.4 vs 2.7%, respectively) but less than Herefords (5.3%) which had the most intramuscular fat. Carcass characteristics including Quality and Yield grade parameters are reported elsewhere (Koch et al., 1987). Differences in intramuscular fat between Bison, Hereford and Brahman samples, although present, do not appear to explain the differences present in phospholipid composition. When PL content is expressed on a fat free basis, identical trends are observed. Link et al. (1970) demonstrated that although the amount of lipid in muscle increased from 2.56% to 15.23% of the wet tissue, during finishing, the amount of PL (relative to the weight of wet tissue rather than as a portion of total lipid) remained constant at a mean level of 535 mg/100 gm wet tissue.

The PC + LPE fraction was by far the most predominant PL present making up from 50-53% of total PL. LPC and PE are also predominant PL comprising 16-20 and 9-11% respectively. PA, PG, CL and PI together accounted for 6-8% while SPH comprised 8-9% and PS 4-5% of total PL.

Nakanishi and Suyama (1966) reported that the relative distribution of individual PL in lean and fattened beef are about the same even though large differences were seen in total lipids. The PL profiles of L. dorsi muscle reported here are not grossly different from other published studies (Nakanishi and Suyama, 1966; Simon and Rouser, 1969) with the exception of higher LPC and lower PE concentrations reported in this study. The variation is due in part to differences in the chromatographic method employed. Native plasmologens generally coelute with their corresponding phosphatidyl compounds in TLC procedures (Marinette, 1967) whereas, in HPLC methods involving acidic solvents the vinyl either linkage of plasmologens is hydrolysed resulting in the formation of lyso compounds (Kaduce et al., 1983). Since LPE coelutes with PC in this system, the concentration of each component is unknown.

Davenport (1964) found the ethanolamine fraction of beef neck muscle to be composed of 29% phosphatidyl and 71% plasmologen and, as such, LPE may be a major contributor to the PL content of the PC + LPE fraction. Further research is necessary to evaluate the contribution of the plasmologens.

Samples of steak from those same Bison were found to have a more intense off-flavor and aftertaste than samples from either Hereford or Brahman carcasses as evaluated by a Modified Descriptive Attribute (MDA) panel. Overall means of the sensory notes and means of species group for the MDA and QDA panels are presented in Table 3.

The off-flavor of steak was found to increase (become more intense) with increasing levels of PE, PC + LPE, LPC and Total PL (Table 4). Similar trends were exhibited for aftertaste with the correlation with LPC being significant at the 1.0% level and PE and PC + LPE at the 5.0% level.

In order to further characterize these flavor differences, samples were evaluated by a trained Quantitative Descriptive Attribute (QDA) panel. Samples of L. dorsi from Bison were more intense in ammonia, bitter, gamey, liverish, old, rotten and sour flavor notes and less intense in the bland flavor characteristic than those from Hereford or Brahman cattle. Little difference was noted between samples of Hereford and Brahman origin with Hereford samples being more intense in the gamey and greasy flavor notes and lower in the bland characteristic than samples from Brahman. Additionally, there were no significant differences in the bloody, browned, charred or metallic flavor notes tested (Table 3).

As in the case of the MDA panel results, PE, PC + LPE, LPC and total PL appeared to be correlated most closely with the QDA flavor notes (Table 3). As the PE content in muscle tissue increased, there was an increase in the intensity of the ammonia, bitter, gamey, liverish, and rotten flavor notes and

Table 3—Overall mean sensory panel intensity scores of L. dorsi muscle and means for individuals species group from the overall mean

Flavor note	Overall mean	Species group		
		Bison	Hereford	Brahman
MDA[a]				
Off-flavor	2.83	2.33[c]	3.02[d]	3.11[d]
Aftertaste	3.16	2.66[c]	3.34[d]	3.46[d]
QDA[b]				
Ammonia	1.63	3.33[d]	1.01[c]	0.67[c]
Bitter	1.10	1.79[d]	0.78[c]	0.79[c]
Bland	0.66	0.12[c]	0.72[d]	1.14[e]
Bloody	0.68	0.84[c]	0.69[c]	0.52[c]
Browned	1.54	1.13[c]	1.94[c]	1.48[c]
Charred	1.19	1.46[c]	1.23[c]	0.87[c]
Gamey	0.60	1.08[e]	0.55[d]	0.18[c]
Greasy	1.07	0.65[c]	1.75[d]	0.65[c]
Liverish	1.11	1.97[d]	0.84[c]	0.56[c]
Metallic	1.24	1.61[c]	1.11[c]	1.01[c]
Old	0.93	1.44[d]	0.71[c]	0.68[c]
Rotten	0.34	0.81[d]	0.16[c]	0.09[c]
Sour	1.76	2.20[d]	1.50[c]	1.64[c,d]

[a] MDA = modified descriptive analysis with 1 = intense flavor and 4 = no flavor.

[b] QDA = quantitative descriptive analysis with a value of 0 = none and 12 = extremely intense.

[c,d,e] Means bearing different superscripts on the same line differ significantly (P ≤ 0.05).

Table 4—Correlation coefficients of phospholipid composition (mg/100 gm lean tissue) and sensory notes[a] of L. dorsi muscles from Bison, Hereford, and Brahman cattle

Phospholipid[b] fraction	Off-flavor	After-taste	Ammonia	Bitter	Bland	Bloody	Browned	Charred	Gamey	Greasy	Liverish	Metallic	Old	Rotten	Sour
NL+PA+PG															
PG+CL+PI	−0.270	−0.162	0.206	0.241	−0.231	−0.034	−0.143	0.024	0.146	−0.242	0.296	0.115	0.231	0.159	−0.316
PS	−0.128	−0.016	0.184	0.151	−0.124	−0.094	−0.269	−0.045	0.116	−0.229	0.202	0.084	0.214	0.153	−0.125
PE	−0.416*	−0.329	0.543**	0.356*	−0.379*	−0.062	−0.261	0.086	0.431*	−0.330	0.448*	0.284	0.269	0.492**	0.077
PC+LPE	−0.381*	−0.340*	0.476**	0.246	−0.242	−0.177	−0.146	0.213	0.426*	−0.364*	0.320	0.188	0.128	0.466**	0.025
LPC	−0.489**	−0.445*	0.472**	0.288	−0.413*	−0.028	−0.325	0.126	0.397*	−0.279	0.498**	0.222	0.378*	0.575**	0.188
SPH	−0.228	−0.046	0.115	0.237	−0.047	−0.022	−0.192	0.104	−0.028	−0.249	0.142	−0.005	−0.052	0.088	−0.122
TOTAL	−0.376*	−0.243	0.394*	0.220	−0.284	−0.082	−0.223	0.169	0.272	−0.343*	0.364*	0.124	0.206	0.416*	0.004

[a] Sensory scores for off-flavor and aftertaste range from 1 = intense to 4 = none. Scores for ammonia through sour range from 1 = none to 8 = extremely intense.
[b] See Table 2 for key to abbreviations.
* Significant at the P ≤ 0.05 level.
** Significant at the P ≤ 0.01 level.

Table 5—Relative percent fatty acid composition of total fatty acids in the phospholipid fractions of L. dorsi muscles from Bison, Hereford and Braham cattle combined

Fatty acid component	NL+PA+ PG+CI+PI	Phospholipid fraction[a]				
		PS	PE	PC+LPE	LPC	SPH
14:0	3.72e	2.03d	2.03d	0.64c	1.16c	2.21d
14:1	0.30d	0.01c	0.01c	0.01c	0.01c	0.01c
16:0	30.91f	21.12d	19.49c,d	31.83f	16.14c	25.56e
18:0	22.38d,e	22.24d,e	24.67e	9.72c	11.67c	20.68d
18:1	35.41c,d	39.98d	34.31c,d	32.20c	36.59c,d	37.31c,d
18:2	3.40c,d	2.21c	3.69c,d	13.01e	18.55f	4.33d
20:0	0.72c	3.32e,f	1.66d,e	0.69c	1.32c	3.74f
18:3	0.16c	0.11c	0.24c	0.67d	0.98e	0.21c
20:1	0.94c	2.81d	2.53c,d	1.01c	1.23c,d	1.85c,d
20:2	0.08c	0.02c	0.44c	0.06c	0.11c	0.02c
20:3	0.01c	0.01c	0.53c	1.24d	1.44d	0.01c
22:0	0.00c	0.42c	0.44c	0.27c	0.24c	0.01c
20:4	0.81c	0.28c	5.59d	6.97e	7.88e	0.37c
22:1	0.42c	1.83d	1.28c,d	0.56c	0.60c	1.18c,d
24:0	0.00c	0.04c	0.31c	0.01c	0.08c	0.06c
24:1	0.18c	1.57e	1.00d,e	0.47c,d	0.68c,d	0.58c,d
22:6	0.55c	2.01e	1.79d,e	0.64c	1.32d	1.91d,e
Total SFA[b]	57.73f	49.17e	48.60e	43.16d	30.61c	52.26e
Total UFA[b]	42.26c	50.84d	51.41d	56.84e	69.39f	47.21d
Total PUFA[b]	5.01c	4.64c	12.28d	22.59e	30.28f	6.85c

[a] NL — neutral lipid; PA — phosphatidic acid; PG — phosphatidylglycerol; CL — cardiolipin PI — phosphatidylinositol; PS — phosphatidylserine; PE — phosphatidyle-thanolamine; PC — phosphatidylcholine; LPE — lysophosphatidylethanolamine; LPC — lysophosphatidylcholine; SPH — sphingomyelin.
[b] SFA = saturated fatty acids; UFA = unsaturated fatty acids; PUFA = polyunsaturated fatty acids.
c,d,e,f Means bearing different superscripts on the same line differ significantly (P ≤ 0.05).

a decrease in the bland character of the sample. Increases in PC+LPE resulted in an increase in the sensory perception of the ammonia, gamey and rotten flavor notes and a decrease in the greasy flavor characteristic. Increases in LPC resulted in increased intensities of the ammonia, gamey, liverish, old and rotten flavor notes and a decrease in the bland character. Total PL content was only correlated to the ammonia, liverish and rotten characteristics.

Melton et al. (1982) used the greasy (beef fat), liverish, metallic and sour notes to characterize the influences of forage versus grain feeding. Forage-fed beef had a higher intensity of the sour note and low intensity scores for the greasy (beef fat) note and were considered to exhibit an off-flavor in general.

The bitter, bloody, browned, greasy (cooked beef fat), old (cardboard), and sour flavor notes are among those proposed by Lynch et al. (1986) and Johnson and Civelle (1986) for characterization of WOF attributes in meat. As samples increased in intensity of WOF the cooked beef fat (greasy) and browned flavor notes were typically lost while the cardboard (old) characteristic was intensified. These results implicate the PL fraction of Bison tissue as the probable source of the off-flavor and aftertaste experienced by the MDA panel.

Several researchers have demonstrated that the stability of PUFA to cooking and storage may be related to the nitrogen moiety which makes up the PL (Love and Pearson, 1971; Keller and Kinsella, 1973). It is thus evident that an examination of the individual PL fractions rather than a profile of the total lipid fraction may be more revealing when evaluating the influence of lipid composition on flavor.

The fatty acid composition of each of the individual PL fractions for Bison, Hereford and Brahman cattle combined are present in Table 5. Palmitic, stearic, and oleic acids comprise the major portion of total fatty acids in each PL fraction. The LPC fraction contained the highest percent of total UFA and total PUFA followed by PC+LPE and then PE. The other fractions, NL+PA+PG+CL+PI, PS and SPH contained lower percentages of PUFA although PS and SPH were high in docosahexaenoic (22:6) acid. The greater PUFA composition of LPC is due to higher percentages of linoleic, linolenic, eicosatrienoic (20:3) and arachidonic acids. The PE fraction contained lower percentages of 18:2, 18:3, 20:3 and 20:4 than either the PC+LPE or LPC fractions. Increases in PUFA composition were as a result of a decrease in palmitic acid for PE, oleic acid for PC+LPE and both palmitic and oleic acids for LPC. No other definitive analyses of the fatty acid composition of individual PL of beef skeletal muscle appears to be available. Hornstein et al. (1961) determined fatty acid profiles of crude "cephalin" and "lecithin-sphingomyelin" fractions from bovine skeletal muscle and indicated that "cephalin" contained somewhat higher proportions of 18:0 and 20:4 fatty acids than did "lecithin-sphingomyelin" which was characterized by greater amounts of 16:0, 18:1 and 18:2 fatty acids. Differences between these results and those reported in the present study again reflect the chromatographic method used.

The unsaturated fatty acids shown in Table 5 can be responsible for oxidized flavor during the storage of beef (Igene et al., 1980); warmed-over flavor in meats (Pearson et al., 1977) and are degraded during the cooking of beef (Keller and

Table 6—Relative percent fatty acid composition of total fatty acids of PE[a] in L. dorsi muscle from Bison, Hereford and Brahman carcasses

Fatty acid components	Bison	Hereford	Brahman
14:0	1.43	2.37	2.21
14:1	0.00	0.00	0.0
16:0	18.01	19.65	20.85
18:0	25.43	25.09	23.46
18:1	34.83	34.79	33.30
18:2	4.45	3.61	2.76
20:0	1.61	1.61	1.78
18:3	0.13	0.24	0.34
20:1	2.13	2.18	3.34
20:2	1.25	0.00	0.16
20:3	0.13	0.00	1.57
22:0	0.12	0.61	0.57
20:4	6.82	6.28	3.54
22:1	0.97	1.19	1.71
24:0	0.00	0.01	0.98
24:1	1.13	0.56	1.40
22:6	1.56	1.81	2.00
Total SFA[b]	46.59	49.34	49.86
Total UFA[b]	53.41	50.66	50.14
Total PUFA[b]	14.36	11.94	10.38

[a] Phosphatidylethanolamine.

[b] SFA = saturated fatty acids; UFA = unsaturated fatty acids; PUFA = polyunsaturated fatty acids.

Table 7—Relative percent fatty acid composition of total fatty acids of PC+LPE[a] in L. dorsi muscle from Bison, Hereford and Brahman carcasses

Fatty acid components	Bison	Hereford	Brahman
14:0	0.57	0.70	0.66
14:1	0.00	0.00	0.00
16:0	32.00c,d	33.45d	29.60c
18:0	9.05	9.66	10.45
18:1	30.07	32.90	33.22
18:2	15.36d	11.38c	12.60c,d
20:0	0.44	0.94	0.64
18:3	0.79	0.58	0.68
20:1	0.79	0.72	1.56
20:2	0.00	0.00	0.07
20:3	0.52c	1.45d	1.72d
22:0	0.00	0.00	0.87
20:4	8.85d	6.64c	5.50c
22:1	0.44	0.41	0.87
24:0	0.00	0.00	0.04
24:1	0.27	0.42	0.72
22:6	0.72	0.58	0.53
Total SFA[b]	42.19	44.75	42.26
Total UFA[b]	57.81	55.27	57.73
Total PUFA[b]	26.24d	20.82c	21.10c

[a] PC — phosphatidylcholine; LPE — lysophosphatidylethanolamine.

[b] SFA = saturated fatty acids; UFA = unsaturated fatty acids; PUFA = polyunsaturated fatty acids.

c,d Means bearing different superscripts on the same line differ significantly (P ≤ 0.05).

Table 8—Means for percentage composition of fatty acids of LPC[a] in L. dorsi muscle from Bison, Hereford and Brahman carcasses

Fatty acid components	Bison	Hereford	Brahman
14:0	0.55	1.17	1.78
14:1	0.00	0.00	0.00
16:0	12.90	17.51	17.76
18:0	9.49	12.48	12.86
18:1	37.16	32.90	33.68
18:2	22.42	16.85	16.72
20:0	0.44	1.78	1.65
18:3	1.03	0.60	1.38
20:1	1.34	0.49	2.01
20:2	0.28	0.18	0.06
20:3	0.58c	1.76d	1.93d
22:0	0.00	0.00	0.76
20:4	11.49d	6.85c	5.53c
22:1	0.80	0.18	0.89
24:0	0.16	0.08	0.00
24:1	0.40	0.22	1.51
22:6	0.97	1.49	1.47
Total SFA[b]	23.54	33.02	34.81
Total UFA[b]	76.47	66.99	65.18
Total PUFA[b]	36.77	27.55	27.09

[a] LPC — lysophosphatidylcholine.

[b] SFA = saturated fatty acids; UFA = unsaturated fatty acids; PUFA = polyunsaturated fatty acids.

c,d Means bearing different superscripts on the same line differ significantly (P ≤ 0.05).

Kinsella, 1973). According to Melton (1983) the thermal oxidation of meat with high concentrations of these unsaturated fatty acids could contribute to higher intensities of undesirable flavors in beef.

Lean tissue from Asian water buffalo has been shown to be higher in PUFA than that from beef (Sinclair et al., 1982). As discussed previously, bison contains more total PL (in particular the PL known to contain the highest levels of PUFA, namely PE, PC+LPE and LPC) than beef. When the fatty acid profiles of these individual PL fractions for Bison, Hereford and Brahman samples were examined, differences in PUFA content which may explain their correlation with sensory response were evident. There was no effect of genetic differences between the three bovine species on the fatty acid composition of PE (Table 6). It appears as though PE is related to sensory response strictly in that it contains a high percentage of PUFA as compared to the other PL fractions. Bison samples contained more 20:4 and total PUFA than Hereford or Brahman samples and more 18:2 than Hereford samples in the PC+LPE fraction (Table 7). Bison was found to contain more 20:4 than either

Brahman or Hereford in the LPC fraction (Table 8). Differences (P ≤ 0.05) were also evident in several of the other PL fractions. Brahman cattle had more nervonic (24:1) acid in the NL+PA+PG+CL+PI fraction than either Bison or Hereford, and Bison had less 14:0 in that fraction. Hereford samples had greater concentrations of arachidic (20:0) acid and less nervonic acid in the PS fraction than did Bison or Brahman, and Brahman had more nervonic acid than did Bison. Bison samples contained less 18:1 in the SPH fraction than either Hereford or Brahman samples. O'Kelly (1985) stated that at the same feed intake, Brahman-cross animals had more microbially synthesized lipid than British cattle with a resultant increased efficiency of utilization of metabolized energy for maintenance. Long chain fatty acids passing from the rumen to the small intestine arise as a result of microbial synthesis of fatty acids de novo as well as microbial modification of the lipids present in the diet. It is suggested that the genetic differences found when animals are consuming equal diets arise largely from the absorption of different amounts of endogenously produced fatty acids.

SUMMARY & CONCLUSIONS

DATA PRESENTED here confirm that the FA composition of lean tissue can be influenced by breed-type and that L. dorsi samples from Bison (in this case North American Bison) contain more PUFA than either Hereford or Brahman cattle. Bison samples exhibited higher off-flavor and aftertaste than either Hereford or Brahman with the off-flavor being characterized by increased levels of ammonia, bitter, gamey, liverish, old, rotten, and sour flavor notes. Samples from Bison contained higher levels of PE, PC+LPE, LPC, and total PL than either Hereford or Brahman. These PL fractions were identified as containing the greatest quantities of total unsaturated and polyunsaturated fatty acids. Individual PL composition, as well as total PL composition may, therefore, be very important in the flavor quality of muscle foods.

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Effects of Meat Temperature, Particle Size, and Grinding Systems on Removal of Bone Chips from Ground Meat

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ABSTRACT

BONE CHIPS (9.5 mm or 13 mm) were added to ground meat batches (45.3 kg) at 0.5%. Meat batches were tempered to −2.2°C and 3.3°C to evaluate effect of temperature during grinding. Three commercial bone elimination systems were used for comparison. Grinding at −2.2°C was not significantly (P<0.05) different from 3.3°C for bone in the final product but more bone remained in the grinder barrel after grinding at 3.3°C. The large chips were more readily fractured and passed to the final product. The channelled central system passed significantly (P<0.05) more bone into the final product than the others. With all bone removal systems, the small chips were more readily removed from the grinder through the removal system.

INTRODUCTION

DURING the past several years, ground beef consumption has increased, partly due to the popularity of fast-food chains. Also, ground beef appears to have other advantages such as ease of preparation, price and a popular preference for the product (Glover, 1968). In 1986, per capita consumption of hamburger alone was 29.3 pounds (13.3 kg) (Anonymous, 1987). Consumption of comminuted meats, however, is not limited to ground beef. Other ground meats such as dried and semi-dried sausages, fresh pork sausage and cooked sausages have gained in popularity during the past several years.

Hamburger and other ground or comminuted meats are usually obtained from economically low value cuts or trimmings. These cuts or trimmings are usually high in connective tissue, which can often influence the acceptance of ground beef (Cross et al., 1976). Along with the possibility of a high degree of connective tissue, ground meat also contains foreign materials such as bone or bone chips that were not removed at the boning tables. It is estimated that 0.1% of all meat product applied to grinders may consist of bone or bone chips (Roeger et al., 1983). Since dense materials such as bone or gristle are sometimes found in ground meats, dental injuries have occurred with the ingestion of commercial products that contained hard particles (Fishman, 1984).

Currently, there are several bone/connective tissue removal systems that are commercially available to meat processors. The systems claim to improve product quality by removing most of the dense materials. There are two general types of removal systems, central removal and peripheral removal.

Central removal systems take advantage of a naturally occurring deposition or accumulation of the dense materials at or near the central portion of the grinder plate. The dense materials are passed through removal ports at the center of the plate, with subsequent passage through tubing into a separate receptacle away from the product being ground. It is generally believed that increased flow through the tubing (controlled by back pressure) of the removal system results in a more complete removal of bone chips. However, there is also an increase in the loss of red meat, which will adversely affect profits.

Most manufacturers of the removal systems recommend adjusting the system to achieve a product loss of 0.25%.

The second general type of bone removal system is the peripheral removal concept. These systems operate by pushing the dense materials to the outer periphery of the plate where they can be deposited into an orifice with subsequent removal. These systems usually have a knife assembly that is curved rearward to the direction of rotation, which will push the chips to the outer regions of the knife-plate interface where they can be removed.

Despite commercial claims, there is very little information dealing with the performance of the different systems available to meat processors. There are many variables that could have an effect on the efficiency of these systems. One of these variables is meat temperature. Many processors incorporate frozen meat blocks into their meat systems, and a very cold meat temperature could have an effect on the amount of bone that is cut and passed into the final product. Another consideration is that of initial grind size. Cross et al. (1980) found that beef initially ground through a 1.9 cm plate or larger was unacceptably tough and high in subjectively detected connective tissue. However, virtually no studies have been performed to evaluate the effect of pregrinding size on the amount of bone that is passed into the product at the final grind nor on the amount of bone removed through the removal system. When raw materials are preground through a large-hole plate, it is likely that bone particles will be created that are at least as large as holes in the pregrind plate. Therefore, size of the pregrind could have an effect on the amount of bone removed through the removal system as well as the amount of bone that is fragmented by the grinder and passed through to the final product. While a ground meat system is likely to contain bone chips of many different sizes, it is not clear if the size of a bone chip has an effect on the tendency of the chip to be sheared or cut and passed through the plate into the final product.

The objective of this study was to evaluate the effectiveness of three commercially available bone removal systems at different meat temperatures and with different sized bone chips. Systems and variables were evaluated by measuring the removal of a known quantity of bone deliberately added to the meat mixture.

MATERIALS & METHODS

Preparation and analysis

USDA Choice chuck rolls were purchased from a commercial source (Carriage House, Ames, IA) and visually inspected for any remaining bone or cartilage pieces, with subsequent removal of these obvious pieces. The meat was ground through a 13 mm plate and separated into ten 45.3 kg batches for each grinder system (30 batches in total) before the addition of bone chips.

Bone chip preparation. Bones were obtained (ISU Meat Laboratory) from rib bones of choice cattle. The bones were prepared by first trimming away all extraneous material. The ribs were frozen at −36°C and subsequently ground through a Buffalo No. 66 grinder equipped with a kidney plate. The ground bone was then sieved through a 14 mm screen. The fraction that passed through the 14 mm screen was again sieved through a 12 mm screen to eliminate smaller chips and slivers and retain those that were uniform in shape and approxi-

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mately 13 mm in diameter. This size of chip would be very representative of that potentially found in meat systems prebroken through a 13 mm or slightly larger plate. This chip size was designated as a large (LG) chip size. Another chip size category was included to represent a meat system prebroken through a 9.5 mm plate and designated as small (SM) chips. The small (SM) chips were generated in the same manner using screens of 8 mm and 11 mm to separate chips that were approximately 9.5 mm in diameter. All the bone chips were then boiled in water for 5 min to remove any adhering cartilage or connective tissue, washed with hot water to separate the connective tissue from the bone and then allowed to dry at room temperature for 2 hr. Samples of bone were analyzed for moisture and fat (AOAC, 1975), placed in plastic bags and frozen at −36°C until they were mixed with the meat.

Mixing. Ten 45.3 kg batches of ground meat were used in each of the three grinders per replication with four of the batches serving as controls (no bone added). The remaining six batches were randomly assigned to receive 0.5% of either LG or SM bone chips and either cold or warm meat temperature treatments. The thirty batches of meat applied to the grinders consisted of the following five designations each at cold and at warm meat temperatures: C1 = no bone added, no removal system; C2 = no bone added, active removal system; C3 = small bone added, nonfunctional removal system; SM = small bone added, active removal system; LG = large bone added, active removal system.

Each of the 45.3 kg batches were individually placed into a Leland Food Mixer (Model 1000A, Detroit, MI) mixed for 2 min, and sampled for proximate analysis. The bone chips were added, and the meat-bone mixture was mixed for an additional 2 min. Each batch was then placed into a covered plastic tub and prepared for chilling.

Chilling. Each batch of the meat/meat-bone mixture was tempered to one of two different temperatures for final grinding. The cold (−2.2°C) treatments were placed into a freezer operating at −30°C for 9 hr. The batches were then removed from the freezer and allowed to temper in a 10°C room for approximately 2 hr. The batches were briefly mixed and subsequently ground when an average temperature of −2.2°C was obtained. The warm (3.3°C) treatments were prepared by placing the meat/meat-bone mixtures into a cooler operating at −2.2°C, and the temperature was monitored in the same manner as the cold treatments.

Grinding. The final grinding of the meat batches utilized a 3.2 mm hole plate in the three different grinder/bone elimination systems that were under study. These consisted of: PR- Peripheral Bone Removal - 250 mm grinder barrel with worm auger operating at 190 rpm (no load); CA- Central Axis Bone Removal - 217 mm grinder barrel with worm auger operating at 150 rpm (no load); CC- Channelled Central Bone Removal - 217 mm grinder barrel with worm auger operating at 150 rpm (no load).

To treat all machines alike, each bone removal system was adjusted to achieve approximately 1% material exhausted from the bone remover. Grinder operating conditions (rpm, exhaust rate) were established according to respective manufacturers' recommendations. The maximum output of the bone removal system on the PR machine was approximately 1%; therefore, the other two systems were adjusted accordingly. The final grinding was carried out in such a way as to allow the machine to reach operating conditions as much as possible. Twenty-five percent of the meat batch was ground and set aside. The actual sampling was then performed on the middle 50% of each batch as it was ground. Random samples were obtained from the material ground through the plate (Final Product-FP) and from the material exhausted through the bone removal system (Exhausted Material-EM). The final 25% of each batch was then ground and set aside. The grinder was disassembled, and samples of the material retained in the barrel after grinding were obtained (Barrel Residue-BR).

Atomic absorption analysis. The final product (FP) was analyzed for calcium with the use of a Perkin-Elmer Atomic Absorption Spectrophotometer (Model 460, Norwalk, CT) as outlined in AOAC (1975). Since the final product was passed through the grinder plate, any bone residue was very finely divided, making sampling for this relatively sensitive calcium measure more feasible. To provide the most representative sample possible, random grab samples were collected from the final ground product to give a composite 4.5 kg sample which was thoroughly mixed. A random 0.45 kg portion was subsequently collected and further blended using a Waring Blendor before triplicate 2-g samples of the mixture were obtained for final preparation. The two-gram samples collected were suspended in 30 mL of concentrated nitric acid overnight. The samples were then boiled on a hot plate for approximately 30 min to completely digest all organic matter. Ten milliliters of perchloric acid was added

and the samples again placed on a hot plate and heated until heavy white perchloric fumes appeared. The sample was then transferred to a 100 mL volumetric flask; 5 mL of concentrated nitric acid was added and made to volume with distilled water. A 10 mL aliquot of this solution was transferred to a 100 mL volumetric flask, mixed with 20 mL of a 5% lanthanum oxide solution and made to volume with distilled water. This solution was then submitted to the atomic absorption spectrophotometer for measurement of calcium in parts per million. The measurement of calcium in parts per million was used as an indicator of the amount of bone that was passed through the plate into the final product. Calcium in parts per million was converted to a bone equivalency by using the measured calcium content of 21.4% for the bone chips as used in this experiment.

Bone chip analysis. Bone chip content of the FP, EM and BR fractions from each of the three grinders was also analyzed in duplicate by alkaline digestion. The bone content was determined by mixing a random 50 g sample of the meat/meat-bone mixture with 200 mL of a 0.49 M NaOH solution and heated for 30 min on a Thermolyne Nuova II Stir Plate (Dubuque, IA) to a final temperature of 86-90°C. Agitation was achieved by means of a magnetic stir bar. The solution was allowed to stand for 2 min to allow for fat separation. The fat was aspirated from the top of the mixture through a vacuum hose. The remaining solution was then successively washed three times with 500 mL boiling water. Between washings, the bone/connective tissue was allowed to settle before aspiration. The particulate material was drawn through filter paper and dried overnight in a vacuum oven at 100°C before residue weight was measured.

The weight loss of the chips due to moisture and fat losses in analysis were corrected by adding back original moisture and fat of the chips based on moisture and fat analysis of the chips alone. The final results were expressed as a percentage of the original weight of bone chips added to the meat mixture.

Statistical analysis

The experiment was replicated twice and results were analyzed using a $2 \times 5 \times 3$ factorial design with two grinding temperatures, five bone chip treatments (three treatments containing bone) and three grinder/bone removal systems. For samples in the alkaline digestion analysis, only the three treatments that contained bone were analyzed statistically. The statistical analysis was performed in this manner because the treatments that did not contain bone were used to determine the background levels of dense connective tissue recovered from the product. The dense connective tissue was largely undigested by the alkaline treatment and, consequently, subtracting the background level gave the true bone residue in treated samples. In the atomic absorption analysis, all five treatments were analyzed statistically. In this situation, the treatments that did not contain bone were used as indicators of the amount of inherent calcium naturally present in the meat. The Statistical Analysis System (SAS, 1985) was used to calculate treatment means and detection of treatment differences. Least significant difference was used as a method of mean separation.

RESULTS

Atomic absorption analysis

Analysis for calcium by atomic absorption (Fig. 1), in general, demonstrated increased calcium in the final product from added bone. The amount of bone found was dependent upon both the removal system used and the bone chip size.

However, the C3 (added bone, nonfunctional removal system) treatment with the channelled central removal system was lower in calcium than either the SM or LG treatments (added bone, functional system) of the same removal system. This indicated less shearing of chips by the nonfunctional system and greater residue remaining in the grinder barrel as indicated by bone chip content in Table 1. The large chips in this system resulted in the most calcium and evident bone chips in the final product (Fig. 1). The effect of all treatments on the three different bone removal systems as shown in Fig. 1 indicate that the level of bone that was cut or sheared at the knife-plate interface and passed through the plate into the final product was very dependent upon both chip size and the particular system used.

The data in Fig. 1 were converted to a bone equivalency in Table 2 to better demonstrate the amount of bone that was
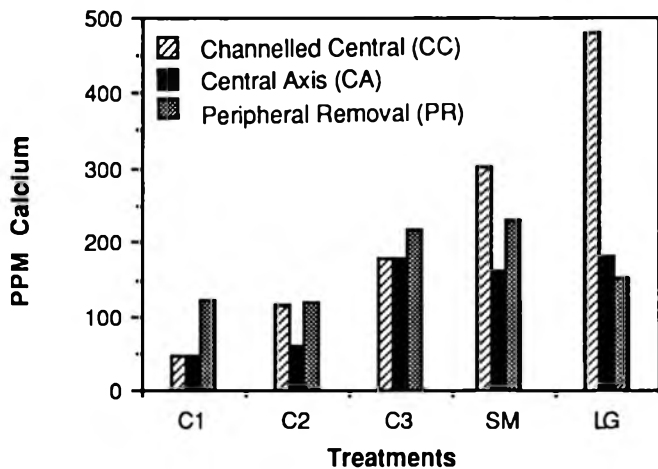
Fig. 1—Effect of bone chip size and bone removal system on bone chip removal from meat (measured as parts per million of calcium in the final product-FP) C1 = No bone added, no bone removal; C2 = No bone added, active bone removal; C3 = 0.5% small bone added, no bone removal; SM = 0.5% small bone added, active bone removal; LG = 0.5% large bone added, active bone removal.

sheared and passed through the grinder to the final product. The calcium naturally present in the meat was subtracted from the total calcium concentration detected and conversion to a bone equivalency was based on the percentage calcium present in the bone (21.4%) that was added to each of the treatments. In this comparison (Table 2), the LG bone chips seemed most detrimental in the channelled central removal system, with 37.24% of the added bone being found in the final product. The peripheral system seemed to handle the LG chips most easily, with only 2.92% of the added bone found in the final product. The C3 treatment (added bone, nonfunctional removal system) was not significantly higher for bone content in the final product in most cases. Evidently, the grinder plate alone tends to screen out particles from the final product to a large extent; one result of which was a large amount of residue

Table 1—Effect of bone size on mean values for bone chip shearing (FP), bone removal (EM) and bone retention in the grinder (BR) during grinding of meat[c]

| Item | Treatment | | | |
	C3[d]	SM[e]	LG[f]	s.e.[g]
Final product	10.65[a]	9.52[a]	18.80[b]	2.38
Exhausted material	—	15.62[a]	9.85[b]	1.49
Barrel residue	72.95[a]	59.44[b]	54.27[b]	2.96

[a,b] Means within a row that bear unlike superscripts are significantly different (P<0.05).
[c] Values in table represent percentages of the 0.5% bone that was originally added.
[d] C3 = 0.5% small bone added with no bone removal
[e] SM = 0.5% small bone added with active bone removal
[f] LG = 0.5% large bone added with active bone removal
[g] s.e. = standard error

Table 2—Percent of added bone in the final product (FP) determined by atomic absorption spectrophotometry[c]

| Removal system | Treatment | | | |
	C3[d]	SM[e]	LG[f]	s.e.[g]
Channelled central	9.12[a]	20.78[a,b]	37.24[b]	5.95
Central axis	11.79[a]	10.14[a]	12.01[a]	2.10
Peripheral removal	9.08[a]	10.40[a]	2.92[a]	3.69

[a,b] Means within a row that bear unlike superscripts are significantly different (P<0.05).
[c] Values in table represent percentages of the 0.5% bone that was originally added. Percentage bone was calculated by substracting ppm calcium that were naturally present in meat, then converted to bone equivalent.
[d] C3 = 0.5% small bone added with no bone removal
[e] SM = 0.5% small bone added with active bone removal
[f] LG = 0.5% large bone added with active bone removal
[g] s.e. = standard error, based on triplicate sample values

within the barrel (Table 1). Comparison of the replications showed no significant differences between the two replications for the levels of calcium in the final product from the various treatments. Consequently, representative sampling for the atomic absorption analysis was achieved since the two replications were not different.

Alkaline digestion analysis

The alkaline digestion method for bone chips (Table 1) also showed that the LG treatment resulted in the most bone (P<0.05) in the final product. Small chips resulted in less bone in the final product and did not differ from C3 (added bone, nonfunctional removal system). The SM treatment also resulted in significantly (P<0.05) higher bone content in the exhausted material than did the LG treatment. Logically, since the final product was low in bone and there was no exhausted material, the barrel residue of the C3 treatment contained significantly (P<0.05) more bone than either SM or LG treatments, which were similar to each other.

The PR system resulted in significantly (P<0.05) less bone in the final product than either the CA or CC systems (Table 3). The PR system may have performed better in this respect because this system incorporated a grinding plate that did not have a bone remover inherent to the plate. Therefore, the chips rode the knife-plate interface more readily without being cut or fractured. The CA system removed the highest percentage of bone from the product as exhausted material. This was significantly (P<0.05) greater than the PR system but not more than the CC system, which was intermediate between the other two. It was visually obvious that both the CA and CC bone removal systems became plugged with bone after a short period of grinder operation. When a system becomes plugged and exhausted material ceases to flow, the system becomes useless in removal of bone chips. The PR system, across all treatments, retained 71.94% of the added bone in the barrel residue, which was significantly (P<0.05) more bone in the barrel at the end of the grinding than the other two systems, which were not significantly (P>0.05) different from each other.

Grinding temperature did not significantly (P>0.05) affect the amount of bone in the final product (Table 4). However, a greater amount of bone was found in the exhausted material when grinding meat at −2.2°C compared to product ground at 3.3°C. In addition, a greater amount of bone (P>0.05) was found in the grinder barrel after grinding meat at 3.3°C.

Table 3—Effect of bone removal systems on mean values for bone shearing (FP), bone removal (EM) and bone retention in the grinder (BR) during grinding of meat[c]

| Item | Bone removal systems | | | |
	PR[d]	CA[e]	CC[f]	s.e.[g]
Final product	6.87[a]	14.92[b]	17.18[b]	2.38
Exhausted material	10.03[a]	16.57[b]	11.60[ab]	1.82
Barrel residue	71.94[a]	56.20[b]	58.52[b]	2.96

[a,b] Means within a row that bear unlike superscripts are significantly different (P<0.05).
[c] Values in table represent percentages of the 0.5% bone that was originally added.
[d] PR = Peripheral removal system
[e] CA = Central axis removal system
[f] CC = Channelled central removal system
[g] s.e. = standard error

Table 4—Effect of grinding temperature on mean values for bone shearing (FP), bone removal (EM) and bone retention in the grinder (BR) during grinding of meat[c]

| Item | Temperature of meat at grinding | | |
	−2.2°C	3.3°C	s.e.
Final product	11.22[a]	14.76[a]	1.95
Exhausted material	16.77[a]	8.70[b]	1.49
Barrel residue	58.35[a]	66.08[b]	2.41

[a,b] Means within a row that bear unlike superscripts differ significantly (P<0.05).
[c] Values in table represent percentages of the 0.5% bone that was originally added.

Individual removal system performance

Treatment differences. Table 5 indicates the performance of each machine and individual removal system within the different treatments. The PR system resulted in the least amount of bone in the final product. However, within each removal system, no significant (P>0.05) differences for bone in final product were observed between the treatments. When these data were pooled for overall comparison, however, the large bone chips (LG treatment) resulted in significantly (P>0.05) greater amounts of bone in the final product (Table 2).

The differences in the amount of bone in the exhausted material within each removal system are also shown in Table 5. Treatment C3 resulted in no values because it had no functional bone removal. Once again, no significant (P>0.05) differences were observed within each system. When the data were pooled as in Table 2, the SM treatment had significantly more bone in the exhausted material.

Some differences for bone in the barrel residue (Table 5) were observed. Generally, the C3 treatment resulted in the most bone from the channelled central and central axis systems, being significantly (P<0.05) greater than the LG treatment. None of the three treatments were significantly (P>0.05) different in the case of the peripheral removal system.

Temperature differences

The differences in grinding temperatures were also separated within each machine and are shown in Table 6. While there were significant differences overall in amounts of bone in the exhausted material and barrel residue as shown in Table 4; within each machine, the amount of bone in exhausted material was not different. The only significant (P<0.05) difference in the barrel residue comparisons was for the peripheral removal system where more bone was found at 3.3°C. However, the trend toward more bone separation in exhausted material at −2.2°C was obvious as was the trend in the barrel residue toward more bone accumulation at 3.3°C.

Comparison of atomic absorption and alkaline digestion methods

Because the final product was analyzed for bone content by the two methods of atomic absorption and alkaline digestion, it was of interest to compare these two methods. When the data were averaged over all three bone removal systems (Table 7), the percentage bone as detected by the two methods was similar over all the treatments. The standard error for the atomic absorption analysis was lower in this experiment than the standard error determined for the alkaline digestion method. The atomic absorption method may be more precise but is also more tedious and time consuming. The alkaline digestion method, on the other hand, is a more rapid method for screening numerous samples.

DISCUSSION

AT THE BEGINNING of this study, it was anticipated that bone added to a meat system and subsequently ground with no bone removal system would represent the most undesirable approach with respect to the passing of bone into the final product. However, this study revealed that such a system (C3, 0.5% added bone, nonfunctional removal system) was less likely to grind or fracture the bone and contribute to bone in the final product. The LG (large chips) treatment, which had an active bone removal system resulted in more bone in the final product than did the C3 treatment. C3 was not significantly (P>0.05) higher in final product bone content compared with the SM (small chips) treatment. This result was similar to that found by Berry (1985).

These results have two implications. First, bone chips found in a meat system will more readily ride the knife-plate interface without being reduced in size if the grinding plate has a smooth, uniform surface, such as one without grooves or channels meant to remove the bone chips. A conventional grinder plate does not, for example, have a channel in which the chips could become lodged and consequently cut or sheared, nor does it contain large orifices near the center of the plate where the chips could also become lodged and sheared. The reduced shearing from a smooth knife-plate interface also lends support to the age-old recommendation to keep all grinding plates sharp. Sharpening plates regularly, probably will decrease the amount of bone shearing by creating a surface that will allow the bone chips to ride the interface with less likelihood of being cut or sheared. Certainly, these bone chips need to be removed as quickly as possible, or shearing may occur as chips build up on the plate surface. By creating an optimum avenue for bone removal along with a "smooth" plate, improved bone removal may be obtained.

Table 5—Effect of bone chip size on the amount of bone detected in the different fractions from the grinders with bone removal systems[c]

Item	Treatment			
	C3[d]	SM[e]	LG[f]	s.e.[g]
Final product				
Channelled central	14.32[a]	14.97[a]	22.23[a]	3.83
Central axis	14.32[a]	6.17[a]	24.27[a]	5.40
Peripheral removal	3.30[a]	7.42[a]	9.90[a]	3.66
Exhausted material				
Channelled central	−	14.32[a]	8.88[a]	3.30
Central axis	−	23.18[a]	9.96[a]	1.81
Peripheral removal	−	9.35[a]	10.71[a]	2.48
Barrel residue				
Channelled central	71.04[a]	56.31[a]	48.19[b]	4.23
Central axis	71.04[a]	50.16[b]	47.39[b]	3.32
Peripheral removal	76.75[a]	71.84[a]	67.22[a]	3.19

[a,b] Means within a row that bear unlike superscripts are significantly different (P<0.05).
[c] Values in table represent percentages of the 0.5% bone that was originally added.
[d] C3 = 0.5% small bone added, no bone removal
[e] SM = 0.5% small bone added, active bone removal
[f] LG = 0.5% large bone added, active bone removal
[g] s.e. = standard error

Table 6—Effect of meat temperature on the amount of bone detected in the different fractions from the grinders with bone removal systems[c]

Item	Temperature of grinding		
	−2.2°C	3.3°C	s.e.
Final product			
Channelled central	14.92[a]	19.43[a]	3.13
Central axis	10.10[a]	19.74[a]	4.41
Peripheral removal	8.64[a]	5.08[a]	2.99
Exhausted material			
Channelled central	18.33[a]	4.87[a]	3.30
Central axis	18.61[a]	14.53[a]	1.81
Peripheral removal	13.36[a]	6.70[a]	2.48
Barrel residue			
Channelled central	58.25[a]	58.78[a]	3.45
Central axis	53.97[a]	58.42[a]	2.71
Peripheral removal	62.82[a]	81.05[b]	2.61

[a,b] Means within a row that bear unlike superscripts differ significantly (P<0.05)
[c] Values in table represent percentages of the 0.5% bone that was originally added

Table 7—Comparison of mean values obtained for bone content in the final product (FP) as determined by both atomic absorption and alkaline digestion methods[c]

Analysis method	Treatment			
	C3[d]	SM[e]	LG[f]	s.e.[g]
Alkaline digestion	10.65[a]	9.52[a]	18.80[b]	3.37
Atomic absorption	10.00[a]	13.77[ab]	17.39[b]	2.34

[a,b] Means within a row that bear unlike superscripts differ significantly (P<0.05).
[c] Values in table represent percentages of the 0.5% bone that was originally added.
[d] C3 = 0.5% small bone added, no bone removal
[e] SM = 0.5% small bone added, active bone removal
[f] LG = 0.5% large bone added, active bone removal
[g] s.e. = standard error

Secondly, comparison of all removal systems for the effect of bone chip size showed that large chips (13 mm diameter in this case) were sheared and fractured more frequently than small (9.5 mm diameter) chips. These data imply that a small prebreak grind size would be advantageous for subsequent final grinding where bone removal systems would be used.

The effect of bone removal system on the amount of bone found in the three different fractions from the grinders showed that the CA and CC systems passed significantly (P<0.05) more bone to the final product than did the PR system. The PR system relies on a grinding plate that does not have an inherent channel in the plate or large orifices near the center of the plate for bone removal. Instead, the PR plate resembles a conventional plate with the bone removal system at the outer periphery of the knife-plate interface. Therefore, this plate has less tendency to shear and reduce the bone to a small enough size that it can pass through the plate to the final product. At the same time, it is commonly observed that, when removing grinder plates after operation, bone chips and connective tissue tend to congregate near the hub or center of the plate. An important part of peripheral removal systems might also be design accommodations which allow or encourage a movement of chips to the plate periphery without excess shearing.

The CA system resulted in significantly (P<0.05) more bone removal, with 16.57% of the added bone in exhausted material while the PR system resulted in 10.03%. The CC system was intermediate between the other systems with 11.60% of the added bone exhausted. These values indicate that there was a major accumulation of dense materials within the grinder barrel and that removal might occur only after a critical point of accumulation or operating pressure was reached. It was also observed in this study that after a reasonable period of operation, both the CA and CC removal systems became plugged with bone and rendered nonfunctional. The PR system used in this study, however, did not become plugged and continued to operate without the lodging of bone chips that occurred in the other systems.

No significant (P>0.05) effect of grinding temperature on the amount of bone found in the final product occurred. However, a difference was observed in the exhausted material, with the −2.2°C grinding temperature showing a greater bone removal than the 3.3°C grinding. In addition, more bone was retained in the grinder barrel at 3.3°C. These data indicate that the warmer grinding temperature was less conducive to bone chip separation and that there might be potential for more bone in the final product during sustained operations. Certainly, there was a trend in this study toward more bone residue in the final product when grinding at 3.3°C even though the differences were not significant.

SUMMARY & CONCLUSIONS

BONE CHIP SIZE was an important factor in determining the amount of bone removed by the bone removal systems as well as the amount of bone passed into the final ground meat product.

A smaller pregrind such as 9.5 mm for the bones was more beneficial than pregrinding with 13 or 19 mm plates because small bone chips had less tendency to be sheared at the knife-plate interface. The small bone chips were also more readily removed through the removal system. The peripheral removal system used in this study passed the least amount of bone to the final product and did not become plugged with bone chips after prolonged periods of use. The channelled central system was affected most by different-sized bone chips with the large chips proving to be most detrimental. The question of whether temperature has an effect on the cutting of bone and subsequent passage through the plate is still a concern. Grinding at a meat temperature of −2.2°C or 3.3°C did not change the amount of bone detected in the final product but more bone was retained in the barrel of the grinder at 3.3°C. After sustained operation, a greater retention of bone within the grinder was likely to contribute to bone in the final product if not cleaned out or removed from the grinder. It was also observed, that the cold grinding temperature resulted in a higher percentage of bone chips removed by the bone removal system.

While this study was shown that several factors can influence the effectiveness of bone removal systems during meat grinding, the performance of all these systems was less than ideal. There is a considerable need for more information on pressure gradients, migration patterns and bone chip localization during grinder operation to facilitate development of improved bone removal systems.

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Effects of Pre-chilling, Freezing Rate, and Storage Time on Beef Patty Quality

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ABSTRACT

The effects of pre-chilling (air at −10°C or CO₂ snow), freezing method or rate (liquid N₂ immersion, CO₂ snow and air blast at −25°C or −14°C) and frozen storage at −17°C for 6–9, 24–30, 56–63 or 88–98 days on ground beef patty quality were studied. The patty quality was determined by evaluating color, water-holding capacity (WHC), pH, shrinkage, 2-thiobarbituric acid and textural properties. The liquid N₂ immersion freezing resulted in significantly higher cooking and total shrink losses, a paler appearance and lower WHC. A significant reduction was noted in patty quality during frozen storage as indicated by significant increases in storage loss, shear values, hardness, gumminess, chewiness, surface reflectance, and TBA. The CO₂ snow pre-chilling provided lower total losses and more intense surface redness.

INTRODUCTION

THE APPLICATION of freezing for the preservation of foods has been practiced for several years to maintain its quality during storage, distribution and marketing. Although meat freezing is generally conceded to cause tissue damage and some quality loss, it remains the method of preference for long-term storage. In North America, the hamburger, also known as the ground beef patty, has become an integral part of the diet. Palatability associated with beef patties has been shown to be a function of such factors as freezing rate (Sebranek et al., 1978), mechanical treatment of raw materials (Berry et al., 1981), composition (Cross et al., 1976), precooking (Joseph et al., 1980) and the use of pre-rigor beef (Jacobs and Sebranek, 1980).

It has been considered for many years that foods which are "quick frozen," yield optimum quality. At present, this subject is surrounded by some controversy. Terms which are generally used in expressing freezing rates include, 'sharp' or 'slow', 'rapid' or 'quick' and 'ultrarapid'. A wide degree of variation is evident in the methods of expression and the rate associated with each term. When one author may define as slow freezing, another may define as rapid (Fennema and Powrie, 1964). According to the International Institute of Refrigeration (IIR, 1972), the freezing rate of a food mass is the ratio between the minimum distance from the surface to the thermal center (slowest cooling point), and the time required for the thermal center to reach a temperature of 10°C lower than the temperature of initial ice formation at the thermal center, once the surface attains a temperature of 0°C. There seems to be much ambiguity in the literature concerning the relationship between water-holding capacity (WHC) and rate of freezing.

The objective of this study was to determine the effects of pre-chilling, freezing method or rate, and frozen storage period on shrinkage, texture, pH, WHC, surface color, and 2-thiobarbituric acid (TBA) values on ground beef patties.

METHODS & MATERIAL

A 2 × 4 × 4 FACTORIAL randomized block design involving two replications was used to study the effects of pre-chilling (cold air at

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−10°C or CO₂ snow), freezing method (liquid N₂ immersion, CO₂ snow, air blast at −25°C or −14°C) and frozen storage at −17°C for 6–9, 24–30, 56–63 or 88–98 days on ground beef patty quality.

Pattie preparation

The beef for each replication was obtained from a different carcass, aged at 2 ± 2°C for 2 days. Lean meat and fat were separated from the chucks of each carcass and were both coarse ground separately through a 6mm plate using a Hobart grinder (Model 4532). A Butcher Boy Mixer (Model L50) was used for mixing. Proximate analysis of lean and fat samples were determined by AOAC (1984). The lean and fat were combined to form four 20 kg groups of desired composition. The mean moisture and fat contents of the patties were 58.0% and 22.7% in the first replication, and 59.5% and 21.5%, respectively, in the second replication. Meat batches were reground through a 3.2 mm plate. Two of the batches were remixed with the addition of about 1.3 kg of CO₂ snow, while the remaining two were remixed after being chilled in an air blast freezer at about −10 ± 2°C for 1.25 to 2 hr. The temperature of the batches following the CO₂ and air chilling treatments were 1 ± 1°C and 4.5 ± 1°C, respectively. Patties were formed using a Hollymatic patty maker (Model 200-U).

Freezing methods

Liquid N₂. Four patties were laid on evenly spaced stainless steel circular mesh grids and submerged in a Dewar flask filled with liquid nitrogen.

CO₂ snow. A plastic foam box was filled with about 2.5 cm of CO₂ snow and for each freezing, patties were laid on the CO₂ and covered with another layer of snow.

Air blast freezing methods. Patties were laid on wire trays placed on a trolley. The trolley was placed in a freezer at −14°C or −25°C. The surface heat transfer coefficients were 23 and 31 W/(m.K) for −14°C and −25°C, respectively.

In all methods, patties were frozen from 2°C to −10°C. The final temperature was controlled after measuring the freezing times by placing thermocouple probes at the geometric center of the patties. These are given in Table 1.

Storage

The patties were packaged in polyethylene-lined cardboard boxes, interleafed with wax paper prior to frozen storage and kept in an air blast freezer at −17 ± 2°C.

Quality determinations

Samples were removed from the freezer upon the completion of the frozen storage periods and placed in a cooler at 2 ± 2°C for thawing. Freezing loss (FL) represented the ratio of the change in patty mass as a result of freezing to the mass of the patty before freezing. Storage loss (SL) was described by the change in patty mass as a result of

Table 1 — Experimental test conditions for ground beef patty freezing (average for all experiments ± s.d.)

Freezing method	Medium temp. (°C)	Patty thickness, (cm)	Observed freezing time (sec)	Freezing rate (cm/hr)
Slow air blast	−14 ± 1	1.10 ± 0.04	3939 ± 385	0.5 ± 0.05
Fast air blast	−25 ± 2	1.12 ± 0.04	2047 ± 298	1.0 ± 0.14
CO₂ snow contact	−78	1.13 ± 0.05	129 ± 26	16.5 ± 2.97
N₂ immersion	−196	1.13 ± 0.07	21.57 ± 8.75	97.3 ± 30.8

frozen storage and thawing. Cooking loss (CL) was described by the change in patty mass as a result of cooking. Patties were grilled at 191°C for 2.5 min on one side, followed by 2 min on the other side. The cooked patties were placed in cardboard boxes lined with paper towels. The patties were cooled for 34 ± 3 min at room temperature prior to weighing. The ratio of the mass difference over the mass of the patties prior to freezing represented cooking losses. Total loss (TL) represented the combined total for freezing, cooking and storage losses.

Texture analysis included a texture profile analysis (TPA) (Bourne, 1978) on an Instron testing machine (Model TM) and the Warner Bratzler shear (WBS) evaluation (Nusbaum et al., 1983). These were determined on core samples, 2.2 cm in diameter and 1 cm in average thickness, taken from cooked patties. The WBS method was used to measure the maximum force required to shear patty cores. The sensitivity of the system was set at 10%. In the case of TPA analysis, a chart speed of 10 cm/min, and cross-head speeds of 1 or 5 cm/min were used. Samples were compressed to 75% of their original height in each cycle. The following parameters were calculated from the resulting profiles: hardness (HARD), cohesiveness (COH), elasticity (EL), gumminess (GUM) and chewiness (CHEW) (Bourne, 1978).

Samples for pH determination were prepared by blending 10g of patty with 100mL distilled water. A modified version of the Wardlaw et al. (1973) method was used for determining the WHC. Surface color measurements were made using a Spectrogard Color System (Model 96) with a Hunter-Lab color scale. Three color parameters were measured (Frye et al., 1985). These included "Lh" (Col 1), "ah" (Col 2) and "bh" (Col 3) where 'COL 1' indicates the degree of whiteness; "COL 2" the intensities of red and green with lower values in the -ve range indicating higher degrees of green; and "COL 3" the intensities of yellow and blue, with lower values in the -ve range representing higher degrees of blue.

A modified method of the TBA test described by Tarladgis et al. (1960) was used in estimating the degree of oxidative rancidity. Values for TBA were expressed as absorbance units, with higher numbers representing higher degrees of rancidity.

Data analysis

Each of the three independent variables, prechilling method (PR), freezing method (MET) and frozen storage period (STOR) was iden-

tified by indicator variables for statistical analysis, i.e., ANOVA and regression analysis (SAS, 1985). For freezing methods, 1, 2, 3 and 4 were assigned to the slow air blast, fast air blast, CO_2 snow contact and liquid N_2 immersion freezing methods, respectively. In the case of storage period, 1, 2, 3 and 4 were assigned to the 6–9, 24–30, 56–63 and 88–98 day storage periods, respectively. For pre-chilling, the air and CO_2 snow treatments were represented by 1 and 2, respectively.

RESULTS & DISCUSSION

IN TABLE 1 ARE LISTED the measured and calculated operating conditions as well as freezing times and rates. The freezing time was defined as the time required to lower the temperature of a particular patty from 1°C to −10°C at the geometric center. Consequently, freezing rate was expressed by the ratio of half the patty thickness to the freezing time.

Shrinkage

Significant differences were found between freezing methods for freezing losses ($P < 0.0001$) and cooking losses ($P < 0.01$), between storage time and storage losses ($P < 0.0001$), and between pre-freezing and total losses ($P < 0.05$) (Table 2). The patties, frozen by the two cryogenic methods, had lower freezing losses. The highest freezing loss occurred with the slow air blast method (Table 3). This is in agreement with Sebranek et al. (1978) and Nusbaum et al. (1983) for beef patties. Cooking losses were significantly higher for the patties frozen by liquid N_2 immersion as compared to those frozen by CO_2 snow contact. These findings disagree with Sebranek (1980) and Nusbaum et al. (1983) for beef patties but are in agreement with Jakobsson and Bengtsson (1969) for 20 mm thick beef slices and with Lind et al. (1971) for lamb chops. Cryogenic immersion freezing caused fragmentation and fracturing of the patties allowing more surface area to be exposed for evaporative losses during cooking. Total losses were, thus, significantly higher for N_2 freezing (Table 3). Carrol et al. (1981) concluded that a greater degree of damage occurs in meat microstructure when freezing takes place at very high rates than at slower rates.

Storage shrink increased with frozen storage time (Table 3), caused by higher evaporative losses due to increased storage time. Miller et al. (1980) also reported increasing amounts of exudate from both ground beef and pork samples as a function of storage time at −18°C. Air pre-chilling caused higher ($P < 0.05$) total losses than CO_2 pre-chilling.

In Table 4 are shown appropriate regression models for the shrinkage parameters. The freezing losses decreased as a function of freezing rate (Eq. 1). The storage losses increased as a function of storage time (Eq. 2), with air pre-chilling re-

Table 2—Analysis of variance for shrinkage parameters

Source	df	Freezing	Storage	Cooking	Total
		Sum of squares for different losses			
Replication (R)	1	0.77***	0.04	109.2***	110***
Pre-chill (P)	1	0.18	1.10	2.61	13**
Freezing method (F)	3	37.2 ***	0.56	66**	7
R × P × F	8	0.84***	3.51*	21.4	10
Storage period	3	0.03	8.66***	8.2	9
Error	36–39	0.39	7.5	75.3	89
Total	52–55	40.5	21.2	298	265

* P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001, df = degree of freedom.

Table 3—Least square means of different properties of ground beef patties for different treatments

Properties[c]	Storage periods[a]				Freezing methods[b]			
	1	2	3	4	1	2	3	4
Freezing loss, %	–	–	–	–	2.0	1.7	0.1	0.1
Storage loss, %	0.7	1.2	1.5	1.7	–	–	–	–
Cooking loss, %	–	–	–	–	34.4	34.6	35.9	37.7
WBS, kg	1.7	1.8	1.9	2.1	–	–	–	–
Hardness, N/cm2	36.9	37.3	41.6	54.1	–	–	–	–
Cohesiveness	0.46	0.46	0.43	0.39	–	–	–	–
Elasticity, cm	3.4	3.7	3.6	3.5	–	–	–	–
Gumminess, N/cm2	16.5	17.1	17.9	20.4	–	–	–	–
Chewiness, N/cm	5.5	6.3	6.8	7.3	–	–	–	–
pH	5.81	5.88	5.84	5.82	5.87	5.86	5.79	5.86
WHC, %	13.3	15.1	13.3	13.1	14.3	14.8	13.5	10.0
COL 1	38.1	37.4	37.1	40.0	36.6	38.3	39.1	38.5
COL 2	8.5	8.6	9.3	9.0	–	–	–	–
COL 3	–	–	–	–	10.2	10.3	10.9	10.6
TBA number	0.15	0.16	0.18	0.19	–	–	–	–

[a] Storage periods 1, 2, 3, and 4 were assigned to the 6–9, 24–30, 56–63 and 88–98 day storage, respectively.
[b] Freezing methods 1, 2, 3 and 4 were assigned to the slow air blast, fast air blast, CO_2 snow and liquid N_2 immersion freezing methods, respectively.
[c] WBS = Warner Bratzler shear value, WHC = water holding capacity; COL 1, COL 2 and COL 3 are various Hunter Color Lab scale values.

Table 5—Analysis of variance for textural parameters

Source	df	Sum of squares for different parameters					
		W.B. Shear	Hardness	Cohesiveness	Elasticity	Gumminess	Chewiness
Replication (R)	1	7.5***	1409***	0.01**	1.7***	346***	91***
Pre-chill (P)	1	0.01	0.01	0.00	0.01	0.5	0.1
Freezing method (F)	3	0.06	191	0.00	0.27	27	8.0
R × P × F	8	0.8**	806	0.00	0.96	168*	33
Storage period	3	1.0***	2167***	0.04***	0.72*	97*	22*
Error	36–39	1.5	3032	0.06	3.1	351	73
Total	52–55	10.9	7866	0.12	6.9	1039	238

* P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001. df = degree of freedom.

sulting in higher losses than CO_2 pre-chilling. The cooking losses increased as a function of freezing rate (Eq. 3). The lowest cooking losses occurred from commercial system (slow air blast freezing and CO_2 pre-chilling) which indicates that the indirect cryogenic freezing, used commercially, resulted in lower cooking losses. The total losses increased as a function of storage time, with larger values expected for the higher freezing rates and CO_2 pre-chilling treatment. The optimal conditions for minimum total losses were at PR = 1, MET = 1, and STOR = 1 (Eq. 4).

Texture

The Warner Bratzler shear values (WBS) were significantly increased by storage period (P<0.0001) (Table 5). The WBS were significantly higher after the final period (Table 3). These findings are in agreement with Jakobsson and Bengtsson (1973) for beef slices, Neer and Mandigo (1977) for pork sausages stored at −23°C, and Roberts et al. (1976) for beef steaks stored at −10°C. Verma et al. (1985), however, found a significant decrease in the WBS for sausages during a storage period of 52 wk at −18°C; Bannister et al. (1971) found no significant changes for pork chops stored in household freezers. The WBS values were not affected by freezing method,

which is in agreement with Carrol et al. (1981) for beef semitendinosus samples. Equations in Table 6 indicate that the optimum conditions for the lowest WBS were at PR = 1 and STOR = 1.

Significant differences existed between storage periods for all TPA parameters, i.e., HARD (P<0.001), COH (P<0.005), GUM (P<0.05), and CHEW (P<0.05) (Table 5). Hardness followed a trend similar to WBS. Ockerman and Organisciak (1979) found similar results for beef steaks, but Miller et al. (1980) found the increase in tenderness for frankfurter with storage time at −18°C. Cohesiveness decreased during the first and the last storage periods, which is in agreement with Ockerman and Organisciak (1979) for beef steaks. The gumminess and chewiness increased between the first and fourth storage periods, while elasticity peaked significantly between the first and second storage periods (Table 3).

The regression models are given in Table 4. The hardness, gumminess and chewiness increased with storage time for both pre-chilling treatments. The cohesiveness decreased with storage time while elasticity increased with the increase in PR, MET, and STOR values. The optimal conditions for the lowest values of hardness, gumminess and chewiness were: PR = 1 and STOR = 1 for hardness, PR = 2 and STOR = 1 for gumminess, and STOR = 1 for chewiness.

pH

The pH of the patties was significantly lower with the CO_2 snow freezing. The pH peaked between the first and second periods of storage before dropping, which is in agreement with Ockerman and Organisciak (1979) for restructured beef steaks (Table 3). Equation 11 in Table 4 shows that pH increased slightly but gradually with increase in freezing rates in the case of air pre-chilling but decreased gradually with increase in the rates in the case of CO_2 snow prechilling.

WHC

The WHC was significantly affected by the freezing method (P < 0.05) (Table 6). The N_2 freezing resulted in a significantly lower WHC than the other methods, which was due to higher cooking losses (Table 3). This is in disagreement with Jakobsson and Bengtsson (1969) for beef patties. However, Sebranek (1980) found no significant differences in beef patties frozen by air blast or CO_2 and N_2 spray freezings. The WHC peaked significantly between the first and second storage periods. This trend was similar to pH changes and thus indicated that WHC increased with the increase in pH, once the isoelectric point had been attained. This is in agreement with Miller et al. (1980) for ground beef, while, Sebranek et al. (1979) did not observe any consistent trend in the WHC of beef patties with frozen storage period at −29°C. Equation 12 in Table 4 indicates that WHC is expected to be at its highest and lowest values with the fast air blast freezing (MET = 2) and the N_2 freezing (MET = 4), respectively, for either PR = 1 or 2.

TABLE 4—Regression models of various properties of ground beef patties as a function of pre-chilling (PR), freezing method (MET) and storage period (STOR)[a]

1. Freezing loss (%) = −0.52 (MET) + 4.22 (PR) − 1.35 (PR)² − 0.20 (PR) (MET)
   R² = 0.93, MSE = 0.15, df = 49
2. Storage loss (%) = 0.94 (STOR) −0.08 (STOR)² − 0.12 (STOR)(PR)
   R² = 0.90, MSE = 0.21, df = 53
3. Cooking loss (%) = 51.59 (PR) − 17.37 (PR)² + 0.22 (MET)²
   R² = 0.997, MSE = 4.1, df = 53
4. Total loss (%) = 3.57 (STOR) + 5.13 (MET) + 10.82 (PR)
   R² = 0.95, MSE = 74.9, df = 50
5. WBS (kg) = 2.35 (PR) + 0.12 (STOR) − 0.77 (PR)²
   R² = 0.95, MSE = 0.19, df = 53
6. Hardness (N/cm2) = 51.08 (PR) + 1.14 (STOR)² − 17.14 (PR)²
   R² = 0.94, MSE = 116.5, df = 50
7. Cohesiveness = 0.70 (PR) − 0.005 (STOR)² − 0.23 (PR)²
   R² = 0.99, MSE = 0.002, df = 50
8. Elasticity (cm) = 0.38 (STOR) + 0.38 (MET) + 1.11 (PR)
   R² = 0.94, MSE = 0.74, df = 50
9. Gumminess (N/cm2) = 24.09 (PR) + 0.24 (STOR)² − 8.01 (PR)²
   R² = 0.95, MSE = 18.9, df = 50
10. Chewiness (N/cm) = 5.06 (STOR) − 0.85 (STOR)²
    R² = 0.90, MSE = 5.0, df = 51
11. pH = 0.11 (MET) + 8.57 (PR) − 2.78 (PR)² − 0.10 (PR) (MET)
    R² = 0.999, MSE = 0.02, df = 52
12. WHC = 3.51 (MET) + 19.40 (PR) − 6.95 (PR)² − 0.98 (MET)²
    R² = 0.95, MSE = 11.5, df = 50
13. COL 1 = 56.38 (PR) − 19.59 (PR)² + 0.76 (PR) (MET)
    R² = 0.99, MSE = 18.5, df = 53
14. COL 2 = 11.41 (PR) − 3.42 (PR)² + 0.07 (STOR) (MET)
    R² = 0.98, MSE = 1.4, df = 53
15. COL 3 = 0.24 (MET) + 14.90 (PR) − 4.97 (PR)²
    R² = 0.99, MSE = 1.4, df = 53
16. TBA = 0.13 (STOR) − 0.02 (STOR)²
    R² = 0.80, MSE = 0.01, df = 54

[a] R² = coefficient of determination; MSE = mean sum of square of error; df = degree of freedom; WBS = Warner Bratzler shear value; WHC = water-holding capacity; TBA = 2-thiobarbituric acid values; and COL 1, COL 2, and COL 3 are Hunter Color Lab scale values.

Surface color

There were significant changes between the storage periods and whiteness (COL 1) (P<0.01) and redness (COL 2)

Table 6 — Analysis of variance for functional properties

Source	df	Sum of squares for different parameters[a]					
		pH	WHC	COL 1	COL 2	COL 3	TBA
Replication	1	0.67***	88**	687***	20***	61***	0.4***
Pre-chill (P)	1	0.03	26	15	17	0.1	0.0
Freezing method (F)	3	0.06	116*	56	3.6	5**	0.0
R x P x F	8	0.13*	74	62	35***	1	0.01*
Storage period	3	0.04	31	71**	5**	1	0.02***
Error	36–39	0.23	370	165	13	10	0.02
Total	52–55	1.16	693	1063	93	79	0.4

[a] WHC = water-holding capacity, TBA = 2-thiobarbituric acid value; COL 1, COL 2, and COL 3 are Hunter Color Lab scale values.

* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, df = degree of freedom.

($P < 0.01$), between freezing methods and yellowness (COL 3) ($P < 0.01$), and between the pre-chilling and COL 2 ($P < 0.1$) (Table 6). The COL 1 peaked significantly between the third and final storage period indicating that the color faded during overall storage. This is in agreement with Sebranek et al. (1979) for beef patties. The COL 2 peaked significantly between the second and third storage periods. This signified an increase in redness. The COL 1 was significantly lower in the slow air blast freezing compared to other methods (Table 3). The COL 3 values were significantly higher for CO_2 snow freezing than air blast freezings. The COL 2 was higher ($P < 0.08$) in CO_2 pre-chilling. Regression equations in Table 4 show that COL 1 and COL 3 increased with increase in freezing rates for both pre-chilling treatments. The COL 2 increased with the increase in storage time, with higher values for the higher freezing rates and the CO_2 pre-chilling.

TBA

The TBA values were significantly ($P < 0.0001$) dependent on storage period (Table 6). The TBA increased as a function of storage time, indicating some fat oxidation during storage (Table 3). This is in agreement with Miller et al. (1980), Awad et al. (1968) and Verma et al. (1985) for both ground beef and beef muscles. There were no significant differences between the TBA for the freezing methods which is in disagreement with Sebranek et al. (1979) for beef patties. Equation 16 in Table 4 indicates that TBA increased steadily with the increase in storage time, regardless of the freezing or pre-chilling methods.

SUMMARY AND CONCLUSIONS

LIQUID N_2 immersion freezing resulted in a poorer quality beef patty than other methods. Patty quality diminished with frozen storage time at $-17 \pm 2°C$. This was shown by adverse changes in several of the quality parameters. The type of pre-chilling method did not affect patty quality significantly. The patties frozen by N_2 immersion exhibited lower WHC and freezing losses, but higher cooking and total losses. The benefits of fast freezing could be obtained if the freezing rate were collected so that fractures would not occur. Presently, commercial systems using cryogenics are not immersion systems. Commercial freezers do not use liquid N_2 at the liquid temperature, but vapor is used to run at some higher temperatures. A spray system might be better than the direct immersion. The advantage associated with fast freezing may be more applicable with intact cuts of meat where cellular damage has not yet taken place.

Storage losses increased significantly during storage period. However, neither cooking nor total losses increased during storage. Patty tenderness decreased during storage, as shown by the increased values of WBS, hardness, gumminess, and chewiness. The cohesiveness, however, decreased, indicating a loss of firmness with time. Surface reflectance peaked significantly between about 8 and 13 wk of storage, indicating that the patties faded during storage time. TBA number increased, suggesting an increase in oxidative rancidity.

Carbon dioxide pre-chilling resulted in significantly lower freezing, storage and total losses, and less pale color with a more red appearance. WHC was the highest in air pre-chilling.

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Variation in Myoglobin Denaturation and Color of Cooked Beef, Pork, and Turkey Meat as Influenced by pH, Sodium Chloride, Sodium Tripolyphosphate, and Cooking Temperature

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ABSTRACT

This study investigated the effects of pH (5.50–7.00), sodium chloride concentration (0.0–3.0%), and sodium tripolyphosphate concentration (0.0 and 0.5%) on the percent myoglobin denatured (PMD) in beef, pork and turkey muscle when heated to temperatures between 55 and 83°C. At most temperatures studied, the presence of sodium chloride and sodium tripolyphosphate increased the PMD. In contrast, high pH markedly decreased the PMD ($P < 0.05$). The effect of pH on PMD was similar for all three species studied and, in all cases, was sufficient to produce obvious color differences in the cooked muscles.

INTRODUCTION

VARIATION in the color of precooked meat products cooked to the same internal temperature has been a problem in the meat industry for over 30 years (Pool, 1956; Anonymous, 1983; Hunt and Kropf, 1987). This problem occurs sporadically in precooked meat products and is characterized by variations in redness in highly pigmented muscles such as beef muscle (Anonymous, 1983) and variations in pinkness in the less pigmented poultry muscles (Cornforth et al. 1986). Although the cause of this problem has not been determined, two possibilities exist: (1) the myoglobin has been converted to a pink hemochrome during heating, or (2) the myoglobin has not been completely denatured.

Several researchers have suggested that nitrosohemochrome is the cause of this color problem. Nitrosohemochrome, the pigment responsible for the characteristic color of cured meat, is produced when either nitrate, nitrite or nitrous oxides are present in meat during cooking. Suggested sources of this contamination in precooked meat products are extremely varied and include the following: (1) nitrate or nitrite contamination from processing equipment and water supply (Brant, 1984); (2) nitrate or nitrite in the diet (Froning et al., 1967); (3) nitric oxide from freezing equipment (Everson, 1984); (4) nitric oxide from exhaust fumes inhaled by animals just before slaughter (Froning, 1983); and (5) nitric oxide produced in gas-fired ovens (Pool, 1956). However, recent research using reflectance spectroscopy to characterize the pigments present in commercially prepared precooked turkey breast has indicated that nitrosohemochrome is not the pigment responsible for this color defect (Cornforth et al., 1986).

Other types of hemochromes may be responsible for this color defect. Under appropriate conditions denatured myoglobin can react with certain amino acids, denatured proteins, and other nitrogen containing substances to produce pink hemochromes (Drabkin and Austin, 1935; Barron, 1937; Dymicky et al., 1975).

It is unclear why this pink hemochrome should form only sporadically in precooked meat products. One recent explanation is that the pink hemochrome is formed by the reaction, under reducing conditions, between the heme from myoglobin

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and nicotinamide normally present in muscle and that the sporadic formation of the pigment is due to variation in nicotinamide concentration in the muscle (Cornforth et al., 1986). However, data in this research did not fully support this conclusion since the spectrum of the pigment found in turkey muscle with this color defect did not completely match that of nicotinamide hemochrome.

The pink color defect may also be due to incomplete denaturation of myoglobin during cooking. A recent study showed that, even when cooked to the same internal temperature, high-pH beef, pork and turkey muscle (pH > 6.0) was redder than low-pH muscle (pH 5.5) and appeared undercooked (Schmidt and Trout, 1984). The explanation suggested for this behavior was that high pH reduced the amount of myoglobin denatured at a given temperature. Although this theory has not been verified, the pink color problem appears to only occur in commercially-produced cooked turkey muscle which have a cooked pH greater than 6.3 (Trout, 1988).

Other explanations for this color problem have been suggested. These include: (1) carbon monoxide contamination from either gas-fired ovens (Pool, 1956) or exhaust fumes (Froning, 1983); (2) addition of dried egg albumen during processing (Froning et al., 1968a); (3) preslaughter stress leading to elevated muscle cytochrome concentration (Babji et al., 1982)); and (4) natural variation in the concentration of muscle myoglobin (Froning et al., 1968b). Although any of these factors may be responsible for isolated occurrences, they cannot fully explain the widespread nature of the problem.

Additives used in precooked meat products such as salt (sodium chloride) and sodium tripolyphosphate may also cause color problems since both additives can alter the denaturation behavior of proteins such as myoglobin (von Hippel and Schleich, 1969). It is also not clear whether this problem occurs with meat from all species since most research has been done with turkey meat where the defect is most prevalent.

The objectives of this study were: (1) to characterize the pink pigment present in cooked high-pH meat, and (2) to quantify the effect of pH, sodium chloride and sodium tripolyphosphate on the color and amount of undenatured myoglobin in cooked beef, pork and turkey meat.

MATERIALS & METHODS

Meat and additives

The beef (top round), pork (ham) and turkey (50% breast-50% thigh) meat used in this study were obtained 48 hr postmortem from commercially slaughtered market animals, except in experiment 4. The meat was hand-deboned when necessary and trimmed of all visual fat and connective tissue. Three samples (10g) were taken from each muscle for pH measurement to ensure that the pH of each muscle was normal (5.4–5.8). Meat from each species was ground separately through a 2.5 cm and then a 0.3 cm plate in a mixer/grinder (Model 4146, Hobart Co., Troy OH), mixed thoroughly by hand, weighed into approximately 600g lots, vacuum packaged and frozen at −30°C until used (within 3 months). All meat preparation and grinding were carried out in a refrigerated room (8°C).

With experiment 4, pork loin chops were purchased from local

supermarkets. Chops were selected that were either pink (i.e., typical of normal fresh pork) or medium to dark red (i.e. typical of DFD [Dark Firm and Dry] pork). The *Longisiimus dorsi* muscle was removed from the chops, ground and packaged as described above. Average pH of muscle from pink chops and red chops was 5.50 and 6.50, respectively.

Chemicals were analytical reagent grade sodium chloride, 1.0M sodium hydroxide, and 1.0M lactic acid, food grade sodium tripolyphosphate (Courtesy of FMC corporation, Philadelphia, PA) and deionized water.

Procedures

The procedures outlined below were used in all five experiments in this study. Deviations from these procedures are listed in the respective treatment sections.

Sample preparation and heating. Meat was thawed for 36 hr at 2°C before use. Treatments for experiments 1-4 were prepared by mixing the meat (400 g) and ingredients in a Kitchen Aid Mixer (Model K45SS, Hobart Co., Troy OH) for 2 min on speed two and then 1 min on speed three. To obtain the pH levels described in the treatment section, predetermined amounts of either 1.0 M NaOH or 1.0 M lactic acid were added to the treatments during the initial stages of mixing as previously described (Trout and Schmidt, 1986). Deionized water (9.0%), sodium chloride and sodium tripolyphosphate (when used) were also added during the initial stages of mixing. Sodium chloride was added dry while sodium tripolyphosphate was dissolved in all the water. The acid and base were diluted immediately before addition by mixing with either water or phosphate solution. Once prepared, the meat was placed in polyethylene bags and equilibrated at 3°C for 12-14 hr. Then, 30 g aliquots from each treatment were weighed into 50 mL plastic centrifuge tubes and centrifuged at 100 × *g* for 2 min to remove air pockets.

For each temperature studied, three tubes from each treatment were heated in a thermostatically-controlled water bath in which water temperature was maintained at 2.0 ± 0.5°C above the required end-point temperature. Internal temperature of samples was monitored using thermocouples (O.F. Ecklund Inc., Cape Coral, FL) placed in the geometric center of two tubes which had been handled in a manner similar to treatment tubes. In all cases, samples reached the predetermined temperature within 20 to 25 min. Tubes were cooled in an ice slush for 30 min and stored in a cooler (0-1°C) for 1 to 2 hr until analyzed for PMD and percent metmyoglobin.

With experiment 5, the meat was prepared and heated as described above except that larger quantities were used; 1 kg meat was used instead of 400 g, and 225g of meat mixture was filled into 211 × 300 cans.

Analysis

All heated samples were analyzed for PMD and percent metmyoglobin; pH was measured on all samples before heating and samples in experiment 5 were analyzed for visual color.

Percent myoglobin denatured and percent metmyoglobin. Undenatured myoglobin was extracted from samples with cold (0°C) 0.04M, phosphate buffer pH 6.8 (Wariss, 1979). With heated samples, myoglobin was extracted by blending the complete sample with the phosphate buffer for 1 min. on high speed using an Osterizer Imperial Blender (John Oster Mfg. Co., Milwaukee, WI). With unheated samples, a 10 g sample was homogenized with phosphate buffer for 20 sec. on setting 4 using a Brinkman homogenizer (Westbury, NY) fitted with a pt10 probe generator. Sample to buffer ratio used for extraction of the different samples was as follows; heated pork and turkey, 1:4; heated beef, 1:5; and all unheated samples 1:10. Homogenates were centrifuged for 30 min (50,000 × *g*, 5°C) and the supernatant was filtered through a Whatman No. 1 filter paper. Absorbance of the filtrate was measured at 525, 572 and 700 nm using a Beckman (Fullerton, CA) model 25 double beam spectrophotometer. Percent metmyoglobin and myoglobin concentration were calculated using the following formula (Krzywicki, 1979):

$$Metmyoglobin(\%) = \{1.395 - [(A_{572} - A_{700})/(A_{525} - A_{700})]\} \times 100$$

$$Myoglobin(mg/mL) = (A_{525} - A_{700}) \times 2.303 \times dilution\ factor$$

where A_λ = Absorbance at λnm.
PMD was calculated using the following formula:

$$PMD = [1 - (myoglobin\ conc\ after\ heating/myoglobin\ conc\ before\ heating)] \times 100$$

pH. pH was measured by blending a 15-g sample with 150 mL deionized water for 2 min on high speed using an Osterizer Blender (John Oster Mfg. Co., Milwaukee, WI). The pH of the resultant suspension was measured with a Fisher Accumet pH meter (Model 805, Fisher Scientific, Pittsburgh, PA) equipped with a Corning combination electrode (Cat. No. 34107, Corning, Medfield, MA).

Visual appraisal. The surface color of the cooked samples in Experiment 5 was determined using a 10-member trained sensory panel. Training sessions were conducted with samples which were either extremely pink, moderately pink or showed no sign of pinkness. The cooked samples were prepared for evaluation by removing 2.0 cm thick slices from the center of each sample, covering each slice with plastic interleave paper and equilibrating it at 5°C for 30 min. Samples were evaluated under Warm White fluorescent lighting that provided 70 ft-candles at the counter surface. Panel members were instructed to rate each sample on a scale of 1 to 6 (1 = no pink, 6 = extremely pink).

Treatments

Experiment 1. Ground beef with normal pH (5.50) and ground beef in which the pH had been adjusted to pH 6.50 was either not heated or heated to 52, 59, 66, or 73 °C. Undenatured myoglobin was extracted from the samples as described in the procedure section and the pigment in the clear supernatant was reduced by addition of 1-2 mg dithionite per mL. The spectrum of the clarified supernatant was measured between 450-700 nm.

Experiment 2. The effects of sodium chloride concentration (0.0, 1.0, 2.0, and 3.0%) on the percent myoglobin denatured (PMD) and percent metmyoglobin in ground beef samples cooked to 52, 59, 66, 73, and 80°C were measured.

Experiment 3. The effects of species (pork, turkey, and beef) addition of sodium tripolyphosphate (beef plus 0.5% sodium tripolyphosphate) and pH (5.50, 6.00, 6.50 and 7.00) on PMD and percent metmyoglobin in ground meat samples cooked to 55, 62, 69, 76, and 83°C were investigated.

Experiment 4. PMD and percent metmyoglobin were determined in pork of normal pH (5.50) adjusted to pH 6.50 and pork with a natural pH of 6.50 that was heated to 59, 66, 73, and 80°C.

Experiment 5. Samples of beef, pork and turkey meat, at both pH 5.50 and pH 6.50, were heated to 55, 62, 69, 76, and 90°C and evaluated by a trained sensory panel to determine pinkness and analyzed for PMD and percent metmyoglobin.

Experimental design and statistical analysis

Experiment 1. This experiment was replicated three times. Absorbance values were averaged every 2 nm between 450 and 700 nm.

Experiment 2 and 4. The experimental design used was a split-block design with three replications (blocks); treatments were the main effects and heating temperature was the subunit.

Experiment 3. The experimental design used was a completely balanced incomplete split-block design with three replications; treatments were the main effects and heating temperature was the subunit. The sixteen treatments were arranged as a 4 × 4 factorial [i.e., four species (beef, beef plus 0.5% tripolyphosphate, pork and turkey) by four pH levels (5.50, 6.00, 6.50 and 7.00)]. Within each replicate, species were assigned to one of two completely balanced incomplete blocks and all treatments (i.e., species-pH combinations) in a block were prepared and analyzed on the same day (Bose, 1939).

Experiment 5. The relationship between PMD and color score was determined for each species using an all possible subsets multiple regression procedure. The regression equations were analyzed by case analysis to check the adequacy of the fitted model (Weisberg, 1980).

All variables in experiments 2-4 were analyzed by analysis of variance. When F values were significant (P<0.05), Fisher's Least Significant Difference test was used to determine differences between treatment means (Snedecor and Cochran, 1976).

RESULTS & DISCUSSION

Experiment 1

There was no difference in absorbance spectra between myoglobin extracted from heated beef samples, at both pH 5.50 and pH 6.50, and myoglobin extracted from unheated samples (Fig. 1). Moreover, the λ_{min} (562 and 514 nm) and λ_{max} (580 and 542 nm) values of the spectra from both heated and un-
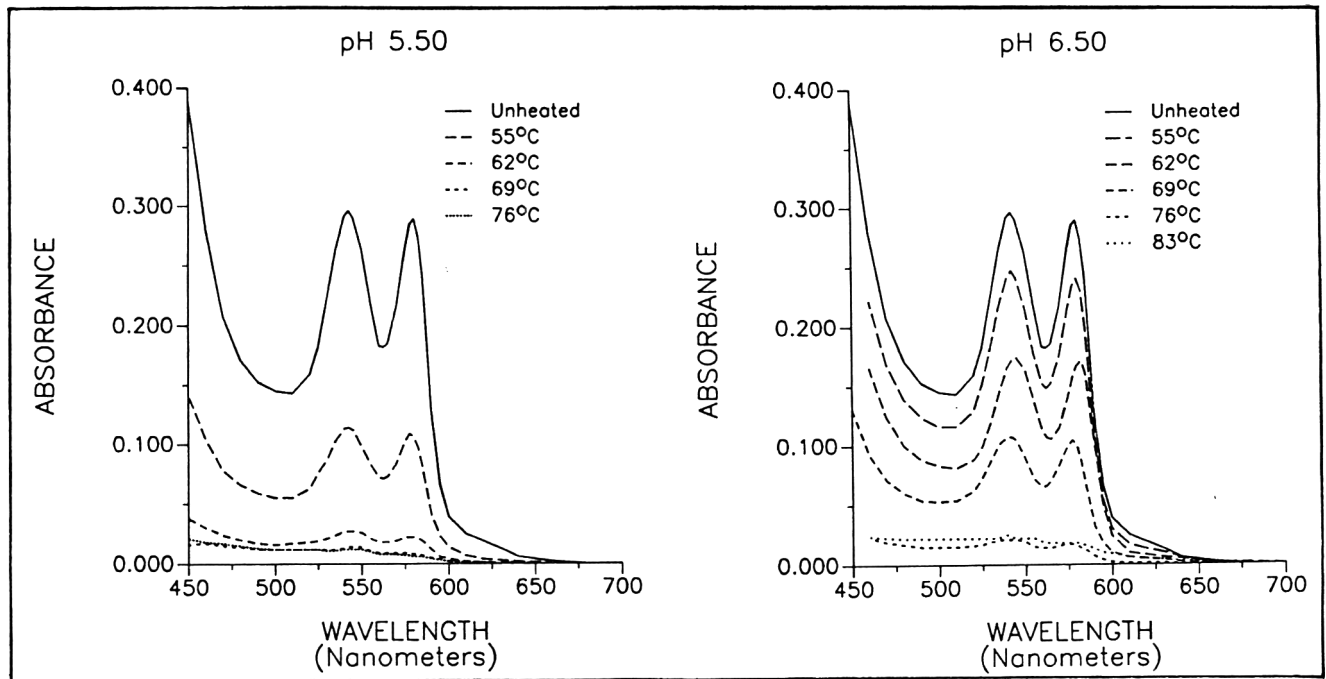
Fig. 1 — Effect of heating temperature on absorbance spectra of extracts from heated beef muscle after reduction with dithionite; muscle pH 5.50 and 6.50.
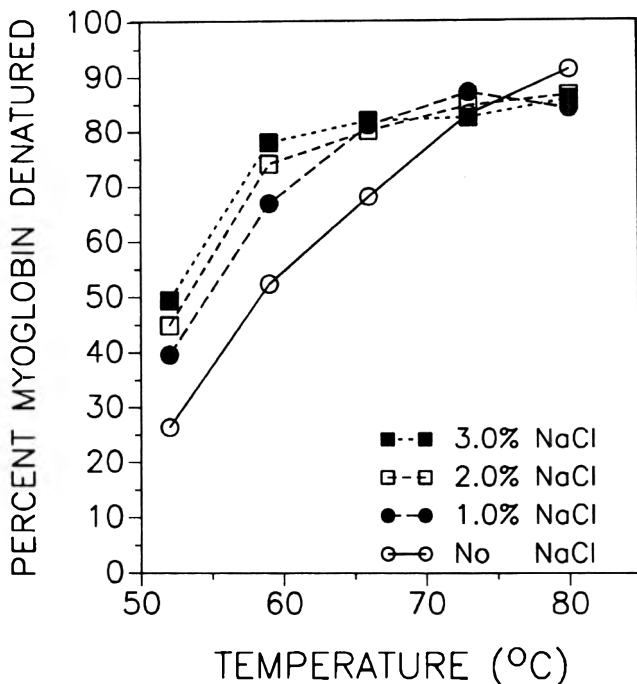
Fig. 2 — Effect of sodium chloride concentration and heating temperature on percent myoglobin denatured (PMD) in beef muscle. $LSD_{(5\%)}$ for comparison between treatments is 6.2%.

heated samples were similar to those previously reported by Bowen (1949) for horse heart oxymyoglobin (λ_{min}, 564 and 510 nm; λ_{max} 582 and 544 nm). The results of the present study support the findings of Bernofsky et al. (1959) in that higher heating temperatures decrease the amount of undenatured myoglobin present in cooked meat.

As expected, increasing the heating temperature decreased the absorbance of extracts at all wavelengths; however, the decrease in absorbance was more rapid at pH 5.50 than at pH 6.50 (Fig. 1). Thus, these results indicate that the more intense red color observed in cooked high-pH beef muscle is due, at least in part, to a higher concentration of undenatured myoglobin. However, because the samples were reduced with dithionite before their spectra were measured, it could not be determined if the difference in color was also partly due to variation in the metmyoglobin concentration.

Experiment 2

Percent metmyoglobin. In this and subsequent experiments where percent metmyoglobin was measured, the treatments had no effect (P>0.05) on the percent metmyoglobin in the samples after heating.

Percent myoglobin denatured. The presence of sodium chloride in the cooked ground beef increased the PMD, particularly at the lower three temperatures (52, 59, 66°C) (Fig. 2). Moreover, at 52 and 59°C, PMD increased linearly with increasing sodium chloride concentration (P<0.05). Therefore, in the normal temperature range in which meat is cooked, sodium chloride will decrease rather than increase the pinkness of cooked meat products.

Experiment 3

pH adjustment. Analysis of variance indicated there was no difference in pH (P>0.05) between any of the treatments (i.e., between beef, beef plus tripolyphosphate, pork and turkey) at any of the four pH levels. The mean and standard deviation of the pH values at the four different pH levels studied, averaged over treatment, were as follows: 5.52 ± 0.04; 6.03 ± 0.03; 6.47 ± 0.04; and 6.99 ± 0.06, respectively.

Percent myoglobin denatured. Temperature had a significant effect on PMD (P<0.05); PMD increased with increasing temperature for all four treatments at all four pH levels (Fig. 3). This general trend of decreasing PMD with increasing temperature is similar to that previously reported by Bernofsky et al. (1959) and Helmke and Froning (1971).

Of the variables studied, pH had the greatest effect on PMD. At a given temperature, PMD decreased with increasing pH (Fig. 3). Although there were significant (P<0.01) treatment by temperature and treatment by pH by temperature interac-

BEEF

BEEF + STP
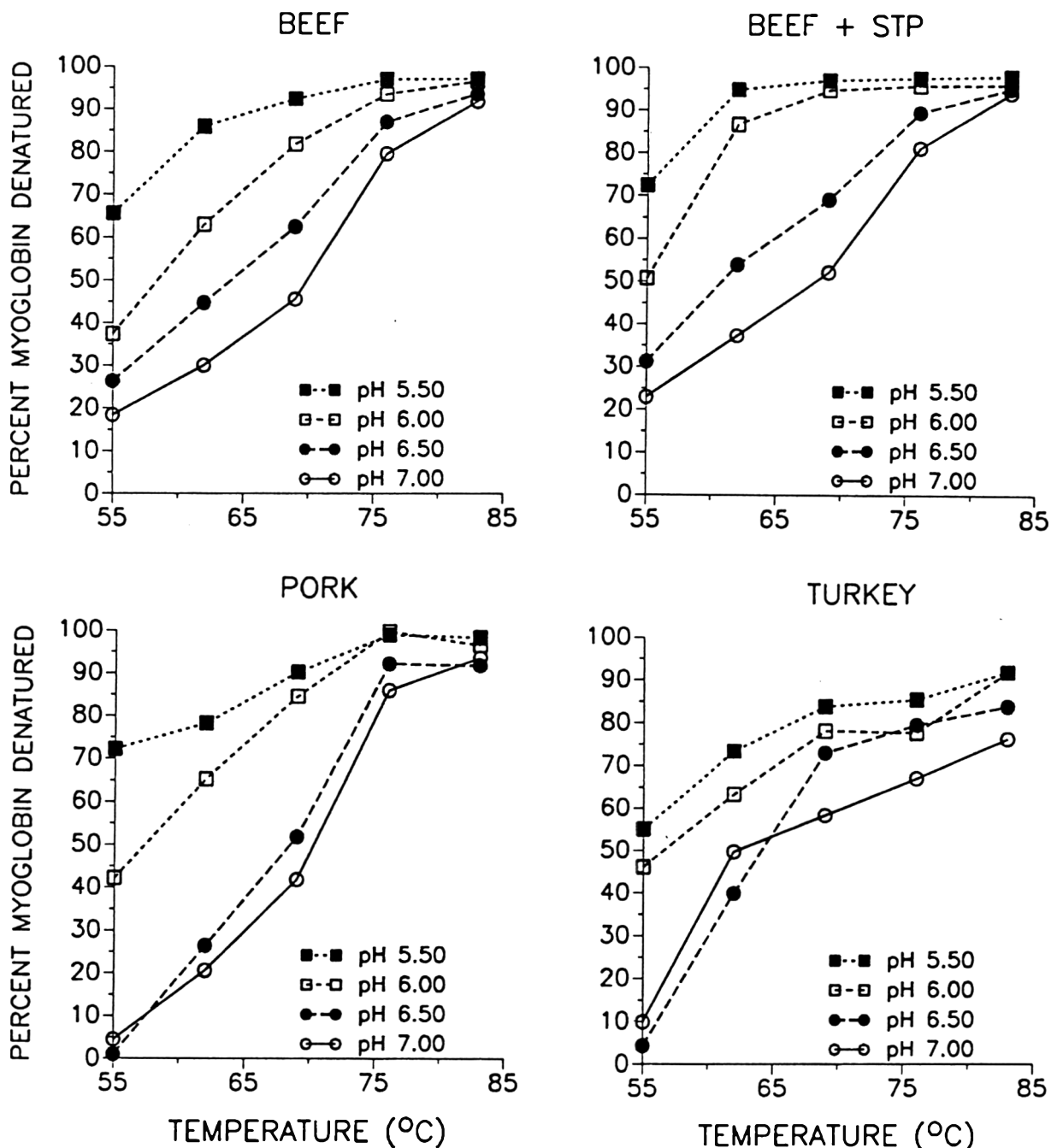
PORK

TURKEY

Fig. 3 — Effect of pH and heating temperature on percent myoglobin denatured (PMD) for beef muscle, beef muscle plus 0.5% sodium tripolyphosphate (STP), pork muscle, and turkey muscle. $LSD_{(5\%)}$ for comparison between treatments is 6.8%.

tions, these effects were small in comparison to that produced by pH and the pH by temperature interaction. Consequently, the overall shape of the curves in Fig. 3 were similar at all four pH levels with all four treatments. It can, therefore, be inferred that pH had a similar protective effect on the denaturation behavior of myoglobin from all three species. Moreover, there was no interaction $(P > 0.05)$ between tripolyphosphate and pH which changed the protective effect pH had on the denaturation of bovine myoglobin.

Although there was no interaction between tripolyphosphate and pH, the presence of tripolyphosphate increased $(P < 0.05)$ the amount of myoglobin denatured by between 5–10% at all four pH levels at the four lower temperatures. This effect of tripolyphosphate was similar to that observed with sodium chloride in the earlier experiment and was probably due to either an anion- or cation-promoted alteration in the denaturation behavior of myoglobin (von Hippel and Schleich, 1969).

Increasing the pH markedly decreased the PMD at a given temperature and, except at temperatures where the myoglobin was completely denatured, this effect increased with increasing pH. The effect of pH was most pronounced at the lower temperatures (55 and 62°C) where the PMD was between 3- and 14-fold greater at pH 5.50 than at pH 7.00. The effect of pH on PMD gradually diminished as the temperature increased, but there were still significant differences $(P < 0.05)$ between the pH 5.50 and pH 7.00 treatments at all but the highest temperature studied (83°C) (Fig. 3).

Because of the protective effect high pH had on myoglobin denaturation, the temperature at which most of the myoglobin was denatured increased with increasing pH until the pH reached 6.50 and then plateaued. With turkey muscle, however, this plateauing effect was less evident. This may have been because turbidity developed in the extracts from the pH 7.00 turkey muscle at the higher temperatures (69, 76 and 83°C). This

Table 1—Color scores of cooked beef muscle, beef muscle plus 0.5% sodium tripolyphosphate, pork muscle and turkey muscle in experiment 3 calculated from their percent myoglobin denatured (PMD) values

Species	pH	Temperature (°C)				
		55	62	69	76	83
Beef	5.50	3.6[a][b]	1.8	1.2	0.7	0.7
	6.00	5.2	3.8	2.2	1.1	0.7
	6.50	5.6	4.9	3.8	1.7	1.0
	7.00	5.8	5.5	4.8	2.4	1.2
Beef	5.50	3.0	0.9	0.7	0.6	0.5
Plus	6.00	4.6	1.8	0.9	0.8	0.8
0.5%	6.50	5.5	4.4	3.3	1.5	0.9
TPP	7.00	5.7	5.2	4.5	2.3	1.0
Pork	5.50	2.2	1.9	1.3	0.8	0.8
	6.00	3.7	2.5	1.5	0.7	0.9
	6.50	5.8	4.5	3.2	1.2	1.2
	7.00	5.6	4.8	3.7	1.5	1.1
Turkey	5.50	2.8	1.6	0.9	0.8	0.4
	6.00	3.4	2.2	1.3	1.3	0.4
	6.50	6.1	3.8	1.6	1.2	0.9
	7.00	5.7	3.1	2.6	2.0	1.4

[a] Scale: 1-6 (1 = No pink; 6 = Extremely pink).

[b] The color scores were calculated from their PMD values using the regression equations developed in experiment 5 which relate PMD to color score.

resulted in misleadingly high myoglobin concentrations and, hence, misleadingly low PMD values. The effect of pH on PMD observed in this study is consistent with the earlier observations that both extracted turkey myoglobin (Janky and Froning, 1973) and purified bluefin tuna myoglobin (Matsuura et al., 1959) exhibit minimum denaturation at pH 6.50-7.00.

From a practical point of view, these results indicate that considerable variation in the color of commercially produced cooked meat products can be the result of normal variation in muscle pH and processing procedures. For example, at 65°C, which would be regarded as an average cooking temperature for precooked meat products, about twice as much myoglobin was denatured at pH 5.50 as at pH 7.00. Although the average pH of most meat is 5.4-5.8 it is not uncommon for the pH to be as high as 6.50, particularly in meat from stress-prone animals such as pigs and turkeys. Moreover, the pH of precooked products may be increased by a further 0.3-0.4 units by the addition of phosphates, such as sodium tripolyphosphate, (Trout and Schmidt, 1984) which are commonly used in these products. Consequently, the pH of the cooked meat products may be as high as 7.0.

Experiment 4

High pH had the same effect on PMD regardless of how the pH was obtained. There was no significant difference ($P > 0.05$) in PMD between treatments with a natural pH of 6.50 and those in which the pH had been adjusted from 5.50 to 6.50. The PMD values for the natural pH and the adjusted pH treatments at 58, 65, 73, and 79°C were as follows: 17.0 and 15.3, 33.8 and 35.4, 70.9 and 65.1, and 83.6 and 87.0, respectively (the LSD [least significant difference] for comparison between treatments was 8.2%). Thus, it would be expected that the denaturation behavior of myoglobin in meat with naturally high pH should be similar to that described in the previous three experiments where the pH of the meat was adjustment.

Experiment 5

Because of the large number of treatments in the previous experiments (> 300 observations), it was not possible to measure the color scores for the treatments. Therefore, the following multiple regression equations which relate PMD values to color score were developed:

Beef: Color Score = PMD^2 × −0.000567
+ 6.023 $R^2 = 0.894$

Pork: Color Score = PMD × −0.051
+ 5.864 $R^2 = 0.899$
Turkey: Color Score = PMD × −0.065
+ 6.348 $R^2 = 0.910$

These equations were used to calculate the color scores of the treatments in Experiment 3. The calculated color scores in Table 1 show that the PMD values determined in Experiment 3 represent a very broad range of cooked colors. Moreover, the difference in calculated color scores between treatments at pH 5.50 and those at pH 7.00 is as much as 3.4 points on a six point scale. This difference would be easily discernible by the average consumer.

One interesting observation in the sensory evaluation of these samples was that at high pH (6.50), the less pigmented pork and turkey samples were still slightly pink even when heated to 90°C. The pink color persisted even though myoglobin analysis of the samples indicated there was no soluble myoglobin or other soluble pink pigment present. This pink color was probably due to the formation of a pink hemochrome. One observation that supports this hypothesis was that the pink color was mainly observed at high temperatures. High cooking temperatures increase the reducing environment in muscle (Hamm, 1977) which is essential for the formation of the pink hemochrome found in cooked turkey breast (Cornforth et al., 1986). Moreover, this pink hemochrome was insoluble in phosphate buffer (Cornforth et al., 1986) which was the buffer used in this experiment to extract myoglobin.

CONCLUSION

THE PINK COLOR found in fully cooked high-pH meat products appears to be due to two different effects: (a) incomplete denaturation of myoglobin at low temperatures (< 76°C) and (b) formation of a pink hemochrome at higher temperatures (> 76°C). Sodium chloride decreased the pinkness of precooked meat products. Consequently, variations in sodium chloride concentration in cooked meat products will produce marked variations in cooked color. Sodium tripolyphosphate increased the pinkness of cooked meat products. Although the tripolyphosphate ion reduces the pinkness of cooked meat products, the increase in pH produced by the tripolyphosphate more than compensates for this effect.

Hence, to prevent excessive pinkness in precooked meat products, meat processors should avoid using meat with abnormally high pH (> 6.0) and avoid using alkaline phosphates, particularly if the meat pH is above normal. If phosphates are to be used, it is recommended that a neutral blend of phosphates (such as a mixture of sodium acid pyrophosphate and sodium tripolyphosphate or sodium acid pyrophosphate and tetrasodium pyrophosphate) be used so that the phosphates do not increase the pH.

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Effects of Collagen and Alkaline Phosphate on Time of Chopping, Emulsion Stability and Protein Solubility of Fine-Cut Meat Systems

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ABSTRACT

Four meat emulsions were prepared with two levels of collagen and alkaline phosphate. Samples were taken at five different chopping temperatures and evaluated for total chopping time, emulsion stability and protein solubility. Adding additional collagen to meat emulsions shortened total chopping time, and decreased emulsion stability but had no effect on protein solubility. The addition of 0.25% sodium tripolyphosphate (STPP) improved emulsion stability but resulted in no significant (p>0.05) change in chopping time or protein solubility. The high-collagen-phosphate (HCP) treatment resulted in less fat, gel-liquid, solid and total cookout when compared with high-collagen no-phosphate (HC) and low-collagen no- phosphate (LC) treatments. Maximum emulsion stability was obtained at 13°C.

INTRODUCTION

THE FUNCTIONALITY of myofibrillar proteins in finely comminuted meat systems hinges on innate quality characteristics such as rigor state and pH. Other factors involved in emulsion stability include additives, mechanical treatment and thermal processing (Schut, 1976).

However, stromal protein, more specifically collagen, makes up a significant portion of muscle. Because collagen tends to react much differently than myofibrillar proteins under similar conditions, the level of stromal proteins in meat products has often been restricted. Wiley et al. (1979) and Kramlich (1971) have reported that the use of high-collagen meat should be limited to a maximum of 15% and 25%, respectively, of the meat block to prevent emulsion breakdown and gelatin pockets. The solubility and type of collagen have been found to play an important role in determining the specific restriction level (Schalk, 1981).

Collagen use in emulsified products has been investigated by numerous researchers. Jones et al. (1982) found a decrease in emulsion stability when up to 40% of the meat block was replaced with tripe. On the contrary, Rao and Henrickson (1983) reported no differences in functional parameters such as cooking yield and expressible juice in bologna that had lean meat replaced with hide collagen at the 20% level.

The stability of finely comminuted sausage products depends, not only on meat protein characteristics, but also on the addition of selected nonmeat ingredients. Since the expanded approval of selected phosphates (Anonymous, 1982), their use has become important to the meat industry.

Polyphosphates are known to decrease the amount of water lost and increase the strength of protein-protein binding in meat products upon cooking. Knipe et al. (1985a) and Trout and Schmidt (1984) reported that the extent to which this occurred depended on the type and concentration of polyphosphate used and the concentration of other added salts.

Hamm (1960) indicated that phosphates, contrary to sodium chloride, have a shrinking effect on "gelatin" rather than a

swelling effect. Ranganayaki et al. (1982) found that sodium acid pyrophosphate and tetrasodium pyrophosphate were more detrimental to hydration of bovine hide collagen in the pH range of 5 to 8 than sodium hexametaphosphate or tripolyphosphate. At pHs below 5.0 all phosphates allowed for greater hydration with the exception of hexametaphosphate. Upon heating bovine hide collagen to 70°C, in the presence of sodium tripolyphosphate, tetrasodium pyrophosphate and sodium acid pyrophosphate, the hydration capacity decreased linearly with increased pH from pH 4 to 10. The addition of salt caused no change in magnitude of hydration capacity of hide collagen upon heating to 70°C.

The stability of sausage emulsions is also dependent on mechanical factors such as chopping time and temperature. For pork fat, a final chopping temperature of 13–16°C is generally recommended (Schut, 1976). Swift et al. (1968) showed that the stability of meat emulsions depended on the melting characteristics of the fat.

During chopping, raw meat ingredients are reduced in size. It has been reported that connective tissue and muscle fibers are more resistant than lipids to structural changes by mechanical action (Hansen, 1960). Swasdee et al. (1982) observed little change in collagen fibers from 2 to 12 min of chopping and concluded that most visible changes occurred during cooking rather than chopping.

Therefore, questions remain concerning the effects of collagen in comminuted meat products, particularly, the interaction between collagen and inorganic phosphates relative to emulsion stability.

The objectives of this study were: (a) to determine the effect of muscle collagen on emulsion stability, (b) to investigate the role of alkaline phosphates on low- and high-collagen products and (c) to establish time-temperature chopping curves as related to emulsion stability.

MATERIALS & METHODS

Sample formulation and preparation

Cow meat was purchased from a commercial source (Amend Pack, Des Moines, IA). Half the cow meat was desinewed, and the other half was left intact before defatting at the Iowa State University Meat Laboratory. Pork trim (approximately 50% lean) and pork backfat were ground through 9.5-mm (3/8") plates on a Weiler and Company Grinder (Model 6) and mixed separately. Each treatment group was formulated to 28% fat (Table 1) and held overnight in a −2°C cooler.

The collagen, containing both epimysium and perimysium, was removed from the cow meat as described above a maximum of 20 hr before use, covered to prevent dehydration and placed in a −2°C cooler. The collagen trim used was not characterized but is representative of collagen trim used in meat processing. Sodium tripolyphosphate (STPP) was used as the phosphate source in this study.

Meat emulsions were prepared in a Krämer Grebe (VSM65) bowl chopper using a six-knife head operated in a 12.7°C processing room. Before each treatment group was manufactured, crushed ice was chopped in an attempt to standardize the bowl temperature. The cow meat and pork trim were chopped for 1 min (low speed) before the addition of sodium chloride, STPP and half the ice. After an additional 2 min of chopping (high speed), the pork fat, collagen and the remaining ice were added. Chopping continued until the emulsion reached a tem-

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Table 1 — Ingredient formulation by treatment

Ingredients (kg)	Treatments			
	Low collagen 0.00% STPP[a]	Low collagen 0.25% STPP	High collagen 0.00% STPP	High collagen 0.25% STPP
Intact Cow Meat	—	—	3.63	3.63
Desinewed Cow Meat	5.26	5.26	—	—
Pork Trim (50.0% Fat)	5.44	5.44	5.44	5.44
Pork Fat (100.0% Fat)	2.29	2.29	2.09	2.09
Collagen Trim (10.0% Fat)	—	—	1.81	1.81
Ice	4.26	4.26	4.26	4.26
Sodium Chloride	0.41	0.41	0.41	0.41
STPP	—	0.044	—	0.044

[a] Sodium tripolyphosphate.

perature of 10°C, at which time a sample was removed. Subsequent samples were taken at 13, 16, 18 and 21°C in the chopping cycle. Four replications of each treatment were completed. Total time of chopping for each sample taken within a treatment group was recorded by using a Fisher Scientific Company 3-channel alarm timer. Raw emulsion temperature was the determining factor as to when samples were taken and was monitored with a Whatman Temperature μ-Sensor Thermometer.

Emulsion stability

A modification of the method by Townsend et al. (1968) as described by Knipe et al., (1988) was used to determine emulsion stability.

Soluble protein

The salt-soluble protein (SSP) content was determined by a modification of the method by Saffle and Galbreath (1964) as described by Knipe et al., (1985a).

Proximate analysis

Moisture, protein and fat contents of the raw emulsions were determined by AOAC methods (1984).

Hydroxyproline

Collagen protein content was determined according to Stegemann and Stalder (1967).

Statistical analysis

The Statistical Analysis System (SAS Institute, Inc., 1985) was used to determine means, standard errors and analysis of variance. A split-plot experimental design was employed, with least significant difference used to separate the means.

RESULTS

THE EFFECT of collagen and STPP on emulsion stability showed that low-collagen emulsions had significantly (P < 0.05) less total and gel-liquid cookout than high-collagen emulsions (Table 2). The addition of 0.25% STPP also significantly (P < 0.01) reduced these two fractions. Low-collagen and STPP treatments caused no significant difference (P > 0.05) in soluble protein levels.

For collagen*STPP treatments, high-collagen emulsions had more total cookout than low-collagen emulsions with or without the addition of STPP (Fig. 1). Conversely, the high-collagen phosphate (HCP) treatment released less exudate than the low-collagen no-phosphate (LC) treatment upon cooking. The LC group also had the most fat cookout of any treatment.

The effect of chopping temperature on emulsion stability showed a maximum stability at 13°C (Fig. 2). Fat, gel-liquid and total cookout was lowest at 13°C and highest at 21°C when averaged over all collagen and STPP treatments. A similar decrease in fat cookout occurred at 18°C, whereas gel-liquid and total cookout increased as chopping temperature increased.

Fat release over the five chopping temperatures was not significantly (p > 0.05) different (Fig. 2). Significant differences (p < 0.05) in gel-liquid cookout were not found at 10°, 13° or 16°C. However, gel-liquid cookout was significantly (p < 0.05) less at 13°C than at 18° or 21°C. Total cookout paralleled gel-liquid cookout, with the exception that total cookout at 21°C was significantly greater than at all other chopping temperatures at the 0.05 confidence level.

Comparing the effects of added collagen and STPP on emulsion stability at different chopping temperatures showed that low-collagen phosphate (LCP), HCP, and high-collagen no-phosphate (HC) treatments resulted in the least total cookout at a chopping temperature of 13°C (Fig. 3). Conversely, the LC treatment released the least amount of cookout at 10°C. The LCP curve exhibited the lowest total cookout at each of the five chopping temperatures and remained stable throughout the chopping cycle. The other three treatments, in the order of decreasing stability were LC < HCP < HC at 10°C and HCP < HC < LC at 21°C. HCP released less exudate than either LC or HC at chopping temperatures between 13° and 21°C. HC exhibited a large decrease in total cookout at 13°C.

The comparison of collagen and STPP on chopping time indicated that high-collagen treatments took less time to chop than low-collagen treatments (Table 3). Also, addition of STPP increased the chopping time needed to reach a sampling temperature. This difference however, was much greater for the low-collagen treatments than for high-collagen treatments.

DISCUSSION

THE RESULTS indicated that high levels of skeletal muscle collagen will decrease cooking stability when used in meat emulsions. These results are similar to those found by Gillett (1987). High collagen treatments released significantly (p < 0.05) greater volumes of fat, gel-liquid, solid and total fractions (Table 2) than did low-collagen treatments. This could be attributed to the heating and subsequent gelatinization of collagen with the loss of fat and water binding between 60° and 80°C (Moller, 1980; Wu et al., 1982; Bendall and Restall, 1983).

The addition of 0.25% STPP was more important to emulsion stability than level of collagen and showed significance at the 0.01 confidence level. The use of alkaline phosphates in a standard meat batter will improve water binding, mainly by increasing ionic strength (Trout and Schmidt, 1984; Hamm, 1970) and pH (Knipe et al., 1985a; Trout and Schmidt, 1984; Hamm, 1970). STPP had no effect on protein solubility, which is contrary to Prusa and Bowers, (1984) and Knipe et al. (1985a); however, less myofibrillar protein was available in this study for extraction after replacement with stromal proteins. While alkaline phosphates may have detrimental effects on pure collagen hydration and solubility (Ranganayaki et al., 1982), the level of collagen added to the meat emulsions in this study allowed for phosphates to improve emulsion stability.

The effect of chopping temperature (Fig. 2) on emulsion stability agrees with Schut (1976) and Helmer and Saffle (1963). Cookout was minimized at 13°C and was highest at 21°C in all fractions owing either to overchopping and melting of fats

Treatment[b]	Total cookout (mL)[c]	Gel-liquid (mL)[c]	Fat (mL)[c]	Solids (mL)[c]	mg Soluble protein per g emulsion
Low Collagen	1.68*	1.54*	0.17	0.04	74.50
High Collagen	2.87	2.63	0.20	0.04	66.58
S.E.	0.31	0.29	0.07	0.006	4.56
0.00% STPP	3.39**	3.14**	0.25	0.07**	69.43
0.25% STPP	1.16	1.03	0.12	0.02	71.85
S.E.	0.31	0.29	0.07	0.006	4.56

[a] Sodium tripolyphosphate.
[b] Mean values for total cookout, gel-liquid, fat, solids, and mg soluble protein, N = 40 per treatment mean.
[c] mLs per 34g emulsion sample.
* Significant (p < 0.05).
** Highly significant (p < 0.01).
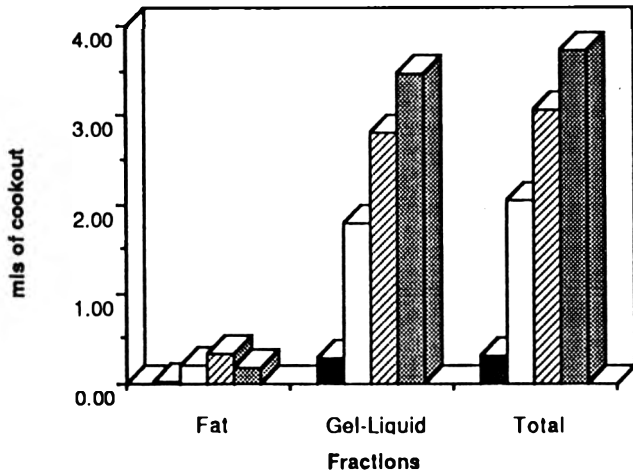
Fig. 1—Emulsion stability (mL per 34g emulsion sample) of collagen plus STPP (sodium tripolyphosphate) treatment means averaged over all chopping temperatures. Mean values for fat, gel-liquid, and total, N = 20 per treatment mean. ■ Low-collagen with STPP; ▨ High-collagen with STPP; ▨ Low-collagen; ▨ High-collagen.

Fig. 2—Effect of chopping temperature on emulsion stability (mL per 34g emulsion) averaged over all treatments; Fat □−□, gel-liquid △−△, and total ■−■ cook-out at each chopping temperature. [f]Mean values on a curve followed by different letters (c,d and e) are significantly different (p<0.05); [g]Mean values for fat, gel-liquid, and total cookout, N = 16 per treatment mean.
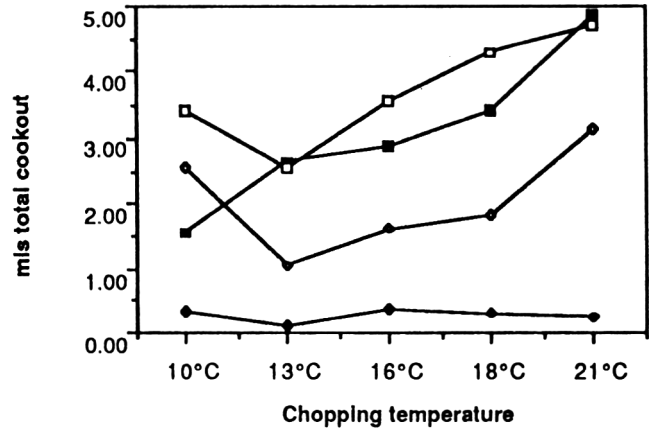
Fig. 3—Effects of collagen and STPP (sodium tripolyphosphate) on meat emulsion stability (mL per 34g meat emulsion); treatment means, (Mean values). N = 4 per treatment mean. ◆−◆ Low-collagen with STPP; ◇−◇ High-collagen with STPP; ■−■ Low-collagen; □−□ High-collagen.

Table 3—Treatment means[a] of the cumulative chopping time (min) needed to reach sample temperature[b]

Treatments		Sample temperature				
Collagen	STPP	10°C	13°C	16°C	18°C	21°C
Low	0.00%	6.54	8.60	10.44	12.38	17.00
Low	0.25%	7.42	9.35	11.85	14.16	19.10
High	0.00%	6.52	7.95	9.34	10.73	13.39
High	0.25%	6.56	7.85	9.46	10.88	13.58

[a] Mean values for each sample temperature group, N = 4 per treatment mean.
[b] Sample of emulsion is taken at each temperature in a continuous system within each treatment group.

temperature on collagen fibers may not be as important as the role of cooking temperature.

The LCP treatment had the least cookout of any treatment, which was expected, and did not show signs of emulsion breakdown (Fig. 3). Again, when HCP is compared with both LC and HC, the STPP stabilized the emulsion and offered more handling flexibility during chopping. The use of selected phosphates lowers viscosity (Knipe et al. 1985b) and results in longer chopping times needed to reach desired chopping temperatures.

It could be postulated that collagen decreases, and STPP increases, the amount of chopping time needed for a given temperature rise in raw emulsions (Table 3). The addition of 0.25% STPP increased chopping time more for low-collagen treatments than for high-collagen treatments. This is most likely due to the higher proportion of collagen in the high-collagen treatments. As the collagen level increases, one would expect an increase in frictional heat buildup due to the fibrous nature of collagen. These two factors should be taken into consider-

(Helmer and Saffle, 1963; Schut, 1976) or to overextracting myofibrillar proteins forming a brittle protein shell around fat globules (Jones and Mandigo, 1982). The effect of chopping

ation when time is the final criterion for meat emulsion production.

From these results, we conclude that alkaline phosphates are not detrimental to high collagen meat emulsions as previously suggested in the literature. The high-collagen-phosphate treatment resulted in greater emulsion stability than the low-collagen treatment. The destabilizing effect of high-collagen meat ingredients on meat emulsions may be partly due to the fact that collagen increases emulsion temperatures during chopping which would reduce total chopping time. Sodium tripolyphosphate would help compensate for final emulsion temperature increases when using emulsion mills on high collagen meat ingredients by allowing for greater chopping time to reach specific final temperatures.

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Oxidative Effects of Meat Grinder Wear on Lipids and Myoglobin in Commercial Fresh Pork Sausage

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ABSTRACT

Pork ground before and after the replacement of worn meat grinder plates and knives was formulated as commercial fresh pork sausage containing antioxidants and processed into 454-g chubs. The sausage was frozen at −15°C for 4, 8, 12, and 16 wk and monitored for oxidation after 1 day, 2 wk and 3 wk of postfrozen refrigeration at 1°C. Estimated average grinder metal in sausage was 136 ppb. Meat temperature rise during grinding was higher (P<0.05) with the worn hardware (2.3°C vs 1.6°C). Hunter "a" values were greater (P<0.05) with the sharp than with the worn equipment. 2-Thiobarbituric acid (TBA) numbers did not differ with equipment state of wear. Significant correlations (P<0.05) indicated in inverse relationship between the oxidation of lipids and myoglobin.

INTRODUCTION

OXIDATIVE RANCIDITY is one of the most significant factors affecting fresh sausage shelf life. Myoglobin oxidation causes the detrimental pigment changes, which may also influence consumer acceptance. Grinding significantly increases the surface exposure of oxidizable meat components, making them more susceptible to oxidation by catalysts and incorporated oxygen during subsequent storage.

Reagan et al. (1983) examined the effects of grinding methods (plate hole size) on lipid and myoglobin oxidation of fresh pork sausage, theorizing that greater myoglobin surface area resulted in faster oxidation. As wear of meat grinder hardware progresses, the equipment becomes less capable of shearing the product, thereby promoting smearing and increasing meat surface area. In addition, increased temperature adversely affects oxidative stability (Lillard, 1985). Increased friction from wear may increase processing temperatures of the lipids and myoglobin, promoting greater initiation of oxidation.

Metals are recognized as potent catalysts of lipid oxidation (Pearson et al., 1983; Marcuse and Fredriksson, 1971; Govindarajan, 1973; MacDonald et al., 1980; Tichivangana and Morrissey, 1985) as well as of myoglobin oxidation (Snyder and Skrdlant, 1966; Fox, 1968; Govindarajan, 1973). The accidental addition of metals from processing equipment has been reported (Moskovits and Kielsmeier, 1960; Lundberg, 1962; Sato and Hegarty, 1971). Iron is the major constituent of steel used to fabricate meat grinder hardware (Hess, 1986). Minute amounts of iron, ranging from 1 to 300 parts per million (ppm), have been demonstrated to catalyze fatty acid oxidation in model systems (Moskovits and Kielsmeier, 1960; Lundberg, 1962; Sato and Hegarty, 1971; Govindarajan, 1973; MacDonald et al., 1980).

Though antioxidant addition is common in commercial production, ground pork or fresh pork sausage used in studies examining lipid oxidation typically has not been formulated with antioxidants (Owen and Lawrie, 1975; Judge and Aberle, 1980; Lopez-Lorenzo et al., 1980; Chiang et al., 1981; Drerup et al., 1981; Reagan et al., 1983; Rhee et al., 1983; Tay et al., 1983; Yasosky et al., 1984). In this study, antioxidants

were included in the pork sausage to allow analysis of a typical commercial product.

The objectives of this inquiry were to: (1) assess the effects of meat grinder wear on lipid and myoglobin oxidation in commercial fresh pork sausage chubs (formulated with antioxidants) after frozen storage and subsequent refrigerated holding, (2) quantitate the temperature rise in ground meat due to worn and sharp grinder hardware, as well as the actual meat grinder plate and knife wear, to assist in explaining potential treatment differences and (3) calculate a correlation between the oxidation of lipids and myoglobin in a comminuted pork product.

MATERIALS & METHODS

Raw materials and formulation

All commercial fresh pork sausage was fabricated on the production line of a major meat processor. Lean and fat pork trimmings were stored at 0°C for 1–2 days prior to processing. Sausage batches required 680.4 kg trimmings; target fat content was 40%. Additional ingredients (based on "meat block" percentages) were water (3%), salt (1.7%), dextrose (0.75%) and seasoning mixture (0.10%).

Seasoning was a Liquispice® Flavoring System (aquaresin) Pork Sausage Seasoning (O.M. Ingredients Co., Madison, WI), privately formulated for the processor. Listed ingredients were: water, natural flavoring, citric acid, butylated hydroxyanisole (BHT), butylated hydroxytoluene (BHT), propyl gallate (PG), mono/diglycerides and xanthan gum. Antioxidant content was designed to be slightly less than 0.02% of the fat content in the final product.

Production and processing investigations

Three replications of the experiment were conducted on the days scheduled for the weekly replacement of the grinder plate and knife inserts after a target volume of 45,360 kg meat had been processed through the grinder. The "worn" treatment was the last sausage batch of the weekly wear period; the "sharp" treatment was the first sausage batch of the new wear period ground through the renewed plate and new knives. Lean and fat trimmings were ground separately through a Weiler & Company (model 11099) grinder (Weiler & Co., Whitewater, WI) equipped with a 4.76-mm Speco Triumph® (D2 hardened steel) plate (Speco Inc., Schiller Park, IL) and four Keene No. 12 (Keene Corp., St. Louis, MO) replaceable knife inserts installed on a four-fingered knife holder.

To determine temperature rise during grinding, pregrind temperatures (immediately before trimmings entered the grinder screw auger) as well as postgrind temperatures (immediately after coming out of the plate) were measured for random, representative samples of lean and fat trimmings with a Keithley thermocouple digital thermometer, model RS-18102 (Markson Science, Phoenix, AZ).

After the last batch was ground through the worn hardware, the worn plate was replaced with a renewed plate. Knife inserts were also replaced with new inserts. Before installation, weights were taken of the new inserts (nearest 0.01 g) and of the renewed replacement plate (nearest 1g). After removal, the worn plate and knife inserts were washed, dried and weighed as they had been before installation 1 week before; the weight difference due to wear was then calculated. The worn plates were renewed on a PIECO model 100 plate and surface grinder (Pieco Inc., Manchester, IA); worn knife inserts were discarded.

Ground trimmings were transferred to a Rietz ribbon-type mixer, model RS-23-K5405 (Rietz Mfg. Co., Santa Rosa, CA). Immediately after start of mixing, the seasoning mixture and other ingredients were added. Total mixing time was 2 min, during which time carbon diox-

ide "snow" was intermittently injected into the mixer for a total period of 65 sec.

After completion of mixing for each respective treatment batch, approximately 23 kg sausage mixture was taken. The sausage mixtures were stuffed under vacuum (−1.00 atm) through a Handtman stuffer, model 325 (T. W. Kutter, Avon, MA), into 7.6-cm-diameter (flat width) oxygen permeable Teepak Unilox® polyroll stock film (2.5-mil thickness; oxygen transmission rate = 260 cm³/1000 cm²/24 hr) Teepak, Oak Brook, IL)forming 454-g chubs 5.8 cm in diameter and 19.1 cm long. These were packaged, 12 chubs per box, into cardboard boxes and placed in the processor's warehouse maintained at −15°C. After 2 weeks, the sausage was transferred in styrofoam coolers to an Iowa State University Meats Laboratory freezer maintained at −15°C.

Instrumental and chemical analyses

Lipid and moisture content, pH, lipid oxidation and color analyses were performed the day after processing of each replication. Thereafter, chubs were removed from frozen storage at 4-wk intervals (4, 8, 12, and 16 wk) and placed into refrigerated storage (1°C). Enough chubs were removed to allow analysis of lipid oxidation and metmyoglobin formation on two chubs per treatment immediately (day 1), and two per week for three consecutive weeks (week 1, 2, and 3) of postfrozen refrigerated storage.

The fresh pork sausage chubs were tempered at −15°C for approximately 3 hr before analysis, firming the chubs to promote clean slicing. Three cross-sectional slices 1.6 cm in thickness, one from each one-third section (i.e., middle section and two end sections), were obtained per chub for color analyses. Exposure to air was allowed for 30 min.

Color analysis of the slices was performed with a Hunterlab LabScan Spectrocolorimeter (Hunter Associates Laboratory, Inc., Reston VA) utilizing the "L" (lightness), "a" (redness), "b" (yellowness) color system. Illuminant A (representing incandescent lamplight) and 10° observer were selected for all readings. A 5.5-cm sample port insert was used. The instrument was standardized with its calibrated white tile (X = 81.60, Y = 86.68, Z = 91.18 (equivalent to "L" = 93.10, "a" = −1.15, "b" = 1.26)). Values from the six slices per treatment were averaged to give one "L", "a" and "b" treatment value after each storage interval. The "L", "a" and "a"/"b" values were used for statistical comparison.

Metmyoglobin formation, progressing toward the chub center with storage time, was measured with a caliper around the circumference of the slices. The distance from the outside of the chub to the inward boundary of metmyoglobin "ring" was measured. Six measurements were taken; a representative mode value for each treatment was rounded to the nearest 0.05 cm and recorded. "Ring' measurements were correlated with Hunterlab color values at the end of the study for evaluation as a noninstrumental tool to monitor color changes in the chubs.

Remaining portions of the chubs were analyzed for lipid oxidation by the 2-thiobarbituric acid (TBA) method as described by Tarladgis et al. (1960). Lipid and moisture contents were determined by using AOAC procedures (AOAC, 1970). Readings for pH were taken with a Fisher Accumet® ion analyzer, model 750 (Fisher Scientific Co., Pittsburg, PA), after 1 min of constant swirling of 10 g product in 90 mL distilled water.

Sensory evaluation

Two sensory evaluation panels were used to compare detectable oxidative rancidity from main treatment and/or storage effects. The first occurred after 2 wk of refrigerated storage preceded by 12 wk of frozen storage; the second panel was after 2 wk of postfrozen refrigerated storage preceded by 16 weeks of frozen storage. Sensory evaluation panels were conducted as triangle taste tests at the Iowa State University Meats Laboratory. Participants were untrained volunteer students and staff; a minimum of 38 panelists, a majority of whom were males, age 21–30, were involved in each of the panels.

Fresh pork sausage chubs selected for the sensory evaluation panels were tempered (as for the color evaluations) and then sliced into 1.6-cm-thick slices on a Hobart model 1712 slicer (Hobart Corp., Troy, OH). Slices were fried for 4 1/2 min on each side on a gas griddle set at 135°C. Cooked slices were cut into eighths and transferred into chafing dishes. The panelists were presented the cooked samples via the chafing dishes placed on tables under incandescent light. The chafing dishes were divided into three compartments, each compartment labeled with a random 3-digit number. Each chafing dish con-

tained a triangle test; panelists were offered no more than two triangle tests per panel. Warm-up samples were offered before actual testing. During testing, participants were allowed to freely select as many test samples as required. Panelists were asked to make comparisons based solely on flavor. Water was provided for rinsing between samples.

Statistical analysis

Experimental design was a split-split-plot arrangement. Each plot or replication was divided into the specific treatments for each experiment (e.g., worn and sharp). Each of these treatments was then divided into the various frozen storage periods, considered to be the subtreatments. Each frozen storage period was further divided into the postfrozen refrigeration periods. labeled as the subdivided subtreatments.

Because the primary objective was to compare treatment storage stability, statistical analysis of treatment effects preceding storage (initial values) was separate from that of sausage subjected to storage; initial values served as a benchmark to compare sausage after frozen plus refrigerated storage. Average initial treatment values were derived from the initial treatment means of each replication.

Analysis of variance and correlations of the Hunterlab evaluations, TBA test and metmyoglobin ring measurements were performed with the Statistical Analysis System (SAS Institute Inc., 1982). Significance between means was tested with SAS by using both the least significant difference (LSD) and Duncan multiple range tests. Sensory evaluation panel results were compared with significance tables reported by Larmond (1977) and Roessler et al. (1978) to detect significance.

RESULTS & DISCUSSION

Main treatment processing analysis

Worn equipment caused a significantly higher temperature rise ($P < 0.05$) than renewed plates and sharp knives (+2.3°C versus +1.6°C). Average pregrind temperature for the trimmings was 6.3°C. According to Hess (1986), as grinder plate and knives become worn, the rate of wear with concomitant dullness increases, requiring more work to force meat through the meat grinder system. The direct effect of the temperature increases on oxidative stability is questionable; the significantly higher temperature does indicate greater friction, possibly resulting in increased smearing of meat ground with the worn hardware. Smearing would theoretically increase surface area, thereby increasing susceptibility to oxidation.

Metal loss from wear was determined only at the end of each wear period; an overall average rate of wear over the total production period was calculated. Weight loss per period from plate wear averaged 4g (plates weighed 6000g to 7000g) and therefore was minimal (<0.1%), whereas the percentage weight loss from knife wear approximated 2–3% (2.66g from four knives collectively weighting approximately 100g). The relatively inexpensive, replaceable knives are manufactured to wear faster than the more expensive plate (Hess, 1986). An average of 51,257 kg of meat was processed through the grinder during each wear period. Grinder metal content in the ground sausage mixture was estimated to average 136 ppb (0.007 kg metal/ 51,257 kg meat); actual deposit rates through the wear period would be expected to gradually increase as wear progressed (Hess, 1986).

Assessment of metal (iron) addition on lipid oxidation in model systems in the literature typically has involved concentrations greater than parts per billion. Researchers have reported prooxidant activity from the addition of 5 ppm of various metals to a sausage mixture (Moskovits and Kielsmeier, 1960), 1 ppm iron to fats (Lundberg, 1962), 300 ppm iron powder to water-extracted ground beef (Sato and Hegarty, 1971), 1 ppm iron added to freshly ground beef (Govindarajan, 1973) and 2 ppm iron added to pure linoleic acid (MacDonald et al., 1980). In comparison, Tay et al. (1983) reported total endogenous iron content of ground pork muscle as 32.7-33.0 ppm; free iron (nonheme) accounted for 4.4-5.3 ppm of this total. Relatively speaking 136 ppb added metal seems quite minimal

compared with concentrations used in studies assessing oxidative potential of added metals or of metals naturally occurring in meat.

Product compositional analysis

Average lipid content, moisture content and pH value of the commercial fresh pork sausage utilized in this study were 34.9% 53.4%, and pH 6.0, respectively.

Main treatment analysis

Initial analyses. Initial TBA analyses yielded some limited oxidation after only 1 day of refrigerated storage after processing (Table 1). Although meat trimmings used by the processor were held 1–2 days before processing at refrigerated temperature (0°C), actual pregrind temperatures for the trimmings averaged 6.2°C. This meat temperature may have prompted initiation of lipid oxidation during this holding period.

Increasing Hunter "a" and "a"/"b" values indicate increasing redness (oxymyoglobin) in meat appearance; decreasing values signify increased metmyoglobin presence (Snyder, 1964; Strange et al., 1974; Eagerman et al., 1977; Chastain et al., 1982; Reagan et al., 1983). Initial Hunter "a" and "a"/"b" values for the worn treatment were greater (P<0.05) than for the sharp treatment (Table 1), indicating a greater proportion of oxymyoglobin in the lean of the worn treatment. The sharp treatment was lighter (P<0.05), exhibiting greater "L" values. Reagan el al. (1983) concluded that greater surface area of meat particles increased myoglobin exposure area. Smear (from less clean shearing) resulting from worn hardware would be expected to produce a similar coloration, allowing more thorough oxygenation of myoglobin in the worn treatment and offering a possible explanation for the redder appearance. No measurable metmyoglobin "ring" was observed on the initial samples.

Analyses after storage. Commercial fresh pork sausage from the meat processor undergoes frozen and subsequent refrigerated storage between time of processing and ultimate consumer selection. To duplicate these conditions, treatments were compared for oxidative changes after frozen plus refrigerated storage.

Lipid oxidation after storage was not affected by treatment; TBA numbers from both the worn and sharp treatment were similar (Table 2). Hunter "a" values were greater (P<0.05) for the sharp treatment than for the worn treatment, indicating more redness. This paralleled the treatment effects on Hunter "a"/"b", which approached significance (P=0.08), as did

those effects on metmyoglobin "ring" formation (P=0.06); the worn treatment yielded a lower mean "a"/"b" ratio and a higher mean metmyoglobin "ring" measurement.

Thus, the worn treatment may have detrimentally affected color of commercial fresh pork sausage during storage. A greater myoglobin surface area possibly resulted from the worn equipment, making it initially more susceptible to oxygenation (Table 1) and also more susceptible to oxidation later after storage (Table 2). Increased incorporation of oxygen may have caused earlier expiration of endogenous reducing capacity, and the greater surface area could have enhanced the vulnerability of myoglobin to oxidation catalysts. Reagan et al. (1983) observed that grinding with plates having smaller hole size significantly decreased Hunter "a" after refrigerated storage and suggested that the increased oxidation was due to increased myoglobin surface area of smaller particles.

Preceding sensory evaluation, the sausage was refrigerated 2 wk after frozen storage to duplicate handling at the retail and consumer levels. Only the sensory panels from replication three significantly detected the odd samples (P<0.05 for 12 + 2 wk and P<0.01 for 16 + 2 wk). Therefore, treatment did not have a significant role in detectable off-flavor development because of the infrequency with which significant detection between treatments occurred. Possible explanations are inadequate duration of storage periods before sensory evaluation or that antioxidant activity may have prevented the panelists from detecting treatment oxidative differences. Researchers have reported significant increases in lipid oxidation in the absence of antioxidants for frozen processed pork products after 30 days of frozen storage at −15°C (Huffman et al., 1981) and during a 90-day frozen storage at −23°C (Keeton, 1983). However, Chastain et al. (1982) reported that the use of antioxidants in restructured beef/pork steaks controlled lipid oxidation at significantly lower levels than that of the controls (no antioxidants) throughout a frozen storage period of 20 wk at −10°C.

Subtreatment analysis

Some variation of the analyzed oxidative factors was attributed to statistically significant subtreatment (frozen storage) and subdivided subtreatment effects (postfrozen refrigerated storage). Because no significant interactions existed between treatment and length of frozen storage, between treatment and length of postfrozen refrigerated storage or between frozen storage and postfrozen refrigerated storage, frozen storage and postfrozen refrigerated storage were viewed as characteristic of commercial fresh pork sausage with antioxidants.

Average initial values for TBA number, Hunter "a", Hunter "a"/"b", metmyoglobin "ring" and Hunter "L" prior to frozen storage are included for reference in Table 3. Since a goal was to test differences after frozen storage, these initial values were not included in the statistical analysis of values taken after storage.

Table 1 – Average initial treatment values before storage[a,b]

Treatment	TBA number[c]	Hunter "a"	Hunter "a"/"b"	Metmyoglobin "ring"[d]	Hunter "L"
Worn	0.115e	18.66e	2.41e	0.00e	60.61f
Sharp	0.118e	16.59f	2.15f	0.00e	64.27e
S.E.	0.021	0.64	0.09		0.99

[a] N = 6 per treatment mean
[b] Means within each column with different letters are significantly different (P<0.05).
[c] Expressed as mg malonaldehyde/kg food sample.
[d] Expressed in cm.

Table 2 – Meat grinder wear effects on oxidative stability of commercial fresh pork sausage after frozen plus postfrozen refrigerated storage[a,b]

Treatment	TBA number[c]	Hunter "a"	Hunter "a"/"b"	Metmyoglobin "ring"[d]	Hunter "L"
Worn	0.306e	14.51f	1.97e	1.35e	62.67e
Sharp	0.323e	15.31e	2.06e	1.30e	61.49e
S.E.	0.021	0.08	0.02	0.01	0.41

[a] N = 48 per treatment mean
[b] Means within each column with different letters are significantly different (P<0.05)
[c] Expressed as mg malonaldehyde/kg food sample.
[d] Expressed in cm.

Table 3 – Effects of frozen storage on commercial fresh pork sausage[a,b]

Frozen storage	TBA number[c]	Hunter "a"	Hunter "a"/"b"	Metmyoglobin "ring"[d]	Hunter "L"
Initial[e]	0.116	17.62	2.28	0.00	62.44
4 wk	0.237f	15.07fg	2.07f	1.29f	62.62f
8 wk	0.251f	14.77fg	2.00fg	1.30f	62.54f
12 wk	0.273f	14.33g	1.93g	1.32f	62.20f
16 wk	0.498f	15.47f	2.05f	1.38f	60.98f
S.E.	0.071	0.26	0.03	0.04	0.88

[a] N = 24 per subtreatment mean.
[b] Means within each column with different letters are significantly different (P<0.05).
[c] Expressed as mg malonaldehyde/kg food sample.
[d] Expressed in cm.
[e] N = 12. Initial values not statistically analyzed with frozen storage values.

Lipid oxidation, as measured by TBA numbers, was not affected (P = 0.07) by length of frozen storage (Table 3).

Length of frozen storage impacted upon meat color; Hunter "a" values and Hunter "a"/"b" values both were related (P < 0.05) to duration of frozen storage (Table 3). Though generally decreasing with storage, an increase in these values occurred from 12 to 16 wk of frozen storage; the reason behind this apparent color improvement is unclear. The largest decreases in Hunter "a" values and Hunter "a"/"b" were after 4 wk of frozen storage, decreasing about 2.55 and 0.21, respectively, from the initial values. Metmyoglobin "ring" formation increased by 1.29 cm during the same initial frozen storage period, reflecting the greatest change between periods throughout the study.

Subdivided subtreatment analysis

After each frozen-storage period, the sausage was further subjected to periods of postfrozen refrigerated (1°C) storage, ranging from 1 day to 3 wk. Hunter "a"/"b" values were affected by postfrozen refrigerated storage (P < 0.01) though Hunter "a" values were not (Table 4). With the exception of 2 wk postfrozen refrigeration, Hunter "a"/"b" values gradually increased with storage. Variation in metmyoglobin "ring" attributed to length of postfrozen refrigeration was very highly significant (P < 0.001), initially increasing and then decreasing after prolonged storage. These results resembled a similar study of commercial fresh pork sausage (Wanous et al., 1989) in which Hunter "a" and Hunter "a"/"b" values increased and, metmyoglobin "ring" formation decreased over 3 wk of postfrozen refrigeration.

Noteworthy was the improvement in color after 3 weeks of postfrozen refrigerated storage. Hunter "a"/"b" ratios were higher than after 2 wk of storage (P < 0.05) and were similar to the highest postfrozen refrigerated values. Correspondingly, metmyoglobin "ring" measurements were lower (P < 0.05) at 3 wk of postfrozen refrigerated storage than for any of the shorter refrigerated periods. It was also after 3 wk of postfrozen refrigerated storage that a slight off-odor became evident.

From results reported in the literature (Butler et al., 1953; Robach and Costilow, 1961; Morley, 1971; Satterlee and Hansmeyer, 1974), it is possible that this metmyoglobin reduction was due to aerobic bacterial proliferation. Demand for available oxygen may have been increased, thereby making it unavailable for reaction with myoglobin, or the production of metabolic reductants may have converted metmyoglobin to the reduced myoglobin forms (Fox, 1968; Price, 1971). However, no microbiological data were obtained in this study to form a basis for such conclusions.

Correlations of lipid and myoglobin oxidation

TBA number and Hunter "a" values, as well as TBA numbers and Hunter "a"/"b" values, were positively (r = +0.26 and +0.24, respectively) and significantly (P < 0.05. N = 96) correlated, indicating an inverse relationship between lipid and

myoglobin oxidation. Other workers have concluded that lipid oxidation was not directly related to metmyoglobin formation in ground pork (Yasosky et al., 1984), pork muscle (Ordonez and Ledward, 1977) or beef muscle (Ledward and MacFarlane, 1971). These findings contrast with those of Rhee et al. (1983), who calculated significant and negative correlations (r = −0.91; P < 0.005) between Hunter "a" values and TBA numbers in frozen ground pork.

Correlations between metmyoglobin "ring" and Hunter "a" values, and metmyoglobin "ring" and Hunter "a"/"b" values, were negative (r = −0.37 and −0.41, respectively) and very highly significant (P < 0.001, N = 96). This supports other research (Snyder, 1964; Reagan et al., 1983) regarding the use of Hunter "a"/"b" ratios for metmyoglobin measurement. Additionally, measurement of circumferential metmyoglobin migration in cross-sectional slices of 454-g sausage chubs allows an estimation of myoglobin oxidative changes when instrumental color analysis is not possible.

CONCLUSIONS

WORN MEAT GRINDER HARDWARE caused greater myoglobin oxidation in sausage after frozen storage than sharp hardware. Worn hardware also caused greater grinding temperature increases than the sharp equipment, indicating more friction from increased flow restriction. A relationship may exist between restricted grinder flow and myoglobin oxidation based upon increased surface area from smear and/or relatively longer contact with hardware during grinding.

During frozen storage, the largest single increase in metmyoglobin formation in commercial fresh pork sausage chubs occurred after the first 4 wk. After 3 wk of postfrozen refrigeration, internal color of commercial fresh pork sausage chubs significantly improved, regardless of length of frozen storage.

Lipid oxidation and metmyoglobin formation were inversely related, supporting the concept that in spite of correlations between the two oxidations, a consistent interdependence may not exist.

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Table 4—Effects of postfrozen refrigeration on commercial fresh pork sausage[a,b]

Postfrozen refrigeration	TBA number[c]	Hunter "a"	Hunter "a"/"b"	Metmyoglobin "ring"[d]	Hunter "L"
1 day	0.272e	14.72e	1.99fg	1.22f	62.52e
1 wk	0.288e	15.23e	2.05ef	1.56e	62.15e
2 wk	0.315e	14.52e	1.94g	1.53e	61.99e
3 wk	0.383e	15.16e	2.08e	1.00g	61.67e
S.E.	0.042	0.23	0.03	0.06	0.49

[a] N = 24 per subdivided subtreatment mean
[b] Means within each column with different letters are significantly different (P < 0.05)
[c] Expressed as mg malonaldehyde/kg food sample.
[d] Expressed in cm.

Pallet Location and Freezing Rate Effects on the Oxidation of Lipids and Myoglobin in Commercial Fresh Pork Sausage

MICHAEL P. WANOUS, DENNIS G. OLSON, and ALLEN A. KRAFT

ABSTRACT

Effects of freezing rates on oxidation of commercial fresh pork sausage chubs were evaluated in association with location extremes on a production pallet. Three treatments requiring 9 hr, 2.4 days and 6.8 days for temperature declines from 7°C to −15°C were tested. Sausage chubs were analyzed after 4, 8, 12, 16, and 20 wk of frozen (−15°C) plus 3 wk of postfrozen refrigerated (1°C) storage. 2-Thiobarbituric acid (TBA) numbers did not increase with slower freezing (P>0.05). Hunter "a" values were greater (P<0.05) for the two slower rates. Overall, pallet location of boxed chubs minimally affected oxidative stability. Significant correlations (P<0.05) suggested a positive relationship between lipid and myoglobin oxidation.

INTRODUCTION

ACCORDING to the American Meat Institute (1987), 880,795,000 pounds of fresh pork sausage were processed under federal inspection in the United States during 1986. The major portion of production is in chubs, usually in units of 340g, 454g, 2.27 kg and 4.54 kg (Rust, 1975, 1985).

The rate of temperature decline of fresh pork sausage in commercial meat processing establishments that box, palletize and place the product into frozen storage immediately after stuffing is directly dependent upon heat exchange allowed by the packaging, combined with pallet location. Preliminary investigations in a commercial facility revealed that the time required for boxed chubs to achieve freezer temperature required from 2 days to 1 wk, depending on pallet location.

Detrimental effects of temperature have been reported for meat lipid oxidation (Keskinel et al., 1964; Ledward and MacFarlane, 1971; Halliday, 1972; Drerup et al., 1981) as well as for myoglobin oxidation (Brown and Mebine, 1969; Hood, 1980). Extended refrigerated storage preceding freezing has been shown to accelerate lipid and myoglobin oxidation after subsequent frozen storage (Ledward and MacFarlane, 1971). The implications of freezing rate variability on differences in product oxidative stability and, therefore, shelf life need to be assessed.

The objectives of this study were to: (1) evaluate the effects of postprocessing freezing rate on lipid and myoglobin oxidation as related to pallet location in boxed commercial fresh pork sausage chubs, (2) analyze sausage formulated with antioxidants after periods of frozen plus refrigerated storage, and (3) calculate a correlation between the oxidations of lipids and myoglobin in a comminuted pork product.

MATERIALS & METHODS

Formulation and production

The commercial fresh pork sausage mixture was ground and mixed on the production line of a meat processor. Trimmings were ground through a Weiler & Company (model 11099) grinder (Weiler & Co.,

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Whitewater, WI) equipped with a 4.76-mm plate. Target product fat content was 40%. Mixing was performed for 2 min in a Rietz ribbon-type mixer, model RS-23-K5405 (Rietz Mfg. Co., Santa Rosa, CA) during which carbon dioxide "snow" was injected intermittently for a total of 65 sec. Seasoning mixture and other ingredients were immediately added after start of mixing. The ingredient formulation, to include the incorporation of antioxidants, was identical to that of a previous study (Wanous et al., 1989).

After mixing, approximately 73 kg sausage mixture from a 680.4-kg batch were placed into plastic-lined styrofoam ice chests and transported to the Iowa State University (ISU) Meats Laboratory. Stuffing was performed under vacuum (−0.95 atm) with a Vemag Robot 500, type 128, continuous vacuum stuffer (Robert Reiser Co., Canton, MA) into oxygen-permeable film, forming 454-g chubs. Chubs were randomly assigned to one of three freezing rate treatments ("single", "boxed", and "skid"; treatments described later) in which the heat exchange rates were directly controlled via packaging insulation. All treatments were placed into an ISU Meats Lab blast freezer (−15°C) where they remained throughout the frozen storage periods.

Copper/constantan thermocouple probes were inserted into three randomly selected chubs from each treatment to monitor temperature decline to −15°C. A Digistrip II, model DR-3C, digital computerized temperature recorder (Kaye Instruments, Bedford, MA) recorded readings every 30 min. Treatment temperature at any given time was calculated as an average of the temperatures of the three representative chubs of that treatment; overall treatment freezing rates were averaged from the three replications.

Treatment descriptions

The "single" treatment was introduced as an extreme in which initial heat exchange would not be limited by packing into boxes. Chubs assigned to the "single" treatment were placed individually in wire-mesh meat freezer baskets suspended on a freezing cart to allow full air circulation. After all treatment temperatures had decreased to −15°C, chubs assigned to the "single" treatment were placed into cardboard boxes (2 rows deep × 6 chubs/row = 12/box) typically used by the processor, measuring approximately 20.3 cm wide by 33.0 cm long by 12.7 cm deep.

The "boxed" treatment represented maximum heat exchange with boxed sausage. Chubs were placed into cardboard boxes (2 rows deep × 6 chubs/row = 12/box), described for the "single" treatment, immediately after stuffing. These boxes were then placed into the wire-mesh freezing baskets (1-2 boxes/basket) and positioned onto the freezing cart supports, allowing air circulation around each box.

The "skid" treatment simulated heat exchange occurring in boxes situated in the middle of a processor's production skid or pallet. Chubs were immediately placed inside cardboard boxes as with the "boxed" treatment. Two boxes were stacked, and 3.8-cm-thick styrofoam was placed around all four sides of this two-box set. Boxed, refrigerated ground meat (about 23 kg/box) was placed on the top and bottom of the two-box set; plastic freezer spacing of 3.8-cm gap separated the boxed ground meat and sausage boxes. This complex was then placed on a pallet inside the freezer.

Product analyses

Lipid and moisture content, pH, lipid oxidation and color analyses were performed the day after processing of each replication. Thereafter, chubs were removed from frozen storage at 4-wk intervals (4, 8, 12, 16, and 20 wk) and placed into refrigerated storage (1°C); lipid oxidation and metmyoglobin formation were monitored on two chubs per treatment immediately (day 1), and two per week thereafter for 3

consecutive weeks (wk 1, 2, and 3) of postfrozen refrigerated storage. Lipid oxidation was estimated by the 2-thiobarbituric acid (TBA) distillation method (Tarladgis et al., 1960). Cross-sectional metmyoglobin formation was compared with Hunter "L", "a" and "a"/"b" values. Use of a caliper to estimate metmyoglobin formation, measuring circumferential metmyoglobin "ring" formation progressing inward with storage time on cross-sectional slices, was also evaluated. Specific procedural details of analyses performed are outlined in prior work (Wanous et al., 1989).

Sensory evaluation panels (triangle taste tests) compared detectable oxidative rancidity from main treatment effects after 20 wk of frozen plus 2 wk of postfrozen refrigerated storage. Panels were at the ISU Meats Lab; participants were untrained volunteer students and staff. A minimum of 35 panelists, a majority of whom were males, age 21–30, were involved in each of the panels.

Fresh pork sausage chubs selected for the sensory panels were tempered (as for the color evaluations) and then sliced into 1.6-cm-thick slices on a Hobart model 1712 slicer (Hobart Corp., Troy, OH). Slices were fried for 4 1/2 min on each side on a gas griddle set at 135°C. Cooked slices were cut into eighths and transferred into chafing dishes divided into three compartments labeled with random 3-digit numbers; each dish contained one triangle test. Panelists selected samples directly from the chafing dishes, under incandescent light, and were allowed to select as many samples as required. Water was provided for rinsing between samples. Warm-up samples were offered before testing. Panelists were asked to make comparisons based solely on flavor. Three triangle tests per panel were required so that all three treatments could be compared with one other.

Statistical analysis

The experimental design was a split-split-plot arrangement with a total of three replications. Each plot or replication was split into the specific treatments (e.g., "single", "boxed" and "skid"). Treatments were partitioned into the various frozen storage periods considered to be the subtreatments. Frozen storage periods were subdivided into the postfrozen refrigeration periods, labeled as the subdivided subtreatments.

Analysis of variance and correlations of the Hunterlab evaluations, TBA tests and metmyoglobin ring measurements were performed with the Statistical Analysis System (SAS Institute Inc., 1982). Significance between means was tested with SAS by using both the least significant difference (LSD) and Duncan multiple range tests. Sensory evaluation panel results were compared with significance tables reported by Larmond (1977) and Roessler et al. (1978) to detect significance.

RESULTS & DISCUSSION

Treatment freezing rates

The temperature declines of all three treatments are depicted in Fig. 1.

The use of styrofoam in the "skid" treatment permitted a relatively accurate duplication of commercial conditions, re-
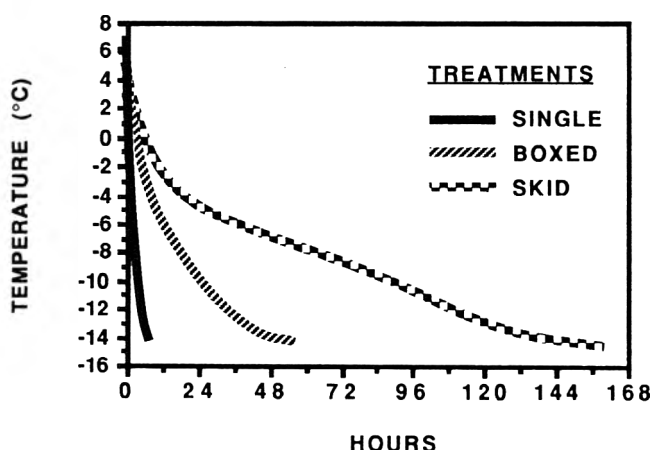
Fig. 1. — Freezing rates of unboxed fresh pork sausage ("single") and boxed sausage duplicating two production pallet location extremes, outside ("boxed") and center ("skid").

quiring approximately 6.8 days to reach −15°C (Fig. 1). Calculations by an ISU faculty food engineer had determined that a layer of 3.8-cm thick plastic foam used in the "skid" treatment would closely approximate the thermal conductivity of boxed sausage surrounding the inner boxes of a palletized layer. Preliminary measurements taken on a center box of a full pallet in the freezer of the processor (who collaborated in this study) indicated that 6 to 7 days were required to achieve the freezer temperature (−15°C) from typical processing temperatures (7°C).

Product compositional analysis

Average values for lipid content, moisture content, protein content and pH of the commercial fresh pork sausage utilized in this study were 32.7%, 54.0%, 11.2%, and pH 5.8, respectively.

Effects of freezing rate (Main treatment analysis)

Many processors' commercial fresh pork sausage typically undergoes frozen and subsequent refrigerated storage between the time of processing and ultimate consumer selection from the retailer's display. To duplicate commercial handling practices, treatments were compared for oxidative changes after frozen plus refrigerated storage.

Lipid oxidation, as measured by TBA numbers, was not affected by rate of freezing (P > 0.05).

Hunter "a" values for the "single" treatment were less (P < 0.05) than those of the two slower freezing treatments (Table 1), indicating less redness (oxymyoglobin) for the fastest freezing rate treatment; no differences were noted between the two slower freezing rates. Hunter "a"/"b" and metmyoglobin "ring" treatment means, however, were not different (P > 0.05). Sensory evaluation panelists were unable to detect any treatment differences. For the most part, it seemed that pallet location of boxed chubs was not critical to oxidative stability.

Fennema (1971) suggested that maximum rates of nonenzymatic oxidative reactions in foods may occur in the subfreezing range of −1°C to −15°C. The "skid" treatment sausage spent approximately 5 days in the −5°C to −15°C range. However, faster rates of lipid oxidation for raw meats (Keskinel et al., 1964) and fresh pork sausage (Halliday, 1972; Drerup et al., 1981) have been reported to occur at refrigeration temperatures (−1°C to 5°C) than at frozen temperatures (−12°C to −20°C). Investigations into temperature dependence of oxymyoglobin oxidation reaction rates have yielded temperature coefficient Q_{10} values of 2-10 for temperatures ranging from −2°C to 22°C (Brown and Mebine, 1969; Hood, 1980). That initial freezing rates were relatively fast, with all three treatments achieving −5°C within 24 hr, may have accounted for the lack of significant treatment oxidative differences.

Treatment freezing rates in this study were controlled by packaging; the "boxed" and "skid" treatments were packaged in boxes sealed with tape and thus were limited in their

Table 1 — Pallet location and freezing rate effects on the oxidative stability of commercial fresh pork sausage after frozen plus postfrozen refrigerated storage[a,b]

Treatment	TBA number[c]	Hunter "a"	Hunter "a"/"b"	Metmyoglobin "ring"[d]
Single	0.499e	14.34f	2.02e	1.21e
Boxed	0.523e	14.77e	2.05e	1.22e
Skid	0.530e	14.80e	2.03e	1.23e
S.E.	0.031	0.06	0.01	0.01

[a] N = 60 per treatment mean.
[b] Means within each column with different letters are significantly different(P < 0.05).
[c] Expressed as mg malonaldehyde/kg food sample.
[d] Expressed in cm.

oxygen exposure. Though the "single" treatment was allowed to freeze faster through better heat exchange, air circulated freely around the chubs during the first week; this may have initiated lipid oxidation and also promoted earlier consumption of endogenous myoglobin-reducing capacity, thereby minimizing any oxidative advantages through faster freezing.

Antioxidant activity and inadequate duration of storage periods may have prevented treatment effects from manifesting themselves. Chastain et al. (1982) reported that the use of antioxidants in restructured beef/pork steaks controlled lipid oxidation at significantly lower levels than those of the controls (no antioxidants) throughout a frozen storage period of 20 wk at −10°C. Wanous et al. (1989) questioned whether antioxidant effectiveness masked treatment oxidative differences in commercial fresh pork sausage after 16 wk frozen (−15°C) plus 3 wk refrigerated (1°C) storage.

Effects of frozen storage (Subtreatment analysis)

Differences existed in oxidative factors between subtreatments (frozen storage) and subdivided subtreatments (postfrozen refrigerated storage). No interactions existed between treatment and length of frozen storage, between treatment and length of postfrozen refrigerated storage or between frozen storage and postfrozen refrigerated storage. Because no significant interactions occurred, frozen storage and postfrozen refrigerated storage were viewed as characteristic of commercial fresh pork sausage with antioxidants.

Initial values for TBA number, Hunter "a", Hunter "a"/ "b" and metmyoglobin "ring" prior to frozen storage are given in Table 2. These initial values were not included in the statistical analysis of values taken after storage.

TBA numbers were related (P < 0.001) to length of frozen storage; from 4 wk though 12 wk of frozen storage they increased rapidly, reaching a maximum at 12 and 16 wk (Table 2). After 20 wk of frozen storage, however, the TBA numbers dropped significantly from the levels seen in each of the previous two frozen storage periods. According to many researchers, TBA numbers do not always increase throughout storage of muscle foods (Melton, 1983). A reduction of TBA reactive substances (TBARS) at freezer temperatures due to protein binding has been suggested to occur with advanced lipid oxidation, decreasing the availability of TBARS and thereby underestimating the extent of oxidation (Kwon et al., 1965; Buttkus, 1967, 1969; Witte et al., 1970; Gray, 1978; Gokalp et al., 1983).

Hunter "a" and Hunter "a"/"b" values decreased (P < 0.001) over the 20-week frozen storage period (Table 2). Metmyoglobin "ring" formation also varied (P < 0.001) with duration of frozen storage and gradually increased with storage.

The largest decreases in Hunter "a" values and Hunter "a"/ "b" were seen after the initial 4 wk of frozen storage. Metmyoglobin "ring" formation increase during the same initial period reflected the greatest change among storage periods.

Similar results were seen in a previous study (Wanous et al., 1989).

Effects of postfrozen refrigeration (Subdivided subtreatment analysis)

After each frozen storage period, the commercial fresh pork sausage was further subjected to periods of postfrozen refrigerated (1°C) storage, ranging from 1 day to 3 wk. Variation in TBA numbers attributed to postfrozen refrigeration storage was significant (P < 0.01) (Table 3). TBA numbers from 2 and 3 wk of storage were greater (P < 0.05) than those from 1 day and from 1 wk.

Both Hunter "a" and Hunter "a"/"b" increased with refrigerated storage (P < 0.001), indicating an enhancement of color with time. Metmyoglobin "ring" formation decreased (P < 0.001) after 2 wk of postfrozen refrigerated storage and abruptly dropped to the lowest level of all periods after 3 wk of refrigeration (P < 0.05). The possibility that aerobic bacterial proliferation and/or bacterial metabolic reductant production may have converted the myoglobin to a reduced form has been advanced as a reason for this color improvement (Wanous et al., 1989).

Correlations of lipid and myoglobin oxidation

TBA number and Hunter "a" values were negatively correlated (r = −0.17; P < 0.05, N = 180); the correlation between TBA numbers and Hunter "a"/"b" values was similar (r = −0.13) and approached significance (P = 0.08, N = 180). Rhee et al. (1983) also reported a significant and negative correlation (r = −0.91; P < 0.005) between Hunter "a" values and TBA numbers for frozen ground pork, again indicating a positive relationship between lipid and myoglobin oxidation. Highly significant correlations between malonaldehyde and metmyoglobin formation have also been reported for refrigerated ground beef (Hutchins et al., 1967; Juhl, 1979).

Other studies have concluded that lipid oxidation was not directly related to metmyoglobin formation in pork muscle (Ordonez and Ledward, 1977), ground pork (Yasosky et al., 1984), commercial fresh pork sausage (Wanous et al., 1989) or beef muscle (Ledward and MacFarlane, 1971). Therefore, the existence of an actual interdependence between lipid and myoglobin oxidation seems questionable.

Correlations also were calculated between the Hunter values and metmyoglobin "ring" measurements to determine the usefulness of the "ring" measurement for estimating myoglobin oxidative status in fresh pork sausage chubs. Correlations between the metmyoglobin "ring" and Hunter "a", as well as between metmyoglobin "ring" and Hunter "a"/"b", were negative (r = −0.53 and −0.64, respectively) and very highly significant (P < 0.001, N = 180). The higher correlations between the measured metmyoglobin "ring" and Hunter "a"/ "b" (versus Hunter "a") in this study and in a previous study (Wanous et al., 1989) support other researchers (Snyder, 1964; Reagan et al., 1983) regarding the use of Hunter "a"/"b" ratios in metmyoglobin estimation. Furthermore, physical

Table 2 − Effects of frozen storage on commercial fresh pork sausage[a,b]

Frozen storage	TBA number[c]	Hunter "a"	Hunter "a"/"b"	Metmyoglobin "ring"[d]
Initial[e]	0.195	18.27	2.35	0.00
4 wk	0.261h	15.19f	2.11f	1.13i
8 wk	0.336h	14.76g	2.06fg	1.17hi
12 wk	0.747f	14.71g	2.02gh	1.21gh
16 wk	0.713f	14.10h	1.99hi	1.28fg
20 wk	0.529g	14.44gh	1.97i	1.30f
S.E.	0.037	0.13	0.02	0.02

[a] N = 36 per subtreatment mean.
[b] Means within each column with different letters are significantly different (P < 0.05).
[c] Expressed as mg malonaldehyde/kg food sample.
[d] Expressed in cm.
[e] N = 6. Initial values not statistically analyzed with frozen storage values.

Table 3 − Effects of postfrozen refrigeration on commercial fresh pork sauage[a,b]

Postfrozen refrigeration	TBA number[c]	Hunter "a"	Hunter "a"/"b"	Metmyoglobin "ring"[d]
1 day	0.456f	13.75g	1.90g	1.42e
1 wk	0.453f	14.70f	2.04f	1.52e
2 wk	0.561e	14.91ef	2.06f	1.23f
3 wk	0.598e	15.20e	2.13e	0.71g
SE	0.034	0.14	0.01	0.04

[a] N = 45 per sub-subtreatment mean.
[b] Means within each column with different letters are significantly different (P < 0.05).
[c] Expressed as mg malonaldehyde/kg food sample.
[d] Expressed in cm.

measurement of circumferential metmyoglobin migration for monitoring myoglobin oxidative changes in cross-sectional slices of commercial fresh pork sausage chubs enables an approximation of metmyoglobin when instrumental color analysis is not possible.

CONCLUSIONS

PALLET LOCATION exerted minimal effects on the oxidative stability of boxed commercial fresh pork sausage formulated with antioxidants. Though the unboxed sausage underwent the fastest freezing rate, its color values (Hunter"a") were lower than the boxed treatments after storage.

During frozen storage, the largest single increase in metmyoglobin formation occurred after the first 4 wk. After 3 wk of postfrozen refrigeration, the internal color of commercial fresh pork sausage chubs had improved to its best level, regardless of length of frozen storage.

Lipid oxidation and metmyoglobin formation were directly related.

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Replacement of Sodium Chloride by Modified Potassium Chloride (Cocrystalized Disodium-5'-Inosinate and Disodium-5'-Guanylate with Potassium Chloride) in Fresh Pork Sausages: Acceptability Testing using Signal Detection Measures

G. PASIN, M. O'MAHONY, G. YORK, B. WEITZEL, L. GABRIEL, and G. ZEIDLER

―――――――――― ABSTRACT ――――――――――

Experiments were developed to evaluate the effect of replacement of NaCl with modified KCl on the hedonic ratings for fresh pork sausage patties. Results clearly indicated that the replacement of up to 75% (w/w) of the salt by modified KCl were significantly well accepted. The incorporation of any level of MSG decreased the overall acceptance level of modified KCl to 50% (w/w). With the incorporation of two additional spice levels, the acceptance level of modified KCl did not improve much beyond 75% (w/w).

INTRODUCTION

HIGH SODIUM INTAKE has been identified as one possible contributor to the development of hypertension, a condition that occurs in 10–20% of the United States population (Pearson and Wolzak, 1982). An estimated 60 million adult Americans have some degree of elevated blood pressure, including 60–70% of those aged 60 or above. It has been established that under normal circumstances we consume more sodium than is necessary for our needs. It has been estimated that the average American consumes about 10–12g salt a day, which is equivalent to 3900–4000 mg sodium (FASEB, 1979).

This intake level is 20–25 times greater than the minimum adult requirement (IFT, 1980). Medical and public opinion is increasingly inclining to the view that a general reduction of salt intake is desirable as a health maintenance measure and for treatment of hypertension and other cardiovascular diseases (Sebranek et al., 1983). Recommendations to reduce dietary sodium levels were made by the USDA (1980), as part of their Dietary Guidelines for Americans.

Processed meat products contribute about 25% of the total sodium in our diet and the meat industry is searching for ways to reduce the salt content of some of the processed meat products. Today, one of the most popular meat products in the U.S. is fresh pork sausage; such a product is salted and seasoned.

The reduction of salt from processed meat products is complicated by the fact that sodium chloride possesses technical functions such as antimicrobial properties, shelf life extension, solubilization of proteins, development of texture and improvement of yield and flavor. Reduction of salt level results in adverse effects on these properties (Ingram and Kitchell, 1967; Kastner and Kropf, 1986). The replacement of salt by substitutes that could also preserve these technical functions was a subject of investigation for some time (Maurer, 1983; Terrell, 1983; Barbut et al., 1988). However, current commercial applications do not exceed a 20–30% reduction of salt. This reduction level is not significant enough from a nutritional point of view.

The greatest difficulty encountered with sodium restriction has been the taste. People find unsalted foods less pleasant and, thus less acceptable. The replacement of NaCl is difficult because NaCl imparts a unique taste property that has a sharp, biting, desirable taste which is not easily duplicated. Although a number of "salt-substitutes" have been developed (Frank and Mickelsen, 1970); the NaCl replacement that has been most commonly used, thus far, has been KCl. The physical properties of KCl make it a good candidate for a salt substitute. However, KCl does not taste like NaCl.

An alternate salt replacement to KCl is modified KCl. The product used in this research was a proprietary brand marketed by Takeda Central Research Lab, Japan, which was prepared by cocrystalization of disodium-5'-inosinate and disodium-5'-guanylate and then incorporated with KCl in a proprietary manner by Takeda.

The objectives of this study were to (1) evaluate the effects of replacing NaCl with modified KCl containing cocrystalized disodium 5-inosinate and disodium 5-guanylate on the hedonic ratings of consumers for fresh pork sausage patties, and (2) examine the masking effect of different levels of monosodium glutamate (MSG) and spices commonly existing in pork sausage formulas.

MATERIALS & METHODS

MODIFIED POTASSIUM CHLORIDE with disodium 5'-inosinate and disodium 5'-guanylate and MSG were prepared at the Takeda Central Research Lab., Japan, a subsidiary of Takeda Chemical Industries, Tokyo, Japan. The sodium chloride as well as all other spices used were food grade.

Experiments were conducted to establish the effect of replacement of NaCl by modified KCl on the degree of liking of pork sausages. The first experiment was designed to see the influence of different levels of MSG incorporated with modified KCl as a salt replacement. The modified KCl was mixed with three levels of monosodium glutamate 0, 3.75, and 7.5% (w/w). The final mixture was used to replace salt in pork sausage formulations at levels from 0 to 100% (w/w) of the total salt used. The second experiment was designed to find the effect of different spice levels incorporated with modified KCl and NaCl on the acceptability of salt replacement (modified KCl). The level of disodium 5'-inosinate and disodium 5'-guanylate, known by the commercial name, Ribotide, was 1.0% (w/w). The modified KCl was mixed with three levels of spices. The final mixture was used to replace salt in pork sausage formulas at levels from 0 to 100% (w/w) of the total salt used.

Preparation

Pork sausage patties were prepared by adding sage, brown sugar, black pepper, red pepper, and salt or salt substitute (Table 1). Approximately 7 mm thick, uniform, round patties were made and formed by a manual patty maker. The patties were placed in nonstick trays and 2–3 tablespoons of water were added. The trays were then covered with aluminum foil and cooked in a conventional oven at 320°F for 20 min. Later the excess juice was drained, the patties uncovered and

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Table 1—Pork sausage patties original formula

Ingredient	(% w/w)
Ground pork	94.15
Water	2.82
Sage (crushed)	0.19
Brown sugar	0.79
Black pepper (fine ground)	0.08
Red pepper	0.04
Salt or salt substitute	2.00
Total	**100.07**

Table 2—Formulation of NaCl/modified KCl mixes at different levels of MSG

MSG (% w/w)	Salt levels (% w/w)	
	NaCl (% w/w)	Modified KCl (% w/w)
0	100	0
	75	25
	50	50
	25	75
	0	100
3.75	—	—
	75	25
	50	50
	25	75
	0	100
7.5	—	—
	75	25
	50	50
	25	75
	0	100

Table 3—Formulation of NaCl/modified KCl mixes at different levels of spice mix

Spice Levels	Salt levels (% w/w)	
	NaCl (% w/w)	Modified KCl (% w/w)
Same as in the original formula (Table 1)	100	0
	25	75
	20	80
	15	85
	10	90
	5	95
	0	100
+5% (w/w) increment from the original formula	100	0
	25	75
	20	80
	15	85
	10	90
	5	95
	0	100
+10% (w/w) increment from the original formula	100	0
	25	75
	20	80
	15	85
	10	90
	5	95
	0	100

left for browning by turning them over once. Patties were later evaluated for sensory acceptability.

For the first experiment (Table 2), 13 patties were prepared at three different levels (0, 3.75, 7.5% (w/w)) of MSG. Salt and salt replacers were used in 5 combinations as follows (NaCl:KCl); 100:0, 75:25, 50:50, 25:75, 0:100. One hundred percent (w/w) NaCl with 0% (w/w) MSG was used as a control.

In the second experiment (Table 3), 21 patties were prepared at 3 different levels of spices. The first spice level was exactly the same as that in the original formula (as in Experiment I), the second and third spice levels were +5% (w/w) and +10% (w/w) increments from the original formula (Table 1).

Sensory evaluation

Pork sausage patties were heated in the microwave oven for 40 sec before presentation. Each formulation was presented to the judges in

Table 4—Effect of MSG: R-index values giving degree of liking of control product over reformulated, with significance levels[a]

Salt levels (% w/w)		MSG levels % (w/w)		
		0	3.75	7.5
Salt:	100	50.0: control product, original formulation		
Salt replacement:	0			
Salt:	75	54.3	55.5	54.1
Salt replacement:	25	(0.49)	(0.35)	(0.51)
Salt:	50	56.2	56.6	54.7
Salt replacement:	50	(0.29)	(0.26)	(0.44)
Salt:	25	55.5	68.8	65.8
Salt replacement:	75	(0.35)	(0.0009)	(0.005)
Salt:	0	69.2	73.9	72.9
Salt replacement:	100	(0.0008)	(0.00006)	(0.00006)

[a] Two tailed significance of difference of R-index value from the chance level (50.0) given in parentheses: Rank sums test (Leach, 1979; O'Mahony, 1986). This table gives the degree of preference (in R-index values) from the control product over the reformulation. High values indicate that the reformulation is not preferred. Low values (not significantly different from 50%) indicate no difference in liking from the control product.

a white plastic portion cup (S.E. Rykof and Co.). All samples (approx. 10g) were presented in random order, and cups were coded with three digit numbers to avoid number bias. Each judge was asked to take a bite of unsalted cracker and rinse his mouth before each sample.

For the first experiment 51 judges (23 M, 28 F; aged range 19–65) and for the second experiment 71 judges (35 M, 36F; age range 19–71) were called. Judges were staff and students at the Univ. of California, Davis. No judges were more experienced with patty tasting than ordinary consumers.

To measure degree of liking for the patties, signal detection measures (Green and Swets, 1966; O'Mahony, 1979; 1988) were adopted for hedonic judgements. These measures were chosen in preference to the traditional nine-point hedonic scale. They have the advantage that they yield numerical hedonic measures without the judge ever having to make a numerical estimation himself. Judges are biased estimators and such biases violate the statistical assumptions required for parametric statistical analysis (O'Mahony, 1982); this is avoided using signal detection.

Judges were required to rate each formula as either 'like strongly,' 'like moderately,' 'dislike moderately,' or 'dislike strongly.' From the judges' data, a signal detection index called the R-index (Brown, 1974; O'Mahony, 1979; 1988) can be computed. In this study the R-index is defined as the probability of the group of judges liking the control product (100% w/w NaCl with 0% w/w KCl, 0% w/w MSG) more than the formulation under consideration when both are presented pairwise. This probability is 100% if the control product is always preferred by the group. The probability (R-index) will be 50% if there is no preference for the control over the formulation under consideration; both will have an equal (50%) chance of being liked more. If the control product is liked less than the formulation under consideration, the R-index would drop below 50%, but in this study it did not. Typically, values ranged between 50–100%; the higher the value, the more the control product was liked compared with the new formulation.

RESULTS & DISCUSSION

THE R-INDEX VALUES obtained for the patty formulations in Experiments I and II are given in Table 4 and 5, respectively. The results presented in Table 4 (treatments with various NaCl, modified KCl and MSG levels) show R-index values greater than 50%, indicating that the control product always had higher hedonic ratings than the test formulation under consideration. Yet, some values were not significantly greater than 50%, indicating that neither the control nor the test formulation had significantly higher hedonic ratings. It is for these low R-index values that the salt replacement would seem to have no significant effect on liking. The table clearly indicates that no significant differences in liking occurred for patties made with 0% (w/w) MSG up to 75% (w/w) salt replacement. However, when MSG was incorporated into the formulations at 3.75% (w/w) and 7.5% (w/w), the overall liking for salt replacement patties was significantly less than for the control (R-index val-

Table 5—Effect of spice levels: R-index values giving degree of liking of original product over reformulation with significance levels[a]

Salt levels (% w/w)		Original spice levels	+5% (w/w) Increment of original formula	+10% (w/w) Increment of original formula
			Spice levels	
Salt:	100	50.0 Control	51.0	52.1[b]
Salt replacement:	0		(0.89)	
Salt:	25	55.3	51.4	52.9
Salt replacement:	75	(0.28)	(0.82)	(0.58)
Salt:	20	59.1	55.9	57.6
Salt replacement:	80	(0.06)	(0.23)	(0.12)
Salt:	15	60.9	58.2	58.9
Salt replacement:	85	(0.02)	(0.09)	(0.07)
Salt:	10	67.5	61.6	63.3
Salt replacement:	90	(0.0003)	(0.01)	(0.005)
Salt:	5	64.7	62.2	62.4
Salt replacement:	95	(0.002)	(0.011)	(0.01)
Salt:	0	64.7	66.3	67.2
Salt replacement:	100	(0.002)	(0.0007)	(0.0004)

[a] Two tailed significance of difference of R-index value from the chance level (50.0) given in parentheses: Rank sums test (Leach, 1979; O'Mahony, 1986).

[b] Values indicate the degree of liking for the control formulation over reformulations when both are given in paired comparison. The value marked 'b' indicates that the reformulation was preferred and the R-index gives the degree of liking for the reformulation over the control.

ues significantly greater than 50%) once salt replacement was at levels above 50% (w/w). These results showed that the addition of MSG had no beneficial effect on the liking for salt replacement. On the contrary, patties prepared with MSG became less liked at a lower KCl replacement level.

After establishing the fact that the addition of MSG did not allow the level of salt replacement to be increased without the product being liked less than the control, an experiment was designed to investigate whether additional spice levels would affect the hedonic ratings given for salt replacement. The results in Table 5, obtained from 71 judges, clearly indicate that when compared to the original spice level, additional +5% (w/w) and +10% (w/w) increments of spice did not raise the level of KCl substitution at which the reformulation still remained equally liked as the control. At 75% (w/w) salt replacement for all spice levels, the reformulations were still not liked significantly less than the control (R-index not significantly greater than 50%). At higher replacement levels the reformulations were liked less. In other words, the higher spice levels did not mask the taste of the modified KCl salt substitute. Again, as in the first experiment, up to 75% (w/w) salt replacement elicited no adverse effects.

From this investigation it can be concluded that it was possible to reduce the NaCl by 75% (w/w) in pork sausage patties and replace it with modified KCl without affecting hedonic

ratings for the product. The addition of MSG in these products also decreased the acceptable level of salt replacement and additional spices used here did not affect it.

Because up to 75% (w/w) of the NaCl can be acceptably replaced by modified KCl, a significant reduction of the average NaCl consumption in meat products can be achieved. These results showed that low sodium products were feasible and provided an indication that modified KCl could be used in other food products where NaCl reduction was wanted. With the acceptance level for modified KCl established, further research is needed to investigate other functional properties of modified KCl at this level in meat products, such as injected ham and bacon, as well as meat emulsion products.

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Survival of *Listeria monocytogenes* on Processed Poultry

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————————————— ABSTRACT —————————————

The potential for *Listeria monocytogenes* to survive and proliferate on processed poultry was investigated. Chicken breasts were inoculated with *L. monocytogenes*, cooked to one of five different internal temperatures, then either vacuum-packaged or wrapped in an oxygen permeable film and stored for up to four wk at 4°C and 10°C. Survivors were encountered at each cooking temperature employed. The magnitude of lethality was directly related to the cooking temperature. No significant increase in microbial populations occurred through 2 wk of storage. Bacterial populations in samples that were overwrapped increased significantly by week 4, except for the 160°F (71.1°C) treatment. For samples which were vacuum-packaged, only the bacterial population in the 150°F (65.6°C) treatment significantly increased. Differences due to packaging were observed at 4°C storage but not at 10°C storage.

INTRODUCTION

RECENT EVIDENCE has linked food containing *Listeria monocytogenes* to documented outbreaks of listeriosis (CDC, 1985; Fleming et al., 1985; Schlech et al., 1983). Individuals particularly at risk include pregnant women, newborns or infants, and immunocompromised persons. It is important to note that the mortality rate within these groups is approximately 30%.

L. monocytogenes is a Gram positive asporogenous rod, grows at refrigeration temperatures, is motile at 25°C and is quite ubiquitous throughout the environment. The organism is carried by 3% of the general population (Schlech, 1988), has been isolated from cultivated as well as uncultivated soils (Brackett, 1988; Weis and Seeliger, 1975) and isolated from foods such as milk, red meats, poultry, seafood, vegetables and fruits (Marth, 1988). *L. monocytogenes* has been isolated from 40 or more mammalian species and at least 17 avian species including domesticated chickens (Bailey, 1988). The microorganism has also been recovered from raw and treated sewage (Al-Ghazali and Al-Azawi, 1986; Watkins and Sleath, 1981) and from effluents of abattoirs and poultry packing plants (Watkins and Sleath, 1981). In poultry, listeriosis is not usually responsible for pathological problems or product losses, yet broilers are fecal carriers of *Listeria* and in turn contaminate the litter, the environment, and ultimately the product (Bailey, 1988). Kwantes and Issac (1974) isolated *L. monocytogenes* from 57% of market poultry sampled in 1971 and 33% of product sampled in 1974. Bailey (1988) found a 23% incidence rate of *L. monocytogenes* on broiler chickens with high variability among different sample types.

L. monocytogenes may survive and proliferate in improperly processed products. This microorganism can grow at temperatures as low as 4°C and has been reported to survive various heat treatments (Bryan, 1986). New insights into the survivability of this foodborne pathogen may be obtained which would be of future benefit as processors continue to develop new or modify existing products. The objective of this research was to determine the effect different cooking temperatures, packaging, and storage conditions had on the survivability of *L. monocytogenes* on processed chicken.

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MATERIALS & METHODS

LISTERIA MONOCYTOGENES (Scott A) was maintained on trypticase soy agar slants at 4°C. In preparation for inoculation onto fresh chicken, trypticase soy broth cultures were grown for 24 hr at 37°C.

Fresh, raw boneless chicken breasts were purchased at an Athens, GA grocery and transported to the laboratory on ice within 30 min. A 4 × 4 cm section of the chicken breast surface was marked and a 0.1 mL inoculum was applied to the surface as evenly as possible with a micropipet. Inoculation levels were approximately 10^5–10^6 microorganisms/g chicken. The inoculated chicken was held at 37°C for 1-1/2 hr to allow possible attachment to or colonization by the organisms on the meat tissue. Once the incubation period expired, a sample was analyzed by the spread plate technique to determine the initial population of microorganisms per gram. A Precision (Chicago, IL) lab oven was operated at 255°C to heat the product portions by dry heat. Cooking times were varied so as to obtain internal endpoint temperatures of 150, 160, 165, 170, and 180°F (65.6, 71.1, 73.9, 76.7, 82.2°C), respectively. This yielded both partially cooked and fully cooked products. Internal temperature was monitored using thermocouples and a microprocessor unit.

One-half of all heated samples were vacuum-packaged (20 mmHg) aseptically in separate portions after the cooking process. Samples not vacuum-packaged were wrapped in an oxygen permeable 0.0007 mil film (Resinite packaging film; North Andover, MA).

After processing, three samples of each cooking treatment were analyzed immediately for surviving microorganisms. The remaining samples were stored at one of two temperatures (4°C and 10°C) for 4 wk. After 1, 2 and 4 wk of storage at the appropriate temperature, a sample was removed and analyzed for *L. monocytogenes*.

A 20-g sample was placed into 180 mL potassium phosphate buffer (0.1M, pH 7.0) and processed in a blender for 1 min. Serial dilutions were prepared and 0.1 mL plated onto Lithium Chloride-Phenylethanol-Moxalactam Agar (LPM agar; Lee and McClain, 1986) by the spread plate technique. Plates were incubated for 24 hrs at 37°C prior to counting presumptive *L. monocytogenes* colonies.

Randomly selected colonies from each sample were screened for confirmation as *L. monocytogenes* by checking the isolate's Gram reaction, morphology, catalase and oxidase reactions, and motility. Isolates of uncertain identity were confirmed by the tests described by Lee and McClain (1986).

Except for the 165°F (73.9°C) treatments which were done three times, each cooking and packaging treatment was done twice. Means of the log counts were analyzed statistically. Analysis of variance (ANOVA) procedures were used to determine if significant differences existed between microbial numbers during the storage period. If analysis of variance showed a significant difference ($p < 0.05$) among microbial counts, then mean counts were separated using Duncan's multiple range test (SAS, 1982).

RESULTS & DISCUSSION

AT EACH TEMPERATURE, survivors of the initial *L. monocytogenes* inoculum were encountered on product stored at 4°C (Table 1). In the table, the precook column refers to samples analyzed prior to heat treatment, whereas the week 0 samples were analyzed immediately following the heat treatment. There are several possible explanations to explain why survivors existed in treatments that should have been sufficient to kill the microorganism. The cooking temperatures were insufficient to totally eliminate the high population of *L. monocytogenes* that were inoculated onto the tissue. Secondly, dry heat which was used in this study, was a less effective method to kill microorganisms than a moist heating method. Additionally, evaporation occurring at the surface of the chicken breast

Table 1—Log plate counts of Listeria monocytogenes *from samples of* chicken cooked to various internal temperatures (Samples were held at 4°C until analyzed)

Cook temp		Precook	Film overwrapped			
(°F)	(°C)		Week 0	Week 1	Week 2	Week 4
150	65.6	6.97	4.14[a]	4.92[a]	3.17[b]	6.28[c]
160	71.1	5.33	3.56[a]	1.91[a]	3.54[a]	2.73[a]
165	73.9	5.19	0.82[a]	ND[*]	0.17[a]	4.05[b]
170	76.7	6.12	0.85[a]	ND	ND	3.01[b]
180	82.2	5.35	0.50[a]	0.69[a]	0.79[a]	2.28[b]

Cook temp		Precook	Vacuum Packaged			
(°F)	(°C)		Week 0	Week 1	Week 2	Week 4
150	65.6	6.97	4.14[a]	4.33[a]	4.04[a]	5.26[b]
160	71.1	5.33	3.56[a]	1.44[a]	3.01[a]	2.21[a]
165	73.9	5.19	0.82[a]	1.51[a]	1.13[a]	1.98[a]
170	76.7	6.12	0.85[a]	ND	ND	1.01[a]
180	82.2	5.35	0.50[a]	ND	0.85[a]	ND

[a,b,c] Values in the same row with the same letter are not significantly different (p<0.05)
[*] ND = no growth detected by direct plating.

Table 2—Log plate counts of Listeria monocytogenes *from samples of* chicken cooked to various internal temperatures (Samples were held at 10°C until analyzed)

Cook temp		Precook	Film overwrapped			
(°F)	(°C)		Week 0	Week 1	Week 2	Week 4
150	65.6	6.97	4.14[a]	7.08[b]	7.35[b]	7.52[b]
160	71.1	5.33	3.56[a]	7.04[b]	7.48[b]	8.04[c]
165	73.9	5.19	0.82[a]	4.98[b]	7.58[c]	7.73[c]
170	76.7	6.12	0.85[a]	6.83[bc]	6.49[b]	7.76[c]
180	82.2	5.35	0.50[a]	6.99[b]	7.52[b]	7.64[b]

Cook temp		Precook	Vacuum packaged			
(°F)	(°C)		Week 0	Week 1	Week 2	Week 4
150	65.6	6.97	4.14[a]	4.85[b]	5.51[c]	6.37[d]
160	71.1	5.33	3.56[a]	4.21[b]	6.81[c]	6.83[c]
165	73.9	5.19	0.82[a]	4.39[b]	6.62[b]	4.75[b]
170	76.7	6.12	0.85[a]	6.47[b]	5.87[b]	6.72[b]
180	82.2	5.35	0.50[a]	4.35[b]	6.90[c]	7.39[c]

[a,b,c,d] Values in the same row with the same letter are not significantly different (P<0.05)

due to the dry heating method could be favoring survival of the organism. As the meat is cooked, the surface will have a lower water activity (a_w) due to drying of the surface tissue. As meat cooks, juices flow from the interior to the surface and can buffer the surface from increasing temperatures. Blankenship (1978), Blankenship and Craven (1982), and Blankenship et al. (1980) noticed similar evaporative phenomenon involving various microorganisms on beef roasts and chicken meat.

A direct relationship was demonstrated between the increasing cooking temperature and the magnitude of lethality. At the three higher cooking temperatures, the L. monocytogenes population in freshly processed samples was reduced 4–5 logs, while at the lower cooking temperatures cell numbers were only reduced approx. 1.8 to 2.8 logs. At 4°C, no significant increase in microbial populations occurred through 2 weeks of storage except on overwrapped product heated to 150°F (65.6°C). Bacterial populations in samples that were overwrapped increased significantly by week 4, except for the 160°F (71.1°C) treatment. For samples which were vacuum-packaged, only the bacterial population in the 150°F (65.6°C) treatment significantly increased. Most poultry processors use some sort of

film overwrap, and vacuum packaging has become a popular type of packaging. Care should be taken to ensure that a microaerophilic microorganism such as L. monocytogenes is not able to survive and proliferate in this type of environment.

Significant increases in microbial numbers occurred within the first week on chicken portions stored at 10°C as opposed to a 4-wk period before significant increases occurred at 4°C storage (Table 2). While the organism was able to survive at the lower storage temperature (4°C), the 10°C storage temperature was more conducive for multiplication of the microbe. At 10°C storage, packaging did not appear to influence the microbial growth rate. In all of these samples, microbial numbers significantly increased by week 4. The microbial population in samples from each cooking treatment increased between week 0 and week 4 in a similar manner regardless of the package type.

Presently, cooking temperatures for fully cooked poultry products appear adequate. This is based partially on the finding that temperatures of 165°F (73.9°C) or greater reduced the L. monocytogenes population by at least 4-5 logs. A. population of L. monocytogenes of this magnitude would not be expected on raw chicken tissue. In addition, the dry heat method utilized might not be the most lethal form of heating available. The physiological characteristics of L. monocytogenes which enable its survival and growth should be recognized by food scientists seeking new product the packaging alternatives.

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Quality Characteristics of Restructured Turkey Meat with Variable Alginate/Calcium-Lactate Ratios

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──────────── ABSTRACT ────────────

Restructured ground turkey and turkey breast meat patties, formulated with combinations of 0-1.0% sodium alginate, 0-0.1875% calcium carbonate and 0 0.6% lactate, were compared to a no additive control. The ratio of sodium alginate to calcium carbonate was held constant at 5.3 to 1. Optimal sensory and instrumental raw and cooked product bind was achieved with combinations of 0.4-1.0% sodium alginate, 0.0750-0.1875% calcium carbonate and 0.6% lactate. All restructured products with the algin/calcium binder had higher (p<0.05) cook yields than the no additive control. Sensory aroma, flavor and juiciness of cooked products were not different (p>0.05) between treatments.

INTRODUCTION

CONVENTIONAL RESTURCTURED meat products are based on sausage technology. They rely on extraction of myofibrillar proteins by salt (NaCl), phosphate and mechanical action, and subsequent formation of heat-set protein gel matrix for binding (Pepper and Schmidt, 1975; Gillett et al., 1977; Theno et al., 1978; Solomon and Schmidt, 1980; Schmidt and Trout, 1982).

Disadvantages of this technology include: (1) the necessity of marketing the product in either the cooked or frozen state; (2) increased potential for oxidative rancidity and discoloration; and (3) sodium content concerns for sodium-sensitive hypertensive consumers.

An algin/calcium gel system, which does not include salt or phosphate, has been found to bind restructured meats in the raw or cooked state, and to prevent the negative effects of salt on product quality (Means and Schmidt, 1986; Means et al., 1987; Schmidt and Means, 1986). The algin/calcium gel restructured meat products may be marketed uncooked, cooked, refrigerated or frozen.

The algin/calcium gel restructured meat technology has been patented (Schmidt and Means, 1986) and approved by the Food Safety and Inspection Service of the United State Department of Agriculture for use in red meats (USDA, 1986). Means and Schmidt (1986) examined various combinations of alginate, calcium carbonate and sodium erythorbate for use in restructured beef. Recently lactate (encapsulated lactic acid/calcium lactate) has been substituted for sodium erythorbate. Restructured meat products formulated with 0.6% algin, 0.1% calcium carbonate and 0.15% lactate have been found to possess acceptable palatability and cohesion characteristics (Clarke et al., 1988). Recent exploratory studies, however, have shown that increased levels of lactate may produce considerable improvement in the sensory and cohesion properties of algin/calcium gel restructured products (Schmidt, 1987) which could provide benefits for both the industry and the consumer.

The process offers the meat industry an opportunity to: (1) increase its market share through extension of product line; (2) add value to underutilized meat sources; and (3) better target consumer health concerns. The algin/calcium restructured product offers the consumer flexibility in form and composi-

tion. Also, the product can be displayed with other fresh meat cuts in refrigerated retail meat cases.

The potential exists for the application of the algin/calcium gel system in restructured meats other then red meats. The objective of this study was to evaluate the effects of three levels of lactate on the palatability, cohension and cook yield of two types of restructured turkey meat formulated with algin and calcium carbonate.

MATERIALS & METHODS

Raw materials

Fresh (48 hr postmortem) ground turkey meat, comprised of light and dark trim from the carcass, and turkey breast pieces were purchased from Longmont Foods Company (Longmont, CO). The turkey breast pieces were ground through a 1.3 cm plate. Twenty percent of the ground breast pieces were then ground a second time through a 0.48 cm plate. An 80:20 ratio of coarse to fine ground breast meat was used to formulate the various treatments. The ground turkey was used to formulate treatments without futher comminution.

Treatments

Treatments, formulated in batches of 500g meat portions, were based on varying levels of sodium alginate (algin) (Manugel DMB, Kelco, Division of Merck and Company, Inc., San Diego, CA) at 0-1% lactac (Cap-Shurc Encapsulated lactic ac d, LCL-135-50; 30% lactic acid; 20% calcium lactate; 50% hydrogenated vegetable oil, Balchem Corp., Slate Hill, NY) at 0-0.6%, and calcium carbonate (Gamma Sperse 80, Georgia Marble Co., Tate, GA) at 0-0.1875% (Table 1). The ratio of algin to calcium carbonate was held constant at 5.3 to 1 based on work by Means and Schmid (1986). Dry ingredients were mixed at low speed into each meat portion with a Kitchen Aid mixer (Model K45-SS, Hobart Co., Troy, OH) according to the following sequence: 30 sec for algin, 30 sec for calcium carbonate and 60 sec for lactate. Two min of mixing was allowed for meat without added ingredients (controls). The mixtures were stuffed with a caulking gun into 6.5 cm diameter cellulose casings immediately after mixing, and were stored at 4°C for 48 hr.

The turkey products were then cut into 0.75 cm thick slices, using a Choice-cut kitchen slicer (Oster Mfg. Co., Milwaukee, WI), and slices from all treatments were randomly allotted to various objective and subjective evaluations.

Table 1 — Variables and experimental design

Treatment code[y]	Ingredients and level (%)[x]		
	Algin	Lactate	CaCO₃
LL1	0.2	0.3	0.0375
LL2	0.4	0.3	0.0750
LL3	0.6	0.3	0.1125
LL4	0.8	0.3	0.1500
LL5	1.0	0.3	0.1875
HL1	0.2	0.6	0.0375
HL2	0.4	0.6	0.0750
HL3	0.6	0.6	0.1125
HL4	0.8	0.6	0.1500
HL5	1.0	0.6	0.1875
C	0	0	0

[x] Algin = sodium alginate, Lactate = encapsulated lactic acid/calcium lactate. CaCO₃ = calcium carbonate

[y] First two letters of treatment code refer to lactate level (LL = low lactate, HL = high lactate), number refers to algin level (1 = 0.2%, 5 = 1.0%)

C = control

All procedures were replicated on two separate batches of ground turkey meat and two batches of turkey breast meat pieces.

Instrumental evaluation

Two slices per treatment weighed before and after grilling to determine cook yield, which was expressed as (weight of cooked slice ÷ weight of raw slice) × 100. Samples were grilled 2 min, (1 min per side), to an internal temperature of approximately 75°C using a Presto electric griddle, Model 0690001 (National Presto Ind., Inc., Eau Claire, WI).

The peak force (Newtons) required to break 0.75 cm thick raw and cooked restructured turkey meat slices with a 1.8 cm diameter steel ball (Field et al., 1984) was determined using a J.J. Lloyd Model T5002 tensile tester (Pacific Scientific Santa Ana, CA) at a crosshead speed of 110 mm/min. Two raw and cooked room temperature (25°C) samples per treatment were evaluated.

Sensory evaluation

Four trained panelists assessed a variety of raw and cooked product attributes in a pass-through cubicle-type sensory evaluation room. Two training sessions were held prior to actual product evaluation. Representative raw and cooked samples were examined and discussed with regard to the product characteristics being evaluated. Samples were cooked as for instrumental evaluation and evaluated at room temperature (25°C). Samples were presented individually in a random manner and were examined under fluorescent lights (Econ-o-Watt Cool White, F40CW/RS/EW-II, Philips, U.S.A.). Six point descriptive scales were used to evaluate bind of raw product, and aroma, flavor, bind and juiciness of cooked product according to the methods of Means and Schmidt, (1986).

Statistical analysis

An analysis of variance for a completely random design was conducted for each variable measured in each of the two replicates for each meat type. When differences ($p < 0.05$) occurred, treatment means were separated by Duncan's New Multiple Range Test (Steel and Torrie, 1980).

RESULTS & DISCUSSION

RESULTS for ground turkey meat are presented in Tables 2 and 3, while results for turkey breast meat are presented in Tables 4 and 5. Restructured ground turkey and turkey breast meats had greater ($p < 0.05$) bind scores in the raw state than

the no additive control. Restructured ground turkey meat with ≥ 0.6% algin and turkey breast meat with ≥ 0.4% algin had higher ($p < 0.05$) raw bind scores than products with 0.2% al-

Table 3 — Ground turkey meat instrumental evaluation results

Treatment code[w]	Raw bind(N)[x]	Cooked bind(N)	Cook yield(%)[y]
LL1	1.34[d]	2.20[cde]	82.73[fg]
LL2	1.29[d]	2.75[bcde]	83.83[efg]
LL3	1.59[cd]	1.89[de]	84.93[def]
LL4	2.44[b]	2.90[bcd]	88.55[bc]
LL5	2.03[bc]	1.80[e]	89.90[ab]
HL1	0.91[de]	1.73[e]	81.38[g]
HL2	2.09[bc]	3.05[bc]	86.50[cde]
HL3	2.37[b]	2.92[bcd]	86.75[cd]
HL4	2.69[b]	3.52[ab]	90.15[ab]
HL5	4.28[a]	4.38[a]	91.80[a]
C	0.28[e]	1.99[cde]	77.68[h]
	(0.22)[z]	(0.33)	(0.90)

[a-h] Means in a column with the same letter are not significantly different, P>0.05.
[w] First two letters of treatment code refer to lactate level (LL = low lactate, HL = high lactate), number refers to algin level (1 = 0.2%, 5 = 1.0%).
C = control.
[x] N = Peak Newton Force to break a 0.75 cm thick sample.
[y] % Cook Yield = (Cooked wt/Raw wt) × 100
[z] (standard error of mean).

Table 4 — Turkey breast meat sensory evaluation results for raw bind and several cooked parameters

Treatment code[y]	Raw bind	Panel scores[x] Cooked bind	Aroma	Flavor	Juiciness
LL1	1.63[e]	3.63[bc]	5.13[a]	5.13[a]	3.63[a]
LL2	2.50[cd]	4.00[abc]	5.50[a]	5.38[a]	3.50[a]
LL3	3.25[bc]	4.63[ab]	5.50[a]	5.25[a]	3.88[a]
LL4	3.13[bc]	4.88[a]	5.50[a]	5.13[a]	3.88[a]
LL5	3.75[ab]	4.38[ab]	5.57[a]	5.00[a]	3.50[a]
HL1	2.13[de]	4.25[abc]	5.38[a]	5.25[a]	3.63[a]
HL2	3.13[bc]	4.38[ab]	5.63[a]	5.50[a]	3.75[a]
HL3	3.75[ab]	4.63[ab]	5.63[a]	5.00[a]	3.88[a]
HL4	4.13[a]	4.25[abc]	5.38[a]	4.88[a]	3.63[a]
HL5	4.13[a]	4.63[ab]	5.50[a]	5.25[a]	3.88[a]
C	1.25[f]	3.25[c]	5.75[a]	5.00[a]	3.75[a]
	(0.25)[z]	(0.34)	(0.26)	(0.28)	(0.33)

[a-f] Means in a column with the same letter are not significantly different, P>0.05.
[x] Raw bind, 1 = virtually no bind; 6 = very strong bind, comparable to intact muscle.
Cooked bind, 1 = virtually no bind; 6 = very strong bind, comparable to intact muscle.
Aroma, 1 = very strong off odor(s); 6 = fresh grilled meat aroma, no off odor(s).
Flavor, 1 = very strong off flavor(s); 6 = natural grilled meat flavor, no off flavor(s).
Juiciness, 1 = extremely dry; 6 = extremely juicy.
[y] First two letters of treatment code refer to lactate level (LL = low lactate, HL = high lactate), number refers to algin level (1 = 0.2%, 5 = 1.0%).
C = control.
[z] (standard error of mean).

Table 2 — Ground turkey meat sensory evaluation results for raw bind and several cooked parameters

Treatment code[y]	Raw bind	Panel scores[x] Cooked bind	Aroma	Flavor	Juiciness
LL1	2.00[d]	3.75[bc]	5.63[a]	4.88[a]	3.88[a]
LL2	1.88[d]	3.50[c]	5.25[a]	5.13[a]	3.38[a]
LL3	3.25[c]	3.75[bc]	5.75[a]	5.25[a]	4.25[a]
LL4	4.00[b]	3.00[c]	5.63[a]	4.75[a]	3.38[a]
LL5	3.88[bc]	3.13[c]	5.63[a]	4.88[a]	3.88[a]
HL1	2.13[d]	3.50[c]	5.50[a]	4.75[a]	3.63[a]
HL2	3.75[bc]	4.63[a]	5.50[a]	4.88[a]	3.39[a]
HL3	4.13[b]	3.88[abc]	5.75[a]	5.25[a]	3.75[a]
HL4	4.25[ab]	3.88[abc]	5.63[a]	4.63[a]	3.88[a]
HL5	4.88[a]	4.50[ab]	5.50[a]	4.75[a]	3.88[a]
C	1.00[e]	3.13[c]	5.75[a]	5.25[a]	4.13[a]
	(0.22)[z]	(0.26)	(0.23)	(0.27)	(0.32)

[a-e] Means in a column with the same letter are not significantly different, P>0.05.
[x] Raw bind, 1 = virtually no bind; 6 = very strong bind, comparable to intact muscle.
Cooked bind, 1 = virtually no bind; 6 = very strong bind, comparable to intact muscle.
Aroma, 1 = very strong off odor(s); 6 = Fresh grilled meat aroma, no off odor(s).
Flavor, 1 = very strong off flavor(s); 6 = natural grilled meat flavor, no off flavor(s).
Juiciness 1 = extremely dry; 6 = extremely juicy.
[y] First two letters of treatment code refer to lactate level (LL = low lactate, HL = high lactate), number refers to algin level (1 = 0.2%, 5 = 1.0%).
C = control.
[z] (Standard error of mean).

Table 5 — Turkey breast meat instrumental evaluation results

Treatment code[w]	Raw bind(N)[x]	Cooked bind(N)	Cook yield(%)[y]
LL1	0.94[d]	3.05[cd]	88.45[bcd]
LL2	1.27[cd]	4.14[abcd]	90.85[ab]
LL3	1.90[bc]	3.34[cd]	87.50[bcd]
LL4	2.11[b]	3.67[bcd]	90.55[abc]
LL5	2.19[ab]	3.19[cd]	91.05[ab]
HL1	1.17[d]	4.83[abc]	87.13[cd]
HL2	2.40[ab]	4.49[abc]	89.13[abc]
HL3	2.47[ab]	5.43[ab]	91.20[ab]
HL4	2.47[ab]	4.81[abc]	91.30[a]
HL5	2.91[a]	5.70[a]	90.55[abc]
C	0.17[a]	2.69[d]	84.15[e]
	(0.24)[z]	(0.58)	(1.14)

[a-e] Means in a column with the same letter are not significantly different, P>0.05.
[w] First two letters of treatment code refer to lactate level (LL = low lactate, HL = high lactate), number refers to algin level (1 = 0.2%, 5 = 1.0%).
C = control.
[x] N = Peak Newton Force to break a 0.75 cm thick sample.
[y] % Cook Yield = (Cooked wt/Raw wt) × 100.
[z] (standard error of mean).

gin. Cooked product bind scores of restructured ground turkey meat treatments HL2 and HL5 were greater (p<0.05) than that of the no additive control product. The remaining restructured treatments did not differ (p>0.05) from the control. For turkey breast meat, cooked product bind scores of LL3, LL4, LL5, HL2, HL3 and HL5 were higher (p<0.05) than for the no additive control. Thus, the restructured turkey breast meat had consistently more acceptable sensory cook bind scores than did ground turkey meat, when compared to their respective control treatments.

Clarke et al. (1988) found that sensory bind scores of raw restructured beef, containing a binder mixture composed of algin, calcium carbonate and lactate, increased with increasing level of binder ingredients up to 1.13%, but indicated no further increase in a raw bind at a higher binder level. In the same study, 0 and 0.28% binder levels produced similar cooked product bind scores, but these were lower (p<0.05) than treatments containing ≥ 0.5% algin/calcium binder. Means and Schmidt (1986) found that raw bind scores of restructured beef treatments were greater than the no additive control. but were not different from each other. Means and Schmidt (1986) and Means et al. (1987) reported no differences between cooked product bind values of restructured beef treatments.

Raw restructured ground turkey and turkey breast meats had higher (p<0.05) breaking strength peak forces than the no additive control. The force needed to break raw samples of ground turkey meat increased nearly fivefold between 0.2 and 1.0% algin, with 0.6% lactate. The magnitude of increase in peak force for raw turkey breast meat between 0.2 and 1.0% algin was approximately twofold, with either 0.3 or 0.6% lactate. This difference may be due to increased surface area available for meat-algin/calcium system interaction in the more finely comminuted ground turkey. Only two restructured ground turkey treatments, LH4 and HL5, had greater (p<0.05) breaking strength values for cooked products than the no additive control. In contrast, all cooked restructured turkey breast meat treatments containing 0.6% lactate (HL1, HL2, HL3. HL4, HL5) had higher (p<0.05) peak forces than the control, but were not different (p>0.05) from each other.

A previous study indicated that instrumental penetration scores for algin/calcium restructured raw beef increased with binder level up to 0.85%, but were not different between binder levels >0.85% (Clarke et al., 1988). In the same study, treatment differences were not observed for cooked penetration force.

Restructured ground turkey and turkey breast meats had greater (p<0.05) cook yields than the no additive control. Increasing the algin/calcium carbonate level in ground turkey meat resulted in a gradual increase in cook yield with both 0.3 and 0.6% lactate. Few treatment differences were found in the cook yields of restructured turkey breast meat.

Clarke et al. (1988) reported that cook yield of restructured beef increased between 0 and 0.57% algin/calcium binder, but not between binder levels >0.57%, and attributed this increased cook yield to probable inhibition of moisture migration with increased binding. In contrast, Means and Schmidt (1986) and Means et al. (1987) found no differences in cook yield between algin/calcium restructured and no additive control treatments. Variable experimental methods and conditions may contribute to this disparity in results.

Panelists detected no treatment differences (p>0.05) for aroma, flavor or juiciness of cooked ground turkey or turkey breast meat products. Means and Schmidt (1986) and Means et al. (1987) also found no treatment differences in aroma

scores between cooked algin/calcium restructured and no additive control beef treatments. However, in the same studies, flavor scores of restructured products were lower (p<0.05) than the no additive control. Means and Schmidt (1986) attributed the lower flavor scores to algin which had not reacted with calcium ions, and thus had not gelled. The utilization of higher levels of lactate in the current study may have improved product flavor by decreasing the level of unreacted algin. Increased gelation, resulting from increased algin/calcium interaction, may also influence product bind and cook yield, as well as other product characteristics.

Further study of the algin/calcium gel system interaction with various meat systems may lead to a better understanding of the influence of ingredient levels, meat types and processing methods and conditions on the final product.

CONCLUSIONS

THE ALGIN/CALCIUM restructuring system can be used to produce palatable turkey meat products with sufficient bind to be marketed uncooked, cooked, refrigerated or frozen. Optimal product characteristics were achieved with combinations of 0.4–1.0% algin, 0.0750–0.1875% calcium carbonate and 0.6% lactate. However, further study may be necessary to determine optimal conditions for commercial production. Development of new poultry meat products, using the algin/calcium restructuring system, may be beneficial to both industry and consumers through increased marketing and product alternatives.

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Yields, Color and Compositions of Washed, Kneaded and Heated Mechanically Deboned Poultry Meat

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ABSTRACT

Kamaboko-like products, prepared with NaCl and phosphate buffer wash solutions, had higher yields compared to those prepared with Na-acetate and polyphosphate solutions. Products prepared with polyphosphate wash solution were lightest in color. Higher fat in mechanically deboned poultry meat caused lower yields than lower fat. There were no interactions (P>0.05) between the temperatures and salt concentrations of the wash solutions on yields of products washed with various concentrations of NaCl. Products prepared with NaCl wash solutions at 2–4°C had higher yields than those prepared at 20–22°C. Generally, products prepared with 1.0% or 1.5% NaCl wash solutions had higher Hunter "L" values and lower Hunter "a" values when compared to those prepared with 0 or 0.5% NaCl washing solutions.

INTRODUCTION

RECENTLY, the National Broiler Council surveyed that further-processed poultry products now account for 20.0% of marketing and will increase to 30.7% by 1990, and perhaps, as much as 39.1% by early 1995 (Wabeck, 1987). The residual poultry parts from further processing such as turkey frames, chicken backs, necks and breast frames are increasing due to increasing demand for processed poultry products. The development of mechanical deboners has enabled more efficient utilization of residual parts. Mechanically deboned poultry meat (MDPM) is usually used in a variety of products such as salami, turkey treats, frankfurters, combination rolls and patties (Froning, 1976). The ever-increasing supply of MDPM and demand for animal protein have heightened the interest in the development of new products.

Since surimi-based products such as kamaboko were introduced into the USA several years ago, their unique chewy texture provided people with a substitute for crabmeat, shrimp, lobster and other natural seafoods. The market for surimi-based products has grown from 0.9 million kg in 1979 to 13.2 million kg in 1983 (NMFS, 1984). Fish contain more myofibrillar protein which contributes to the chewy texture of surimi (Matsumoto, 1978). The MDPM is high in protein, especially myofibrillar protein (Scopes, 1970). Theoretically, it should be a good ingredient for surimi-based foods.

Kamaboko or fish cake is a typical seafood in Japan in the form of protein gel. Washing, grinding with salt and heating are the three fundamental steps in processing kamaboko from minced fish (Suzuki, 1981). Minced fish is extracted with water to remove undesirable substances such as pigments, blood and fat. Pigments impart undesired color and catalyze lipid oxidation (Lanier et al., 1982). The washing process may also increase the concentration of myofibrillar protein (actomyosin), thereby, improving gel strength and elasticity (Lee, 1984).

This study was designed to examine the suitability of utilizing MDPM in the manufacturing of kamaboko-like products. The effects of MDPM sources and types of wash solutions on yields, color and compositions of kamaboko-like products were evaluated.

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MATERIALS & METHODS

Mechanically deboned poultry meat

Frozen mechanically deboned poultry meat (MDPM) from broiler breast frames (BF), broiler breast frames plus backs and necks with skin (BN), and broiler breast frames (45%) and backs (55%) (BB) were obtained from a commercial poultry processing plant. The frozen MDPM samples were stored at −20°C for 2 months. Samples were thawed at 4°C for 24 hr prior to use.

Preparation of kamaboko-like products from MDPM

Washing. For each 100g of MDPM from BB, 600 mL of the following wash solutions were added separately and stirred for 5 min: 0.5% NaCl, 0.5% Na-acetate, 0.5% Kena (a commercial polyphosphate, a mixture of sodium tripolyphosphate and sodium hexametaphosphate, Stauffer Chemical Co., Westport, CT), and 0.038M phosphate buffer at pH 8. The mixture was then centrifuged at 907Xg force for 10 min. After discarding the supernatants, the residues were washed and centrifuged two more times at 20°C.

MDPM from BF, BN and BB were washed as previously described with 0.5% NaCl solution at 20–22°C. MDPM from BF was also washed with 2–4°C and 20–22°C tap water containing 0, 0.5, 1.0, and 1.5% table salt.

Kneading and heating. The washed MDPM samples, as obtained previously, were weighed and 2% (w/w) table salt was added to each. After hand-kneading with a spatula for 10 min at 2–4°C, the MDPM samples were aged by storing at 34°C for 2 hr. The washed and aged samples were placed in aluminum foil lined baking pans and heat in a preheated conventional oven at 90°C for 30 min (Suzuki, 1981).

Analyses

After heating, the final product was cooled at room temperature (20°C) for 30 min and then weighed. The percent overall yield was obtained by dividing the final product weight by the raw material weight and multiplying by 100. Moisture, lipid, protein and ash contents determined in accordance with AOAC (1984) methods.

The colors of top and bottom surfaces of kamaboko-like products were measured with a Hunter Model D-25 Color and Color Difference Meter (Hunter Associates Laboratory, Fairfax, VA). To insure proper surface illumination, the samples were measured by covering the aperture and applying pressure. Standard plate #6274 with a Hunter "L" value of 92.4, "a" value of −0.7, and "b" value of −0.9 was used for reference. All studies were quadruplicated and data were subjected to analysis of variance (Steel and Torrie, 1980). The means (P<0.05) were separated by Duncan's Multiple Range Test (Duncan, 1955).

RESULTS & DISCUSSION

THE YIELD of kamaboko-like products made from MDPM varied with the types of wash solutions used in the preparation (Table 1). Products prepared with NaCl and phosphate buffer solutions had higher (P<0.05) yields than those prepared with 0.5% Na-acetate and 0.5% Kena solutions. No difference (P<0.05) in yield was observed between 0.5% NaCl and 0.038M phosphate buffer wash solutions. It was noticed that the total composition data were less than 100%. The limited number of observations and/or the possible presence of additives such as cryoprotectant might be responsible.

Kamaboko-like products prepared with 0.5% Kena washing solution were lightest in color (higher Hunter "L" values) compared to those prepared with the other solutions (Table 1). Also, the 0.5% Kena solution resulted in a product with less

Table 1 — Effects of different washing solutions on yields and Hunter color values of kamaboko-like products[a]

Type of washing solution	Yield (%)	Hunter color values					
		"L"		"a"		"b"	
		Top surface	Bottom surface	Top surface	Bottom surface	Top surface	Bottom surface
0.5% NaCl	56.08A	56.26cB	62.61aC	2.04aA	0.79bB	14.33aB	13.24bB
0.5% Na-acetate	43.03B	48.75bD	56.03aD	2.24aA	2.68aA	15.35aB	12.17bC
0.5% Kena	40.10B	65.04aA	65.85aA	0.90aB	0.64aB	15.17aB	14.40bA
0.038M phosphate buffer with pH 8	54.83A	58.87bB	64.19aB	0.95aB	0.13bC	17.00aA	13.97bA

[a] Mean of four observations from mechanically deboned broiler breast frame (45%) and back (55%) meat (BB). Means within a column not fo lowed by the same capital letter (ABCD) are significantly different (P<0.05).

Means within a row of a color subgroup not followed by the same lower case letter (a,b) are significantly different (P<0.05).

Table 2 — Yields of kamaboko-like products as affected by the composition of raw MDPM[a]

Source	Composition of raw MDPM (%)				Yield (%)
	Moisture	Fat	Protein	Ash	
BF[b]	66.95b	12.54c	13.74a	1.06a	71.23a
BN[c]	65.95b	19.63b	12.91b	0.71b	70.30a
BB[d]	57.48a	24.72a	12.27c	0.70b	56.08b

[a] Mean of four observations. Products were prepared with 0.5% NaCl washing solution. Means within a column not followed by the same lower case letter (a-c) are significantly different (P<0.05)

[b] Mechanically deboned poultry meat from broiler breast frames (BF).

[c] Mechanically deboned poultry meat from broiler breast frames, backs and necks with skin (BN).

[d] Mechanically deboned poultry meat from broiler breast frames (45%) and backs (55%) (BB).

Table 4 — Yields of kamaboko-like products as affected by temperature and NaCl content of washing solutions[a]

Washing solution temperature (°C)	NaCl (%)			
	0	0 5	1.0	1.5
2-4	87.73a	72.13bcd	71.18bcd	76.40b
20-22	74.20bc	69.38bcd	67.25cd	65.53d

[a] Means of four observations from mechanically deboned broiler breast frames meat (BF). Means not followed by the same lowercase letter (a-d) are significantly different (P<0.05).

redness (Hunter "a" values). It appeared that 0.5% Na-acetate solution was the least effective in pigment extraction when compared to the other solutions. Generally, kamaboko-like products were browner in color on the top surface possibly due to reducing sugars or a browning reaction of oven heating. The pigments responsible for the color of MDPM are myoglobin and hemoglobin (Froning, 1976). Water washing was used in early attempts to extract these heme pigments from meat. Hernandez et al. (1986) extracted heme pigments from MDPM by using 0.04M phosphate buffer at pH 8.0 and observed the color characteristics as being 61.5 for Hunter "L" values, 4.6 for Hunter "a" values and 14.85 for Hunter "b" values, compared to 40.69, 12.85, and 11.76 for Hunter "L", "a", and"b" values, respectively, for controls.

The composition of raw MDPM varied according to source and influenced processing yields (Table 2). The MDPM from BB had the highest fat which resulted in the lowest processing yield. The removal of fat during the washing procedure might be partially responsible for this difference.

The protein of kamaboko-like products was slightly lower than those of their raw MDPM counterparts (Tables 2 and 3). The data on protein of raw MDPM agreed with those reported by Froning (1976). In the preparation of fish surimi, Babbitt (1986) reported the protein of washed minced flesh from Alaskan Pollock as 10.75%. Lee (1984) indicated that the wash process in surimi manufacturing could remove sarcoplasmic protein (water-soluble protein).

Table 3 — Composition of kamaboko-like products made from various MDPM washed with 0.5% NaCl[a]

Source	Composition (%)			
	Moisture	Fat	Protein	Ash
BF[b]	78.95a	2.25b	12.40a	3.54a
BN[c]	79.35a	2.43b	12.47a	2.74c
BB[d]	77.40a	3.85a	11.11a	3.10b

[a] Mean of four observations Means within a column not followed by the same lower case letter (a-c) are significantly different (P<0.05).

[b] Mechanically deboned poultry meat from broiler breast frames (BF).

[c] Mechanically deboned poultry meat from broiler breast frames, backs and necks with skin (BN).

[d] Mechanically deboned poultry meat from broiler breast frames (45%) and backs (55%) (BB).

The composition of kamaboko-like products varied with the fat of the raw material (Tables 2 and 3). Froning (1970) stated that bone marrow and skin were responsible for large increases in the fat of MDPM. Regardless of source, ash of kamaboko-like products was higher than that of raw MDPM (Tables 2 and 3), because 2% NaCl was incorporated into the washed MDPM during kneading. Regardless of the types of raw MDPM, no difference (P>0.05) in moisture of kamaboko-like products was observed.

Analysis of variance for yield of kamaboko-like products indicated that there were significant (P<0.05) temperature and salt effects. However, there was no interaction (P>0.05) between temperature and salt concentrations. The MDPM washed with NaCl at 2-4°C had a higher yield than that washed at 20-22°C (Table 4). Lowered wash solution temperature should produce products with better microbial quality. There were no differences (P>0.05) in yields among the various NaCl solutions compared to the NaCl-free solution at 20-22°C. However, kamaboko-like products prepared with 2-4°C tap water washing had higher (P<0.05) yields than those prepared with various NaCl solutions.

A difference (P<0.05) in Hunter "L" values of top and bottom surfaces of kamaboko-like products among various salt concentrations was observed. Generally, for both surfaces of kamaboko-like products, there was no interaction between temperature and salt concentrations on Hunter "L" values. Hunter "L" values increased as the concentration of salt increased (Table 5).

Salt concentration and wash solution temperature affected (P<0.05) Hunter "a" values on both top and bottom surfaces of kamaboko-like products. In addition, there was an interaction between wash solution temperature and salt concentrations for Hunter "a" values of the top surface of the products. MDPM washed with solutions containing NaCl was much less red than that washed with tap water. Generally, the more concentrated was the salt washing solution, the less redness was there in the kamaboko-like products.

The MDPM washed with solutions containing salt resulted in kamaboko-like products with higher Hunter "b" values on both surfaces when compared to those washed with tap water. Generally, based on color, kamaboko-like products prepared with 20-22°C wash solutions removed more pigments than those prepared with 2-4°C wash solutions (Table 5). Poel (1949) and Fleming et al. (1960) reported that plain water was not very effective in extracting the heme pigments from meat.

The concentration of salt in the wash solution affected

Table 5—Hunter color values of kamaboko-like products on top and bottom surfaces prepared with various NaCl washing solutions[a,b]

Surface	NaCl (%)	"L"		"a"		"b"	
		2-4°C	20-22°C	2-4°C	20-22°C	2-4°C	20-22°C
Top	0	37.36d	38.68d	10.00a	3.60b	11.05b	10.92b
	0.5	54.43c	57.58bc	3.38bc	0.86cd	13.24a	13.26a
	1.0	59.78ab	59.78ab	1.15bcd	0.57d	13.47a	14.53a
	1.5	60.70ab	63.48a	0.94cd	−0.74d	13.38a	13.46a
Bottom	0	43.13e	42.75e	7.23a	4.60ab	10.75b	10.71b
	0.5	56.26d	59.96cd	2.41bc	0.88cd	12.68a	12.70a
	1.0	61.89bc	63.88ab	1.00cd	−0.97d	13.13a	12.92a
	1.5	62.71ab	65.27a	0.95cd	−1.12d	13.01a	12.74a

[a] No interaction between salt concentrations and temperature.

[b] Means of four observations from mechanically deboned broiler breast frames meat (BF). Means within a subgroup not followed by the same lower case letter (a-e) are significantly different (P<0.05).

Table 6—Composition of kamaboko-like products as affected by temperature and salt concentration[a,b]

Conc. of NaCl (%)	Moisture (%)		Fat (%)		Protein (%)		Ash (%)		Soluble ash (%)	
	2-4°C	20-22°C	2-4°C	20-22°C	2-4°C	20-22°C	2-4°C	20-22°C	2-4°C	20-22°C
0	80.68a	80.52ab	3.43a	2.89ab	12.17ab	13.06a	3.07cd	2.62d	2.98g	2.53h
0.5	78.95bc	78.82c	2.25ab	2.17ab	12.40ab	12.95a	3.54bc	3.32bc	3.46d	3.20f
1.0	80.65a	80.82a	2.22ab	1.38b	9.70cd	11.58ab	3.72abc	3.52bc	3.67c	3.43e
1.5	81.88a	81.52a	2.41ab	1.66ab	8.99d	10.96bc	4.28a	3.98abc	4.18a	3.81b

[a] No interaction between salt concentrations and temperature.

[b] Means of four observations from mechanically deboned broiler breast frames meat (BF). Means within a subgroup not followed by the same lower case letter (a-h) are significantly different (P<0.05).

(P<0.05) the moisture content of kamaboko-like products. Salt concentration and temperature also affected (P<0.05) the protein of kamaboko-like products. The MDPM washed with 1.0% or 1.5% NaCl solutions contained less protein compared to 0.5% or tap water solutions (Table 6). Lee (1984) reported that solubilization of myofibrillar protein occurred when too much salt was used during surimi manufacturing. One may note that the lipid content of kamaboko-like products was not affected by either salt in the wash solution or by the wash solution temperature (Table 6).

Ash of kamaboko-like products was higher than that of the raw material (Tables 2 and 6). As expected, the ash of kamaboko-like products increased as the salt concentration of the wash solution increased. The addition of 2% NaCl during the kneading process also contributed to increased ash readings. Soluble ash data (Table 6) may verify this hypothesis. Rasekh et al. (1980) reported little difference in proximate composition between water-washed and unwashed minced fresh croaker.

SUMMARY

THE YIELD of kamaboko-like products made from MDPM varied with the types of wash solutions used in the preparation. MDPM prepared with NaCl and phosphate buffer wash solutions had higher (P<0.05) yields than those prepared with 0.5% Na-acetate and 0.5% Kena solutions. No difference (P<0.05) in yield was observed between MDPM washed in 0.5% NaCl or 0.038M phosphate buffer solutions.

The yields of kamaboko-like products prepared with 2–4°C wash solutions were higher than those prepared with 20–22°C wash solutions. Kamaboko-like products prepared with 1.0% or 1.5% NaCl wash solutions showed higher yields than those prepared with 0 or 0.5% NaCl solutions. Also, kamaboko-like products washed with 1.0% or 1.5% NaCl tended to be lighter in color with less redness. Therefore, based on this study, the manufacture of kamaboko-like products from MDPM prepared

with 1.0% or 1.5% NaCl wash solutions at 2–4°C would be recommended.

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Textural Attributes of Mechanically and Cryogenically Frozen Whole Crayfish (*Procambarus clarkii*)

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—————————— ABSTRACT ——————————

Textural quality of quick frozen and conventionally frozen whole crayfish was evaluated. Toughness, as measured by Kramer shear press, increased significantly (p ≤ 0.05) as a result of freezing. Between 6 and 16 wk of frozen storage, toughness increased (p ≤ 0.05) in seasoned (SCF) samples but remained the same in all other treatments. In all treatments, toughness declined after 16 wk of frozen storage. Individually quick frozen (IQF) samples were more tender (p ≤ 0.05) than conventionally frozen (CF) samples. Seasoned crayfish had the greatest degree of toughness (p ≤ 0.05). Extractable protein decreased (p ≤ 0.05) during frozen storage, with IQF samples showing the smallest changes. SCF samples had significantly (p ≤ 0.05) lower moisture retention and more (p ≤ 0.05) lipid oxidation.

INTRODUCTION

THE EMERGENCE of "Cajun Cuisine" in national and international foodservice markets has induced a large increase in the demand for traditional Louisiana food products such as crayfish (Barnett, 1985). This increase necessitated alternate approaches to traditional processing and marketing practices. A major problem that confronts the crayfish industry is the seasonal supply of product. Because the foodservice industry demands a consistent product year round, methods of extending the supply of crayfish beyond the 6-month harvesting season is mandatory.

The availability of crayfish tail meat has been successfully extended through frozen storage. However, freezing of whole crayfish has been discounted due to degradation problems associated with the hepatopancreatic tissue (fat). This is mainly exhibited in a mushy texture after relatively short periods of iced storage, although deterioration during extended frozen storage may also occur (Godber et al., 1986). A variety of proteolytic enzymes has been identified in the hepatopancreas of the fresh water prawn (Lee et al., 1980) and fiddler crab (Eisen and Jeffery, 1969), and tail meat of prawns has been found to have superior quality after storage compared with whole animals (Hale and Waters, 1981). Recent research in Hawaii indicates that the enzyme responsible for the tissue softening in iced prawns is a collagenase (Baranowski et al., 1984; Nip et al., 1985). Marshall et al. (1987) have suggested the existence of proteolytic enzymes in the hepatopancreatic tissue of crayfish and have indicated the importance of blanching for maintenance of textural quality.

Freezing crayfish also could cause toughening of the texture as noted with many other seafood products (Sikorski et al., 1976). The mechanism for such occurrences is not well understood; however, denaturation and aggregation of protein have been suggested as a possible explanation. This may be due to mechanical damage associated with the rate of freezing such as ice crystal formation and ion concentration effects, or interaction with chemical components such as formaldehyde or lipid oxidation products (Sikorski et al., 1976).

The factors that contribute to textural deterioration in frozen whole crayfish must be determined. The potential of fast freezing by a cryogenic liquid nitrogen (LN) method to reduce textural problems also should be considered. Therefore, the objectives of this study were to evaluate the textural problems associated with freezing whole crayfish and to determine if cryogenic freezing could reduce textural problems.

MATERIALS & METHODS

Processing

Live whole crayfish (harvested from the Belle River, LA, area) were processed on three consecutive days (each day representing a replication, i.e. n = 3). Live crayfish were purged, heat processed for 5 min in boiling water and frozen by one of four processes: packaged, conventionally still frozen (CF); seasoned, packaged, conventionally still frozen (SCF); packaged, cryogenically frozen (PQF); and individually cryogenically frozen before packaging (IQF).

A vacuum packaging machine (Koch, Kansas City, MO) was used to package all treatments. An attempt to obtain a vacuum failed, however, due to the puncture of the bags by the exoskeleton of the crayfish. Approximately 1.8 kg quantities of crayfish were packaged in 25.4 × 35.6 cm polyethylene bags (W.R. Grace, Duncan, S.C.). The CF samples were placed in a commercial still freezer at −23°C. The SCF samples were seasoned with a dry commercial mix, which consisted primarily of salt and cayenne pepper, following the heat treatment and then frozen in a manner similar to CF. PQF samples were packaged and frozen cryogenically using a liquid nitrogen tunnel freezer, whereas IQF samples were placed individually into the liquid nitrogen tunnel, frozen and then packaged. Both cryogenic treatments were stored in a commercial still freezer at −23°C. Samples from each treatment were analyzed initially and at 6, 12, 16, 20 and 24 wk following overnight thawing at 4°C.

Moisture

Total moisture was determined in duplicate aliquots by gravimetric means following overnight drying at 102°C to constant weight. The method of Jauregui et al. (1981) was used to determine expressible moisture. The weight of the sample minus the weight of expressed moisture times the original moisture was considered the retained moisture. This analysis was done 5 times for each treatment.

Instron analysis

The Kramer shear attachment (10 blade) of an Instron Universal Testing Machine Model 1122 was used to measure differences in toughness. Accurately weighed samples (n = 5) of approximately 15g were placed in the cell and the shear force measured with a crosshead and chart speed of 20 mm/min and 100 kg full scale load.

Extractable protein nitrogen

Extractable protein was determined according to the procedure developed by Dyer et al. (1950). Total protein and the protein concentration of the supernatant were determined in duplicate by the Kjeldahl method (AOAC, 1980) and Micro-Biuret method (Itzhaki and Gill, 1964), respectively.

Lipid oxidation

Lipid oxidation was determined using the 2-thiobarbaturic acid (TBA) method as described by Tarladgis et al. (1960). Absorbance was read at 532nm using a Gilford Response Spectrophotometer. TBA values were determined, after establishing a standard curve using graded concentrations of tetramethoxypropane, as the average of two determinations for each of two distillates per sample.

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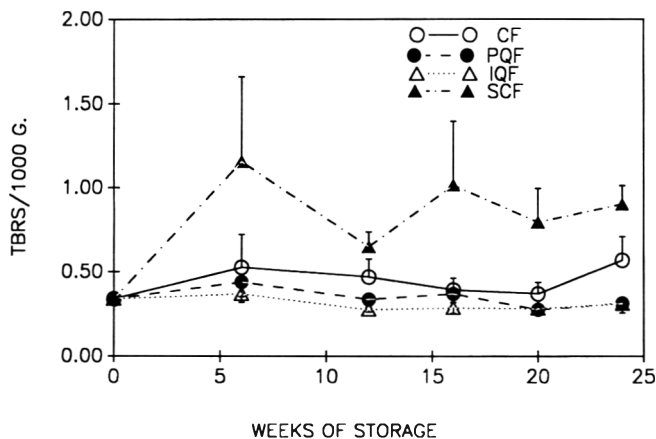
Fig. 1—Changes in TBA value (thiobarbituric reactive substances, TBRS/1000g) during frozen storage of crayfish frozen by different methods. Each point represents the mean (n = 3) ± SEM. Conventionally frozen (CF), seasoned conventionally frozen (SCF), packaged quick frozen (PQF), individually quick frozen (IQF).
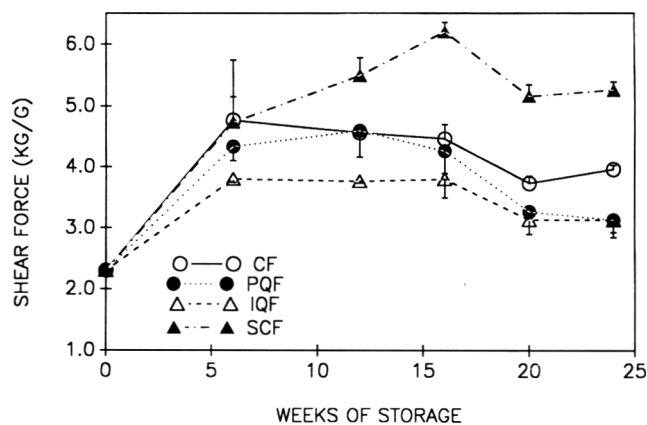

Fig. 2—Changes in Kramer shear resistance during frozen storage of crayfish by different methods. Each point represents the mean (n = 3) ± SEM. Conventionally frozen (CF), seasoned conventionally frozen (SCF), packaged quick frozen (PQF), individually quick frozen (IQF).

Statistical analysis

A randomized complete block design (RCBD) was employed. Replications (n = 3) were blocked and treatments were assigned in a 4 × 5 factorial arrangement within each block plus a zero time control. Data were analyzed using the Statistical Analysis System (SAS, 1985). Differences between the means were determined by least significant difference (LSD).

RESULTS & DISCUSSION

TEXTURAL QUALITY was evaluated primarily on the basis of physical measures of moisture and shear resistance. Certain insights into the mechanisms for the textural changes were obtained through analyses of protein and oxidation conditions. IQF samples had lower (p ≤ 0.05) shear values than the conventionally frozen samples. SCF samples had the greatest (p ≤ 0.05) shear resistance (Table 1) and the least amount of retained moisture.

A relationship between toughening of seafood products during frozen storage and reduced extractable protein has been suggested (Sikorski et al., 1976). The mechanism for this relationship is less well understood. Most researchers attribute this occurrence to the denaturation and aggregation of protein (Sikorski et al., 1976). During the freezing process changes in ionic strength and pH occur due to concentration of the aqueous phase as freezing progresses from the outside portions of the muscle inward. This may then promote the denaturation of proteins through alterations in hydrophobic interactions, allowing more ready formation of new linkages or associations between proteins and possibly other constituents within the protein matrix (Babbitt et al., 1972; Karel et al., 1975). The end result is that the protein component becomes less extractable and may cause texture to become tougher. Associated with freeze denaturation of the proteins is a lessened ability to retain moisture (Samson et al., 1985). SCF samples demonstrated the relationship between toughness and lower retained moisture.

The fact that IQF samples had superior textural properties was most probably related to the rapid rate of freezing, which would reduce the effects of aqueous phase concentration. Reid et al. (1987) found that quick frozen samples of rockfish had higher levels of extractable protein than slow frozen samples. They also noted that ice crystal formation was highly influenced by freezing rate, with more numerous nucleation sites and smaller crystals as freezing rate increased.

The explanation for the inferior textural quality of SCF samples is probably related to ionic effects associated with the high salt content of the seasoning, which would be intensified

Table 1—Main effect of freezing on chemical and physical attributes in whole crayfish

	Freezing method			
Attribute	CF	SCF	PQF	IQF
Moisture (%)	77.8[b]	72.1[c]	78.2[b]	78.9[a]
Expressible moisture (%)[d]	51.7[ab]	53.0[b]	51.6[ab]	51.4[a]
Retained moisture (%)[e]	51.8[a]	36.7[b]	53.2[a]	55.9[a]
Kramer shear (kg/g)	4.30[b]	5.37[c]	3.92[ab]	3.53[a]
Extractable protein (%)	7.4[ab]	7.7[ab]	7.0[b]	8.3[a]
TBA (mg TBRS/kg)	0.47[a]	0.91[b]	0.35[a]	0.31[a]

[a,b,c] Means (n = 15 summed among all storage times) in the same row with the same superscript are not different (p ≥ 0.05). Conventionally frozen (CF), seasoned conventionally frozen (SCF), packaged quick frozen (PQF), individually quick frozen (IQF). Thiobarbituric acid is TBA and TBA reactive substances in TBRS.
[d] % of sample
[e] % of total moisture

due to the concentration effect during freezing. These changes resulted in reduced water holding capacity of proteins and inferior textural quality. SCF samples had higher levels of extractable proteins than would have been expected based on moisture retention and shear resistance but this may have been due to a greater ionic strength of extraction solution as a result of residual salt from the seasoning.

Another theory for textural deterioration of proteinaceous foods holds that lipid oxidation products interact with proteins to form insoluble aggregates (Karel, 1973; Karel et al., 1975). Lipid oxidation, as measured by the TBA test, was appreciable only in the SCF treatment (Table 1), which varied erratically over time (Fig. 1). In all other treatments, TBA numbers remained fairly constant throughout storage. These findings would indicate that lipid oxidation may play a role in the toughening of crayfish texture during frozen storage but the only consistent relationship was with SCF samples, which were both most oxidized and toughest. Differences in texture between the other treatments could not be explained on the basis of oxidation. However, Castell (1971) has pointed out that low levels of TBA reactive compounds could indicate that these compounds are interacting with the protein component. Further research is necessary to clarify the precise relationship.

Texture of crayfish became tougher (p ≤ 0.05) as a result of 6 wk of frozen storage for all treatments (Fig. 2). It then remained unchanged between 6 and 16 wk, except for the SCF samples, which continued to become tougher during the first 16 wk of storage. Between 16 and 20 wk of frozen storage, there was a significant (p ≤ 0.05) decline in shear force for PQF and SCF treatments with a more modest (N.S) decline for CF (p ≤ 0.10) and IQF (p ≤ 0.13) treatments (Fig. 2). Beyond 20 wk of frozen storage, there was no change in tex-

Fig. 3—Changes in moisture retention during frozen storage of crayfish frozen by different methods. Each point represents the mean (n = 3) ± SEM. Conventionally frozen (CF), seasoned conventionally frozen (SCF), packaged quick frozen (PQF), individually quick frozen (IQF).

Fig. 4—Changes in extractable protein during frozen storage of crayfish frozen by different methods. Each point represents the mean (n = 3) ± SEM. Conventionally frozen (CF), seasoned conventionally frozen (SCF), packaged quick frozen (PQF), individually quick frozen (IQF).

ture. Water-holding capacity, as a function of retained moisture (Fig. 3), declined (p ≤ 0.05) after 6 weeks of storage in all treatments except PQF, which did not evidence a significant reduction in retained moisture until 16 wk of storage. During extended storage, IQF samples, which unexplainably had a large decrease in retained moisture after 6 weeks of storage, were found to have the greatest retained moisture. However, only SCF treatments had significantly (p ≤ 0.05) lower retained moisture than the other treatments at each storage period. Extractable protein declined (p ≤ 0.05) after 6 weeks of frozen storage in all treatments. Thereafter, a gradual decline occurred, with significant differences (p ≤ 0.05) from the 6 week levels, occurring after 20 weeks for SCF, and after 24 weeks for CF and IQF (Fig. 4).

Textural changes in seafood during frozen storage have been associated with toughness, chewiness, rubberiness or stringiness (Connell, 1964). The initial rise in shear force resistance was suggestive of a similar type of change in crayfish texture. The fact that moisture retention and extractable protein also declined after 6 weeks of frozen storage provided physicochemical support for an alteration in proteins that might have caused these textural changes. However, the results of the present study showed a curvilinear relationship between shear force

and storage, despite a linear reduction in extractable protein. This occurrence indicated that other factors were involved in textural changes during frozen storage. Work by Godber et al. (1986) and Marshall (1988) suggested residual enzyme activity during frozen storage of crayfish tail meat. Whole crayfish, with hepatopancreas in close proximity to tail meat, would be expected to be even more susceptible to proteolytic attack during frozen storage. This likely was the cause of the reduction in shear force resistance after 16 weeks of storage. These findings emphasize the possible importance of the endogenous proteolytic enzymes of crayfish to textural changes during frozen storage. The full impact of these enzymes to crayfish textural quality is being evaluated currently.

Data obtained in this research provide several important insights with regard to freezing crayfish. First, fast freezing by a cryogenic method might improve the textural quality of whole crayfish during frozen storage. Secondly, seasoning crayfish before freezing imparted major textural problems as a result of freezing. Finally, the texture of whole frozen crayfish is influenced by freezing as well as residual proteolytic enzyme activity, which made it difficult to distinguish specific textural defects.

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Effect of Processing on Proximate Composition and Mineral Content of Sea Cucumbers (*Parastichopus* spp.)

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―――――――――― ABSTRACT ――――――――――

The nutrient content, texture and holothurin content of two sea cucumber species were determined for fresh, dried and canned products. Fresh sea cucumber contained 89–91% moisture, 5–6% protein, 0.3% fat, 3% ash and 0.3% carbohydrate; dried sea cucumber contained 2–6% moisture, 61–70% protein, 2–3% fat, 16–24% ash, and 2–3% carbohydrate. Fresh sea cucumber contained 90 mg of holothurin per 100g of tissue. Canned sea cucumber contained moisture and protein levels similar to those generally found in marine finfish. Processing methods for canned sea cucumber resulted in a significant ($P<0.01$) decrease in potassium and increase in sodium. No significant differences ($P<0.01$) were found in the texture of canned products made from fresh-cooked, salted-cooked and cooked-dried raw material.

INTRODUCTION

SEA CUCUMBERS are an important food in the Indo-Pacific region, where they are marketed fresh and processed as trepang (Philippines), iriko (Japan) and beche de mer (Halstead, 1965; Tanikawa, 1985). The sea cucumber *Stichopus japonicus* is commonly consumed in Japan and is one of the few species that has been studied biochemically in detail (Borgstrom, 1962; Tanikawa, 1955). Sea cucumbers are usually marketed as frozen, cooked-dried, cooked-salted, and cooked-salted-dried products (Zaitsev et al., 1969).

The California sea cucumber (*Parastichopus californicus*) and the warty sea cucumber (*P. parvimensis*) are common off the California coast. Though there is a small commercial fishery for both species, sea cucumbers are not marketed widely. Both species are considered an under-utilized fishery resource in the U.S.; and *P. parvimensis* is felt to yield more desirable processed products (NMFS, 1983).

The objective of this study was to determine the effect of processing on the proximate composition, selected mineral and holothurin content of sea cucumbers. The texture of canned sea cucumber processed from fresh, dried and salted raw materials was also compared.

MATERIALS & METHODS

Materials

Sea cucumbers (*P. parvimensis* and *P. californicus*), 15-30 cm long, were harvested by commercial trawl vessels in the Santa Barbara channel. Iced sea cucumbers were shipped by truck to Oakland and delivered to Davis for analysis within 24 hr after being harvested.

Processing

Fresh sea cucumbers were eviscerated, washed, and sorted. Six sea cucumbers were ground in a heavy duty food grinder and pooled for proximate and mineral analyses. The remaining sea cucumbers were frozen at −20°C for later processing.

Cooked-dried sea cucumbers were boiled in 3% NaCl for 1 hr, rinsed in cold running water for 6 hr and dried in a gravity convection oven at 60°C for 36 hr. Six cooked-dried samples were pulverized in

a Micro-Mill grinding mill (Bel-Art Products) and pooled for nutritional and mineral analyses. Before being canned, cooked-dried sea cucumbers were soaked in water (50°C initially) for 24 hr at room temperature.

Salted sea cucumbers were dry-salted and held at 4°C for 2 wk. Before being canned, salted sea cucumbers were rinsed in cold running water for 6 hr and boiled in tap water for 30 min.

Before being canned, frozen sea cucumbers were thawed under cold running water and boiled in a 3% citric acid/sodium citrate buffer at pH 3.5 for 1 hr. Acidification prior to thermal processing coagulates proteins and prevents deformation during retorting (Tanikawa, 1955).

Sea cucumbers were canned in 473 mL glass jars in a 3% citric acid/sodium citrate solution at pH 3.5 and processed in a steam cabinet to an internal temperature of 90.6°C for 5 min. The jars were cooled in air to ambient temperature. Six sea cucumbers were taken from each jar, ground and pooled for nutritional and mineral analyses.

Nutrient analyses

Moisture was determined by the method of Baardseth and Haug (1953); total nitrogen by the method of Carlson (1978); protein by a standard procedure (AOAC, 1984); and total lipid by the method of Bligh and Dyer (1959). After carbohydrate extraction (AOAC, 1984), total carbohydrates were determined from a standard curve using glucose, phenol and sulfuric acid as references (Dubois et al., 1956). For mineral analyses, samples were ashed at 550°C (AOAC, 1984) and dissolved in 20% nitric acid. Minerals were determined by atomic absorption spectrophotometry according to Muys (1984). Determinations were made in triplicate.

Texture measurements

Samples for texture measurement were taken from the middle of the sea cucumber body with a 1.5 cm cork borer. Texture measurements were made on an Instron Model 1122 under the following conditions: extension measurement, 20 mm; crosshead speed, 100 mm/min; chart speed, 200 mm/min; load range, 0-2 kg; probe, 0.32 cm diameter flat head. The work required to puncture each sample was determined from the area under each curve. Four measurements were made on each sea cucumber.

Holothurin analyses

Holothurin was determined by a boiling water extraction and ethanol crystallization of the saponins (Yamanouchi, 1955).

Statistical analyses

Significant differences ($P<0.01$) between treatments were determined using analysis of variance (Steel and Torrie, 1980) and Duncan's multiple range test (Duncan, 1955).

RESULTS & DISCUSSION

THE PROXIMATE COMPOSITION of fresh and cooked-dried *P. parvimensis* and *P. californicus* (Table 1) is similar to that reported for *S. japonicus*, *Cucumaria frondosa* and other sea cucumber species (DHEW, 1972; Sidwell, 1981; Ke et al., 1983; Mottet, 1976; Zaitsev et al., 1969). Raw *P. parvimensis* had significantly higher moisture and ash than *P. californicus*. Sea cucumbers generally contain higher moisture and lower protein than marine fish and shellfish, but reported proximate compositional data for fresh sea cucumbers vary greatly: mois-

Table 1 – Effect of processing on the proximate composition of sea cucumber

Process	Species	Moisture (%)	Protein (%)	Eat (%)	Ash (%)	Carbohydrate (%)
Raw	P. parvimensis	90.8(1.1)[a]	4.7(1.4)	0.3(0.1)	3.4(0.3)	0.3(0.1)
	P. californicus	88.8(0.4)	5.9(0.2)	0.3(<0.1)	2.6(0.4)	0.2(0.1)
Dried	P. parvimensis	2.0(<0.1)	60.7(4.6)	2.4(1.4)	23.9(0.3)	2.4(0.2)
	P. californicus	5.5(0.4)	69.6(1.4)	2.8(0.5)	16.1(0.4)	2.7(<0.1)
Canned	P. parvimensis[b]	73.8(0.7)	20.4(1.1)	1.1(<0.1)	1.3(0.2)	0.2(<0.1)
	P. parvimensis[c]	83.1(1.8)	10.3(0.4)	0.6(0.1)	2.0(0.1)	0.2(<0.1)
	P. parvimensis[d]	76.8(0.7)	16.5(0.5)	0.9(0.1)	1.6(0.1)	0.3(<0.1)

[a] Mean (standard deviation).
[b] Fresh-cooked.
[c] Salted-cooked.
[d] Cooked-dried.

Table 2 – Effect of processing on the mineral content of sea cucumber

Process	Species	Na	K	Ca (mg/100g)	P	Mg	Fe	Zn (ppm)	Cu
Raw	P. parvimensis	16(7)[a]	47(11)	95(24)	14(4)	11(3)	21.5(3.3)	2.9(2.2)	1.3(0.4)
	P. californicus	19(1)	54(4)	67(15)	25(7)	13(1)	71.6(9.2)	2.3(0.1)	1.0(0.2)
Dried	P. parvimensis	132(21)	286(36)	668(191)	100(8)	58(6)	134.1(25.0)	25.2(3.6)	6.6(1.2)
	P. californicus	39(7)	208(15)	652(42)	120(49)	31(4)	668.1(11.8)	38.2(1.5)	4.2(0.1)
Canned	P. parvimensis[b]	326(16)	6(1)	64(4)	35(6)	19(2)	15.5(2.0)	<0.1	0.9(<0.1)
	P. parvimensis[c]	808(33)	12(8)	35(8)	39(3)	17(1)	58.9(20.2)	0.1(0.1)	3.2(1.2)
	P. parvimensis[d]	410(9)	7(2)	90(6)	46(8)	23(4)	17.9(1.4)	<0.1	0.6(0.1)

[a] Mena (standard deviation).
[b] Fresh-cooked.
[c] Salted-cooked.
[d] Cooked-dried.

Table 3 – Effect of processing on holothurin concentrations in sea cucumber tissue

Process	Species	Holothurin (mg/100g)
Raw	P. parvimensis	90[a] (<10)
	P. californicus	90 (<10)
Dried	P. parvimensis	2.670 (<10)
	P. californicus	1.980 (<10)
Canned	P. parvimensis[b]	90 (<10)
	P. parvimensis[c]	140 (<10)
	P. parvimensis[d]	110 (<10)

[a] Mean (standard deviation).
[b] Fresh-cooked.
[c] Salted-cooked.
[d] Cooked-dried.

ture (82–92.6%), protein (2.5–13.8%), fat (0.1–0.9%), ash (1.5–4.3%) and carbohydrate (0–2.2%) (DHEW, 1972; Mottet, 1976; Sidwell, 1981). These variations may be due to seasonal variations in feeding behavior (Mottet, 1976) or to individual or geographical variations.

Dried and canned sea cucumber products contained less ash than could be accounted for by the amount of moisture lost. The low ash in these products was probably due to the loss of mineral constituents during the cooking process. A similar reduction in ash during steaming has been observed with mussels (Slabyj and Carpenter, 1977). The apparent increase of protein in processed sea cucumbers, compared to the raw material, reflects the changes in ash and moisture.

The mineral content of raw sea cucumber and the effect of processing on the concentrations of these minerals are shown in Table 2. P. californicus had significantly higher Fe than P. parvimensis. Previously reported values for other species of raw sea cucumber include: Ca, 34–67 mg/100g; P, 11–14 mg/100g; Fe, 3.0–46 ppm; and Cu, 9.6–28.0 ppm (DHEW, 1972; Sidwell, 1981; Mottet, 1976). No information is available for Na, K, Mg and Zn in raw sea cucumbers. The Ca in our samples was higher and the Cu was lower than the few values previously reported. Ca has been reported to be present in sea cucumbers in large quantities (Zaitsev et al., 1969), and the

difference in values could reflect geographic and/or seasonal variations.

Drying caused a 2- to 10-fold increase in mineral content. There was a significant loss of K and a 20- to 51-fold increase in Na during cooking/canning. The increase in Na in canned sea cucumbers was due to the 3% NaCl or Na Citrate buffer used to boil the sea cucumbers prior to canning, to the Na citrate used to pack the canning jars, or to the NaCl used to dry salt the cooked-dried sea cucumbers.

The work required to puncture canned P. parvimensis body wall ranged from 57.2 to 106.4 g-cm. There was no significant difference in the texture of canned sea cucumber made from fresh-cooked, salted-cooked and cooked-dried raw material.

Holothurin is a generic term used to describe steroid saponins isolated from sea cucumbers. These heat-stable saponins may have tumor inhibitory properties and have been found, in large doses, to be toxic to many animal species (Yamanouchi, 1955; Baslow, 1969). There are no reported cases of toxicity of holothurins to humans. The holothurin content of fresh sea cucumber (Table 3) was slightly higher than the 500 to 800 mg/kg reported for Holothuria vagabunda tissue (Yamanouchi. 1955). The increase in holothurin concentration in dried and canned sea cucumber products was due to moisture and ash loss during processing.

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Distribution of the Lipid Classes and Eicosapentaenoic (20:5) and Docosahexaenoic (22:6) Acids in Different Sites in Blue Mackerel (*Scomber australasicus*) Fillets

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——————— ABSTRACT ———————

The distribution of lipid in various sites of the edible fillets of the blue mackerel (*Scomber australasicus*) was determined. Specific details of the lipid composition of the dark and white muscles were provided by column and thin layer chromatography. Dark muscle contained 20% lipid whereas white muscle had only 4% lipid. However, white muscle lipid was relatively much higher in phospholipids; 19% vs 10% in dark muscle lipid. The major phospholipids were phosphatidylcholine, phosphatidyl ethanolamine, and sphingomyelin. These were distributed in similar proportions in both muscles, although the white muscle phospholipids included more polyunsaturated [eicosapentaenoic (EPA) and docosahexaenoic (DHA)] acids than those of the dark muscle.

INTRODUCTION

OVER THE last 20 years, interest in the human consumption of fish has increased dramatically. Seafood is now regarded as an excellent source of protein and important polyunsaturated fatty acids. Two of these acids, eicosapentaenoic (EPA) (20:5) and docosahexaenoic (DHA) (22:6) are claimed (Dyerberg and Jorgensen, 1982) to be beneficial for human health by reducing the risk of cardiovascular diseases that have become rather prevalent in western society.

For these reasons, the demand for further investigation of many other marine resources has been called for and deep-sea fish located at a depth of approximately 1–1.2 km are now being exploited. Of those species caught in New Zealand waters, many are rich in protein (Vlieg, 1982b) and polyunsaturated fatty acids within the ω3-series (Body, 1983). Other more abundant deep-sea species like orange roughy (*Hoplostethus atlanticus*) (Hayaski and Takagi, 1980; Body, 1982; Buisson et al., 1982; Body et al., 1985) contain high levels of wax esters (>90%) rather than equivalent quantities of triacylglycerol moieties as their lipids. Unfortunately, these marine wax esters (Body et al., 1985) do not contain adequate amounts of the required ω3-series of polyunsaturated fatty acids and consequently do not promote prevention of cardiovascular disorders.

These deep-sea resources are not unlimited so further exploration of other species of commercial value caught in all depths of New Zealand waters is essential (Vlieg and Body, 1988). One of these species is the blue mackerel (*Scomber australasicus*), a member of the Scombridae family, widely spread in the Pacific Ocean between 45°N and 45°S. It appears in the New Zealand fishing area throughout the year yielding an annual catch of about 1500—2000 tons (Weeber, 1987). Other related mackerel species (Jones, 1983) *S. scombrus* (confined to both sides of the North Atlantic Ocean) and *S. japonicus* (located in the South Atlantic, Mediterranean, Indian and North Pacific Oceans) are similarly good resources for the fishing industry. Those of the Northern Hemisphere have been subjected to extensive study (Ackman and Eaton, 1971;

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Hardy and Keay, 1972; Ohtsuru et al., 1984) but only incidental lipid information is available about the blue mackerel (Pickston et al., 1982; Vlieg, 1982a).

This paper will report comparative details of the lipid composition of blue mackerel lipid and its distribution in different sites of the edible flesh. Indication of the specific distribution of ω3-polyunsaturated fatty acids in the different lipid classes in both the dark and white muscle tissues will be included. This is important because when preparing the acceptable appearance of the edible flesh by deep filleting to attract the consumer, substantial layers of oily dark colored layers are discarded. An extended investigation of the lipid-fatty acid composition of these off-cuts will detect if any recoverable material of commerical value is available.

MATERIALS & METHODS

Samples

Commerical quantities of blue mackerel were landed off the east coast of the North Island (near Gisborne) in 1982 and 6 average sized (mean length 50 cm) specimens within the 1.6–2.5 kg weight range were frozen and air-freighted to the laboratory. When they were thawed, the edible flesh was removed for inspection and prepared for lipid analytical investigations.

Fillets from each specimen were subdivided into two groups: (1) A strip of flesh (2 cm wide) taken from a central portion of one fillet from each pair was cut into 0.5 cm sections commencing from the skin layer to the flesh closet towards the bone. (2) The whole of the dark and white muscles was dissected from the matching fillets, and, respectively, minced and bulked together before separate extraction and analysis.

Distribution of lipid

The lipid content of individual fillet fractions was determined by homogenizing and extracting with $CHCl_3$-CH_3OH (2:1 v/v) (Folch et al, 1957). The recovered $CHCl_3$-phase was taken to dryness under reduced pressure at 45°C until constant weight, then dissolved in $CHCl_3$ and stored at −20°C until required for further analyses.

Lipid composition

The fractionation of the whole dark and white muscle lipid extracts into their various classes was performed by loading SiO_2 columns (35 g; 28.0 × 2.0 cm) with approximately 350 mg lipid extract as described before (Body, 1985). Initially, $CHCl_3$ (300 mL) eluted the neutral lipids (cholesteryl esters/triacylglycerol/cholesterol), and CH_3OH (400 mL) recovered the polar lipids. These could be further resolved by preparative thin layer chromatography (TLC) with plates coated with 0.3 mm silica gel G and developed with $CHCl_3$-CH_3OH-H_2O (65:25:4 v/v). The developed plates were sprayed with 2',7'-dichlorofluorescein, and the observed bands scraped-off and extracted with $CHCl_3$-CH_3OH-H_2O (10:10:1 v/v). Throughout this work the identification of the different lipid classes collected was confirmed by comparing their chromatographic properties with those of authentic standards by TLC under identical conditions.

Fatty acid composition

The fatty acid content of the total lipid extracts or isolated individual lipid classes was released as methyl esters by transesterification re-

actions with the BCl_3-CH_3OH reagent (Morrison and Smith, 1964) and analysed by gas liquid chromatography (GLC). The instrument used (Pye-Unicam 104, Cambridge, England) was equipped with polar (10% EGSS-X) and nonpolar (3% JXR) columns with identification procedures as outlined previously (Body, 1985).

Cholesterol

The fraction with cholesterol as eluted from the SiO_2 column also contained other lipid constituents. These were identified as unesterified fatty acids and fatty alcohols. After treatment with diazomethane (Schlenk and Gellerman, 1960), the methyl esters were easily separated from the non-saponifiable matter by means of SiO_2 (3 g) column chromatography and the residuals (cholesterol and fatty alcohols) could be directly resolved by GLC with 3% JXR as the liquid phase. All the required operating conditions have been described earlier (Body, 1985).

RESULTS & DISCUSSION

THE DISTRIBUTION PATTERNS of lipid and ω-3 fatty acids in the blue mackerel fillets at various depths are shown in Table 1. The lipid content follows the same pattern of diminishing with depth common to related species, skipjack tuna (*Katsuwonus pelamis*)(Vlieg et al., 1983) and slender tuna (*Allothunnus fallai*) Vlieg, 1984) and this is paralleled by the distribution of the principal ω3-fatty acids (i.e., 20:5 and 22:6). However, it was noticed that this pattern only applied if the data were expressed in term of g ω3-fatty acids/100g edible flesh. If these proportions of ω3-fatty acids are classified as a percentage of the total fatty acid content at each site, their concentration increases with depth. One format of ω3-fatty acid data is suitable for dietitians whereas the other would be more applicable for biochemical studies.

Comparatively, the dark meat (the first 0.5 cm depth under the skin, Table 1), represented 20% of the fillets while the white meat was as much as 80% of the edible flesh. The general composition of these muscles is shown in Table 2. Dark muscle tissues contained at least five times more lipid than their related white muscle tissues. These porportions have been investigated over a period of seasons, and it was demonstrated by Leu et al. (1981) that in autumn, the Atlantic mackerel (*S. scombrus*) dark muscles had their highest lipid content. In some instances, these lipids are reported to be also enriched with phospholipids (Ackman and Eaton, 1971). These observations were not found in the current work although changes in the proportions of some other individual lipid constituents, i.e., more cholesterol and total ω3-fatty acids did occur (Table 2).

Lipid composition of dark and white muscle

Further details of lipid class variations associated with the dark and white muscle are presented in Table 3. Although the lipid in white muscle contained 18.8% phospholipid compared with 9.9% in the dark muscle, these differences were compensated by those of the triacylglycerols, 83.9% (dark muscle) and 76.2% (white muscle). The relative amounts of cholesterol (when expressed as a percentage of the total lipid extract) were the same in both forms of muscle. When the cholesterol data are given in g/100g tissue, however, they differ by more than a factor of 5 (Table 2). As expected (Gray and Macfarlane, 1961; Addison et al., 1968; Patton, 1975), phosphatidylcholine was the predominant phospholipid followed by phosphatidylethanolamine, sphingomyelin, and cardiolipin. The comparative proportion of these individual phospholipid compounds was very similar in both muscle tissues.

Fatty acid composition of dark and white muscle lipid classes

The overall distribution of phospholipid fatty acids indicates the dark muscle tissues contain more saturated fatty acids than the white muscle tissues (Table 4). This data confirmed earlier observations of Wessels and Spark (1973), when they compared the iodine values of fatty acids from similar sources. This finding is further confirmed when compared with the different phospholipid class fatty acids of both dark and white freshwater fish tissues (Mai and Kinsella, 1979). Their data are in agreement with the fatty acid profile of blue mackerel dark and white muscle individual phospholipid components shown in Table 4. This explains why the concentration of polyunsaturated (ω3-series) fatty acids increases in the different site depths of edible flesh demonstrated in Table 1.) Not only is this due to the greater proportion of phospholipids present in the total lipids, but the inner layer of white muscle individual phospholipids also contains higher proportions of unsaturated fatty acids.

However, the appearance of increased or reduced levels of unsaturated fatty acids in the tissues of fish also varied according to the habitat of the species studied. It has been reported (Lewis, 1967; Patton, 1975; Cossins and Macdonald, 1983) that deep-sea fish tissues contain more unsaturated fatty acids than those caught near the surface. The fatty acid profile the pelagic fish can also fluctuate according to different climates. Antarctic fish, compared with tropical fish, have increased levels of poly- and monoenoic fatty acids (Patton, 1975); this pattern change of unsaturated fatty acids is particularly evident with the individual phospholipid classes. More recent investigation of Australian fish (Gibson, 1983; Sinclair et al., 1983, 1986) show that tropical fish also contain higher levels of ω6-series of unsaturated fatty acids, specifically eicosate-

Table 1—*Distribution of ω3 fatty acids and their related ω3/ω6 ratios in the blue mackerel fillets at various depths in relation to their corresponding lipid contents*[a]

Depth of fillet from exterior (cm)	Wt % lipid	g ω3-fatty acids/ 100g flesh	Wt % ω3-fatty acids/Total fatty acids	Ratio ω3/ω6
0.5	26.9 ± 9.5	4.4 ± 0.3	17.7 ± 4.3	7.5 ± 0.2
1.0	8.3 ± 2.5	2.2 ± 0.3	27.5 ± 2.0	11.5 ± 1.2
1.5	3.7 ± 1.8	1.0 ± 0.4	28.9 ± 3.7	13.4 ± 0.4
2.0	2.0 ± 0.6	0.8 ± 0.3	37.9 ± 4.6	22.5 ± 4.5
2.5	2.2 ± 0.8	0.9 ± 0.3	39.4 ± 0.5	22.2 ± 2.6

[a] Expressed as mean of six samples ± standard deviation.

Table 2—*General characteristics of blue mackerel fillets*[a]

	g/100g Tissues	
	Dark muscle	White muscle
Lipid	19.6	3.9
Protein[h]	13.6	20.8
Cholesterol	0.510	0.094
Fatty acids		
20:5 ω3	1.9	0.21
22:6 ω3	2.49	0.54
Total ω3 fatty acids	4.05	0.78
Ratio ω3/ω6	6.4	7.9

[a] Derived from pooled samples from six fish.
[h] Estimated as fat-free residue recovered from lipid extraction.

Table 3—*Lipid composition of blue mackerel fillets*[a]

	Wt (%)	
Components	Dark muscle	White muscle
Cholesteryl esters/hydrocarbons	0.8	0.6
Triacylglycerol	83.9	76.2
Fatty acid[b]	2.6	1.8
Cholesterol[b]	2.6	2.4
Fatty alcohol[b]	0.2	0.2
Cardiolipin	0.3	1.0
Phosphatidylethanolamine	1.9	4.0
Phosphatidylcholine	6.2	11.3
Sphingomyelin	1.5	2.5

[a] Determined gravimetrically following chromatographic techniques described in the text and related to percentage of total lipids recovered.
[b] Resolved by GLC.

Table 4—Fatty acid composition of the major lipid classes of blue mackerel fillets[a]

| | Weight (%) | | | | | | | | | |
| | Dark muscle | | | | | White muscle | | | | |
Fatty acid	TG[b]	Card	PE	PC	Sph	TG	Card	PE	PC	Sph
n-Saturated										
14:0	4.1	4.2	0.8	1.3	1.8	4.5	3.4	0.4	0.8	1.1
15:0	0.6	0.6	0.2	0.4	0.5	0.6	0.5	0.2	0.4	0.4
16:0	19.6	23.5	7.7	24.2	30.4	18.9	13.5	5.3	23.4	23.6
17:0	0.7	0.1	0.8	1.0	1.5	0.7	tr	0.5	0.5	0.4
18:0	6.1	8.6	40.7	17.9	28.4	5.7	6.8	21.6	6.3	10.9
20:0	0.3	0.3	0.5	0.2	0.3	0.3	0.2	0.3	0.1	0.2
(%) Saturated)	(31.4)	(37.3)	(50.7)	(45.0)	(62.9)	(30.7)	(24.4)	(28.3)	(31.5)	(36.6)
n-Unsaturated										
14:1 ω5	0.3	1.0	0.3	0.1	0.3	0.3	0.3	0.1	–	0.1
15:1 ω6	0.2	0.7	0.8	0.5	1.0	0.1	0.6	1.9	0.5	1.7
16:1 ω7	4.8	5.2	1.3	2.1	2.3	4.9	3.6	0.7	1.6	1.6
17:1 ω8	0.8	1.2	1.0	0.5	1.0	0.9	0.9	1.4	0.4	0.5
18:1 ω9	27.4	30.7	12.4	22.2	19.9	27.8	32.6	11.8	15.7	27.7
18:2 ω6	2.5	3.9	1.2	1.4	1.3	2.7	9.8	2.6	2.6	6.9
20:1 ω9	2.6	3.4	1.1	1.2	1.2	2.8	5.7	1.6	1.4	3.8
20:5 ω3	6.6	1.4	3.8	6.2	1.2	7.1	1.9	5.5	7.5	1.9
22:1 ω11	1.6	0.2	1.8	2.0	1.0	1.3	0.8	1.9	1.7	1.1
22:4 ω6	0.9	–	0.2	0.4	–	0.8	0.8	0.5	0.4	–
22:5 ω3	1.8	–	1.1	1.5	0.4	1.6	–	1.4	1.8	0.7
22:6 ω3	14.3	9.6	20.6	15.0	4.9	13.5	13.1	39.7	32.7	10.3
24:1 ω9	–	–	–	–	0.6	–	–	–	–	6.0
Ratio ω3/ω6	6.7	2.8	18.2	12.6	5.0	6.3	1.4	15.0	42.0	1.9

[a] Related to percentage of total fatty acids.

[b] Abbreviations: TG (triacylglyercol); Card (cardiolipin); PE (phosphatidylethanolamine); PC (phosphatidylcholine); Sph (sphingomyelin); tr (traces, less than 0.1%); (–) not detected.

traenoic (20:4) acid, than those from temperate areas. Semi-Antarctic species contain higher proportions of ω3-series of polyunsaturated fatty acids.

The fatty acid composition of the neutral lipids (triacylglycerol) isolated from both the dark and white blue mackerel muscles did not change (Table 4) and confirmed other observations (Mai and Kinsella, 1979). This result is different from the fatty acid changes of the comparable triacylglycerol content of adipose tissues at different body depths of mammals (Body, 1988). The inner adipose tissues of ruminants had reduced levels of unsaturated fatty acids in their triacylglycerol fraction compared with the outer surface adipose tissues. A similar variation of fatty acids also applies to the inner and outer phospholipid muscle layers of rabbits (Bick et al., 1987) but this pattern is the opposite of that with the blue mackerel muscle phospholipid layers of fatty acids (Table 4).

The fatty acid profile in most of the marine phospholipid classes follows the pattern typical of mammal tissues (White, 1973). In both the dark and white blue mackerel muscle phospholipids, phosphatidylethanolamine contained less 16:0 than 18:0; the converse was observed in the fatty acid pattern of phosphatidylcholine. Furthermore, phosphatidylethanolamine contained slightly more 22:6 and 20:5 than phosphatidylcholine. The white muscle sphingomyelin fraction had a measurable level of tetracosenoic (24:1) acid which was specific to this group of lipids, although only a small quantity appeared in the dark muscle sphingomyelin fatty acids. These similarities of the fatty acid in these major phospholipids indicated that they played the same role in the fish muscle cell membranes as they did in other living organisms. The only difference appeared to arise from the fish cardiolipin fatty acids when they were compared with those associated with mammalian tissues. Rat liver mitochondria (Gray, 1964) and pig lung (Body and Gray, 1967) cardiolipin fatty acid are dominated by high concentrations (48–84%) of octadecadienoic (18:2) acid. This fatty acid does not appear at the same extent in the fish cardiolipin fraction isolated from blue mackerel tissues and confirms earlier findings reported (Gray and Macfarlane, 1961; Cossins and Macdonald, 1986).

The availability of any mackerel species available is beneficial to the fishing industry when provided in moderate abundance (Leu et al., 1981; Weeber, 1987), and at least 45 - 50% of the body weight is edible as fillets. The high amount of

lipid present is also advantageous for canning of products (Leu et al., 1981), and the fatty acids are suitable for the consumers concerned about reducing their risk of cardiovascular disorders. However, there are serious drawbacks in the industrial handling of mackerel. The spoilage of mackerel fillets is more rapid than fillets of many other fish and particular care is required for storage at sea before they are landed, and special attention for the preservation (shelf-life) of the consumable and end-products (Ke et al., 1976, 1977b, 1978).

These problems do vary when different parts of the edible mackerel fillets are being treated (Ke et al., 1977a). Dark muscle lipids are more susceptible to autoxidation degradation than the corresponding white muscle lipids (Ke and Ackman, 1976), due to the higher amount of Pro-oxidant catalysts (organic Fe content) in the dark meats (14.9 ppm in wet tissues), compared with white meats (0.2 ppm in wet tissues). This also explains why the outer dark muscle and skin of mackerel fillets are often discarded.

However, with the public awareness of the value of fish diets rich in ω3-polyunsaturated fatty acids to improve health (Dyerberg et al., 1978; Fogerty et al., 1986; Nestel, 1987), the much richer dark flesh (Tables 1 and 2) could be more attractive. It could be also used as a source of an extractable fish oil suitable for an enrichment in ω3-fatty acids process by urea fractionation (Ackman et al., 1988). These resultant fatty acids could be used in nutritional or pharmaceutical products.

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Effect of Modified Manufacturing Parameters on the Quality of Cheddar Cheese made from Ultrafiltered (UF) Milk

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———————————— ABSTRACT ————————————

Cheddar cheese was made from milk concentrated twofold by ultra-filtration (UF). Lowering the cooking and cheddaring temperature from 39°C to 35°C resulted in faster acid development, promoted more proteolysis, caused faster degradation of lactose, and contributed smoother body and texture to the cheese. Starter culture at 2% by weight of unconcentrated milk in combination with low cooking and cheddaring temperature reduced pH at faster rate and shortened the cheese making time by approximately 45 min, compared to cheese made using the traditional temperature (39°C). For the traditional temperature (39°C) of cooking and cheddaring, the addition of 0.2 mL/kg rennet of unconcentrated milk produced the same rate of proteolysis in both control and cheese made from UF retentate. Composition (fat, protein, salt and moisture) and yield of the UF cheeses with modified temperature treatments were not significantly different from control.

INTRODUCTION

ULTRAFILTRATION (UF) technology has been applied to cheese manufacture in many ways over the past 20 years. UF milk offers the advantages of increased plant efficiency, capacity and savings in energy and labor costs. In the Maubois, Mocquot and Vassal (MMV) process (Maubois et al., 1969), pre-cheese (UF retentate) is converted to cheese without any whey drainage or curd handling. This process is successful for some fresh and soft ripened cheese varieties such as Cream, Quark, Feta, and Camembert (Winwood, 1983; Mahaut et al., 1982; Puhan and Gallman, 1980; Covacevich and Kosikowski, 1977; Hansen, 1977; Maubois and Mocquot, 1975). These cheese varieties have relatively low total solids and do not require rigorous pH control. Manufacture of Cheddar and other hard ripened cheeses by the MMV process have not yet been successful.

The Low Concentrated Retentate (LCR) process uses milk concentrated to 1.5 - 2.5 times its original solids by UF and cheese is made by traditional procedures with minor modifications. The LCR process allows the conventional methods and equipment to be used which is important to a cheese plant considering an expansion in capacity but having space limitations. The LCR process is used to manufacture Camembert in France and Cottage cheese in the USA (Kosikowski et al., 1985; Matthews et al., 1976; Maubois and Mocquot, 1975). Another process, Medium Concentrated Retentate (MCR) with total solids 3 to 5 times the solids of original milk has been used to manufacture Feta cheese with open structured curd. Attempts have been made in Britain to prepare Cheddar by the MCR process (Green, 1985).

Cheddar cheese made from UF milk retentate (UF Cheddar cheese) is generally hard, crumbly, grainy, mealy, less springy, more sticky and bland without typical Cheddar flavor (Green et al., 1981; Green, 1985). Cheddar cheeses prepared from 1.5X and 2.0X UF retentate using the traditional procedure were harder, more rubbery, crumbly, chewy and grainy and less dense and less adhesive than control cheese (Pabari et al., 1988). The defects may be related to the effects of UF on the

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buffer capacity of retentate (Brule et al., 1974), the distribution of milk components between serum and dispersed phases, the activities of ripening enzymes and the initial gel structure (Green et al., 1981).

Conversion of lactose to lactic acid is an essential step for attaining proper pH, flavor and taste of the cheese; high residual lactose in the final cheese may result in poor cheese quality (Van Slyke and Price, 1949; Davis, 1965). Lactose in UF Cheddar cheese was reported to vary from 0.84% to 2.15% before pressing (Sutherland and Jameson, 1981). Kealey and Kosikowski (1985) also reported high lactose and other sugars in UF Cheddar cheese after 1 day manufacture (1.95% to 2.93%). The high lactose may be due to a high concentration of lactose in the serum phase of UF milk retentate as the retention coefficient of lactose is about 10% (Glover, 1985) or to altered rates of fermentation.

The increased buffer capacity of the retentate was found to increase the Cheddar cheese-making time in the manufacture of hard cheese from UF milk and is a second limiting factor (Covacevich and Kosikowski, 1979). Pre-acidification of milk prior to ultrafiltration has been used to achieve the proper final pH and overcome textural problems (Sutherland and Jameson, 1981).

Additional rennet above the amount needed to cause coagulation (0.2mL/L) may be necessary to obtain a normal ripening rate in UF Cheddar cheese (Green et al., 1981; Bynum and Barbano, 1985; Creamer et al., 1987) because cheese flavor and rate of proteolysis are proportional to the amount of rennet retained in the curd.

Temperature reduction during setting slows the coagulation process and decreases curd firmness (Scott-Blair and Burnett, 1959). This suggests that faster coagulation due to higher concentration can be offset by reducing the coagulation temperature about 2–5°C. A lower temperature of cooking may also retard syneresis resulting in higher moisture cheese with softer texture, because cooking helps to squeeze out whey and toughen curd cubes (Potter, 1986).

The objectives of this present study were to: (1) improve quality of Cheddar cheese from UF milk by using lower setting, cooking and cheddaring temperatures; (2) offset the effect of increased buffer capacity of the UF milk by addition of higher amounts of starter culture; and (3) overcome the slow ripening rate and produce characteristic flavor by adding rennet on the basis of the original amount of milk.

MATERIAL & METHODS

MILK was obtained from the Elora Dairy Farm of the Univ. of Guelph and was pasteurized at 62°C for 30 min. Ultrafiltration was carried out at 50°C in a spiral wound polysulfone membrane with 3–5,000 molecular weight cut-off (manufactured by Osmonics, Hopkins, MN). The milk was immediately cooled to below 10°C and stored overnight at the same temperature.

Cheese manufacture

Cheese was made in four rectangular, double-jacketed, steam-heated vats of 10L capacity, each containing about 6.5 kg of twofold UF milk except in case of control cheese which was made using about 12.0 kg of regular pasteurized milk. The milk was standardized to a

```
                    RAW MILK
                       |
        PASTEURIZE (62°C, 30 MIN)
                       |
          ULTRAFILTER (50°C, 2 X)
                       |
      STANDARDIZE (PROTEIN/FAT RATIO 0.96)
                       |
   HEAT MILK TO SETTING TEMPERATURE (TABLE 1)
                       |
     ADD STARTER CULTURE (2.0% OF ORIGINAL MILK)
                       |
               RIPEN (45 MIN)
                       |
     ADD RENNET (0.2mL/kg OF ORIGINAL MILK)
                       |
              SET (20 - 25 MIN)
                       |
          CUT THE COAGULUM (8 - 10mm)
                       |
   HEAT THE CURD TO COOKING TEMPERATURE (TABLE 1)
     IN ABOUT 30 MINUTES WITH GENTLE STIRRING
                       |
         HOLD UNTIL pH REACHES 6.0-6.1
                       |
     LET CURD SETTLE, DRAIN OFF THE WHEY
                       |
  CUT INTO BLOCKS, CHEDDAR UNTIL pH REACHES 5.4-5.5
                       |
   MILL AND SALT (2.5% NaCl BASED ON CURD WEIGHT)
                       |
               FILL HOOPS
                       |
     PRESS OVER NIGHT AT 150- 200 K PASCALS
                       |
     PACK IN POLYETHENE BAGS UNDER VACCUM
                       |
          STORE AT 8.5°C OR 15°C
```
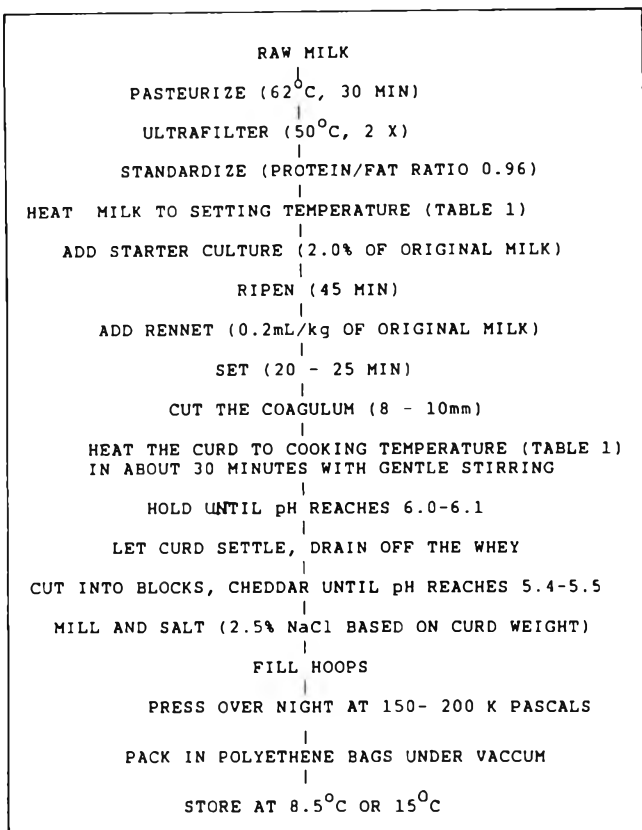
Fig. 1 — Cheddar cheese manufacturing procedure.

protein/fat ratio of 0.96 by adding low heat, nonfat dry milk. Lactic starter culture (mesophilic, Marschall brand supplied by Miles Laboratories, Madison, WI) was prepared by inoculating 2-fold concentrated milk with 1% frozen starter and incubating overnight at 32°C till the pH dropped to 4.6. This culture was added at the rate of 2% by weight of regular milk for control cheese and at the rate of 4% by weight of UF milk for UF Cheddar cheese. Single strength calf rennet (Dairyland Food Laboratories, Waukesha, WI) was used at the rate of 0.2 mL/kg of regular milk for control cheese and 0.4 mL/kg of UF milk for UF cheese. The cheese making procedure of Irvine (1986) was followed excepting modifications of temperatures of setting, cooking and cheddaring as shown in Table 1. A flow diagram of milk treatment and cheese manufacture is shown in Fig. 1. The entire experiment was replicated four times.

Analyses

The analysis of milk composition before and after UF was performed using a Multispec milk analyser in the Ontario Central Milk Testing Laboratory, Guelph. UF retentate was diluted with permeate before fat and protein determination. Cheese fat was determined by the Mojonnier (1984) method and lactose by a beta-galactosidase enzyme assay (Mannheim, 1986).

Table 1 — Modified temperature treatments used in cheese manufacture

	Temperature (°C)	
Treatment	Setting	Cooking & Cheddaring
Control: 30-39[a]	30	39
2X: 28-35 UF[b]	28	35
2X: 30-35 UF[c]	30	35
2X: 30-39 UF[d]	30	39

[a] Control cheese made from pasteurized milk using 30°C for setting and 39°C for cooking and cheddaring.
[b] UF cheese made from twofold UF retentate using 28°C for setting and 35°C for cooking and cheddaring.
[c] UF cheese made from twofold UF retentate using 30°C for setting and 35°C for cooking and cheddaring.
[d] UF cheese made from twofold UF retentate using 30°C for setting and 39°C for cooking and cheddaring.

The extent of proteolysis was assessed by release of free amino acids, determined by 2,4,6-trinitrobenzene sulfonic acid (TNBS) reaction with amines (Kuchroo, 1983). This method has been found to give a better measure of the hydrolysis of caseins by enzymes than determination of TCA-soluble nitrogen (Tam and Whitaker, 1972). The analysis of nitrogen was carried out by the micro-Kjeldahl method. Calcium, sodium and magnesium were analysed by atomic absorption (Varian model 125) in the Land Resource Science Laboratory, University of Guelph. A radiometer pH meter (Model pH M84) and combination electrode (Type GK2401C) were used to record the pH. Analyses of pH and lactose were made after 1, 15, 30, 45 and 60 days of ripening. The TNBS test was carried out after 30, 60 and 90 days of ripening.

Sensory evaluation was performed after 90 days by 5 expert panelists using a 9 point hedonic scale (9 = liked extremely, 1 = disliked extremely).

Statistical analysis

The data were analyzed using an analysis of variance "Split plot design" (SAS, 1985) as shown in Table 2. Error terms for main plot treatments (trt), subplot levels of time and treatment-time interactions were trt rep, time rep and time trt rep, respectively. If the treatment effect was found significant, then a t-test (LSD) was performed to test the comparison wise error rate within means of treatments and replicates.

The response variables tested were lactose, pH, free amino acids moisture, sensory score, fat, protein, calcium, salt in moisture ratio and cheese yield. The levels of time were 5 for both pH and lactose and 3 for free amino acids (TNBS).

RESULTS & DISCUSSION

Effect of setting temperature on curd formation

Setting temperature of the milk affected the rate of coagulation and curd firmness. The UF milk formed curd within 18–20 min when set at 30°C but took about 5 min more at 28°C. During cutting, the UF curd was firmer than the control. The curd formed at 28°C tended to clump slightly more during cooking, and the curd size was reduced due to frequent breaking of the curd by manual agitation. Green (1985) reported that lowering the coagulation temperature improved the curd structure of cheese made from 4X UF milk and produced less grainy but more sticky cheese; however, this was not observed on 2X milk in this experiment. Keeping the setting temperature at 30°C appeared to be better for easy handling of the curd.

Effect of cooking and cheddaring temperature

The pH drop during cooking and cheddaring was faster for UF cheese curd held at 35°C than at 39°C (Table 3). The time required to reach pH 6.1 (whey drainage) was similar for all treatments. However the pH drop to 5.4 (milling point) was about 45 min. faster for curd cheddared at 35°C than at 39°C. This suggested that a lower (35°C) cheddaring temperature would partially offset the effects of high buffering capacity probably because of more rapid growth of mesophilic lactic starters at 35°C than at 39°C (Robinsin, 1981). Less syneresis from the curd and comparatively smooth, soft texture were observed in cheese curd cooked and cheddared at 35°C than

Table 2 — Split plot design of analysis of variance

Sources	Degree of freedom
Main plot comparison	
Treatment (Trt)	3
Replicates (Rep)	3
Main-Plot Error Trt Rep	9
Sub-plot comparison	
Time	4
Time Rep	12
Time Trt	12
Time Trt Rep	36
Total	79

Table 3 – Cheese manufacturing time with different treatments[a]

| Treatments | Time taken from addition of culture to: | |
	Whey drainage[b] (min)	Milling[c] (min)
Control	230 ± 20	420 ± 51
2X: 28-35 UF	218 ± 16	401 ± 37
2X: 30-35 UF	214 ± 14	395 ± 39
2X: 30-39 UF	219 ± 11	438 ± 26

[a] See Table 1 for treatments. The data are based on the average of 4 replicates.
[b] Whey drainage at pH 6.1.
[c] Milling at pH 5.4-5.5.
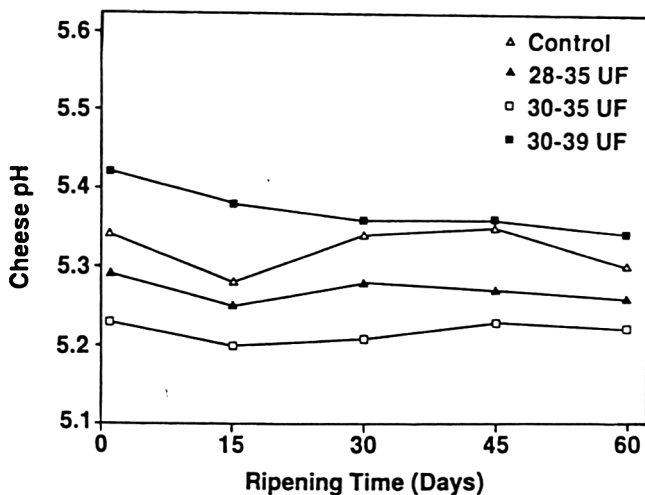
Fig. 2 – pH change in UF Cheddar cheese during ripening at 8.5°C.
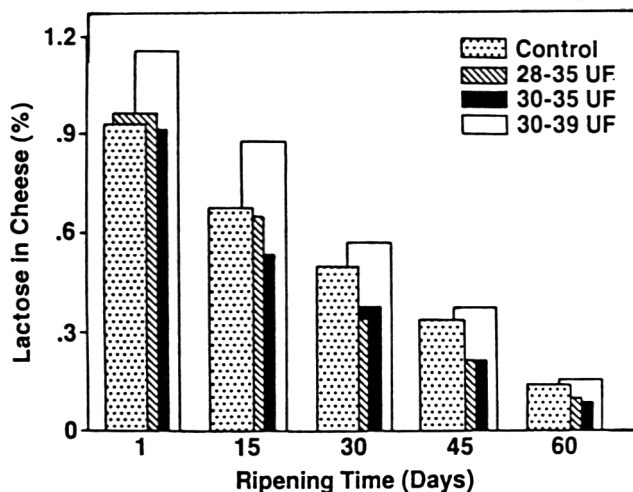
Fig. 3 – Lactose (%) degradation in the UF and control Cheddar cheeses during ripening at 8.5°C.

at 39°C. Thus, low cooking and cheddaring temperature may be a useful tool in improving UF cheese texture.

Change of pH during ripening

The pH reduction was greater in UF cheese made using lower cooking and cheddaring temperature (35°C) than using conventional temperature (39°C) (Fig. 2). The pH of the UF cheeses varied from 5.23–5.42 (1 day old) and 5.22–5.34 (60 days old). The pH was proportional to lactose measured in cheese 1 day after manufacture. For example, the cheese with high pH (5.42) had higher lactose (1.16%) and cheese with a low pH (5.23) had lower lactose (0.91%) (Fig. 2 and 3). This suggested that high pH was attributed to comparatively lower degradation of lactose during cheese manufacture. The pH de-

creased until 15 days of storage and then started increasing (Fig. 2) but the effect of storage time on pH was not statistically significant (P>0.05). The pH is expected to increase during ripening because of the neutralizing effect of alkaline products of protein degradation (Webb et al., 1974). The pH values were similar to values reported in the literature for traditional Cheddar cheese, which ranges from 4.80 to 5.29 after 1 day and 5.40 to 5.60 after 60 days of ripening (Dolby et al., 1937; Czulak et al., 1969). The pH values of UF Cheddar cheese were reported to range from 5.40 to 5.97 (Green et al., 1981; Sutherland and Jameson, 1981). High pH may be due to low acidity of curd at milling and high buffer capacity of the UF curd (Lawrence and Gillles, (1982). Their data suggested that adding more starter culture and lowering cooking and cheddaring temperature could reduce the UF cheese pH to a satisfactory level.

Degradation of lactose during ripening

Lactose degradation was significantly faster in cheese cooked and cheddared at 35°C (Fig. 3) than in UF cheese made using the traditional temperature of 39°C (P<0.01). The lactose degradation was significant with time (P<0.05) and its concentration ranged from 0.911–1.158% (1 day) and 0.085–0.157% after 60 days of ripening. The literature values also vary from 0 to 1% at different ripening times: 0.24–0.28% for 1 day old (Dolby et al., 1937); 0.43% for 7 days old (Harvey et al., 1981); 0.34% for 59 days old (Czulak et al., 1969). The variations may be due to different types of starter, seasonal variation in milk lactose, differences in salt-in-moisture ratios in final cheese (Turner and Thomas, 1980), different analytical techniques and differences in holding time of the curd in the whey before the whey is drained (Dolby, 1941; Lawrence and Gilles, 1982). If curd is kept in the whey for longer periods of time, diffusion of lactose from whey to curd may increase residual lactose in the cheese (Dolby, 1941; Czulak et al., 1969, 1970). However, this is questionable because the diffusion coefficient of large lactose molecules is small and the difference in lactose concentration between curd and whey is probably too small to allow an appreciable amount of diffusion (Lewis, 1974).

Cheese with high lactose at the time of hooping may develop low pH while maturing (Dolby et al., 1937) and may have a greater risk in off-flavor development due to nonstarter bacteira (Lawrence and Gilles, 1982). However, this was not observed in this experiment, probably because the high buffer capacity of UF cheese neutralized the effect as described by Huffman and Kristoffersen (1984). Lactose degradation followed similar trends in cheese treated with the same cooking and cheddaring temperature (Fig. 3). These data suggested thet lactose degradation in UF cheese could be enhanced by lowering the cooking and cheddaring temperature.

Effect on composition

Composition of cheese made from UF milk with modified temperatures was not significantly different with regard to fat, moisture, protein and salt in moisture ratio, when compared with control (Table 4). Moisture in non-fat substance (MNFS), salt in moisture (S/M) ratios and fat in dry matter (FDM) were within ranges for premium quality cheese (S/M = 4.0 - 6.0%, MNFS = 52 - 56% and FDM = 50 - 56%) as described by Lawrence et al. (1984). This suggested that a cooking and cheddaring temperature of 35°C could be adopted for cheese manufacture, while maintaining cheese composition within standard limits. Although moisture retention averaged about 1.5% higher in 30-35 UF cheese than 30-39 UF cheese, the difference was not statistically significant (P>0.05).

Calcium concentrations in UF cheese with low temperature of cooking and cheddaring were similar to the control (0.88 and 0.91%, respectively). Calcium was highest (0.96%) in UF

Table 4—Composition (%) of cheese made from UF milk with modified temperature treatments[a]

Treatments	Fat	Moisture	Calcium	Protein	S/M[b]	MNFS[c]	FDM[d]
Control	34.04	36.59	0.90	26.71	4.42	55.44	5'.60
2X: 28-35 UF	34.86	36.00	0.91	26.55	4.28	55.25	53.51
2X: 30-35 UF	33.76	37.04	0.88	26.55	4.27	55.90	50.96
2X: 30-39 UF	35.60	35.60	0.96	27.43	4.27	54.20	52.30

[a] See Table 1 for treatments. The data are based on the average of 4 replicates

[b] S/M = Salt in moisture ratio

[c] MNFS = Moisture in non-fat substance

[d] FDM = Fat in dry matter

Cheddar made using conventional processing temperatures, but the difference was only marginally significant ($p < 0.1$). More calcium may be due to high pH and high buffer capacity of the curd because calcium retention in cheese is directly proportional to the acidity at the time of draining the whey (Czulak et al., 1989). Calcium values in this experiment were higher than values reported by Kindstedt and Kosikowski (1988) which ranged from 0.606–0.83%. Hill and Ferrier (1988) observed calcium values ranging from 0.60–0.95% with a mean of 0.76% for Canadian Cheddar cheese. Higher calcium in UF cheese may be responsible for altered cheese structure.

Effect on yield

Yield of the UF cheese ranged from 20.35 - 20.81% of the concentrated UF retentate (Table 5). The moisture adjusted yield (based on 37% moisture) was similar among all treatments, indicating that Cheddar cheese yield was not increased by using 2X concentrated milk.

Effect on proteolysis

Proteolysis converts the rubbery textured green cheese to the smooth body typical of Cheddar cheese. Proteolysis of cheese measured as free amino acids was significantly higher in 30–35 UF (Table 1) cheese ($p < 0.01$) than in the control and other UF cheese. This increase was significant with time ($P < 0.05$). Proteolysis was faster during the first 4 weeks of ripening, which caused a slight pH increase during 15 to 30 days (Fig. 2 and 4). More free amino acids were released in the first 4 weeks of ripening (0.121 - 0.145 mmoles/g) compared to values reported in the literature. Hickey et al. (1983) reported the accumulation of free amino acids as 0.058 mmoles/g in control and 0.019 mmoles/g in UF Cheddar cheese after 8 weeks of ripening using S. cremoris as starter culture. These values were increased to 0.275 mmoles/g and 0.120 mmoles/g, using a special strain of L. helveticus as starter culture. In this experiment, 28-35 UF and 30-35 UF cheeses accumulated 0.167 and 0.181 mmoles/g respectively after 8 weeks, which was significantly higher ($P < 0.05$) than control (0.157 mmoles/g). The higher amount of proteolysis may have resulted from the greater amount of both starter culture and rennet, and retention of slightly more moisture (Hickey et al., 1983; Green et al., 1981; Lowrie and Lawrence, 1972). Proteolysis is influenced by pH, ratios of salt to moisture and of the moisture to casein and the temperature of ripening (Lawrence et al., 1983). Low

Table 5—Yield (%) of cheese made from different temperature treatments[a]

Treatments	Gross yield[b]	Corrected yield[c]	Moisture adjusted yield[d]
Control	10.58	10.58	10.54
2X: 28-35 UF	20.58	10.24	10.45
2X: 30-35 UF	20.81	10.40	10.40
2X: 30-39 UF	20.35	10.17	10.40

[a] See Table 1 for treatments. The data based on the average of 4 replicates

[b] Gross yield is based on the weight of the control and UF milk used for cheese manufacture.

[c] Corrected yield is gross yield/concentration factor

[d] Moisture adjusted yield is based on 37% moisture
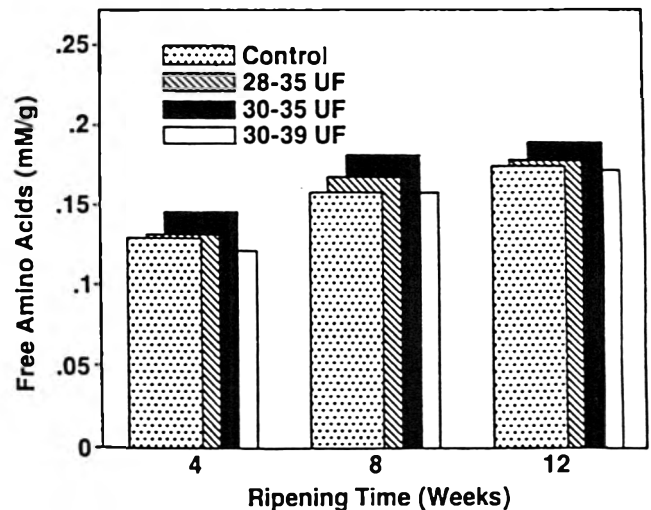
Fig. 4—Proteolysis in UF and control Cheedar cheese measured as free amino acids in mmoles/g during ripening at 8.5°C.

Table 6—Sensory score in cheese manufactured at different temperatures[a]

Treatments	Sensory Score
Control	6.65[b]
2X: 28-35 UF	6.15[b,c]
2X: 30-35 UF	5.90[c]
2X: 30-39 UF	5.92[c]

[a] See Table 1 for treatments. The data are based on the average of 4 replicates.

[b,c] Means with the same letters are not significantly different (P>0.05)

pH increases rennet activity and also solubilizes the calcium phosphate which increases the rate of proteolysis (O'Keeffe et al., 1975).

Sensory evaluation

Cheese was graded for flavor, taste, body and texture, using a 9-point hedonic scale. The sensory score of control cheese was higher (6.65) than UF cheese (5.90 - 6.15) (Table 6), but this difference was only marginally significant ($P < 0.07$). Sensory scores were not significantly different within UF cheeses ($P > 0.05$), in spite of differences in chemical parameters such as accumulation of free amino acids. The 30-35 UF cheese was criticized for slight acid flavor after 3 months of ripening, but its body and texture were consistently better than other UF treatments.

CONCLUSION

SATISFACTORY CHEESE was made from 2X UF milk by addition of a higher amount of starter culture (2% of original milk) and more rennet (0.2 mL/kg of unconcentrated milk) and by using typical setting, cooking, and cheddaring temperatures. UF cheese manufactured using lower cooking and cheddaring temperature (35°C) significantly increased the proteolysis and lactose degradation ($P < 0.01$) when compared to UF and control cheese cooked and cheddared at 39°C. The lower tem-

perature also imparted smoother body and texture to the cheese and reduced the cheese making time by increasing the rate of acid development. Lowering the setting temperature from 30 to 28°C, had no effect on the overall cheese quality. The yield of 30–35 UF cheese was slightly higher than 30–39 UF cheese, but the moisture adjusted yield was similar in all treatments. Sensory score was not significantly different amount treatments (P>0.05). Decreased cooking and cheddaring temperatures might also facilitate Cheddar cheese manufacture from more concentrated UF retentates.

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A Novel Approach to Quantify the Amount of Formaldehyde Added to Milk in Grana Padano Cheese Production

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─────────────── ABSTRACT ───────────────

Little information is available on the reaction products of formaldehyde (FA) with milk components. An isoelectric focusing (IEF) method was developed which detected a unique protein band with pI = 6.7 in defatted Grana Padano cheese samples prepared with FA. The quantity of FA (ppm) added to milk may be extrapolated by calculating the amount of protein associated with this band. The IEF method was rapid, simple, highly specific, sensitive, reproducible, inexpensive and easy to utilized in routine controls.

INTRODUCTION

FORMALDEHYDE (FA) is used as a bacteriostatic agent only in the production of the typical Italian cheese, Grana Padano, which is made with milk from dairy cattle of the Po Valley (Ministerial Decree, 1986; EEC Council Directive, 1978). For most of the year, the cattle in this area are fed on siloed fodder which improves milk yield, but at the same time causes an increase in spores of the Clostridium genus (Bottazzi and Corradini, 1987). These spores form clusters, which favor their germination and reproduction during the one year cheese ripening time, causing fermentation of lactic acid, lactate and lactose with the production of butyric acid, acetic acid and gas. As a result, holes or eyes develop inside the cheese causing cracking. To avoid this, FA as an antibacterial agent is added to the milk during the cheese-making process. In the case of Grana Padano cheese, 15-25 ppm FA (depending on the season) is added to the milk before it is placed in trays for separation of the curds and whey. This quantity is sufficient to inhibit and delay the massive growth of microflora in the milk (Bottazzi, 1964; Bottazzi et al., 1966; Rossi, 1964) without preventing the fat content from rising to the surface or affecting the natural microbial fermentation necessary to produce good quality cheese (Rossi, 1964; Bottazzi and Dell'Aglio, 1974).

The amount of free FA which remains in cheese after ripening is very low, below 0.5 ppm (Resmini et al., 1980b), and is not detectable by the Italian Official Method (a colorimetric method which uses chromotropic acid as a reactant) for evaluation of additive residues (Ministerial Decree, 1972). However, formaldehyde is a highly reactive chemical which readily combines with DNA, RNA, proteins and amino acids (Chaw et al., 1980; Hemminki, 1982; Siomin et al., 1973). The possible formation of reaction products of FA and milk components was previously investigated and it was demonstrated that ^{14}C-FA added to milk during the production of Grana Padano cheese reacted very rapidly with casein (Resmini et al., 1980b) and preferentially with histidine, the NH_2 terminal amino acid of the $gamma_2$-casein sequence (Resmini et al., 1980a; Restani et al., 1988). The presence of a unique FA-casein complex in FA-treated cheese thus could be used as an indicator in the early stages of ripening for FA treatment of milk used in Grana Padano cheese production. A method based in detection of this FA-casein complex would be useful

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in monitoring FA use in cheese production. The purpose of this study was to develop an isoelectric focusing procedure to detect the presence of reaction products of FA and milk proteins in Grana Padano cheese and to correlate the amount of such protein complex with the amount of FA added.

MATERIALS & METHODS

Cheese preparations

Grana Padano cheese produced with and without different amounts of FA (0, 5, 10, 15, 20 ppm) was kindly supplied by "Consorzio per la tutela del formaggio Grana Padano" Soresina, Italy. Two batches (each weighing 35 kg) for each FA concentration were prepared. Radioactive grana cheese, made using ^{14}C-FA (17.6 mCi/mmole; Amersham International, Bucks, UK) according to the standard procedures of Grana Padano cheese making, was kindly supplied by Prof. P. Resmini.

Sample preparation

Cheese samples (1g each) were chopped and extracted with diethyl ether (1:25, w:v; Merck, Darmstadt, FRG) to remove lipids. After sedimentation, the supernatants were removed and 10 mg of each pellet was resolubilized with 8 M urea (250 μL, Bio Rad Laboratories, Richmond, CA, USA). Samples were then ready to be loaded on isoelectric focusing polyacrylamide gel.

Isoelectric focusing (IEF)

Gels for IEF had the following final concentration: 7% acrylamide, 0.19% N,N'- methylene-bis-acrylamide, 5% carrier ampholytes pH 5-8, 6M Urea, 0.02% ammonium persulfate, 0.03% N,N,N',N'-tetramethylethylendiamine (TEMED). Samples (30 μL = 1.2 mg cheese) were loaded on gel, using cellulose application pieces, in the anodic area. When ^{14}C-Grana Padano was loaded on gel, 1.2 mg cheese corresponded to 22,000 dpm. IEF analyses were performed at 8°C (Multitemp II, LKB, Bromma, Sweden) and at constant power (15 W, Microdrive 5, LKB) for 4 hr, measuring the pH gradient at 0.5 cm intervals. Gels were dyed with Fast Green, overnight (16 hr) at room temperature (22.0° ± 2.0) (Gorovsky, et al., 1970).

Autoradiographies of dried gels were carried out for 30 days at 4°C with Kodak DEF-5, X-ray film (Eastman Kodak Comp. Rochester, N.Y., USA).

The protein amount associated with each band was evaluated by analyzing IEF gel with a laser densitometer (Ultroscan XL, LKB, Bromma, Sweden). Peak areas were calculated with the LKB 2400 Gel Scan XL Software Package, using an Olivetti Personal Computer M 24.

The quantification of band with pI = 6.7 was expressed as:

$$\frac{\text{Area of band}}{\text{Total area of gamma caseins}} \times 100$$

Two-dimensional electrophoresis

In the first dimension, IEF of ^{14}C-Grana Padano cheese (1.2 mg corresponding to 22,000 dpm) was performed as described above. After IEF, a gel strip (containing gamma caseins) was loaded on ureapolyacrylamide gel electrophoresis (Urea-PAGE: 2nd dimension) and analysed in parallel with ^{14}C-Grana Padano cheese (30 μg ^{14}C-Grana Padano cheese resolubilized in 30 μL 8M urea). The Running gel had the following final concentration: 10% acrylamide, 0.28% N,N'-methylene-bis-acrylamide, 4.9M urea, 0.35M tri-hydroxymethyl am-
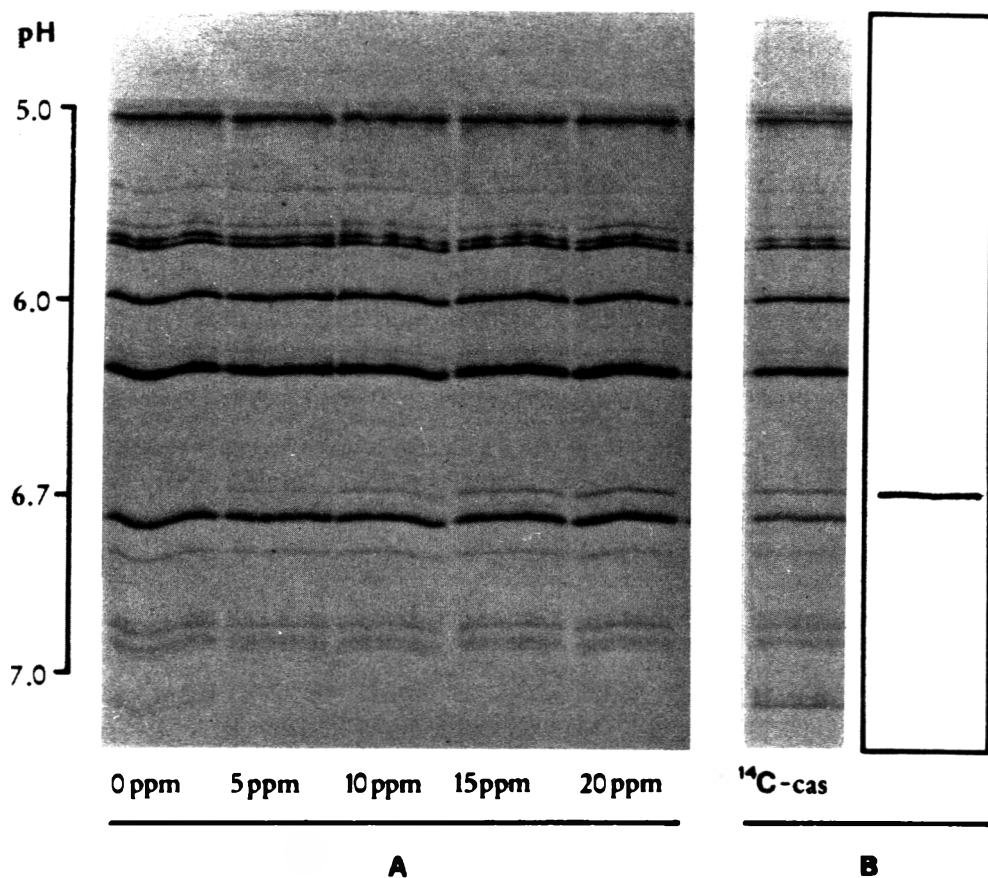
Fig. 1 — (A) IEF (^{14}C-caseins) and autoradiography (^{14}C) of ^{14}C-Grana Padano cheese produced with ^{14}C-FA (formaldehyde). ^{14}C-cheese was loaded on the same gel as samples shown in B. (B) IEF of cheeses produced with and without formaldehyde (0 to 25 ppm added to milk). Gel preparation, running and staining were performed as described in Materials & Methods.

Table 1 — Correlation between ppm of formaldehyde (FA) added to milk and protein associated with the band at pI = 6.7

ppm FA added to milk	Protein associated with the band at pI = 6.7[a] % ± S.E.	ppm FA calculated[b] $\bar{x}$ ± S.E.
0 (20)[c]	0.0	0.0
5 (7)	3.8 ± 0.3	5.4 ± 0.4
10 (7)	6.7 ± 0.3	9.7 ± 1.3
15 (7)	10.8 ± 0.5	15.8 ± 1.5
20 (7)	13.2 ± 0.9	19.4 ± 2.6

[a] Expressed as: $\dfrac{\text{Area of band with pI} = 6.7}{\text{Total area of gamma caseins}} \times 100$

[b] The amount of FA was calculated by using the curve:

$$Y = (0.669 \pm 0.029) X + (0.193 \pm 0.111)$$

[c] The figures in parenthesis indicate the number of cheese samples analyzed. Samples were prepared from at least two different batches of Grana Padano cheese.

inomethane (TRIS)-HCl buffer pH 8.5, 0.02% ammonium persulfate and 0.03% TEMED. The Stacking gel had the following final concentration: 4.5% acrylamide, 0.13% N,N'-methylene-bis-acrylamide, 4.9M urea, 0.12M TRIS-HCl pH 7.5, 0.04% ammonium persulfate, 0.03% TEMED. All chemical products were purchased from Bio Rad Laboratories, Richmond, CA. After the electrophoretic run, the gel was fixed overnight with 12% trichloroacetic acid (Merck, Darmstadt, FRG), to precipitate the proteins and to eliminate the carrier ampholytes; Coomassie Blue G 250 (Bio Rad Laboratories, Richmond, CA, USA) was used as dye. Autoradiographies of dried gels (Gel Dryer, LKB, Bromma, Sweden) were performed as described above.

RESULTS

GAMMA$_2$-CASEIN, which contains histidine at the amino terminal end, appeared to be the most reactive milk component for FA interaction, yielding spinancine (1,2,3,4 tetrahydro-H-imidazo [4,5 c] pyridine-6-carboxylic acid) (Restani et al., 1988). Modification of gamma$_2$ pI was further suggested as a consequence of the reaction described.
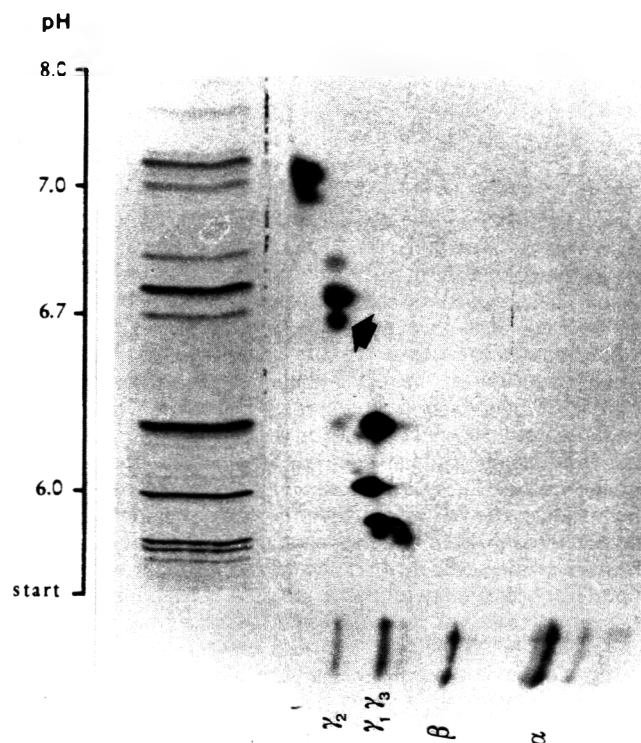
Fig. 2 — Two dimensional electrophoresis of ^{14}C-Grana Padano cheese. Arrow indicates the unique radioactive spot, detected in the gel. Gel preparation, running, staining and autoradiography were performed as described in Materials & Methods.

To verify this hypothesis, gamma fraction pIs were evaluated, using the isoelectric focusing technique. Defatted Grana Padano cheese samples (produced with and without FA) were loaded on 6M urea polyacrylamide gel containing the suitable pH gradient. The pH range 5-8 was chosen since it has been reported that pIs of gamma caseins are close to 7 (Trieu-Cuot, 1981).

The protein patterns obtained after IEF are shown in Fig.1B; Grana Padano cheeses produced with FA had a band with pI = 6.7, which was not detectable in cheeses prepared without FA. ^{14}C-Grana cheese (produced with ^{14}C-FA) was run in parallel with unlabeled cheese samples to localize the interaction products, namely the ^{14}C-IEF bands, to confirm that the band observed was a reaction product of FA and casein. The autoradiography of dried IEF gel showed that all the radioactivity recovered in gamma caseins was associated with the band with pI = 6.7 (Fig.1A). To identify the new band as the modified gamma$_2$-casein, a two-dimensional electrophoresis was performed using ^{14}C-Grana Padano cheese. The band with pI = 6.7 had mobility similar to gamma$_2$-casein (Fig. 2) and the identification was supported by the autoradiography of dried gel. In fact, the unique radioactive spot, observed after two-dimensional electrophoresis, and indicated by an arrow in Fig. 2, corresponds to the band with pI = 6.7 (1st dimension) and to gamma$_2$-casein (2nd dimension). To correlate the amount of FA added to milk (during the production of Grana Padano cheese) and the protein quantity associated with the band at pI = 6.7, the gels, obtained by loading cheese samples prepared with increasing amounts of FA, were analyzed with a laser densitometer. The ratios of the areas of the protein associated with the band at pI = 6.7 and the total gamma casein area were plotted against the ppm of FA and a straight line was obtained (Table 1) indicating that formation of the modified gamma$_2$-casein increased linearly with the increase of FA concentration in the cheese preparation. Regression analysis (x = ppm; y = ratio between area of band with pI = 6.7 and total gamma casein area) gave:

$$Y = (0.669 \pm 0.029) X + (0.193 \pm 0.111)$$
$$r^2 = 0.988.$$

DISCUSSION

ALTHOUGH it is generally accepted that the amount of free FA which remains in Grana Padano cheese after ripening is not of toxicological significance (WHO, 1974; Galli, 1983), a more specific method of control is necessary to avoid the improper use of this chemical. Considering the high reactivity of FA and the low sensitivity of the Italian Official Method (I.O.M.), available analytical methodology is inadequate to certify and quantify free or combined FA in cheese. Previous results (Resmini et al., 1980a, b; Restani et al., 1988) demonstrated that FA, added to milk, reacted very rapidly with gamma$_2$-casein during the cheese-making process. In particular, histidine, the NH$_2$-terminal amino acid of gamma$_2$-casein, combined with FA, giving rise to spinacine, a cyclic derivative (Restani et al., 1988). Since the amino terminal group of a peptidic sequence may influence the protein pI, a modification of gamma$_2$-casein pI was suggested. On this basis, IEF with the suitable pH range (pH 5-8) was performed to detect the presence of the hypothesized new band. Grana Padano cheeses produced with FA presented a new band with pI = 6.7, undetectable in cheeses prepared without the food additive. The new band was analyzed and indentified as modified gamma$_2$-casein. In fact, in the ^{14}C-Grana Padano cheese IEF pattern, since the band with pI = 6.7 was labeled, it must represent a reaction product of ^{14}C-FA and one of the milk proteins. Moreover, performing two-dimensional electrophoresis, the band

with pI = 6.7 behaved like gamma$_2$-casein as previously hypothesized.

This protein could represent a valid marker to identify and quantify the presence of FA in Grana Padano cheese. The IEF technique described above is highly selective, sensitive, reproducible and shows a significant correlation between FA added to milk and the amount of protein associated with the band at pI = 6.7 ($r^2 = 0.988$). Therefore the amount of modified gamma$_2$-casein relative to the total gamma caseins is an indirect method to quantify FA added to milk. Because of these characteristics, the method is appropriate for verification of the proper use of FA in Grana Padano cheese production. It is necessary to underline that I.O.M. is not able to differentiate the amounts of FA normally added to milk (from 15 to 25 ppm) because the free FA residues are always undetectable after the ripening period.

In conclusion, the IEF technique permitted the use of cheese as such, in the first period of ripening, without changing its properties and intrinsic characteristics. It was accurate and reproducible in the range of concentrations tested on the basis of the regression curve obtained on analyzing several cheese samples. Moreover, it was rapid (16 samples in 24 hr including the time for the preparation of the gels and for scanning the gels), simple (only extraction of lipids is required), relatively inexpensive and easy to utilize in routine controls. As a consequence, the use of this new method is suggested as an alternative to conventional methods.

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Uracil as a Potentially Useful Indicator of Spoilage in Egg Products

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ABSTRACT

The relationship between sensory acceptability of egg products and their uracil content was investigated. Pasteurized liquid whole egg, albumen, and yolk were allowed to deteriorate for 5–8 days at temperatures not exceeding 22°C. Periodically a panel of USDA inspectors evaluated the products' odors and samples were frozen for analysis. Uracil and uridine were determined by high performance liquid chromatography. Uridine contents decreased and uracil contents increased as the products deteriorated. Spoilage odors were detected only in products containing ≥ 1.7 μg/g uracil. Analysis for uracil is potentially useful for objectively detecting egg decomposition.

INTRODUCTION

UNDER THE EGG PRODUCTS INSPECTION ACT, USDA's Agricultural Marketing Service provides mandatory inspection for wholesomeness in plants processing liquid, frozen, or dried egg products and condemns products found to be adulterated. Adulteration of egg products due to decomposition is detected mainly by their odor as perceived by trained and licensed egg products inspectors. A simple, objective method that could be used to confirm the subjective sensory classification would be highly desirable.

Several compounds have been suggested as chemical indices of egg decomposition. The development of spoilage odors in egg products has been found to be associated with the appearance or increased concentrations of ethanol (Rayner et al., 1980; Brown et al., 1986), dimethyl sulfide (Brown et al., 1986), and formic, acetic, lactic, and succinic acids (Lepper et al., 1956; Hillig et al., 1960; Littman et al., 1982a). The organic acids are known to be imperfect criteria for decomposition, since some egg samples rejected on the basis of odor do not have abnormal concentrations of any of the four acids. Nevertheless, there has been sufficient interest in their use as spoilage indicators to prompt development of official methods for determining them in eggs using gas chromatography (GC) (AOAC, 1984). Different AOAC methods are required for the volatile acids and the nonvolatile ones, and both methods require relatively complex sample preparation. GC methods proposed more recently (Littman et al., 1982b; Stijve and Diserens, 1987) share these disadvantages. During preliminary work on use of high-performance liquid chromatography (HPLC) for simultaneously determining volatile and nonvolatile acids in egg products, it was observed that the most striking feature of chromatograms for spoiled products was a large peak that was identified (Morris, 1986) as uracil. The study reported here was conducted to ascertain the possibility of using uracil as an indicator of spoilage in egg products.

MATERIALS & METHODS

Egg products

Fresh, pasteurized liquid egg products—whole, yolk, and albumen—were obtained from processing plants designated as B, C, N, and S. Batches of product were placed in 30-lb lacquered tins, leaving

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a headspace to enhance odor detection. They were allowed to deteriorate for 5-8 days, during which time they were moved to warmer or cooler environments to accelerate or retard spoilage. Product temperature ranges during this period were 8-22°C for one group of products (cans 1-25) and 7-17°C for another group (cans 26-35). At intervals, the contents of each can were stirred aseptically and evaluated for odor. After each sensory analysis, one or more 6-oz samples were transferred aseptically to plastic cups and stored in a freezer.

Sensory evaluation

The sensory panel consisted of five trained and licensed USDA egg products inspectors. Each panelist classified the product's odor as satisfactory or eggy—the categories including products that are acceptable for human consumption—or as slightly sour, sour, slightly putrid, or putrid—odors that would result in product condemnation. Cans containing products in which spoilage odors had developed were isolated to prevent interference from cross-odors.

Chemical analysis

Samples were kept at temperatures no higher than -20°C until they were analyzed. A portion of the sample was obtained with the aid of an electric drill. Whole egg and albumen were analyzed for uracil, uridine, and formic acid by HPLC as described previously (Morris, 1987). Yolk was analyzed by the same procedure except that the mixture of yolk and precipitant was centrifuged for 20 min rather than 10 min. In calculating contents of analytes in the product, the weight of precipitated protein and lipid was estimated as 23.29% of the whole egg weight, 43.17% of the yolk weight, or 10.14% of the albumen weight (Posati and Orr, 1976).

RESULTS & DISCUSSION

CHANGES in uracil and uridine contents in whole egg (can 12) as spoilage odors developed are illustrated in Fig. 1. At 36 hr the uracil concentration had increased from 0.002 to 0.029 μmol/g. At this point all five members of the sensory panel still considered the product acceptable, but four of them classified its odor as eggy. During the next 14 hr there was a rapid, linear increase in uracil concentration as increasing num-

Fig. 1 — Effect of storage at 8–22°C on uracil (●—●) and uridine (○—○) concentrations in pasteurized whole egg and on number of panelists judging product unacceptable (▲—▲).

Table 1 — Uracil and uridine contents of pasteurized whole egg before and after first detection of spoilage odor

Can[a]	Storage Time (hr)	Storage Temperature (°C)	Odor category[b] Acceptable Sat	E	Unacceptable SS	S	Concentration (µg/g) Uridine	Uracil
1B	24		5	0	0	0	31.6	0.4
	36	13-22	0	1	4	0	30.8	4.9
8N	36		1	4	0	0	22.4	0.7
	40	13-22	0	2	3	0	33.5	5.0
11B	24		5	0	0	0	45.6	0.3
	36	13-22	0	0	5	0	45.4	5.4
12C	24		4	1	0	0	24.3	0.3
	36		1	4	0	0	24.0	3.2
	40	13-22	0	4	1	0	21.9	11.3
14S	13		4	1	0	0	24.·	0.6
	20		5	0	0	0	30.7	3.7
	24	13-22	0	2	1	2	20.2	3.9
17C	24		4	1	0	0	24.5	0.9
	36	13-22	0	1	3	1	2.5	13.8
18N	24		5	0	0	0	16.5	0.3
	36	13-22	0	0	2	3	17.6	13.5
28B	52		0	5	0	0	40.1	0.2
	67	7-17	0	0	5	0	30.9	2.7
30B	52		0	5	0	0	25.4	0.2
	67	7-17	0	3	2	0	28.0	2.0
31B	52		0	5	0	0	94.1	0.7
	67	7-17	0	0	5	0	27.8	2.7
35B	52		0	5	0	0	23.9	0.2
	67	7-17	0	2	3	0	24.3	1.7

[a] B, C, N, S = processing plants.
[b] Number of panelists classifying the product's odor as satisfactory (Sat), eggy (E), slightly sour (SS), or sour (S).

bers of panelists judged the product unacceptable. The rapid decrease in uridine concentration during the last half of this 14-hr period suggests that part of the uracil formed was derived from uridine. Other possible precursors, the uracil nucleotides, could not be determined by the HPLC method used because they would have eluted near the void volume with other substances excluded from the column packing material. After 24 hr, however, the total area of peaks in this early part of the chromatogram began to decrease· rapidly.

Results of sensory evaluations and chemical analyses of 11 batches of pasteurized whole egg when they were first judged unacceptable by any panelist, and at the last previous sampling, are shown in Table 1. (The original batch numbering system is retained to facilitate comparisons with results of GC analyses of the same products (Brown et al., 1986).) In all batches the uracil concentration had increased at least to 1.7 µg/g when a panelist first detected a slightly sour odor. For nine batches the last sample of product judged acceptable by all panelists had a uracil concentration below 1µg/g. The only samples without spoilage odors in which the uracil concentration was unusually high were the 36-hr sample from can 12 and the 20-hr sample from can 14. The former also contained more than the trace of dimethyl sulfide usually found in acceptable egg products (Brown et al., 1986). Storage at the lower temperature range not only led to slower development of spoilage odors but also appears to have resulted in lower uracil concentrations when these odors were first detected.

Data on development of spoilage odors and chemical changes in two batches of yolk (Table 2) followed patterns typical of data for whole egg stored at the same temperature.

Spoilage odors developed more slowly in the three batches of albumen (Table 3), as would be expected in view of albumen's ability to retard certain types of microbial growth (Elliott and Hobbs, 1980). In all three batches, the uracil concentration had increased to more than 5 µg/g when a panelist first judged it unacceptable. In can 21, there was a sharp increase to this level with the first detection of unacceptable odor, but in the other two batches this increase was detected before the spoilage odor. The three samples with unusually high uracil concentrations for their odor category—the 116-hr and 120-hr samples

from can 23 and the 116-hr sample from can 22—also contained substantial amounts of dimethyl sulfide (Brown et al., 1986). The formic acid concentration in the latter sample was 124 µg/g, a level high enough to constitute evidence of spoilage (Lepper et al., 1956).

A total of 46 samples from 16 batches were analyzed. The data are summarized in Table 4. They clearly establish the sensitivity of uracil as an indicator of spoilage in egg products: all 22 samples in which spoilage odors were detected contained at least 1.7 µg/g uracil. In five samples the uracil concentration had increased to this level but all five panelists still judged the product acceptable. The latter finding may have been due to the uncertainty to be expected in data based on subjective judgment, to formation of uracil at an early stage of the decomposition, before unpleasant odors developed, and/or to variations in the microflora present. Additional evidence that at least four of these five samples had undergone decomposition is that their contents of other spoilage indicators were unusually high for acceptable products, as mentioned above. Exceptions to the rule that spoilage odors develop as microorganisms grow in egg products are well known, and proprietary processes for controlled bacterial fermentation of albumen to remove glucose are based on them (Elliott and Hobbs, 1980; Hill and Sebring, 1986). A sample of albumen desugared by such a process contained 22.4 µg/g uracil, 1.0 µg/g uridine, and 505 µg/g formic acid—levels that would be normal in a sour product. However, if a similar fermentation was responsible for the relatively high uracil contents in the five samples in question, it was followed by a more usual type of decomposition: detection of spoilage odors followed within 4 hr for whole egg and within 4-20 hr for albumen.

No additional evidence of a causal relationship between bacterial growth and uracil formation in egg products has been sought, but the existence of such a relationship would not be surprising. Enzymes catalyzing uracil formation from exogenous nucleosides are distributed in a variety of microorganisms (Wang et al., 1983; Utagawa et al., 1985). Hicks et al. (1985), discussing the only previous investigation linking an increase in uracil content to deterioration of a food product, suggested that the uracil formation accompanying a decrease in the flavor

Table 2—Uracil and uridine contents of pasteurized yolk before and after first detection of spoilage odor

Can[a]	Storage time[b] (hr)	Odor category[c]				Concentration (µg/g)	
		Acceptable		Unacceptable		Uridine	Uracil
		Sat	E	SS	S		
24N	24	4	1	0	0	19.3	0.3
	36	0	1	3	1	1.5	8.1
25N	24	4	1	0	0	21.9	0.2
	36	0	4	1	0	0.9	7.9

[a] N = processing plant.
[b] The storage temperature was 13-22°C.
[c] Number of panelists classifying the product's odor as satisfactory (Sat), eggy (E), slightly sour (SS), or sour (S).

Table 3—Changes in uracil and uridine concentrations in pasteurized albumen during spoilage

Can[a]	Storage		Odor category[b]				Concentration (µg/g)	
	Time (hr)	Temperature (°C)	Acceptable		Unacceptable		Uridine	Uracil
			Sat	E	SS	S		
21S	116		0	5	0	0	13.2	1.1
	120	13-22	0	5	0	0	11.1	1.3
	136	8-22	0	2	3	0	1.5	5.9
22S	0		5	0	0	0	–	0.0
	92		2	3	0	0	17.0	0.9
	116		0	5	0	0	1.7	6.3
	120	13-22	0	1	4	0	1.5	6.3
	183	8-22	0	0	0	5	–	24.6
23S	92		1	4	0	0	17.7	0.4
	116		0	5	0	0	8.8	5.2
	120	13-22	0	5	0	0	2.4	5.4
	136	8-22	0	3	1	1	2.1	6.7

[a] S = processing plant.
[b] Number of panelists classifying the product's odor as satisfactory (Sat), eggy (E), slightly sour (SS), or sour (S).

Table 4—Relationship between uracil concentration and sensory acceptability for all (46) samples

Uracil concentration (µg/g)	Unacceptable ratings		
	0	1-2	3-5
0.0 - 1.3	19	0	0
1.7 - 6.3	5	1	8
6.7 - 28.8	0	3	10

quality of goats' milk resulted from the activity of enzymes produced by spoilage organisms. On the other hand, Parris et al. (1983) found that analogous changes in inosine monophosphate and hypoxanthine concentrations in stored meat, which had been suggested as chemical indicators of decreasing quality, are results of meat enzymes and not microbial activity. Spoilage of fish has often been measured in terms of the accumulation of hypoxanthine and other products of adenosine triphosphate degradation (Gill et al., 1987); the hypoxanthine formation initially is due to endogenous enzymes but subsequently to bacterial action also (Jones and Murray, 1962).

We have shown that uracil is potentially useful as an indicator of decomposition in egg products. To translate these findings into a practical test for confirming sensory classification will require that ranges of uracil concentrations indicating acceptability or unacceptability of various products be more clearly established; the relatively high uracil contents in some acceptable albumen samples suggest that different ranges may be appropriate for different products. Uracil's thermal stability and nonvolatile nature may make it useful for determining whether dried egg products were prepared from acceptable liquid products.

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Chromatographic Analyses of Xanthophylls in Egg Yolks from Laying Hens Fed Turf Bermudagrass (*Cynodon dactylon*) Meal

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ABSTRACT

The characterization of xanthophylls in egg yolks from laying hens fed turf bermudagrass (*Cynodon dactylon*) meal was studied. Laying hens were fed 2 wk on a milo-soybean meal diet to deplete the birds' xanthophyll stores from developing ova prior to introduction of 9% turf bermudagrass. After an additional 4 wk the various carotenoids present in egg yolks were determined by open-column, thin-layer, and high-performance liquid chromatography. Chromatographic analyses suggest that the yolk color was due mainly to the presence of free lutein and zeaxanthin at a ratio of 4:1. Neoxanthin and violaxanthin were present in minor amounts. The presence of β-carotene was not detected.

INTRODUCTION

THE COLOR of egg yolks or the degree of pigmentation is important in table eggs for direct consumption by the consumer as well as for various food manufacturers. Yolk color is dependent upon the dietary concentration of carotenoids, primarily xanthophylls, and their subsequent absorption and deposition into the developing ova by the laying hen. In addition to imparting color to egg yolks, there is increasing evidence that carotenoids such as β-carotene are anticarcinogenic (Peto et al., 1981; Alam and Alam, 1987; Dedari et al., 1987; Pastorino et al., 1987) as well as immunoenhancing (Alexander et al., 1985; Bendich and Shapiro, 1986).

Marusich et al. (1960) found that canthaxanthin, isozeaxanthin, isozeaxanthin diacetate, and capsanthin imparted an orange tinge to the yolks which was not acceptable. Williams et al. (1963) reported that there was a greater deposition of carotenoids in eggs from hens receiving corn than from hens receiving alfalfa or corn gluten meal. Nelson and Baptist (1968) reported that carotenoid pigments extracted from anaerobic mud, algae, tomato paste, lobster shells, and bacteria were deposited in egg yolks. These pigments include lutein, myoxanthophyll, lycopene, astaxanthin, and bacterioruberin. Marusich and Bauernfeind (1970) demonstrated that xanthophylls varied in their ability to pigment yolks. Buchecker and Eugster (1979) found the deposition of 3'-oxolutein in egg yolks receiving a diet containing lutein. Tyczkowski et al. (1986) also found the deposition of 3'-oxolutein in egg yolks of chickens fed a diet high in lutein and free of detectable 3'-oxolutein.

The primary xanthophyll sources for egg yolk deposition in the past decade have been corn, corn gluten meal, and dehydrated alfalfa meal. Dehydrated grasses such as Coastal bermudagrass and turf bermudagrass also represent potential xanthophyll sources for commercial poultry feeds. The utilization of Coastal bermudagrass as a pigmenting agent to laying hens has been well documented (Barnett and Morgan, 1959; Wheeler and Turk, 1963; Wilkinson and Barbee, 1968; Marusich and Bauernfeind, 1970; Burdick and Fletcher, 1984; Fletcher et al., 1985). Turf bermudagrass has also recently been evaluated as a pigmenting source for laying hens (Chen

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and Bailey, 1988; Bailey and Chen, 1988a). The purpose of this study was to determine the various xanthophylls present in egg yolks from laying hens fed turf bermudagrass meal.

MATERIALS & METHODS

Instrumentation

The HPLC instrument consisted of an IBM LC/9533 Ternary Gradient Liquid Chromatograph (Danbury, CT, U.S.A.) with an IBM UV-VIS fixed wavelength detector employing a 440 nm filter and a Waters Nova-Pak C18 stainless-steel column (3.9 mm i.d. × 15 cm). A solvent system of water:acetonitrile:chloroform (2:83:15) (Chen and Bailey, 1987) pumped at a flow rate of 1.0 mL/min was used. Relative carotenoid concentrations were determined by triangulation of the chromatograph peaks. Spectrophotometric determinations were made with a Beckman DU-6 Spectrophotometer (Irvine, CA, U.S.A.).

Materials

Neoxanthin and violaxanthin standards were prepared from saponified turf bermudagrass extract by thin-layer chromatography (TLC) as described by Chen and Bailey (1987). Lutein and zeaxanthin standards were prepared from corn by open-column chromatography using a method similar to that described by Quackenbush et al. (1961). The silica gel G TLC plates were purchased from Whatman (Clifton, NJ). All solvents were purchased from Fisher Scientific Co. (Pittsburgh, Pa. U.S.A.). Acetonitrile, water, and chloroform were HPLC grade. Acetone, hexane, toluene, ethanol, methanol, ethyl acetate, petroleum ether (b.p. 37.9–55.4 C), and benzene were ACS grade. HPLC grade solvents were filtered through a 0.2-um membrane filter and degassed under vacuum prior to use.

Turf bermudagrass was fresh-cut from Maxim Productions in South Central Texas. The grass was stored in a dark, ventilated room to dry for one week before grinding into fine materials with a hammer mill fitted with a 16 mm screen. A total of 120 laying hens, 25 wk of age, were depleted of xanthophylls on a grain sorghum basal diet for 14 days. After depletion, the birds were separated into eight groups of 15 hens. Diets containing 9% turf bermudagrass as the sole xanthophyll source (Table 1) were then randomly assigned to four groups. The remaining 4 groups were maintained on the grain sorghum basal diet. Eggs were collected once a week for yolk color analysis. The eggs were opened and the yolk was separated intact from the albumin and rolled across a moistened paper towel to remove the chalazae and any adhering albumen. The color was then scored visually using a 1984 Roche color fan (Nutley, NJ). After 4 wk, four eggs from each group of the hens fed turf bermudagrass were collected for carotenoid analyses.

Preparation of lutein and zeaxanthin standards from the saponified corn extract

A 30-g aliquot of freshly ground corn was extracted with 75 mL hexane:acetone:ethanol:toluene (10:7:6:7) in a 250 mL vol. flask. The resulting slurry was saponified by adding 10 mL 40% methanolic KOH under N₂ overnight. Seventy-five mL hexane was added to the flask, which was then diluted to volume with 10% Na₂SO₄, mixed and let stand for 1 hr before decanting 10 mL of the hexane layer onto a column containing a mixture of diatomaceous earth and adsorptive magnesia at 1:1 ratio. The total hydrocarbon fraction was eluted with hexane:acetone (90:10). The zeinoxanthin and cryptoxanthin fraction were eluted with hexane:acetone:methanol at a ratio of 89:10:1. The lutein and zeaxanthin fraction were next eluted with

Table 1—Composition of milo and bermudagrass diets

	Turf bermudagrass meal (%)	
Ingredients	0	9
Ground grain sorghum	69.61	60.54
Dehulled soybean meal	19.81	18.02
Turf bermudagrass	–	9.00
Oystershell flour	7.98	7.66
Defluorinated phosphate	1.92	1.92
Animal-vegetable fat	3.41	2.24
Vitamin premix[a]	0.25	0.25
Sodium chloride	0.20	0.13
DL-methionine	0.18	0.18
Manganese sulfate	0.025	0.025
Zinc oxide	0.025	0.025
Calculated analysis:		
Metabolizable energy, kcal/kg	2772	2772
Protein, %	16.60	16.60
Calcium, %	3.70	3.70
Available phosphorus, %	0.50	0.50
Methionine, %	0.38	0.38
Methionine + cystine, %	0.60	0.60
Lysine, %	0.76	0.76
Tryptophan, %	0.21	0.23
Xanthophyll (mg/kg)	–	35.68

[a] Vitamin premix provides (per kg of diet): vitamin A, 11,000 IU; vitamin D_3, 1,100 ICU; vitamin E, 11 IU; riboflavin, 4.4 mg; Ca pantothenate, 12 mg; nicotinic acid, 44 mg; choline C1, 220 mg; vitamin B_{12}, 6.6 μg; vitamin B_6, 2.2 mg; menadione, 1.1 mg (as MSBC); folic acid, 0.55 mg; d-biotin, 0.11 mg; thiamin, 2.2 mg (as thiamin mononitrate); ethoxyquin, 125 mg.

Fig. 1—Time course effects of turf bermudagrass on egg yolk pigmentation.

Table 2—Chromatographic fractions and identification of lutein and zeaxanthin standards from saponified corn extract

	Major components	
	Lutein	Zeaxanthin
Eluant[a]	88.5:10:1.5	88:10:2
	Visible spectra[b]	
Lambda max observed	419,445,475	424,449,480
Lambda max reported[c]	418,444,474	422,450,481
	R_f	
Observed	0.58	0.51
Reported[c]	0.57	0.53

[a] Ratio of hexane:acetone:methanol in percent by volume.
[b] Spectra were obtained by scanning with a Bechman DU-6 spectrophotometer. The solvent used was methanol.
[c] Reported data of visible spectra and R_f are from a reference by Davies (1976).

hexane:acetone:methanol at a ratio of 88.5:10:1.5 and 88:10:2, respectively. Each fraction was further purified on silica gel G TLC plates by employing a solvent system of methanol:ethyl acetate:benzene (5:20:75) (Davies, 1976). R_f values were determined and each band was then eluted with acetone under slight suction and evaporated to dryness. The lutein and zeaxanthin standards were dissolved in methanol to determine the visible spectra with a Beckman DU-6 Spectrophotometer, and peak purity was assessed with the IBM LC/9533 Ternary Gradient Liquid Chromatograph.

Preparation and extraction procedure of egg yolks

Four samples of egg yolks (2g) from each replicate were extracted with 30 mL hexane:acetone:ethanol:toluene (10:7:6:7) in a 100 mL vol. flask. Two samples were saponified by adding 2 mL 40% methanolic KOH to each sample under N_2 overnight while the other two samples were extracted without saponification. Thirty mL hexane was added to each flask which was then diluted to volume with 10% sodium sulfate and mixed. After 1 hr, 10 mL of the upper phase was evaporated to dryness and dissolved in 10 mL chloroform. This extract was then filtered through a 0.2-μm membrane filter. Ten μL extract was injected onto the HPLC column and developed with water:acetonitrile:chloroform (2:83:15) with a solvent flow rate of 1.0 mL/min. Samples were monitored at 440 nm with a sensitivity at 0.05 a.u.f.s. All the sample preparations were conducted under diffused light and samples were kept under N_2 whenever possible during the procedure. Identification of the various carotenoids in egg yolks was verified by comparison of retention time with standards and co-chromatography with added standards.

Separation and identification of various carotenoids in egg yolks by TLC

A solvent system of methanol:ethyl acetate:benzene (5:20:75) (Davies, 1976) was used to separate the various carotenoids in concentrated egg yolk extracts on silica gel G TLC plates. Individual carotenoids were characterized by comparing their R_f values and absorption spectra with values reported in the literature (Davies, 1976). For co-chromatographic test, neoxanthin, violaxanthin, lutein and zeaxanthin were used as reference standards.

RESULTS & DISCUSSION

EGG YOLKS were essentially devoid of pigment after 14 days on the grain sorghum diet as evidenced by a Roche color score of approximately 1 (Fig. 1). Egg yolks from hens fed the control diet remained at this level of pigmentation for the next

4 wk. Hens fed 9% turf bermudagrass laid eggs with Roche color scores of approximately 7 after only 1 wk of feeding, leveled off by 2 wk, and remained there for the remainder of the study. This clearly indicates that the vast majority of yolk pigments came directly from the diet rather than minor stores that may have been present in the skin or liver and is in agreement with the results of Williams et al., 1963.

Xanthophylls in egg yolks

Lutein and zeaxanthin standards were prepared from corn because it contains relatively high amounts of lutein and zeaxanthin (Quackenbush et al., 1961). The chromatographic fractions and identification data of lutein and zeaxanthin standards from the saponified corn extract are shown in Table 2. The ester fraction was not found because it was removed by saponification. The eluant used in our experiment was a bit different from that used by Quackenbush et al. (1961). These authors used hexane-acetone (90:10) to elute all hydrocarbon and monohydroxy carotenoids. In our laboratory, however, we found that hecane-acetone (90:10) could only elute total hydrocarbons and not the monohydroxy carotenoids. This was confirmed by comparing retention times of monohydroxy carotenoids on HPLC chromatogram and solvent systems used for separation of carotenes and xanthophylls in dried plant materials and mixed feeds by AOAC (1980). This may be due to the difference in adsorption affinity of pigments on the colum even under the condition of using the same packing material made by different companies. After the hydrocarbon fraction was eluted it was possible to separate xanthophylls into five well-defined bands by employing a solvent system of hexane:acetone:methanol (89:10:1). The first and second bands were the monohydroxy carotenoids, zeinoxanthin, and cryp-

toxanthin, respectively. They were identified according to a report by Quackenbush et al. (1961). The third and fourth bands were dihydroxy carotenoids, namely lutein and zeaxanthin. They were identified by determining the visible spectra and R_f values, and comparing with those reported in the literature (Davies, 1976).

The chromatogram of carotenoid standards is shown in Fig. 2. Although the solvent system of water:acetonitrile:chloroform (2:83:15) provided a clear separation of these standards there were some minor peaks present. These peaks were probably lutein and zeaxanthin isomers originally present in corn extract.

The chromatograms of egg yolks with and without saponification, respectively are shown in Fig. 3. The peaks were identified by comparing retention time with standards and co-chromatography with added standards. Both saponified and unsaponified egg yolks showed the same chromatogram, which implied only free xanthophylls were present in the egg yolks. This is in agreement with a report by Philip et al. (1976) who found that the egg yolk xanthophylls from different diets containing lutein fatty acid esters were free lutein. Lutein monoester elutes after free lutein and prior to β-carotene with a retention time of approximately 6 min on our HPLC system (data unpublished). The identification data for the pigments of egg yolks separated by TLC are shown in Table 3. The identification of carotenoids was further confirmed by co-chromatography of egg yolk extract with standards prepared from corn extract on silica gel G TLC plates. The average ratio of lutein to zeaxanthin in egg yolks was about 4:1 (Table 4).

The major carotenoids present in turf bermudagrass are neoxanthin, violaxanthin, lutein, and β-carotene (Chen and
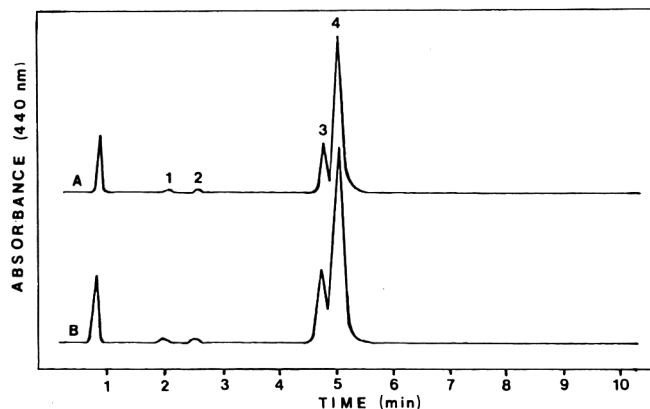
Table 3 — TLC identification of carotenoids in egg yolks from laying hens fed 9% turf bermudagrass meal

	Carotenoids	
	Lutein	Zeaxanthin
	Visible spectra[a]	
Lambda max observed	420,445,475	425,450,480
Lambda max reported[b]	418,444,474	422,450,481
	R_f	
Observed	0.58	0.52
Reported[b]	0.57	0.53
Color	light orange	light orange

[a] Spectra were obtained by scanning with a Bechman DU-6 spectrophotometer. The solvent used was methanol.

[b] Reported data of visible spectra and R_f value are from a reference by Davies (1976).

Table 4 — Ratio of lutein to zeaxanthin in egg yolks from laying hens fed 9% turf bermudagrass meal[a]

Replicate	Lutein:Zeaxanthin	Area ratio
1	4.88 ± 0.07	83 : 17
2	4.26 ± 0.02	81 : 19
3	3.35 ± 0.17	77 : 23
4	2.57 ± 0.12	72 : 28

[a] The values for the ratios represent four randomly selected eggs from each replicate.

Bailey, 1987). Zeaxanthin is also present at lower concentrations (Bailey and Chen, 1988b). Of these carotenoids lutein and zeaxanthin are the most effective yolk pigmentors (Brockman and Volker, 1934; Gillam and Heilbron, 1935; Peterson et al., 1939; Williams et al., 1963; Philip et al., 1976; Tyczkowski and Hamilton, 1986). The significant amounts of zeaxanthin present in egg yolks from hens fed turf bermudagrass may be the result of increased efficiency of deposition. Williams et al., (1963) reported that zeaxanthin was more efficiently utilized than lutein for deposition in the egg yolk and Peterson et al. (1939) found that purified zeaxanthin was deposited in egg yolk approximately twice as efficiently as purified lutein. Quackenbush et al. (1965) also reported that the depth of color produced was slightly less for lutein than for zeaxanthin in skin pigmentation.

Interestingly, we were not able to identify β-carotene in the egg yolk fractions even though it has been reported in turf bermudagrass (Chen and Bailey, 1987). This may be because β-carotene is largely converted to retinal in the intestinal mucosa during absorption. We have analyzed egg yolks from hens fed yellow corn and found substantial amounts of β-carotene (unpublished data).

In conclusion turf bermudagrass was found to be an effective yolk pigmentor. The xanthophylls primarily responsible for the yolk color were lutein and zeaxanthin, present at a ratio of about 4:1. β-Carotene could not be identified in the egg yolks from hens fed 9% turf bermudagrass, probably because of conversion to retinal.

Fig. 2 — A chromatogram of carotenoid standards by employing an isocratic solvent system of water:acetonitrile:chloroform (2:83:15). Peaks: 1 = neoxanthin; 2 = violaxanthin; 3 = zea xanthin; 4 = lutein.

Fig. 3 — Chromatograms of saponified (A) and unsaponified (B) egg yolks from laying hens fed 9% turf bermudagrass meal. Peaks: 1 = neoxanthin; 2 = violaxanthin; 3 = zeaxanthin; 4 = lutein.

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Effects of pH on Calcium, Zinc, and Phytate Solubilities and Complexes Following In Vitro Digestions of Soy Protein Isolate

ELAINE T. CHAMPAGNE and BRIAN Q. PHILLIPPY

─────────── ABSTRACT ───────────

High intraluminal gastric pH values in young infants could affect the bioavailability of zinc in soy protein isolate formula. This investigation examined the effects of pH on the solubilities and complexes of calcium, zinc and phytate following in vitro pepsin and pepsin-pancreatin digestions of soy protein isolate. The results of these in vitro studies suggested that (1) high intraluminal gastric pH values could lead to the formation of insoluble calcium-zinc-phytate complexes in the stomach following ingestion of soy protein isolate and (2) high intraluminal gastric pH values would only affect resultant soy zinc solubility in the small intestine in the absence of exogenous calcium or zinc but not in the presence of same.

INTRODUCTION

LOW SECRETION of hydrochloric acid by gastric parietal cells occurs in young infants (Agunod et al., 1969) and in approximately 30% of the elderly population (Russell, 1986). The consequences of reduced hydrochloric acid secretion are raised intraluminal gastric pH values, reduced pepsin formation from pepsinogen and reduced pepsin activity. Raised intraluminal gastric pH values could affect the denaturing of food proteins and the breaking of intermolecular bonds that occurs with high acidity. In the case of phytate-containing plant foods, raised intraluminal gastric pH values could lead to the formation of insoluble mineral-phytates and protein-mineral-phytates in the stomach. Low pepsin formation and activity could prevent the digestion of food proteins to polypeptides in the stomach and the subsequent complete breakdown to amino acids and smaller peptides in the small intestine following digestion by pancreatin. Poor digestion of protein-mineral and protein-mineral-phytate complexes could affect the release of the minerals for absorption at intestinal sites.

Soy protein isolate formulas are fed to infants with digestive problems and frequently serve as the sole or primary form of nourishment for the infant from birth to about age six months. Zinc bioavailability from soy formulas has been found to be much lower than from human milk or cow's milk (Momcilovic et al., 1976; Sandstrom et al., 1983a,b; Lonnerdal et al., 1984; Casey et al., 1981) due to the phytate contents of the former (Lonnerdal et al., 1984). These observations raise concern that the absorption of zinc from soy protein isolate formulas by infants may be lowered further in those with low gastric hydrochloric acid secretion.

The objective of this investigation was to examine the effects of pH on the solubilities and complexes of the endogenous calcium, zinc and phytate in soy protein isolate following in vitro pepsin and pepsin-pancreatin digestions of the protein. In view of the calcium content of soy formulas and the known potentiation of zinc binding by phytate in the presence of calcium (Byrd and Matrone, 1965; Oberleas, 1973; Graf and Eaton, 1984), the effects of exogenous calcium on the resultant solubility profiles of the digests were also examined.

MATERIALS & METHODS

Materials

Soy protein isolate [Ralston Purina Protein 1711 (pH 7)] was obtained courtesy of Protein Technologies International (St. Louis, MO). The isolate contained 2.0 mg calcium/g soy protein (49.9 micromoles calcium/g soy protein), 0.04 mg zinc/g soy protein (0.6 micromoles zinc/g soy protein and 1.30% phytic acid (19.7 micromoles phytic acid/g soy protein). Calcium chloride and zinc sulfate solutions (Dilut-It, J. T. Baker Chemical Co.) were used as exogenous mineral sources and for preparing atomic absorption standards. Porcine pepsin enzyme (110 units activity/mg solid), pancreatin [3 × U.S.P. (represents protease activity digesting 75 mg of casein/mg pancreatin in 60 min at pH 7.5 and 40°C)] and bile extract were purchased from the Sigma Chemical Company (St. Louis, MO).

Sample preparations: Treatments with and without pepsin

Samples were prepared by magnetically stirring 2.0g soy protein isolate, 0.877g sodium chloride and distilled-deionized water (100 mL minus the volumes of calcium chloride and zinc sulfate solutions) for 10 min. Portions of standard calcium chloride and zinc sulfate solutions were then added. The slurries were stirred for 15 min following which their pH values were adjusted with either 5N HCl or 10N NaOH. Each sample was stirred and its pH monitored for an additional 30 min. Sufficient amounts of pepsin were then added to 50 mL of each protein slurry to produce 6000 units of pepsin activity/g protein (wet wt) at pH 2. This quantity was determined from pepsin activities as measured by several other investigators (Crews et al., 1983; Hazell et al., 1978; Lock and Bender, 1980; Nelson and Potter, 1980; Rao and Prabhavathi, 1978; Rizk and Clydesdale, 1985). The calcium and zinc contents of a control containing pepsin and sodium chloride adjusted to pH 2 with 5N HCl were negligible. Each sample (with and without pepsin) was incubated for 2 hr in a water bath maintained at 38°C. Following incubation each pair of samples was immediately transferred to 50-mL centrifuge tubes and centrifuged for 20 min at 18,000 × g. On sample sets used for determining soluble protein bound calcium, zinc and phytate, each supernatant was then decanted into a 50-mL Amicon Ultrafiltration Cell (Model 8050). Ultrafiltration was conducted with a PM 10 membrane (molecular weight cut-off of 10,000 daltons) at a constant pressure of 0.8 atm while slowly magnetically stirring the samples.

Sample preparations: Pepsin-pancreatin digestions

Initial sample preparation was as above, except that pepsin was added to the entire sample (100 mL). Following the 2 hr incubation, the pH value of each sample was adjusted to 7.0 by slowly adding sodium bicarbonate (8.4 g/100 mL, 38°C) dropwise while magnetically stirring. The pH values of the samples were monitored for 15 min. Pancreatin (40 mg) and bile extract (250 mg) were added to each sample and the samples were returned to the water bath for an additional 2 hr. The quantities of pancreatin and bile extract used were based on the amounts used in earlier studies (Hazell et al., 1978; Miller et al., 1981; Akeson and Stahmann, 1964). The zinc and calcium contents of a control containing pancreatin and bile extract adjusted to pH 7 with sodium bicarbonate were 0.4 ppm and 0.04 ppm, respectively. The zinc and calcium solubility data were corrected for these contributions. Following incubation the samples were centrifuged and one half of each sample was ultrafiltered as described above.

Calcium and zinc determinations

Calcium and zinc concentrations of the supernatants and filtrates were determined by atomic absorption spectroscopy.

Phytate determination

The phytate contents of the supernatants and filtrates were isolated by a modification of the ion-exchange method of Ellis and Morris (1983). Ten milliliters of supernatant or filtrate was applied to 0.8 × 4 cm plastic columns which contained 0.35g of Dowex 1 − > 8, 200–400 mesh (Biorad, Richmond, CA). Inorganic phosphate was eluted from the columns with 10 mL 0.1M HCl or 0.1M NaCl for samples with and without pepsin and pepsin-pancreatin digested samples, respectively. The inositol phosphates were eluted from the columns with 10 mL of 2.0M HCl or 1.0M NaCl for samples with and without pepsin and pepsin-pancreatin digested samples, respectively. Recoveries were 97–100%. It was not necessary to add EDTA to the samples to prevent low phytate recovery from the ion-exchange columns, as suggested by Ellis and Morris (1983). The phosphorus contents of the eluents were determined by a modification of the Fiske-Subarrow method (Fiske and Subarrow, 1925) following sulfuric-nitric acid (3:2, v/v) digestion. Phytate concentrations were calculated based on the relative amounts of the hexa- and lower inositol phosphates found in samples representative of the various treatments as determined by the gradient ion chromatography method of Phillippy and Blanc (1988). The relative amounts of the hexa- and penta-inositol phosphates were the same before and after all treatments: approximately 87% phytic acid, 12% DL-myo-inositol 1, 2, 4, 5, 6-pentakisphosphate and 1% DL-myo-inositol 1, 2, 3, 4, 5-pentakisphosphate. No lower inositol phosphates were detected. No hydrolysis of phytic acid during the treatments to increase the relative amount of DL-myo-inositol 1, 2, 3, 4, 5-pentakisphosphate was evident.

Protein determination

The protein concentrations of the supernatants and filtrates were determined by Waddell's method (Waddell, 1956; Murphy and Kies, 1960; Wolf, 1983) which is based on the difference in absorption at 215 nm and 225 nm. This method primarily depends on quantitating peptide linkages and is largely unaffected by variations of protein composition and structure (Wolf, 1983).

Reliability of data

The solubility data represent averages from triplicate runs on each series of experiments. In each experiment, duplicate analyses were obtained on each sample. The average deviations in the percentages of soluble zinc, calcium, phytate and protein from the mean values were approximately ±1%, ±1%, ±2% and ±2%, respectively. The Student's t test was used to determine significance at the F < 0.05 probability level (Shoemaker et al., 1974).

RESULTS

Pepsin treatments of soy protein isolate as functions of pH

Solubility profiles. The solubility profiles of calcium, zinc and phytate following treatments of soy protein isolate with pepsin as functions of pH are shown in Fig. 1, 2 and 3, respectively. The zinc and phytate solubility profiles are those of the zinc and phytate endogenous to the soy protein isolate. The effects of the calcium concentrations of the soy protein isolate slurries on calcium, zinc and phytate solubilities are shown for calcium concentrations of 2.0 (endogenous calcium level), 3.2, 7.0, 9.5 and 30.0 mg calcium/g soy protein isolate. The calcium and zinc solubility profiles of samples prepared with calcium and zinc concentrations mimicking those in soy protein isolate formulas (0.24 mg zinc and 30.0 mg calcium/g soy protein isolate) were identical (P > 0.05) to those shown in Fig. 1 and 2 for samples containing 30.0 mg calcium/g soy and no exogenous zinc, except that a small amount (2–5%) of zinc remained soluble in the calcium-zinc supplemented samples at pH values in the 5.0–7.0 range. Phytate solubilities in these samples containing exogenous calcium and zinc were 11% and 9% higher at pH values 2.0 and 3.0, respectively, than in the samples containing 30.0 mg calcium/g soy and no exogenous zinc. The presence of exogenous zinc along with the exogenous calcium made no significant differences (P >
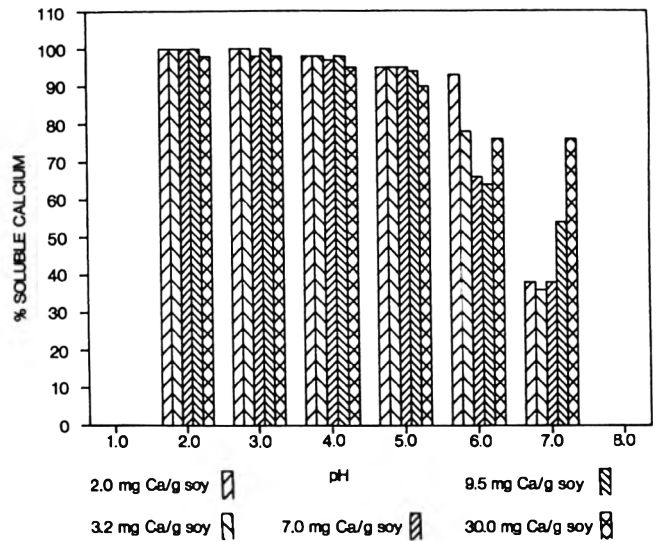
Fig. 1 — *Solubility profiles of calcium as function of pH of pepsin treatments of soy protein isolate. Endogenous calcium = 2.0 mg Ca/g soy.*

0.05) in phytate solubilities in the pH 4.0–7.0 range when compared with soy protein isolate slurries containing solely exogenous calcium.

Treatments with and without pepsin. There were no significant differences (P > 0.05) in the amounts of calcium solubilized in samples with pepsin (Fig. 1) compared to samples without pepsin (data not shown) for pH values in the 2.0–7.0 range. In the case of zinc, there were no significant differences (P > 0.05) in zinc solubilities in samples with pepsin (Fig. 2) compared to samples without pepsin (data not shown) for pH values 2.0, 3.0, 6.0, and 7.0. At pH values 4.0 and 5.0 soy zinc solubilities were approximately 7% and 18% lower, respectively, in the samples without pepsin than in samples with pepsin. When no pepsin was present, phytate solubilities were approximately 35%, 30%, 25%, 6%, and 10% lower at pH values 2.0, 3.0, 4.0, 6.0 and 7.0, respectively, than in samples with pepsin (Fig. 3).

The protein solubilities of the samples with and without pepsin relative to the protein solubility of the pancreatin digest which was initially treated with pepsin at pH value 2.0 are

Fig. 2 — *Solubility profiles of soy zinc as function of pH of pepsin treatments of soy protein isolate. Endogenous calcium = 2.0 mg Ca/g soy.*

Fig. 3—Solubility profiles of soy phytate as function of pH of pepsin treatments of soy protein isolate. Endogenous calcium = 2.0 mg Ca/g soy.

Table 3—Soluble calcium, zinc, and phytate bound to soluble protein having molecular weights larger than 10,000 daltons

pH[a]	Ca Level	Zn Level	Percent		
	(mg/g soy)		Ca	Zn	PA[b]
6.0	2.0	0.04	25	64	30
	3.2	0.04	18	45	37
	7.0	0.04	14	–	30
	9.5	0.04	14	–	24
	30.0	0.04	10	–	41
	30.0	0.24	11	>95	88
7.0	2.0	0.04	11	50	20
	3.2	0.04	14	62	33
	7.0	0.04	21	–	36
	9.5	0.04	26	–	75
	30.0	0.04	23	–	67
	30.0	0.24	21	>95	82

[a] pH value of pepsin treatment
[b] PA = phytate

Pepsin-pancreatin digestions of soy protein isolate as functions of pH of initial pepsin treatment

The solubility profiles of calcium, zinc, and phytate, respectively, following pancreatin digestion at pH 7.0 of soy protein isolate samples (with and without exogenous calcium) which were initially treated with pepsin at pH values in the 2.0–7.0 range are shown in Fig. 4, 5 and 6. A comparison of Fig. 4, 5 and 6 with Fig. 1, 2 and 3 permits an evaluation of the changes in calcium, zinc and phytate solubilities, respectively, following pancreatin digestion of the pepsin-treated samples. Pancreatin digestion of the pepsin-treated samples containing 0.24 mg zinc and 30.0 mg calcium/g soy protein isolate resulted in approximately 91%, 14% and 9% of the calcium, zinc, and phytate being soluble, regardless of the pH value of the initial pepsin treatment.

The protein solubilities of the pepsin-pancreatin digests relative to the protein solubility of the pancreatin digested sample initially treated with pepsin at pH 2.0 are listed in Table 1. There was approximately a 20%–35% increase in protein solubilities following pancreatin digestion of the samples initially treated with pepsin. The presence of exogenous calcium and/or zinc had no significant effects (P > 0.05) on resultant protein solubilities.

The percentages of the soluble protein having molecular weights larger than 10,000 daltons in the pepsin-pancreatin

listed in Table 1. Table 2 lists the percentages of soluble protein in the samples with and without pepsin having molecular weights larger than 10,000 daltons. These data give an indication of how efficient the pepsin treatment was in digesting the soy protein as a function of pH. The levels of calcium and zinc in the samples had no significant effects (P > 0.05) on protein solubility and on the molecular weight distribution of the soluble protein (data not shown). There was no evidence of significant binding (P > 0.05) of the soluble calcium, zinc or phytate with the soluble soy protein larger than 10,000 daltons for pH values in the 2.0–5.0 range. Table 3 lists the percentages of the soluble calcium, zinc and phytate that were bound to the soluble soy protein having molecular weights larger than 10,000 daltons in samples treated with pepsin at pH values 6.0 and 7.0.

Table 1 — Relative protein solubilities in samples treated with and without pepsin and with pepsin followed by pancreatin

pH[a]	Relative solubilities (%)[b]		
	+ Pepsin	– Pepsin	+ Pepsin/Pancreatin[c]
2.0	83	50	100
3.0	61	42	90
4.0	53	37	87
5.0	50	38	86
6.0	48	48	85
7.0	61	63	82

[a] pH values of initial treatment of samples with or without pepsin.
[b] Solubilities expressed relative to protein solubility of sample digested with pancreatin following initial treatment with pepsin at pH 2.0 (set at 100%).
[c] Samples treated with pepsin at indicated pH values followed by pancreatin digestion at pH 7.0.

Table 2 — Soluble protein having molecular weights larger than 10,000 daltons

pH[a]	% of soluble protein MW > 10,000 daltons		
	+ Pepsin	– Pepsin	+ Pepsin/Pancreatin[b]
2.0	17	55	11
3.0	33	68	14
4.0	30	52	22
5.0	33	44	20
6.0	48	65	16
7.0	75	78	21

[a] pH values of initial treatment of samples with or without pepsin.
[b] Samples treated with pepsin at indicated pH values followed by pancreatin digestion at pH 7.0.

Fig. 4—Solubility profiles of calcium following pancreatin digestions at pH 7.0 of soy protein isolate initially treated with pepsin at pH values in the 2.0–7.0 range. Endogenous calcium = 2.0 mg Ca/g soy.

Fig. 5—Solubility profiles of soy zinc following pancreatin digestions at pH 7.0 of soy protein isolate initially treated with pepsin at pH values in the 2.0–7.0 range. Endogenous calcium = 2.0 mg Ca/g soy.

digests are listed in Table 2. These data give an indication of the effect of the pH of the initial pepsin treatment on the extent of protein digestion following treatment with pancreatin.

DISCUSSION

Evaluation of the possible effects of gastric pH values on soy calcium, zinc and phytate solubilities in the stomach

The solubility profiles in Fig. 1, 2, and 3 represent the solubilities of calcium, zinc and phytate in the stomach as functions of gastric juice pH following pepsin treatments of soy protein isolate containing various amounts of calcium. At normal intraluminal gastric pH values (2.0–3.0), all of the calcium (both endogenous and exogenous) and soy zinc were soluble. Likewise, all of the calcium and zinc in soy protein isolate supplemented with both calcium (28.0 mg/g soy) and zinc (0.20 mg/g soy) were soluble at pH values 2.0 and 3.0 At these low pH values where pepsin activity is high, calcium
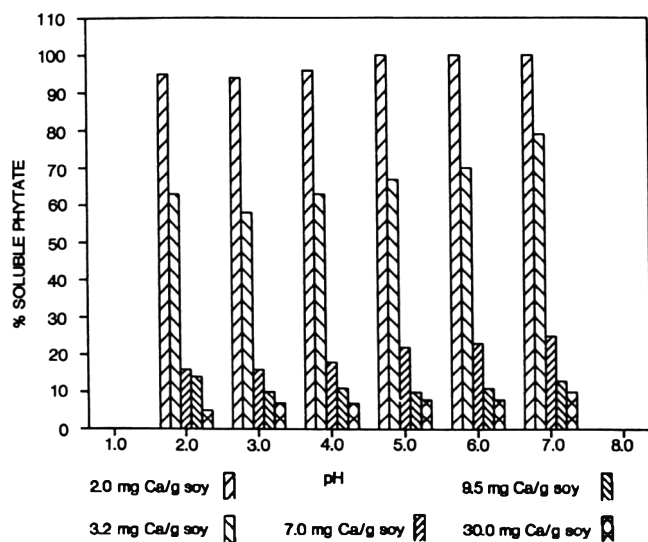
Fig. 6.—Solubility profiles of soy phytate following pancreatin digestions at pH 7.0 of soy protein isolate initially treated with pepsin at pH values in the 2.0–7.0 range. Endogenous calcium = 2.0 mg Ca/g soy.

and zinc solubilities depended entirely on the pH of the pepsin treatment and not on the digestion of the protein by pepsin. There was no binding of the soluble zinc or calcium to the soluble protein having molecular weights larger than 10,000 daltons at pH values 2.0 and 3.0. In contrast, phytate solubility was a function of both pH and the extent of protein proteolysis by pepsin for pH values in the 2.0–4.0 range. The differences in phytate solubilities in samples with and without pepsin were indications that phytate was present as insoluble phytate-protein complexes at these low pH values. Phytic acid forms insoluble complexes with proteins at pH values below the isoelectric point of the protein (Cheryan, 1980), which lies around pH 5 for soy protein. Pepsin digestion of the soy protein would solubilize the protein and thus the phytate. In this study calcium and zinc ions appeared to dissociate the insoluble phytate-protein complexes at pH values in the 2.0–4.0 range forming soluble calcium-zinc-phytates. This dissociation has been explained previously in terms of competition between divalent cations and the positively charged sites of the protein for binding the phosphate groups of phytate (Okubo et al., 1976)

Phytate solubility was higher in the pepsin-treated samples at pH values 6.0 and 7.0 than in the samples without pepsin. It is suggested that pepsin binds the phytate forming soluble pepsin-zinc-phytate complexes. Above the isoelectric point of a protein (enzyme), protein-phytate complexation can occur through an intermediary cation (Cheryan, 1980). The binding of zinc by pepsin has been studied elsewhere (Klotz and Ming, 1954; Kirchgessner et al., 1976).

Soy zinc solubilities in the pepsin-treated samples declined rapidly at higher pH values to a minimum of 5% at pH value 7.0 in samples without exogenous calcium. Endogenous calcium solubilities remained high (93–98%) at pH values in the 4.0–6.0 range and then dropped sharply to 38% at pH value 7.0. As shown in Table 3, some of the soluble zinc and calcium in the pepsin-treated samples was bound to the soluble soy proteins having molecular weights larger than 10,000 daltons at pH values 6.0 and 7.0. It is possible that this soluble calcium and zinc was bound as protein-calcium-zinc-phytate complexes.

The lower zinc solubilities observed at pH values 4.0 and 5.0 in samples without pepsin than in samples with pepsin were an indication of an association of zinc with the insoluble soy protein. Since the proteolytic activity of pepsin is negligible at pH values above 5.0, an evaluation of whether calcium and zinc were associated with the insoluble soy protein at pH values 6.0 and 7.0 could not be obtained from comparisons of the solubility data for samples with and without pepsin. The insoluble soy zinc at pH values 5.0 and 6.0 in samples without exogenous calcium was not present as protein-calcium-zinc-phytate complexes, because phytate solubility was 100% in these samples.

The presence of exogenous calcium did not significantly (P > 0.05) affect calcium and soy zinc solubilities for pH values in the 2.0–5.0 range, with the exception of no soy zinc being soluble at pH value 5.0 when the calcium was 30.0 mg/g soy. With this high calcium concentration at pH 5.0 and at pH values 6.0 and 7.0 in samples with exogenous calcium, the soy zinc coprecipitated with the calcium as calcium-zinc-phytates. Likewise, in samples having zinc and calcium concentrations of 0.24 mg and 30.0 mg/g soy, respectively, nearly all (95–98%) of the zinc precipitated as calcium-zinc-phytates at pH values in the 5.0–7.0 range. The exogenous calcium did not precipitate the zinc as protein-calcium-zinc-phytates, since the concentration of calcium and zinc did not significantly (P > 0.05) affect resultant protein solubilities. This observation differs from that of de Rham and Jost (1979). They observed a decrease in soy flour protein solubility from 80% to 20% with a calcium concentration of 10 mM (Ca/phytate molar ratio = 18). This corresponds to the addition here of 7.5 mg calcium/g soy isolate to give a Ca/phytate molar ratio of 18. Soy

flour contains other constituents (e.g., starch) that could possibly bind the protein-mineral-phytate comp.exes forming insoluble species (Thompson, 1986).

The observation that insoluble soy protein-calcium-zinc-phytate complexes were not present at pH values 5.0 and 6.0 in samples with and without exogenous calcium could have nutritional significance for infants with intraluminal gastric pH values in this range. It has been suggested that zinc associated with a protein-phytate complex is even less bioavailable than zinc associated solely with phytate due to the poor digestibility of these ternary complexes (Prattley et al., 1982).

These data suggested that at the zinc and calcium concentrations found in soy protein isolate infant formulas (0.24 mg zinc and 30 mg calcium/g soy protein or higher), all or nearly all of the zinc (endogenous and exogenous) would be present as insoluble calcium-zinc-phytates in the stomachs of infants with intraluminal gastric pH values of 5.0 or higher.

Evaluation of possible effects of gastric pH values on soy calcium, zinc, and phytate solubilities in the small intestine

The solubility data in Fig. 4, 5 and 6 represent possible calcium, zinc and phytate solubilities in the small intestine following pancreatin digestion of soy protein isolate as a function of gastric juice pH. Phytate solubility following pepsin-pancreatin digestion of the soy protein isolate containing no exogenous calcium or zinc was high (94–100%). Apparently, raising the pH to 7.0 prior to adding pancreatin led to the dissociation of the insoluble protein/polypeptide-phytate complexes that were present at pH values below the protein (polypeptide) isoelectric point. Thus, even though the degree of protein proteolysis to polypeptides achieved by pepsin affected phytate solubilities following treatment with just pepsin, it did not influence resultant phytate solubilities following digestion by pancreatin. The absence of insoluble calcium-zinc-phytate formation upon raising the pH to 7.0 is an indication that the endogenous calcium content of the soy protein isolate was insufficient to cause precipitation of the calcium-zinc-phytates. All of the soluble zinc, 70% of the soluble calcium and 90% of the soluble phytate passed a 10,000 dalton molecular weight cut-off filter. Some of the phytate which passed the filter was possibly in the form of amino acid/low molecular weight peptide-calcium-zinc-phytate complexes. The bioavailability of zinc and calcium from such complexes (assuming that they exist) is not known.

Resultant phytate solubilities in the pepsin-pancreatin digests containing exogenous calcium are a reflection of (1) formation of soluble amino acid/polypeptide-calcium-zinc-phytates along with (2) formation of insoluble calcium-zinc-phytates. Sufficient calcium was present in these digests to precipitate calcium-zinc-phytates.

The lower solubilities of calcium and zinc following digestion of the pH 2.0–6.0 pepsin digests with pancreatin compared with treatments solely with pepsin were partially due to the formation of insoluble calcium and zinc hydroxides as the pH values of the digests were raised to 7.0 with sodium bicarbonate. Insoluble calcium and zinc hydroxide formation occurs at pH values greater than 6.0 (Britton, 1925). Also, the carbonate anion forms insoluble salts with calcium at pH 7.0 (Rendleman, 1982a). Bile salts have been shown (Rendleman, 1982b) to form insoluble complexes with zinc but not with calcium. This may explain the larger lowering of zinc solubility compared to calcium solubility following digestion by pancreatin. The increase in calcium and zinc solubilities resulting from pancreatin digestion of samples initially treated with pepsin at pH 7.0 was a reflection of the release of these elements from insoluble protein complexes by the action of pancreatin.

The pH of the initial pepsin treatment affected resultant calcium and soy zinc solubilities following pancreatin digestion

in the samples without added calcium, as shown in Fig. 4 and 5, respectively. Calcium and soy zinc solubilities decreased from approximately 94% to 70% and 45% to 20%, respectively, as the pH values of the initial pepsin treatments increased from 2.0–7.0. In the pancreatin-treated samples containing 3.2 mg calcium/g soy protein isolate, calcium solubility was 59% and 48% in pH 2.0 and pH 3.0–7.0 pepsin-treated samples, respectively. Zinc solubility was approximately 10% in these samples containing 3.2 mg calcium/g soy protein isolate, regardless of the pH of the initial pepsin treatment. In the other samples containing exogenous calcium, the pH of the initial pepsin treatment had no significant effects (P > 0.05) on resulting calcium and soy zinc solubilities. Furthermore, resulting calcium and zinc solubilities were unaffected by the pH value of the initial pepsin treatment when soy protein isolate was supplemented with zinc (0.20 mg/g soy protein isolate) instead of calcium (data not shown). In samples supplemented with 0.20 mg zinc and 28.0 mg calcium/g soy protein isolate (mimicking zinc and calcium concentrations found in soy formulas), 14% of the total zinc was soluble following pancreatin digestion, regardless of the pH of the initial pepsin treatment. Considering the solubility criterion of bioavailability, it appears that high gastric pH values could have a small effect on calcium bioavailability and possibly a marked effect on zinc bioavailability from unsupplemented soy protein isolate. Although calcium supplementation led to soy zinc solubilities in the pepsin-pancreatin digests being unaffected by the pH values of the initial pepsin treatments in the pH 2.0–5.0 range, calcium supplementation alone would not be beneficial in regards to zinc availability. Soluble soy zinc was lower in all of the pepsin-pancreatin digests containing exogenous calcium than in any of the digests without exogenous calcium (including those initially treated with pepsin at high pH values). Only 3–5% of the soy zinc was soluble in the pepsin-pancreatin digests having calcium concentration of 30.0 mg/g soy as found in soy protein isolate formulas.

In conclusion, the solubilization of zinc from soy protein isolate following pepsin treatments appeared to depend on pH along with the amount of calcium present. The extent of proteolysis of the protein, as determined by the amount and activity of the pepsin present, only influenced zinc solubility at pH values 4.0 and 5.0. The results of these in vitro studies suggest that high intraluminal gastric pH values could lead to the formation of insoluble calcium-zinc-phytate complexes in the stomach following the ingestion of soy protein isolate. However, high intraluminal gastric pH values would only affect resultant soy protein isolate zinc solubility and thus availability in the small intestine in the absence of exogenous calcium or zinc but not in the presence of same. Since soy protein isolate formulas are supplemented with calcium and zinc, gastric pH values are possibly of no consequence to the amount of zinc absorbed by infants.

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Effect of Soybean Maturity on Storage Stability and Process Quality

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——————————— ABSTRACT ———————————

Hack and Williams 82 soybeans from four maturation stages were studied for storage stability and process quality. As compared to mature beans, trypsin inhibitor, urease and lipoxygenase (LA) activities were lower in immature seeds, but free fatty acid (FFA) was higher and oil was greener. During storage for six months, LA decreased and FFA increased at a faster rate in immature than in mature soybeans. Crude oil and protein contents were similar, regardless of maturation or storage time. Both 7S and 11S proteins increased with maturation but the 7S/11S ratio decreased. There was no change in protein during storage.

INTRODUCTION

MOST SOYBEANS are processed for oil extraction and high protein meal production. Soybeans are important constituents of both human and animal diets; the protein is of high-quality compared to other oilseeds and the oil has a lower level of saturated fatty acids than most oils of plant origin.

Raw soybeans contain many biologically active factors. Trypsin inhibitor and lipoxygenase are major factors responsible for poor protein digestibility and beany off-flavor, respectively. For ruminant feed containing urea, ammonia toxicity may occur due to urease present in raw soybeans. The trypsin inhibitor (Collins and Sanders, 1976), lipoxygenase and urease (Iwanicki, 1971) activities have been reported to be increased during soybean maturation. However, several articles contained conflicting results concerning the activities during maturation as well as storage (Sternberg, 1985; Urbanski et al., 1980).

Yao et al. (1983) obtained Williams soybeans at different maturation stages by spraying with paraquat which arrests maturation. They found that crude oils of immature beans were greener in color and higher in free fatty acid (FFA) content than those of mature beans. Upon storage, the FFA content increased at a faster rate in the immature beans than that in mature beans.

Soy protein is a good source of all essential amino acids except methionine, a sulfur-containing amino acid. Because most of the methionine has been shown to be present in 11S protein fraction (Krober and Carter, 1966), it has been proposed that decreasing the 7S/11S ratio would be one way to improve the quality of soy protein. Furthermore, the 7S/11S ratio may also affect the overall functional properties of the soy protein in various food systems. Thus, an improved protein may be obtained by controlling maturation.

Soybeans are frequently subjected to weather and field damage. Even in a normal year, harvested soybeans consist of not only mature sound soybeans but also immature or green soybeans. According to recent grading standards for soybeans (Federal Grain Inspection Service, 1985), the immature, green seeds are discolored seeds which are considered "damaged." After harvesting, the soybeans have to be stored for a period of time before processing. Therefore, in the processing of whole soybeans into oil and meal, one should be concerned with

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changes in the quality of the raw material during both maturation and storage.

The objective of this work was to investigate the quality of oil and meal obtained from Hack and Williams 82 soybeans at four maturation stages and after storage for zero, three and six months. The scope of the study included the effects of maturation and storage on: (a) trypsin inhibitor, lipoxygenase and urease activities; (b) crude oil content, free fatty acid content and oil color; and (c) total protein, 7S and 11S proteins, and 7S/11S ratio.

MATERIALS & METHODS

Sample preparation

Two soybean varieties, Williams 82 and Hack, were planted on the south farms of the Univ. of Illinois at Urbana-Champaign on May 22 and 29, 1986, respectively. Four maturation stages of pod samples were harvested and depodded by hand; the Williams 82 soybeans were harvested at 43, 50, 57, and 64 days whereas the Hack soybeans were harvested at 57, 64, 71, and 78 days after flowering.

The seeds were dried in a through-flow air drier (Blue M Electric Company, Blue Island, IL) at ambient temperature to a moisture content of 10% or below. The dried beans were sealed in air in No. 2 C-enamel cans and stored at 23 to 25°C. The samples were analyzed at 0, 3, and 6 months of storage. All analyses were in triplicate.

Proximate analyses

For moisture content, a sample was ground in a water-cooled micro-mill (Tekmar Company, Cincinnati, OH) and moisture content determined by heating in a vacuum oven for 24 hr at 60°C and 28 inches Hg vacuum. The oil content was determined by extraction with petroleum ether according to the AOCS (1984). The free fatty acid content in the crude oil was determined by titration with 0.25 N NaOH according to the AOCS (1984). The color of the crude oil was determined by the photometric index (AOCS, 1984); absorbance was determined with a sepctrophotometer (model UV-260, Shimadzu Corp., Kyoto, Japan). Nitrogen content was determined by the micro-Kjeldahl procedure (AOAC, 1984) and protein content calculated as 6.25 times the nitrogen.

Enzyme determinations

Trypsin inhibitor (TI) activity was determined by the method of Kakade et al. (1974), with a modification by Hamerstrand et al. (1981). The rate of enzymatic hydrolysis was followed by reading the absorbance at 410 nm in the Shimadzu spectrophotometer. The TI activity was expressed as mg TI per g of defatted soybean.

The activity of liposygenase-1 was determined according to a spectrophotometric method of Surrey (1964) with a modification by Lao (1971). The amount of conjugated dienes was measured by reading the absorbance at 234 nm in the Shimadzu spectrophotometer. Activity was calculated from the rate of increase in dienes and related to the amount of dry soybean solids present per mL reaction mixture. Therefore, unit activity was expressed as the change in absorbancy per min from 1 mg defatted soy solid (Rice et al., 1981; Rice, 1972).

Urease activity was determined by the AOCS (1984) method. The activity was reported as the difference in pH between the test and blank samples.

Fractionation of 7S and 11S proteins

This was conducted by applying the method of Thanh and Shibasaki (1976). The basis of this procedure was the difference in solubility of the 7S and 11S proteins in a dilute Tris-HCl buffer (0.03 M).

Table 1—Moisture content of Hack and Williams 82 soybeans harvested at four maturation stages before and after drying

Maturation stages	Hack soybeans		Williams 82 soybeans	
	At harvest	After drying	At harvest	After drying
	Moisture content (%)			
1 (immature)	68.53[a]	8.02[a]	69.66[a]	7.81[a]
2	64.21[b]	7.23[b]	62.68[b]	7.44[b]
3	59.40[c]	6.46[c]	58.19[c]	6.64[c]
4 (mature)	40.85[d]	5.64[d]	55.75[d]	6.07[d]

[a-d] Means in the same column bearing different superscripts differ significantly at the 5% level.

Statistical analyses

Data were analyzed according to Steel and Torrie (1980) for analysis of variance and F-test at the 5% level. If the F-test proved significant, the least significant difference procedure was applied to determine significant differences among treatment means.

RESULTS & DISCUSSION

Moisture content

High moisture seeds favor enzymatic reactions, such as lipoxygenase activity (Ferrier, 1976). Therefore, the moisture content of soybean seeds was reduced below 10% before storage. However, the lower the maturity at harvest, the higher the moisture content (Table 1). This translates into a longer drying time for the more immature soybeans. For instance, the moisture content of immature beans was about 8.0% after drying for a period of 12 days, whereas that of mature beans was about 6.0% after drying for only 5 days.

Trypsin inhibitor activity

Trypsin inhibitor from raw soybeans causes pancreatic hypertrophy and growth inhibition in various animals (Rackis, 1974). During maturation, trypsin inhibitor (TI) activity increased significantly ($P < 0.05$) for both Hack and Williams 82 soybeans (Table 2). Collins and Sanders (1976) also reported that the TI activity for Kanrich, Verde, Soylima and Dare soybeans increased as the soybeans matured. The TI was active not only in mature beans but also in immature ones. Thus, both the mature and immature beans must be treated to improve their nutritional value.

For both Hack and Williams 82 soybeans, the TI activity at each maturation stage showed no significant ($P > 0.05$) change during storage (Table 2). Thus, the larger amounts of trypsin inhibitor present in the more mature beans would require a longer period of heat treatment for inactivation.

Lipoxygenase activity

Lipoxygenase catalyzes the oxidation of unsaturated fatty acids, resulting in rancidity, off-flavor and poor storage stability (Eskin et al., 1977). This enzyme becomes especially active when the beans are damaged, but its activity is much reduced at a low moisture (Snyder and Kwon, 1987). During maturation, lipoxygenase-1 activity increased significantly ($P < 0.05$) from stage 1 to stage 3 for both Hack and Williams 82 soybeans (Table 3). No significant difference in the LA was found as the beans developed from stage 3 to stage 4. Similar results were also found in other varieties of soybeans such as Beeson and Disoy varieties (Sternberg, 1985; Iwanicki, 1971).

During three-month storage of both Hack and Williams 82 soybeans at room temperature, the LA for all stages of maturation decreased rapidly. The LA decreased at a faster rate in immature than that in mature soybeans (Table 3). There was no significant decrease in activity between three- and six-month storage for the Hack variety but it continued to decrease in the Williams 82. According to Urbanski et al. (1980), the LA of mature Clark 63 seeds remained fairly constant during 14-month storage under the same condition. This would indicate that the effect of storage on the soybean LA is dependent on variety.

Urease activity

Soybean processing plants are typically using urease activity, ranging from 0.15 to 0.25, as an index to indicate proper heat treatment for animal feeds as well as for trypsin inhibitor inactivation for human diets (Snyder and Kwon, 1987; Wright, 1981; McNaughton et al., 1981). For both varieties, urease activity was significantly ($P < 0.05$) lower in the immature beans at stage 1 than in the mature ones at stages 3 and 4 (Table 4). The enzyme was quite stable under this storage condition. Therefore, the soybeans of all maturation stages and storage periods require heat treatment because the urease activity exceeded the limits.

Crude oil content

Oil and protein levels are the most important factors for soybean processors (Donhowe, 1988). Researchers have long suggested using oil content as the additional characteristics of the grading standard. Then, immature beans low in oil content will be considered less valuable. For both varieties, the oil content increased significantly ($P < 0.05$) from stage 1 to stage 3, and remained relatively constant as the beans developed further (Table 5). A similar trend also had been found in other varieties of soybeans (Yazdi-Samadi et al., 1977). There was no significant change in the oil content during 6-month storage regardless of maturity.

Free fatty acid content

Immature beans are high in both moisture and free fatty acid contents; these favor the lipoxygenase reaction (Ferrier, 1976). As the beans matured, free fatty acid (FFA) contents decreased significantly ($P < 0.05$) for both Hack and Williams 82 soybeans (Table 6). For the Hack soybeans, six-month storage of

Table 2—Effect of maturity and storage time on trypsin inhibitor (TI) activity of Hack and Williams 82 soybeans[1]

Maturation stages	Hack soybeans			Williams 82 soybeans		
	Storage time (months)					
	0	3	6	0	3	6
	TI activity (mg/ g defatted solid)					
1	19.61[a]	18.83[a]	18.80[a]	21.75[a]	20.85[a]	21.10[a]
2	24.34[b]	22.80[b]	23.50[b]	22.89[b]	21.41[b]	22.20[b]
3	26.18[c]	25.83[c]	25.54[c]	24.34[c]	23.17[c]	23.25[c]
4	28.42[d]	27.35[d]	27.44[d]	25.88[d]	24.53[d]	24.88[d]

[1] Means with a common underline in the same horizontal row do not differ significantly at the 5% level.
[a-d] Means in the same column bearing different superscripts differ significantly at the 5% level.

Table 3—Effect of maturity and storage time on lipoxygenase activity of Hack and Williams 82 soybeans[z]

	Hack soybeans			Williams 82 soybeans		
	Storage time (months)					
	0	3	6	0	3	6
Maturation stages	Lipoxygenase activity (units/ mg defatted solid)					
1	17.07[a]	4.31[a]	4.10[a]	21.57[a]	4.21[a]	3.37[a]
2	21.47[b]	11.53[b]	10.05[b]	25.91[b]	9.57[b]	6.35[b]
3	25.35[c]	19.41[c]	18.05[c]	33.96[c]	21.60[c]	17.42[c]
4	25.35[c]	18.85[c]	18.14[c]	33.47[c]	22.56[c]	16.81[c]

[z] Means with a common underline in the same horizontal row do not differ significantly at the 5% level.
[a-c] Means in the same column bearing different superscripts differ significantly at the 5% level.

Table 4—Effect of maturity and storage time on urease activity of Hack and Williams 82 soybeans[z]

	Hack soybeans			Williams 82 soybeans		
	Storage time (months)					
	0	3	6	0	3	6
Maturation stages	Urease activity (pH difference)					
1	1.95[a]	1.95[a]	1.98[a]	1.97[a]	2.00[a]	1.95[a]
2	2.03[ab]	2.05[ab]	2.04[ab]	2.02[ab]	2.04[ab]	2.04[ab]
3	2.07[b]	2.10[b]	2.12[b]	2.06[b]	2.11[b]	2.10[b]
4	2.07[b]	2.11[b]	2.10[b]	2.04[ab]	2.10[b]	2.08[b]

[z] Means with a common underline in the same horizontal row do not differ significantly at the 5% level.
[a,b] Means in the same column bearing different superscripts differ significantly at the 5% level.

Table 5—Effect of maturity and storage time on oil content of Hack and Williams 82 soybeans[z]

	Hack soybeans			Williams 82 soybeans		
	Storage time (months)					
	0	3	6	0	3	6
Maturation stages	Oil content (%, dry basis)					
1	20.42[a]	20.30[a]	20.35[a]	18.77[a]	19.10[a]	19.04[a]
2	21.54[ab]	21.58[ab]	21.60[ab]	21.68[ab]	21.48[ab]	21.50[ab]
3	22.34[b]	22.41[b]	22.53[b]	22.63[b]	22.74[b]	22.81[b]
4	22.44[b]	22.52[b]	22.50[b]	22.64[b]	22.86[b]	22.75[b]

[z] Means with a common underline in the same horizontal row do not differ significantly at the 5% level.
[a,b] Means in the same column bearing different superscripts differ significantly at the 5% level.

Table 6—Effect of maturity and storage time on free fatty acid (FFA) content of Hack and Williams 82 crude soybean oils[z]

	Hack soybeans			Williams 82 soybeans		
	Storage time (months)					
	0	3	6	0	3	6
Maturation stages	FFA content (g oleic acid/100g oil)					
1	0.76[a]	1.91[a]	2.26[a]	0.58[a]	1.63[a]	2.00[a]
2	0.58[b]	1.23[b]	1.50[b]	0.50[b]	1.15[b]	1.46[b]
3	0.22[c]	0.44[c]	0.50[c]	0.29[c]	0.32[c]	0.34[c]
4	0.15[d]	0.18[d]	0.18[d]	0.13[d]	0.15[d]	0.16[d]

[z] Means with a common underline in the same horizontal row do not differ significantly at the 5% level.
[a-d] Means in the same column bearing different superscripts differ significantly at the 5% level.

Table 7—Effect of maturity and storage time on color of Hack and Williams 82 crude soybean oils[z]

	Hack soybeans			Williams 82 soybeans		
	Storage time (months)					
	0	3	6	0	3	6
Maturation stages	Photometric index					
1	376[a]	380[a]	378[a]	367[a]	355[a]	362[a]
2	353[b]	355[b]	352[b]	346[b]	342[b]	348[b]
3	251[c]	255[c]	251[c]	260[c]	267[c]	261[c]
4	205[d]	203[d]	206[d]	211[d]	215[d]	213[d]

[z] Means with a common underline in the same horizontal row do not differ significantly at the 5% level.
[a-d] Means in the same column bearing different superscripts differ significantly at the 5% level.

Table 8—Effect of maturity and storage time on protein content of Hack and Williams 82 soybeans[1]

Maturation stages	Hack soybeans		Williams 82 soybeans	
	Storage time (months)			
	0	6	0	6
	Protein content (%, dry basis)			
1	40.36[a]	40.06[a]	42.55[a]	42.88[a]
2	41.21[a]	41.39[a]	42.43[a]	42.30[a]
3	41.58[a]	41.63[a]	43.30[a]	43.35[a]
4	41.47[a]	41.56[a]	43.28[a]	43.17[a]

[1] Means with a common underline in the same horizontal row do not differ significantly at the 5% level.
[a] Means in the same column bearing different superscripts differ significantly at the 5% level.

stages 1 and 2 soybeans resulted in a significant (P<0.05) increase in FFA content. However, the FFA content at stages 3 and 4 remained the same, except for the rapid increase of the FFA content for the stage 3 samples during the first three months of the storage. Similar results were also found in the Williams 82 soybenas. It is obvious that the FFA content increased at a faster rate in the immature beans rather than in the mature ones. The moisture content of soybeans decreased significantly (P<0.05) with maturation (Table 1); therefore, the variation in moisture may also affect the change in the FFA content during storage.

According to Urbanski et al. (1980), the crude oil from freeze-damaged soybeans had less storage stability than that from undamaged soybeans, due to a more rapid increase in FFA content. Yao et al. (1983) also reported that, upon storage, the immature soybeans increased significantly ir FFA content, while the mature beans increased slightly in the FFA content.

Color of crude oil

When oil is removed from soybeans, it must be refined before it can be used for human consumption. During refining, color material must be removed and the more color material present, the lower the yield of salable oil. As soybeans developed from maturation stage 1 to stage 4, photometric index of the crude oil samples decreased significantly for both Hack and Williams 82 soybeans (Table 7). The high photometric index of oil is due to the presence of green color. The chlorophyll content of oil was reported to be responsible for the green color in the oil (MacMillan and Melvin, 1955; Sander, 1946).

During six-month storage, the photometric index of oils from all samples remained relativbely constant. This indicates that the green color of the oil from the immature beans did not disappear during storage. Urbanski et al. (1980) also reported that the green color of freeze-damaged soybean oil did not dissipate during 14-month storage at the same condition.

Protein content

For both soybean varieties, the protein content on a dry weight basis did not increase significantly during maturation, and they were relatively constant upon storage (Table 8). According to Awolumate (1983), soybeans exhibited rapid accumulation of total protein per seed basis during the first half of seed development, but slowed and gradually approached zero additional accumulation at the time of maturity. Therefore, the increase in protein per seed was primarily due to the increase in the seed size with maturation. However, he also observed that protein content on a dry seed weight basis changed very little with maturation as found here.

Major storage proteins

As the Hack soybeans matured, 7S and 11S proteins increased significantly (Table 9). The 7S protein content was higher than the 11S protein, regardless of maturation and storage time. A similar trend to those of the above results were also observed for the Williams 82 soybeans (Table 10).

As calculated from the data in Tables 8, 9 and 10, for Hack soybeans, the 7S and 11S proteins accounted for only 42.6% of the total seed protein at the earliest stage, but for about 68.6% at the mature stage. In case of Williams 82 seeds, the combination of both major proteins accounted for 35.0 % and 64.3 % of the total protein at stages 1 and 4, respectively. In mature soybeans of Hack and Williams 82 varieties, the 11S fraction made up 30.2% and 25.1% of total protein, while the 7S fraction was about 38.4% and 39.2%, respectively. According to Wolf and Cowan (1975), the 11S protein makes up 25-35% of total protein, whereas the 7S protein constitutes 30-35% of the total soy protein. Derbyshire et al. (1976) reported that the content of these two storage proteins were about 70% of the total seed protein.

The ratio of 7S/11S proteins decreased as the beans developed (Table 9 and 10). The ratio was not changed during storage, regardless of maturation. However, the 7S/11S ratio of the Williams 82 soybeans was higher than that of the Hack variety, irrespective of maturation and storage time. The reduction of the 7S/11S ratio with maturation indicates that most of the 7S protein began to accumulate at an early stage of the seed development, while the 11S protein accumulated at a later stage. A similar result was also observed in Enrei soybeans (Kondo et al., 1986). Since most of the methionine has been reported to the present in the 11S protein (Krober and Carter, 1966), the results of this study suggest that the more mature soybeans will be higher in methionine.

However, Yao et al. (1983) reported that the 7S/11S ratio of Williams soybeans was increased from 0.3 to 0.6 during maturation. According to Taira and Taira (1972), 7S/11S ratios of mature soybeans among 30 cultivars averaged over three environments ranged from 0.7 to 1.4. The accumulation of these storage proteins within the seeds may be influenced by maturation and variety as well as environment. Murphy and Resurreccion (1984) stated that genetics has an influence on

Table 9—Effect of maturity and storage time on 7S and 11S protein contents, and 7S/11S ratio of Hack soybeans[1]

Maturation stages	Storage time (months)					
	0	6	0	6	0	6
	7S Content (%, dry basis)		11S Content (%, dry basis)		7S/11S Ratio	
1	12.46[a]	12.22[a]	4.74[a]	4.60[a]	2.63[a]	2.66[a]
2	13.17[b]	13.03[b]	8.03[b]	7.95[b]	1.64[b]	1.64[b]
3	15.54[c]	15.64[c]	11.55[c]	11.45[c]	1.35[c]	1.37[c]
4	15.93[c]	15.87[c]	12.52[d]	12.64[d]	1.27[d]	1.26[d]

[1] Means with a common underline in the same horizontal row do not differ significantly at the 5% level.
[a-d] Means in the same column bearing different superscripts differ significantly at the 5% level.

Table 10—Effect of maturity and storage time on 7S and 11S protein contents, and 7S/11S ratio of Williams 82 soybeans[z]

| Maturation stages | Storge time (months) | | | | | |
| | 0 | 6 | 0 | 6 | 0 | 6 |
	7S Content (%, dry basis)		11S Content (%, dry basis)		7S/11S Ratio	
1	11.75[a]	11.50[a]	3.15[a]	3.22[a]	3.73[a]	3.57[a]
2	12.03[b]	12.13[b]	4.69[b]	4.52[b]	2.57[b]	2.68[b]
3	14.30[c]	14.38[c]	9.28[c]	9.35[c]	1.54[c]	1.55[c]
4	16.96[d]	16.85[d]	10.88[d]	10.78[d]	1.56[c]	1.56[c]

[z] Means with a common underline in the same horizontal row do not differ significantly at the 5% level.
[a-d] Means in the same column bearing different superscripts differ significantly at the 5% level.

the expression of these two proteins, but to a lesser extent than environment.

CONCLUSION

THE QUALITY of crude oil and meal from the mature soybean seeds was superior to those from the immature ones. The crude oil content was relatively constant during maturation and 6-month storage. However, the oil from the immature beans was high in FFA content and green in color. Upon storage for 6 months, the FFA content extracted from the immature beans increased significantly whereas the green color remained unchanged. These problems would contribute to significant loss of the oil after a refining process. TI, urease and lipoxygenase activities increased with maturation but they remained constant during six-month storage, except for a decrease of lipoxygenase activity. Protein content was similar regardless of maturation and storage time. Both 7S and 11S contents increased with maturation but 7S/11S ratios decreased with maturation and varied with variety. Thus, maturation and varietal differences may affect functional properties of soybean protein in food systems.

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Preparation and Functional Properties of Enzymatically Deamidated Soy Proteins

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—————————— ABSTRACT ——————————

Heat-denatured soy protein was hydrolyzed by Alcalase to 2.0% or 4.0% degree of hydrolysis (DH), heated again at 100°C and deamidated with *B. circulans* peptidoglutaminase. The extent of deamidation was 6.0% and 8.2% for 2.0 DH hydrolysates and 12.8% and 16.0% for 4.0 DH hydrolysates heated for 15 and 30 min, respectively. Deamidation increased protein solubility and substantially enhanced emulsifying activity under mildly acidic (pH 4–6) as well as alkaline conditions. Deamidation improved emulsion stability and foaming power of heat-denatured hydrolysed soy proteins. Enzymatically deamidated soy protein hydrolysates had improved functional properties compared to nondeamidated hydrolysates and the native soy protein.

INTRODUCTION

FUNCTIONAL PROPERTIES of food proteins depend on their conformation in food systems. Kinsella (1976) reviewed the relationship of protein structure to functionality and indicated that altering the chemistry of the food proteins can improve protein functional properties such as solubility, viscosity, gelation, fat emulsification and foaming. The conversion of protein amide groups to carboxyl groups by deamidation improves solubility and other physical properties of proteins under mildly acidic conditions. Improving solubility, emulsifying or foaming properties of soy proteins may enhance their use as functional ingredients in many food systems, including beverages, pourable and nonpourable dressings, whipped toppings, frozen desserts, confections, baked goods and meat.

Modification of food proteins can be carried out by chemical or enzymatic means. Cheetham (1986) reviewed the many benefits of enzymatic food processes and efficiently modifying food proteins. An enzymatic approach to protein deamidation offers several advantages over chemical deamidation, including selectivity and mild deamidating conditions such as neutral pH and room temperature.

Hamada et al. (1988) reported that *B. circulans* peptidoglutaminase (PGase) readily hydrolyzed the amide groups of glutamine residues in soy peptides, but its activity towards the intact protein was small. Subsequent studies by Hamada and Marshall (1988) revealed that treatment of soy protein with heat and proteolytic enzymes significantly increased the degree of PGase deamidation. Unfortunately, enzymatic hydrolysis of soy protein is often accompanied by bitter taste. Low concentrations of bitterness can be obtained by restricting the degree of hydrolysis (DH) to small values, i.e., 3-5% (Adler-Nissen, 1986). Accordingly, protein hydrolysis must be kept under 5% DH to minimize bitter peptide formation. The objectives of this study were to extend previous findings on soy protein deamidation by PGase to prepare an enzymatically deamidated soy protein and to investigate the changes in solubility, emulsification and foaming properties brought about by deamidation.

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MATERIALS & METHODS

NUTRISOY 7B FLAKES were obtained from Archer Daniels Midland Co. (Decatur, IL), Alcalase 2.4 L (2.4 Anson units/g) from Novo Laboratories Inc. (Wilton, CT) and *Bacillus circulans* culture (ATCC # 21590) from the American Type Culture Collection (Rockville, MD). The BCA (bicinchoninic acid) protein assay reagent was purchased from Pierce Chemical Co. (Rockford,IL). Other chemicals were reagent grade or the highest purity obtainable.

Preparation of peptidoglutaminase

Phosphate gel evaluate was prepared from *B. circulans* cell extract, according to Hamada et al. (1988), freeze-dried and stored at −5°C until needed. The lyophilized evaluate contained 25% protein.

Preparation of soy protein substrate and PGase deamidation

A flow chart summarizing the preparation of hydrolyzed, deamidated soy protein is given in Fig. 1. Soy flakes were extracted with water at 25°C for 1 hr, then filtered. The soy protein filtrate was heated at 100°C for 15 min, cooled to 50°C, the pH adjusted to 8.0 and Alcalase 2.4L added to start proteolysis. The extent of hydrolysis was controlled to 2.0% or 4.0% DH by the pH-stat method (Novo Industri A/S, 1978), using an Auto Titrator (Radiometer A/S, Copenhagen). Both the 2.0% and 4.0% DH hydrolysates were divided into two parts. One-half of the hydrolysate was heated to 100° C for 15 min, while the other half was heated to 100°C for 30 min. Each of the heat-treated hydrolysates were deamidated with PGase from the phosphate gel eluate. The hydrolysed, deamidated products were freeze-dried for storage.

Protein

The protein contents of soy protein extracts and hydrolysates were determined by the Kjeldahl method using 6.25 as a conversion factor. The protein content of PGase preparations was determined by the bicinchoninic acid method of Smith et al. (1985).

Degree of hydrolysis (DH)

The percentage of peptide bonds cleaved during proteolysis was determined with a reaction between free amino groups and 2,4,6-trinitrobenzenesulfonic acid (Adler-Nissen, 1979).

Percent deamidation

The extent of deamidation was calculated as the ratio of ammonia released from substrate by PGase to the ammonia released by the soy protein substrate after total amide hydrolysis with 2 N HCl at 100° C for 4 hr (Wilcox, 1967). In the determination of the % deamidation, a correction for free ammonia was applied (Wilcox, 1967).

Protein solubility

Soy protein (1.2%, w/v) was homogenized in 0.2M NaCl using an Omni mixer (Ivan Sorvall Inc., Norwalk, CT) at 4,000 rpm for 1 min at 25° C. The pH of a 5 mL aliquot was adjusted between 2–10 with either 0.2 M NaOH or 0.2 M HCl. Then 0.2M NaCl was added to a volume of 6 mL. Protein dispersions were stirred for 45 min and centrifuged at 2000 × g for 10 min at 25° C. Protein concentration in both the supernatant and the precipitate (dissolved in 0.01 M NaOH)

cred the 0 time sample, and in time intervals up to 20 min, diluted to 1/100 with 0.1% SDS and the absorbance was determined at 500 nm.

Foaming properties

Foaming power and foam stability were determined by the conductimetric method of Kato et al. (1983b). Foams were prepared by sparging 10 mL 0.1% protein solution in 0.1M phosphate buffer, pH 7.5, in a 4 × 40 cm column with air at constant flow rate of 100 cm³ min⁻¹ through a glass sinter, for 15 sec. The conductivity was measured by an electrode cell, connected to a PCMl conductivity meter (Cole-Parmer Instrument Co., Chicago, IL) for 5 min. Foaming power was defined as the maximum conductivity of foams produced after aeration. Foam power value was also considered as conductivity at 0 time (C_0). The ratio of $C_{0.5}$ to C_0 was used to express foam stability, where $C_{0.5}$ is conductivity after 0.5 min, as suggested by Wright and Hemmant (1987).

RESULTS & DISCUSSION

Deamidation of soy protein by peptidoglutaminase

Moist heat, limited proteolysis and PGase treatment were combined to produce soy protein at two degrees of hydrolysis and several levels of deamidation, as depicted in the flow chart (Fig. 1). Moist heat and proteolysis are necessary to render soy protein susceptible to PGase attack (Hamada and Marshall, 1988). The protein content was 57.0% for the freeze-dried water extract of soy flakes and ranged from 52.2% to 53.9% for the lyophilized hydrolysates.

PGase treatment had no effect on the degree of hydrolysis as the DH remained constant at 2.0% or 4.0% after deamidation. The extent of deamidation was 6.0% and 8.2% at 2.0% DH and 12.8% and 16.0% at 4.0% DH when the hydrolysates were heated for 15 and 30 min, respectively. Since heat treatment and proteolysis of soy proteins were used as a means to achieve significant deamidation, heat-treated soy protein hydrolysates were used as controls. Therefore, changes in protein functional properties were due only to deamidation and not heat treatment and proteolysis.

Deamidation and protein solubility

The solubilities of the native soy protein, heat-treated hydrolysates and the deamidated, heat-treated hydrolysates in 0.2M sodium chloride, as a function of pH are presented in Fig. 2. Typical pH solubility profiles were observed for the soy protein (Wolf, 1978) and soy protein hydrolysates (Adler-Nissen, 1986). Upon hydrolysis, protein solubility increased at its isoelectric pH, and the solubility curve changed from a curve with a deep trough to a much flatter curve, of which solubility was not as dependent on pH as the solubility of the native protein. The solubility of 2.0% DH hydrolysates was slightly greater than the solubility of 4.0% DH hydrolysates at pH values 2–9 (Fig. 2). Decreased solubility of the hydrolysates was surprising since early studies of Puski (1975) indicated that solubility increased with increasing protein hydrolysis. The lower solubility of 4.0% DH hydrolysates may reflect changes in protein conformation after heat treatment of soy protein prior to hydrolysis. Adler-Nissen (1986) observed heat-denatured soy protein contained a soluble high molecular weight fraction, consisting of substantial amounts of 2S globulins. Adler-Nissen (1986) also noted a considerable decrease in the protein solubility index of a 6% DH hydrolysate prepared from heat-denatured soy protein and attributed the decrease in protein solubility index to the disappearance of the soluble aggregated fraction.

The pH-solubility of deamidated, soy protein hydrolysates at 2.0% DH was similar to the hydrolysed controls, but the pH-solubility of deamidated, soy protein hydrolysates at 4.0% DH was considerably greater than their respective controls. (Fig. 2). The solubility of deamidated, soy protein hydrolysates increased in the isoelectric pH range of 4 and 5 and in the pH regions of 4-7 as well as under alkaline conditions, as

Fig. 1. — Flow chart summarizing the preparation of enzymatically deamidated soy proteins.

was determined using the bicinchoninic acid method of Smith et al. (1985). The solubility was calculated as follows

$$\% \text{ Solubility } = \text{Absorbance } \frac{\text{Supernatant}}{\text{Supernatant } + \text{ Precipitate}} \times 100$$

Emulsifying properties

Emulsifying activity was determined by the method of Pearce and Kinsella (1978). Emulsions were prepared by homogenizing 5 mL of 0.5% soy protein dispersions in 0.1M NaCl, pH 2-9 and 5 mL soy oil aliquots in an Omni mixer at 10,000 rpm for 30 sec at 20°C. Emulsions (0.2 mL) were diluted to 1/1000 using 0.1% sodium dodecyl sulfate (SDS) and the absorbance at 500 nm was determined. Emulsion stability was determined by the method of Pearce and Kinsella (1978) as modified by Kato et al. (1987). Three milliliters of soy oil and 10 mL of a 0.1% protein dispersion in 0.1M phsophate buffer, pH 8.0 were homogenized in an Omni Mixer at 12,000 rpm for 1 min at 20°C. Emulsions (0.2 mL) were taken from the bottom of the container 15 sec after emulsion formation, which was consid-

Fig. 2. — Solubility of native and modified soy proteins at pH values 2-9: (A) Water extract of soy flakes; (B) 2% DH heated 15 min: Control (○), 6% deamidated (■) or 30 min: Control (●), 8.2% deamidated (▲); (C) 4% DH heated 15 min: Control (○), 12.8% deamidated (■) or 30 min: Control (●), 16.0% deamidated (▲).

a result of deamidation. Deamidation increased the negative charge of the hydrolysates and might disrupt hydrophobic and hydrogen bonds. Structural changes may account for the increase in solubility as suggested by Bigelow (1967) and Leonard and Foster (1961). Although the 4% DH hydrolysate, heated for 15 min was less deamidated than the hydrolysate heated for 30 min, the pH-solubility curve was markedly higher for the former than the latter. Greater hydrophobicity might result from the longer heat treatment. Bigelow (1967) proposed that a balance between charge frequency and hydrophobicity determined protein solubility. Our research confirms the observations of Motoki et al. (1986) that deamidation of a casein fraction by transglutaminase improved solubility at mildly acidic pH of 5.

Emulsification properties of deamidated soy proteins

Emulsifying activity, the ability of protein to serve as emulsifying agent in the formation of a stable emulsion, is expressed as the absorbance of diluted emulsions at 500 nm (Pearce and Kinsella, 1978). The emulsifying activity of the unmodified, heat-treated hydrolysates and the deamidated, heat-treated hydrolysates in the pH range of 2-9 is presented in Fig. 3. Protein hydrolysates exhibited greater emulsifying activity than intact protein. Limited hydrolysis of soy proteins increases emulsifying capacity (Puski, 1975). Emulsifying activity of heat-treated hydrolysates increased with the increase in the rate

of deamidation. Improvement in emulsifying properties may be attributed to the increase in protein solubility, negative charge and surface hydrophobicity (Halling, 1981; Waniska et al., 1981). The profiles of emulsifying activity in the pH range of 2-9 of deamidated hydrolysates resembled the protein solubility curves (Fig. 2), suggesting that the solubility of the protein is a prerequisite for emulsifying activity. Increased solubility of modified soy protein facilitates the formation of a stronger interfacial film (Huang and Kinsella, 1987).

The role of protein hydrophobicity in emulsification (Kato et al., 1983a; Nakai, 1983) was explained as a quantitative relationship between emulsifying properties and surface hydrophobicity. The emulsifying activity of the 4.0% DH hydrolysates, heated for 15 min, increased at pH 8 and 9 (Fig. 3C) but was not affected by deamidation at pH 7 or less. The unchanged emulsifying activity at acid pH was probably due to the decrease in surface hydrophobicity, brought about by the disruption of hydrophobic bonds. Deamidation increased the solubility of the 4.0% DH hydrolysates, presumbaly in part by reducing hydrophobicity. Nakai (1983) suggested that both hydrophobicity and solubility be taken into consideration to explain the emulsifying phenomena of heat-denatured proteins. Waniska et al. (1981) reported that in addition to solubility, electrostatic and hydrophobic interactions are also important in determining emulsifying activity and suggested that a certain degree of tertiary structural stability is needed for optimum emulsion formation. At pH values greater than 7.8, the ap-
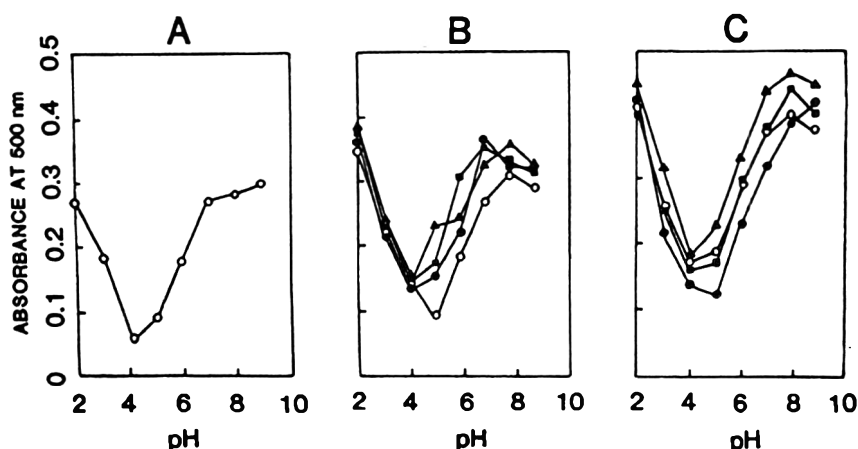
Fig. 3. — Emulsifying activity of native and modified soy proteins at pH values 2-9: (A) Water extract of soy flakes; (B) 2% DH heated 15 min: Control (○), 6% deamidated (■) or 30 min: Control (●), 8.2% deamidated (▲); (C) 4% DH heated 15 min: Control (○), 12.8% deamidated (■) or 30 min: Control (●), 16.0% deamidated (▲).
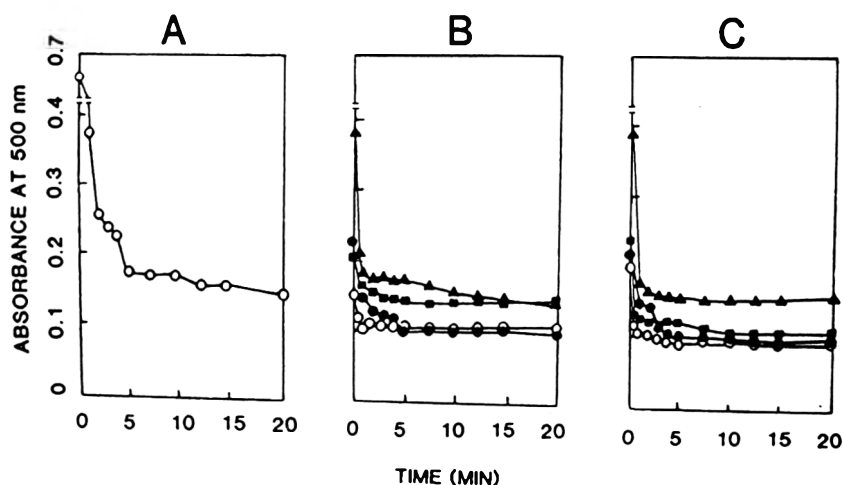
Fig. 4.—Emulsion stability of native and modified soy proteins: (A) water extract of soy flakes; (B) 2% DH heated 15 min: Control (o), 6% deamidated (■) or 30 min: Control (●), 8.2% deamidated (▲); (C) 4% DH heated 15 min: Control (o), 12.8% deamidated (■) or 30 min; Control (●), 16.0% deamidated (▲).

proximate pK' value of the α-amino terminal group, the polypeptide chains are negatively charged. Accordingly, there will no longer be an ionic interaction between the negative charges from the glutamyl carboxyl and the positively charged amino terminal groups. These conformational changes probably restored the surface hydrophobicity and hence increased the emulsification at pH 8 and 9.

The ability of discrete emulsion droplets to remain dispersed without separating is defined as emulsion stability. The time dependent changes in turbidity of diluted emulsions produced from controls and deamidated proteins are given in Fig. 4. Turbidity declined for 1-4 min and stabilized for the remainder of the assay. The emulsion stability of soy protein dispersion decreased upon protelysis. This effect of proteolysis on emulsion stability has been previously reported (Puski, 1975).

Contrary to emulsifying activity data, emulsions obtained from the control hydrolysate heated for 30 min were more stable than emulsions prepared from hydrolysates heated for 15 min, regardless of their DH values or protein solubility. This may indicate that in contrast to emulsifying activity, solubility is not a prerequisite for increased emulsion stability. Smith et al. (1973) described emulsion stability of insoluble proteins as "the insoluble protein particles sufficiently small to collect at the oil-water interface and provide a barrier to coalesence of oil droplets." Emulsion stability of the deamidated, hydrolysed proteins was substantially improved over the 2.0% and 4.0% DH controls, particularly in the hydrolysate deamidated to 16%. The increase in emulsion stability resulting from deamidation may be dependent on the increase in the number of negative charges (Halling, 1981), the surface hydrophobicity and the flexibility of the protein brought about by deamidation Kato et al. (1987). Kato et al. also reported that deamidation improved emulsifying activity and emulsion stability.

Deamidation of soy protein and foaming properties

Assessment of foaming properties of food proteins are generally based on either bubbling or beating. Although Halling (1981) reported the structure of foams obtained by the two methods was quite different, Wright and Hemmant (1987) observed good correlation between bubbling and beating. The foaming power and foam stability of modified soy proteins are presented in Table 1. Pretreatment of soy protein substrate prior to deamidation doubled the foaming power but apparently had no effect on foam stability. Foams from 2.0% DH control hydrolysates heated for 30 min were more stable than 2.0% DH control hydrolysates heated for only 15 min. Changes in foaming properties of control hydrolysates may be a combined effect of limited proteolysis and heat treatment. Soy protein

Table 1 — Foaming properties of modified soy proteins

Soy proteins	Foaming power	Foam stability
Water extract of soy flakes	257.4	0.13
2% DH heated 15 min, control	593.0	0.13
6.0% deamidation	657.4	0.14
2% DH heated 30 min, control	613.8	0.20
8.2% deamidation	633.8	0.19
4% DH heated 15 min, control	627.5	0.13
12.6% deamidation	643.3	0.12
4% DH heated 30 min, control	642.0	0.12
16.0% deamidation	747.5	0.12

with limited hydrolysis exhibits less stability than unhydrolyzed protein, but the foams are formed more readily (Puski, 1975). Kato et al. (1983a) reported that heat denaturation of soy globulins increased foaming power and foam stability due to increased surface hydrophobicity. Comparing control hydrolysates to PGase-treated hydrolysates, deamidation increased the foaming power of hydrolysates but had no apparent effect on foam stability (Table 1). Halling (1981) stated that increased solubility and enhanced electrostatic repulsion between adjacent bubbles may account for the improvement in foaming propeties.

Since soy proteins are used in many foods, the improvement in solubility and emulsification under mildly acidic conditions imparted by deamidation, although not dramatic, will make the deamidated soy protein better suited for a number of food applications such as carbonated or noncarbonated acidic beverages, pourable and nonpourable dressings, and coffee whiteners.

CONCLUSIONS

ENZYMATIC DEAMIDATION of soy protein by peptidoglutaminase generally enhanced protein solubility and emulsifying properties. Foams produced by deamidated protein had greater foaming power but foam stability was unchanged. Deamidated hydrolysates exhibited greater solubility and greater emulsifying properties under mildly acidic conditions than the undeamidated hydrolysates and the native soy protein.

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Objectionable Flavor of Soy Milk Developed during the Soaking of Soybeans and its Control

M. MATSUURA, A. OBATA, and D. FUKUSHIMA

————————————— ABSTRACT —————————————

Daidzein and genistein, which are responsible for the objectionable flavor of soy milk, increased during the soaking of soybeans, the first step of soy milk manufacturing. The maximum production of these isoflavone compounds occurred at 50°C and at pH 6.0 depending upon the temperature and pH of the soaking water. The production of these substances was strongly inhibited by glucono-δ-lactone which was a competitive inhibitor of β-glucosidases. It would appear, therefore, that β-glucosidases were responsible for the production of daidzein and genistein during soaking.

INTRODUCTION

SOY MILK is one of the popular traditional products of soybeans and has been consumed widely in China as a nutritious and economical protein food. However, the soy milk manufactured by the traditional process has not been accepted in Japan, because of its off-flavor which is characterized by a beany flavor and an objectionable aftertaste.

There are several investigations on the objectionable aftertaste of soybeans. Arai et al. (1966) identified a number of phenolic acids from defatted soy flour and showed that these phenolic acids had sour, bitter and astringent taste. Sessa et al. (1976) reported that oxidized phosphatidylcholines contributed to the bitter taste in soy flakes. Huang et al. (1981) identified isoflavone compounds from defatted soy flour, as daidzein (the aglucone of daidzin), genistein (the aglucone of genistin) and glycitein-7-β-glucoside, and stated that these isoflavones might be responsible for the undesirable bitter and astringent flavors of soybean products. Okubo et al. (1983) investigated the flavor of the isoflavones found in soybeans and observed that the isoflavone aglucones have more intense disagreeable flavor than the isoflavone glucosides.

Generally, it has been said that the objectionable aftertaste of foods is attributable to the presence of the polyphenols in the vegetable tissues (Goldstein and Swain, 1963, 1965). Isoflavones are the major phenolic compounds in soybeans (Ahluwalia et al., 1953); the main components are daidzin and genistin which are isoflavone glucosides (Walz, 1931; Naim et al., 1974).

The authors found that daidzein and genistein increased by the action of the β-glucosidases in soybeans during the processing of soy milk manufacturing. Further, the objectionable aftertaste paralleled the quantities of daidzein and genistein in the soy milk. The purpose of this study was to find a method to make soy milk with a very low degree of objectionable aftertaste by clarifying the mechanisms of the development of this aftertaste during the processing.

MATERIALS & METHODS

Sample preparation

Commercially available U. S. whole soybeans (10g) were soaked in 5-fold their weight of distilled water at several different tempera-

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tures and pH values, drained, rinsed with cold water, freeze-dried, ground with a mill, and then defatted with hexane in a Soxhlet extractor for 10 hr, followed by extraction with methanol. The soak water was freeze-dried, and the residue was extracted twice with methanol. The methanol extracts were concentrated to 50 mL under vacuum and filtered through a 0.22μ membrane for the analysis of isoflavone compounds by high performance liquid chromatography.

High performance liquid chromatography

Recently, high performance liquid chromatography (HPLC) has been used for the analysis of isoflavones (Murphy, 1981; Farmakalidis and Murphy, 1984; Eldrige, 1982). The analysis of soybean isoflavones was carried out according to the procedure reported by Eldridge (1982). A Model 209D instrument (Waters Asoc.), composed of a microprocessor-controlled dual pump system (Model 510, Waters Asoc.) and a variable wavelength detector (Model 490, Waters Asoc.) connected to a computing integrator (Chromatocorder 11, Sic Inc., Japan) was used for the HPLC analysis. The Waters 3.9 × 150 mm NOVA PAK-C₁₈® reverse phase column was used throughout the experiments.

The linear gradient elution by acetonitrile-water mixture was applied for the separation. The concentration of the acetonitrile was changed from 5% to 50% linearly for 40 min. The solvent flow rate was 1.0 mL/min. The samples were injected in varied amounts between 10 to 100 μL with a microsyringe. Eluted isoflavones were monitored at 254 nm and the areas of the resultant eluted peaks were integrated by the above-described integrator.

Daidzin, daidzein, genistin, and genistein as standard samples

The standard samples of daidzein and genistin were purchased from Extrasynthese Co., France (formerly Labindustries Laboratory & Research Instruments). The standard genistein was isolated from the hydrolyzate of the genistin using a methanol solution containing hydrochloric acid by the method of Walter (1941) and crystallized from 60% ethanol. The shape of the resultant crystals were white rectangular rods (m.p.298°C). Pure standard daidzin was obtained by the following procedure. Namely, the crude crystals of isoflavone glucosides were separated from defatted soybeans through the modified method of Walter (1941) and dissolved in a small amount of 10% methanol solution. The resultant highly concentrated isoflavone glucoside solution was put on Sephadex G25® (E.M.Merk & Co.), eluted with 10% aqueous methanol solution and crystallized from 80% ethanol to obtain pure daidzin. These crystals were white and needle-shaped (m.p.236°C). These separated isoflavones were checked by the spectral data of Naim et al. (1974) and Huang et al. (1981).

Hydrolysis of p-nitrophenyl-β-D-glucopyranoside by soybean β-glucosidases during soaking

A modified procedure of Heyworth and Walker (1962) was used for determination of the β-glucosidases assay. Whole soybeans (10g) were soaked in an aqueous solution (30 mL) containing p-nitrophenyl-β-D-glucopyranoside (P-NPG, 10mg) at several different temperatures and pH values. The same soybeans (10 g) were soaked in distilled water (30 mL) at the same conditions to serve as a blank. After washing with distilled water, these soybeans were boiled in water (100 mL) for 5 min to inactivate enzyme, cooled, dehulled and dipped in 80 mL of sodium carbonate (0.25M) for 2 hr to extract the p-nitrophenol liberated by the β-glucosidases in the soybeans. The p-nitrophenol in the alkaline extracts was colored yellow. The alkaline extracts were filtered, and the absorbance of the filtrate was measured at 420 nm
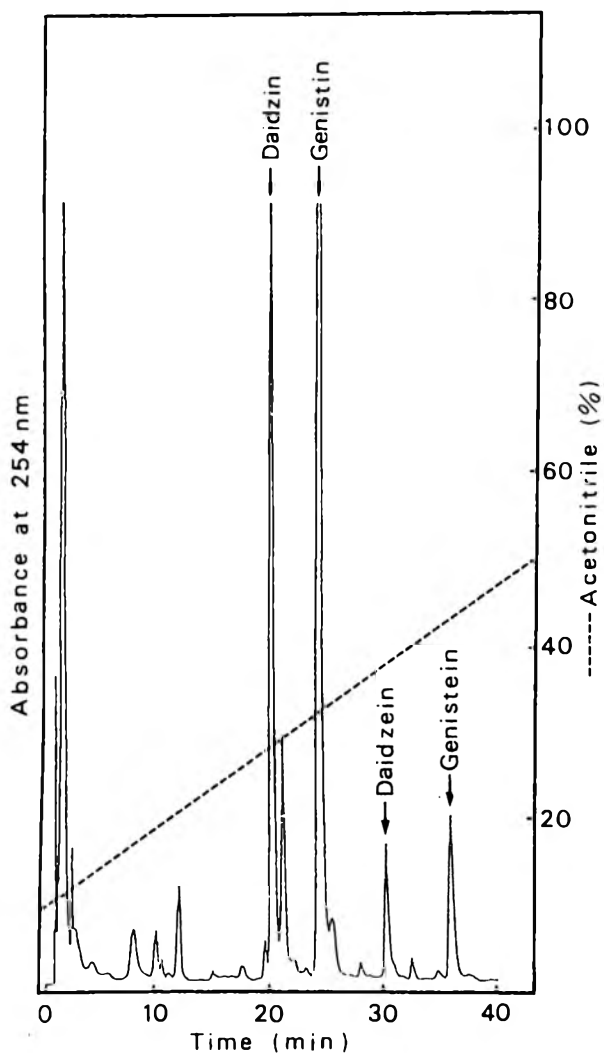
Fig. 1 — HPLC pattern of isoflavones from soybeans.

Table 1 — Change in content of the isoflavones contained in soybeans, before and after soaking

Soybeans	Isoflavone content[a] (mg/100g, dry basis)			
	Daidzin	Genistin	Daidzein	Genistein
Before soaking	115.2	181.1	5.1	4.9
After soaking[b]	97.4	119.8	14.7	24.3

[a] Average of two replicates. Means within the same column are significantly different (p ≤ 0.05).
[b] Sample was soaked at 20°C for 16 hr.

against the blank to determine the amount of p-nitrophenol liberated in the soybeans.

Preparation of soy milk

Whole soybeans (5kg) were soaked at 20°C for 16 hr in aqueous solution (15L) containing glucono-δ-lactone. The soaked soybeans were ground continuously in a horizontal grinder (TK-Homomic line mill, Tokushukikacogyo Co., Japan), with the addition of hot water (40L) at 95°C. The bean mash was heated at 100°C for 0.5 min in a double tube heat-exchanger and filtered by vacuum into a de-aerator through a plate-type filter. The soy milk was homogenized at 6000 psi (first stage pressure) and 500 psi (second stage pressure) with a Gaulin model M3 8TBS. The resultant soy milk contained approximately 3% protein. Charcoal treated tap water was used throughout this process.
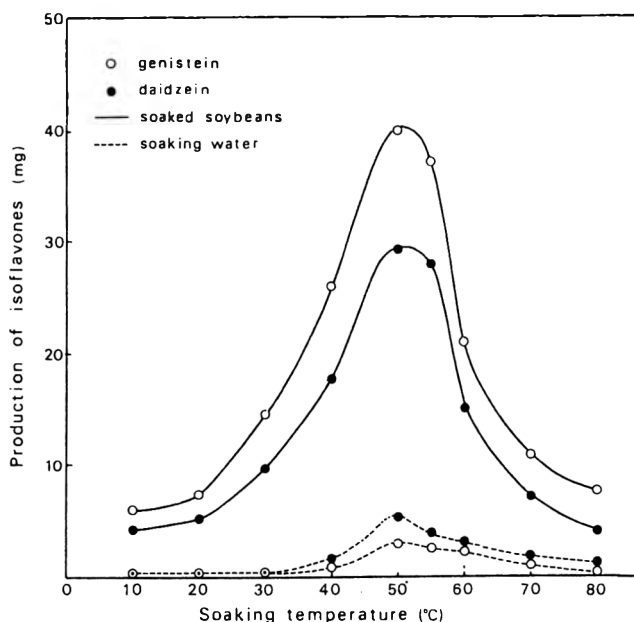
Fig. 2 — Effect of temperature on the production of isoflavones during the soaking. All samples were soaked for 6 hr.
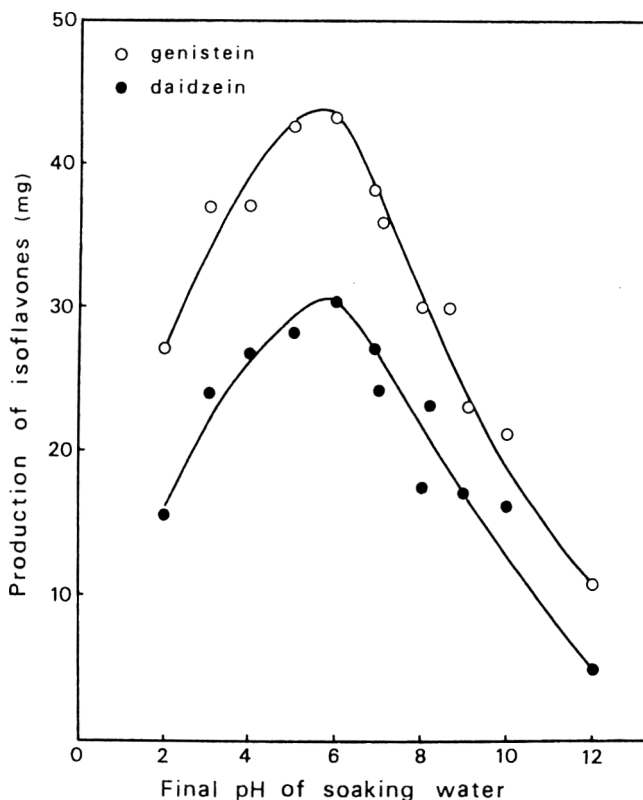
Fig. 3 — Effect of pH on the production of isoflavones in soybeans during the soaking. All samples were soaked at 50°C for 6 hr.

Sensory evaluation

Sensory evaluation was made on 20 mL of the five cultivars of soy milk by a panel of 12 experienced members selected from the laboratory staffs. Panelists evaluated each sample of soy milk for objectionable aftertaste on a five point scale (4 = very strong, 3 = strong, 2 = weak, 1 = almost none and 0 = none). Panelists were given sugar and water to take away any aftertaste between samples.
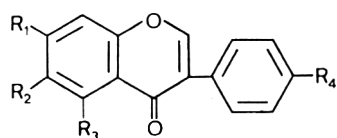
daidzein, $R_1 = OH$; $R_2 = H$; $R_3 = H$; $R_4 = OH$

daidzin, $R_1 = O$-glucosyl ; $R_2 = H$; $R_3 = H$; $R_4 = OH$

genistein, $R_1 = OH$; $R_2 = H$; $R_3 = OH$; $R_4 = OH$

genistin, $R_1 = O$-glucosyl ; $R_2 = H$; $R_3 = OH$; $R_4 = OH$

Fig. 4 — Chemical structures of soybean isoflavones.

Fig. 6 — Effect of pH on the action of β-glucosidases to P-NPG during the soaking of soybeans. All samples were soaked at 20°C for 16 hr.

Fig. 5 — Effect of temperature on the action of β-glucosidases to P-NPG during the soaking of soybeans. All samples were soaked for 6 hr.

RESULTS & DISCUSSION

THE HPLC PATTERN of soybean isoflavones in the linear gradient chromatographic method is shown in Fig.1. The absorbance of each eluted peak was measured every 1 nm between 190 and 400 nm by a learn-scan system (Waters, Model 490 detector) to compare with those of the standard isoflavone samples. As a result, the four peaks designated in Fig.1 were identified as daidzin, genistin, daidzein and genistein. The quantitative analysis of these isoflavones can be made within the range 0.5 to 5 μg.

The change of the isoflavones in the soybeans before and after soaking is shown in Table 1. The ratio of the isoflavone aglucones to the total isoflavones increased from approximately 3.3% to approximately 12.4% after soaking for 16 hr at 20°C.

Naim et al. (1974) reported that 99% of the isoflavones are present in soybeans as glucosides. These data are about the same as the data of "before soaking" in Table 1. However, the isoflavones of "after soaking" decreased markedly. Therefore, these results indicated that the original glucosides contained in the soybeans were hydrolyzed during the soaking. Murphy (1982) also presumes the hydrolysis of isoflavones

Fig. 7 — The production of isoflavones in soybeans during the soaking with and without glucono-δ-lactone at 20°C. The concentration of glucono-δ-lactone in the soaking water is 0.5% (w/ w).

during the water treatments of germinated soybean sprouts and tofu.

The effect of temperature on the production of daidzein and genistein during the soaking of soybeans is shown in Fig.2. The upper two curves show the isoflavone aglucones contained in the soaked soybeans. The amount of daidzein and genistein contained in 100g soaked soybeans (dry basis) changed markedly, depending upon the soaking temperatures, which ranged

	Glucono-δ-lactone (w/w %)		Soy milk		
No.	Soak solution	Amount incorporated into soybeans[a]	Isoflavone content[b] (mg/100mL)		Panel score[c]
			Daidzein	Genistein	
1	Tap water	0	1.6[d]	1.9[d]	3.9[d]
2	0.0015	0.001	1.5[d]	1.8[d]	3.8[d]
3	0.015	0.01	0.9[e]	1.0[e]	2.5[e]
4	0.15	0.1	0.5[f]	0.6[f]	1.5[f]
5	1.5	1.0	0.3[f]	0.4[f]	0.6[f]

[a] Calculated from the amount of the aqueous glucono-δ-lactone solution absorbed in the soybeans after the soaking.
[b] Average of two replicates.
[c] Based on a scale of 0 = no objectionable aftertaste to 4 = very strong objectionable aftertaste.
[d-f] Means with different superscripts within the same column are significantly different (p≤0.05).

from 5-7 mg at 10°C to 30–40 mg at 50°C when soaked for 6 hr. The effect of pH during the soaking at 50°C for 6 hr on the amount of daidzein and genistein produced in soybeans is shown in Fig.3. The Optimum pH for the production of daidzein and genistein is around 6.0 (Fig.3) which is very close to the pH of soy milk. Based on these findings, the mode of formation of daidzein and genistein in this case was presumed to be enzymatic.

Isoflavone glucosides and their aglucones in soybeans are shown in Fig. 4 (Walz, 1931; Ahluwalia et al., 1953; Naim et al., 1974). It can be presumed, from the chemical structure, that the β-glucosidases hydrolyze the isoflavone glucosides during the soaking of the soybeans and increase the amount of daidzein and genistein. Further, the temperature and pH dependent curves observed in Fig. 2 and 3 support the possibility described above. There are several reports on the glucosidases in soybeans (Moustafa and Wong, 1967; Hou et al., 1968, Hösell and Todenhagen, 1980), but no publications on the effect of these enzymes during the soaking of soybeans. To ascertain the action of soybean β-glucosidases during the soaking, P-NPG, a synthetic specific substrate for β-glucosidases, was added to the soaking water.

The effects of the temperature and pH of the soaking water on the action of β-glucosidases to P-NPG during the soaking of soybeans are shown in Fig. 5 and 6. The maximum liberation of p-nitrophenol occurred at 50°C and pH 6.0 of the soaking water. The shapes of these curves were about the same as those of the production of daidzein and genistein in Fig.2 and 3. From these results, it is clear that daidzein and genistein were developed through the hydrolysis of the isoflavone glucosides by the β-glucosidases contained in soybeans during the soaking.

To control the hydrolysis of isoflavone glucosides during soybean soaking, glucono-δ-lactone was added in the soaking water since it is a highly specific competitive inhibitor of β-glucosidases (Reese and Parrish, 1971; Levvy and Snaith, 1972). The production of daidzein and genistein which increased with the soaking time was inhibited by the addition of glucono-δ-lactone (Fig. 7).

The relationship between the objectionable aftertaste of soy milk and the amounts of daidzein and genistein contained in the soaked soybeans is shown in Table 2. According to this table, the degree of the objectionable aftertaste of soy milk increased with the increase in the amounts of the daidzein and genistein the soy milk, indicating that the daidzein and genistein were responsible for the objectionable aftertaste. Thus, the addition of glucono-δ-lactone to the soaking water was necessary to produce soy milk with a very low degree of the objectionable aftertaste.

Glucono-δ-lactone is converted into gluconic acid and glucono-γ-lactone in aqueous solution. The velocity of this conversion is very rapid at a higher pH and temperature (Takahashi

and Mitsumoto, 1963). Preliminary experiments showed that the inhibitory action of glucono-δ-lactone to the β-glucosidases was much stronger than that of glucono-γ-lactone. Therefore, glucono-δ-lactone should be added to the soak water at a low temperature, at which the conversion of glucono-δ-lactone to gluconic acid and glucono-γ-lactone is slow. When larger amounts of glucono-δ-lactone were used, the soybean protein tended to coagulate during the heating process. Within the range of the amounts used in Table 2, no substantial coagulation occurred. But, the condition of No.4 in Table 2 is recommended in the actual procedure.

Thus, a more widely acceptable tasting soy milk can be produced by the following two steps. The first step is the soaking of soybeans in low temperature water with glucono-δ-lactone. In this stage, β-glucosidases are inhibited by the glucono-δ-lactone and as a result the production of daidzein and genistein responsible for the objectionable after taste is depressed. The second step is the hot grinding stage, at which the β-glucosidases are inactivated completely and at the same time the lipoxygenases responsible for the development of the rancid flavor are also inactivated (Wilkens et al., 1967; Nelson et al., 1976).

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Soybean Protease Inhibitors in Foods

C.M. DiPIETRO and I.E. LIENER

──────────────── ABSTRACT ────────────────

The major types of soybean protease inhibitors are the Kunitz inhibitor (KSTI) and the Bowman-Birk inhibitor (BBI). Enzymatic analysis and rocket immunoelectrophoresis were used to specifically quantitate the amounts of KSTI and BBI in various soy foods. Many soy products such as soy flours, soy concentrates and soy isolates contained significant amounts of KSTI and BBI. Some soy foods contained small but still detectable levels of KSTI and BBI.

INTRODUCTION

THE EXISTENCE of an antiproteolytic substance in soybeans was first noted over fifty years ago (Read and Haas, 1938). Since then soybean protease inhibitors (SBPI) have been the subject of numerous scientific studies. A review by Liener and Kakade (1980) summarized the molecular properties of the major forms of SBPI, the classical Kunitz inhibitor (KSTI) and the classical Bowman-Birk inhibitor (BBI). Classical KSTI has a molecular mass of 20100 daltons, contains two disulfide bonds and inhibits trypsin strongly. Classical BBI has a molecular mass of 8000 daltons, contains seven disulfide bonds and strongly inhibits both trypsin and chymotrypsin. Due to their different molecular properties, KSTI and BBI are likely to have different antinutritive effects (Tan-Wilson and Wilson, 1986).

Soybeans are commonly heat treated before ingestion which inactivates a large portion of their SBPI activity (Smith and Circle, 1972). However, some commercially available soybean products contain residual SBPI activity which may equal 5 to 20% of the SBPI activity of raw soy flour (Rackis and Gumbmann, 1981). There is concern that this residual amount of activity could be nutritionally significant (Liener, 1986).

Based on a report that BBI is heat stable when heated in purified form (Birk, 1961) residual SBPI activity has sometimes been attributed exclusively to BBI (Johnson et al., 1980a,b). However, recent research has demonstrated that BBI, as it occurs *in situ*, is easily inactivated during moist heat treatment (DiPietro and Liener, 1988). Thus, residual activity in heated soy products is probably due to a combination of KSTI and BBI. Individual determination of KSTI and BBI would aid in evaluating the potential antinutritive effect of residual SBPI.

Enzymatic methods are widely used for quantitating SBPI, relying on the measurement of a decrease in proteolytic activity when some type of soy-protein derived material is present. Typically, bovine trypsin is used. Several studies have employed such techniques to assess the protease inhibitory activity of soy foods and other foods (Doell et al., 1981; Rackis et al., 1986; Smith et al., 1980). This provides a good overall measure of SBPI in a sample since both inhibitors are active against trypsin but does not individually measure the amounts of KSTI and BBI present.

A study by Sessa and Bietz (1986), which utilized a combination of enzymatic, chromatographic and immunoblotting

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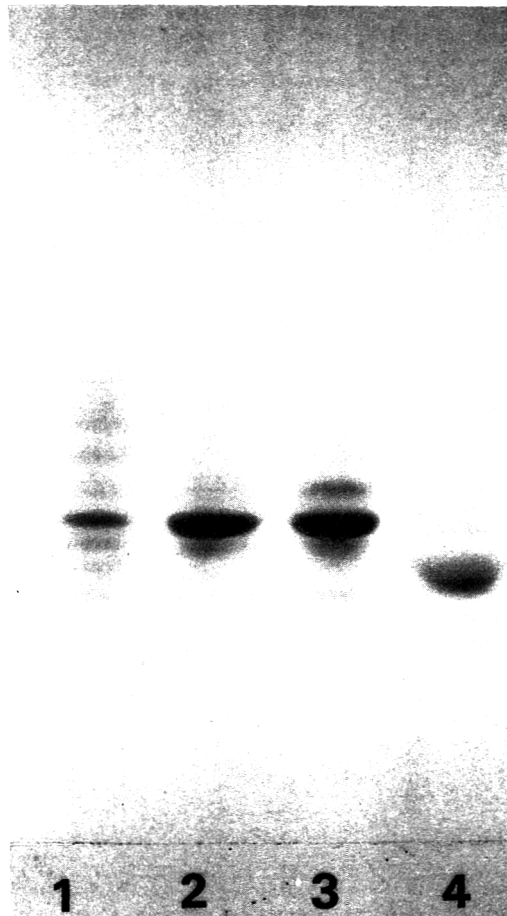
Fig. 1—Polyacrylamide gel electrophoresis of inhibitor preparations. Samples were as follows: Lane 1—25 µg crude soybean protease inhibitor; Lane 2—15 µg purified Kunitz inhibitor; Lane 3—15 µg commercially prepared Kunitz inhibitor; Lane 4—15 µg purified Bowman-Birk inhibitor.

methods, individually characterized the residual KSTI and BBI activity in a heated soy flour. No study has reported individual detection of KSTI and BBI in a wide variety of food products.

The objective of this study was to utilize a combination of enzymatic and immunochemical methods to quantify KSTI and BBI individually in a wide variety of food products.

MATERIALS & METHODS

Purified inhibitors

Classical KSTI was purified from commercial KSTI (Sigma Chemical Co.) by chromatography on DEAE-cellulose (Freed and Ryan, 1978). Classical BBI was purified by a slight modification of the procedure described by Birk et al. (1963) which involved the extraction of raw defatted soy flour with 60% ethanol and precipitation by two volumes acetone as well as sequential chromatography on DEAE- and CM- cellulose. Purity of the inhibitors was verified by PAGE (Ornstein, 1964; Davis, 1964). Stacking gels contained 2.8% acrylamide with an acrylamide: bisacrylamide ratio of 4:1. Separating gels contained 15% acrylamide with an acrylamide: bisacrylamide ratio of

B K SE

Fig. 2—Immunoelectrophoresis. Wells contained purified Bow-man-Birk inhibitor (B), pure Kunitz inhibitor (K), and a crude extract of raw soy flour (SE). Troughs (indicated by black lines) were filled with antiKSTI serum.

Table 1 – Reproducibility of inhibitor analyses

Sample	Replication	Total Inhibitory Activity[a] (IU/mg)	KSTI content (μg/mg)	BBI content (μg/mg)
1	1	267	18.41	5.91
	2	307	17.00	7.12
	3	264	17.90	6.49
	4	307	17.34	7.17
	Average	286 ± 24	17.66 ± 0.62	6.67 ± 0.60
	C.V.[b]	8.39%	3.51%	8.99%
2	1	164	15.96	3.47
	2	167	14.72	3.76
	3	160	13.89	3.69
	4	158	14.33	4.14
	Average	162 ± 4	14.73 ± 0.89	3.76 ± 0.28
	C.V.[b]	2.44%	6.04%	7.45%

[a] Total inhibitor activity was measured by trypsin inhibition. KSTI (Kunitz inhibitor) content was measured by rocket immunoelectrophoresis. B3I (Bowman-Birk inhibitor) content was measured by chymotrypsin inhibition. Results for each replication are the means of triplicate analyses for total inhibitory activity and BBI content and means of duplicate analyses for KSTI content.
[b] Coefficient of variation.

150:1. Gels were 0.75 mm thick and were stained with Coomassie Brilliant Blue R.

Soy product extracts

For soy flours, soy concentrates and soy isolates, 2% extracts were prepared with 0.01N NaOH. For solid soy foods, 6% extracts were prepared with 0.01N NaOH. Liquid soy foods were diluted with an equal volume of 0.02 N NaOH. Extracts were prepared by combining the proper amounts of soy material and NaOH for 2 min with a high shear mixer and then centrifuging the mixture at 10,000xg for 30 min. The supernatant was collected and analyzed for inhibitor content within 5 days. If necessary, before analysis, the extract was passed through a 1.0 μm filter to obtain a clear solution.

Total SBPI activity

1.92 mL buffer (0.05M Tris, 0.02M $CaCl_2$, pH 8.2) was placed in a test tube and 0.040 mL bovine trypsin (Sigma Chemical Co.) in solution (0.4 mg/mL in 0.001N HCl) was added. This mixture was tempered for 5 min in a 37°C bath, then 0.100 mL N-benzoyl-DL-arginine-p-nitroanilide (BAPNA) in dimethyl sulfoxide (20 mg/mL) was added. The mixture was vortexed briefly and returned to the 37°C bath. Exactly 5 min later, 0.50 mL 30% acetic acid was added. Absorbance of the solution (versus a calibration blank) was read at 410 nm. Calibration blanks were prepared by substituting distilled deionized water (DDW) for the sample solution and adding acetic acid before adding BAPNA. Sample blanks were prepared by adding acetic acid before BAPNA. Controls were prepared by substituting DDW for the sample solution. Each sample was assayed in triplicate. Before reading absorbance, some samples were centrifuged to remove material precipitated by the addition of stopping solution. Results were converted to inhibitor units (IU) per mg sample ("as is" basis). One IU was defined as a decrease of 0.01 absorbance units as compared to a control sample.

BBI content

1.92 mL buffer (0.05M Tris, 0.02M $CaCl_2$, pH 8.2) was placed in a test tube and 0.040 mL bovine chymotrypsin (Sigma Chemical Co.) in solution (0.4 mg/mL in 0.001N HCl) was added. This mixture was tempered for 5 min in a 37°C bath, then 0.300 mL N-benzoyl-L-tyrosine-p-nitroanilide (BTPNA) in dimethyl sulfoxide (1.33 mg/mL) was added. The mixture was vortexed briefly and returned to the 37°C bath. Exactly 5 min later, 0.50 mL 30% acetic acid was added. Absorbance of the solution (versus a calibration blank) was read at 410 nm. Calibration blanks were prepared by substituting DDW for the sample solution and adding acetic acid before adding BTPNA. Sample blanks were prepared by adding acetic acid before BTPNA. Controls were prepared by substituting DDW for the sample solution. Each sample was assayed in triplicate. Before reading absorbance, some samples were centrifuged to remove material precipitated by the addition of stopping solution. Results were converted to equivalent micrograms BBI per mg sample ("as is" basis) using a standard curve relating % chymotrypsin inhibited to BBI concentration (10–100 μg/mL).

KSTI content

To generate antisera, KSTI (2 mg/mL in phosphate buffered saline) solution was emulsified with an equal volume of Freund's complete adjuvant (Sigma Chemical Co.) One mL of the emulsion was injected subcutaneously in three sites on the back of a New Zealand White rabbit. Six weeks later, a booster injection (0.5 mL, in Freund's incomplete adjuvant) was given. Seven days later, 50 mL blood was taken via the marginal ear vein. The blood was allowed to clot overnight at 4°C and the serum was collected after centrifugation at 1500xg for 20 min. The serum was stored at −20°C until needed. The titer of the serum used was 1:4 as determined by Ouchterlony immunodiffusion.

Specificity of the serum was determined by immunoelectrophoresis (Weeke, 1973; Mayer and Walker, 1984). Various protein solutions were separated by electrophoresis in a 1% agarose gel. Troughs were then cut in the gel alongside the proteins. The troughs were filled with the serum and allowed to stand for 24 hr at 4°C. The plates were then washed, dried and dyed with Coomassie Brilliant Blue R to aid in visual detection of precipitin bands.

Rocket immunoelectrophoresis (RIEP) was performed according to Freed and Ryan (1978). Results were converted to micrograms KSTI per mg sample ("as is" basis) using a standard curve relating rocket height to KSTI concentration (2.5-40 μg/mL).

RESULTS & DISCUSSION

Purity of inhibitors

The results of PAGE of the purified inhibitors used in this study are shown in Fig. 1. The preparation of classical KSTI
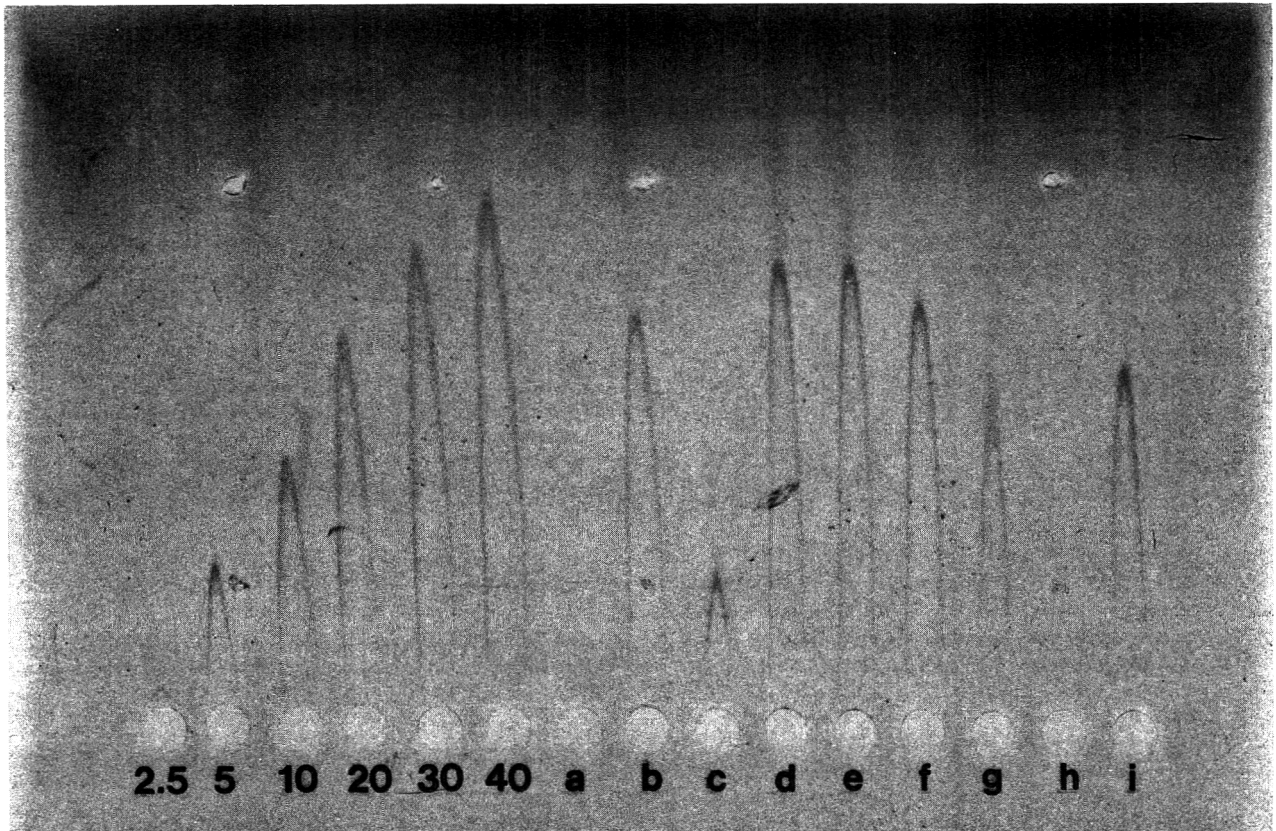
Fig. 3—Rocket immunoelectrophoresis for Kunitz inhibitor (KSTI). Wells with number designations were filled with standard solutions of KSTI. Numbers indicate concentration in μg/mL. Wells with letter designations were filled with the following solutions: well a—Bowman-Birk inhibitor (BBI), 85 μg/mL; well b—extract of raw defatted soy flour, 0.125% w/v; well c—extract of heated defatted soy flour, 0.125% w/v; well d—extract of raw defatted soy flour, 0.125% w/v, spiked with KSTI, 5μg/mL; well e—extract of raw defatted soy flour 0.125% w/v, spiked with KSTI, 5 μg/mL, and BBI, 20 μg/mL; well f—extract of soy concentrate, 0.25% w/v; well g—extract of texturized soy protein, 0.5% w/v; well h—extract of soy protein isolate a, 6% w/v; well i—extract of soy protein isolate b, 0.125% w/v.
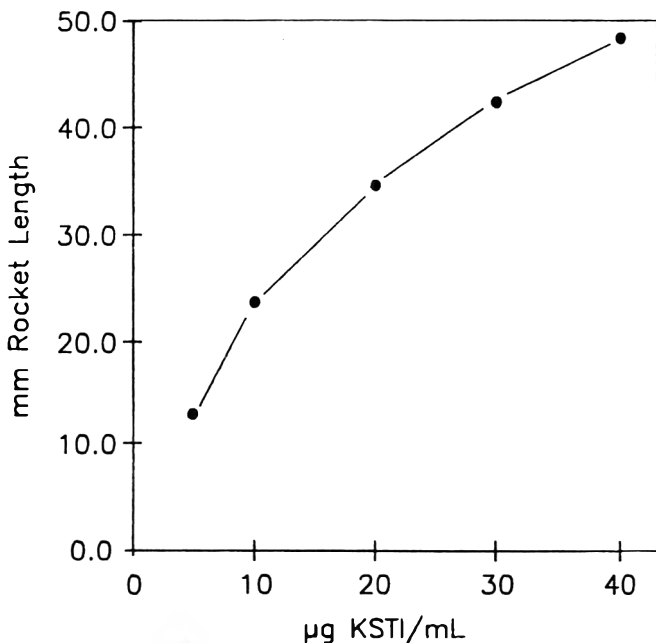
Fig. 4—Rocket immunoelectrophoresis standard curve for Kunitz inhibitor (KSTI). Rocket height (mm) versus KSTI concentration (μg/mL). Each point on the curve represents a single determination for each standard solution of KSTI as shown in Fig. 3.

in Lane 2 was used to generate antibodies. Some minor contaminants were detectable in this preparation of KSTI; however, it did not appear to contain BBI. The preparation of classical BBI in Lane 4 was used to generate standard curves for chymotrypsin inhibitor analysis. No contaminating bands were detectable at up to 75 μg (get not shown).

Charcterization of antisera

An immunoelectrophoresis plate prepared with antiKSTI serum is shown in Fig. 2. The antiKSTI serum reacted with KSTI and a similarly migrating protein in the soy extract but not with BBI or other proteins in the soy extract. This indicates that the serum was specific for KSTI. Previous work (DiPietro and Liener, 1985) demonstrated that thermal inactivation of antienzymatic activity of KSTI corresponded to loss of antibody recognition. A photograph of a KSTI RIEP plate is shown in Fig. 3. The corresponding standard curve is shown in Fig. 4. The shape of this curve represents a typical curve shape for RIEP (Verbruggan, 1975). Laurell and McKay (1981), have noted that to obtain quantitative results with RIEP, rockets produced with unknown samples must have the same morphological characteristics as standard solutions of antigen. Therefore, extracts of various soy materials were applied to the rocket plate. Wells "b" through "i" contained extracts of various soy-containing materials. The rockets of all these solutions have a morphology similar to standard solutions of KSTI. Previous studies have shown RIEP can be used as a quantitative method for KSTI (Freed and Ryan, 1978) as well as many other proteins (Verbruggan, 1975; Gross and Marz, 1988).

Reproducibility of measurements of SBPI activity

Data which illustrate the reproducibility of the measurements of total SBPI activity, KSTI content and BBI content

Table 2—Inhibitor content of soy products[a]

	Total inhibitory activity (IU/mg)	KSTI content (μg/mg)	BBI content (μg/mg)	Ratio of KSTI/BBI
Flours				
A	286.3 ± 4.6	17.40 ± 0.77	4.86 ± 0.05	3.6
B	257.0 ± 4.6	19.16 ± 0.26	4.64 ± 0.12	4.1
C	241.3 ± 6.4	15.51 ± 0.44	4.30 ± 0.38	3.6
D	232.3 ± 4.0	19.60 ± 0.53	2.53 ± 0.08	7.8
E	226.0 ± 8.7	16.39 ± 0.24	3.56 ± 0.23	4.6
F	148.2 ± 4.6	10.03 ± 0.07	3.80 ± 0.15	2.6
G	91.5 ± 4.3	6.13 ± 0.09	1.16 ± 0.06	5.3
H	32.5 ± 1.0	3.47 ± 0.18	0.45 ± 0.08	7.8
I	14.0 ± 0.5	1.13 ± 0.05	0.35 ± 0.07	3.3
J	10.8 ± 1.5	1.51 ± 0.14	<0.23	–
Concentrates				
A	74.2 ± 0.4	6.13 ± 0.57	1.04 ± 0.12	5.9
B	11.9 ± 2.9	1.24 ± 0.01	0.47 ± 0.01	2.6
C	10.3 ± 2.6	1.11 ± 0.10	0.48 ± 0.05	2.3
D	<5.0	<0.50	<0.23	–
Isolates				
A	46.3 ± 0.9	3.61 ± 0.26	0.45 ± 0.02	8.1
B	42.8 ± 1.3	1.50 ± 0.11	2.04 ± 0.07	0.7
C	42.4 ± 0.3	1.52 ± 0.08	1.03 ± 0.05	1.5
D	23.7 ± 0.2	0.46 ± 0.05	0.90 ± 0.04	0.5
E	20.0 ± 0.3	0.69 ± 0.17	0.55 ± 0.01	1.2
F	16.6 ± 0.3	<0.5	0.71 ± 0.07	–
G	16.3 ± 0.2	0.84 ± 0.04	0.91 ± 0.05	–
H	15.7 ± 0.8	0.89 ± 0.09	0.39 ± 0.09	2.3
I	13.6 ± 0.9	<0.50	0.59 ± 0.06	–
J	9.2 ± 0.3	<0.50	0.29 ± 0.01	–
K	8.8 ± 1.0	<0.50	0.29 ± 0.11	–
L	5.2 ± 1.6	<0.50	0.33 ± 0.08	–
M	4.9 ± 1.6	<0.50	0.34 ± 0.10	–

[a] Total inhibitory activity was measured by trypsin inhibition. KSTI (Kunitz inhibitor) content was measured by rocket immunoelectrophoresis. BBI (Bowman-Birk inhibitor) content was measured by chymotrypsin inhibition. The results are means ± standard deviations of triplicate analyses for total inhibitory activity and BBI content and means ± average deviations of duplicate analyses for KSTI content. Samples with values listed as < (less than) a particular amount contained less than a detectable amount of activity as measured by the method indicated. The values listed represent the detection limit for a 2% extract of soy products.

Table 3—Inhibitor content of soy foods[a]

	Total inhibitory activity (IU/mg)[b]	KSTI content (μg/mg)[b]	BBI content (μg/mg)[b]	Ratio of KSTI/BBI
Meat substitutes				
Textured Soy Protein	14.3 ± 0.8	0.96 ± 0.05	0.48 ± 0.07	2.0
Breakfast Strips	3.3 ± 0.3	<0.17	<0.07	–
Firm Tofu A	2.7 ± 0.3	<0.17	0.08 ± 0.04	–
Firm Tofu B	2.4 ± 0.6	<0.17	0.07 ± 0.02	–
Breakfast Pattie	2.2 ± 0.3	<0.17	<0.07	–
Tempeh Burger	2.0 ± 0.2	<0.17	<0.07	–
Infant foods				
Dry Cereal, High Protein	2.7 ± 0.8	0.24 ± 0.01	0.10 ± 0.04	2.4
Jarred Dinner, Chicken	1.5 ± 0.1	<0.17	<0.07	–
Formula, Dehydrated	<1.8	<0.17	<0.07	–
Formula, Liq. Conc. A	0.7 ± 0.1	<0.02	0.11 ± 0.01	–
Formula, Liq. Conc. B	0.4 ± 0.1	<0.02	0.02 ± 0.01	–
Dried Dinner, Chicken	<1.8	<0.17	<0.07	–
Other foods				
Soy Milk, Dehydrated	118.6 ± 3.9	11.30 ± 0.21	1.85 ± 0.07	6.1
Pancake Mix, Wheat/Soy	13.0 ± 0.3	0.99 ± 0.21	0.38 ± 0.01	2.6
Cat Food, Dry	<1.8	<0.17	<0.07	–
RTE Cereal, High Protein	<1.8	<0.17	<0.07	–
Soy Milk, Frozen	0.4 ± 0.1	<0.02	0.02 ± 0.01	–
Dog Food, Dry	<1.8	<0.17	<0.07	–

[a] Total inhibitory activity was measured by trypsin inhibition. KSTI (Kunitz inhibitor) content was measured by rocket immunoelectrophoresis. BBI (Bowman-Birk inhibitor) content was measured by chymotrypsin inhibition. The results are means ± standard deviations of triplicate analyses for total inhibitory activity and BBI content and means ± average deviations of duplicate analyses for KSTI content. Samples with values listed as < (less than) a particular amount contained less than a detectable amount of activity as measured by the method indicated. The values listed represent the detection limit for a 6% extract for solid soy foods or a 50% dilution for liquid soy foods.
[b] Value for liquids are per mL rather than per mg

are shown in Table 1. In all cases, values were reproducible with a coefficient of variation of 10% or less over four separate analyses for each of the SBPI measurements.

SBPI content of soy products

The results of the analyses of 27 soy flours, isolates, and concentrates are shown in Table 2. The highest levels of the inhibitors were found in soy flours. Flour A was known to be raw defatted soy flour and would be expected to have the highest level of inhibitors of all the samples. Flours with lower levels of inhibitors had probably received some heat treatment. For concentrates and isolates, much of the reduction in SBPI activity was probably due to extraction or heat inactivation of SBPI during the normal processing of these products. However, concentrates contained up to 26% of the trypsin inhibitory activity of raw soy flour and isolates up to 16% of the activity of raw soy flour.

Other researchers have reported that the total SBPI content has ranged from 20–50 μg/mg for soy flour, from 5–15 μg/mg for soy concentrate, and from 3–30 μg/mg for soy isolate (Rackis et al., 1986; Smith et al., 1980). These reports were made by equating micrograms trypsin inhibited to micrograms KSTI since KSTI and trypsin have similar molecular mass (20100 daltons and 23800 daltons, respectively). However, in most soy products, a significant but variable portion of the SBPI activity is due to BBI which has a molecular mass of only 8000 daltons. Therefore, results based on equating SBPI with the amount of trypsin inhibited tend to be somewhat higher than the results presented here. The ratio of KSTI to BBI (by weight) in flours which received little heat treatment (Flours A through C) was generally near 4. Since the molecular mass of KSTI is about 2.5 times that of BBI, there are probably about 1.5 moles of KSTI present for each mole of BBI present. The ratio of KSTI to BBI in the other flours concentrates and isolates was at times higher and at other times lower than those of the unheated flours. This is probably due to differences in the way these materials were processed. It is possible that one of the inhibitors is inactivated to a different extent during a particular type of heat treatment. In addition, extraction and precipitation steps in concentrate and isolate production may favor removal of one inhibitor as opposed to the other.

SBPI content soy foods

The results of the analyses of 18 soy containing foods are shown in Table 3. In general, they contain very low amounts of SBPI. In many cases the amounts were too low to give quantitative results by the techniques employed in this study. Of particular interest were the results for dehydrated soy milk which had been purchased at a "health food" store. This product contained almost 50% of the SBPI activity of raw soy flour. It is designed to be consumed without further heating and therefore a consumer could unknowingly ingest a large amount of active protease inhibitors.

Since there is some uncertainty as to the level of SBPI which is injurious to human health (Liener, 1986), a true understanding of implications of the results presented here will require quantitation of the human response to ingestion of active SBPI.

CONCLUSIONS

VARIABLE AMOUNTS of SBPI activity were found in soy flours, concentrates, and isolates. This activity may be due to KSTI, BBI or both KSTI and BBI. In commonly available

Factors Affecting Wheat Flour Amylographic Maximum Viscosity

BLAŽENKA ŠEBEČIĆ

──────── ABSTRACT ────────

To explain differences in amylolytic activity of flour determined by amylograph and by reliable chemical methods, the influence of starch concentration, the physicochemical properties of starch and the effect of α-amylase on the viscosity maximum of flours and isolated starches was studied. Differences in viscosity of flours were partly due to the differences in starch concentration in suspensions. The viscosity maximum of inactivated flour and isolated starch depended on starch physicochemical properties ($r = 0.957$ and $r = 0.992$, respectively) and concentration. The decrease in viscosity maximum due to activity of α-amylase was associated with the amount of hydrolyzed starch ($r = 0.986$) and depended on both physicochemical properties of starch and concentration of α-amylase. Neither the viscosity maximum nor the decrease in viscosity maximum was a reliable indicator of amylolytic activity.

INTRODUCTION

THE AMYLOGRAPH has been used along with the Falling Number test (AACC, 1969) to investigate the gelatinization properties of starches and to determine the diastatic activity or malt index in flour. This viscometric method is also used throughout the world as a means of determination of malt supplementation, sprouting and evaluation of breadmaking properties of wheat or rye flour. However, in comparing amylolytic activity measured by reliable chemical methods with activities determined viscometrically using the amylograph, unacceptably large differences between the two methods are observed. So, although the amylograph has been used for a long time, it is still uncertain what exactly is measured.

Several studies have sought to identify those properties of flour which are responsible for viscosity as measured by the amylograph. The possibility of varietal and environmental influences, effect of enzyme concentration, influence of amyloclastic susceptibility on milling characteristics and milling damage of starch have been considered (Anker and Geddes, 1944; Brown and Harrel, 1944; Selmann and Sumner, 1947; Mathewson and Pomeranz, 1978). The results of these and other investigations indicate that the amylographic viscosity maximum reflects the gelatinization properties of starch and α-amylolytic activity of flour.

To study the role of starch itself in amylographic viscosity of flour, some authors investigated the behavior of different pure starches under amylographic conditions. Mazurs et al. (1957) through graphical analysis of amylographic curves and starch concentration in suspension observed nonlinear interdependence of these two variables, and on the basis of the curve shapes could identify different kinds of starches. Momirović-Čuljat (1970) determined the functional dependence between the maximum of viscosity and starch concentration. This author showed that the maximum of viscosity (MV), expressed in amylographic units, was related to the substrate concentration by $MV = a^x$, where "x" was the substrate concentration in weight percentage (Momirović-Čuljat, 1970). The basis of this exponential function, the constant "a", is specific to each type of starch. Wheat, corn and potato starches had "a" values of 1.80, 2.29 and 2.62, respectively. In the same paper (Momirović-Čuljat, 1970), it was shown that equal amounts of α-amylase added in presence of surplus substrate might exhibit different decreases in viscosity maximum in the same starch sample, depending on the initial concentration of substrate in suspension.

Investigating the effect of protein and starch components on wheat flour amylographic viscosity by inactivation of diastatic activity (pH 2.95), Balint and Momirović-Čuljat (1976) found that viscosity maximum (MV) was a function of the starch concentration (in weight percentage) in slurry (c) and could be expressed as $MV = a^{\sqrt{c}}$, where "a" is a constant value specific to wheat flour $= 11.90$.

Mathewson and Pomeranz (1978) found unacceptable differences between α-amylolytic activity as determined by the amylograph and colorimetric method. Investigating the reasons for these deviations, they found that the same amount of α-amylase added from three malts caused the same viscosity maximum decrease in the same flour, but different flours had different decreases. Therefore, they concluded that environmental conditions and real differences in starch properties influenced the α-amylase susceptibility of starches and consequently the values of amylographic viscosity maximum.

One of the drawbacks of the amylograph is that the concentration of α-amylase is not measured directly. Only the effects of the enzyme and of some other unknown factors on the viscosity of flour suspension are determined.

Another drawback derives from the choice of sample weight. Since diastatic activity determination of flour according to the method 22-10, AACC (1962) is carried out using a definite sample weight (100 g, 14% moisture basis), this ensures the equal dry matter content of flour but does not ensure equal starch concentration in suspensions of different samples. Since Momirović-Čuljat (1970) and Balint and Momirović-Čuljat (1976) reported that the amylographic viscosity maximum depended on starch concentration and not on dry matter concentration, such a choice of sample weight could be a cause of greater or lower errors in amylogram interpretation due to differences in starch concentration of different flour samples.

The objective of this study was to investigate factors responsible for the differences in viscosity of wheat starch and flour as determined by the amylograph, to find out what actually was being measured by this technique and to explain the observed differences in the amylolytic activity determined by two methods.

The principal factors investigated were starch concentration in suspension, starch physicochemical properties and the effect of α-amylase on viscosity as related to starch physicochemical properties. The effect of the protein content and its composition, as well as of damaged starch, starch granulation and its composition were studied.

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MATERIAL & METHODS

FIFTY-ONE SAMPLES of qualitatively very different untreated wheat cultivars harvested in the course of three years in different regions of Yugoslavia were used. Samples were milled on an experimental lab-

oratory mill (about 70% extraction). Moisture was determined by drying (AACC, 1962), starch by the polarimetric method, hydrochloride procedure (Arbeitsgemeinschaft Getreideforschung, 1978) and protein by the Kjeldahl method (AACC, 1962). The granular composition of starch was determined by the quantitative microscopic method (Seidemann, 1966); the damaged starch content of flour, by the photometric method of Williams and Fegol (1969); α-amylase activity, with a Brabender amylograph (AACC, 1962).

To investigate the gelatinization properties of wheat starch without effects from other components, the starch was isolated by manual washing of the dough (prepared in a faringraph bowl to the consistency of 500 farinograph units) and by centrifugation of the resultant suspension, with several washings and separation of tailings. Moisture of the isolated air-dried starch was determined by vacuum drying, 120°C, 4 hr (Schormüller, 1967) and starch polarimetrically (Arbeitsgemeinschaft Getreideforschung, 1978). Amylose and amylopectin were determined photometrically (Dahle, 1971).

Amylographic measurements

To determine "the amylolytic activity" of flour samples, amylograms were run with 60 g flour and 450 mL phosphate-citrate buffer (PCB) pH 5.35; buffer was prepared by dissolving and diluting 14.8g anhydrous disodium phosphate and 10.3g citric acid monohydrate to 1 L with distilled water; 100 mL concentrated buffer was diluted to 1 L with water to obtain buffer with pH range of 5.30–5.35.

To investigate the influence of the starch concentration in flour suspension and the effect of its physicochemical properties on the value of amylographic maximum, amylograms of the flour samples were run under various conditions: (1) 50g flour and 450 mL PCB pH 5.35; (2) 50 g flour and 450 mL PCB pH 2.95 (buffer contained 1.00 g anhydrous disodium phosphate and 6.00 g monohydrate citric acid dissolved in 1 L distilled water); (3) flour and buffer corresponding to 6.5% starch in suspension (PCB, pH 2.95).

Amylograms were run on Brabender amylograph with scale range from 0 up to 1000 AU thus limiting the sample weight to 60 g flour at pH of enzyme activity and 50 g flour at pH of enzyme inactivation. Since the adjustment of flour moisture to 14% was pointless, as previously shown, 60 g and 50 g flour samples, respectively, were used without adjustment of moisture. That simplified the performance without affecting the method drawback, thus prepared suspensions differed in starch concentration as was the case with AACC (1962) method.

To examine the influence of physicochemical properties of starch on the α-amylase susceptibility of starch and consequently on the effect of α-amylase on the amylographic viscosity maximum, the amylograms were run on the isolated starch samples without amylase and with addition of α-amylase: (a) 50 g starch and 450 mL PCB pH 5.9 (buffer solution - 15.5 g anhydrous disodium phosphate and 10.3 g of citric acid monohydrate dissolved in 1 L distilled water; 100 mL of this solution was diluted to 1 L with water to obtain buffer with pH 5.9); (b) 50 g starch and 450 mL PCB pH 5.90 and 5 IU α-amylase (bacterial) added.

Amylograms were run on ten samples of isolated starches using PCB pH 5.9 and 0.25 - 5 IU α-amylase, in a separate experiment to investigate the effect of different concentrations of α-amylase on the viscosity maximum of gelatinized starch.

Bacterial α-amylase was freshly prepared each day by dissolving a definite amount of α-amylase (*Bacillus subtilis*, Merck) at concentration of 150000 IU/g in 0.02M NaCl. Buffer value of 5.9 was chosen after preliminary investigations which indicated that the added α-amylase exhibited the greatest decrease in viscosity (in AU) in suspension made with this buffer. The pH range investigated was 5.5-6.9.

Simple linear correlation analyses and Student's t-test were performed.

RESULTS & DISCUSSION

THE PROTEIN CONTENT of the flour samples ranged from 8.3-14.7% ($\bar{x}$ = 11.4%), starch 64.31-70.61% ($\bar{x}$ = 66.64%), damaged starch 3.30-21.66 Farrand units ($\bar{x}$ = 8.37) (Table 1) and moisture 11.48-13.04%.

Tests of the granular starch composition revealed that on an average 66.30% of the granules were smaller than 10μm (41.00-80.00%), whereas 15.50-48.00% of the granules ($\bar{x}$ = 28.90%) were 10-25 μm. On the average there were 7.60% of the granules larger than 25 μm (2.00-16.00%), 6.60% of which were from 25-35 μm (1.50-15.00%) and 0.00-3.00% of the gran-

ules were more than 35 μm ($\bar{x}$ = 1.00%). This classification was made because many authors emphasize the difference in physical properties of large and small granules, starting from differences in optical properties (Evers, 1971; Czaja, 1972) and behavior during gelatinization (Hill and Dronzek, 1973) to differences in binding of protein material, susceptibility to amylolytic decomposition (Bathgate and Palmer, 1972) and baking characteristics (Kulp, 1972, 1973). Furthermore, investigations performed by Buttrose (1963), Banks and Greenwood (1975) and others showed that granule sizes mostly ranged up to 30 μm, though larger sizes also occurred. Granules up to 10 μm were round, those from 25-35 μm were lenticular. There were also granules between these two pure types in size and shape.

Starch in isolated starch ranged from 78,54-83,74% ($\bar{x}$ = 80,14%) and amylose was 18.80-27.56% ($\bar{x}$ = 23.41%) of the total starch.

Wheat flour amylograms

Amylograms of the tested samples determined on 60g flour and 450 mL phosphate-citrate buffer pH 5.35 (full enzymatic activity) ranged from 0-1000 amylographic units (AU) (Table 1) and suggested large differences in amylolytic activity of flours.

In general, the amylographic maximum is determined by gelatinization properties of starch and by the activity of amylolytic enzymes, mainly α-amylase. To distinguish the effect of amylolytic activity on the maximum of viscosity from the effect of starch gelatinization properties, as well as to investigate the prime factor affecting the amylograph viscometric results, amylograms were run at conditions of complete inactivation of diastatic enzymes (buffer pH 2.95) and at full enzyme activity with buffer pH 5.35, using 50 g of flour and 450 mL of each of the buffers. The data are recorded in Table 2.

Differences in hot-paste viscosity of samples tested in the absence of enzymes showed that the flours differed greatly in the other factors responsible for viscosity, and suggested that the variability of the amylographic maxima of wheat flours at pH of enzyme activity could be in great part due to differences in gelatinization properties of starch or to some other reasons. Consequently, the differences in flour viscosity maxima cannot be interpreted as differences in amylolytic activity of flours, as concluded by Mathewson and Pomeranz (1978), as well. This raises the question of which factors are responsible for the differences in the maximum of viscosity of inactivated flour.

Therefore, further investigations were made to find out how differences in starch concentration and starch properties in tested samples can influence differences of viscosity maxima of inactivated flours and the effect of alpha-amylase.

Starch concentration in suspension and maximum of viscosity value

Since the amylograms were prepared with the same concentration of flour suspension (10%), the differences in the height of amylograms might be due to the differences in starch concentration in suspensions (6.43-7.06%) because of the interdependence of viscosity maximum and starch concentration as indicated by Balint and Momirović-Čuljat (1976). To investigate this possibility, amylograms were made at the buffer pH of enzyme inactivation with flour weight and buffer volume necessary to ensure the same concentration of starch in suspensions (6.50%). Under such conditions, amylograms ranged from 430-675 AU, whereas the amylograms of the 10% flour suspensions ranged from 435-820 AU. Aberration of starch concentration in the 10% flour suspensions (ΔS) ranged from −0.07 to +0.56% in relation to the constant starch concentration of 6.50%. Differences in viscosity maxima due to dif-

Table 1 – Composition[a] and amylographic viscosity maximum of wheat flour samples

Sample	Total protein (N × 5.7) %	Starch %	Amylose (of total starch) %	Damage starch FU[b]	Viscosity maximum (60g flour) AU
1	11.9	65.68	24.70	9.74	970
2	12.3	65.68	23.72	3.61	980
3	12.1	65.67	21.34	4.51	910
4	13.1	65.13	20.30	21.66	520
5	11.8	66.78	22.39	9.59	890
6	11.8	65.41	20.51	6.47	760
7	12.7	66.76	18.70	12.51	840
8	12.3	69.50	23.97	11.40	1000
9	12.1	64.86	24.70	5.67	920
10	12.4	65.13	21.48	12.86	890
11	11.7	65.68	22.46	14.17	850
12	12.5	68.96	22.97	15.23	900
13	13.3	64.57	21.39	15.12	650
14	12.9	67.80	19.49	13.21	750
15	12.6	64.58	20.43	17.64	720
16	9.1	67.87	20.38	4.31	930
17	8.5	67.32	20.19	7.63	670
18	8.3	68.97	29.42	3.76	560
19	9.1	65.68	21.26	7.48	120
20	9.1	65.68	23.79	5.42	940
21	9.2	65.13	23.92	8.84	670
22	8.7	65.68	26.20	7.03	770
23	8.5	67.87	27.35	3.30	1000
24	9.1	66.23	26.34	5.47	850
25	9.7	65.68	25.76	4.46	650
26	12.9	65.13	23.21	11.07	910
27	10.4	68.96	26.53	3.56	170
28	9.3	70.06	22.68	3.30	260
29	10.4	68.42	22.63	3.91	900
30	10.8	67.32	22.84	12.81	0
31	9.8	66.77	22.24	3.61	0
32	8.3	66.77	23.92	3.30	340
33	10.2	66.77	21.69	4.76	360
34	8.9	70.06	21.53	4.31	420
35	9.2	68.97	21.67	3.61	690
36	9.5	70.61	21.81	3.40	670
37	13.0	68.97	25.91	9.76	490
38	14.4	66.78	24.54	9.14	520
39	14.2	67.87	21.53	11.75	290
40	11.9	66.78	24.70	11.86	430
41	14.5	65.68	21.29	7.78	300
42	11.9	68.96	25.64	10.55	290
43	14.7	65.13	26.78	9.89	385
44	12.2	65.68	23.92	7.38	375
45	14.1	64.31	24.70	9.39	500
46	12.6	65.13	22.94	4.51	360
47	12.1	64.59	25.18	12.71	280
48	13.9	64.83	25.18	7.83	60
49	12.5	65.68	26.78	13.46	655
50	13.0	65.68	26.78	10.35	600
51	13.3	64.59	27.56	11.25	410
min	8.3	64.31	18.70	3.30	0
max	14.7	70.61	27.56	21.66	1000
x̄	11.4	66.64	23.38	8.37	595
SD	1.9	1.70	2.29	4.11	250

[a] In original samples.
[b] FU = Farrand units.

whereas the decrease of viscosity maximum due to the action of α-amylase was 75, 90, 100 and 120 AU in the samples marked 8, 10, 9 and 15, respectively.

Physicochemical properties of starch in flour and maximum of viscosity

Assuming that MV $= a^{\sqrt{c}}$, where "a" is a constant value characteristic to wheat flour (11.90), amylograms made at the same starch concentration in suspensions should have the same maximum value. However, the values of viscosity maxima ranging from 430 to 675 AU showed crucial differences in gelatinization properties of starch of different flour samples. By means of the known starch concentration in suspension and the amylographic maximum value (amylograms prepared with 50g of flour, pH 2.95), the basis of exponential function MV $= a^{\sqrt{c}}$ was calculated for each sample. Thus, the values, presented in Table 2 with a_f, ranging between 10.799 and 12.868, represent the characteristics of flour samples. Consequently, the characteristic a_f can be considered as a numerical presentation of the physicochemical properties of starch in the flour under the conditions of amylographic determination.

Such numerical presentation of starch properties in the flour enabled investigation of the influence of the flour properties on amylographic maximum and α-amylase effect, as well as investigation of the factors determining the extent of wheat flour characteristic a_f.

Analysis of the flour viscosity maximum and characteristic a_f interdependence by constant starch concentration rendered a significant positive correlation, r $= 0.957$, which indicated once again a strong influence of the starch properties on the viscosity maximum height. Differences in viscosity maximum values caused by differences in starch concentration in suspensions of different samples by the usual amylographic measurement of amylolytic activity, associated with the variability of maxima of viscosity due to differences in physicochemical properties of starch in different flour samples, with variability due to different effect of α-amylase depending on different initial starch concentration, (Momirović-Ćuljat, 1970) and with different starch susceptibility to amylolysis, make it almost impossible to correctly estimate the α-amylase content on the basis of the viscosity maximum.

The results point to the conclusion that a more precise insight into α-amylolytic activity of flour could probably be obtained by the difference in viscosity maximum of amylograms made at pH value of enzyme inactivation and at the enzyme activity pH (ΔMV - Table 2).

To find out to what extent the decrease in viscosity maximum is a reliable indicator of amylolytic activity of the flour, further investigations and analyses on isolated wheat starch were conducted.

ferences in starch concentration (ΔMV$_s$) ranged from − 15 to + 185 AU (Table 2). The correlation coefficient for the relation ΔMV$_s$/ΔS, r $= 0.985$, confirmed the significant influence of starch concentration in suspension on viscosity maximum and supported the assumption that one of the reasons for the variation of the maxima of inactivated flour samples was the difference in starch concentration in different samples. This also indicated that the deviation of amylolytic activity values, determined amylographically and by chemical methods could be partly attributed to the differences of starch concentration in suspension of discrete samples in the determination of amylolytic activity. The data showed (Table 2) that the differences in viscosity maximum height due to differences in starch concentration might be greater in some samples than the effect of amylolytic activity on the maximum of viscosity in other samples. For example, Δ MV$_s$ of flour sample number 36 (low protein content, high starch content, high Δ S) was 185 AU,

Amylograms of isolated wheat starch

To confirm previous conclusions about influence of starch concentration and starch physicochemical properties on flour viscosity maximum value, amylograms were run on 50g of isolated starch at pH 5.9 without α-amylase and with 5 IU α-amylase added (Table 3). The starch concentration in suspensions ranged from 7.85% to 8.37% and viscosity maxima in absence of amylase from 340 to 1000 AU. The "a" value for isolated starch samples was calculated using exponential function MV $= a^x$ (Momirović-Ćuljat, 1970) with a small modification. Since the author (Momirović-Ćuljat, 1970) investigated functional dependence of the maxima of viscosity and starch concentration in one sample of starch, he could use substrate concentration in slurry (x) in the calculation. In the case of the use of 51 starch samples different in starch concentration, "x" in expression MV $= a^x$ had to be substituted with starch con-

Table 2—Wheat flour amylograms, differences in starch concentrations in suspensions and wheat flour characteristic a_t

Sample	$MV^a_{5.35}$ (10% flour suspension) AU	$MV^a_{2.95}$ (10% flour suspension) AU	ΔMV^b AU	$MV_{2.95}$ (6.5% starch) AU	ΔS^c %	ΔMV_s^d AU	a_t
1	445	550	105	530	+0.07	+ 20	11.729
2	445	570	125	550	+0.07	+ 20	11.894
3	420	560	140	540	+0.07	+ 20	11.815
4	255	500	245	495	+0.01	+ 5	11.417
5	440	640	200	585	+0.18	+ 55	12.187
6	390	530	140	520	+0.04	+ 10	11.620
7	375	530	155	490	+0.18	+ 40	11.337
8	625	700	75	565	+0.45	+135	12.566
9	420	520	100	525	−0.01	− 5	11.653
10	430	520	90	515	+0.01	+ 5	11.594
11	350	570	220	550	+0.07	+ 20	11.894
12	425	590	165	490	+0.39	+100	11.354
13	315	530	215	540	−0.04	− 10	11.805
14	330	520	190	455	+0.28	+ 65	11.043
15	315	435	120	445	−0.04	− 10	10.921
16	410	560	150	490	+0.29	+ 70	11.347
17	355	560	205	500	+0.23	+ 60	11.727
18	320	640	320	530	+0.40	+110	11.982
19	40	560	520	540	+0.07	+ 20	11.812
20	510	610	100	580	+0.07	+ 30	12.150
21	335	530	195	515	+0.01	+ 15	11.566
22	350	540	190	525	+0.07	+ 15	11.646
23	515	660	145	575	+0.29	+ 85	12.086
24	425	590	165	555	+0.12	+ 35	11.931
25	290	540	250	525	+0.07	+ 15	11.646
26	390	520	130	515	+0.01	+ 5	11.594
27	50	650	600	540	+0.40	+110	11.781
28	110	690	580	530	+0.51	+160	11.988
29	110	590	480	500	+0.34	+ 90	11.463
30	0	480	480	430	+0.23	+ 50	10.799
31	0	650	650	595	+0.18	+ 55	12.263
32	165	620	455	570	+0.18	+ 50	12.041
33	170	620	450	570	+0.18	+ 50	12.041
34	230	720	490	565	+0.51	+155	12.009
35	390	820	430	675	+0.40	+145	12.868
36	355	790	435	605	+0.56	+185	12.319
37	260	720	460	595	+0.40	+125	12.248
38	240	620	380	570	+0.18	+ 50	12.038
39	130	680	550	590	+0.29	+ 90	12.225
40	215	670	455	615	+0.18	+ 55	12.405
41	120	670	550	650	+0.07	+ 20	12.668
42	135	730	595	600	+0.40	+130	12.313
43	185	485	300	480	+0.01	+ 5	11.282
44	150	590	440	570	+0.07	+ 20	12.055
45	250	500	250	515	−0.07	− 15	11.596
46	200	650	450	645	+0.01	+ 5	12.654
47	125	540	415	550	−0.04	− 10	11.889
48	0	550	550	555	−0.02	− 5	11.920
49	315	580	265	560	+0.07	+ 20	12.254
50	300	540	240	525	+0.07	+ 15	11.917
51	200	555	355	565	−0.04	− 10	12.294
min.	0	435	75	430	−0.07	− 15	10.799
max.	625	820	650	675	+0.56	+185	12.868
$\bar{x}$	281	594	313	545	+0.16	+ 49	11.875
SD	147	81	170	49	0.17	52	0.434

[a] $MV_{5.35}$ and $MV_{2.95}$ — maximum viscosities obtained with buffer pH 5.35 and pH 2.95, respectively

[b] $\Delta MV = MV_{2.95} − MV_{5.35}$

[c] ΔS = % starch in 10% flour suspension — 6.5% starch.

[d] $\Delta MV_s = MV_{2.95}$ (10% flour suspension) − $MV_{2.95}$ (6.5% starch in suspension)

centration in slurry (c). Therefore, the "a" value for each sample was calculated on the basis of viscosity maximum and percentage of starch concentration in suspension from expression $MV = a^c$, and presented in Table 3 as a_s.

To examine the influence of the isolated starch characteristic a_s on viscosity maximum height, amylograms were run at constant starch concentration, $c = 7.8\%$. Since the amylogram values determined on ten starch samples were equal to those calculated from expression $MV = a^c$ (within the limits of experimental error), in further consideration the data calculated for viscosity maximum of isolated starches at constant starch concentration (7.8%) were used (Table 3).

An analysis of the relation between maximum of viscosity (starch conc = constant) and starch characteristic a_s revealed that $r = 0.992$ (Fig. 1), which again confirmed a significant role of the physicochemical properties of starch in the determination of amylographic maximum. Starch concentration ab-

berations from the constant value of 7.8% ($\Delta S_{i.s.}$) ranged in suspensions from 0.05% to 0.57% ($\bar{x} = 0.21\%$) and were significantly correlated with differences in viscosity maxima ($\Delta MV_{s(i.s.)}$) caused by these abberations ($r = 0.893$). The results confirmed the influence of starch concentration in suspension on the viscosity maximum and consequently the hypothesis that erroneous choice of sample weight might be one of the causes of the differences in viscosity maxima of different flour samples and to the differences in amylolytic activity determined by amylograph and chemical methods.

Susceptibility of starch to α-amylolysis

To investigate how the physicochemical properties of starch influence its α-amylolytic susceptibility, amylograms were run with the same amount (5 IU) of bacterial α-amylase added to a suspension of 50g of isolated starches buffered at pH 5.9.

Table 3 – Amylograms and characteristic a_s of isolated starch

Sample	$MV_{5.9}$ AU	$MV_{5.9}$ (50 g isolated starch) 5 IU α-amylase added AU	ΔMV^b AU	$MV_{5.9}$ (starch = 7.8%) AU	a_s	DMV^c %	hydrolyzed starch %
1	840	120	720	670	2.303	85,71	2.33
2	570	125	445	510	2.224	78,07	1.90
3	550	145	405	475	2.203	73,64	1.67
4	550	0	550	470	2.202	100.00	—
5	790	225	565	755	2.339	71,52	1.10
6	620	120	500	570	2.256	80,65	2.02
7	550	80	470	455	2.191	85,45	2.46
8	900	280	620	695	2.296	68,89	1.41
9	560	115	445	410	2.161	79,46	2.05
10	590	105	485	565	2.253	82,20	2.13
11	530	0	530	455	2.192	100,00	—
12	580	100	480	440	2.182	82,76	2.25
13	600	90	510	525	2.233	85,00	2.36
14	510	80	430	470	2.200	84,31	2.35
15	560	95	465	535	2.238	83,03	2.20
16	610	110	500	570	2.256	81,97	2.11
17	800	225	575	665	2.302	71,87	1.52
18	760	225	535	725	2.327	70,39	1.44
19	570	125	445	425	2.171	78,07	1.96
20	820	280	540	670	2.302	65,85	1.29
21	600	130	470	485	2.209	78,33	1.93
22	660	260	400	445	2.171	60,60	1.20
23	700	220	480	560	2.251	68,57	1.43
24	770	265	505	655	2.297	65,58	1.28
25	520	125	395	485	2.208	75,96	1.87
26	340	85	255	320	2.095	75,00	1.87
27	780	365	415	585	2.263	53,20	0.93
28	960	540	420	895	2.390	43,75	0.66
29	760	320	440	580	2.262	57,89	1.06
30	700	285	415	585	2.264	59,28	1.10
31	660	280	380	615	2.279	57,57	1.04
32	720	240	480	670	2.304	66.67	1.32
33	730	310	420	665	2.302	57,53	1.03
34	790	340	450	755	2.338	56,96	0.99
35	950	480	470	615	2.278	49,47	0.61
36	860	400	460	670	2.303	53,48	0.92
37	830	310	520	775	2.346	62,65	1.16
38	760	295	465	595	2.268	61,18	1.16
39	930	405	525	720	2.325	56,45	0.99
40	740	220	520	690	2.312	70,27	1.45
41	850	300	550	660	2.299	64.70	1.25
42	880	365	515	820	2.364	58,52	1.02
43	810	230	580	630	2.285	71,60	1.52
44	620	205	415	510	2.224	66,94	1.39
45	660	240	420	565	2.253	63,64	1.25
46	1000	580	420	710	2.320	42,00	0.65
47	710	225	485	665	2.300	68,31	1.38
48	900	400	500	800	2.356	55,56	0,95
49	770	275	495	550	2.247	64,29	1.27
50	780	280	500	665	2.301	64,10	1.23
51	820	290	530	590	2.264	64,63	1.27
min	340	0	255	320	2.095	12,00	0.51
max	1000	580	720	895	2.390	100.00	2.36
$\bar{x}$	715	234	475	599	2.265	69,10	1.46
SD	142	117	85	118	0.060	11,20	0.50

[a] $MV_{5.9}$ — viscosity maxima obtained with buffer pH 5.9

[b] ΔMV_α = $MV_{5.9}$ (in absence of α-amylase) − $MV_{5.9}$ (5 IU α-amylase added)

[c] DMV — decrease in viscosity maximum

The amount of added α-amylase was chosen to obtain the range of the viscosity maximum decrease of the same order of magnitude as ΔMV of the tested flour samples. The viscosity maxima ranged from 0 to 580 AU (Table 3). As evident from the data, the decrease in viscosity maxima differed greatly with the same amount of α-amylase. The decrease in viscosity maxima (ΔMV_α, Table 3) ranging from 255–720 AU suggested significant differences in the α-amylase susceptibility of starches.

Statistical analysis of the interdependence between the viscosity maximum decrease (ΔMV_α) and untreated starch maximum viscosity showed that the higher the viscosity maximum of untreated starch, the greater ΔMV_α (r = 0.415). That might be misleading since the dependence leads to the conclusion that starches with the higher characteristic, i.e., higher viscosity maximum, are more susceptible to the activity of α-

amylase, contrary to the known fact that native starches have high amylographic maxima and are less susceptible to the amylolysis than damaged starches with lower viscosity maxima. However, this apparent illogic can be explained by the exponential dependence of MV on starch concentration and linear dependence of MV on the starch characteristic a_s. Namely, the viscosity decrease of slurry in amylographic determination is caused by α-amylolytic hydrolysis of starch, i.e. by decrease of starch concentration in slurry. Since MV of starch depended on the starch characteristic, a_s, and starch concentration according to expression $MV = a^c$, the equal decrease in concentration of starch having high characteristic a_s will cause a larger decrease in MV than the starch with low characteristic a_s. For example, the starch sample No. 28 with the characteristic $a_s = 2.390$ and with MV value equal to 895 AU at 7.8%
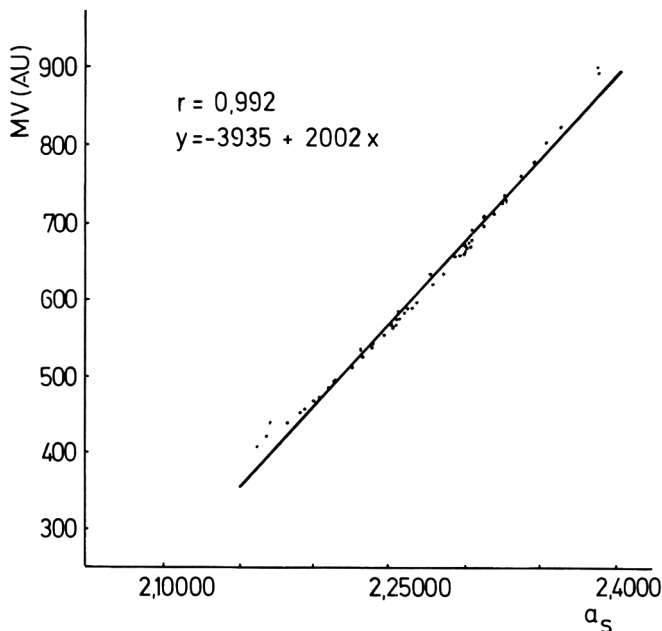
Fig. 1 — Dependence of viscosity maximum (MV) on starch characteristic a_s.

Fig. 2 — Interdependence between decrease of viscosity maximum (%DMV) and amount of hydrolyzed starch.

starch in suspension would have MV value equal to 121 AU at 5.5% starch in suspension, ΔMV then being 774 AU. Starch sample No. 26 with the characteristic a_s = 2.095 and MV value 320 AU at 7.8% starch in suspension would have MV value 58 AU at 5.5% starch in suspension, ΔMV then being 262 AU. Since ΔMV expressed in AU is obviously an unsuitable indicator of the effect of α-amylase, the viscosity maximum decrease was expressed as a percentage of the viscosity maximum of untreated starch, and shown in Table 3 as DMV%.

Susceptibility of starch to α-amylolysis thus presented shows the real extent of the viscosity maximum decrease and correlates negatively with both, the maximum of untreated starch (r = -0.506) and starch characteristic a_s (r = -0.509). It was further found that starch susceptibility to α-amylolysis was partly determined by the amylose content (r = -0.342), as well as by the damaged starch content (r = 0.584). The granular composition of starch affects neither ΔMV nor DMV%.

To examine if DMV% was a reliable indicator of starch α-amylase susceptibility, the expression MV = a^c was used to calculate the decrease in starch concentration in suspension of different samples due to the activity of the equal amount (5 IU) of bacterial α-amylase. The values ranged from 0.51–2.36% (Table 3). The two samples, for which it was impossible to calculate starch concentration due to a 100% viscosity decrease were not included.

The analysis of the relationship between the amount of hydrolyzed starch (%) and maximum of viscosity decrease (DMV%) at constant α-amylase concentration (r = 0.968) shows that DMV% is a correct indicator of the changes in hot-paste viscosity (Fig. 2) but reflects the amount of hydrolyzed starch only and not the α-amylolytic activity. In this case, it can be regarded only as a measure of starch susceptibility to α-amylolysis under the conditions of amylographic determination. The coefficient of correlation between the amount of hydrolyzed starch and ΔMV$_\alpha$, r = -0.436, confirms once again that ΔMV (expressed in AU) cannot be used either as an indicator of starch susceptibility or that of changes in starch hot-paste in the presence of α-amylase.

To investigate if differences in starch concentration in suspensions of different samples influenced the value of DMV%, amylograms were run on some samples with suspension at 7.8% starch concentration and with 5 IU of α-amylase added. The decreases of viscosity maxima, obtained with 5 IU of α-amylase added in suspensions equal in starch concentration,
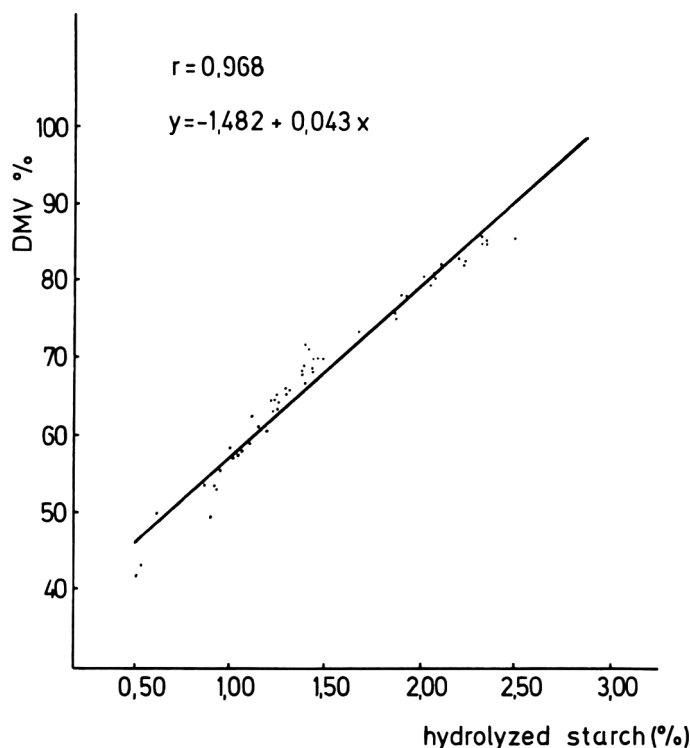
were compared with the viscosity maxima decreases in suspensions different in starch concentration, prepared with 50g of isolated starch and 5 IU α-amylase added. Differences in ΔMV, due to differences in starch concentration in differently prepared suspensions, were, for example, 65 AU and 235 AU at samples 28 and 35, respectively. The DMV% were equal (43.75% and 49.47%, respectively) in both cases irrespective of the starch concentration in suspensions.

This confirmed the influence of initial starch concentration on the effect of α-amylase expressed in AU, as indicated by Momirović-Čuljat (1970) but not on the amount of hydrolyzed starch and consequently on DMV%. So, DMV% seemed to be a very practical indicator of α-amylase effect since its determination did not demand adjusting of starch concentration to constant value.

Viscosity maximum decrease in relation to starch characteristic and α-amylase concentration

Differences in the effects of the same amount of α-amylase, i.e., differences in starch susceptibility to α-amylolysis, were the result of the differences in the physicochemical properties of starches. This was evident from the analysis of the relations — the amount of hydrolyzed starch/a_s (r = -0.631) and amount of hydrolyzed starch/MV of untreated starch (r = -0.643), respectively. However, according to the correlation coefficients, the effect of α-amylase activity was not unambiguously determined by the properties of starch.

Therefore, in a separate experiment the effect of different concentrations of α-amylase on different samples of isolated starch was investigated. Amylograms were determined on 50g starch in 450 mL PCB pH 5.9, without amylase and with addition of 0.25-5 IU α-amylase. The effect of different α-amylase concentrations on the decrease of viscosity maximum of ten different samples is presented in Table 4 and Fig. 3, respectively. The data suggested that the effect of α-amylase depended on both the starch properties and the amount of α-amylase present.

The data in Table 4 also explain the unacceptably large

Table 4—Dependence of the decrease of maximum of viscosity (DMV%) on £-amylase concentration[a]

α-amylase IU	DMV%[b]
0.25	9.1 – 19.3
0.50	18.6 – 32.7
1.00	24.6 – 43.0
1.50	30.5 – 50.0
2.00	35.9 – 54.5
2.50	38.5 – 59.2
3.00	42.0 – 62.7
3.50	45.2 – 66.1
4.00	48.0 – 68.3
4.50	50.4 – 71.3
5.00	53.3 – 72.7

[a] N = 10 samples of isolated starches different in characteristic a_s value

[b] % DMV — the decrease of maximum of viscosity expressed as percent of untreated starch maximum of viscosity

variation between viscosity and α-amylase concentration determined by a reliable colorimetric method, as reported by Mathewson and Pomeranz (1978). Obviously, the same effect may be achieved in various samples even at concentrations of α-amylase differing by as much as 200%.

Factors affecting flour characteristic a_f

Further analyses were made to find out how the main components of flour, starch and proteins affect flour characteristic a_f, and consequently viscosity maximum. The protein concentration in a slurry, at constant starch concentration of 6.5%, ranged between 0.81% and 1.46%. Investigating the effect of protein fractions (determined after Bietz and Wall, 1975) on the parameters of some rheological tests (unpublished data)

with the same samples in another experiment, the influence of those fractions on a_f and amylographic viscosity maximum was also studied. The effect of starch granular composition was analyzed as well.

According to correlation coefficients presented in Table 5, the characteristic of flour a_f and viscosity maximum (MV) depend on damaged starch content and susceptibility of starch to amylolysis (DMV%), as well as on the characteristic a_s and viscosity maximum of isolated starch (MV_s). Neither the content of amylose and amylopectin nor the granular composition of starch nor the content of proteins in suspension nor the content of discrete protein fractions showed any significant influence on the value of flour characteristic and amylographic maximum.

In summary, the amylographic maximum being a function of several factors such as starch concentration in suspension, starch physicochemical properties and concentration of α-amylase, cannot be interpreted as an indicator of α-amylolytic activity. Neither can the decrease in viscosity maximum (DMV%), expressed as a difference between the maxima of inactivated flour viscosity and the viscosity of the flour at full enzymatic activity be used as an indicator of amylolytic activity of flour, since the same DMV% value may be achieved in various samples even at the concentrations of α-amylase differing by as much as 200%. However, DMV% can be used as a good indicator of the effect of α-amylase in flour and starch hot-paste during the amylographic measurement because it is significantly related to the amount of hydrolyzed starch. The data suggest, furthermore, that physicochemical properties of starch are a primary factor in determining amylographic viscosity maximum, since they significantly influence the amylographic maximum of pure starch and starch susceptibility to amylolysis, as well as the effect of α-amylase on starch de-
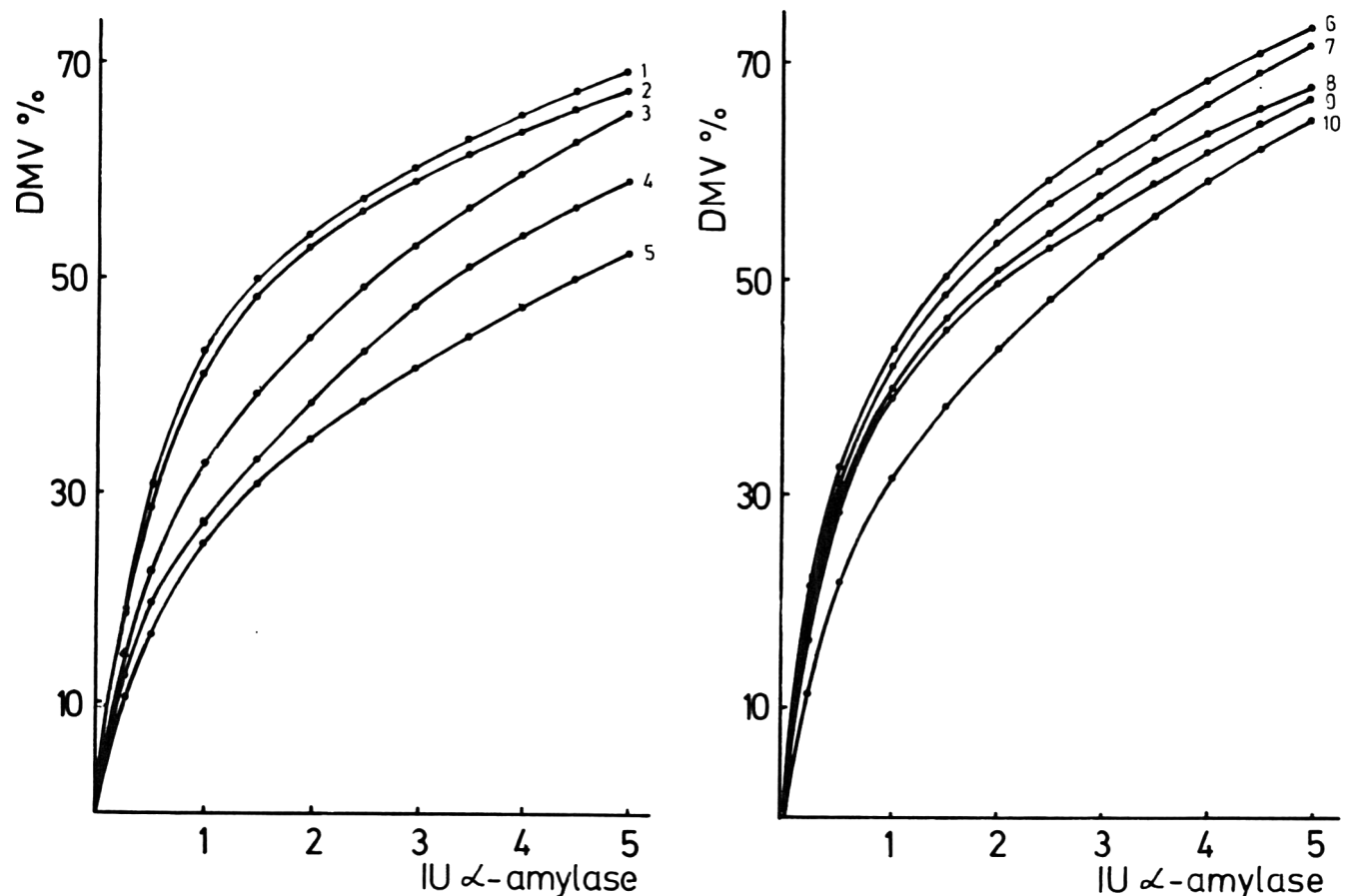
Fig. 3—Interdependence of DMV% and α-amylase concentration. (Numbers 1–10, 10 samples of isolated starch different in characteristic a_s value).

Table 5—Dependence of flour characteristic (a_f) and maximum viscosity (MV) on wheat flour starch and protein component (coefficients of correlation)

	MV_s^a AU	Amylose %	α-Amylase susceptibility DMV%	a_s	Damaged starch FU^b	Protein in suspension %
a_f	0.431*	0.199NS	−0.507*	0.445*	−0.410*	0.003NS
MV	0.385*	0.120NS	−0.494*	0.398*	−0.456*	−0.066NS

[a] % of starch = const.
[b] FU = Farrand units
* $p < 0.005$; NS = not significant

composition. The results presented explain the large differences between the amylolytic activity determined by chemical method and by amylograph and indicate the need of further research to find out the exact relation between α-amylase concentration, decrease of viscosity maximum and physicochemical properties of starch. In that case an accurate measurement of the amylolytic activity by this simple and easy to perform viscosimetric method would be possible.

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processed soy foods, at least trace amounts of SBPI were often present. As was the case with flours, concentrates, and isolates, this activity may be due to KSTI or BBI alone or a combination of KSTI and BBI.

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Direct Use of Apple Pomace in Bakery Products

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ABSTRACT

DRUM-DRIED APPLE POMACE (DDAP), an edible by-product from single pass metallic membrane ultrafiltration of apple puree, was examined for composition and utilization as a source of sugar and dietary fiber in bakery products. Total dietary fiber (TDF) of freeze-dried apple pomace (FDAP) and DDAP was 35.29% and 33.24%, respectively. Total sugar of the apple pomace concentrate (APC), FDAP, and DDAP was 54.34, 46.26, and 36.71%, respectively. Sensory evaluation demonstrated that the experimental muffins with 50% (w/w) of the plain wheat bran substituted by powdered apple pomace were significantly (p < 0.01) more desirable than the control bran muffins. In addition, experimental moon cookies with flaked apple pomace substituted for 40% (w/w) of the all-purpose flour in the crust and 40% (w/w) of the quick-cooking oats in the filling were significantly (p < 0.01) more desirable than the control moon cookies.

INTRODUCTION

APPLE POMACE is the primary by-product of the apple juice industry. A conventional apple juice process will remove about 75% of the fresh weight of the apple as juice (Sargent, 1984). More than 500 apple juice processing plants in the United States produce a total of about 1.3 million metric tons of apple pomace each year, and it is likely that annual disposal fees exceed $10 million (Jewell and Cummings, 1984). This disposal problem is rapidly becoming more difficult to solve. The use of apple pomace as an animal feed has been a conventional approach. Apple pomace is currently used for extraction of pectin (Jain et al., 1984) and other hydrocolloids (Walter et al., 1977, 1986) and to fertilize damaged soils (Stapleton, 1982). The estimation of savings in fossil fuel costs from combusting apple pomace was discussed by Sargent (1984) and Sargent et al. (1986). Recently, Hang (1987) demonstrated that apple pomace, which has a high moisture and sugar content, can be fermented to produce citric acid or alcohol.

Morris (1985) indicated that apples and pears have the highest crude fiber content of all the fruits. Edible fiber has also been extracted from apple pomace (Walter et al., 1985), and apple fiber has been incorporated into cookies, granola bars, and muffins to produce high fiber bakery products (Deuel, 1986). However, in most foods the fiber material has a poor flavor, which alters the normal flavor of the products. Often, a loss of appearance, texture and mouthfeel are noted by the consumers (Beereboom, 1979). These problems can be solved through the use of flavors, such as vanilla (Belshaw, 1978), spices or molasses. Problems with texture and appearance still persist with such products.

The purposes of this study were to determine the compositional changes in apple pomace after different drying processes, to utilize the apple pomace as a source of sugar and dietary fiber by incorporating dried apple pomace directly into bakery products and to determine the sensory values of these baked products.

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MATERIALS & METHODS

Preparation of dried apple pomace

Apple pomace concentrate (APC) and apple juice were obtained from single pass, tubular metallic membrane ultrafiltration which was similar to the system described by Thomas et al. (1986, 1987). Juice yield was 85%; no filtration aids were utilized. APC consisted of the entire insoluble portions of the apple, including seeds, skins and cores, since the entire fruit was comminuted to a puree before filtration.

A double-drum drier and a freeze drier were used for the preparation of dried apple pomace. APC was passed through a double-drum drier (15.3 cm diameter × 19.1 cm long) with a drum blade opening of 0.051 cm and an internal pressure of 80 psi (162.8°C surface temperature) at 2.5 rpm. Also, the APC was dried with a Virtis freeze dryer (Virtis Inc., Gardiner, NY). The vacuum was operated between 50 and 100 millitorr (Keeling and James, 1986).

Both drum-dried apple pomace (DDAP) and freeze dried apple pomace (FDAP) were ground in a Wiley Mill (Arthur H. Thomas Co., Philadelphia, PA) with a 20 mesh screen before chemical analyses.

Sugar analysis

One gram samples of APC, FDAP, and DDAP were accurately weighed. The samples were extracted with 100 mL of 80% alcohol at 70-80°C for 25 min (Hurst et al., 1979). Sucrose, glucose and fructose were determined by high performance liquid chromatography (HPLC) (Waters Associates, Millford, MA), using a Bio-Rad HPX-87H⁻ column (300 × 7.8 mm) at 25°C. A 4.6 × 40 mm precolumn packed with AMINEX HPX-87H (Bio-Rad Labs, Richmond, CA) was used. HPLC-grade-water for the mobile phase was filtered through a 0.45 μm filter and degassed under vacuum. The sugars (20 μL) were eluted with 0.01% H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min and detected using a Waters model 410 differential refractive index detector. The sensitivity of the detector was set at 32X and the scale factor was set at 20.

Determination of fiber

Methods have been developed for the determination of water insoluble fiber, such as neutral-detergent fiber (NDF) and acid-detergent fiber (ADF), which refer to partially fractionated plant residues. According to Kurasawa et al. (1982), NDF from a food sample contains cellulose, hemicellulose and lignin as cell wall constituents. ADF contains most of the cellulose, lignin, a portion of the pectin substances and variable but small amounts of the hemicelluloses. The NDF, ADF, cellulose, hemicellulose and lignin of FDAP and DDAP were determined by the methods of Van Soest (1963a, b) and Van Soest and Wine (1967). The hemicellulose was obtained by subtracting the ADF from the NDF, and the value of cellulose was estimated by the difference between the values of ADF and lignin. For the lignin assay, ADF was decomposed by treating with 72% (w/w) H_2SO_4 at room temperature for 3 hr with the amount of the residue remaining measured as lignin. TDF content of the apple pomace was determined according to the enzyme digestion procedure of Novo Biolabs (Anonymous, 1987), except the volume of alcohol was increased to 450 mL. Pectin was calculated by the difference of TDF and NDF.

Muffins and moon cookies preparation

In this study, there were two kinds of bakery products made from the DDAP. The formulation of control bran muffins is presented in Table 1. Experimental muffins were prepared by substituting 50% (w/w) of the plain wheat bran (20 mesh) with the powdered (20 mesh) apple pomace in the control muffin formula. The plain wheat bran was hydrated with apple juice and applesauce until it was moist. The dried ingredients were sifted together twice and mixed with melted

Table 1 — Formulation of control bran muffins

Ingredient	Amount (g)	(%)
Plain wheat bran[a]	105.00	9.93
Whole wheat flour	120.00	11.36
All-purpose flour	55.00	5.21
Margarine	56.80	5.38
Molasses	41.00	3.88
Brown sugar	27.50	2.60
Apple juice	62.00	5.86
Applesauce (unsweetened)	340.00	32.16
Eggs	216.00	20.43
Baking Powder	15.40	1.46
Salt	3.35	0.31
Cinnamon	2.40	0.23
Nutmeg	1.60	0.15
Clove	0.56	0.05
Mace	0.40	0.04
Ginger	0.71	0.07
Topping:		
Cinnamon	1.20	0.13
Sugar	4.20	0.40

[a] In experimental muffins, 52.50g of the plain wheat bran was replaced with an equal weight of the powdered apple pomace.

Table 2 — Formulation of control oriental moon cookies

Ingredient	Amount (g)	(%)
Crust:		
All-purpose flour[a]	156.25	43.16
Egg	110.00	30.80
Granular sugar	25.00	7.00
Vegetable oil	25.00	7.00
Water	15.00	4.21
Nonfat milk powder	17.00	4.76
Cinnamon	4.80	1.34
Salt	1.65	0.46
Filling:		
Quick oatmeal[a] (uncooked)	66.70	17.92
Unsalted sunflower seeds	36.20	9.73
Unprocessed sesame seeds	18.75	5.04
Unprocessed chopped peanuts	31.00	8.60
Raisins	41.00	11.00
Brown sugar	55.00	14.78
Margarine	56.80	15.26
Molasses	61.50	16.53
Salt	1.65	0.44
Vanilla	2.50	0.67

[a] In experimental moon cookies, 62.50g of the all-purpose flour in the crust and 26.70g of the quick-cooking oats in the filling were replaced by equal weights of the flaked apple pomace. Sugar was reduced by 37.50% (w/w) in the crust and 37.50% (w/w) in the filling.

Table 3 — Effect of different drying processes on sugar content of apple pomace

Constituent	% Apple pomace (Dry wt basis)		
	Concentrated	Freeze-dried	Drum-dried
Sucrose	11.16[a]	5.17[b]	4.39[c]
Glucose	13.35[a]	11.40[b]	10.55[b]
Fructose	30.05[a]	28.90[a]	21.85[b]
Glucose/Sucrose	1.17[y]	2.26[x]	2.18[x]
Fructose/Sucrose	2.70[b]	5.59[a]	4.80[a]
Total sugar	54.34[x]	46.62[x]	36.71[y]

[abc] For each constituent, any values with the same letters are not significantly (p > 0.05) different.

[xy] For each constituent, any values with the same letters are not significantly (p > 0.01) different.

margarine, molasses, beaten eggs and wheat bran batter, stirring only until combined (about 28 stokes). The batter was evenly portioned into 24 greased, 2½ in. muffin pan cups. A sugar and cinnamon mixture was spread on the top of each muffin. The muffins were baked at 176.7°C (350°F) for 25 min or until brown.

The formulation of control moon cookies is presented in Table 2. In the experimental moon cookies, flaked apple pomace was substituted for 40% (w/w) of the all-purpose flour in the crust and 40% (w/w) of the quick-cooking oats in the filling of a control moon cookie formula. Also, the adjustments of sugar were made to compensate for

the differences in sugar (36.7%) coming from apple pomace. Therefore, the amount of sugar added in both of the crust and the filling was reduced 36.7% (w/w) from the control moon cookie formula. There were two procedures for preparing the moon cookies. For the crust, the eggs were beaten with an electric mixer at a high speed for 60 sec. Sugar and vegetable oil were added gradually and beaten for 9 min before adding vanilla. The dried ingredients were sifted together and mixed into the beaten eggs. The dough for the crust was covered with wax paper and set aside for 1 hr, then divided into 24 portions. For the filling, the quick-cooking oats, chopped peanuts, and sesame seeds were baked at 176.7°C (350°F) for 3 min. The margarine, brown sugar, and molasses were melted over medium heat and mixed with all the dried ingredients and the vanilla. The filling batter was divided into 24 portions and placed into each of the crust portions. A design was pressed on each cookie by using a greased cookie maker. One egg yolk was mixed with one tablespoon of water and brushed onto each of the moon cookies which were baked at 204.4°C (400°F) for 12 min or until browned.

Nutrient analyses

Nutrient and TDF content of the muffins and the moon cookies were determined by the methods of Nutritionist III (Anonymous, 1985) and Novo Biolab enzyme digestion procedures (Anonymous, 1987), respectively.

Sensory evaluation

All the samples were examined in individual booths equipped with daylight fluorescent lamps. Sensory evaluations of the muffins were conducted with 24 panelists (untrained) in a triangle test and a preference test. In the triangle test, the panelists were presented with three coded samples and were asked to indentify the differences, moisture and the preference among samples. For the moon cookies, a panel of 21 untrained participants was used for the sensory evaluation. When testing for preference, the panelists were presented with two coded samples and were asked to determine their preferences of appearance, sweetness, consistency and the overall preference.

Statistical analyses

Statistical analyses were conducted using the analysis of variance with the general linear model procedure (SAS, 1985). Sugar and fiber data means and the sensory evaluation analyses were conducted using a Least Significant Difference Test at the 1% and 5% level of probability.

RESULTS & DISCUSSION

THE SUCROSE and glucose contents (Table 3) of the FDAP and the DDAP were significantly lower (p < 0.05) than that of the APC. The fructose content (Table 3) in DDAP was significantly lower (p < 0.05) than in APC and FDAP. These results indicated that sucrose inversion and fructose destruction were occurring at the elevated temperature of drum drying (Lee and Nagy, 1988). The glucose content in FDAP and DDAP showed no significant difference (p > 0.05), but the fructose content in FDAP was significantly higher (p < 0.05) than that in DDAP, indicating that there was a greater loss of fructose than glucose at higher temperatures. Total sugar of the APC and the FDAP showed no significant difference (p > 0.05), but the total sugar of DDAP was significantly (p < 0.01) lower than that of APC and FDAP. This indicated that freeze drying provided the least compositional change in the sugar of the product.

Results of the fiber analyses (TDF, NDF, ADF, pectin, hemicellulose, cellulose and lignin) are given in Table 4. TDF of the FDAP and the DDAP was 35.9% and 33.24%, respectively. The NDF was 29.53% for FDAP and 29.20% for DDAP. The values of pectin were determined by the difference of TDF and NDF. Pectin in the DDAP (3.71%) was significantly lower (p < 0.05) than in the FDAP (6.09%), due to heat degradation of the apple pectin during drum drying. The NDF, ADF, hemicellulose, cellulose and lignin of these FDAP and DDAP showed no significant difference (p > 0.05), but the TDF in FDAP

Table 4 — Fiber content of apple pomace

| Constituent | % Apple pomace (Dry wt basis) | |
	Freeze-dried	Drum-dried
Total dietary fiber (TDF)	35.29[a]	33.24[b]
Neutral-detergent fiber (NDF)	29.53[a]	29.20[a]
Acid-detergent fiber (ADF)	24.39[a]	25.11[a]
Pectin[c]	6.09[a]	3.71[b]
Hemicellulose	4.69[a]	4.09[a]
Cellulose	16.67[a]	16.44[a]
Lignin	8.44[a]	8.87[a]

[ab] For each constituent, any values with the same letters are not significantly (p > 0.05) different.
[c] TDF - NDF.

Table 5 — Nutrient composition of muffins

| | Control | | Experimental | |
Constituent	38.0g[a]	%	38.0g[a]	%
Calorie (Kcal)	83.6	–	84.3	–
Carbohydrate	11.3	29.7	11.7	30.8
Protein	2.5	6.6	2.2	5.9
Fat	3.2	8.4	3.2	8.4
Total dietary fiber (Dry wt basis)	6.8*	17.9	7.1*	18.7
Cholesterol (mg)	49.3	–	49.3	–
Sodium (mg)	155.0	–	155.0	–

[a] Per muffin.
* TDF values are not significantly (p > 0.05) different.

Table 6 — Nutrient composition of moon cookies

| | Control | | Experimental | |
Constituent	25.0g[a]	%	25.0g[a]	%
Calorie (Kcal)	115.0	–	105.0	–
Carbohydrate	14.3	57.2	12.0	46.8
Protein	2.8	11.2	2.5	10.0
Fat	5.6	22.4	5.6	23.2
Total dietary fiber (Dry wt basis)	2.9[b]	11.6	4.6[c]	18.4
Cholesterol (mg)	25.0	–	25.0	–
Sodium (mg)	90.0	–	90.0	–

[a] Per moon cookie.
[b,c] For TDF, any values with the same letters are not significantly (p > 0.05) different.

Table 7 — Preference of 24 untrained panelists for muffins

| | Control | Experimental |
Attributes	(% of panelists)	
Moisture		
Too moist	21.4	14.3
Just right	57.2	85.7
Too dry	21.4	0.0
Overall Preference	20.8	79.2[a]

[a] Significant preference at the 1% level.

Table 8 — Preference of 21 untrained panelists for moon cookies

| | Control | Experimental |
Attributes	(% of panelists)	
Appearance	14.0	86.0
Sweeteners		
Too sweet	14.3	4.8
Just right	57.1	85.7
Lacks sweetness	28.6	9.5
Consistency		
Too moist	4.8	0.0
Just right	28.6	66.7
Too dry	66.7	33.3
Overall preference	14.0	86.0[a]

[a] Significant preference at the 1% level.

was significantly higher (p < 0.05) than that in DDAP. The apple pomace was primarily composed of cellulose, whereas wheat bran is composed primarily of hemicellulose. Oatmeal contains mostly soluble dietary fiber, such as soluble glucans (Schneeman, 1987).

DDAP was used in the baking products, because the process of drum drying is more economical than freeze drying. The sugar analyses indicated that the FDAP was better than the DDAP in regards to sugar composition, but the DDAP provided essentially the same levels of fiber as the FDAP (Table 4). Fiber becomes most significant in the apple pomace, since some juice processes will remove essentially all of the sugar from the apple by counter-current extractions. The microcomputer program from Nutritionist III (Anonymous, 1985) for the nutrient analyses of the muffins (Table 5) showed that the experimental muffins contained slightly more calories than the control muffins, due to the sugar content of the apple pomace. TDF in the experimental muffin (7.12%) and the control muffin (6.80%) showed no significant difference (p > 0.05).

The nutrient composition of the moon cookies is shown in Table 6. Each of the experimental moon cookies contained 1.7g more total dietary fiber, 2.3g less carbohydrate and 10.0 less calories than each of the control moon cookies, due to a larger fiber content of the apple pomace than that of the quick-cooking oats and the all-purpose flour. By following the sugar reducing adjustments in the moon cookie formula, an experimental moon cookie was obtained with higher fiber and lower calories than the control moon cookie.

The average difference between experimental and control

bran muffins in a triangle test was 2.07 (1 = no difference, 2 = slightly, 3 = extremely) and was significant at p < 0.01. The taste panelists distinguished that the experimental muffins were good in texture and flavor and were sweeter, softer and more moist than the control bran muffins. The overall preference (Table 7) showed that the muffins made by incorporating apple pomace were significantly more desirable than the controls (p < 0.01). For the preference test of the moon cookies, the panelists distinguished that the control moon cookies were too dry and less sweet than the experimental moon cookies. However, the experimental moon cookies were judged to be slightly more moist and also good in sweetness and in consistency (Table 8). The color and appearance of the experimental moon cookies were judged better defined and more appealing than the control moon cookies. The explanation may be due to the DDAP keeping the uniformity of the shape and the crust color of the moon cookies. The overall preference showed that the moon cookies made by incorporating apple pomace were significantly more desirable than the controls (p < 0.01). These evaluations indicated that high fiber bakery products having better taste, texture and appeal than products made from more conventional fiber sources can be produced by incorporating DDAP.

These results demonstrated the value of using apple pomace directly in bakery products. The high dietary fiber and fruit sugar content of the apple pomace could provide consumers with an alternative source of fiber and give bakers an option for reducing the amount of sugar and other fiber added to their products. In addition, this utilization of apple pomace could provide a savings to the juice processor through both the sale of pomace and the elimination of disposal costs.

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Effects of Heat Transfer by Radiation and Convection on Browning of Cookies at Baking

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———————— ABSTRACT ————————

The effects of modes of heat transfer (radiation or convection) on the baking color development of food were studied. An experimental baking oven that could be altered to two heat transfer modes was designed; the ratio of heat by radiative transfer to total heat transferred was about 30% or 70%. The glucose-glutamate solutions were heated at different air temperatures to measure the browning rates to calculate the activation energies. Cookies were baked at 200°C to measure the lightness of color on the surface and the surface temperature. It was clarified that the development of color depended on the temperature only.

INTRODUCTION

PRESENTLY, various types of ovens are available both in the home and in the food industry. Heat is transferred to the food surface in a hot oven mainly by radiation and convection. Some ovens incorporate radiative sources such as electric heaters, other ovens incorporate a fan to circulate hot air. However, in these ovens, the time required to cook food may differ even at the same air temperature, and the product properties, the surface color and the volume, may differ depending upon the oven type. Supposedly, this is due to the difference of heat transfer characteristics.

In oven cooking, the parameters which affect the development of the product surface color are temperature, air velocity, humidity and individual heat transfer mode. In several studies, the effects of temperature, air velocity and humidity on the surface color have been discussed (Skjöldebrand and Öste, 1979; Holtz et al., 1984; Paloheimo et al., 1984; Sato et al., 1987). Standing (1974) examined the major effects of individual heat transfer modes in biscuit baking and suggested the radiative heat transfer might play the most important role in surface color development; however, this suggestion is only an inference.

In previous papers (Shibukawa, 1984, 1985), sponge cakes and cookies were baked at the same air temperature with various types of ovens, and the relationships of the heating abilities of ovens with the product properties were investigated. The time required to cook was highly correlated with the heat transfer coefficient; therefore, the cooking time was estimated from heat transfer coefficient and air temperature. On the other hand, the surface color that developed depended both on the heat transfer coefficient and the ratio of radiative heat transfer to total heat transfer. From these results, the effectiveness of radiative heat transfer in the development of surface color was suggested. However, in these reports, the dimension of ovens, the material of the oven wall and the method of heating were not the same. Therefore, the effects of heat transfer mode on the browning reaction were not clear.

The purpose of this study was to evaluate the effectiveness of radiative and convective heat transfer for the browning of

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glucose-glutamate solutions and of cookies. First, the oven was modified to alter the heat transfer mode, radiative or convective, and the heating abilities were measured. Secondly, the effects of the heat transfer mode on the browning reaction in aqueous solution and on solid surface of cookies were investigated.

MATERIALS & METHODS

Apparatus

The oven used in this study is shown in Fig. 1. The baking chamber, 290 × 382 × 330 mm, was provided with three shelves. The maximum output power of each electric heater was 1.2 kW. A fan was provided on the back wall. The three heaters and the fan were operated optionally for the purpose of the experiment. The oven was operated in two modes: radiative heat transfer mode (RAD) and convective heat transfer mode (CON). In the RAD mode the upper and lower heaters were alternated every 10 sec. In the CON mode the back heater and the fan were operated at the same time. Air temperature at the center of the oven was measured with chromel-alumel thermocouple.

Measurement of heating ability

The apparent heat transfer coefficient was determined using the method of Sato et al. (1987). However, in this study, a copper cylinder was used instead of the aluminum one.

A unique method was used in the measurement of the ratio of radiative heat transfer to total heat transfer (Fig. 2). Two copper cylinders which had different surface conditions but the same shape were heated simultaneously. One cylinder was coated with nickel; the other was coated with heat-resistant black paint. The emissivities of the surfaces were assumed from the literature (Perry and Chilton, 1973) to be about 0.3 and 0.87, respectively. The experiment was repeated four times for two heat transfer modes at the air temperature of 200°C.

Solution test

Monosodium glutamate (Ajinomoto Co., Ltd., Tokyo, Japan) (14.41g) and glucose (Junsei Chemical Co., Ltd., Tokyo, Japan) (6.76g) were dissolved in 40 mL of boiled water in a 100 mL beaker. The beaker containing a freshly prepared sample solution was placed in the oven with a central air temperature, 180°C, 200°C, 220°C or 240°C. After the sample solution was heated for a scheduled time, it was taken out of the oven, weighed and distilled water equal to the weight loss was added. The beaker was covered with polyethylene wrapfilm to prevent further evaporation and quickly cooled with tap water. The scheduled time for heating was determined on condition that the amount of evaporation of water was not greater than 20% of the weight of the initial solution. Thus, the scheduled times were not the same according to the fixed air temperature and the heating mode. For
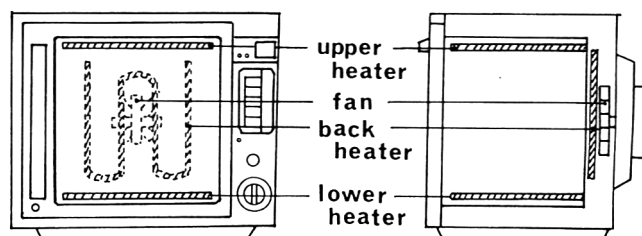
Fig. 1 — Diagram of oven used in experiments.

upper heater
fan
back heater
lower heater
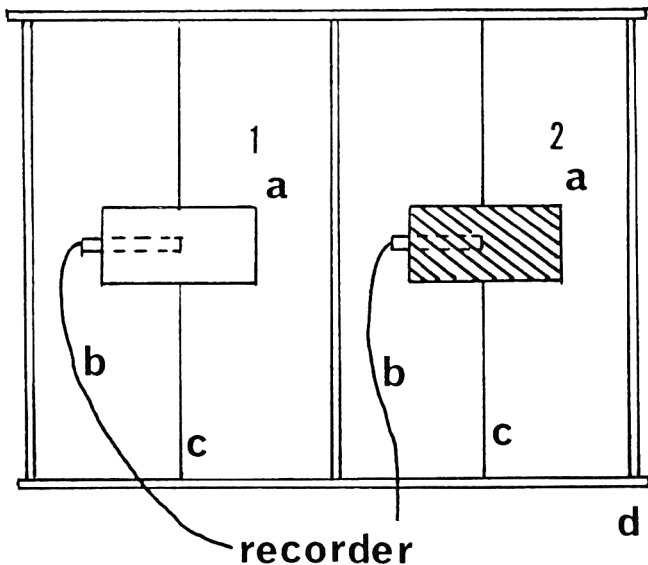
Fig. 2—Diagram of device to measure the ratio of heat transferred by radiation to total heat transferred: (a) Copper cylinder (1, painted black; 2, nickel coated); (b) Thermocouple; (c) Thin wire; (d) Frame.
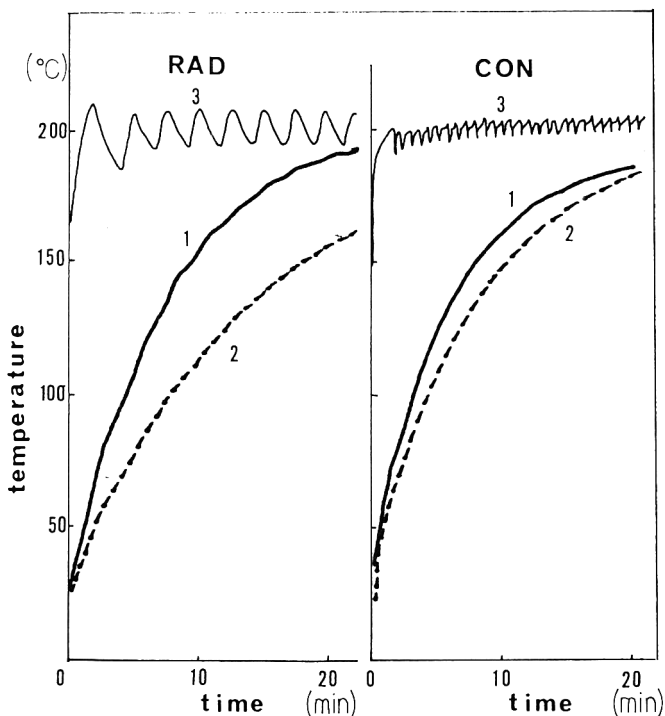
Fig. 3—Temperature rising curve at the center of two cylinders: (1) Temperature of cylinder coated with heat-resistant black paint; (2) Temperature of cylinder coated with nickel; (3) Air temperature.

Fig. 4—Browning pigment formation in solution at various temperatures (logarithm of absorbance vs heating time).

Table 1—Brown pigment formation rate constants

	Heat transfer mode	Air temperature (°C)				Ea (kcal/mol)
		180	200	220	240	
Final soln temp (°C)	RAD	94.3	98.6	102.9	105.4	
	CON	91.9	96.7	101.7	104.1	
Rate constant (1/min)	RAD	0.115	0.175	0.207	0.253	16.5
	CON	0.125	0.163	0.210	0.263	
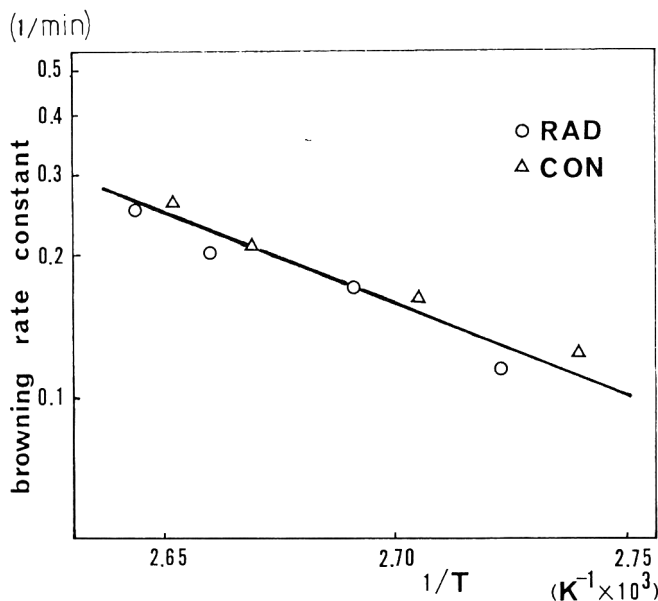
Fig. 5—Arrhenius plot (logarithm of the rate constant vs the reciprocal of the absolute temperature) for browning of solutions.

example, in case of CON, 13, 18, 23, and 28 min at 180°C; 13, 15, 18, 20, and 23 min at 200°C. The experiment was repeated four times for each heating condition. The development of brown pigment was measured at 550 nm with a spectrophotometer (Simazu Seisakusho Ltd., Kyoto, Japan, Spectronic 20).

5-Hydroxymethylfurfural (HMF), which was one of intermediate products of browning reaction, was also measured to estimate the velocity of browning reaction by the thiobarbituric acid (TBA) method (Keeney and Bassette, 1959). Five milliliters of 40% trichloroacetic acid and 2 mL of 0.05M TBA solution were added to 5 mL of the diluted sample (HMF concentration 1 − 6 × 10⁻⁵M). The solution was kept at 38°C for 40 min, and the developed color was measured at 445 nm. HMF concentration of the solution was obtained from the calibration curve of the standard solution.

Food test

The cookies were made from a dough containing 100 flour, 20g baking margarine, 50g sugar, 20 mL milk, and 5g eggs. The dough was mixed according to a standardized procedure. It was sheeted to about 3 mm in thickness and cut into disk pieces about 40 mm in diameter. One piece was placed on Pyrex glass disk, 3 mm in thickness and 50 mm in diameter and baked in the oven at an air temperature of 200°C. The baking times were 5, 8, 13, 15, and 19 min. At each baking time, the cookie was taken out of the oven and within 3 sec its surface temperature was measured with a radiation thermometer (Nippon Denshi Co., Tokyo Japan, JTG-3200). With this thermometer, temperatures at many points (512×240) in one view could be measured simultaneously. From these point temperatures, the average temperature of a cookie's surface was calculated with attached soft-
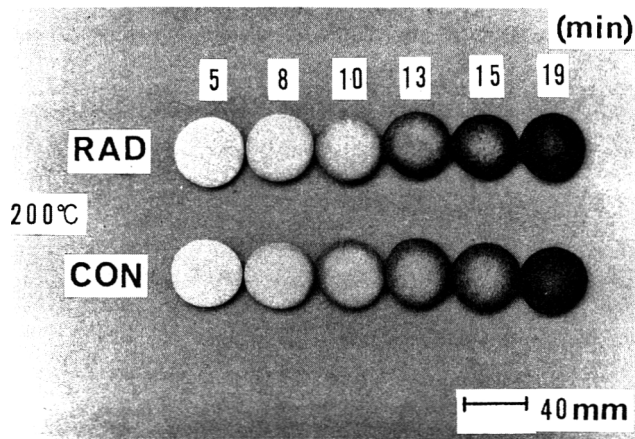
Fig. 6—Surface color of cookies baked at air temperature, 200°C, by two different heat transfer modes.
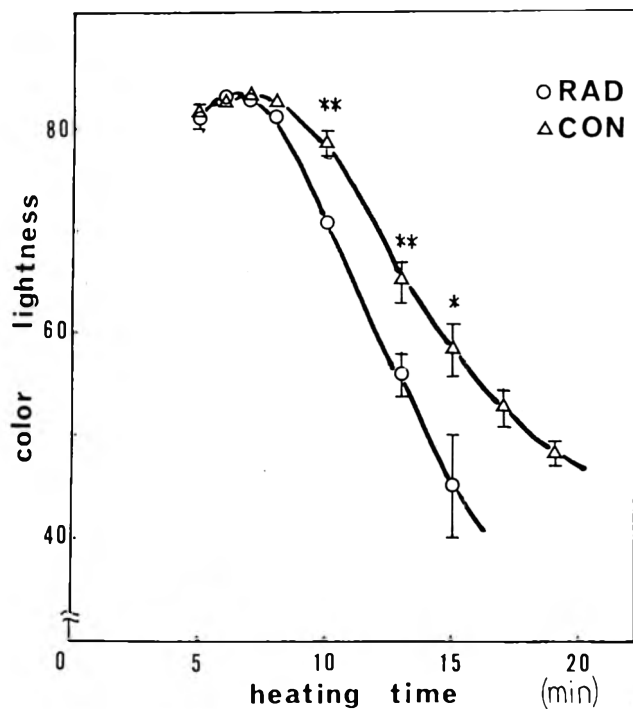
Fig. 8—Surface temperature of cookies as a function of heating time. Each point is the average of four replicates and the vertical line represents ± standard deviation. (*: P<0.05; **: P<0.01).

Fig. 7—Color lightness as a function of heating time. Each point is the averge of four replicates and the vertical line represents ± standard deviation. (*: P<0.05; **: P<0.01).
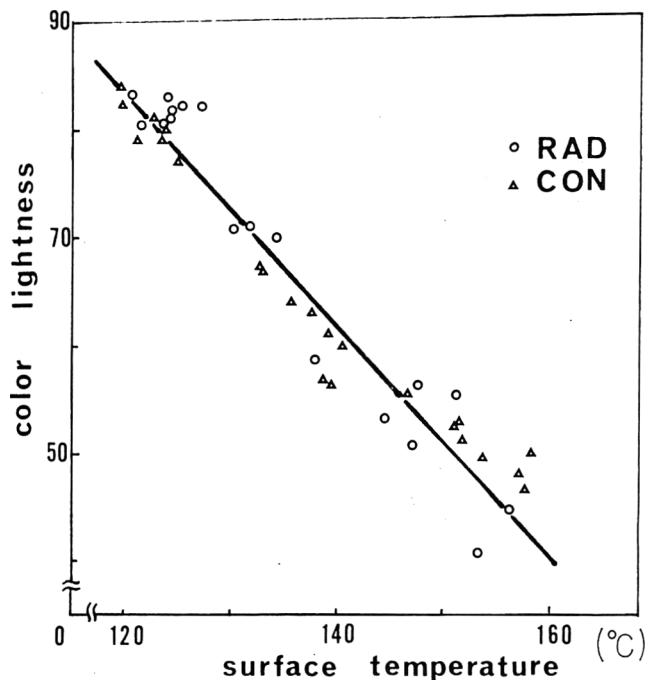
Fig. 9—Color lightness as a function of surface temperature.

ware (TG-SWA). The lightness (L value) of color of the surface was measured using a color difference meter (Nippon Denshoku Kogyo, Tokyo, Japan, ND-101DP). The target range of measurement was the circle, 30 mm in diameter, at the center of a cookie's surface. The L value was obtained as the average of the target range. According to Uniform Chromaticity Scale (U.C.S) system, the L values of pure black and pure white were 0 and 100, respectively. The experiment was repeated four times at each baking time.

Theory

The mechanism of heat transfer to products in the oven included convective heat transfer from hot air and radiative heat transfer from the electric heaters' surface. The expression for convective heat transfer is as follows:

$$Qc = hc \cdot A \cdot (Tg - Ts) \qquad (1)$$

In case a small sample is enclosed by a significantly large heating surface, the expression for radiative heat transfer is as follows (Holman, 1981):

$$Qr = \sigma \cdot A \cdot \epsilon \cdot (Tr^4 - Ts^4) \qquad (2)$$

In this study, the expression for total heat transfer to the sample using expressions (1) and (2) was as follows:

$$Q = Qc + Qr \qquad (3)$$

The thermal conductivity of copper (334 kcal/m·hr·°C (Perry and Chilton, 1973)) is so high that the center temperature of the cylinder is assumed to be equal to the surface temperature. Therefore, at the same center temperature of two cylinders, T°C, total heat transfered to each cylinder is expressed as follows:

$$Q_1 = Qc + Qr_1 \qquad (4)$$

$$Q_2 = Qc + Qr_2 = Qc + (\epsilon_2/\epsilon_1)\cdot Qr_1 \quad (5)$$

The expression for total heat transferred must be equal to that for total heat absorbed. The expression for heat absorbed is as follow:

$$Q = m\cdot c\cdot dT/dt \quad (6)$$

Temperature was measured using the chromel-alumel thermocouple inserted into the center of the cylinder. From the temperature-time curve obtained, both Q_1 and Q_2 can be determined with expression (6). The value of Qr_1 is obtained from expressions (4) and (5). Thus, we define the ratio of radiative heat transfer to total heat transfer, ϕ, as in expression (7).

$$\phi = (Qr_1 / Q_1) \times 100 \quad (7)$$

Identification of terms in expressions are given in the nomenclature section.

RESULTS & DISCUSSIONS

Heating ability

The measured apparent heat transfer coefficients for RAD and CON were 26 ± 1.4 and 25 ± 2.1 kcal/m²·hr·°C, respectively; there was no significant difference between them (probability level, $P < 0.05$). An example of the temperature-time curve of two different cylinders is shown in Fig. 3. From this curve, the heat absorbed by each cylinder was calculated, and the ratio of radiative heat transfer to total heat transfer, ϕ, was determined. The values for RAD and CON were 69 ± 3.8 % and 29 ± 7.3 %, respectively. These heating abilities were measured only at air temperature of 200°C. However, in a previous paper (Shibukawa, 1985), it was confirmed that the change in the ratio of radiative heat transfer to total heat transfer in the range of 180–240°C was negligible.

Solution test

The plot of the extent of browning as a function of time at 180°C, 200°C, 220°C, and 240°C is shown in Fig. 4. In this figure the heating time was the period after the solutions reached a stable temperature, because the sample temperature at the beginning of heating was about 80°C and then increased to a certain temperature. In Fig. 4, the logarithm of absorbance was in proportion to the time of heating, showing that the development of brown pigment was of the first order reaction, which agreed with the work by Marquez and Anon (1986). Rate constants for brown pigment formation in samples heated at various temperatures are listed in Table 1; they were obtained from the slopes of regression equations estimated by least squares method. For all cases examined, the correlation coefficients, r, were greater than 0.93. The rate constants at two heat transfer modes were plotted on an Arrhenius-type plot vs the reciprocal of the temperature (Fig. 5). Linear regression (Snedecor and Cochran, 1985) showed that the regression equations for RAD and CON should be pooled ($P < 0.05$); one regression equation is given in Fig. 5. From this result, the value calculated for the activation energy with glucose/glutamate system was 16.5 kcal/mol which was in good agreement with values obtained in other browning reactions (Cole, 1967; Stamp and Labuza, 1983).

The quantity of 5-HMF was found to be proportional to the absorbance at 550 nm. No significant difference was observed ($P < 0.05$) in the amount of 5-HMF accumulated during heating with two modes. According to these results, individual heat transfer modes had no effect on the mechanism of browning reaction in the solution.

Food test

The surface of cookies after baking at 200°C, arranged according to the passage of time is shown in Fig. 6. The photograph shows that the color on the surface developed from the edge to the center, and there was a difference in the degree of the development of color with each heat transfer mode. These color changes were the passage of heat time were evaluated by L value and are shown in Fig. 7. The lightness, L, decreased rapidly after about 8 min. Though the increase of the L value was observed at the early stage of heating, this seemed to be due to the drying of the surface of the cookie dough.

In spite of the same air temperature under the conditions which had about the same apparent heat transfer coefficient (25 and 26 kcal/m²·hr·°C), the rate of development of the surface color differed with two heat transfer modes. After 10 min, the difference was significant and the surface color heated with RAD was darker than that heated with CON. Also, when the surface temperature of cookies was measured with a radiation thermometer for the two heat transfer modes. there was a significant difference after 6 min. The surface temperature heated with RAD was higher than that heated with CON at the same air temperature, 200°C (Fig. 8). This result suggested that, for cookies, the heat transfer coefficient of RAD was greater than that of CON. Since the radiative heat transfer coefficient was measured with a black copper cylinder anc used for cookies, the discrepancy would be due to the differences in the surface property between cookies and the copper cylinder, the emissivity and the penetration of the electromagnetic waves. However, further analysis of measurements of the true radiative heat transfer coefficient for food is necessary.

On the other hand, the development of color was highly correlated to the surface temperature of cookies as shown in Fig. 9 ($r = -0.97$). The higher was the surface temperature, the darker was the color of surface, independent of heat transfer mode. Consequently, it was confirmed that, not only for the solution but also for the solid cookies, the development of color depended only on the temperature. The difference in the rates of color development between the two heat transfer modes was due to the difference in the rate of increase of surface temperature, even with the same air temperature in the oven.

NOMENCLATURE

A	heat transfer area (m²)
c	specific heat (kcal/kg·°C)
hc	heat transfer coefficient of convective heat transfer (kcal/m²·hr·°C)
m	weight (kg)
t	time (hr)
T	temperature (°C)
Tg	air temperature (°C)
Tr	temperature of radiant surface (°C)
Ts	temperature of product surface (°C)
Q	the rate of heat transferred to or absorbed by cylinder (kcal/hr)
Q_1	the rate of total heat transferred to cylinder coated with heat-resistant black paint (kcal/hr)
Q_2	the rate of total heat transferred to cylinder coated with nickel (kcal/hr)
Qc	the rate of heat transferred by convection (kcal/hr)
Qr	the rate of heat transferred by radiation (kcal/hr)
Qr_1, Qr_2	the rate of heat transferred by radiation to cylinder coated with heat-resistant black paint and with nickel, respectively (kcal/hr)
ϵ_1	emissivity of cylinder coated with heat-resistant black paint
ϵ_2	emissivity of cylinder coated with nickel
σ	Stefan-Boltzman constant, 4.88×10^{-8} (kcal/m²·hr·k⁴)
ϕ	the ratio of heat transferred by radiation to total heat transferred (%)

Sensory Studies of High Potency Multiple Sweetener Systems for Shortbread Cookies with and without Polydextrose

HYESOOK LIM, CAROLE S. SETSER, and SANG SOOK KIM

──────────── ABSTRACT ────────────

Time-intensity (TI) sweetness and bitterness curves determined for six potent sweetener combinations with and without polydextrose were compared to sucrose in shortbread cookies. Hardness, fracturability and cohesiveness of shortbreads were determined Sweetener combinations of aspartame/cyclamate, aspartame/cyclamate/saccharin, acesulfame K/saccharin, aspartame/saccharin/acesulfame K, acesulfame K/aspartame and aspartame/saccharin gave sweetener TI profiles similar to that of sucrose. Bitterness TI profiles were similar for all potent sweetener combinations but higher than for sucrose. Polydextrose increased hardness and fracturability and decreased cohesiveness of cookies compared to those made with high potency sweeteners without polydextrose. Textural characteristics of polydextrose cookies approached those of sucrose shortbreads.

INTRODUCTION

USE OF TWO or three potent sweeteners together has been found to offer several advantages over using each of the individual sweeteners in food products (Miller, 1987; Bakal, 1987; Gelardi, 1987). Synergistic, multiple sweetener combinations cut costs and improve product taste and stability, because the combinations can overcome limitations of the individual sweeteners (Gelardi, 1987). Studies have shown that aspartame/saccharin (APM/SAC), aspartame/cyclamate (APM/CYC), cyclamate/saccharin (CYC/SAC), acesulfame K/aspartame (ACK/APM), acesulfame K/cyclamate (ACK/CYC) and aspartame/saccharin/cyclamate (APM/SAC/CYC) combinations are effective in providing stability and superior taste profiles in tabletop sweeteners, diet soft drinks, dry beverage mixes and chewing gum (Yamaguchi et al., 1970: Anon., 1985a,b; Vetsch, 1985; Von Rymon Lipinski, 1985; Anon., 1986; Bakal, 1986; Gelardi, 1987).

In evaluating new synthetic sweeteners, Swartz and Furia (1977) identified several important issues: intensity, taste-timing properties and response in food systems. Time-intensity (TI) studies of sweeteners (Dubois et al., 1977; Larson-Powers and Pangborn, 1978; Swartz, 1980; DuBois and Lee, 1983; Kelling and Halpern, 1983; Harrison and Bernhard, 1984; Yoshida, 1986) indicated that different sensory stimuli display unique time courses of perception from onset through maximum intensity to extinction. Nabors and Gelardi (1986) reported that the ideal sweetener should be a least as sweet as sucrose without any unusual sweetness profile, such as a delayed onset or persistence in sweetness, which might lead to off-tastes. In other words, an accepted sweetener or sweetener combination needs to emulate the TI curve of sucrose. The TI response has been measured by varied techniques, from using stop watches to computers (Neilson, 1957; Jellinek, 1964; McNulty and Moskowitz, 1974; Larson-Powers and Pangborn, 1978; Birch et al., 1980; Schmitt et al., 1984; Lee, 1985; Yoshida, 1986). Several time-related events can be extracted from TI response curves (Lee and Pangborn, 1986).

Removal of sugars from food formulations yields products

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lacking more than sweetness and calories. Polydextrose is a nonsweet, low calorie (about 1 kcal/g) carbohydrate that can substitute for sugars as a bulking agent and carrier ingredient (McCormick, 1987). Polydextrose has improved the texture of some non-carbohydrate sweetened baked products (Torres and Thomas, 1981; Smiles, 1982; Neville and Setser, 1986). However, in cakes, bitterness increased as polydextrose increased, even though sweetener levels were held constant (Neville and Setser, 1986).

This study extended work with high potency sweeteners used alone in model systems (Redlinger and Setser, 1987a,b) to using combinations of the potent sweeteners in shortbread cookies. Sweetness and bitterness TI profiles of the sweetener combinations, with and without polydextrose, were compared to those of sucrose in shortbread cookies to note sensory responses to the sweetener system. The polydextrose-bulked and non-bulked, shortbread cookies without sucrose were compared texturally to cookies with sucrose.

MATERIALS & METHODS

Sample preparation

Sweeteners used in this study were sucrose (solutions: Fischer Scientific; shortbread: C & H), aspartame (NutraSweet), calcium cyclamate (Abbott Laboratories), acesulfame K (Hoechst, AG) and sodium saccharin (Sherwin Williams Co.). All-purpose flour (Gold Medal, General Mills, Inc.), polydextrose (Pfizer, Inc.) and hydrogenated shortening (Crisco, Procter and Gamble) were used to prepare the shortbread. Distilled, deionized (d/d) water was used for all products.

Combinations, levels and sweetener proportions were based on results from (1) preliminary difference testing to determine proportions and combinations that would give solutions equal in sweetness to a 5% sucrose solution, and (2) ranking tests to ascertain levels of combinations approximately equi-sweet to 25% (based on total weight of formula, twb) sucrose in a cookie. Levels and combinations used are given in Table 1. Shortbread type cookies were prepared according to procedures of Redlinger and Setser (1987b). In some cases, 25g polydextrose were added after beating the shortening-water-sweetener system 15 sec at medium speed. The mixture was beaten 20 additional sec at medium speed and 20 sec at high speed, following the polydextrose addition and before flour was incorporated. Cookie doughs

Table 1 — Levels of sweeteners in shortbread cookies

Sweetener	Approximate ratio	Percentage (twb)[a]
Sucrose		25.0
APM/CYC	1:5	0.0585
		0.2915
APM/CYC/SAC	10:49:1	0.0545
		0.2725
		0.0055
ACK/SAC	18:1	0.0236
		0.0013
APM/SAC/ACK	10:1:2	0.0750
		0.0075
		0.0140
ACK/APM	1:2	0.0345
		0.0745
APM/SAC	10:1	0.1165
		0.0115

[a] twb = based on weight of total formula

were refrigerated (7 ± 1°C) overnight in Ziploc® plastic bags before being baked.

Sensory analysis

Sample presentation. Refrigerated cookie doughs were allowed to come to room temperature (25 ± 1°C), shaped into a ball, and rolled between waxed paper sheets over a 14.5 cm diam plastic mold. Unbaked cookies were marked in 2.2 cm² sections, avoiding the perimeter by using a specially made plastic template. Cookies were baked on a Cushionnaire™ insulated aluminum baking sheet in a rotary hearth oven (National Manufacturing Co., Lincoln, NE) at 350°F (177°C). Baking time for experimental cookies was 22 min without polydextrose and 17 min for cookies with polydextrose. Baked cookies were stored overnight in sealed, odor-free plastic containers at room temperature.

Evaluation. Five professional panelists from the KSU Sensory Analysis Center were oriented to the product and evaluation techniques in six 1-hr sessions. Informed consent was obtained from panelists prior to their participation. Unstructured line scales of 0 (none) to 60 (intense), corresponding to a 6 in scale divided into tenths, were used to determine sweetness and bitterness intensity every 5 sec for up to 85 sec after ingestion. Five sec were allowed after initial ingestion of the cookie as lag time before sweetness or bitterness intensities were recorded. Panelists established anchors for sweetness and bitterness based on sucrose and caffeine solutions, respectively. For sweetness, 5, 10, and 15% sucrose solutions were anchors for scale values of 15, 40, and 60, respectively. Caffeine solutions of 0.01%, 0.05% and 0.1% represented values of 5, 40, and 60, respectively. Monadic presentation of three samples/session was randomized for each panelist. Assessors used d/d water, raw apples and raw carrots between each sample to ensure that no residual sweetness of bitterness remained. Intensity line scales for textural attributes of hardness, fracturability and cohesiveness were anchored at the ends by none (0) and extreme (60). These attributes were evaluated using procedures and references given by Muñoz (1986). All assessments were performed in a controlled environment in individual partitioned booths at 20°C. Panels were conducted mid-morning.

Data analysis

Sweetness and bitterness intensities for each time were analyzed for differences using analysis of variance (SAS, 1986). If significant differences were found, mean separation by least significant differences was used.

The TI curves were drawn using the mean values for each time from three replications (reps) for the five evaluations (panelists) per rep. Areas under the curves for each sweetener combination were measured using a polar planimeter. The time to reach maximum intensity for sweetness and bitterness for each sweetener was read directly from the composite TI curves. Maximum intensities of each rep were determined, and significant differences were calculated using analysis of variance.

RESULTS & DISCUSSION

THE F-RATIOS (Table 2) for the taste attributes, bitterness and sweetness, indicated highly significant differences (p < 0.001) among sweetener combinations. All potent sweetener combinations gave TI curves for sweetness similar to that of sucrose, with (Fig. 1) or without polydextrose (Fig. 2), except the ACK/SAC system. Intensity of this combination was lower (p < 0.05) than all other combinations at all times of evaluation. Sweetness profiles of shortbread cookies made with and without

Table 2 — F-ratios from statistical analyses of shortbread for taste and textural attributes

Attribute	Treatment	Trt × Error		df
		Panel	Panel	
Bitterness	17.90**	3.49*	2.36***	2077
Sweetness	30.79***	5.42***	1.69*	2077
Hardness	93.50***	20.39***	1.16	104
Fracturability	57.95***	14.76***	1.31	104
Cohesiveness	77.38***	1.37	1.61*	104

* Significant at p < 0.05
*** significant at p < 0.001.
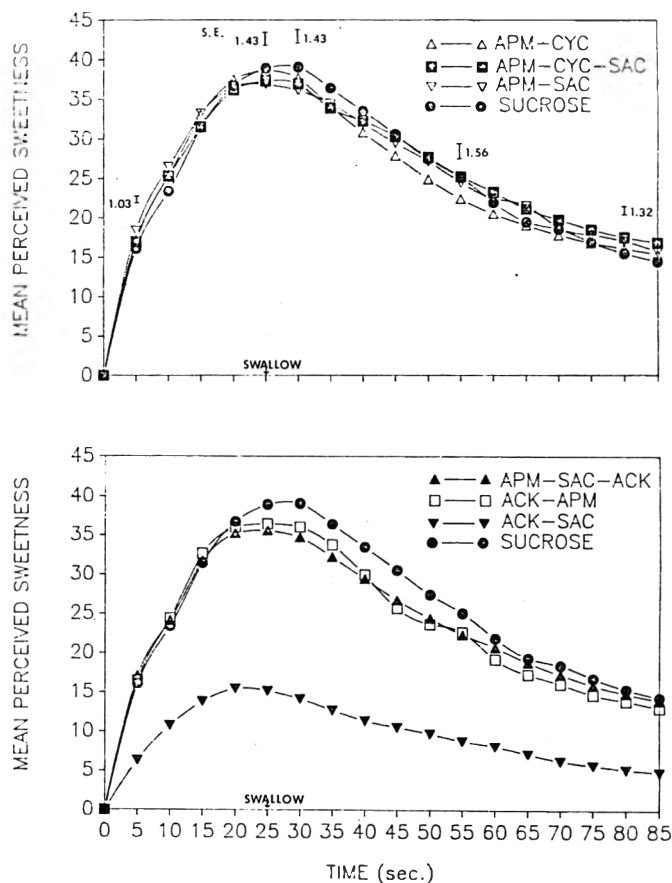
Fig. 1. — Composite (n = 3) time-intensity curves for sweetness of combinations of high potency sweeteners in shortbread without polydextrose. Sweetener abbreviations are aspartame, APM; acesulfame K, ACK; cyclamate, CYC and saccharin, SAC.

polydextrose were similar, except that the intensities generally were slightly higher with polydextrose. However, comparisons of maximum sweetness intensities for corresponding sweetener systems with and without polydextrose (Table 3) indicated that sweetness was not significantly higher with polydextrose.

Maximum sweetness intensity for sucrose was reached 25 sec after ingestion (Table 3). Even though some high potency sweetener combinations reached maximum intensity slightly later (30 sec) than sucrose, the difference between 25 sec and 30 sec was considered small. For sweetness, the maximum intensities of APM/SAC/ACK without polydextrose and for APM/CYC, APM/CYC/SAC, ACK/APM and APM/SAC, with or without polydextrose, were the same as that of sucrose.

As stated earlier, the combination of ACK/SAC showed an exceptionally low maximum intensity (Fig. 1 and 2, Table 3). In previous work, ACK and SAC were heat stable (Anon., 1986). The low intensity of sweetness likely resulted from usage of a low sweetener level. If the level was increased, this combination might be similar to other systems for sweetness, but bitterness curves likely would be higher as well. Difficulties in establishing equi-sweet systems in preliminary testing were compounded by the accompanying bitter taste of cyclamate and saccharin, which Helgren et al. (1955) found increased with concentration. Establishing equi-sweet concentrations for these two sweeteners has been difficult in previous studies also (Larson-Powers and Pangborn, 1978; Redlinger and Setser, 1987a; Tunaley et al., 1987). At high concentrations, sweetness functions of potent sweeteners have been shown to decrease with bitter side-tastes becoming predominant (Moskowitz, 1970).

Profiles for bitterness of the potent sweeteners were similar to each other, and all intense sweetener combinations had significantly higher bitterness scores than sucrose (Fig. 3 and 4).
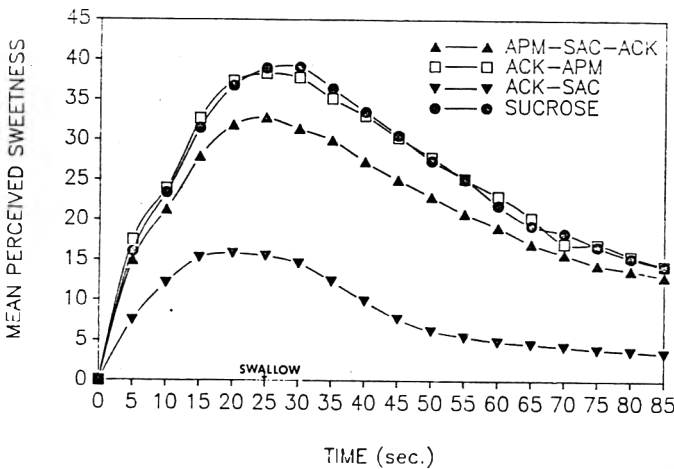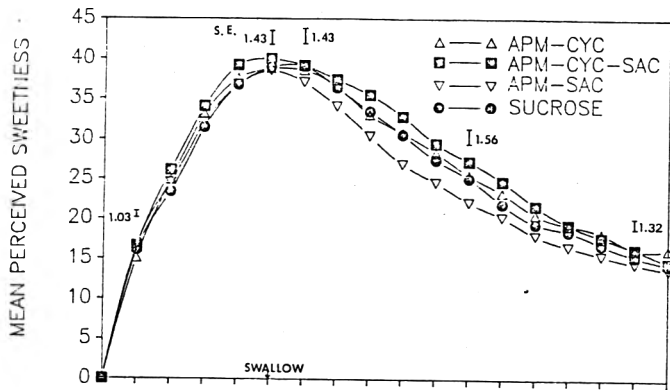
Fig. 2. — Composite (n = 3) time-intensity curves for sweetness of combinations of high potency sweeteners in shortbread with polydextrose. Sweetener abbreviations are aspartame, APM; acesulfame K, ACK; cyclamate, CYC and saccharin, SAC.

Fig. 3. — Composite (n = 3) time-intensity curves for bitterness of combinations of high potency sweeteners in shortbread without polydextrose. Sweetener abbreviations are aspartame, APM; acesulfame K, ACK; cyclamate, CYC and saccharin, SAC.

Fig. 4. — Composite (n = 3) time-intensity curves for bitterness of combinations of high potency sweeteners in shortbread with polydextrose. Sweetener abbreviations are aspartame, APM; acesulfame K, ACK; cyclamate, CYC and saccharin, SAC.

Table 3 — Some indices derived from time-intensity curves for sweetness and bitterness of shortbread made with several potent sweetener combinations

Treatment	Time(sec) to reach maximum intensity		Max. intensity[a]		Curve area (cm²)[b]	
	Sweet	Bitter	Sweet	Bitter	Sweet	Bitter
Sucrose	30	25	39.1ab	3.3f	14.99	1.03
Intense sweeteners[c]						
w/ polydex.						
APM/CYC	25	30	38.8ab	37.4a	13.27	11.90
APM/CYC/SAC	25	25	40.0a	38.7a	15.59	12.08
ACK/SAC	25	25	15.5d	16.8de	4.63	5.06
APM/SAC/ACK	25	25	32.7c	22.1cd	11.89	6.39
ACK/APM	25	30	38.3ab	26.5bc	13.49	7.81
APM/SAC	25	25	38.5ab	21.9cd	12.97	7.42
w/o polydex.						
APM/CYC	25	25	38.6ab	32.'ab	13.09	9.43
APM/CYC/SAC	25	25	37.3ab	28.4bc	12.67	9.38
ACK/SAC	25	30	15.1d	10.6e	5.00	3.72
APM/SAC/ACK	25	30	35.6bc	16.2de	12.50	5.49
ACK/APM	25	25	36.5abc	16.7de	12.46	5.82
APM/SAC	25	25	36.9ab	16.9d	13.49	6.05

[a] Means in a column that have common letters are not significantly different, p = 0.05.
[b] Areas measured are from curves drawn using mean of three reps and five evaluations/rep for each time.
[c] Proportions of sweeteners used given in Table 1.

Systems that had the same sweetness as sucrose with low levels of bitterness in shortbread without polydextrose were APM/SAC, APM/SAC/ACK and ACK/APM. The APM/CYC and APM/CYC/SAC systems with polydextrose were significantly higher in bitterness than other combinations (Fig. 4). Total areas under the curves indicated that bitterness increased in
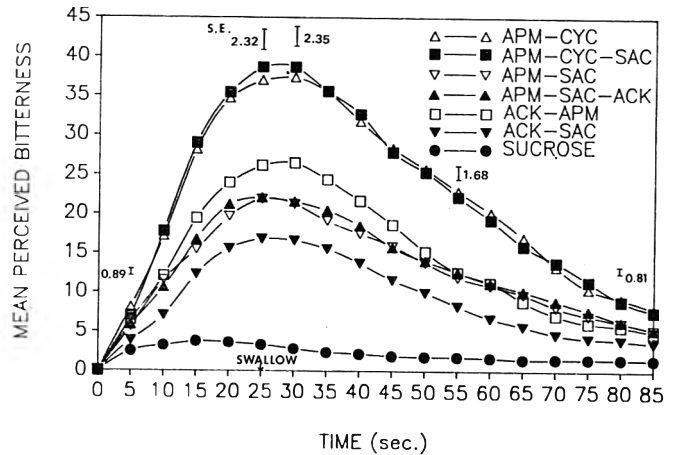
each system when polydextrose was included in the formulation. Maximum bitterness was significantly higher in some cases (APM/CYC/SAC and ACK/APM) when polydextrose was added to the system (Table 3). This generally agrees with findings in layer cakes (Neville and Setser, 1986). Undoubtedly, the scores were higher than would be obtained using the general population, because panelists in this study had both extensive training and experience to make them highly sensitive to "off" taste factors such as bitter.

The statistical analyses of shortbread for textural attributes are given in Table 2. All of the textural attributes, hardness, fracturability and cohesiveness, showed highly significant (p < 0.001) differences for treatment. No consistent relationship of the high potency sweetener combinations to textural attributes of the shortbread cookies was found in these studies. The effects of polydextrose on the mean scores for textural attributes of shortbread with potent sweeteners are shown in Table 4. The shortbread that was made with sucrose was the hardest and most fracturable. Polydextrose is used for a reduced-calorie bulking, bodying or texturing agent in baked products. The slightly higher viscosity compared to sucrose and the amorphous structure of polydextrose provide improved mouthfeel and viscosity (Smiles, 1982). However, these properties can cause spreadability to increase too much in cookies. Generally, a substitution of polydextrose for one-third or less of the sugar, or a total of 15% twb, in the batter is advised

Table 4—Mean values[a] for textural attributes of shortbread with potent sweeteners with and without polydextrose

Treatment	Hardness	Fracturability	Cohesiveness
Sucrose	41.83a	28.77a	15.39b
Intense sweeteners[b]			
w/ polydex			
APM/CYC	31.14b	24.09b	12.79bc
APM/CYC/SAC	27.37cd	21.46bc	11.47bc
ACK/SAC	26.05cd	21.83bc	7.33c
APM/SAC/ACK	27.90bcd	22.48bc	9.08c
ACK/APM	29.24bc	20.15c	8.47c
APM/SAC	25.35d	20.10c	11.07bc
w/o polydex.			
APM/CYC	9.87c	8.24d	34.42a
APM/CYC/SAC	7.68e	6.84d	35.18a
ACK/SAC	7.23e	5.65d	36.24a
APM/SAC/ACK	6.87e	5.48d	37.73a
ACK/APM	8.37e	6.22d	35.45a
APM/SAC	7.55e	5.59d	38.19a

[a] Means that have common letters in column are not significantly different, $p = 0.05$; LSD for hardness, fracturability, and cohesiveness = 3.64, 3.35, and 4.36, respectively.

[b] Proportions of sweeteners used given in Table 1

(LaBell, 1985). The 25 g polydextrose that was used in this experiment was approximately 12% of the total dry weight of the ingredients or about a 36% sucrose substitution. Results given in Table 4 indicated that the polydextrose increased hardness and fracturability and decreased cohesiveness compared to the cookies made without polydextrose. The shortbread made with polydextrose had textural characteristics more similar to shortbread made with sucrose than the shortbread without polydextrose.

Cohesiveness was measured as the cohesiveness of mass with mastication rather than cohesiveness of the shortbread cookie itself. This was defined as how well the product held together or retained its identity during mastication. Cohesiveness can be developed by thermal, salivary and mechanical action during mastication (Muñoz, 1986). The non-sucrose shortbread cookies that were made without polydextrose had very high cohesiveness scores. These studies have shown that all but one of the high potency sweetening systems studied gave TI curves for sweetness similar to that of sucrose, but gave TI curves for bitterness higher than that of sucrose. Polydextrose added to cookies tended to increase both maximum bitterness and total sweetness and bitterness as assessed by areas under the TI curves. Polydextrose increased hardness and fracturability and decreased cohesiveness of intensely sweetened shortbreads. Cookies with polydextrose had textural characteristics more similar to those of sucrose-sweetened shortbread than cookies without sucrose or polydextrose.

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Effect of Packaging Materials on the Chemical Composition of Potatoes

BARRY GOSSELIN and NELL I. MONDY

——————————— ABSTRACT ———————————

Russet Burbank and Chieftain potatoes were packaged in either mesh, paper or polyethylene bags and compared in chemical composition after 1, 4, and 8 wk of storage at 20°C. Potatoes were analyzed for weight loss, discoloration, phenols, ascorbic acid, glycoalkaloids and nitrate-nitrogen. Potatoes packaged in polyethylene were lowest in weight loss, ascorbic acid and nitrate-nitrogen and highest in discoloration, phenols and glycoalkaloids than those packaged in mesh or paper. Potatoes packaged in paper were lowest in discoloration and phenols and highest in ascorbic acid. There were no significant differences in weight loss, glycoalkaloids or nitrate-nitrogen between potatoes packaged in mesh on paper.

INTRODUCTION

POTATOES are packaged in a variety of containers for the public. The most common types of packaging materials used for potatoes are double walled, wet strength Kraft paper bags and perforated polyethylene bags. The effect of packaging material on tuber weight loss and microbial spoilage has been studied. Potatoes packaged in polyethylene were lowest in weight loss but highest in microbial spoilage than those packaged in mesh, paper, and other types of materials (Lutz et al., 1951; Hardenburg, 1954; Chapogas and Hale, 1960; Ginn et al., 1963; Varis, 1973). Alban and Tussing (1946) reported that potatoes packaged in mesh were higher in weight loss than potatoes packaged in paper. Hardenburg (1949), however, found no significant differences in weight loss between potatoes packaged in mesh or paper.

Very few reports were found on the effect of packaging materials on the chemical composition of potatoes. Enzymatic discoloration (blackspot) is one of the most serious problems facing the potato industry. Chapogas and Hale (1960) observed greater discoloration in potatoes packaged in polyethylene compared to paper as a result of bruising. Ginn et al. (1963), however, found less discoloration as a result of bruising in potatoes packaged in polyethylene than those packaged in paper. Enzymatic discoloration has been correlated (+ 0.83) with tuber phenolic content (Mondy et al., 1967) and phenols have been shown to give potatoes bitter off-flavors (Mondy et al., 1971). No studies were found on the effect of packaging material on the phenolic content of potatoes.

Ascorbic acid is an important nutrient in the diet of man. One hundred fifty grams of raw potatoes can supply as much as 90% of the USRDA for ascorbic acid (Augustin et al., 1978). However, ascorbic acid is rapidly lost during storage (Augustin et al., 1975). No reports were found concerning the effect of packaging materials on ascorbic acid levels in potatoes. Packaging that reduces ascorbic acid loss during storage would be beneficial to consumers.

Glycoalkaloids are naturally occurring toxicants in potatoes. Over 2000 individual cases of gylcoalkaloid poisoning and 29 deaths involving potatoes have been recorded (Morris and Lee,

1984). Exposure to light has been found to increase the glycoalkaloid content of potatoes (Jadhav and Salunkhe, 1975). The glycoalkaloid content of potatoes packaged in various colored polyethylene and cellophane films was found to be dependent on the amount of light transmitted through the packaging material (Gull and Isenberg, 1960; Wu and Salunke, 1975). No studies were found concerning the effect of the packaging material without light exposure on the glycoalkaloid content of potatoes.

Nitrates are also naturally occurring toxicants in potatoes. High concentrations of nitrates in the food supply is of great concern because of their role in the formation of nitrite. Nitrites have been shown to react with secondary and tertiary amines to form carcinogenic and mutagenic N-nitroso compounds (Walters et al., 1979). White (1975) estimated that potatoes contributed 14% of the per capita consumption of nitrates in the US. No reports were found regarding the effect of packaging material on the nitrate content of potatoes.

This study was conducted to examine the effect of three different packaging materials (mesh, paper, and polyethylene) on the weight loss, discoloration and phenolic, ascorbic acid, total glycoalkaloid (TGA) and nitrate-nitrogen (NO_3-N) contents of potatoes during storage.

MATERIALS & METHODS

RUSSET BURBANK and Chieftain potatoes were grown at the Cornell Vegetable Research Farm in Riverhead, Long Island using standard cultural practices. After harvest, potatoes were washed, dried and stored 4 mo. in the dark at 5°C, 95% RH prior to the study.

Potatoes were divided into 10 lb (4.54 kg) lots and packaged in either (1) paper mesh bags, (2) double wall, wet strength Kraft paper with 3 × 6 1/2 inch mesh window or (3) 2 mil polyethylene bags with twenty-four 1/4 inch perforations. Samples were stored in the dark at room temperature (20°C, 70% RH) for 1, 4 and 8 weeks.

Tubers were cut longitudinally from bud to stem end in order to include both apical and basal portions. Slices were subsequently separated into cortex and pith sections along the vascular ring. Cortex tissue, including the periderm, was used to each analysis since it is the area of highest metabolic activity, highest phenol and glycoalkaloid concentration and the area most affected by tuber discoloration. Both cortex and pith tissues were used for the analysis of ascorbic acid and nitrate-nitrogen contents, since significant levels of each compound are located in both tissues. Four tubers were used in each determination and all analyses were performed in duplicate for each storage period and variety.

Weight loss

Each lot of potatoes was weighed prior to packaging and following storage to determine the percent weight loss.

Enzymatic discoloration

Enzymatic discoloration of potatoes was determined using the Hunter Color Difference Meter as described by Monday et al. (1985).

Phenolic content

Phenolic content was determined colorimetrically using Folin-Denis reagent ethanol extracts of potato tissue (Mondy et al., 1985).

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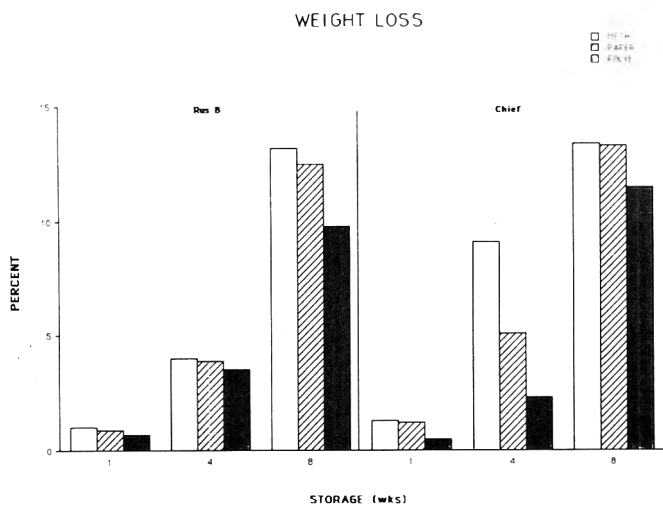
WEIGHT LOSS

Fig. 1—Weight loss of Russet Burbank (Rus B) and Chieftain (Chief) potatoes packaged in mesh, paper or polyethylene (POLYE) bags.

Ascorbic acid content

Ascorbic acid content was determined using a modified Iodate titration method (Samotus et al., 1982).

Total glycoalkaloid (TGA) content

Total glycoalkaloids were extracted using chloroform-methanol (1:2, v/v) and precipitated using concentrated ammonium hydroxide (Bushway et al., 1980). Total glycoalkaloids were quantified by nonaqueous titration using bromphenol blue (Fitzpatrick et al., 1978).

Nitrate-nitrogen (NO$_3$-N)

Nitrate-nitrogen level was determined colorimetrically using phenoldisulfonic acid on aqueous extracts of potato tissue (Mondy and Ponnampalam, 1985).

Statistical analysis

Statistical significance of data was determined using analysis of variance with protected LSD test (Steel and Torrie, 1980).

RESULTS & DISCUSSION

BOTH RUSSET BURBANK and Chieftain potatoes packaged in polyethylene bags had significantly (p < 0.05) less weight loss than those packaged in mesh or paper (Fig. 1). Similar findings for polyethylene bags have been previously reported (Lutz et al., 1951; Hardenburg, 1954; Chapogas and Hale, 1960; Ginn et al., 1963; Varis, 1973). No significant difference was found in weight loss between potatoes packaged in mesh or paper bags. These findings are in agreement with Hardenburg (1949).

Potatoes packaged in polyethylene were significantly higher in phenolic content (p < 0.01) and discoloration (p < 0.05), followed by those packaged in mesh and then paper (Fig. 2). Chapogus and Hale (1960) also found greater enzymatic discoloration in potatoes packaged in polyethylene compared to those packaged in paper. The insides of the polyethylene bags were high in moisture and a few tubers developed slight surface mold growth. However, any potatoes infested with mold were discarded prior to chemical analyses. The high moisture environment in the polyethylene bags may have produced a stress on the tubers and resulted in high phenolic levels and discoloration.

Potatoes packaged in polyethylene bags had significantly higher (p < 0.05) ascorbic acid loss than tubers packaged in mesh or paper (Fig. 3). The pith was significantly higher (p

COLOR   PHENOLS

Fig. 2—Effect of packaging Russet Burbank (Rus B) and Chieftain (Chief) potatoes in mesh, paper or polyethylene (POLYE) bags on color and phenols. Lower Rd values indicate greater discoloration of potatoes.
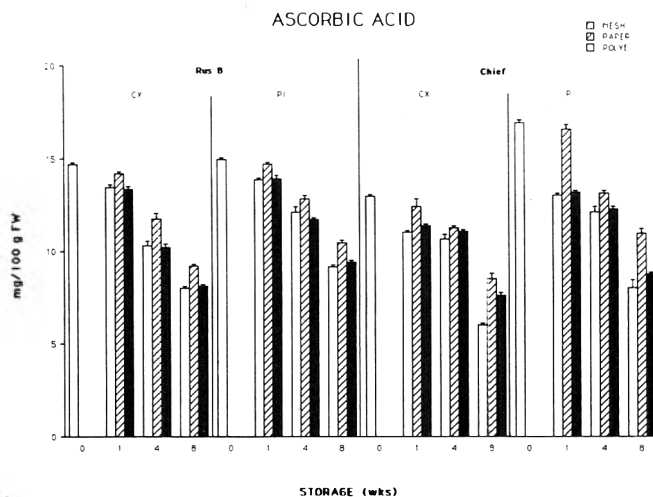
ASCORBIC ACID

Fig. 3—Ascorbic acid content of cortex (CX) and pith (PI) tissues of Russet Burbank (Rus B) and Chieftain (Chief) potatoes packaged in mesh, paper or polyethylene (POLYE) bags.

< 0.01) in ascorbic acid content than cortex tissue. Ascorbic acid levels may have also been influenced by the high moisture environment observed in the polyethylene bags.

The TGA content of potatoes packaged in polyethylene was significantly higher (p < 0.01) than that of potatoes packaged in mesh or paper (Fig. 4). No significant difference was found in the TGA content of potatoes packaged in paper or mesh. Since potatoes were stored in the dark, light emittence through the packaging material was not a factor in TGA accumulation of potatoes. The high moisture environment of the polyethylene bag may have stressed the potatoes and resulted in higher TGA levels than those packaged in the other two materials. Alteration of the packaging environment using vacuum packaging was also found to affect light-induced TGA synthesis (Wu and Salunkhe, 1975).

The NO$_3$-N content of potatoes packaged in polyethylene was significantly lower (p < 0.05) than that of those packaged in mesh or paper (Fig. 5). No significant differences were found in the NO$_3$-N content of potatoes packaged in mesh or paper. These findings were true for both cortex and pith tissues. The cortex was significantly higher (p < 0.01) in NO$_3$-N than the pith.
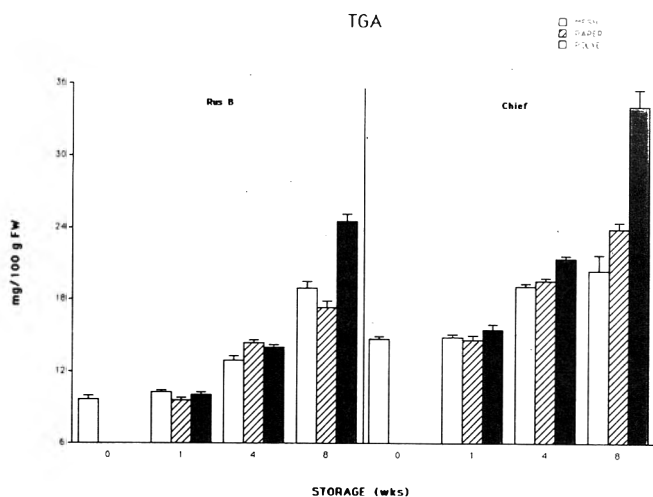
Fig. 4—Total glycoalkaloid (TGA) content of Russet Burbank (Rus B) and Chieftain (Chief) potatoes packaged in mesh, paper or polyethylene (POLYE) bags.
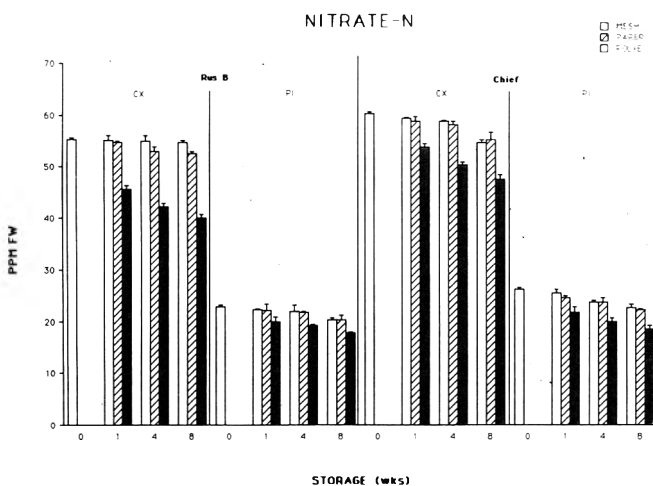
Fig. 5—Nitrate-N content of cortex (CX) and pith (PI) tissues of Russet Burbank I (Rus B) and Chieftain (Chief) potatoes packaged in mesh, paper or polyethylene (POLYE) bags.

Packaging potatoes in Kraft paper bags resulted in the higher quality potatoes since they were lower in discoloration, phenols, TGA and NO_3-N and higher in ascorbic acid content as compared to polyethylene bags. More study is needed to determine the effect of different packaging environments on the chemical composition of potatoes. Providing more perforations to polyethylene bags would alter the packaging environment and could improve potato quality. Tubers packaged in mesh

bags were not highly different in chemical composition from those packaged in paper.

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Effects of pH Adjustment and Subsequent Heat Treatment on the Formation of Volatile Compounds of Garlic

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─────────── ABSTRACT ───────────

Effects of pH adjustment during the blending of garlic cloves and subsequence heat treatment on the formation of volatile compounds of garlic were studied by means of gas chromatography (GC). The formation of the two isomeric cyclic compounds 3-vinyl-[4H]-1,2-dithiin and 2-vinyl-[4H]-1,3-dithiin, which were artifacts from allicin, was favored around pH 5.5. Formation of diallyl trisulfide, methyl allyl trisulfide, cis-1-propenyl allyl disulfide, isobutyl isothiocyanate, 2,4-dimethylfuran, 1,3-dithiane, aniline, methyl allyl sulfide, dimethyl disulfide and 3-methyl-2-cyclopentene-1-thione were favored in neutral or weak acidic conditions, whereas formation of diallyl disulfide, diallyl sulfide, methyl allyl disulfide, propenylthiol, propyl allyl disulfide and 1,2-epithiopropane were favored around pH 9.0.

INTRODUCTION

GARLIC (*Allium sativum* Linn.) has been prized for its flavor and pungency for many centuries. It was evident at an early stage that the ordorous compounds of interest were not present in the plant as such but were formed enzymically when the cellular tissue was disrupted. Cavallito and Bailey (1944) described the isolation of the odoriferous antibacterial substance, allicin (diallyl thiosulfinate), from extraction of garlic with ethanol at room temperature. Stoll and Seebeck (1948) reported that intact garlic cloves contain 0.24% by weight S-allylcysteine S-oxide (alliin), a colorless odorless solid, and an enzyme allinase which converts alliin into allicin. In addition to alliin, three (possibly four) γ-L-glutamyl derivatives of S-alkenyl cysteine sulfoxides have been identified in garlic (Virtanen, 1965). These compounds are not cleaved by allinase; they represent only "potentially available" flavor. Consequently, peptidases and transpeptidases which "release" these secondary flavor precursors to primary flavor precursors, that is thiosulfinates, are important enhancers of the aroma of garlic and its products (Virtanen, 1965).

The crude cell-free garlic enzyme solution, of unspecified purity, utilized by Stoll and Seebeck (1947, 1948), showed a broad pH optimum of 5 to 8 and a temperature optimum of 37 °C, under the conditions used. Using protamine and ammonium sulfate as precipitating agents, followed by fractionation on G-200 Sephadex, Mazelis and Crews (1968) obtained a sixfold purification of the enzyme and reconfirmed the observations of Stoll and Seebeck (1947, 1948). The purified enzyme possessed a pH optimum of 6.5 with S-methyl-L-cysteine sulfoxide as substrate. The formation of allicin is also favored around pH 6.5 (Yu and Wu, 1989a). Allicin is very unstable and will decompose even at room temperature (Brodnitz et al., 1971). Decomposition of allicin proceeds by several pathways (Block, 1985). In one of these allicin self-decomposes to form two isomeric cyclic compounds, 2-vinyl-[4H]-1,3-dithiin and 3-vinyl-[4H]-1,2-dithiin. Allicin also decomposed into 2-vinyl-[4H]-1,3-dithiin and 3-vinyl-[4H]-1,2-dithiin during gas

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chromatography (Yu and Wu, 1989). Several methods have been reported for the quantitation of thiosulfinates (Barnard and Cole, 1959; Carson and Wong, 1959; Watanabe and Komada, 1966; Nakata et al., 1970). The best method for measuring the flavor and aroma probably is gas chromatography (GC) under carefully controlled conditions (Bernhard, 1968); however, the method is imperfect in that it measures secondary compounds of the enzymatic action and the relative contribution of these compounds to overall flavor and aroma is not known. In this study, GC was used to determine the effects of pH adjustment during the blending of garlic cloves and subsequent heat treatment on the formation of volatile compounds of garlic.

MATERIALS & METHODS

Material and chemicals

Garlic cloves, of unknown origin, were purchased locally. 2-Vinyl-[4H]-1,3-dithiin and 3-vinyl-[4H]-1,2-dithiin were synthesized by the method of Bock et al. (1982).

Sample preparation

A total of 200g fresh peeled garlic cloves was blended with 800 mL distilled water for 5 min in a Waring Blendor; the pH of the whole mixture during blending was maintained at a constant value by adding 0.5M sodium hydroxide or 0.5M hydrochloric acid solution. The pH value of the mixtures ranged from 2.0 to 10.0 with 1.0 unit/interval. Volatiles of each mixture were extracted for 1.5 hr by using the modified Likens-Nickerson apparatus (Romer and Renner, 1974), in which steam is the heat source; diethyl ether (40 mL, 99.5%, E. Merck) was used as the extracting solvent. Dipropyl disulfide (Wako, 1 mL, 2.502g in 100 mL diethyl ether) was then added to the extracts as an internal standard. The extracts were dried with anhydrous Na_2SO_4 and then injected into the gas chromatograph without further concentration to avoid any loss. Controlled experiments were conducted using blanched peeled garlic cloves (97 °C, 10 min) under the same conditions.

Gas chromatography

A shimadzu GC-9A gas chromatograph equipped with a flame ionization detector (FID) and a 50 m × 0.22 mm fused silica column (Chrompack International, B. V.), coated with CP-Wax 52 CB, was used. The oven temperature was programmed from 50 °C to 200 °C at 2 °C/min. The injector and detector temperatures were 250 °C. The carrier gas was nitrogen at a flow rate of 0.75 mL/min. The data were recorded on a Shimadzu C-R3A integrator. Values reported were the average of two analyses. The linear retention indices of the volatile components were calculated with n-paraffins (C8-C25, Alltech Associates) as references (Majlet et al., 1974).

Gas chromatography-Mass spectrometry

Gas chromatography-mass spectrometry was conducted with a Hewlett-Packard 5985B system. The gas chromatograph was installed with a fused silica capillary column (CP-Wax 52 CB; 50 m × 0.32 mm). Operational parameters were as follows: carrier gas, helium; ionization voltage, 70 eV; ion source temperature, 200 °C.

Fig. 1 — Capillary gas chromatograms of volatile components of garlic formed at (A) pH 2.0, (B) pH 6.0, and (C) pH 10.0.

Fig. 2 — Effects of pH on artifacts formed from allicin. (A) 2-Vinyl-[4H]-1,3-dithiin, $Y = -20.1216 + 16.5679 X - 1.50358 X^2$, $R = 0.90$; (B) 3-vinyl-[4H]-1,2-dithiin, $Y = -7.57056 + 6.68977 X - 0.60498 X^2$, $R = 0.88$.

RESULTS & DISCUSSION

THE CAPILLARY GAS CHROMATOGRAMS of volatile components of garlic that were formed at pH 2 (A), pH 6 (B), and pH 10 (C) are shown in Fig. 1. A comparison of the volatile constituents of the different pH samples are shown in Table 1. Identification of these compounds was reported previously (Yu et al., 1989).

The control experiments were conducted by sampling the blanched peeled garlic cloves (97 °C, 10 min) instead of fresh peeled garlic cloves; only a small amount of volatiles (ca. 148 ppm) could be extracted. The result indicated that (a) the small amount of volatiles detected might not be due to the enzymic reactions and that (b) enzymes involved in the formation of volatiles were inactivated by the heat treatment.

The effects of pH on the formation of 2-vinyl-[4H]-1,3-dithiin and 3-vinyl-[4H]-1,2-dithiin which were confirmed as the major artifacts from allicin during gas chromatography by Block (1985) and Yu and Wu (1989b) are shown in Fig. 2. Formation of these two compounds was favored around pH 5.5, which was inconsistent with nonheated disrupted garlic. Formation of these two compounds was favored around pH 6.5 in the unheated disrupted garlic when analysed by GC, i.e., the optimum pH for the formation of allicin was around pH 6.5 (Yu and Wu, 1989a). The reason for the shift of optimum pH in the formation of these two cyclic compounds might be that allicin was more stable in acidic conditions than in others under the heat treatment. More allicin was formed at pH 6.5 than at pH 5.5 but it decomposed even faster at pH 6.5 than at pH 5.5 with heat.

The effects of pH and subsequent heat treatments on the formation of diallyl trisulfide, methyl allyl trisulfide, cis-1-propenyl allyl disulfide, isobutyl isothiocyanate, 2,4-dimethyl-furan, 1,3-dithiane, aniline, methyl allyl sulfide, dimethyl disulfide and 3-methyl-2-cyclopentene-1-thione are shown in Fig. 3. The formation of these compounds was maximum in the neutral pH range. Among these compounds, diallyl trisulfide
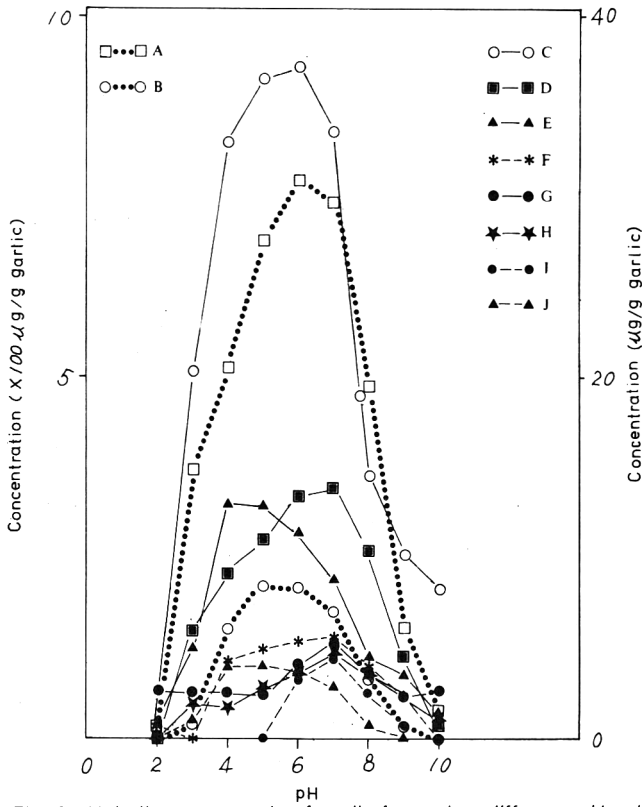
Fig. 3—Volatile compounds of garlic formed at different pH values and subsequent heat treatment: (A) diallyl trisulfide; (B) methyl allyl trisulfide; (C) cis-1-propenyl allyl disulfide; (D) isobutyl isothiocyanate; (E) 2,4-dimethylfuran; (F) 1,3-dithiane; (G) aniline; (H) methyl allyl sulfide; (I) dimethyl disulfide; (J) 3-methyl-2-cyclopentene-1-thione.
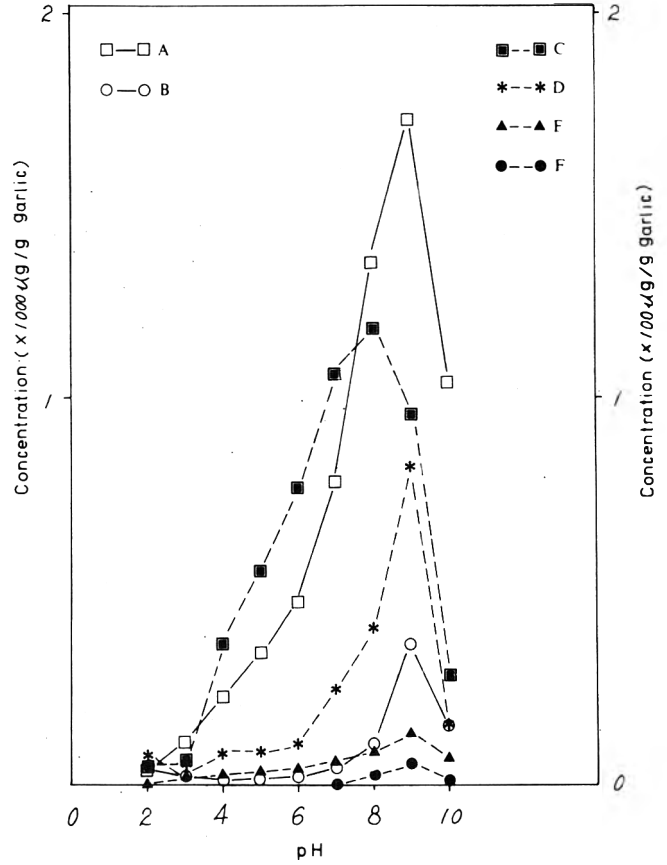
Fig. 4—Volatile compounds of garlic formed at different pH values and subsequent heat treatment. (A) diallyl disulfide; (B) diallyl sulfide; (C) methyl allyl disulfide; (D) propeneylthiol; (E) propyl allyl disulfide; (F) 1,2-epithiopropane.

Table 1—Effects of pH and subsequent heat treatment on the formation of volatile compounds of garlic

Peak no.[a]	Compound[b]	I[c] (CP-Wax 52 CB)	Blank[d]	Yield[e], × 10⁻⁶g/g of garlic bulb at pH 2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0
2	propenylthiol	–	–[f]	7.75	3.05	8.29	8.44	10.54	24.62	39.63	82.23	14.71
3	1,2-epithiopropane	924	–	–	–	–	–	–	–	2.43	5.20	1.46
4	methyl allyl sulfide	956	–	–	1.88	1.78	2.75	3.65	4.72	3.57	2.31	1.16
5	dimethyl disulfide	1077	–	–	–	–	–	3.26	4.52	2.49	0.85	–
6	2,4-dimethylfuran	1093	–	0.92	5.04	12.97	12.85	11.41	8.74	4.45	3.52	1.41
7	2-propen-1-ol	1125	91.30	278.15	154.15	17.82	17.92	46.02	59.87	48.38	26.79	29.17
8	diallyl sulfide	1148	4.28	47.79	23.17	13.66	14.19	17.17	43.46	105.01	363.29	156.43
9	tetrahydro-2,5-dimethylthiophene	1197	–	–	–	–	–	–	–	0.44	0.53	0.37
10	C6H10S [m/e,45(100),42(86), 43(86),29(86),55(64), 73(48),71(28),64(28)]	1233	–	–	–	–	–	2.59	0.57	1.48	1.95	0.57
11	methyl propyl disulfide	1252	–	–	–	–	–	–	–	0.75	0.69	–
12	3-methyl-2-cyclopentene-1-thione	1261	–	–	1.02	3.86	3.95	3.63	2.82	0.74	–	–
14	methyl allyl disulfide	1282	–	5.42	4.98	35.58	55.03	75.32	106.94	118.35	95.10	27.85
15	1,3-dithiane	1296	–	0.65	–	4.27	4.88	5.33	5.59	3.95	2.34	–
16	aniline	1328	2.46	2.66	2.57	2.50	2.29	3.94	5.27	3.50	2.40	2.75
18	dimethyl trisulfide	1380	–	–	0.88	1.15	1.22	–	1.22	1.13	1.31	1.16
19	propyl allyl disulfide	1432	–	0.53	1.12	1.78	2.59	3.70	5.28	8.26	12.81	6.50
20	cis-1-propenyl allyl disulfide	1471	–	1.04	20.27	33.10	36.47	37.09	33.49	14.45	10.19	8.31
21	diallyl disulfide	1490	38.37	39.80	108.46	227.56	340.44	472.54	781.77	1349.80	1723.94	1039.51
22	1,2-dithiocyclopentane	1519	–	–	–	1.58	1.85	2.10	1.70	0.91	–	–
23	unknown [m/e,103(100),104(64), 45(39),119(16),39(15), 69(11),105(11),74(8)]	1532	–	–	12.31	12.75	8.73	6.06	2.77	1.59	–	–
25	methyl allyl trisulfide	1593	–	3.00	20.45	153.26	213.16	207.48	176.90	82.40	16.07	2.44
28	isobutyl isothiocyanate	1753	–	–	5.90	9.27	11.16	13.46	13.83	10.50	4.79	0.81
29	3-vinyl-[4H]-1,2-dithiin	1761	–	–	11.08	10.32	10.64	10.30	8.96	4.26	1.86	–
31	diallyl trisulfide	1806	11.09	15.53	373.57	511.29	682.62	770.31	742.98	485.72	155.72	39.30
32	unknown [m/e,138(100),111(92), 109(64),110(62),95(60), 123(50),77(48),151(36)]	1851	–	0.93	4.98	–	–	–	–	–	–	–
33	2-vinyl-[4H]-1,3-dithiin	1872	–	–	21.32	30.10	22.15	24.63	21.42	11.04	4.16	–
	Total		147.50	404.17	776.20	1092.89	1453.33	1730.53	2057.44	2305.23	2518.05	1333.91

[a] Number refers to Fig. 1.
[b] Number in parentheses indicates relative percentage.
[c] Calculated Kovats' retention indices.
[d] Garlic cloves which were blanched in boiling water for 10 min.
[e] Average of two experiments using dipropyl disulfide as internal standard.
[f] Nondetected.

and methyl allyl trisulfide were the two most abundant compounds formed. Formation of diallyl trisulfide was favored around pH 9.0 when solvent extraction was used instead of steam distillation to obtain the flavor extract from garlic (Yu and Wu, 1989a). The reasons for the shift in optimum pH formation of diallyl trisulfide might be that it was more stable at pH around 6.0 than at other pH values under the heat treatment or that allicin was decomposed more easily to the trisulfide at pH around 6.0 than at any other pH under heat treatment.

The effects of pH and subsequent heat treatment on the formation of diallyl disulfide, diallyl sulfide, methyl allyl disulfide, propenylthiol, propyl allyl disulfide and 1,2-epithiopropane are shown in Fig. 4. Formation of these compounds was maximum around pH 9.0. Among these compounds, diallyl disulfide and diallyl sulfide were the two most abundant compounds formed. Schwimmer and Austin (1971) found that gamma-glutamyl transpeptidase (GGT) acts optimally at pH 9.0. It was postulated that these compounds reached the highest concentrations around pH 9.0 because allicin decomposed into these compounds more easily around pH 9.0 than at any other pH or that the formation of these compounds at pH around 9.0 was due to high activity of GGT. Possibly the GGT in garlic also has an optimum pH at 9.0; at pH around 9.0, GGT catalyzed the transfer of γ-L-glutamyl-S-allyl sulfoxide to alliin easily. Alliin was then converted into allicin by allinase; at these alkali conditions, allicin decomposed easily to the sulfur compounds. Comparison of the changes of volatile compounds extracted by diethyl ether from disrupted garlic at different pH values (Yu and Wu, 1989a) with those from the same samples recovered by steam distillation for 1.5 hr in this study, indicated that the composition of volatile compounds prepared by these two methods were significantly different. Nonheated garlic volatiles contained about 80% allicin in total volatiles, but the heated sample contained less than 4% allicin. However, diallyl disulfide, diallyl sulfide, diallyl trisulfide and other sulfur-containing compounds increased significantly. Diallyl disulfide became the major component formed at pH 9.0. It can be concluded that during the steam distillation of garlic homogenates most of allicin decomposed to sulfides;

both of the heat treatments and the pH values affected the formation of these sulfides.

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Butyric Acid Spoilage of Fermented Cucumbers

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ABSTRACT

Small cucumbers brined to equalize at 2.3% NaCl in an anaerobic tank underwent a normal primary lactic acid fermentation that resulted in 1.09% titratable acidity (as lactic) and pH 3.7. Nine months later the product was observed to have spoiled, as evidenced by an unpleasant odor. Products formed during spoilage in order of concentration were acetic acid > butyric acid > n-propanol > propionic acid. No lactic acid remained. No botulinal toxin was detected. *Clostridium tertium* was identified as contributing to the spoilage, but did not produce propionic acid or n-propanol under test conditions. Evidence indicated that unidentified bacteria, possibly propionibacteria sp., degraded lactic acid causing a rise in pH which allowed *C. tertium* to grow.

INTRODUCTION

THE PRESERVATION of pickling cucumbers by brine fermentation and storage in bulk containers constitutes approximately 40% of the total crop grown in the U.S. Processors could greatly benefit from brining procedures that minimize salt (NaCl) usage. Much of the salt used for storage of fermented cucumbers must be leached from the brine-stock pickles during further processing. Disposal of salt poses environmental and economic concerns to the pickle industry. The concerns could be increased with current proposals by federal and state agencies for limitation of chloride in waterways into which salt and other processing wastes may be discharged.

Two major functions of salt in the brining of cucumbers include the inhibition of softening enzyme activity (Bell and Etchells, 1961) and control of microbial activity. Recent studies have indicated that the firmness of brined cucumbers can be retained at appreciably lower salt concentrations than traditionally used (Fleming et al., 1987). This optimism is due to recent studies on the use of calcium salts to retard cucumber softening (Fleming et al., 1978; Buescher et al., 1979; Tang and McFeeters, 1983) and the use of anaerobic tanking procedures to preclude entrance of rainwater, foreign matter and aerobic microorganisms (Fleming et al., 1983; 1988).

Higher salt concentrations (10.6–15.8%, w/w) in cucumber brines suppress growth of lactic acid bacteria, but allow growth of bacteria of the genus *Enterobacter* (formerly *Aerobacter*) and yeasts (Etchells and Jones, 1943). The latter two groups of microorganisms produce large amounts of gas, which can result in bloater damage to the brine-stock pickles (Jones and Etchells, 1943). A lower salt concentration (5.3%) was shown to favor growth of lactic acid bacteria and suppress growth of *Enterobacter* and yeasts. Thus, the current commercial use of salt concentrations of 5 to 8% for fermentation and 8 to 16% for storage has evolved as a compromise to achieve a desirable fermentation and maintain a desirably textured product.

In a recent pilot-scale experiment employing anaerobic tank

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technology (Fleming et al., 1988) at low concentration of salt (2.3%), we observed an unusual spoilage problem of fermented cucumbers. The cucumbers underwent a normal lactic acid fermentation which terminated at pH 3.7. During storage, lactic acid was depleted and acetic acid, butyric acid, propionic acid, and n-propanol were formed. The objective of this study was to characterize chemical changes that occurred during fermentation and spoilage, and to identify spoilage microorganisms.

MATERIALS & METHODS

SIZE 1B (2.4 to 2.7 cm diameter) pickling cucumbers were washed with an Osborn U-Brush Washer (model 810–273) and loaded into a dome top fiberglass tank (model CM-CFV-5, Warner Fiberglass Products, Belding, MI) as previously described (Fleming et al., 1983). The tank (4500L total volume) was filled with 60% cucumbers and 40% brine, by volume. The cover brine was added to the tank first and consisted of 0.045M Ca(OH)$_2$, 0.8% acetic acid (added as vinegar) and 6.6% NaCl; the pH was 4.7. The brining procedure was generally that described earlier (Fleming et al., 1988) except for differences in salt concentration and addition of the *Lactobacillus plantarum* culture (isolate MOP7 from fermenting cucumbers) to the brine after addition of cucumbers. The *L. plantarum* culture was grown overnight in MRS broth and added to give about 6×10^5 cells per mL brined cucumbers. The brined cucumbers were purged with nitrogen at the rate of 15 SCFH (standard cubic feet per hour) during the fermentation; thereafter purging was discontinued and the tank headspace held under a slight nitrogen pressure for the storage period (Fleming et al., 1983).

Chemical analyses of raw cucumbers and of fermentation brines were determined by HPLC as previously described for sugars, organic acids and ethanol (McFeeters et al., 1984). To confirm the identity of presumptive peaks of butyric acid, propionic acid and n-propanol samples were also chromatographed on a Bio-Rad HPX-87H cation exchange column with 0.01N H$_2$SO$_4$ as the eluent and both refractive index detection with a Waters model 401 detector and UV detection at 210 nm with a Varian Varichrom UV detector. In addition, samples were chromatographed using a Hewlett Packard 5390 GLC equipped with a 1 meter GP 60/80 Carbopack C/0.3% Carbowax 20M/0.1% H$_3$PO$_4$ column (Supelco no. 1-1825) and an FID detector in order to confirm the identity of these compounds by gas chromatography. Brine samples were injected directly onto the column without derivatization. Helium at a flow rate of 50 mL/min was used as the carrier gas. The column temperature was 120°C. Detector and injector temperatures were 200°C. Titratable acidity, pH, reducing sugar, and salt concentration were determined as described earlier (Fleming et al., 1984). Fermentation balances were calculated by general procedures described by Wood (1961).

Brine samples of spoiled pickles were taken in screw capped tubes which were kept anaerobically (GasPak jar, BBL Microbiology Systems, Cockeysville, MD) overnight at room temperature. The brines were viewed by phase contrast microscopy (500X) for characterization of microbial cell morphology. Samples of brine were heated at 80°C for 15 min. The samples were then streaked onto anaerobic egg agar (Kautter and Lynt, 1978) and incubated anaerobically for 3 days at 30°C. Representative colonies were picked into tubes of thioglycollate broth. The tubes were incubated anaerobically at 30°C for 7 days. Six isolates thus obtained were subjected to identification procedures as described by Holdeman et al. (1977). The procedure of Cato et al. (1982) was followed for determination of the electrophoretic pattern of cell proteins to assist with identification of *Clostridium* isolates.

A sample of brine from the spoiled pickles was sent to the CDC Botulism Laboratory (Atlanta, GA) for determination of the presence of botulinal toxin. The product was then acidified to inhibit further

fermentation and to reduce a potential safety hazard. Concentrated H₂SO₄ (13L of 36N) was slowly added to the tank over a 1 hr period through a funnel in the top of the tank. The brine was circulated by nitrogen purging. The pH was 1.6 after acidification. Sodium benzoate (24L of 33%) was added to further stabilize the product. After determination that the product did not contain botulinal toxin, the tank contents were removed and properly discarded.

RESULTS

THE CUCUMBERS were brined on September 20 and underwent a typical primary fermentation which resulted in 1.09% titratable acidity (as lactic acid), pH 3.7 and no fermentable sugar after 30 days (Table 1). The NaCl concentration equalized at 2.3%. The product was determined to be of excellent quality after fermentation (late October) in terms of firmness, absence of bloater damage, flavor, and general appearance. The brined cucumbers were stored over the winter months in the tank in which they were fermented with no observed changes. On June 3 of the following year (256 days after brining), a sample of cucumbers and brine was removed from the tank for quality evaluation and experimental product formulation and was still of excellent quality. No samples for chemical analyses were taken at this time. The tank was returned to the anaerobic storage mode by flushing the headspace with nitrogen and maintaining a nitrogen blanket under a slight positive pressure. On the following July 10 (293 days after brining), the fermented cucumbers were observed to be spoiled, as evidenced by a strong butyric acid odor and frothing and overflow of the brine. The fermented cucumbers remained firm and intact. A secondary fermentation apparently had occurred as the temperature of the tank contents increased during the summer. At this time, the acidity was only 0.59%, and the pH was 4.8 (Table 1). A sample of the brine was determined not to contain botulinal toxin by the CDC Botulism Laboratory (Atlanta, GA).

HPLC analysis of the brine revealed that none of the lactic acid formed during primary fermentation was present in the spoiled pickles and that acetic acid, butyric acid, propionic acid, and n-propanol had been formed. Butyric acid, propionic acid, and n-propanol were identified by their retention times on HPLC and GLC compared to standard compounds. Retention times matched the standards on a Bio-Rad HPX-87H cation exchange column and on a C₁₈ reversed phase column using HPLC. On the cation exchange column, the peaks presumptively identified as butyric and propionic acids by refractive index detection also gave peaks with a UV detector at 210 nm, as would be expected for carboxylic acids. Also as expected, the presumptive n-propanol peak gave no response on the UV detector. GLC of the brine samples also showed peaks with the same retention times as standard compounds. The composition of the raw cucumber/cover brine mixture and the brine after primary fermentation (30 days after brining) and after spoilage (293 days after brining) is summarized in Table 2. Carbon recovery after primary fermentation was 109.8%, which is consistent with previously reported carbon recovery of cucumbers fermented by this method (Fleming et al., 1988). The hydrogen/oxygen ratio of substrates before fermentation

Table 1 — Brine chemistry of the cucumber fermentation[a]

Days after brining	pH	Acid (%)	NaCl (%)	Sugar (%)
Primary fermentation				
0	4.73	0.35	5.5	0.0
1	4.38	0.37	2.5	0.4
2	3.72	0.48	2.3	0.1
3	3.67	0.88	2.3	0.0
5	3.67	1.10	2.4	0.0
8	3.62	1.11	2.3	0.0
13	3.59	1.12	2.3	0.0
30	2.68	1.09	2.4	0.0
After spoilage				
293	4.80	0.59	2.3	0.0

[a] Concentrations are expressed as %, w/v.

Table 2 — Substrates and products of the cucumber fermentation

Compound	Before fermentation	After primary fermentation	After spoilage
Concentration of compounds, mM			
Glucose	27.1	ND	ND
Fructose	34.3	ND	ND
Malic acid	12.6	ND	ND
Acetic acid	53.2 (0.0)[a]	63.7 (12.5)[a]	105.3 (39.6)[a]
Lactic acid	ND[b]	140.1	ND
Ethanol	ND	7.3	1.7
Propionic acid	ND	ND	8.1
Propanol	ND	ND	34.5
Butyric acid	ND	ND	38.7
Elemental recoveries, %			
Carbon		109.8[c]	79.4[d]
Hydrogen (H)		115.0[c]	85.9[d]
Oxygen (O)		104.9[c]	46.2[d]
Hydrogen/oxygen ratio of compounds, mM			
	1.88	2.06	3.84

[a] Values in parentheses are those used in calculations of elemental recoveries and reflect the assumption that acetic acid was not a substrate for products measured.

[b] ND = none detected.

[c] Recoveries based on composition of compounds before and after fermentation.

[d] Recoveries based on composition of compounds after primary fermentation and after spoilage.

Table 3 — Effect of pH on fermentation of spent cucumber brine by Clostridium tertium[a]

Adjusted pH	Visual growth	Lactic acid	Acetic acid	Butyric acid
		Brine components after incubation, mM		
3.7 (control)	−	121.8	56.1	ND
3.7	−	123.8	58.0	ND
4.1	−	120.1	55.6	ND
4.8	±	106.9	50.1	ND
6.0[b]	+ +	ND[d]	6.5	86.8
7.0[c]	+ +	1.5	7.4	99.7

[a] Cucumber brine after primary fermentation was filter-sterilized, pH adjusted and inoculated with C. tertium isolates. Observations were made after incubation at 30°C for 6 days.

[b] Carbon recovery, 74.0%, based on pH 3.7 control (uninoculated).

[c] Carbon recovery, 86.1%, based on pH 3.7 control (uninoculated).

[d] ND = none detected.

was 1.88, and this ratio increased slightly to 2.06 for primary fermentation products (Table 2). Carbon recovery after spoilage was only 79.4% (based on products formed during primary fermentation serving as substrates for the spoilage fermentation), and the hydrogen/oxygen ratio of products formed was 3.84.

Phase contrast microscopy of the brine revealed the presence of rod-shaped bacteria, some of which were swollen with a terminal, refractile spore. Six anaerobes isolated from the brine were all identified as Clostridium tertium, based on identification procedures as described in the Anaerobe Laboratory Manual (Holdeman et al., 1977). The isolates grew both aerobically and anaerobically in PYG broth; however, spores were formed only under anaerobic conditions. The isolates formed large amounts of gas, were Gram-positive and nontoxic by i.p. injection of mice. The isolates produced acids in PYG broth in the following decreasing order of concentration: acetic, butyric, lactic, formic, and succinic. The electrophoretic pattern of proteins (Cato et al., 1982) of cells grown in BHI broth plus Tween and NaHCO₃ was similar for all isolates (data not shown). This electrophoretic pattern, when compared to previously described reference strains of Clostridium species (Cato et al., 1982), was most similar to C. tertium.

Effect of pH on growth and acid metabolism of isolated clostridia (a combination of 8 isolates) in spent cucumber brine is given in Table 3. The spent cucumber brine was taken from the tank before the spoilage occurred. No growth of the isolates or changes in acid composition occurred at pH 3.7 or 4.1 during anaerobic incubation at 30°C for 6 days. At pH 4.8,

slight growth and a decrease in lactic and acetic acids were observed. No butyric acid was detected, although there were slight reductions in concentration of lactic and acetic acids. At pH 6 and 7, heavy growth occurred. Also, lactic and acetic acids were nearly depleted, and butyric acid had been formed. Carbon recovery as butyric acid, based on lactic and acetic acids as substrates, was 74% at pH 6 and 86.1% at pH 7.

DISCUSSION

REASONS for fermented cucumbers undergoing subsequent spoilage by butyric acid bacteria in the present case are unclear. However, two contributing factors may be the relatively low concentration of NaCl used (2.3%) and the small sized cucumbers to which soil readily adheres. The low NaCl concentration did not adversely affect the primary lactic acid fermentation, but apparently allowed growth of spoilage organisms after primary fermentation. Since clostridia were implicated in the chemical changes, the question of their origin is raised. Although the cucumbers were washed, it is likely that clostridia-bearing soil was present on the fruit.

The addition of acetic acid to the cover brine should be considered as possibly contributing to growth of clostridia in the spoiled pickles. Various species of clostridia produce butyric acid from lactic acid, but the presence of acetate was shown to be necessary for this conversion by *C. lacto-aceto-philum* (Bhat and Barker, 1947) and by *C. tyrobutyricum* (Bryant and Burkey, 1956).

While the NaCl concentration was low for commercial cucumber fermentations, the concentration was typical for sauerkraut fermentation (2 to 3% NaCl; Pederson and Albury, 1969). Although butyric acid has been found in sauerkraut (Vorbeck et al., 1961), we are unaware of any instance where it was formed with the concomitant depletion of lactic acid, as in the present case.

Butyric acid formation in brined olives has been attributed to two distinct types of microbial action, both of which result in malodorous products. In one type, *C. butyricum* and a closely related group of four other *Clostridium* species produce butyric acid from sugars during the primary stage of fermentation (Gilliland and Vaughn, 1943). From a total of 50 isolates that grew in 1% NaCl (in liver infusion agar), only one (*C. aceto-butylicum*) grew at 6% NaCl. These authors indicated that olive industry experience has shown that a concentration of 7 to 8% salt in olive brines is sufficient to prevent this type of spoilage. All isolates grew in tryptone glucose broth with sodium thioglycollate at pH 7.0, 17 grew at pH 4.5 and none grew at pH 4.3 (Gilliland and Vaughn, 1943). The broth pH was adjusted with Na_2HPO_4 and citric acid.

In a second type of malodorous olive fermentation, "zapatera" spoilage results from decomposition of organic acids at a time when little or no sugar is present and the lactic acid fermentation stops before the pH has decreased below pH 4.5 (Kawatomari and Vaughn, 1956). Lactic and acetic acids constitute the acids in normal fermentations, but butyric, propionic, formic, valeric, caprylic, and caproic acids have been reported to occur in "zapatera" olives (Delmouzos et al., 1953). Propionic and butyric acids occurred most frequently. From a total of 270 *Clostridium* isolates from "zapatera" olives, 138 were identified as *C. sporogenes* and 77 as *C. bifermentans* (Kawatomari and Vaughn, 1956). Other identified species were *C. butyricum, C. multifermentans, C. beijerinckii, C. pasteurianum,* and *C. sphenoides.* Of 39 isolates tested for salt tolerance, all grew at 1% NaCl, but only 4 grew at 8% NaCl (all were *C. sporogenes*). Of 36 isolates tested for pH tolerance, all grew at pH 6.0 but only 1 grew at pH 4.5 (*C. pasteurianum.* Kawatomari and Vaughn (1956) stated the "zapatera" spoilage can be prevented based on olive industry experience, if the brine pH is 3.8 or below. Interestingly, however, none of the above isolates from olives produced propionic acid under the conditions tested (Plastourgos and Vaughn, 1957).

Plastourgos and Vaughn (1957) isolated 68 cultures of propionic acid bacteria from commercial olives with indications of "zapatera" spoilage. Of these isolates, 46 were indentified as *Propionibacterium zeal* and 22 as *P. pentosaceum.* All isolates grew at 7% salt in lactate medium. The minimum pH tolerated by the isolates was pH 4.8 to 5.2. The maximum pH for growth was 7.2 to 8.0. These authors conjectured that propionic acid bacteria appeared first in the sequence of microorganisms responsible for the development of "zapatera," caused a rise in pH and thus permitted *Clostridium* species to grow. They cautioned against complete acceptance of this hypothesis, however, since Kawatomari and Vaughn (1956) had previously shown that many species of *Clostridium* are as tolerant to low pH as the two species of *Propionibacterium* tested.

More recently, Borbolla y Alcala et al. (1975) and Rejano Navarro et al. (1978) reported the formation of propionic acid in Sevillian olives and suggested that acidity, salt concentration and pH can be controlled to prevent its formation. Gonzalez Cancho et al. (1980) identified 26 isolates of propionic acid-producing bacteria from Spanish-style green olives as belonging to the species *Propionibacterium acnes.* At pH 7.0, 11% NaCl was required to prevent growth of these isolates; at pH 5.1, 9% NaCl was required; and at pH 3.5, no NaCl was required. Borbolla y Alcala and Rejano Navarro (1981) characterized the fermentation of Sevillian olives into four stages, with the formation of acetic and propionic acids occurring at the expense of lactic acid in the fourth phase. They suggested that the resultant rise from this conversion could encourage the spoilage problem, "zapatera." They concluded that this fourth phase can be prevented by sufficiently high salt concentration and sufficiently low pH.

In an attempt to reconstruct the sequence of microbial changes that resulted in the spoilage of fermented cucumbers, we propose the following: The cucumbers underwent a normal lactic acid fermentation with no evidence of acids other than acetic (including that added) and lactic being present after primary fermentation. Upon subsequent storage, the lactic acid was slowly degraded to propionic and acetic acids (by unidentified bacteria, possibly propionic acid bacteria) with resultant rise in pH. This rise in pH eventually allowed growth of *C. tertium* (and perhaps other clostridia).

We do not believe that clostridia directly converted lactic acid to butyric acid for two reasons. First, the isolated *C. tertium* did not grow or produce butyric acid in the spent cucumber brine at pH 4.1 and below, whereas, the pickles were originally pH 3.7. Secondly, *C. tertium* did not produce propionic and acetic acids in the spent brine when allowed to ferment at pH 4.8 and above, whereas, the spoiled pickles contained large amounts of each acid. Plastourgos and Vaughn (1957) also found that *Clostridium* isolates from "zapatera" olives did not produce propionic acid. They concluded that an intermediate propionic acid fermentation occurred. Propanol also was present in the spoiled pickles, but we did not determine whether its formation occurred during propionic acid fermentation or butyric acid fermentation.

We have been unable to duplicate the spoilage problem. The isolated *C. tertium* does not grow in the spent cucumber brine without pH adjustment. Currently, we are attempting to isolate and characterize propionic acid bacteria from fermented pickles held under low salt conditions.

Limiting conditions for microbial stability of fermented cucumbers during anaerobic storage are yet to be established. It is likely that a combination of factors are involved including salt concentration, pH, acidity, and perhaps others. We have not previously observed butyric acid spoilage of larger sized cucumbers (3.5 to 5.1 cm) that were fermented and stored at 2.7 to 4.4% salt by the procedure described herein. Our observations represent more than 20 pilot-scale and 14 commer-

cial-scale fermentations over the past 5 years. However, we have observed a rise in pH accompanied by formation of propionic acid and n-propanol during storage under laboratory conditions of a few samples from the tanks.

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An Evaluation of Marinated Southern Peas (*Vigna unguiculata*)

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ABSTRACT

Cooked southern peas (*Vigna unguiculata*) of two levels of maturities were soaked in sweet- or hot-flavored marinades. Marinade treatments and maturity level of the peas affected most attributes tested. Treated peas had less moisture, protein and nitrogen-free extract and more fat, crude fiber, ash and caloric content than unmarinated peas. Luminosity was reduced, redness was varied and yellowness was increased by marinating. Firmness of marinated peas was increased. PER was not affected and averaged 1.83. pH was lowered from 6.6 to 3.9: acidity was raised from 0.1% to 1.9%. Neither treatment nor maturity affected the microbiological counts. Samples were scored as "like slightly."

INTRODUCTION

IN RECENT YEARS there has been an increase in the number of restaurants and fast food chain outlets that offer the salad bar to their customers. In 1975, a Gallup survey was made to indicate what customers of restaurants wanted and what brought them back as customers (Anon., 1975). Almost one-half of all respondents selected the self-service salad bar as the feature preferred. Top merchandizers strive continually to add new food items to make the salad bar more interesting (Anon., 1978b). The salad bar concept has been adopted by restaurants as a means of expanding business and meeting consumer demands (Anon., 1974, 1976, 1978a).

There has been concern that fast food restaurants were not offering nutritious menu items (Appledorf, 1980). By offering an array of fresh or processed fruits and vegetables on a salad bar, restaurants could offer the consumer alternatives to traditional items being served. The southern pea (referred to hereafter as peas) is one food item that would offer variety and an excellent source of protein and other nutrients. The objectives of this experiment were to develop the peas as a salad bar item by marinating in a liquid that included vinegar, oil and spices and to determine the effect of marinades and level of maturity of the peas on the chemical, physical and sensory characteristics of the peas.

MATERIALS & METHODS

PEAS (*Vigna unguiculata*, 'California Blackeye') were grown and harvested by hand at the mature, succulent maturity on the experimental farm, Univ. of Tennessee, Knoxville, by personnel of the Plant & Soil Science Dept. 'California Blackeye' peas of the mature, dry level of maturity were purchased from a local wholesaler. Dehydrated green pepper flakes, onion powder, garlic powder, ground black pepper, salt and sugar were used to prepare the marinade. The spices were obtained from Spicecraft, Inc. (St. Louis, MO).

Processing of the peas

Mature, succulent peas were shelled with a roller-type sheller (Taylor Mfg. Co., Moultrie, GA), blanched in 87°C water for 3 min, and individually quick frozen at -35°C in an air blast freezer. The peas

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were packaged in plastic bags and stored at -17°C until used. All peas were utilized before the end of 6 months. For use, mature, dry peas were rehydrated in tap water at room temperature, and the frozen mature, succulent peas were allowed to thaw to room temperature. Peas of both maturity levels, in batches of 2721g, were placed into stainless steel blanching baskets and processed in a retort at 103.5 kPa (15 psi) at 121°C for 12 min. Water was added to the bottom of the retort below the basket of peas to allow for development of an atmosphere of water vapor. After the tenderization step, the peas were placed into plastic containers, covered with tap water of room temperature and allowed to continue rehydrating for 3 hr. Peas were drained and weighed into 907g batches. One batch from each maturity level was not marinated (control). The other batches were used for the marinade treatments.

Preparation of the marinades

Marinades of two flavors were prepared, one sweet-flavored (SF) and one hot-flavored (HF). Formulations are presented in Table 1. The ingredients were mixed together, brought to a boil, removed from the heat immediately and allowed to cool. The marinades were held in plastic containers at 7°C for 24 hr.

Marinating the peas

Tenderized, rehydrated peas were placed into plastic containers and covered with marinade. Batches of 907 g peas of both maturity levels were marinated in each of the two marinades to produce four treatments. Each treatment was replicated three times. The unmarinated and marinated samples of each maturity levels were stored in plastic containers. Marinating consisted of holding the peas in the marinade for 16 hr at 7°C. After the period of holding, the remaining liquid was drained off the peas, and both the peas and the marinades were kept separately for analyses. No effort was made to remove the particles of spices from the drained peas.

Chemical analysis

Proximate analysis was made on samples of all treatments according to AOAC (1980) methods and reported on the dry weight basis. Gross energy value was determined with a Parr Oxygen Bomb Calorimeter (model 124) (Anon., 1960). Approximately 0.8g ground freeze-dried sample was tested, and energy content was reported as kilo-Joules (kJ)/100g sample on a dry weight basis.

Physical analysis

Color of the peas, recorded as L, "a" and "b" values, was measured with a Hunter Color Meter (model D25D2M) (Anon., 1979). The meter was standardized against a beige tile (Hunter standard C2-

Table 1 — Formulations for hot and sweet marinades used to flavor southern peas

Ingredient	Sweet flavor (g)	Hot flavor (g)
Vinegar, distilled 5%	720	800
Oil, vegetable salad	208	232
Sugar	208	80
Onion powder	48	64
Green bell pepper flakes	64	48
Garlic powder	12	12
Jalapeño pepper granules	–	8.64
Salt	8	8
Black pepper, ground	4.8	4.8

21128), possessing L, "a" and "b" values of 77.0, -1.6, and 21.0, respectively. Peas were held in a cuvette with an optical glass bottom for measurement.

Firmness of 60g samples of peas was determined with an Instron machine (model 1132). The peas were placed in a Kramer shear-compression cell for measurement. A 500-kg capacity load cell was used, and the machine was operated at crosshead and chart speeds of 10 cm per min. Readings were recorded as maximum force required to shear the sample.

pH and titratable acidity measurements were made on a slurry that had been prepared by crushing 10g peas in 40 mL boiled, deionized water (AOAC, 1980). pH was measured with a Fisher Accumet pH meter, model 600. For the acidity measurement, the titration was carried to pH 8.1. Titratable acidity was calculated as acetic acid.

Microbiological examinations were made on samples of raw, unmarinated and marinated peas and on the marinades (APHA, 1984). Preparation consisted of adding 99 mL sterile 0.1% peptone buffer to 11g peas or 11 mL marinade. The peas and buffer were homogenized for 30 sec in a Stomacher (model BA 6021). The marinade and buffer were mixed by shaking. Dilutions of 10^{-1} to 10^{-4} were spread-plated for aerobic plate count on standard methods agar, for coliforms on violet red bile agar and for yeasts and molds on acidified potato dextrose agar, using approved methods of The American Public Health Association (APHA, 1984). Counts were reported as colony forming units (CFU) per g or mL sample. All media were purchased from Difco (Detroit, MI).

Protein efficiency ratio

Protein efficiency ratio (PER) was determined by the AOAC (1980) method. Sprague-Dawley albino rats weighing 40–50g with a mean weight of 45g at the beginning of the experiment were used. Rats were selected by a random numbers procedure to distribute animals of the different weights among the treatments. Ten rats were fed per treatment (diet containing peas of one of the two levels of maturity of which were either raw, unmarinated or marinated). Individual metal cages (0.8 m³) with mesh bottoms were used to house the rats. Temperature was held at 21 ± 2°C. PER for a reference diet of casein was determined and adjusted to 2.5. All diets were isocaloric at 1611 kJ/100g. Rats were weighted at 2-day intervals for 28 days.

Sensory evaluation

Marinated peas of both levels of maturity were evaluated by an untrained sensory panel of 52 members, consisting of faculty, staff and students of food-oriented departments. Testing was conducted in a laboratory designed for sensory testing, according to methods and conditions suggested by IFT (1971), Kramer (1973), and Larmond (1977). Samples were evaluated on an 8-point hedonic scale for preference with 1 = "dislike extremely" and 8 = "like extremely." Samples were served in a randomized order, illuminated with cool white fluorescent light and evaluated at mid-afternoon. Each panelist was presented one sample (peas with no liquid) at a time and instructed to evaluate the sample both alone and with lettuce. The material was expectorated after testing. Panelists used a slice of apple and water to cleanse the mouth between evaluations. Panelists were asked if they liked hot, spicy foods. Hedonic responses of panelists who stated no to the question were not included in analysis of the data. Panelists were also asked if they liked salads and if they selected foods from salad bars in restaurants.

Experimental design and data analysis

The data of each test were analyzed by analysis of variance as factorials of a completely randomized block (Little and Hills, 1978). The statistical analysis system (SAS, 1982) was used to analyze the data. Experimental models for moisture, PER, color, firmness, microbiological, and sensory tests were reduced by removing non-significant (P > 0.05) variables from the analysis and recalculated. Significance among means was determined by the Student-Newman-Kuel means separation test.

RESULTS & DISCUSSION

IN MOST CASES, the analysis of variance for the individual sets of data showed that treatment (unmarinated and two marinades) and maturity (mature, dry and mature, succulent) of peas had significant effects (P < 0.01) on the attribute under investigation. Because of these findings, treatment means will be presented within each maturity where applicable. Where maturity was not a significant factor, the fact will be stated.

Proximate composition and caloric content

Composition of previously tenderized peas was changed by soaking the peas in marinade (Table 2.) The changes may be considered, in part, the result of the apparently higher osmotic pressures of the marinades (higher than that of water) and the solubilities and absorptive capacities of the pea and marinade components. The 16-hr period of marinating obviously allowed the exchange of certain soluble substances between the peas and the marinades.

The marinade treatments reduced the amount of moisture in the peas in comparison to the unmarinated peas with one exception (Table 2). As exception, the moisture of the mature, dry peas from the HF marinade (higher vinegar and oil content) was not different from the moisture of unmarinated peas. Of the marinated treatments of SF marinade resulted in the greater loss of moisture. This finding might be the result of higher osmotic pressure of the SF marinade that was produced by the higher sugar content.

The marinades had profound effects on the protein and fat contents of the peas (Table 2). Marinated peas contained less protein and more fat than unmarinated peas. Realistically, the lower protein probably reflected a decrease in the proportional amount of protein (on dry weight basis) as the amount of fat was being increased. Peas of the SF marinade contained the smaller amount of protein. pH and isoelectric point (pI) of the proteins were likely changed by the addition of vinegar to the unmarinated peas. No tests were conducted, however, to determine the effect on protein solubility or absorption of ingredients from the marinade. Changes in fat, however, were not as consistent as changes for protein. No difference in fat (mean = 12.8%) was found between peas of the two marinade treatments for mature, succulent peas, but for the mature, dry group, peas of the HF marinade contained the larger amount of fat (15.0% vs 12.2% for peas of SF marinade).

Three of the four marinade treatments yielded peas that contained a higher ash than unmarinated peas (Table 2). The exceptional treatment consisted of mature, dry peas of the SF marinade which contained less ash than the unmarinated peas. It is possible that the capacity of mature, dry peas for binding minerals was less than that of the mature, succulent peas. The nutrient minerals of peas (southern) include important amounts of iron, calcium, magnesium, phosphorus and potassium (USDA, 1984).

A greater amount of crude fiber was found in peas of the HF marinade of both maturities than was found in unmarinated peas (Table 2). This finding was most likely due to the presence of jalapeño pepper and the greater amount of onion powder in the HF marinade. Reportedly, onion powder and jalapeño pepper contain a mean 4.8 and 22.8% crude fiber (dry weight basis), respectively (USDA, 1984). Peas of the SF marinade had an intermediate amount of crude fiber that was not different from that of either of the two other treatments. The magnitude of the experimental values was lower than the 6.31 mg/100g reported for cooked, boiled, drained peas (USDA, 1984).

The NFE content of peas was varied due to treatment effects (Table 2). Peas marinated in HF marinade had a mean 7.4% less NFE than peas of the SF marinade or unmarinated peas, among which no differences were found. The reported value for carbohydrate of similar peas was 64.4% (calculated, dry wt. basis) (USDA, 1984).

The caloric content of peas was increased by marinating but not affected by maturity. Therefore, overall means of each treatment (maturities combined) are presented (Table 2). Peas held in HF marinade had a 2.4% greater caloric content than peas held in the SF marinade. The higher percentage of fat in

Table 2—Proximate composition and caloric content of marinated southern peas

Maturity	Treatment	Moisture* g/100g	Protein**	Fat*	Ash**	Crude fiber**	NFE**	Treatment	Content*** kJ/100g (dry wt basis)
					g/100g (dry wt basis)				
Mature, dry	Unmarinated	66.1a	26.2a	0.9c	2.7b	3.3b	66.9a	Unmarinated	1892f
	Sweet flavor marinade	56.0b	15.8c	12.2b	2.5c	3.4ab	66.1e	Sweet flavor marinade	2022e
	Hot flavor marinade	61.7ab	17.2b	15.0a	2.9a	3.8a	61.1b	Hot flavor marinade	2070d
Mature, succulent	Unmarinated	69.9a	25.3a	0.9b	2.3c	3.6b	67.9a		
	Sweet flavor marinade	57.8c	13.9c	12.5a	2.4b	3.7ab	67.5a		
	Hot flavor marinade	62.5b	16.4b	13.0a	2.9a	4.0a	63.7b		

* N = 9.
** N = 6; NFE = nitrogen-free extract.
*** N = 12; kJ = kiloJoules; kJ 4.184 = kCal.; maturity of peas was not significant.
a-c Means within a column and maturity not sharing the same superscript are different (P<0.01).
d-f Mean not sharing the same superscript are different (P<0.01).

the HF marinade is believed to have contributed to the higher caloric content of the peas. The latter group had a 6.9% greater caloric content than unmarinated peas. Published values indicated that cooked, boiled, drained peas (similar to peas used in this experiment) contained 1611 kj/100g (USDA, 1984).

Color

Soaking the peas in marinade caused small, but significant, changes in all the Hunter color values, with one exception (Table 3). The exception was found for mature, dry peas in which the HF marinade did not change luminosity (L) from that of the unmarinated peas. Realizing the exception, the marinades lowered luminosity and increased yellowness ("b"). Changes in redness ("a") were less systematic; redness was reduced for all marinated treatments of mature, dry peas but was increased for all marinade treatments of mature, succulent peas. Because of the relatively low L and "a" values and the fairly high "b" values (yellowness), one may describe the visual appearance of peas of all treatments as dull gray with a tinge of yellow.

Firmness

Firmness of peas treated in the marinades was greater than firmness of unmarinated peas (Table 4). The SF marinade produced the firmest peas with values averaging 1.5 times greater than values for firmness of unmarinated peas. The HF marinade produced peas with firmness values an average 1.2 times greater than firmness values of unmarinated peas. As a group, mature, dry peas were 1.4 times firmer than mature, succulent peas. All the firmness values of mature, succulent peas were either lower than or similar to the firmness value of unmarinated mature, dry peas. This finding suggested that the greater firmness of marinated mature, dry peas might lower consumer acceptance.

Table 3—Hunter color values of marinated southern peas

Maturity	Marinade treatment	L (luminosity)	"a" (redness)	"b" (yellowness)
Mature, dry	Unmarinated	36.6a	4.7a	10.9b
	Sweet flavor	33.9b	3.6b	14.1a
	Hot flavor	36.5a	3.2b	14.6a
Mature, succulent	Unmarinated	39.6a	1.0b	11.6b
	Sweet flavor	34.5c	1.8a	14.8a
	Hot flavor	37.5b	2.4a	15.3a

* N = 9.
a-c Means within a column and maturity not sharing the same superscript are different (P<0.01).

Table 4—Firmness measurement of marinated southern peas

Maturity	Marinade treatment	Firmness* (kg force/60g)
Mature, dry	Unmarinated	41.4c
	Sweet flavor	62.8a
	Hot flavor	49.9b
Mature succulent	Unmarinated	28.2c
	Sweet flavor	43.9a
	Hot flavor	35.3b

* N = 9.
a-c Means within a maturity not sharing the same superscript are different (P<0.01).

Attempts were made to reduce firmness of the marinated peas by tenderizing the raw peas further with heat. In such attempts the peas became very soft, even to the point of becoming mushy and losing their structural integrity. Since a processor could not handle the softened peas satisfactorily, further tenderization was not attempted.

Flora (1980) found that crowder peas (a type of southern pea) became firmer when acidified with citric acid, a phenomenon he attributed to protein denaturation and decreased hydration of proteins and starches. Turner (1981) reported that peas treated in acetic acid solution were firmer than unacidified peas.

Protein efficiency ratio

No difference in PER was found among peas of the three treatments and between peas of the two maturities. The mean PER of 1.83 is similar to the PER of 1.86 reported by Molina et al. (1976) for a protein concentrate of peas. Proteins of peas possess limiting amounts of the sulfur-containing amino acids, cysteine and methionine (Elias et al., 1964; Evans and Boulter, 1974; Khan et al., 1979; Lee, 1985). This condition contributed to the experimental PER. However, peas can contribute valuable amino acids to the diet, a contribution made more significant when the peas are consumed with other foods which supply larger proportions of the sulfur-containing amino acids.

pH and titratable acidity

As expected, pH of the marinated peas was lowered considerably from that of the unmarinated peas (Table 5). Changes in pH, however, were not similar between the two maturities. Mature, dry peas that were soaked in HF marinade developed a slightly lower pH than peas of the SF marinade treatment; whereas, no difference in pH was found between the two marinade treatments of the mature, succulent peas.

Titratable acidity of the marinated peas was increased from 16.8-fold for the mature, dry peas to 20-fold for the mature,

Maturity	Marinade treatment	Peas, marinated		Marinade	
		pH[*]	Acidity[*] (g acetic acid/100g)	pH[*]	Acidity[*] (g acetic acid/100g)
Mature, dry	Unmarinated	6.6[a]	0.1[c]		
	Sweet flavor	4.0[b]	1.7[b]	3.9[a]	1.8[e]
	Hot flavor	3.9[c]	2.0[a]	3.9[a]	2.0[d]
Mature, succulent	Unmarinated	6.3[a]	0.1[c]		
	Sweet flavor	3.9[b]	1.8[b]	3.9[a]	1.8[b]
	Hot flavor	3.8[b]	2.0[a]	3.9[a]	2.1[a]

[*] N = 6
[a-c] Means within a column and maturity not sharing the same superscript are different (P<0.01)
[d-e] Means not sharing the same superscript are different (P<0.05).

succulent peas over that of unmarinated peas (Table 5). Of the marinated treatments, acidity was higher for peas of the HF marinade. The correlation coefficient between pH and acidity was −0.993 for each maturity.

pH and acidity values of the used marinades are presented in Table 5. pH values did not differ between the marinades within a maturity level. Acidity and amount of vinegar, however, were greater for the HF marinades of both maturity levels. pH values between peas and marinades were similar, indicating that equilibrium had been reached.

Microbiology

Neither marinade treatment nor level of maturity had an effect on the microbiological counts of the peas or marinades. Mean counts (CFU/g) of peas of the two marinade treatments were log 2.69/g for total plate count, log 3.2/g for coliforms and log 3.9/g for yeasts. An estimated mold count of 10/g was found. The used marinades had mean counts of log 3.1 CFU/mL for total plate count, log 2.9/mL for coliforms and log 3.7/mL for yeasts. No molds were found on samples of marinade. Only the onion powder ingredient of the marinades contained detectable microorganisms. Onion powder had coliform counts of log 2.7/g.

Coliform analysis was performed to provide an indication of sanitation and handling of the peas and ingredients since samples were evaluated by a sensory panel. From the magnitude of the coliform counts obtained, it is evident that the processing of such products will require a rigid program of sanitation to assure their safety and shelflife. The low pH did not destroy coliforms which were added with onion powder. Therefore, sources of possible contamination, such as spices, should be monitored, and specifications for ingredients should be established for this product. Heating the freshly prepared mixture of peas and marinade to approximately 70°C should destroy vegetative bacterial cells and most yeasts.

Sensory evaluation

The sensory panelists (N = 39) who stated they liked hot, spicy foods indicated no preference for peas of the two marinade treatments or the two levels of maturity (Table 6). The mean hedonic scores indicated that the panelists expressed a "like slightly" preference for the samples. Scores of this magnitude suggested that the product was liked only a small degree. Such a conclusion may be accepted with some degree of

reservation. A panel that consists of students and faculty of food science may be overly critical in its assessment of sample quality and may tend to assign lower scores than anticipated. Similar panels in this laboratory have assigned scores in the median portion of the scale, even though the samples being evaluated were commercial products that rated highly with the consuming public (Penfield, 1988). Another point may be made regarding acceptability of acidified products. When salad bar items are selected by the diner, a relatively small amount of a particular food item may be taken. It is not unusual for several items to become mixed on the plate, and, consequently, the flavor of a particular food will become part of the mixture of the flavor of all the items. When served under similar conditions, marinated peas might receive higher scores for preference. Of the original panel of 52 members, 96% of the panelists indicated they liked salads, and 92% indicated they selected items from salad bars in restaurants.

SUMMARY & CONCLUSIONS

MARINADE TREATMENT of the southern peas produced significant changes, including compositional, physical and chemical. Maturity of the peas affected most of the attributes tested. The proportional amount of protein in marinated peas was lowered by 34 to 45% as the percentage of fat was being increased more than 14 fold. Along with the fat increase was a concomitant increase in caloric content. Maximum caloric content of peas treated in the HF marinade was 8.6% higher than that of unmarinated peas. Marinades caused slight color changes but large firmness increases. Mature, dry peas were much firmer than mature, succulent peas.

The microbiological counts of the peas indicated that careful attention should be given to prevent contamination of the pea-marinade mixture by either ingredients or by mishandling. Also, the heating of the freshly prepared mixture to approximately 70°C should destroy the vegetative bacteria cells and most yeasts.

Even though the peas received a mean preference score of 5.1 (like slightly), discussion was presented to suggest that this product would be suitable as a salad bar item. The use of marinade to impart flavor to the bland tasting peas was a satisfactory means of preparing peas as a food item. The characteristics of this product should permit its preparation at a central location and distribution to area restaurants for use as a salad bar item.

Table 6 — Preference scores for marinated southern peas

Marinade treatment	Score[*]	Maturity	Score[*]
Sweet flavor	5.3[a]	Mature, dry	5.0[a]
Hot flavor	4.9[a]	Mature, succulent	5.1[a]

[*] N = 39. Scale: 1 = dislike extremely, 4 = dislike slightly, 5 = like slightly, 8 = like extremely
[a] Means within a column are not different (P>0.05)

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Purification and Characterization of Peroxidase Isoenzymes from Green Peas (*Pisum sativum*)

B. HALPIN, R. PRESSEY, J. JEN, and N. MONDY

—————————————— ABSTRACT ——————————————

Peroxidase isoenzymes were purified from green peas with ion-exchange chromatography on DEAE- and S-Sepharose. Three isoenzymes were identified, one neutral (N) and two cationic (C1, C2). N was extremely heat labile, with 50% original activity lost after heating 1.5 min at 25°C. N had Km values (pH 5.0) of 10.2 mM and 2.6 mM for guaiacol and H_2O_2, respectively. C1 and C2 retained activity on heating at 30–70°C. C1 was able to reactivate after thermal inactivation. Km values for guaiacol/H_2O_2 were 10.8 mM/7.2 mM (pH 5.0) and 10.8 mM/4.3 mM (pH 6.0) for C1 and C2, respectively. The three isoenzymes exhibited different peroxidase activities with different H-donors, different sensitivities to cyanide and different abilities to catalyze oxidation of indoleacetic acid.

INTRODUCTION

PEROXIDASE (donor: hydrogen peroxide oxidoreductase, EC 1.11.1.7) appears to be widely distributed in the plant kingdom, having been identified in all higher plants that have been investigated. Peroxidase has traditionally been related to food quality in processing due to its thermal stability (Burnette, 1977; Reed, 1975). The heat inactivation of peroxidase has been shown to be a biphasic process presumably due to the existence of isoenzymes with differing heat stabilities (McLellan and Robinson, 1981; Adams, 1978; Yamamotc et al., 1962).

Conventional blanch processing of vegetables using destruction of peroxidase activity as an indication of adequate blanch has come under increasing scrutiny (Halpin and Lee, 1987; Williams et al., 1986; Böttcher, 1975). Böttcher (1975) noted that peroxidase activity has not been shown to be directly responsible for quality deterioration during frozen storage of vegetables. Work with green peas blanched for various time/temperature combinations and stored at -23°C indicated that peroxidase activity did not correspond with quality under ideal conditions of blanching and frozen storage (Halpin, 1988; Halpin and Lee, 1987). However, other enzymes evaluated as blanch indicators: polyphenol oxidase, lipoxygenase and catalase, were not more appropriate as blanch indicators and peroxidase will therefore continue to serve in this capacity (Halpin, 1988). Thus it is important to study the native peroxidase enzyme in order to gain information which may be useful in further evaluating the enzyme as a blanch indicator. The objective of this study was to purify peroxidase isoenzymes from green peas and to study thermal stability and some kinetic properties of these isoenzymes.

MATERIALS & METHODS

Raw product

Fresh green peas (20 kg) (*Pisum sativum* var. Valero) tenderometer 103, were obtained from a local processing plant in upstate New York

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the morning of harvest in June 1987. The peas were transported to the pilot plant, cleaned, sorted to remove dirt and stones, rinsed with water, drained and frozen on trays at -40°C. Frozen peas were packaged in plastic bags in a cardboard box for storage at -40°C until needed for enzyme purification.

Reagents

Tris (Tris (hydroxymethyl)-aminomethane), polyvinylpyrrolidone (PVP), phenylmethylsulfonylfluoride (PMSF), MES (2-[N-morpholino] ethanesulfonic acid), guaiacol, o-dianisidine, pyrogallol and bovine serum albumin protein standard were from Sigma Chemical Co. Indoleacetic acid was from Kodak Co. and hydrogen peroxide solution (30% w/v) was from Mallinkrodt. Column packing materials DEAE-Sepharose and S-Sepharose were from Pharmacia Inc.

All other compounds were reagent grade and distilled deionized water was used in all experiments.

Peroxidase activity and determination of protein content

Peroxidase activity was determined spectrophotometrically as the change in absorbance at 470 nm as described by Thomas et al. (1981) with some modifications. An aliquot of sample (1-100 μL) was added to 2.5 mL substrate (0.1M K phosphate buffer, pH 6.0, containing 32 mM guaiacol and 8 mM H_2O_2) and the change in absorbance at 470 nm monitored for 3 min at 21°C. One unit of peroxidase activity was defined as a change of 0.1 absorbance unit per min/ mL.

The amount of protein was determined based on the dye-binding method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

Separation and purification

Unless otherwise noted, all procedures were carried out at 4°C. Green peas (125 g) were removed from frozen storage and blended for 3 min in 250 mL chilled 0.02M Tris-HCl buffer, pH 7.8, containing 1.0 M NaCl and 2% (w/v) PVP. The blended slurry was homogenized for 1 min using a Polytron homogenizer and 0.02% (w/v) PMSF, solubilized in a minimal volume of methanol, was added. The pea slurry was stirred for 30 min followed by centrifugation at 8,000g for 30 min. The supernatant obtained on centrifugation was assayed and found to contain >98% of the peroxidase activity.

The crude extract was concentrated by ultrafiltration using an Amicon model 2000 stirred cell with a PM10 membrane to a volume of approximately 60 mL under N_2 at 20 psig. The concentrated sample was dialyzed overnight versus 4L 0.05M Tris-HCl, pH 7.8, with one change of dialysis buffer. The dialyzed sample was centrifuged at 20,000g for 20 min to remove precipitate and the supernatant was applied to a 5 x 12.5 cm column of DEAE-Sepharose previously equilibrated with 0.05M Tris-HCl, pH 7.8. The column was washed with 100 mL 0.05M Tris-HCl (pH 7.8) followed by elution with a 1-L linear gradient of 0-0.5M NaCl in 0.05M Tris-HCl, pH 7.8. Fractions eluting from the column (220 drops, approximately 11.2 mL) were monitored by absorbance at 280 nm and tested for peroxidase activity as follows: a 100 μL aliquot of column fraction was added to 1 mL peroxidase substrate (32 mM guaiacol and 8 mM H_2O_2 in 0.1M K phosphate, pH 6.0). Fractions producing the characteristic red-brown color reaction of active peroxidase were assayed for activity spectrophotometrically at 470 nm by the method of Thomas et al. (1981).

Three peaks of peroxidase activity eluted from the DEAE column, one in the wash volume and two others early in the salt gradient. Fractions comprising each active peak were separately pooled, concentrated under 10 psig N_2 using an Amicon cell with PM10 mem-

brane to approximately 20 mL and dialyzed versus 2 L 0.02M MES (pH 6.0) overnight. The dialyzed samples were centrifuged at 20,000g for 20 min and the supernatant of each was applied separately to an S-Sepharose cation exchange column (2.5 x 14.5 cm) preequilibrated with 0.02M MES, pH 6.0. The column was washed with 40 mL 0.02M MES (pH 6.0) and elution continued with a 400 mL linear gradient of 0-0.5M NaCl in 0.02M MES, pH 6.0. Fractions (220 drops, approximately 11.2 mL) were monitored by absorbance at 280 nm and tested for peroxidase activity. Active fractions were assayed spectrophotometrically for peroxidase activity. Each of the three peaks of peroxidase activity which eluted from the DEAE-Sepharose column corresponded with a single active peroxidase peak on chromatography on S-Sepharose. Each active peak was pooled, concentrated to approximately 5 mL using an Amicon cell with PM10 membrane and stored at -23°C. Samples retained peroxidase activity for at least 6 months under these storage conditions.

Isoelectric focusing

Isoelectric focusing (IEF) was performed using the PhastGel system (Pharmacia Inc.) with a homogeneous polyacrylamide gel and Pharmalyte carrier ampholytes. Two μL aliquots of each peroxidase peak were loaded, using sample applicators, at anode, middle and cathode areas of a PhastGel IEF 3-9. The programmed method separated proteins at 15°C in a run time of approximately 30 min. The IEF gel was stained for peroxidase activity using o-dianisidine as described by McLellan and Robinson (1987a) followed by silver staining as programmed into the PhastGel system. Calibration proteins for pI measurement and their corresponding pI values were: lentil lectin (basic) 8.65, lentil lectin (middle) 8.45, lentil lectin (acidic) 8.15, horse myoglobin (basic) 7.35, horse myoglobin (acidic) 6.85, human carbonic anhydrase B 6.55, bovine carbonic andyrase B 5.85, β-lactoglobulin A 5.20, soybean trypsin inhibitor 4.55, and amyloglucosidase 3.50.

Kinetic studies

Guaiacol as H-donor. The peroxidase assay of Thomas et al. (1981) as previously described was the basis for kinetic studies. For pH studies, 0.05 M Na phosphate, pH 3.0-7.0, and 0.05 M Tris-HCl, pH 7.0-10.0, were employed at 8 mM H_2O_2 and 40 mM guaiacol. For determination of Km values, H_2O_2 concentrations from 0.4 to 80 mM were used at optimum guaiacol concentration in 0.05 M Na phosphate at optimum pH. Concentrations of guaiacol ranging from 1 to 80 mM were employed at optimum H_2O_2 concentration. Lineweaver-Burk plots were prepared to determine Km values for guaiacol (H-donor) and H_2O_2 (substrate).

Pyrogallol as H-donor. Assays with pyrogallol as H-donor were based on the procedure of Jen et al., (1980). For pH studies, 0.05 M Na phosphate, pH 3.0-7.0, and 0.05 M Tris-HCl, pH 7.0-10.0, were employed at 10 mM pyrogallol and 2 mM H_2O_2. For determination of Km values, H_2O_2 concentrations from 0.1 to 100 mM were used at optimum pyrogallol concentration in 0.05 M Na phosphate, pH 6.5 (N), or 0.05 M Tris-HCl, pH 7.0 (C1, C2). Concentrations of pyrogallol ranging from 0.1 to 100 mM were employed at optimum H_2O_2 concentration. Lineweaver-Burk plots were prepared to determine Km values for pyrogallol as H-donor and H_2O_2 as substrate.

Cyanide inhibition. Each isoenzyme was analyzed separately for its sensitivity to cyanide in 0.05 M Na phosphate at optimum pH with 32 mM guaiacol and 8 mM H_2O_2. An aliquot of isoenzyme was added to 2.5 mL 0.05 M Na phosphate containing 32 mM guaiacol. Next, 50 μL KCN was added to a final concentration of 0.08 to 1.62 μM

(N, C1) or 0.8 to 162 μM (C2) and after incubation for 1 min, H_2O_2 was added and change in absorbance at 470 nm measured over 3 min.

IAA oxidase activity. Indoleacetic acid oxidase (IAA oxidase) activity of each isoenzyme was measured using a variation of the procedure of Huang and Haard (1977). An aliquot of isoenzyme (0.1 mL) was added to the reaction mixture to a final volume of 2.0 mL. The pH of the reaction mixture was varied from 3.5 to 7.0. The reaction mixture was shaken in a water bath for 15 min at 20°C, 2.0 mL Salkowski reagent was added, the mixture was stored in the dark for 30 min, and absorbance at 525 nm was recorded. The amount of IAA remaining in the reaction mixture was calculated from a standard curve for 0.05 to 1.0 mM IAA.

Thermal stability. Thermal stability of each peroxidase isoenzyme was evaluated as follows: an aliquot of enzyme was added to 0.5 mL 0.1 M Na phosphate (pH 6.0) in a 12x100 mm test tube. Test tubes were placed in a shaking water bath with slight agitation and heated for a designated time at a set temperature. Heated samples were cooled immediately in ice water and assayed for peroxidase activity by addition of 2.0 mL buffered substrate (32 mM guaiacol and 8 mM H_2O_2 in 0.1 M Na phosphate, pH 6.0) and change in absorbance at 470 nm measured over 3 min. Thermal stability of the crude extract was also evaluated.

Reactivation. The ability of the major isoenzyme, C1, to reactivate after heating was established. Five sets of aliquots of C1 in 0.5 mL 0.1M K phosphate (pH 6.0), were heated in 12x100 mm test tubes in a shaking water bath. Samples were heated for 1, 2, 5, or 10 min at 50°, 60° or 70°C. Heated samples were cooled immediately and one set was assayed for activity as time-zero sample. The four remaining sets were incubated in a water bath at 25°C and assayed for activity after 1, 2, 4 or 14 hr. Enzyme assays were performed as directed for thermal stability studies using guaiacol as H-donor.

RESULTS & DISCUSSION

Separation and purification of isoenzymes

The procedure used to purify the three peroxidase isoenzymes from green peas is summarized in Table 1. Results presented are representative of four separate purification trials. A large amount of 280 nm absorbing material was removed using the DEAE-Sepharose column (Fig. 1) and this is reflected in the large increase in specific activity of each of the 3 active peroxidase fractions. Peroxidase eluted from the anion exchange column either in the column wash (fractions 12-15) or early in the gradient (fractions 16-23 and 27-37). Each of the three active fractions was pooled, concentrated and applied separately to an S-Sepharose cation exchange column.

Further purification of each peak was achieved using the S-Sepharose column. The neutral fraction, N, did not bind to either the DEAE- or S-Sepharose columns but eluted with the wash buffer (Fig. 2). The other two peroxidase isoenzymes bound to the S-Sepharose cation exchange resin and were eluted with increasing concentrations of salt. The peroxidase peak eluting first from the S-Sepharose column at 0.16 M salt was labelled C1 (Fig. 3). C1 was the major isoenzyme in terms of total activity recovered from the crude extract. C2 eluted from the S-Sepharose column at 0.28M salt (Fig. 4) and was the least purified due to a loss of activity in this fraction from the cation exchange column. It appears that fraction 27-37 from the DEAE column also contained some activity representative of N and C1, as each of these isoenzyme activities eluted in

Table 1—Summary of the purification of green pea peroxidases

Sample	Vol.(mL)	Protein (mg/mL)	Total protein (mg)	Activity (units/mL)	Total activity	Specific activity	Fold purif.	% yield
Crude extract	298	9.68	2885	631	188,038	65	1.0	100
Ultrafil. dialyzed	64	28.2	1805	2366	151,424	84	1.3	91
DEAE-Seph								
12-15 (N)	21	0.138	2.91	736	15,467	5318	82	8
16-23 (C1)	17.4	0.71	12.4	1670	29,058	2343	36	15
27-37 (C2)	19.4	1.31	25.4	746	14,472	569	8.8	8
S-Seph.								
3- 4 (N)	6.1	0.008	0.05	91.2	556	11,393	175	0.3
14-16 (C1)	6.0	0.103	0.62	2329	13,973	22,610	348	7
21.25 (C2)	7.9	0.045	0.35	202	1593	4481	69	0.8
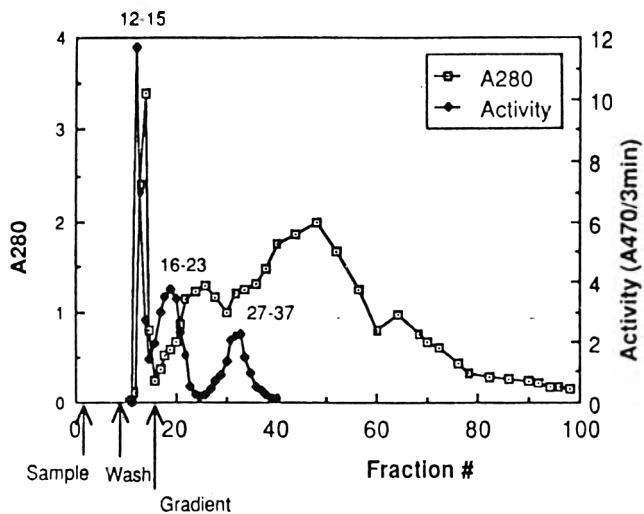
Fig. 1.—Separation of green pea peroxidase on DEAE-Sepharose.
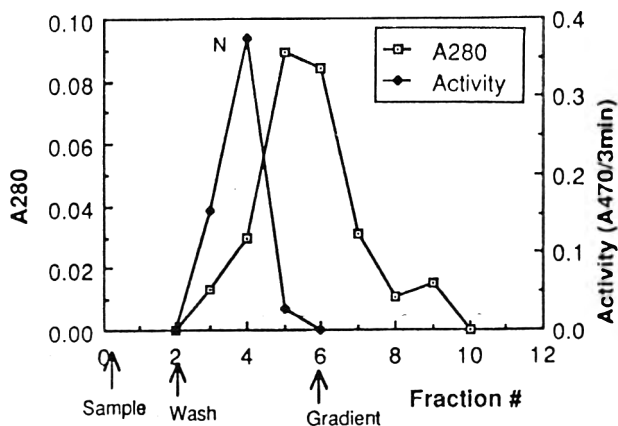
Fig. 2.—Chromatography of DEAE fractions 12–15 on S-Sepharose.
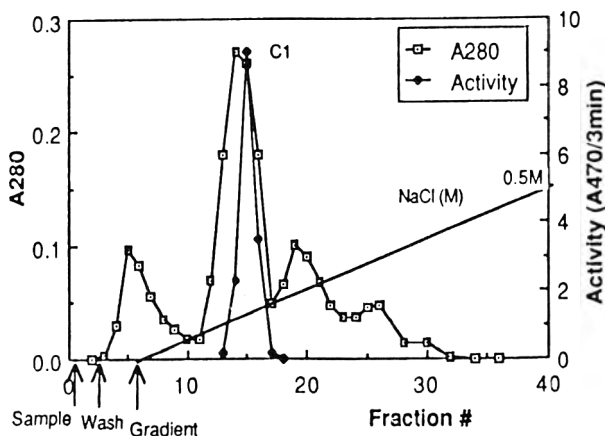
Fig. 3.—Chromatography of DEAE fractions 16–23 on S-Sepharose.

Fig. 4.—Chromatography of DEAE fractions 27–37 on S-Sepharose.
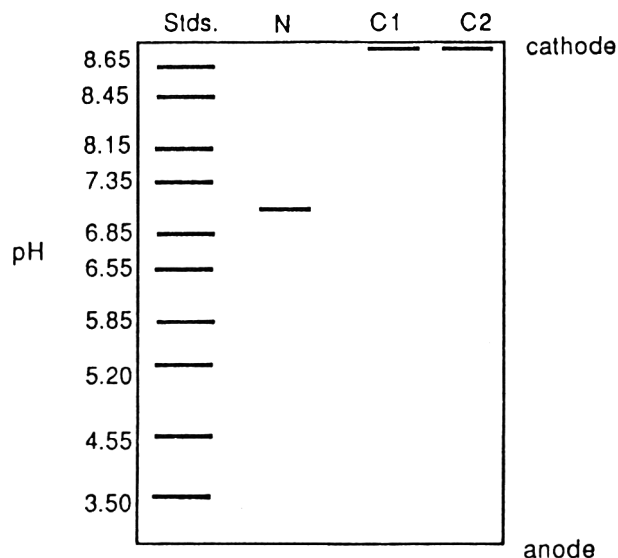
Fig. 5.—Isoelectric focusing pH 3–9 of green pea peroxidase isoenzymes N, C1 and C2

Isoelectric focusing

When subjected to isoelectric focusing at pH 3-9, each of the three isoenzyme fractions separated on the S-Sepharose column appeared as a single band. N migrated to pH 7, while C1 and C2 migrated to the cathode end of the gel indicating a pI value of 9.0 or higher (Fig. 5) Isoelectric points of peroxidases purified from various sources have been established. Acidic perosidases have been identified in turnip root, pI 3.3–3.7 (Welinder and Mazza, 1975); tomato, pI 2-4 (Heidrich et al., 1983); and Brussels sprouts and cabbage, pI 3.7–4.8 (McLellan and Robinson, 1987b; 1983). Neutral isoenzymes have been identified in sweet potato seedlings, pI 6-7.8 (Floris et al., 1984); horseradish, pI 7-9 (Heidrich et al., 1983); and Brussels sprouts and cabbage, pI 5.2-6.2 (McLellan and Robinson, 1987b; 1983); while basic isoperoxidases have been found in turnip root, pI 11.6 (Welinder and Mazza, 1975) and Brussels sprouts and cabbage, pI 8-10 (McLellan and Robinson, 1987b 1983).

addition to C2 from the cation exchange resin. The large amount of protein applied to the DEAE column (> 1g) may have prevented some specific binding to and release from the anion exchange resin, preventing separation from occuring until the cation exchange column. Alternately, charge interconversion of the peak material may have occurred. This would account, at least in part, for the small amount of activity recovered in the C2 isoenzyme fraction.

Characterization with guaiacol

The peroxidase enzyme is specific for H_2O_2 as substrate but operates with a number of H-donor molecules. Each peroxidase isoenzyme was characterized using guaiacol as H-donor and H_2O_2 as substrate. The pH optima for activity were 5.0, 6.0 and 5.0 for N C1 and C2, respectively (Fig. 6). These corresponded well with optimum pH values values for perox-
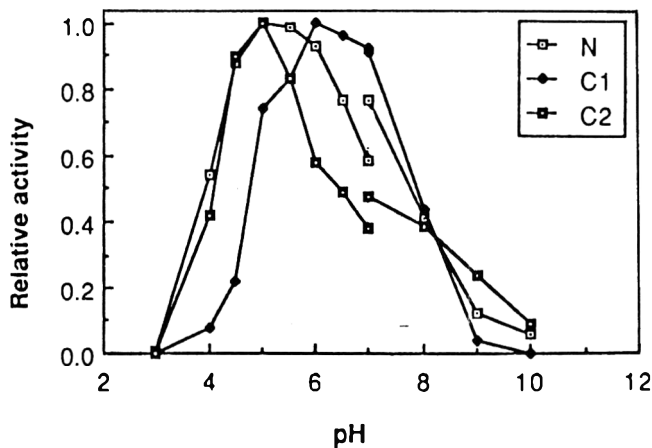
Fig. 6.—pH optima for green pea peroxidase isoenzymes (N, C1 and C2) with guaiacol as H-donor.

Table 2—Km values of peroxidase isoenzymes.

Isoenzyme	Guaiacol (mM)	H_2O_2 (mM)	Pryogallol (mM)	H_2O_2 (mM)
N	10.2	2.6	4.2	2.6
C1	10.8	7.2	6.2	2.8
C2	10.2	4.3	2.0	3.0

idase in soybean pH 5.5 (Sessa and Anderson, 1981); tomato pH 5.3-5.5 (Heidrich et al., 1983; Jen et al., 1980); cauliflower pH 6.5 (Lee and Pennesi, 1984) and sweet potato seedling pH 5.75 (Floris et al., 1984), but were lower than values reported for pea roots pH 6.9 (Fielding and Hall, 1978) and pea seedlings pH 7.7 (Macnicol, 1966).

Apparent Km values from concentration studies with guaiacol as H-donor and H_2O_2 substrate given in Table 2. The three isoenzymes exhibited affinities for guaiacol lower than values reported for pea roots 5 mM (Fielding and Hall, 1978); soybean 5.9 mM (Sessa and Anderson, 1981) and tomato 5-10 mM (Heidrich et al., 1983; Jen et al., 1980; Evans, 1970). Km values for H_2O_2 were, in general, higher than those previously reported in other vegetables: pea roots 0.3 mM (Fielding and Hall, 1978); soybean 0.58 mM (Sessa and Anderson, 1981); and tomato 0.4-0.8 mM (Heidrich et al., 1983; Evans, 1970); and compared well only with the value of 4 mM for tomato peroxidase reported by Jen et al. (1980). C1 was more tolerant of the H_2O_2 substate than the other two isoenzymes and peroxidases from other sources. In general, results suggest that green pea peroxidases possess a greater tolerance for guaiacol and H_2O_2 than noted in other fruits and vegetables. Lineweaver-Burk plots revealed inhibition by H_2O_2 above 24 mM for C2 and 32 mM for N and C1 while guaiacol was inhibitory above 24 mM for C2 and above 40 mM for N and C1.

Characterization with pyrogallol

The three isoenzymes were also characterized by their ability to react with pyrogallol, a naturally occurring phenolic compound, as H-donor. The pH optima for activity with pyrogallol were 6.5, 7.0 (Tris), and 8.0, for N, C1 and C2, respectively (Fig. 7). The pH optima with pyrogallol were sharper than observed with guaiacol and there was wider variation among isoenzymes. Values, however, are similar are similar to those observed for sweet potato seedling pH 7.0 (Floris et al., 1984), and tomato pH 7.5 (Jen et al., 1980; Evans, 1970).

Apparent Km values from concentration studies with pyrogallol as H-donor and H_2O_2 are given in Table 2. The range of Km values for green pea peroxidases is narrower than that reported for tomato fruit 0.5 mM (Evans, 1970) and 10 mM (Jen et al., 1980), indicating a more similar donor affinity among pea isoperoxidases. Apparent Km values for H_2O_2 of

N, C1, and C2 were higher than values of 0.2 and 0.8 mM reported for tomato by Evans (1970) and Jen et al. (1980), respectively. Lineweaver-Burk plots revealed inhibition by H_2O_2 above 50 mM for C1 and 25 mM for N and C2, and by pyrogallol above 100 mM for C1 and C2 and 50 mM for N. Working with roots of green pea seedlings, Fielding and Hall (1978) suggested the possibility that peroxidase isoenzymes perform multiple functions within a cell and as such employ different H-donors. They suggested that multiple active sites on peroxidase enzymes would account for the differences in affinity observed.

Fig. 7.—pH optima for green pea peroxidase isoenzymes (N, C1 and C2) with pyrogallol as H-donor.

Cyanide inhibition

Cyanide inhibits many of the enzymes that contain iron as an essential part of their catalytic mechanism. Competitive cyanide inhibition has been observed for peroxidase from sweet potato seedling (Floris et al., 1984); tomato (Heidrich et al., 1983); soybean and horseradish (Sessa and Anderson, 1981) with Ki values of 0.5 μM, 1.0 μM, 0.15μM and 3.0 μM, respectively.

The three peroxidase isoenzyme reacted differently to treatment with cyanide. Both N and C1 were very sensitive to CN with inhibition above 0.08 μM KCN and calculated Ki values of 1.5 μM, while C2 was more tolerant and exhibited sensitivity to CN only at concentrations above 0.8 μM and with a calculated Ki value of 5.0 μM.

IAA oxidase activity

Indoleacetic acid (IAA) is plant hormone produced in meristematic areas that affects cell growth. It may also function as a naturally occurring ripening retardant. Peroxidase enzymes have been shown to possess IAA oxidase activity and Macnicol (1966) suggested that peroxidase is the major enzyme responsible for IAA oxidase activity in plant extracts, although the relationship between the two enzyme activities in situ has not been conclusively established. The pH optima for the catalysis of IAA oxidation were determined for green pea peroxidases. C1 exhibited IAA oxidase activity over a very broad pH range with an optimum at pH 5.0, while N and C2 had sharper optima at pH 5.5 and the activities dropped rapidly as the pH increased above the optima. Macnicol (1966) had noted similar pH optima for IAA oxidase activity of 4 green pea seedling peroxidase isoenzymes, one neutral (N) and 3 basic (C_1, C_2, C_3), operating maximally at pH 5.5, 4.9, 5.2 and 4.9, respectively. Huang and Haard (1977) noted a very narrow pH range with an optimum at pH 6.1 for IAA oxidase activity in tomato fruit. Chibbar and van Huystee (1984) noted much lower pH optimum for IAA oxidase activity of peanut, pH 3.6 for both anionic and cationic peroxidase isoenzymes.
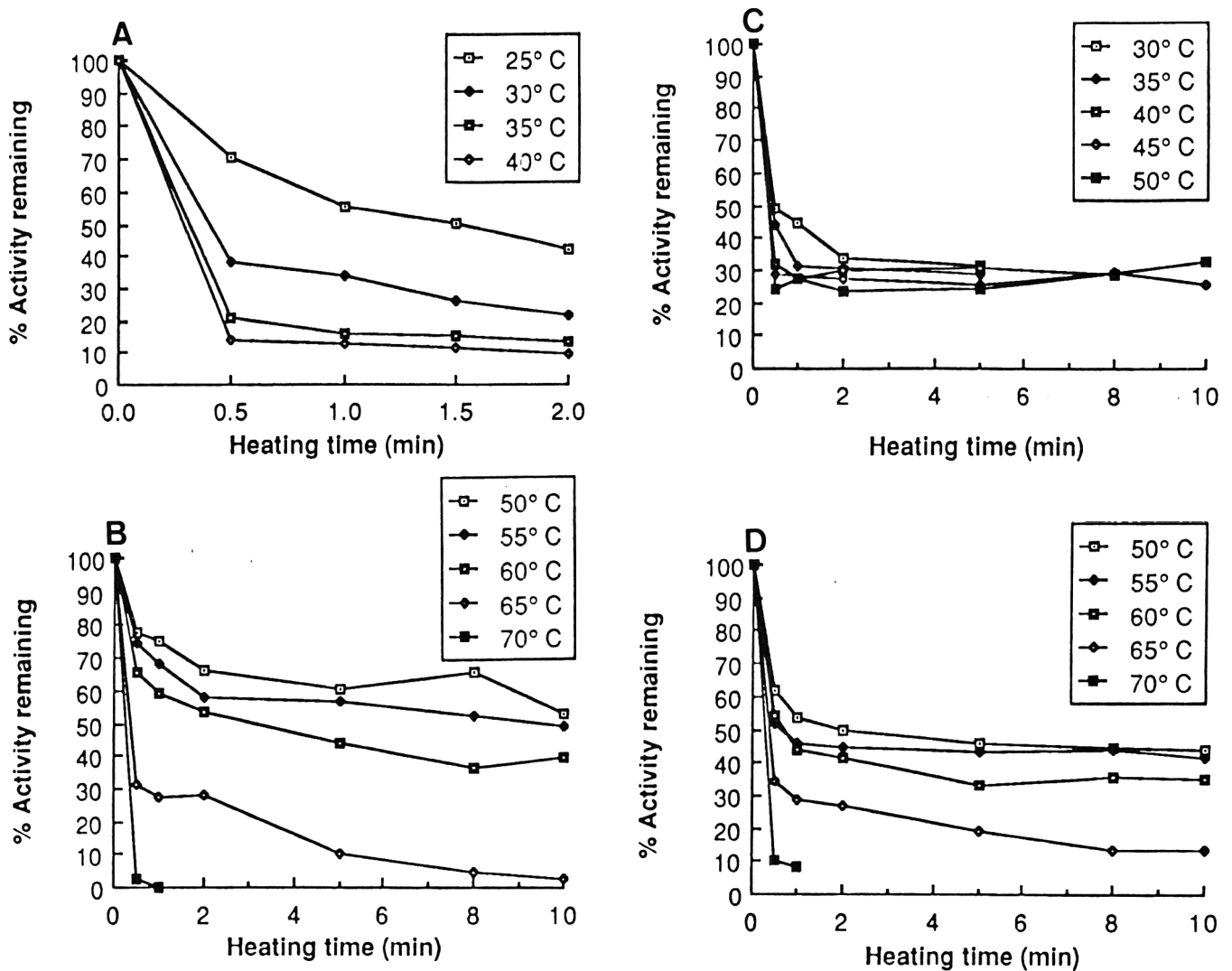
Fig. 8. — Heat inactivation of green pea peroxidases: (a) isoenzyme N; (b) isoenzyme C1; (c) isoenzyme C2; (d) crude extract.

Thermal stability

When the thermal stability of each peroxidase isoenzyme was assayed, N was found to be extremely heat labile with loss of 50% activity after heating 1.5 min at 25°C (Fig. 8A). Isoenzyme C1 was the most heat stable (Fig. 8B) while approximately 25% of C2 activity appeared very resistant to inactivation by heating (Fig. 8C). Thermal stability of the crude extract was similar to that of peroxidase isoenzyme C1 (Fig. 8D).

Each of the isoenzyme components exhibited non-linear rates of heat inactivation. McLellan and Robinson (1981) suggested that different states of the enzyme might explain the occurrence of non-linear inactivation plots. In addition, other authors have attributed non-linear inactivation curves to the formation, during heat treatment, of a new compound of higher thermostability formed from heat denatured enzyme protein and groups of peroxidase that remain active (Vamos-Vigyazo, 1981).

Reactivation of isoenzyme C1 after heating

Reactivation or regeneration of peroxidase activity in heated isoenzymes or crude extracts has been noted by many researchers (Naveh et al., 1982; McLellan and Robinson, 1981; Adams, 1978; Gibriel et al., 1978; Gordon and Alldridge, 1971; Wang and Dimarco, 1972; Wilder, 1962). Reactivation was studied in peroxidase isoenzyme C1 because C1 was the major isoenzyme in terms of activity recovered from the crude extract

and because its thermal stability closely resembled that of the crude extract. A regain of peroxidase activity was noted during incubation of all samples after heating, regardless of time/temperature heating combination (Fig. 9) Maximum reactivation occurred after heating at 50°C. In some instances, activity decreased after an initial reactivation. Joffe and Ball (1962) suggested that the reactivated peroxidase molecule was less stable than the native molecule. Little or no peroxidase activity was detected in aliquots of isoenzyme C1 after heating at 70°C for 1–10 min. However, or incubation at 25°C, reactivation occurred in all cases to 15–30% original. Similarly, MeLellan and Robinson (1981) note that cabbage and Brussels sprout peroxidases, heated at 75°C to the point of inactivation, regained 16% of original activity after 2.5 hr incubation at 30°C.

CONCLUSION

THE THREE PEA ISOPEROXIDASES exhibited pH optima and affinity for H-donor molecules similar to other peroxidases. Differences in kinetic parameters among the isoenzymes may reflect the different roles played by each isoenzyme within the cell.

The thermal stability of the three peroxidase isoenzymes was examined as a means of evaluating potential importance in thermal processing. Isoenzyme N was heat labile and may be rapidly inactivated during processing, even in the presence of protective factors in the cellular milieu. The thermal stability of isoenzyme C1 and the portion of isoenzyme C2 activity
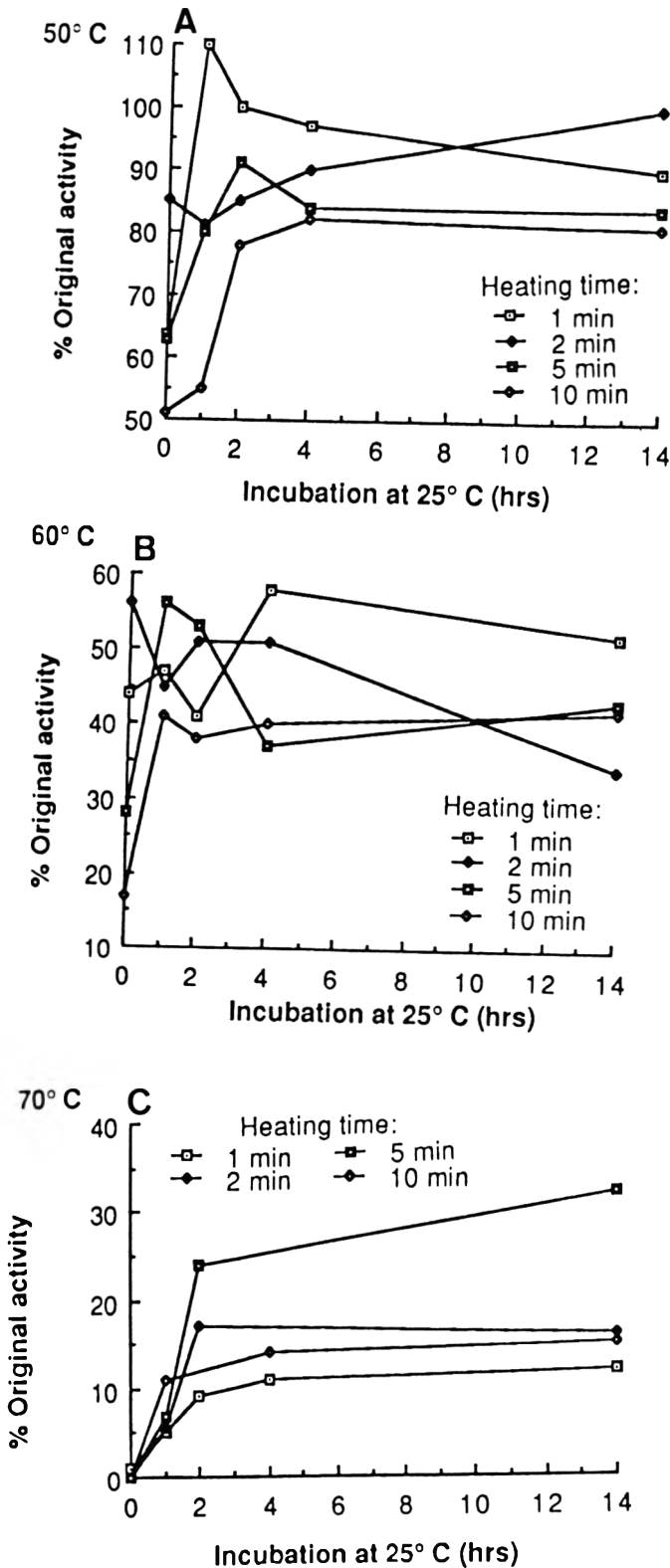
Fig. 9—Reactivation of peroxidase C1 after heating at: (a) 50°C; (b) 60°C; (c) 70°C.

resistant to thermal inactivation may be important in determining the severity of any thermal processing regime. In addition, the ability of isoenzyme C1 to regain activity after thermal inactivation may be important in influencing quality under non-ideal conditions of blanching and frozen storage.

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Inactivation and Injury of *Listeria monocytogenes* in a Minimal Medium as Affected by Benzoic Acid and Incubation Temperature

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—————————— ABSTRACT ——————————

The population of *Listeria monocytogenes* in a minimal medium (lacking a nitrogen source) at pH 5.5 decreased logarithmically during incubation. Rate of death of the pathogen, indicated by D-values, was greatly affected by changes in the temperature of incubation (4–35°C), but to a much lesser extent by the presence of benzoic acid (3000 ppm). Injury of *L. monocytogenes* during incubation was equally detectable on tryptose agar containing 6% salt and McBride Listeria Agar containing 0.5% lithium chloride. A greater degree of injury was detected at the lower than the higher temperatures of incubation, but presence of benzoic acid (3000 ppm) did not seem to affect the extent of injury.

INTRODUCTION

LISTERIA MONOCYTOGENES, a recently recognized foodborne pathogen, is an ubiquitous microorganism, and has been isolated from several raw foods (Gitter, 1976; Hayes et al., 1986; Terplan et al., 1986). Results of several studies (Doyle et al., 1987; Fleming et al., 1985; Schlech et al., 1983) suggest that the failure to properly process foods or post-processing contamination may have caused some of the food-related outbreaks of listeriosis. Some ways to control *L. monocytogenes* in foods and food-processing environments have been studied. In our laboratory we determined the effectiveness of physical factors such as ultraviolet energy (Yousef and Marth, 1988) and heat (El-Shenawy et al., 1989), some food additives such as benzoic and sorbic acid (El-Shenawy and Marth, 1988a, b), and the sanitizing agent, chlorine (El-Kest and Marth, 1988) against *L. monocytogenes*.

Benzoic acid is one of the oldest and most widely used preservatives in the food industry. Foods often preserved by benzoic acid include beverages, bakery products, salad dressings and pickles (Chipley, 1983). According to the 1988 Code of Federal Regulations (Title 21, Sections 184.1021 and 184.1733), benzoic acid is an additive generally regarded as safe (GRAS), and its use in food is permitted up to a maximum concentration of 0.1%. Results of a study (El-Shenawy and Marth, 1988b) indicate that benzoic acid, when present in a nutritious medium (tryptose broth), can inhibit the growth of and sometimes inactivate *L. monocytogenes*. Although some foods support the growth of *L. monocytogenes* (Khan et al., 1973; Rosenow and Marth, 1987), others only permit the pathogen to survive for extended periods during the cold storage of these products (Johnson et al., 1988; Ryser and Marth, 1987).

The degree of recovery of *L. monocytogenes* from foods when using selective media may be affected by the presence of injured cells of this pathogen. Many environmental factors may cause injury to bacterial cells (Ray, 1986); however, little is known about those that may injure cells of *L. monocytogenes*. Therefore, it is necessary to investigate the behavior of nongrowing cultures of *L. monocytogenes* in response to different storage conditions and the presence of food additives. Accordingly, this study was made to determine the survival and injury of *L. monocytogenes* in a minimal medium incubated over a wide range of temperatures and in the presence of benzoic acid.

MATERIALS & METHODS

Organism

L. monocytogenes strain Scott A (clinical isolate, serotype 4b) was used throughout this study. the stock culture of *L. monocytogenes* was maintained through bimonthly transfers onto tryptose agar slants, and storage at 4°C.

Minimal medium

A glucose-citrate medium was prepared to contain glucose (0.5%) and citric acid (0.05 M). For benzoic acid treatments, sufficient sodium benzoate was added so the final medium contained 1000, 2000 or 3000 ppm, calculated as benzoic acid. From their molecular formulae, it is evident that 1g benzoic acid is equivalent to 1.18g sodium benzoate. The pH of this medium was adjusted to 5.5 using a 10% aqueous solution of NaOH. Portions (99 mL each) of the minimal medium were dispensed into 250-mL screwcapped Erlenmeyer flasks which were then autoclaved at 121°C for 15 min.

Plating media

The following plating media were prepared, (1) tryptose agar (TA), (2) tryptose salt agar (TSA) made by adding 5.5% NaCl to TA, so it contained a total of 6% salt, (3) modified McBride Listeria Agar (MMLA); it contained 5g lithium chloride/L prepared medium (normal McBride Listeria Agar contains 0.5 g).

Experimental procedure

Inoculum from a stock culture of *L. monocytogenes* on a TA slant was tranferred into a test tube containing 9 mL tryptose broth (TB), and incubated at 35°C for 24 hr. Subsequently, a similar transfer was done and tubes were incubated as just described. A loopful of the culture was then tranferred into each of 10 screw-capped test tubes, each containing 10 mL TB. The inoculated TB was incubated at 35°C for 24 hr, then centrifuged in a bench-top centrifuge (IEC clinical centrifuge, Damon/IEC Division, Needham Hts., MA) at 1100 × *g* for 10 min. Most of the supernatant medium (ca 9 mL) in each test tube was decanted and the cell pellet was resuspended in the remaining 1 mL medium. Cell suspensions from all test tubes were combined and centrifuged using the conditions just indicated. The supernatant medium was decanted and the cell pellet was suspended in 10 mL fresh minimal medium. This washing procedure was repeated and the resulting cell pellet was suspended in 12 mL minimal medium. Each of the Erlenmeyer flasks containing the minimal medium (with or without benzoic acid) was inoculated with 1 mL washed cell suspension, and incubated at 4, 13, 25 or 35°C for 3 to 34 d, depending on the temperature of incubation. Samples of inoculated minimal medium were taken periodically and plated on one of the three plating media indicated earlier. Two independent trials of the entire experiment were done.

Statistical analysis

Regression analysis was used to calculate D-values for inactivation of *L. monocytogenes* under the various experimental conditions studied. Data (Log_{10} D-values) were analyzed statistically using the split-split plot design (Steel and Torrie, 1983). The following three factors (and their interactions) were included in the model; (1) temperature of incubation (main plot factor); four levels (4, 13, 25 and 35°C) were investigated, (2) presence of benzoic acid (sub-plot factor); two levels (0 and 3000 ppm) of the additive were used, and (3) type of agar

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medium on which *L. monocytogenes* was plated (sub-sub-plot factor); three media (TA, TSA and MMLA) were tested. The general linear model (GLM) procedure of SAS statistical programs (SAS Institute Inc., Cary, NC) was used to analyze the data according to the following model:

$$Y_{ijkl} = \mu + T_i + e_{ij} + B_k + TB_{ik} + s_{ijk} + M + TM_{il} + BM_{kl} + TBM_{ikl} + f_{ijkl}$$

where: Y_{ijkl} is the dependent variable (Log_{10} D-value), T_i is the temperature of incubation, B_k is the level of benzoate and M_l is the plating medium. Log_{10} rather than actual D-values were used in the analysis; this transformation gave stable variance for replicate data over the various levels of the factors studied. The terms e_{ij}, s_{ijk} or f_{ijkl} are the whole-plot, sub-plot, and sub-sub-plot error terms, respectively.

RESULTS & DISCUSSION

NUMBERS OF *L. monocytogenes* in the minimal medium decreased logarithmically during incubation of the various cultures included in this study (representative data are shown in Fig. 1). This warranted use of regression analysis to calculate D-values for inactivation of *L. monocytogenes* in these experiments. The minimal medium lacked a nitrogen source; accordingly, growth of *L. monocytogenes* was not expected.

L. monocytogenes incubated at 4°C in the absence of benzoic acid, was at a nearly resting state (population decreased less than an order of magnitude in 34 days). This behavior is somewhat similar to that reported in some cheeses (Ryser and Marth, 1987; Yousef and Marth, 1987) and sausages (Johnson et al., 1988). The population of the other *L. monocytogenes* cultures decreased at a faster rate, which may be attributed to the higher temperatures of incubation and/or the presence of benzoic acid in the minimal medium.

Statistical analysis showed that temperature of incubation was a highly significant factor (p < 0.01) for survival of *L. monocytogenes* in the minimal medium (Table 1). The log_{10} D-value for inactivation of *L. monocytogenes* decreased as the temperature of incubation increased (Fig. 2). Because the relationship between Log_{10} D-values and temperature of incu-

bation is linear (Fig. 2), calculation of the z-value for inactivation of *L. monocytogenes* is justified. The average z-value for *L. monocytogenes* in the minimal medium (lacking benzoic acid) and plated on TA medium, was 16.6°C. z-Values of 4.3–6.7°C were observed at temperatures commonly used to inactivate milk-borne *L. monocytogenes* by heat (Bradshaw et al., 1985; Bunning et al., 1986; Donnelly and Briggs, 1986; El-Shenawy et al., 1989). The z-value obtained in this study cannot be compared directly with those reported earlier since different media and higher temperatures were used in those investigations. If we assume that the type of medium has a minimal effect on heat inactivation of *L. monocytogenes*, which may not be true, thermal inactivation curves (Log_{10} D-value vs temperature) may not be linear over an extended range of temperatures.

Results of statistical analysis indicate that presence of benzoic acid in the minimal medium marginally decreased ($P = 0.071$) Log_{10} D-value of *L. monocytogenes* (Table 1). In this study, benzoic acid at concentrations smaller than 3000 ppm (1000 and 2000 ppm) inactivated *L. monocytogenes* only minimally, therefore these data were not included in the statistical analysis or shown graphically. From this and results of

Table 1—Statistical analysis of Log_{10} D-values for L. monocytogenes in a minimal medium as affected by incubation temperature (4 levels), the presence of benzoic acid (2 levels) and use of different types of plating media (3 levels)

Source	Degrees of freedom	Mean square	p
Temp of incubation (T)	3	7.4	0.0008
Whole-plot error (e)	4	0.12	
Conc of benzoic acid (B)	1	0.38	0.071
B*T	3	0.061	0.50
Sub-plot error (s)	4	0.064	
Type of medium (M)	2	0.13	0.0001
M*T	6	0.036	0.0001
M*B	2	0.00013	0.96
M*B*T	6	0.0091	0.056
Sub-sub-plot error (f)	16	0.0034	
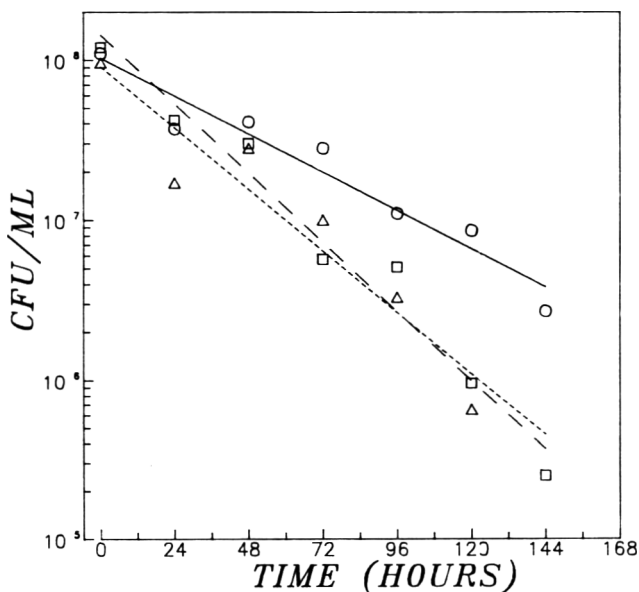
Fig. 1.—Survival of L. monocytogenes *in the minimal medium incubated at 25°C in the absence of benzoic acid (first trial). Samples were plated on tryptose agar (TA), modified McBride Listeria Agar (MMLA), or tryptose salt agar (TSA). Symbols represent actual data and lines represent regression analysis best fit. Symbols and lines are as follows: (○——○, CFU/mL on TA, (□— —□) CFU/mL on MMLA and (△----△) CFU/mL on TSA media.*
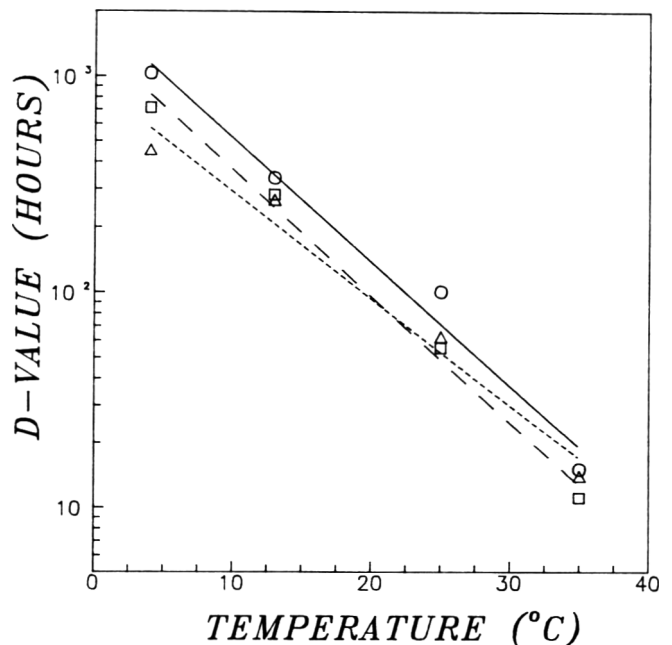
Fig. 2.—D-values for inactivation of L. monocytogenes *in the minimal medium incubated at 4, 13, 25 or 35°C (first trial). Samples were plated on tryptose agar (TA), modified McBride Listeria Agar (MMLA) or tryptose salt agar (TSA). Symbols represent estimated D-values and lines represent regression analysis best fit. Symbols and lines are as follows: (○——○) TA, (□— —□) MMLA, (△----△) TSA media.*

a previous study (El-Shenawy and Marth, 1988b), it is apparent that benzoic acid at concentrations of ca. 1000-3000 ppm had strong bacteriostatic, but relatively modest bactericidal activities against *L. monocytogenes*. The metabolic activity of *L. monocytogenes* is presumably slower in the minimal medium than in a nutritious medium. This may have affected the uptake of benzoic acid by cells of *L. monocytogenes* in the minimal medium, and thus reduced the capacity of the additive to inactivate the pathogen. Our results raise a question about the suitability of benzoic acid alone to control *L. monocytogenes* in foods.

Injury of *L. monocytogenes* was assessed by plating the cultures on each of two restrictive media; TSA and MMLA. Differences in numbers of *L. monocytogenes* obtained with TA and the restrictive media (TSA or MMLA) were assumed to represent the population of injured cells. Although the difference between number of colonies on TA and restrictive media at the time of inoculation were negligible, these differences, in most treatments, progressively increased as the period of incubation increased (Fig. 1). This indicates that the extent of injury of *L. monocytogenes* in these treatments depended on the length of incubation in the minimal medium. Statistical analysis (Table 1) shows that type of plating medium had a significant ($P < 0.01$) general effect on Log_{10} D-values for *L. monocytogenes* in the treatments studied. Results of Fisher's least significant difference (LSD) test at $P = 0.05$ indicate that *L. monocytogenes* plated on TSA or MMLA medium, (compared with plating on TA medium) had significantly smaller Log_{10} D-values. Results also indicate that both restrictive media (TSA and MMLA) were equally effective in inhibiting growth of injured cells of *L. monocytogenes*. Lee and McClain (1986) modified McBride Listeria Agar (MLA) by adding moxalactam and increasing the concentration of lithium chloride from 0.5 to 5.0 g/L medium. This modified medium (LPM) was selectively superior to MLA when used to isolate *L. monocytogenes* from mixed cultures. According to our results, MMLA (which is similar to LPM in lithium chloride concentration) did not permit growth of injured cells of *L. monocytogenes*, hence suitability of both media (MMLA and LPM) for isolation of *L. monocytogenes* from some processed foods is questionable.

The degree of injury was somewhat greater at the lower than at the higher temperatures of incubation (Fig. 2). Thus, incubation temperature significantly affected the extent of injury of *L. monocytogenes*, as indicated by the significant (p < 0.01) medium-temperature interaction (Table 1). In an earlier study (El-Shenawy et al., 1989), temperatures in the range that is commonly used to inactivate *L. monocytogenes* caused injury, the extent of which depended on the temperature used. Cells of *L. monocytogenes* in the minimal media were injured to about the same extent regardless of the absence or presence of benzoic acid (3000 ppm) (Fig. 3). Results of statistical analysis (Table 1) appear to confirm this conclusion since there was no significant interaction between the concentration of benzoic acid and type of plating medium used.

In conclusion, cells of *L. monocytogenes* were inactivated in the minimal medium at a rate that depended on the temperature of incubation. Presence of benzoic acid had relatively little effect on the rate of death of *L. monocytogenes* in the minimal medium. TSA and MMLA were equally suitable media for detecting injury of *L. monocytogenes*. Incubation of the pathogen in the minimal medium caused injury of cells that depended on the temperature of incubation but not the presence of benzoic acid at a concentration of 3000 ppm.

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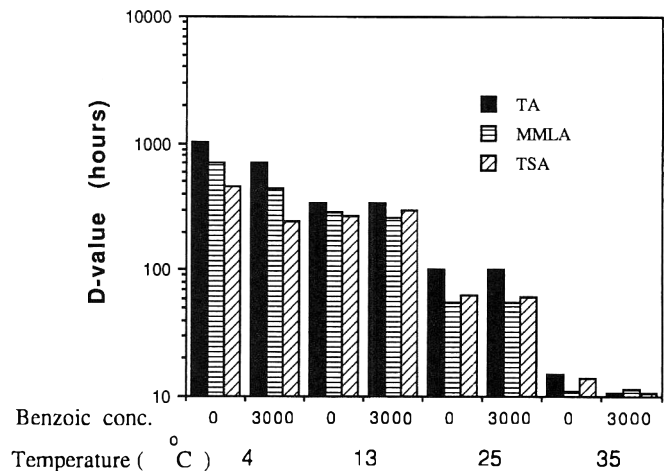
Fig. 3. — D-values for inactivation of L. monocytogenes *in the presence or absence of benzoic acid (3000 ppm), when the bacterium was incubated in the minimal medium at 4, 13, 25 or 35°C (first trial). Samples were plated on tryptose agar (TA), modified McBride Listeria Agar (MMLA), or tryptose salt agar (TSA).*

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Isolation and Characterization of Pectin Methylesterase from Apple Fruit

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─────────── ABSTRACT ───────────

Two forms of the enzyme pectin methylesterase are evidenced in the apple (*Malus communis*). They differ both in their charge and molecular weight. The two enzymes were separated by DEAE-cellulose chromatography. Their molecular weights, determined by gel-filtration, were 55000 and 28000 daltons. The heavier form has been purified at homogeneity and subjected to investigations regarding its activity as a function of the pH and temperature, and determination of its kinetic parameters. The enzyme has a Km value of 1.05 mg/mL for citrus pectin and an optimum activity in the pH range between 6.5-7.5. The enzyme was stable up to 40°C. Incubation for 1 min at 90°C leads to its complete inactivation.

INTRODUCTION

The enzyme pectin methylesterase (PME) (EC 3.1.1.11) catalyzes the hydrolysis of methyl ester groups from the pectin (a polymer of $\alpha - 1.4$ linked galacturonic acid and galacturonic acid methylester). The enzyme is involved in the first step of the fruit-ripening process by producing pectin with a lower degree of methylation which, in turn, becomes the substrate of polygalacturonase. The pectin methylesterase plays a central role in the process of the fruit softening during ripening; the control of its activity, through knowledge of the dependence on such parameters as temperature and pH, is of great practical importance in the food industry for the maintainance of the texture characteristics of industrial fruit puree preparations.

PME has been characterized from various fruit sources (Versteg et al., 1980; Puri et al., 1982; Markovic et al., 1986). The purpose of this study was to investigate the purification and characterization of PME from apple (*Malus communis*).

MATERIALS & METHODS

Reagents

2,4-Pentanedione was obtained from Aldrich Chemical Co. Steinheim. Alcohol oxidase from *P. pastoris* (EC 1.1.3.13) and pectin from citrus fruits was purchased from Sigma Chemical Co. St. Louis. DEAE-cellulose was obtained from Wathman (England). Sephadex G-75 Superfine was purchased from Pharmacia (Sweden).

All other reagents were analytical grade.

Determination of pectin methyl esterase activity

The enzyme activity was determined by methanol production as a consequence of pectin hydrolysis. PME activity was determined by a modification of the method of Klavons and Bennet (1986). The enzyme assay was performed as follows: 20 µL PME was incubated with pectin in 100 µL incubation mixture containing 100 mM sodium phosphate pH 6.5, at 25°C in a screw-cap glass vial. The reaction was allowed to proceed for 5 min, then stopped by heating the mixture at 100°C in water bath for 3 min. The mixture, cooled at 25°C, was diluted to 2 mL with 20 mM Tris-HCl pH 7.5 and 1 unit alcohol oxidase was added. After 15 min at 25°C, 1 mL 20 mM 2,4-penta-

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nedione in 2M ammonium phosphate was added and the mixture placed in a water bath at 60°C for 15 min. The absorbance was measured at 412 mn against a blank made up with the same components without PME. A calibration curve, using methanol as standard, was prepared. The correlation between color developed and methanol concentration was linear up to 0.4 µmoles/mL methanol. The enzyme activity was expressed in Units (µmoles of methanol produced per minute). The pectin used as substrate in all the experiments was exhaustively dialyzed against water. We found that undialyzed pectin gave too high blanks, which made the methanol determination uncertain.

Heat stability of enzyme

Enzyme (1.5 µg) in 20 µL of 20 mM Tris-HCl pH 7.5, 50 mM NaCl, and 5 mM mercaptoethanol were incubated in capped vials for 1 min. at the indicated temperature, immediately cooled in ice and then assayed for activity.

Determination of the molecular weight

The molecular weights of the native pectin methylesterases were determined by gel-filtration chromatography on a Sephadex G-75 superfine column (1.5 × 90 cm) equilibrated in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 5 mM mercaptoethanol. The column was eluted at flow rate of 6 mL/h; 1 mL fractions were collected. The calibration curve was prepared with ovalbumin (Mw = 43000 daltons), carbonic anhydrase (Mw = 30000 daltons), soybean trypsin inhibitor (MW = 20100 daltons), lactalbumin (Mw = 14400 daltons) and nicotinamide-adenine dinucleotide. The void volume was determined with Blue Dextran 2000.

The determination of the molecular weight in denaturing conditions was made by using a SDS polyacrylamide gel electrophoresis technique (SDS-PAGE) according to Laemli (1970). The acrylamide concentration was 12.5%.

Ultracentrifugal measurements

Samples of purified PME were dialysed overnight against 10 mM Tris-HCl pH 7.5 and 100 mM NaCl; N_2 was bubbled through the dialysis solution all through the dialysis period. The molecular weight was determined by equilibrium sedimentation in a Beckman L8-70 ultracentrifuge equipped with a prep-UV scanner using an AN-D aluminium rotor. The measurements were made at two different rotor speeds (10000 and 15000 rev/min) on three cells containing the enzyme at different concentrations, whose initial absorbance values at 280 nm were 0.13, 0.31, and 0.48, respectively. Sedimentation equilibrium as checked by comparing the sedimentation profiles at 6 h intervals. Before the calculations, the profiles were corrected for the baseline absorbance by accelerating the rotor to 42000 rev/min for 6 h and then decelerating to initial speed just before the scan.

Determination of protein concentration

Aliquots were taken at each purification step and dialysed against a solution containing 20 mM Tris-HCl pH 7.5, 50 mM Nacl and 5 mM mercaptoethanol, and the protein concentration was determined according the method of Bradford (1976) using a calibration curve made with bovine serum albumin (Sigma) dissolved in the same buffer.

Extraction of pectin methylesterase

The whole apple fruits (2 kg) were homogenized in water at 4°C (1:2 w/v) and centrifuged at 15000xg for 20 min. The precipitate was washed twice with 2L ice-cold water and then extracted for 24 hr at

Table 1 — Purification steps of pectin methylesterase from apple

Fraction	Total volume (mL)	Units/mL	Total units	Protein (mg/mL)	Total protein (mg)	Units/mg	Recovery	Purification
10% Ammonium sulfate extract	1500	2.5	3750	0.4	600	6.3	100	1
Ammonium sulfate precipitate	200	14.8	2960	1.1	220	13.5	79	2.1
2nd Ammonium sulfate precipitate	25	111	2775	7.4	185	15	74	2.4
DEAE-Cellulose column								
Enz. A	35	47.4	1660	0.9	31.5	52.7	44	8.4
Enz. B	50	11	550	1.2	60	9.2	15	1.5
Sephadex G-75, Enz. A	7	95	665	0.7	4.9	135.7	18	21.5
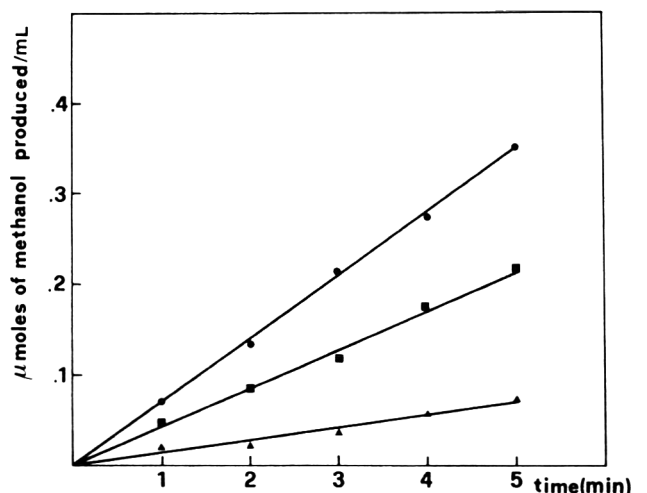
Fig. 1 — Activity of purified pectin methylesterase from apple as a function of incubation time and enzyme concentration: ▲—▲ = 0.3 μg PME; ■—■ = 0.9 μg PME; ●—● = 1.5 μg PME

Fig. 2. — Gel-filtration chromatography on Sephadex G-75 Superfine of a sample containing the two pectin methylesterase forms from apple. The elution pattern was followed at 280 nm (- - -) and the activity was reported as Units/mL (—), where one unit is defined as the μmoles methanol produced per min.

4°C with 1.5L of a buffer containing 200mM Tris-HCl pH 8.2, 10% ammonium sulfate, 10mM EDTA and 5mM mercaptoethanol.

Purification of pectin methylesterase

The suspension was centrifuged, the clarified supernatant was brought to 85% saturation with ammonium sulfate and allowed to stand 1 hr at 4°C. After the centrifugation at 15000xg for 30 min, the pellet was extracted twice with 100 mL 20 mM Tris-HCl pH 7.5 containing 10mM EDTA and 5mM mercaptoethanol. To the protein solution, ammonium sulphate to 85% saturation was added again and the collected precipitate suspended in 20 mL of the same buffer and dialyzed exhaustively at 4°C.

The dialyzed solution was loaded on a DEAE-cellulose column (1 × 20 cm) equilibrated in 20 mM Tris-HCl pH 7.5, 5 mM mercaptoethanol and the column was washed with the same buffer. The retained proteins were eluted with the same buffer containing 300 mM potassium chloride. Two enzymatic activities were detected: the first, coming out unretained, the second released by adding the high salt elution buffer. The fractions constituting the first activity (35 mL) were pooled, concentrated and subjected to gelfiltration by using a Sephadex G-75 superfine column. The fractions from the gel-filtration step were analyzed by SDS-PAGE and those containing the pure enzyme were pooled.

RESULTS & DISCUSSION

THE VARIOUS STEPS utilized to purify the PME are summarized in Table 1. Since the PME is bound to the cell wall, it was difficult to determine with sufficient accuracy the activity of the enzyme in the homogenate of the whole fruit. For this reason the purification steps in Table 1 start from the 10% ammonium sulfate extract. Since the PME assay is based on one point determination, we verified the linearity of the reaction rate as function of the incubation time as well as of the enzyme concentration. The time course of the reaction at different concentrations of the purified enzyme are shown in Fig. 1. The reaction rate was constant up to 5 min and the amount
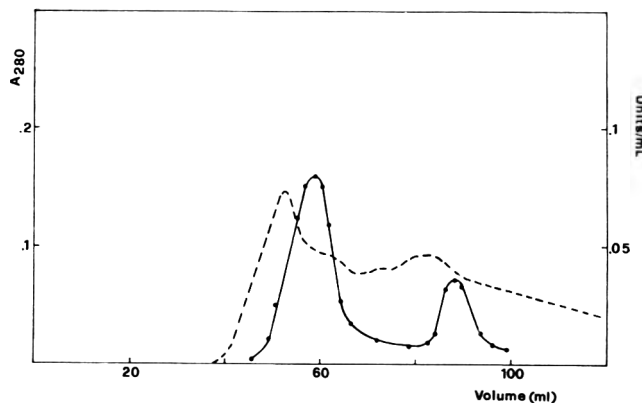
of methanol produced increased linearly with the enzyme concentration. Studies on a crude salt extract showed a similar behavior.

The first step of the PME purification entailed ammonium sulfate precipitation. However, a large buffer volume is required to obtain a high recovery of the PME from the precipitate. Hence, in order to concentrate the enzyme solution, the ammonium sulfate procedure was repeated once again.

The DEAE-cellulose chromatography produced the separation of two PME activities, reported in Table 1 as Enz. A (not retained by the column) and Enz. B (released in a step elution with 300 mM potassium chloride).

Enzyme A was purified to homogeneity by gel-filtration chromatography on Sephadex G-75 Superfine. The final enzyme purification was 21.5-fold, which is low considering that the enzyme was purified at homogeneity. However, it should be considered that the starting material for the enzyme purification was not a crude homogenate. In fact, it was prepared by extracting with 10% ammonium sulfate that precipitate obtained after homogenate centrifugation followed by exhaustively washing with water. Thus, all the soluble proteins were removed except those, like PME, bound to the cell wall. This selective extraction by high salt treatment gave low protein recovery and consequently, a relatively high specific activity in the starting enzyme extract.

Using a Sephadex G-75 column calibrated for molecular weight as a last step of purification, we estimated a molecular weight of 55000 daltons for the enzyme not retained on the DEAE-cellulose column, while Enzyme B had a molecular weight of 28000 daltons. A gel-filtration experiment on a sample from the 10% ammonium sulfate extract containing the two PME forms is shown in Fig. 2. The higher molecular weight form was the most abundant, ranging between 65 and 80% of the total PME activity in all the purifications made.

The homogeneity of the purified PME having Mw = 55000 daltons was demonstrated by the presence of a single protein band on SDS-PAGE (Fig. 3). In addition, the presence of only
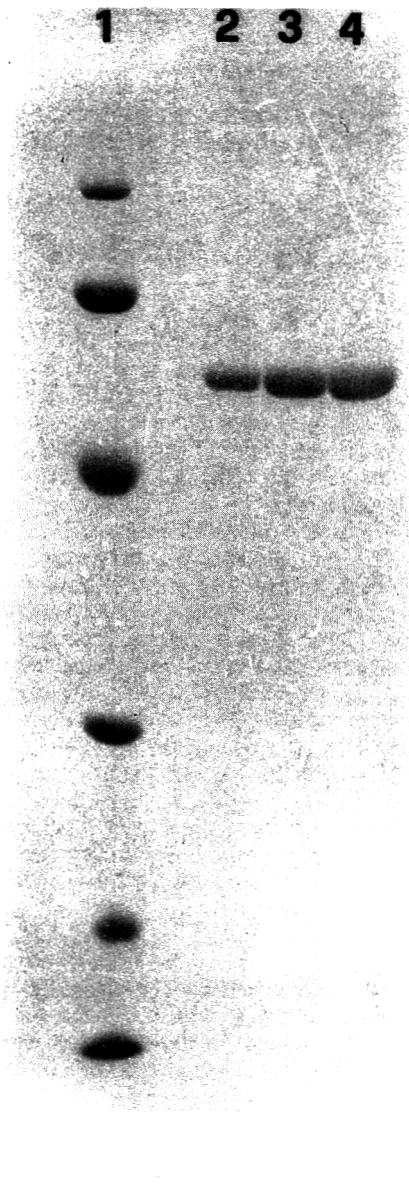
Fig. 3—SDS-PAGE of purified pectin methylesterase from apple. Lane 1: Molecular weight markers (from top to bottom): phosphorylase b (Mw = 94000 daltons), bovine albumin (Mw = 67000 daltons), ovalbumin (Mw = 43000 daltons), carbonic anhydrase (Mw = 30000 daltons), soybean trypsin inhibitor (MW) = 20,100 daltons), lactalbumin (Mw = 14,100 daltons). Lanes 2, 3, 4: 3 μg, 6 μg, 9 μg purified PME, respectively.

Fig. 4—Molecular weight determination of apple Pectin methylesterase by sedimentation equilibrium. The ln of absorbance at 280 nm (ln A) in the cell at distance (r) from the rotation axis, was plotted against the square of that distance. The data were obtained at 15000 rpm (a) and 10000 rpm (b). The solid lines were derived from least-square analysis of the data points.
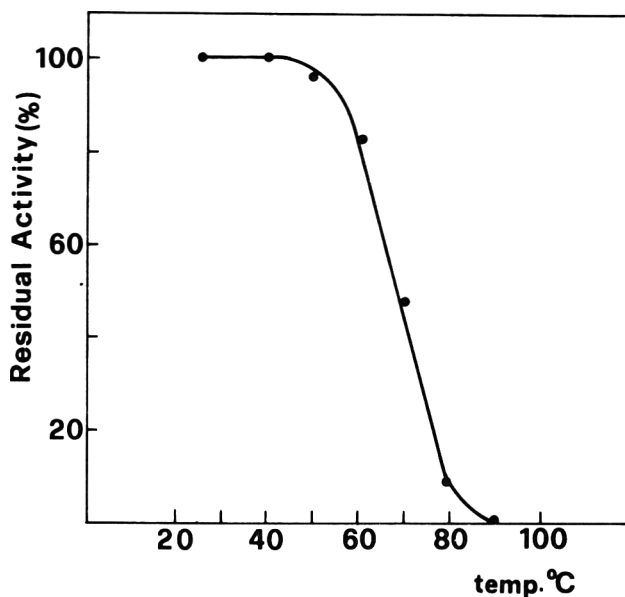
Fig. 5—Heat stability of purified Pectin methylesterase from apple. The values shown are the means of three determinations. The assay conditions are described under "Materials & Methods."

one symmetrical boundary in the sedimentation velocity experiments, as well as the linearity of the plot of ln(Abs) versus r from sedimentation-equilibrium analysis, confirmed that the protein was essentially homogeneous. The equilibrium sedimentation profile at 10000 and 15000 rev/min, plotted as ln(Abs) versus r, are shown in Fig. 4. A least-squares analysis on the data groups obtained both at 10000 and 15000 rev/min gave a regression coefficient, for the straight line, higher than 0.99. A MW of 55000 ± 2000 daltons was calculated from the slopes. The same MW for PME obtained by sedimentation equilibrium was also confirmed by the SDS-PAGE analysis. The molecular weight of 55000 daltons found for the enzyme by gel electrophoresis in denaturing and reducing conditions led to the conclusion that this from was not a dimer of the lighter form with molecular weight of 28000 daltons.

The purified enzyme was subjected to further investigations of its activity as a function of pH, its thermal stability, and some kinetic parameters. The enzyme activity exhibited a strict

pH dependence; in fact one unit of pH variation (from 6 to 5) produced a 60% decrease in activity. Below pH 4 the activity became negligible, whereas the optimum remained constant in the range 6.5–7.5. However, in all other experiments we used in incubation mixture at pH 6.5 since higher pH values resulted in a slow hydrolysis of pectin, thus giving a higher blank due to nonenzymatic methanol production. The activity of the PME as a function of pectin concentration was also investigated. The double reciprocal plot gave a Km value of 1.05 mg/mL for citrus pectin and a Vmax of 0.73 μmol methanol produced per min.

In order to establish the thermal stability of the enzyme, protein solutions were incubated for 1 min at increasing tem-

Postharvest Physiology and Quality Maintenance of Sliced Pear and Strawberry Fruits

JOAN C. ROSEN and ADEL A. KADER

—————————— ABSTRACT ——————————

Sliced strawberries (cvs. 'Pajaro' and 'G-3') and partially ripe pears (cv. 'Bartlett') were dipped in various solutions (citric acid, ascorbic acid, and/or calcium chloride) and stored in air or in controlled atmospheres (CA) for 7 days at 2.5°C followed by one day at 20°C. Fruit slices respired at a higher rate than whole fruits at both temperatures. CA storage suppressed respiration and ethylene production rates of sliced fruits. Firmness of strawberry and pear slices was maintained by storage in air + 12% CO_2 and in a 0.5% O_2 atmosphere, respectively, or by dipping in 1% calcium chloride. These treatments also resulted in lighter colored pear slices than the air control treatment.

INTRODUCTION

SLICED FRUIT can be expected to behave differently from whole fruit during storage because of the response to wounding and damage to the skin. Possible manifestations of slicing fruit include increased ethylene production and respiration rates, accelerated senescene and enzymatic browning.

Many examples of wound-induced ethylene production in fruit and vegetable tissue have been reviewed by Abeles (1973), Lieberman (1979), Yang and Hoffman (1984) and Yang and Pratt (1978). The stimulation of ethylene production by stress typically occurs within a lag of 10-30 min and subsides later after reaching a peak within several hours (Yang and Pratt, 1978; Yang and Hoffman, 1984). However, MacLeod et al. (1976) found that impact-bruised tomatoes exhibited sustained increases in rates of ethylene and CO_2 production.

The respiration of fresh slices is, in most cases, 3 to 5 times that of the intact organ, while aging elicits another comparable increase (Laties, 1978). Palmer and McGlasson (1969) found that transverse slices of green banana fruit exhibited an initial burst of respiration which subsided within 2 hr and a broad peak of "induced" respiration at 15-20 hr.

Various postharvest dip treatments have been utilized to control or alleviate degradative changes and prolong the storage life of fruits and vegetables. Calcium dips were shown to delay ripening (Wills and Tirmazi, 1982; Poovaiah, 1986), retard flesh softening (Bangerth et al., 1972) and retain vitamin C content (Drake and Spayd, 1983). Morris et al. (1985) reported that calcium dips were more effective in firming sliced strawberries than in firming whole strawberries. Calcium treatments reduced browning (Drake and Spayd, 1983) and enzyme activity (Hopfinger et al., 1984). Other dip treatments which retard enzymatic browning include ascorbic acid (Bauernfeind and Pinkert, 1970) and citric acid (Anonymous, 1983).

Controlled atmospheres (CA) retarded senescence (Brecht, 1980; Kader, 1980) and delayed softening of fruit (Knee, 1980). Elevated CO_2 atmospheres slowed down the softening rate of strawberries (Harris and Harvey, 1973); this effect was often noticeable after transfer of the berries to air (Kader, 1986).

CA has also been evaluated as a means of minimizing en-zymatic browning. Murr and Morris (1974) found that storage of mushrooms in 0% O_2 (tank nitrogen) reduced discoloration and o-diphenoloxidase activity for up to 7 days. Browning of shiitake mushrooms was avoided in O_2 concentrations of 2% or less (minamida et al., 1980). Increased CO_2 reduced browning or other discoloration of lettuce (Singh et al., 1972; Siriphanich and Kader, 1985) and snap beans (Buescher and Henderson, 1977).

Previous work with sliced fruits and vegetables indicated that several factors influenced quality. In studies with lettuce, low temperatures (0-2°C), storing dry, minimizing physical damage, use of a sharp blade in a slicing action and minimizing microbial load were effective factors in extending storage life (Bolin et la., 1977; Krahn, 1977). Modified atmospheres of 2-3% O_2 reduced browning and maintained texture and flavor at a high level, whereas chemical treatments, including sodium bisulfite and $CaCl_2$, were of little or no value in extending storage life (Krahn, 1977).

In a study of refrigerated apple slices, Ponting et al. (1972) reported that neither ascorbic acid alone nor calcium alone was effective in preventing discoloration, but the combination treatment was very effective.

In this study, factors affecting the postharvest life of slices of a climacteric fruit ('Bartlett' pear) and a non-climacteric fruit (strawberry) were examined to evaluate the potential use of stored sliced fruits for fresh market consumption. Controlled atmosphere storage and various dip treatments were tested for their effects on the physiological behavior and quality maintenance of sliced pears and strawberries.

MATERIALS & METHODS

Strawberries

Strawberry cultivars 'Pajaro' and 'G-3' were obtained on the day of harvest in Watsonville, CA. They were transported to Davis, CA, in an air-conditioned vehicle and stored at 0°C until preparation for experiments the following morning.

The berries were sorted to remove defective and damaged fruit. Sound fruits were sorted by color to remove berries with less than 2/3 red or riper than full red surface color and matched samples were used for each treatment. Three replicates of 20 berries each were used per treatment.

Slice preparation

The calyx was removed, and the berries were sliced longitudinally into quarters. All utensils were cleaned thoroughly before use. Slices were placed in the inner bowl of a Copco Salad Spinner (Item No. 2555-04), dipped in 4L distilled water for 15 sec (60 sec for experiments evaluating dip treatments), drained 30 sec and then spun to remove excess water.

Pears

'Bartlett' pears (size 120, average fruit weight = 150 g) were obtained from a packinghouse in Lake County, CA, on the day of harvest and transported to Davis, CA, in an air-conditioned vehicle. These pears were used for various tests after storage at −1°C for 2 to 12 wk.

Pears were sorted to eliminate damaged or defective fruit. They were then partially ripened to approximately 44.5N firmness by ex-

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posures to 10 ppm ethylene at 20°C for 48 hr. After ripening, pears were arranged by color from green to yellow, and matched samples were used for the treatments. Slices from five fruits were used per each of thre replicates per treatment.

Slice preparation. Pears were cut into 12 wedges with a Westmark Divisorex corer/slicer. The fruit slices were then washed and spun as described for strawberries.

Storage

After preparation, the fruit slices were stored at 2.5°C for 7 days in glass jars ventilated with humidified air followed by 1 day at 20°C in nonhumidified air.

Experimental treatments

For strawberries, atmospheric treatments of 12% CO_2, 2% O_2 and 2% O_2 + 12% CO_2 were compared to air. All CA-treated fruit were returned to air upon transfer to 20°C on day 7. Dip treatments of 1% ascorbic acid, 1% citric acid, 1% ascorbic acid + 1% citric acid, 0.5% $CaCl_2$, and 1.0% $CaCl_2$ were compared to distilled water.

CA treatments for pears consisted of 0.5, 1.0, or 2.0% O_2. All CA-treated fruit were returned to air upon transfer to 20°C on day 7. Dip treatments were the same as those used on strawberries. In addition, a combination of 0.5% O_2 and 1% $CaCl_2$ was evaluated.

All experimental samples were compared to two controls: (1) untreated sliced fruit, and (2) whole fruit stored under control conditions and sliced on the day of evaluation.

Gas mixing and analysis

Fruits were placed in glass jars under continuous flow of humidified air or gas mixtures at known flow rates. The composition of atmospheres was measured daily with a Carle gas chromatograph model 211 using a thermal conductivity detector for O_2 and CO_2 and a flame ionization detector for ethylene. CO_2 production rates were measured with an Infrared Gas Analyzer, (HORIBA-PIR-2000R) using 10 mL effluent.

Color

A Gardner Color Difference Meter, Model No. XL-23, was used to evaluate external and internal color. It was calibrated with a white plate (X = 84.1, Y = 81.7, Z = 92.9) and the L, a, b scale was used.

The internal color of pears was measured over a 2 cm diameter orifice with a glass plate. Two measurements of each slice or fruit were taken; color of each replicate represents the average of 20 measurements.

Firmness

Strawberries. Firmness of 100 g of strawberries was evaluated in the compression/shear cell of a Food Technology Corporation Texture Test System Model TP-4 utilizing a 1334-N force transducer, a 30-sec ram time, and a 30-sec ram stroke integration time. Integrated area was measured with the Model TI texture integrator and Model TR5 Texturecorder.

Pears. Pear slice firmness was measured with a U.C. Fruit Firmness Tester supplied with an 8 mm tip. Resistance to penetration of the flesh was evaluated on the slice's cut surface, immediately adjacent to the sliced edge of skin.

Calcium content

Frozen fruit samples were dehydrated and 500 mg of fruit powder were digested with 3 mL concentrated nitric acid for 1 hr at 110°C, and 6 ml of 30% hydrogen peroxide were added. After cooling to room temperature, volume was brought to 50 mL with water, samples were filtered and analyzed on a Techtron AA 120 Atomic Absorption Spectrophotometer. A Varian Techtron D 1-30 Digital indicator was used, samples were compared to standard curve and are expressed as mg/100g FW.

RESULTS & DISCUSSION

Strawberries

The respiration rate of slices was greater than that of whole fruit throughout the 8-day storage period (Fig. 1). On day 8 (after transfer to 20°C) there was no difference between treatments. C_2H_4 production was negligible at 2.5°C (data not shown); this is typical of a nonclimacteric fruit. After transfer to 20°C, the C_2H_4 production of both whole and sliced fruits increased dramatically. The ethylene production of sliced fruit was about four times greater than that of whole fruit (0.40 vs 0.12 μL/kg · hr). The increase in C_2H_4 production and CO_2 production in sliced fruit as compared to whole fruit is typical of a wounding response (Abeles, 1973; Laties, 1978).

After 7 days at 2.5°C, CA-stored 'Pajaro' strawberry slices were about the same firmness as air-stored slices and air-stored whole fruit (Table 1). Both 12% CO_2 and 2% O_2 were effective in minimizing loss of firmness of 'G-3' strawberries. These treatments had comparable firmness measurements to whole, freshly sliced fruit. The effect of CA on softening rate appeared to be cultivar-dependent. After transfer to 20°C and air, the 12% CO_2-treated slices were firmer than all other treatments for both cultivars. Kader (1986) reported the effect of CA on firmness retention in strawberries is often noticeable after return to air.

Calcium chloride dips resulted in higher calcium content but there were no differences in fruit firmness initially (Table 2). After 7 days at 2.5°C both 0.5% and 1.0% $CaCl_2$ dips resulted in firmer fruit with higher Ca contents than either water-dipped slices or whole, freshly sliced fruit. Although the calcium con-
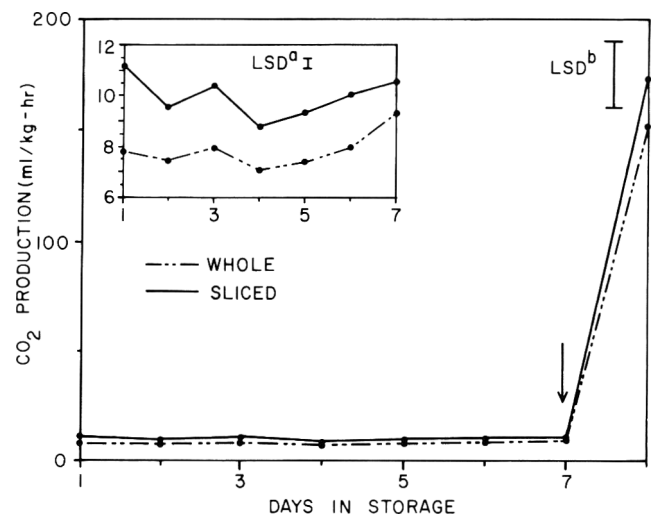
Fig. 1 — CO_2 production by strawberries stored in air at 2.5°C for 7 days (inset) and after transfer (indicated by arrow) to 20°C for 1 day. LSD[a] and LSD[b] are the least significant differences at 0.05 level for days 1 to 7 and day 8, respectively.

Table 1 — Effect of CA storage on firmness (relative shear force) of 'Pajaro' and 'G-3' strawberries after storage at 2.5°C for 7 days and after transfer to 20°C and air for 1 day

Treatment	'Pajaro' Day 7	'Pajaro' Day 8	'G-3' Day 7	'G-3' Day 8
Air - sliced	69.6	58.6[b]	44.9[b]	44.5[b]
12% CO_2	63.6[ab]	70.0[a]	49.5[a]	49.8[a]
2% O_2	56.8[b]	60.3[b]	45.0[b]	38.8[c]
2% O_2 + 12% CO_2	66.9[a]	60.8[b]	48.3[a]	47.3[ab]
Air - whole, freshly sliced	68.1[a]	54.6[b]	49.2[a]	45.1[b]

[abc] Mean separation in columns for each evaluation time by Duncan's multiple range test, 0.05 level.
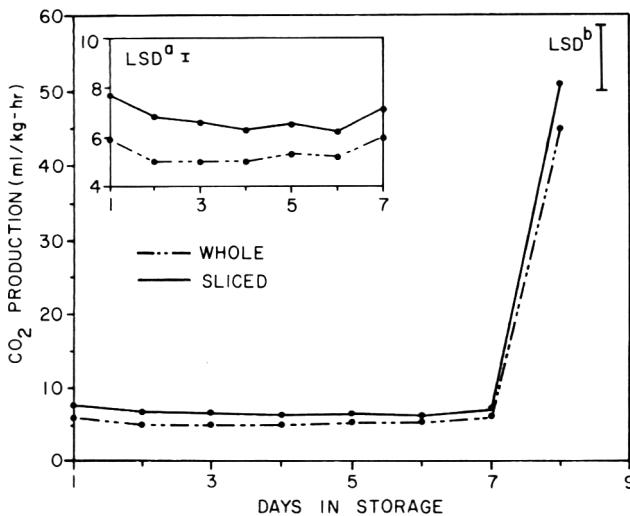
Fig. 2—CO_2 production by pears stored in air at 2.5°C for 7 days (inset) and after transfer (indicated by arrow) to 20°C for 1 day. LSDᵃ and LSDᵇ are the least significant differences at 0.05 level for days 1 to 7 and day 8, respectively.
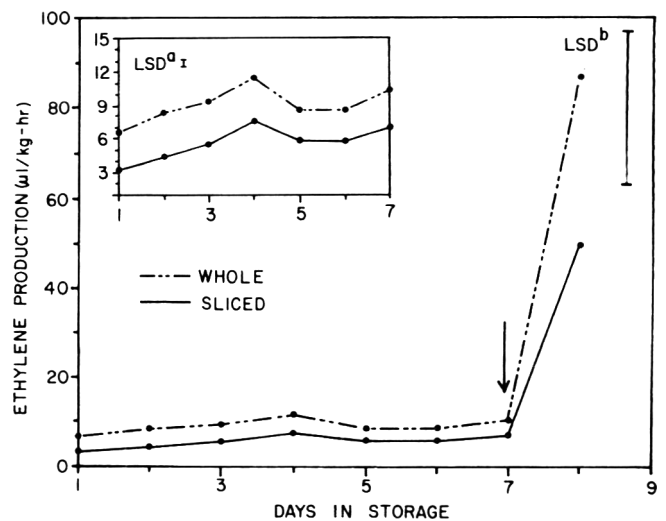
Fig. 3—Ethylene production by pears stored in air at 2.5°C for 7 days before transfer (indicated by arrow) to 20°C for 1 day. LSDᵃ and LSDᵇ are the least significant differences at 0.05 level for days 1 to 7 and day 8, respectively.

Table 2—Effect of $CaCl_2$ dips on firmness (relative shear force) of 'G-3' strawberries stored at 2.5°C for 7 days and at 20°C for 1 day

	Day 0		Day 7		Day 8	
Treatment	Firmness (N)	Ca (mg/100g)	Firmness (N)	Ca (mg/100g)	Firmness (N)	Ca (mg/100g)
Water	52.2	10.3ᶜ	47.1ᵇ	11.4ᶜ	47.3ᵇ	11.0ᶜ
0.5% CaCl₂	54.3	16.7ᵇ	56.3ᵃ	16.7ᵇ	53.4ᵃ	16.8ᵇ
1.0% CaCl₂	53.4	21.4ᵃ	57.0ᵃ	23.1ᵃ	56.7ᵃ	22.8ᵃ
No dip, freshly sliced	51.4	10.1ᶜ	48.9ᵇ	10.4ᶜ	53.7ᵃ	10.7ᶜ

ᵃᵇᶜ Mean separation in columns for each evaluation time by Duncan's multiple range test, 0.05 level.

tent of 1% $CaCl_2$ dipped fruit was greater than that of the 0.5% $CaCl_2$ dipped fruit, their firmness values were not different. After transfer to 20°C, the $CaCl_2$-treated slices were as firm as whole, freshly sliced fruit, and they were firmer than water-dipped slices.

Pears

The sliced pears respired at a higher rate than whole pears throughout the 8 day storage period (Fig. 2). However, the differences were only significant during the 7 days at 2.5°C. The whole fruit consistently produced more C_2H_4 than the sliced fruit throughout the 8-day storage period (Fig. 3). The lack of a wounding response was probably due to the advanced stage of ripeness (postclimacteric stage) and subsequent saturation of the enzyme system responsible for C_2H_4 biosynthesis. Typically, wound-C_2H_4 is observed in pre-climacteric fruit (McGlasson and Pratt, 1964; McGlasson, 1969; Hoffman and Yang, 1982).

Air-stored sliced fruit respired at a higher rate than air-stored whole fruit during the 7 days at 2.5°C (Fig. 4). The low O_2-stored slices respired at a lower rate than the air-stored fruit, although there were no differences in respiration rate among the three low O_2 levels.

C_2H_4 production differed among treatments, with whole fruit producing the most C_2H_4, followed by air-stored slices, 2.0%, 1.0% and 0.5% O_2-stored slices, respectively (Fig. 5). O_2 is required for C_2H_4 production (Adams and Yang, 1979); therefore, one would expect lower O_2 levels to result in lower C_2H_4 production. After transfer to 30°C and air, the whole fruit had a higher level of C_2H_4 production. There were no significant differences among the three O_2 levels.
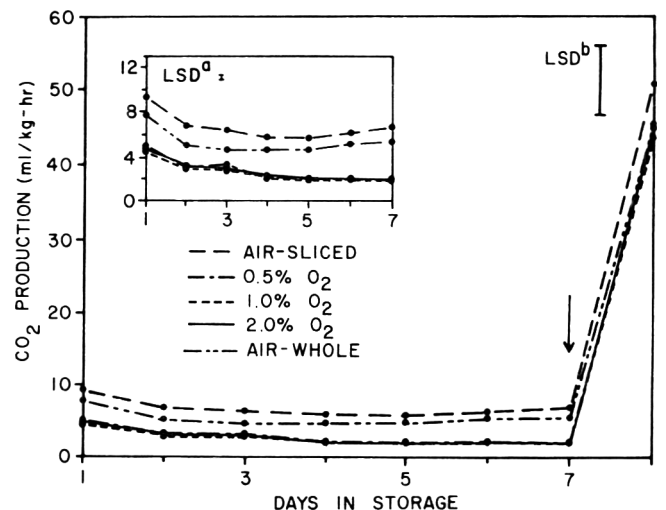
Fig. 4—CO_2 production by pears stored under various O_2 levels at 2.5°C for 7 days (inset) and after transfer (indicated by arrow) to 20°C and air for 1 day. LSDᵃ and LSDᵇ are the least significant differences at 0.05 level for days 1 to 7 and day 8, respectively.

After 8 days, slices stored under 0.5% O_2 were lighter in color than slices stored under air, with "L" values of 68.1 and 65.4, respectively. Also, the 0.5% O_2-stored slices were significantly firmer (34.7N) than the air-stored slices (25.4N) or whole freshly sliced fruit (28.9N) after 7 days at 2.5°C. CA storage has been shown to slow the rate of softening (Knee, 1980).

Slices from the 1.0% $CaCl_2$ dip treatment were lighter in color than water-dipped slices after 7 days at 2.5°C with "L" values of 72.6 and 67.9, respectively. $CaCl_2$ dip treatments were reported to reduce browning in apples (Drake and Spayd, 1983; Hopfinger et al., 1984). Citric acid and ascorbic acid dip treatments did not appear to be effective in minimizing browning (data not shown).

The 1.0% $CaCl_2$ treatment had an immediate firming affect on the slices since they were significantly firmer than water-dipped slices and whole, freshly sliced fruit (Table 3). The observed trend was consistent on day 7 and day 8.

The 1.0% $CaCl_2$ dip and the 0.5% O_2 treatments were found
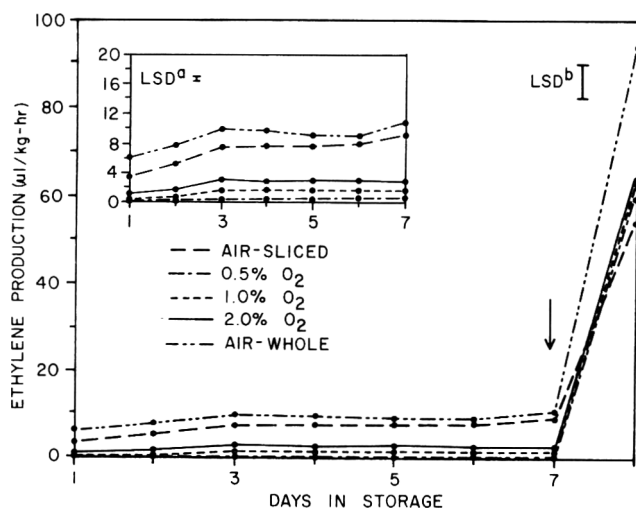
Fig. 5—Ethylene production by pears stored under various O_2 levels at 2.5°C for 7 days (inset) and after transfer (indicated by arrow) to 20°C and air for 1 day. LSD[a] and LSD[b] are the least significant differences at 0.05 level for days 1 to 7 and day 8, respectively.

Table 3—Effect of $CaCl_2$ dips on firmness (penetration force) of 'Bartlett' pears stored at 2.5°C for 7 days and at 20°C for 1 day

	Day 0		Day 7		Day 8	
Treatment	Firmness (N)	Ca (mg/100g)	Firmness (N)	Ca (mg/100g)	Firmness (N)	Ca (mg/100g)
Water	45.4[b]	7.8[b]	20.0[c]	7.3[b]	15.1[c]	6.8[b]
1.0% $CaCl_2$	49.4[a]	20.6[a]	35.1[a]	15.8[a]	28.0[a]	18.9[a]
No dip, freshly sliced	45.4[b]	7.3[b]	27.1[b]	6.8[b]	20.9[b]	6.1[b]

[abc] Mean separation in columns for each evaluation time by Duncan's multiple range test, 0.05 level.

Table 4—Effect of $CaCl_2$ and/or low O_2 on firmness (penetration force) of 'Bartlett' pear slices stored at 2.5°C for 7 days and at 20°C for 1 day

	Firmness (N)	
Treatment	Day 7	Day 8
Water + air	39.1[b]	29.8[c]
1% $CaCl_2$ + air	53.8[a]	39.6[b]
Water + 0.5% O_2	40.5[b]	38.3[b]
1% $CaCl_2$ + 0.5% O_2	59.2[a]	46.3[a]
Whole fruit, freshly sliced	34.7[b]	26.2[c]

[abc] Mean separation in columns for each evaluation time by Duncan's multiple range test, 0.05 level.

to be effective in maintaining firmness and a lighter color of slices. Therefore a combination of these two treatments was evaluated to determine whether they would have a synergistic effect. After 7 days at 2.5°C both $CaCl_2$ treatments resulted in firmer slices, although there was no difference between $CaCl_2$ + air or $CaCl_2$ + 0.5% O_2 (Table 4). On day 8 the combination of 1.0% $CaCl_2$ + 0.5% O_2 resulted in the firmest slices, followed by each of these treatments individually.

CONCLUSIONS

THE MAJOR CONSEQUENCES of slicing strawberries were an increase in CO_2 and C_2H_4 production and a loss of firmness. Storage in 12% CO_2 and $CaCl_2$ dips were effective in maintaining firmness of stored slices.

Slicing pears caused an increase in CO_2 production, but not in C_2H_4 production relative to intact fruit. Symptoms of deterioration due to slicing included browning and a loss of firmness. Storage in 0.5% O_2 was effective in reducing CO_2 and

C_2H_4 production and in minimizing browning and softening. A 1.0% $CaCl_2$ dip was effective in reducing browning and loss of firmness. A combination of 0.5% O_2 and 1.0% $CaCl_2$ resulted in greater maintenance of firmness than either treatment alone after return to air.

Citric acid and/or ascorbic acid dips were not effective in controlling browning. Although these acids are recommended to control browning, their beneficial effects may be short-term.

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Effects of Fruit Storage and Processing on Clarity, Proteins, and Stability of Granny Smith Apple Juice

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─────────── ABSTRACT ───────────

Granny Smith apples were processed into juices and concentrates from fruits which had been stored at 1°C for three months (short-term, ST) and nine months (long-term, LT). Total soluble proteins decreased with storage time of fruits. Juices processed from ST fruits were more resistant to haze formation (heat stability test) than those processed from LT fruits. For juices processed from ST apples, haze formation was related to proteins in the MW range of 21,000 to 31,000 daltons. For juices processed from LT apples, haze formation was not only related to proteins but also to other components in the juices. Fining was recommended to prevent haze and sediment formation.

INTRODUCTION

PROTEINS, independently and in association with phenols, are important in fruit juice turbidity and in post-clarification haze and sediment formation (Van Buren, 1972, 1983; Heatherbell, 1976, 1984; Gierschner et al., 1982; Lea, 1984; Nagel and Schobinger, 1985; Wucherpfenning et al., 1985; Flores et al. 1988). Therefore knowledge of the proteins present in juice is important to juice processors. However, the literature contains little information on the nature of proteins in apple juices. The prolonged storage of apple fruits is a prevalent commercial practice. Change in proteins during storage may influence the stability of juices produced from these fruits.

The objectives of this study were (1) to investigate the effect of prolonged storage of fruits on clarity, proteins and stability; (2) to investigate the effect of processing, particularly fining, on clarity, proteins, and stability; (3) to characterize the heat-unstable protein components; and (4) to investigate the relationship between proteins and juice stability.

MATERIALS & METHODS

Preparation of juices

Granny Smith apples obtained from the Mid-Columbia Experimental Station, Hood River, OR, which had been stored at 1 ± 2°C for three months (13.9 °Brix, pH 3.48) and nine months (13.4 °Brix, pH 3.73) were used. Fruits were processed into juices by standard procedures with and without fining in the pilot plant of the Dept. of Food Science & Technology, Oregon State Univ., Corvallis. Duplicate 30 kg lots of fruit were crushed for each trial and a 1% (v/v) solution of Rohapect D5L pectinase (Rohm Tech., Inc., Malden, MA) was added to a concentration of 50 mg/L and reacted at room temperature (20°C–23°C) for 90 min before pressing. Pressing was carried out in a hydraulic rack and cloth press. Particles were removed from the juice by racking through a 100 micromesh bag. Juices were then heated in an APV HTST unit to 85–90°C for 90 sec and cooled to 40°C. Enzyme clarification consisted of treatment with 100 mg/L each of Rohapect DA1L (a pectinase arabinase mixture; Rohm Tech., Inc., Malden, MA) and Rohapect HT amylase (Rohm Tech., Inc., Malden, MA). Juices were maintained at 45°C until the gel-forming pectin was no longer detected by alcohol test (Anonymous, 1982). Juices were fined with bentonite (sodium bentonite, Volclay), gelatin (Scott Labs., San

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Rafal, CA) and silica sol (clarifying agent C-2, Rohm Tech. Inc.) at equivalent rates of 500 mg/L, 100 mg/L and 300 mg/L, respectively. Approximately 20 min reaction time was allowed between fining treatments. Both fined and unfined portions were filtered with 1.0% SuperCel DE (Manville, Denver, CO) before bottling as bottled juices or concentrating to 70° Brix concentrates. The flow diagram of processing is shown in Fig. 1. Juice samples were obtained from each processing stage and analyzed immediately after processing. Concentrates were reconstituted to single strength juices with the same degree of Brix as bottled juices before analysis.

Determination of clarity

Clarity was measured by visual observation under a strong beam of light and by a Hunter Model D25P-2 Color Difference Meter operated in the transmission haze mode. Sediments in juice samples were resuspended before measurement.

Determination of total soluble proteins

All juice samples were filtered through 0.45 μm membrane before protein determination and gel electrophoresis. Total soluble proteins were then determined by using the modified Bio-Rad procedure as described by Hsu and Heatherbell (1987a).

Heat stability test

The heat stability of the juices was determined by the procedure recommended by Pocock and Rankine (1973). Fifty mL of filtered juices was transferred into 65 mL bottles. Bottles were sealed with screw caps and heated in a 80 °C water bath for 6 hr, held at 4 °C
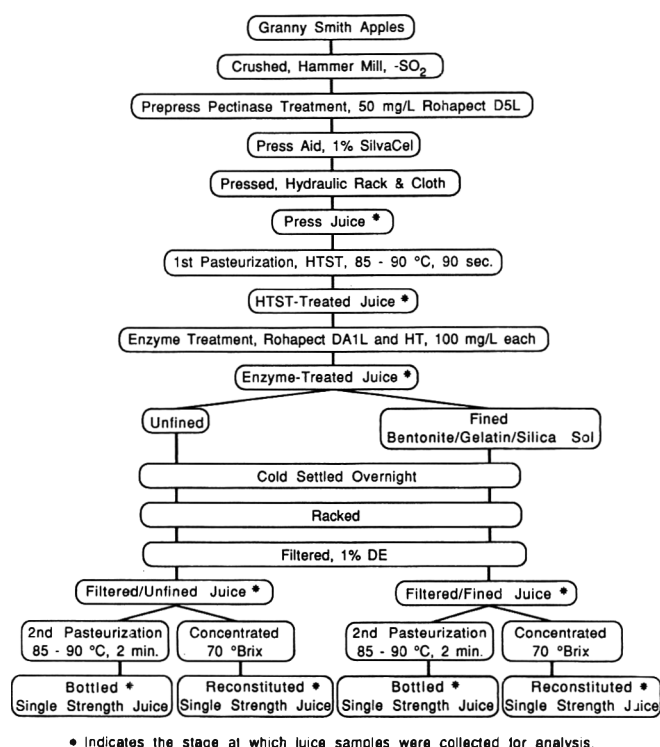
Fig. 1 — Flow diagram of Granny Smith apple juice processing.

for 12 hr and allowed to warm to room temperature. Formation of turbidity was measured by visual observation and by a Hunter Model D25P-2 Color Difference Meter.

Gel electrophoresis

Lithium dodecyl sulfate polyacrylamide gel electrophoresis (LDS PAGE), silver staining, protein blotting and glycoprotein detection were used for determination of protein molecular weights and glycoproteins as described by Hsu and Heatherbell (1987a) with the following modifications: A Bio-Rad protein 16-cm slab cell unit was utilized; a constant current setting of 10 mA per gel was used; and a Bio-Rad silver stain procedure was applied for the gel staining. Eighty μL of filtered juices was applied to gel.

RESULTS & DISCUSSION

Effect on clarity

The effect of fruit storage and processing on clarity of apple juice is shown in Table 1. Pre-filtration processing had similar effects on clarity regardless of the storage time of fruits. Following the enzyme treatment, all juices separated into a partial clarified supernatant and a settled floc. All juices were clear following filtration. The apparent high turbidity in the enzyme-treated juices resulted from the resuspension of settled solids before haze measurement. Fining prevented haze formation in bottled juices processed from short-term stored fruits. Bottled juices processed from long-term stored fruits showed higher haze formation than those processed from short-term stored fruits, and this was not prevented by fining. This might be due to changes in physical condition and chemical composition of fruits and the formation of turbidity precursors during fruit storage.

All juices were clear after filtration (Table 1), however, fining was necessary to stabilize juices from long-term stored fruits to heat stability testing (Table 3). Single strength juices (SSJ) reconstituted from concentrates were free of haze. In comparing the haze formation between bottled and reconstituted juices, pasteurization showed a more severe effect than concentration (Table 1).

Total soluble proteins and heat stability

Juices processed from long-term stored fruits had lower protein than those processed from short-term stored fruits (Table

Table 1—Effect of storage and processing on Hunter haze reading[a] and visible clarity[b] of Granny Smith apple juice

Juice samples[c]	Short-term storage		Long-term storage	
	Unfined	Fined	Unfined	Fined
Press juice	98.7 (+ + + +)		71.3 (+ + + +)	
HTST-treated	98.6 (+ + + +)		95.9 (+ + + +)	
Enzyme-treated	98.7 (+ + + +)		94.6 (+ + + +)	
Filtered	0.1 (−)	0 (−)	0.5 (−)	0.1 (−)
Bottled	2.7 (+)	1.0 (−)	1.5 (+ +)	1.9 (+ +)
Reconstituted	0.5 (−)	0.3 (−)	0.3 (−)	0.2 (−)

[a] Mean of duplicate processing trials.
[b] Clarity of visible observation indicated in brackets ( ): (−) = Clear; (+) = Slight Haze; (+ +) = Moderate Haze; (+ + +) = Strong Haze; (+ + + +) = Extreme Haze.
[c] Refer to Fig. 1.

Table 2—Effect of storage and processing on total soluble protein[a] in Granny Smith apple juice

Juice sample[b]	Short-term storage		Long-term storage	
	Unfined	Fined	Unfined	Fined
Press juice	21.1		5.1	
HTST-treated	39.8		13.8	
Enzyme-treated	26.8		9.9	
Filtered	17.2	12.2	15.6	10.3
Bottled	20.7	15.7	13.5	7.6
Reconstituted	19.9	14.7	15.1	10.2

[a] Mean of duplicate processing trials, mg/L.
[b] Refer to Fig. 1.

Table 3—Effect of storage and processing on Hunter haze reading[a] and visible clarity[b] of Granny Smith apple juice after heat stability testing

Juice samples[c]	Short-term storage		Long-term storage	
	Unfined	Fined	Unfined	Fined
Bottled	0.3 (−)	0 (−)	0.2 (−)	0.3 (−)
Reconstituted	0.3 (−)	0.3 (−)	1.3 (+)	0.2 (−)

[a] Mean of duplicate processing trials.
[b] Clarity of visible observation indicated in brackets ( ): (−) = Clear; (+) = Slight Haze.
[c] Refer to Fig. 1.

2). In general, high temperature short time heat treatment (HTST, first pasteurization) of press juices increased the total soluble proteins in the juice samples. This may be due to extraction of proteins from the fruits' cell walls or heat-induced dissociation of protein-phenolic complexes exposing more detectable proteins or heat-unfolding of protein molecules exposing more detectable reactive sites. However, enzyme treatment and fining reduced the concentration of total proteins. Fining reduced the protein concentration by approximately 5 mg which accounted for approximately 30% of the total proteins compared with unfined juices. Neither pasteurization nor concentration of DE pad filtered juices appeared to influence the level of total proteins.

Effects of fruit storage and processing on clarity of apple juice after heat stability testing are shown in Table 3. Reconstituted SSJ from unfined concentrate processed from long-term stored fruit formed a slight haze after heat stability testing. Reconstituted SSJ processed from short-term stored fruits had higher protein than those processed from long-term stored fruits (Table 2) but were more heat-stable (Table 3). It was concluded that total soluble proteins did not correlate with the juice stability. Similar results were found in wine (Moretti and Berg, 1965; Hsu and Heatherbell, 1987b). Only a part of the protein present was responsible for protein instability rather than total protein.

Characterization of Soluble Proteins

To further understand the behavior of proteins during fruit storage and processing, lithium dodecyl sulfate polyacrylamide gel electrophoresis (LDS PAGE) and protein blotting for glycoprotein (GP) were used to characterize the individual proteins. The electrogram of short-term storage apple juices sampled during processing is shown in Fig. 2. The main protein fractions in press juice had MW in the range of 11,000 to 66,000 daltons (Fig. 2(A)). An increase in the number of protein fractions was detected in HTST treated juices. Protein fractions with MW in the range of 64,000 to 92,000 daltons were mainly GP and were detected in enzyme (arabanase and amylase) clarified juice (Fig. 2(C)). These fractions were not detected in either press juices or HTST-treated juices and were identified as enzyme proteins which were added during arabanase and amylase enzyme treatment. These fractions were not removed by fining and DE pad filtration and remained in the reconstituted SSJ. They were removed only during the second pasteurization at bottling and were not detected in bottled juices (Fig. 2 (E), (H)), (Bottled juices were filtered before gel electrophoresis, refer to Materials & Methods). Therefore, the possibility that certain enzyme protein fractions might remain in clarified juices during processing with the potential to contribute to instability must be considered. Fining removed mainly the low MW fractions of apple proteins particularly those with a MW less than 31,000 (Fig. 2 (D), (G)). In contrast, the second pasteurization tended to remove the high MW (>25,000) fractions (Fig. 2 (D), (E)). The data of heat stability testing showed that only unfined reconstituted SSJ formed haze (Table 3). By comparing the samples E, F, H and I in Fig. 2, the results indicated that protein fractions in the MW range of 21,000 to 31,000 were related to juice instability and haze formation. Similarly, it was recently demonstrated for grape
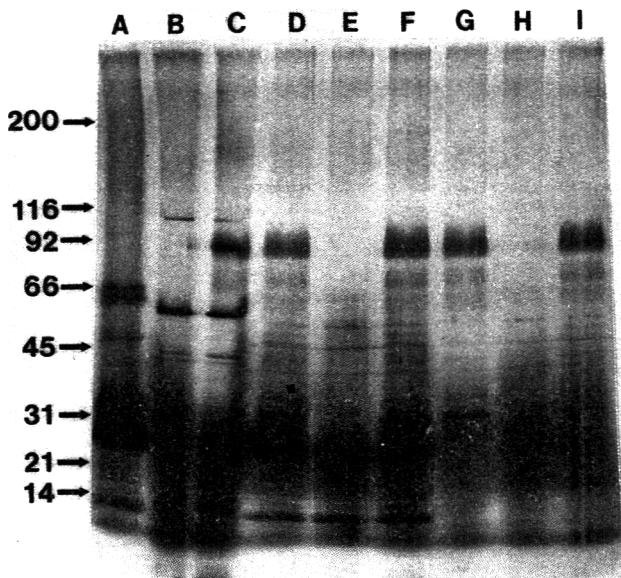
Fig. 2—LDS PAGE of Granny Smith apple juices processed from short-term stored fruits. (A) Press juice. (B) HTST-treated juice. (C) Enzyme-treated juice. (D) Unfined/filtered juice. (E) Unfined bottled juice. (F) Unfined reconstituted juice. (G) Fined/filtered juice. (H) Fined bottled juice. (I) Fined reconstituted juice. 80 μL of each sample was applied to gel. Molecular weights (daltons × 10⁻³) of standards are given on the left side of gel.
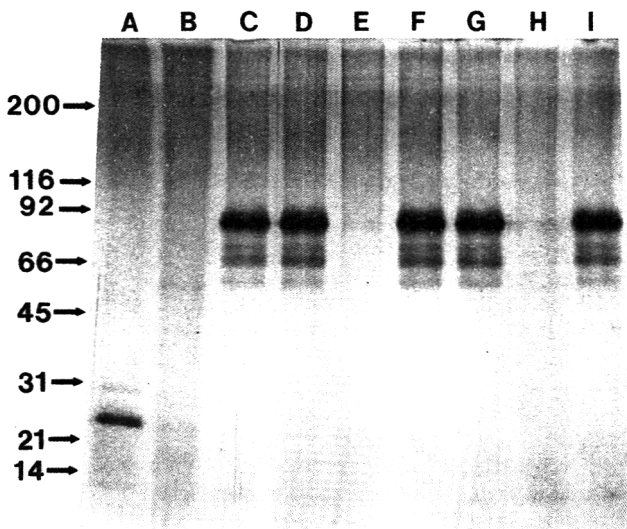
Fig. 3—LDS PAGE of Granny Smith apple juices processed from long-term stored fruits. (A) Press juice, (B) HTST-treated juice. (C) Enzyme-treated juice. (D) Unfined/filtered juice. (E) Unfined bottled juice. (F) Unfined reconstituted juice. (G) Fined/filtered juice. (H) Fined bottled juice. (I) Fined reconstituted juice. 80 μL of each sample was applied to gel. Molecular weights (daltons × 10⁻³) of standards are given on the left side of gel.

juice and wine that it is important to remove fractions of lower MW (12,600–30,000) and lower pI (4.1–5.8) to achieve stability (Hsu and Heatherbell, 1987b; Hsu et al., 1987; Heatherbell and Flores, 1988).

The electrophoretogram of juices from long-term stored apple is shown in Fig. 3. Fewer protein fractions were detected in juices from long-term stored fruits. Only two protein fractions with MW in the range of 21,000 to 31,000 were detected in press juices (Fig. 3(A)). Unlike the electrophoretogram of

short-term stored apple juices, no new fractions were detected in the first pasteurization (HTST) treated juice (Fig. 3(B)). Enzyme fractions were detected in juices after arabanase and amylase treatment as previously described in the short-term storage processing trials. No differences in the protein profile between fined (Fig. 3(G)) and unfined (Fig. 3(D)) samples were detected, even though unfined juice formed a slight haze after heat stability testing. This indicated that haze formation in juice processed from long-term stored apples might not only be related to proteins but might also be related to other components in the juices. In support of this, sediments from bottled juices were collected, analyzed and found to contain proteins, phenols and trace amounts of neutral polysaccharides. Therefore, appropriate comprehensive fining in conjunction with a suitable heat treatment (Heatherbell, 1984) may be necessary to prevent haze and sediment formation in some clarified apple juices and concentrates.

Similar studies on grape juice (Heatherbell and Flores, 1988; Flores et al., 1988) have also indicated that it is not only the amount but also the nature/state of compounds such as proteins, phenols and polysaccharides and their interactions that result in instability. A related study on the phenols present in the juice samples in this study and their changes during processing is being reported elsewhere (Spanos and Wrolstad, 1989). Further research is required at the molecular level to fully understand these events.

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Thermophysical Properties of Clarified Apple Juice as a Function of Concentration and Temperature

D.T. CONSTENLA, J.E. LOZANO, and G.H. CRAPISTE

─────────── ABSTRACT ───────────

Effect of concentration and temperature on thermophysical properties of clarified apple juice was studied. Density, viscosity, specific heat, and thermal conductivity were measured at different conditions, ranging from 20 to 90°C and 12 to 70°Brix. Experimental data were related to the corresponding properties of water and compared with the behavior of sugar solutions. The results obtained were used to derive mathematical models and correlations for predicting these properties as a function of both concentration and temperature.

INTRODUCTION

KNOWLEDGE of the essential thermophysical properties as density, viscosity, specific heat and thermal conductivity is of primary importance to the fruit juice industry. This information is needed for a variety of research and engineering applications. To design and optimize the operation of handling and processing units such as tanks, pumps, pipes, chillers, heaters and evaporators, information on thermophysical and thermodynamical properties is required over a wide range of concentrations and temperatures. Recently the authors of this paper studied the effect of concentration and pressure on the boiling point rise of clarified apple juice and pointed out the need for accurate thermophysical properties data (Crapiste and Lozano, 1988).

Existing experimental values of thermophysical properties for a number of liquid foods have been reviewed by Rha (1975), Polley et al. (1980) and Choi and Okos (1983), but no report covers apple juice extensively. The rheological behavior of apple juice has been studied by Saravacos (1970), Rao et al. (1984) and Ibarz et al. (1987). Information on thermal conductivity of fruit juices, in particular apple juice, has been reported by Riedel (1949) and Ziegler and Rizvi (1985). However, at present no attempt was made to measure and model all the above properties at the conditions usually found in the apple juice industry.

The purpose of the present work was to measure density, viscosity, specific heat, and thermal conductivity of clarified apple juice at different concentrations and temperatures and to develop correlations for predicting these properties under different conditions.

MATERIALS & METHODS

APPLE JUICE at various soluble solid contents, in the range 12–70°Brix, were made by reconstituting with distilled water a 70°Brix concentrate obtained from Coop Julia & Echarren (Buenos Aires, Argentina). The main characteristics of this concentrate are summarized in Table 1 (Crapiste and Lozano, 1988). Soluble solids were measured as °Brix with a bench refractometer at 20.0 ± 0.1°C. Thermophysical properties were determined at different temperatures ranging from 20 to 80°C and accurately controlled to ±0.02°C with a

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Table 1 — Specifications of the apple juice concentrate[a]

Soluble solids, °Brix	12.0
Reducing sugars, g/kg	91.5
Total sugars, g/kg	107.7
Total acidity, g/L	6.0
Total phenolics, ppm	6.9
Total aminoacids, meq/L	7.6

[a] Rediluted to 12°Brix

constant temperature circulation bath (Cole-Parmer Instrument Co., Illinois, USA).

Measurement of density

Density was determined by pycnometric and hydrostatic methods. Both methods were checked with sucrose solutions whose densities at various concentrations and temperatures are known (Honig, 1953; Weast, 1985). In the first method density was determined by weighing the juice contained in standard volumetric pycnometers. The pycnometers of 25 and 50 mL capacity were calibrated with distilled water at each temperature. Corrections for temperature were shown to be negligible. A minimum of five consecutive replicates per sample with a reproducibility better than 0.2% were averaged. To check results, a set of determinations were made with an hydrostatic method based on the measurement of the buoyant force acting on a tare body submerged in the juice. Comparable results were obtained and no significant differences between methods were observed.

Measurement of viscosity

It was found that clarified apple juices behave as a Newtonian fluid over the ranges of concentration and temperatures used in this work (Saravacos, 1970; Ibarz et al., 1987). Kinematic viscosity was determined by using Cannon-Fenske glass capillary viscometers, calibrated at different temperatures with distilled water and aqueous sucrose solutions (Honig, 1953; Perry and Chilton, 1973; Weast, 1985). Efflux times greater than 200 sec were used and kinetic-energy corrections were negligible. The viscosity of each solution was also measured with a Haake Falling Ball Viscometer (Haake Mess-Tecknick GnbH u Co., Germany). Three consecutive measurements, both for flow time and falling time, which were averaged to evaluate viscosity had a reproducibility of about 0.1%.

Determination of thermal conductivity

Thermal conductivity was measured with a thermal conductivity probe, a test body made basically of a line source providing a constant amount of heat and a temperature measuring device. Details of the method have been extensively reviewed in the literature (De Vries and Peck, 1958; Ziebland, 1969; Nagasaka et al., 1973). A complete discussion of the design and operation of the probe and the testing procedure used in this work was reported by Lozano et al. (1979). The thermal conductivity was calculated on the basis of the equation:

$$T - T_1 = Q/4\pi k \, \ln(t/t_1) \qquad (1)$$

where T, T_1, t, and t_1 are temperatures and times corresponding to final and initial running time respectively, Q is the heat produced per unit length of probe and k is the thermal conductivity of the medium. Recorded times and temperatures were correlated through a least squares procedure (Daniel and Wood, 1980) and runs with a coefficient of determination less than 0.99 were rejected. Error due to natural convection was avoided by adding 0.5% xantham gum (Keltrol, Merck & Co. Inc.) to the juice. The probe was tested by determining thermal

conductivity of water (0.5% xantham gum), glycerine and selected water-glycerine solutions (Perry and Chilton, 1973). It was found that the experimental values reproduced the actual values within a ±2%.

Determination of specific heat

The specific heat of the juice was determined with a differential scanning calorimeter (Perkin-Elmer DSC-2) using the procedure for specific heat determination with water as external standard and graphical correction for sloping baseline (Perkin-Elmer, 1979). Samples were weighted with a precision of 0.0001 g and encapsulated into aluminum pans. Temperature was continuously scanned from 20 to 100°C and the heating rate was 10°C/min. Specific heat at any temperature was calculated by:

$$Cp_s = W_w D_s Cp_w / W_s D_w \qquad (2)$$

where Cp, W and D are the specific heat, weight and recorder displacement for sample(s) and water(w) respectively.

Statistical analysis

Experimental data of thermophysical properties were analysed with the BMDP (1983) statistical software which allows the determination of the goodness of fit for multiparametric models and the significance of the regression.

RESULTS & DISCUSSION

Density

Experimental results of density of apple juice at seven selected soluble solids levels ranging from 12 to 68.5°Brix and at 20, 40, 60, and 80°C are represented in Fig. 1. There was a very strong dependence of density with concentration and a noticeable decrease as temperature increased.

Different types of equations have been used to correlate experimental density data of sugar solutions and liquid foods, many of them resulting in complex polynomials in concentration and temperature (Choi and Okos, 1983; Maxwell et al.,

Fig. 1. — Experimental density of apple juice as a function of concentration and temperature. Predictions of Eq. (3) and (6) [— —].

1984). In the present work, several simple equations were proposed and run through the BMPD programs for statistical residual analysis. The following equation resulted as the best fit for the obtained density data of apple juice (with an adjusted squared multiple correlation coefficient $r^2 = 0.9978$ and a standard error of estimate $s = 0.0040$):

$$\rho = 0.82780 + 0.34708 \exp(0.01 X) - 5.479 \times 10^{-4} T \quad (3)$$

where ρ is the density (g/cm³), X the concentration (°Brix) and T the temperature (°K).

A theoretical approach also was proposed by considering the thermodynamic expression for the specific volume of a multicomponent solution (reciprocal of density) in terms of partial specific volumes:

$$V = 1/\rho = \Sigma w_i v_i \qquad (4)$$

where w_i and v_i represent the mass fraction and the partial specific volume of the i-component in solution, respectively. In water solutions, this volume is not necessarily equal to the specific volume of the pure component. Sugars, organic acids and different macromolecules interact with a substantial number of water molecules, resulting in a nonideal solution behavior. In the dilute limit, v_w (water) has contributions mainly from structured free solvent regions while v_s (solute) is affected by hydration and water-solute interactions. In the concentrated limit, v_w is defined by water-solute aggregations, i.e: hydrogen bonded to hydroxyl groups. For these reasons, in sugar solutions both v_w and v_s are functions of concentration and temperature (Taylor and Rowlinson, 1955; Maxwell et al., 1984).

It is important to note that the coefficient of thermal expansion of crystalline glucose and sucrose is about 3.8×10^{-4} °C^{-1} between 20 and 60°C (Maxwell et al., 1984), very close to the average value for pure water at these temperatures (Perry and Chilton, 1973). Similar values, in the range $3.8-4.6 \times 10^{-4}$ °C^{-1}, can be obtained from the experimental data of the density of sugar solutions (Honig, 1953) and apple juice at different concentrations. On this basis, we suggest that the thermal effect on density can be significantly reduced by referring the specific volume to that of pure water v_{wo}, so that Eq. (4) can be written as:

$$V/v_{wo} = v_w/v_{wo} + w_s (v_s - v_w)/v_{wo} \qquad (5)$$

Although, according to the above discussion the partial specific volumes depend on concentration, from a practical point of view a linear relationship like that suggested by Eq.(5) can be used to correlate density data. In that case, the resulting coefficients represent average values of v_i in the range of concentrations under consideration. With apple juice an excellent correlation was obtained ($r^2 = 0.9989$ and $s = 0.0021$) and the final equation can be rearranged as:

$$\rho = \rho_w / (0.992417 - 3.7391 \times 10^{-3} X) \qquad (6)$$

The influence of temperature was also studied by including an additional linear term in Eq.(5) and using statistical analysis. The value obtained for the F-statistic indicates that temperature can be excluded as a variable in the representation. The agreement between density curves evaluated with Eq. (3) and (6) and experimental data are shown in Fig. 1. Equation (6) provides a simple and accurate means to obtain ρ as a function of concentration and temperature. Supplementary measurements showed that predictions of this equation were also extrapolatable to other temperatures in the range 10-90°C.

Viscosity

Viscosity of apple juice increased rapidly with soluble solids and decreased considerably as the temperature was increased from 20 to 80°C, particularly at the higher concentrations (Fig. 2). A reasonable good agreement was obtained when these experimental results were compared with those reported in pre-
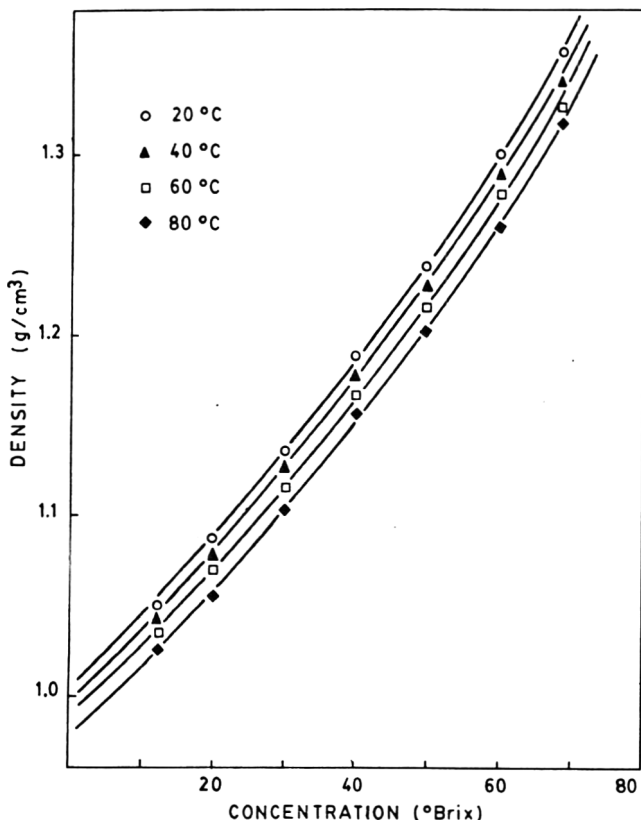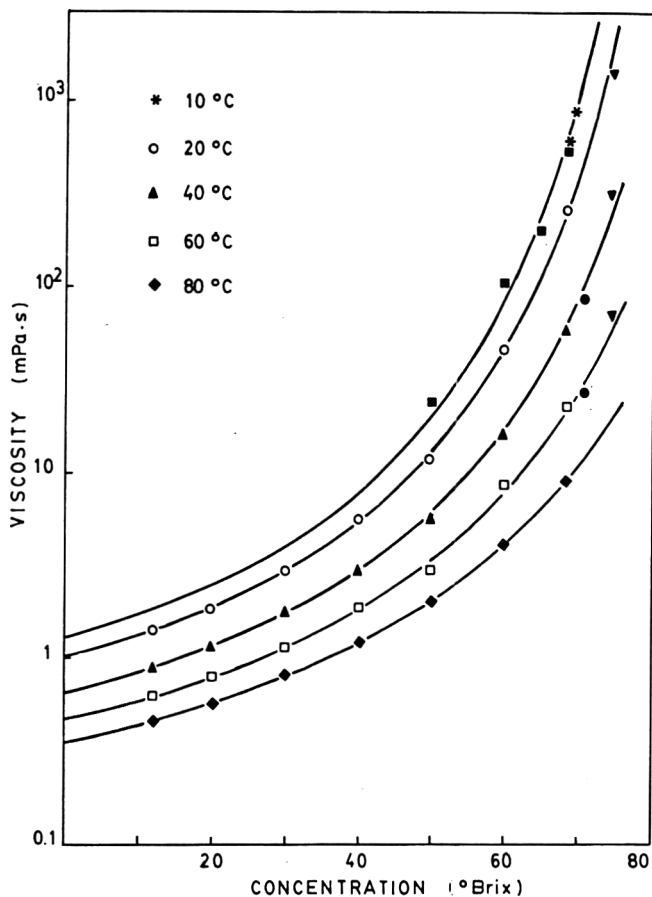
Fig. 2.—Effect of concentration and temperature on viscosity of apple juice. ■10°C (Rao et al., 1984); ●72°Brix (Ibarz et al., 1987); ▼ 75°Brix (Saravacos, 1970). Predictions of Eq. (10) [—].

vious studies on viscosity of apple juice (Saravacos, 1970; Rao et al., 1984; Ibarz et al., 1987).

As in the case of density, the viscosity of a solution is a function of the intermolecular forces and water-solute interactions that restrict the molecular motion. These forces depend upon the intermolecular spacings and the strength of hydrogen bond, and are affected by changes in both concentration and temperature. When more solutes like sugars are dissolved in the solution, the viscosity increases because of the increase in hydrogen bonding with hydroxyl groups and the distortion in the velocity pattern of the liquid by hydrated molecules of solute. When the solution is heated, the viscosity decreases since the thermal energy of the molecules increases and the intermolecular distances increase due to thermal expansion.

The effect of temperature on viscosity can be described by the Arrhenius relationship:

$$\mu = \mu_o \exp (Ea/RT) \qquad (7)$$

where μ is the viscosity (mPa.s or cp), μ_o a preexponential factor, Ea the activation energy of flow (kcal/gmole) and R the gas constant (1.987×10^{-3} kcal/gmole °K). The average values of Ea between 20 and 80°C were evaluated by linear regression analysis and ranged from 4.7 kcal/gmole for a 12°Brix juice to 11.4 kcal/gmole for a 68.5°Brix juice. The magnitudes of Ea obtained in this work are similar to those of sucrose solutions at the same concentrations and comparable to those reported in previous studies (Saravacos, 1970; Rao et al., 1984).

Rao et al. (1984) and Ibarz et al. (1987) reported that the effect of concentration on viscosity of fruit juices at constant temperature can be represented by either an exponential-type or a power-type relationship. However, we found that when concentration was introduced in Eq. (7) very complex expressions for Ea and μ_o resulted. Since these equations are cum-

bersome, a different approach was used. A true solution like sugar solutions and clarified apple juice can be considered as a suspension of very small particles, i.e: hydrated molecules of solute. Several equations have been suggested to describe the effect of concentration on viscosity of suspensions (Perry and Chilton, 1973). Mooney (1951) developed the following semitheoretical expression to evaluate the relative viscosity (ratio of the viscosity μ to that of the suspending fluid μ_{wo}) of concentrated solutions:

$$\ln \mu/\mu_{wo} = 2.5\phi / (1-K\phi) \qquad (8)$$

where ϕ denotes the volume fraction of solids and K is a coefficient which take into account the interactions between particles. This equation reduces to the classic equation of Einstein for an infinitely dilute suspension of spheres and K usually varies from 1 to 1.9 for monodisperse systems (Mooney, 1951; Perry and Chilton, 1973). In order to express the concentration on a weight basis, as °Brix, and to take into account the effect of temperature, Eq. (8) was modified to:

$$\ln \mu/\mu_{wo} = AX / (100-BX) \qquad (9)$$

where the coefficients A and B are functions of temperature. Using Mooney's result for polydisperse suspensions and assuming similar interactions between different particles, it can be proved that for a multicomponent solution Eq. (8) can be extended to:

$$\ln \mu \cdot \mu_{wo} = \Sigma A_i w_i / (1-\Sigma B_i w_i) \qquad (10)$$

It can be shown that a linear dependence of A with the inverse of temperature is needed to be consistent with the Arrhenius representation. On the other hand, a small decrease in the value of B with temperature can be expected because of the reduction in particle interactions due to thermal expansion. On this basis, the following functional relationships for A and B were proposed and fitted with a nonlinear multiparametric program to obtain (s = 0.0417):

$$A = -0.25801 + 817.11/T ; \qquad (11)$$
$$B = 1.8909 - 3.0212 \times 10^{-3} T$$

The viscosity values obtained through Eq. (9) and (11) are compared with the experimental data in Fig. 2 and a very good agreement can be observed. Further determinations of viscosity of apple juice at 68.5 and 69.5°Brix were made to evaluate the applicability of Eq. (9) to predict the behavior at different conditions. Results indicated that at 10°C, a normal temperature for the bulk storage of concentrate apple juice, the estimated error when applying Eq. (9) was less than 5%. At temperatures below 10°C viscosity determinations with the falling ball viscometer were more reproducible and simpler than with the other reported method. As an additional test of the proposed equation, the experimental results of Saravacos (1970) and Ibarz et al. (1987) at higher concentrations and those of Rao et al. (1984) at 10°C are also included in Fig. 2. It can be seen that, in spite of the different sources of data, the predictions are quite satisfactory.

Thermal conductivity

Results of thermal conductivity experiments at 20, 40, and 60°C are shown in Fig. 3. It should be noted that a set of experiments was carried out at 80°C but, since that convective effects became important at this temperature, those runs were rejected by the statistical procedure. The experimental values at 20°C were in agreement with those of Riedel (1949) but lower than those obtained by Ziegler and Rizvi (1985) by the thermal comparator method.

Several equations have been proposed to model thermal conductivity of liquid foods and its dependence on concentration and temperature (Rha, 1975; Cuevas and Cheryan, 1978; Choi and Okos, 1983). It can be seen (Fig. 3) that the thermal
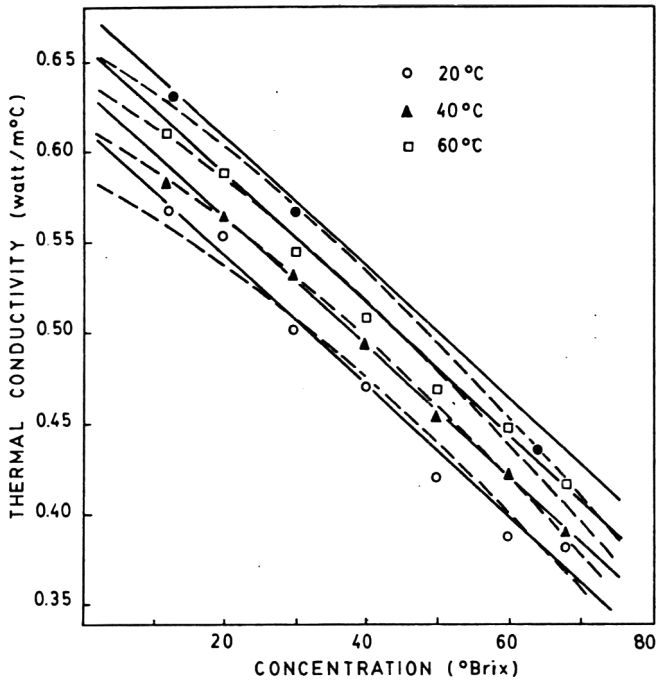
Fig. 3.—Thermal conductivity of apple juice as a function of concentration and temperature. ●80°C (Riedel, 1949). Predictions of Eq. (12) [——]; theoretical predictions of Eq. (15) [------].

conductivity of apple juice decreased linearly with increasing soluble solids, a characteristic of sugar solutions and fruit juices (Riedel, 1949). For simple liquids, thermal conductivity usually changes linearly with temperature (McLaughlin, 1969) and approximately the same behavior was observed in this work. Thus a linear dependence on concentration and temperature was proposed and correlated to obtain ($r^2 = 0.9988$ and $s = 0.0077$):

$$k = 0.27928 - 3.5722 \times 10^{-3} X + 1.1357 \times 10^{-3} T \quad (12)$$

where k represents the thermal conductivity of apple juice (watt/ m°K).

From a theoretical point of view, the thermal conductivity of a solution can be evaluated from the apparent thermal conductivity and the volume fraction of each component as:

$$k = \Sigma k_i \phi_i \quad (13)$$

This additive model is based on the assumption that constituents are positioned in layers parallel to the heat flow. In water solutions, since the transfer of energy by conduction is mainly associated with vibrational mechanisms (McLaughlin, 1969), apparent thermal conductivities depend on temperature (Choi and Okos, 1983) and in a lesser extent on concentration. The most important factor in controlling the temperature dependence of k in liquids is the coefficient of thermal expansion (McLaughlin, 1969). Thus, on the same basis that has been discussed above, we suggest that the effect of temperature can be computed by referring k to the thermal conductivity of pure water k_{wo}. In addition ϕ_i can be expressed in terms of the mass fraction ($\phi_i = v_i w_i / V$) so that Eq. (13) can be rearranged to:

$$k' = k \rho_w / k_{wo} \rho = k'_w + (k'_s - k'_w) w_s \quad (14)$$

where k'_i represents a reduced thermal conductivity ($k'_i = k_i v_i / k_{wo} v_{wo}$). Based on this result, an equation for k' as a linear function of concentration was proposed. As for density, the statistical test indicated that temperature can be eliminated as an additional variable in Eq. (14). The final correlation for apple juice resulted in:

$$k = k_{wo} \rho / \rho_w (0.9789 - 0.007719 X) \quad (15)$$

As is shown in Fig. 3, both Eq. (12) and (15) provide a reasonably good representation of the data within the order of experimental errors. A very good agreement between predictions of Eq. (15) at 80°C and the results of Riedel (1949) was obtained.

Specific heat

Specific heat of apple juice resulted in practically a linear function of soluble solids in the range 6 to 75°Brix (Fig. 4). Also a continuous increase with temperature between 30 and 90°C was observed. Experimental data were correlated to obtain ($r^2 = 0.9900$ and $s = 0.0098$):

$$Cp = 0.80839 - 4.3416 \times 10^{-3} X + 5.6063 \times 10^{-4} T \quad (16)$$

where Cp is the specific heat of juice (cal/g°C). It can be seen in Fig. 4 that Eq. (15) gives a good fit of the experimental data in the entire range of concentrations and temperatures under consideration.

As in the case of density and thermal conductivity, a theoretical approach can be used to predict the specific heat of a solution in terms of partial specific heats of individual components as follow:

$$Cp = \Sigma Cp_i w_i \quad (17)$$

A linear relationship of Cp with concentration like that suggested by Eq. (17) is the basis for most of the existing correlations to evaluate specific heat of liquid foods (Choi and Okos, 1983). It must be taken into account that, because of water-solute interactions, Cp_i differs from the specific heat of pure component and usually changes with the concentration of soluble solids. In fact, the resulting values of Cp_s for sugar solutions are significantly higher than those corresponding to crystalline sugar at the same temperature (Taylor and Rowlinson, 1955; Pancoast and Junk, 1980) while at high water contents Cp_w approximates to the heat capacity of pure water Cp_{wo}. In addition, while Cp_{wo} remains almost constant with temperature, the specific heat of the solution increases with this variable following the same pattern as that of crystalline sugar. This behavior was also observed in apple juice so that no
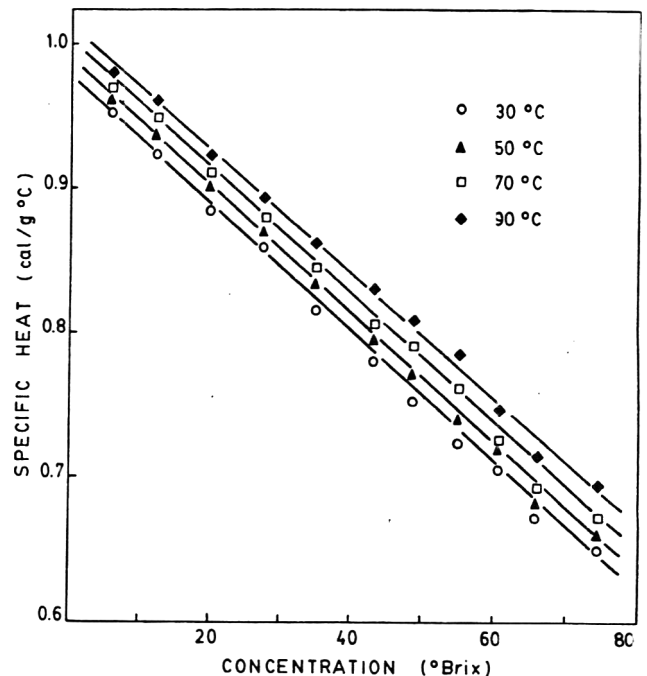
Fig. 4.—Specific heat of apple juice as a function of concentration and temperature. Predictions of Eq. (16) [——].

improvement in the correlation ability of Eq. (17) was obtained by using the ratio Cp/Cp_{wo}.

Compositional effects

Compositional effects on thermophysical properties of fruit juices are not very well established. However, these properties depend on composition and they should be theoretically predicted from the thermophysical properties and fractional composition of individual constituents. Clarified fruit juices contain different amounts of dissolved sugars, salts and organic acids. Composition of apple juices from different sources has been reported by Mattick and Moyer (1983). Fructose, glucose and sucrose are the most important constituents of clarified apple juice, accounting for more than 90% of soluble solids, so that thermophysical properties should be largely determined by the type and concentration of sugars. On this basis, the predictive ability of Eq. (4), (10), (13), and (17) for density, viscosity, thermal conductivity and specific heat respectively was studied. The resulting coefficients of the above equations for different sugars at 20-25°C, as they compare with those obtained for apple juice, are presented in Table 2. Thermophysical properties data for sugar solutions were obtained from Riedel (1949), Honig (1953), Taylor and Rowlinson (1955), Pancoast and Junk (1980) and Weast (1985). The properties of fructose, glucose and sucrose solutions appear to be very similar.

Results indicated that there was a difference at the 1% level between density data of apple juice and sugar solutions. It can be observed (Fig. 5) that at the same concentration expressed as mass fraction, the juice has a density a little higher than the sugar solutions, which means an average specific volume of solids v_s smaller than those corresponding to sugars (Table 2). Thus, it seems that Eq. (4) slightly underpredicts the density of apple juice. If the effect of the minor components is taken into account, a decrease in density should be expected since a typical value of v_s for proteins, the most important minor component in apple juice, is about 0.73 cm³/g (Kunz and Kauzmann, 1974). The observed behavior could be explained by considering the influence of organic acids in the refractometric reading of soluble solids. Millies and Burkin (1984) reported that a reduction up to 3.5% in the refractometric value was found in a concentrated apple juice with a malic acid content similar to that of the juice samples used in this work. Therefore, the reported value of density for a 63.5°Brix could be comparable to a sugar solution of 70.8% soluble solids. No significant differences between densities of grape juice, orange juice and sucrose solutions at 20°C were found by Moresi and Spinosi (1980, 1984), the °Brix reading being corrected by acidity. Thus, it appears that density of fruit juices can be predicted by Eq. (4) using their main sugar composition if the correction by acidity in the refractometric reading is taken into account.

The specific heat of apple juice decreased less rapidly with soluble solids than those of pure sugar solutions (sets of data were statistically different at the 99% confidence level) (Fig.
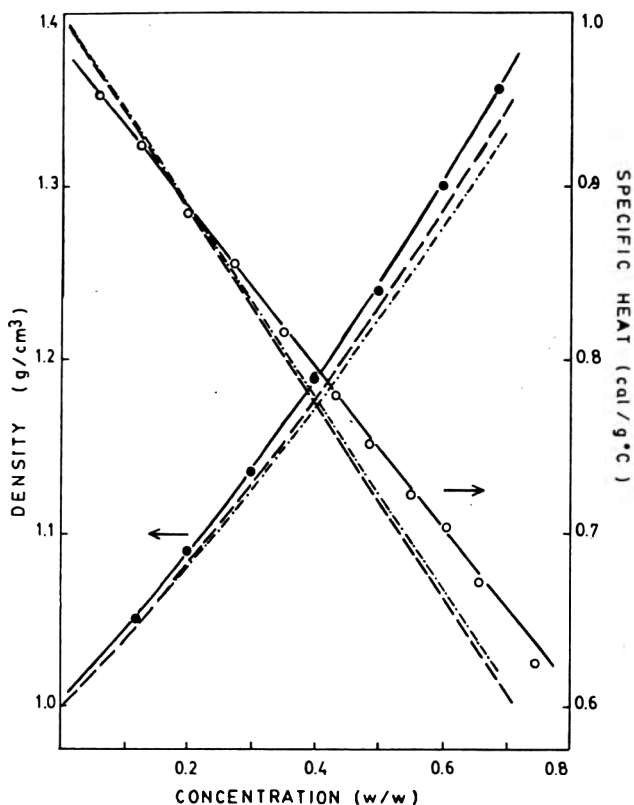
Fig. 5. — Effect of concentration on density and specific heat of apple juice and sugar solutions at 20-25°C. ○ ● apple juice; sucrose solutions [------]; glucose solutions [−·−·−].
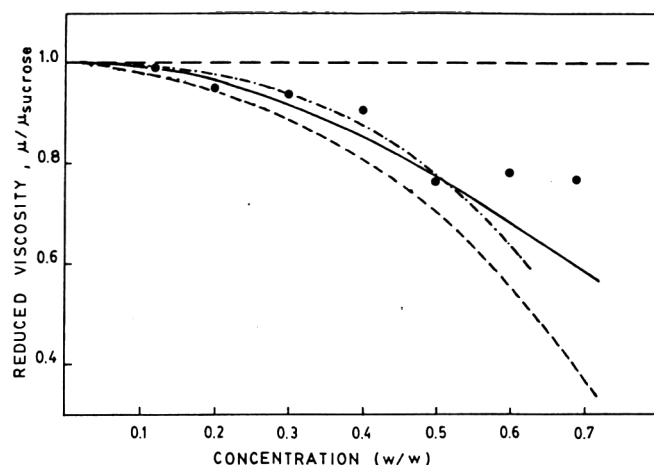
Fig. 6 — Effect of concentration on the reduced viscosity at 20 °C. ● apple juice; glucose solutions [−·−·−]; fructose solutions [----]; Theoretical predictions of Eq. (10) [——].

Table 2 — Values of coefficients for evaluating thermophysical properties with the proposed models at 20°C

Eq.	Coefficient[a]	Sucrose	Glucose	Fructose	Apple juice
4	v_s	0.6261	0.6323	0.6277	0.6145
	v_w	0.9956	0.9962	0.9950	0.9921
9	A	2.6122	2.5617	2.4153	2.5289
	B	1.0381	0.9725	0.9807	1.0052
14	k_s	0.2090	0.2142	–	0.2070
	k_w	0.9790	0.9810	–	0.9789
17	Cp_s	0.4400	0.4425	–	0.5242
	Cp_w	0.9971	0.9989	–	0.9773

[a] v_s = partial specific volume of solute; v_w = partial specific volume of water; A and B = functions of temperature; k_s = thermal conductivity of solute; k_w = thermal conductivity of water; Cp_s = partial specific heat of solute; Cp_w = partial specific heat of water.

5). Moresi and Spinosi (1980, 1984) reported the opposite behavior for orange and grape juices at 20°C and concluded that minor components reduced the Cp values. However, using suggested values for Cpi (Kunz and Kauzmann, 1974; Choi and Okos, 1983) in Eq. (17), it can be estimated that the influence of these components on Cp of fruit juices is practically negligible. Obviously more work is needed in this area to explain the above discrepancies.

On the other hand, comparison between viscosities of apple juice and sugar solutions is shown in Fig. 6. A reduced viscosity using μ of sucrose solutions as reference was used to visualize the differences. It can be seen that viscosity of apple juice lies between the corresponding curves for sucrose and reducing sugars. Predictions of Eq. (10), for a model system

made with sugars in the same proportions present in apple juice, were also plotted in Fig. 6. Equation (10) gives a reasonable estimate of the behavior of apple juice, the difference being associated with the presence of nonsugar organic components which usually tend to increase the viscosity. Some discrepancies can be also attributable to the effect of malic acid on the refractometric measurement of soluble solids (Millies and Burkin, 1984). It was observed that viscosity data of clarified pear juice (Ibarz et al., 1987) also lie between those of sucrose and reducing sugars. On the other hand, grape and orange juices containing some suspended colloids, mainly as pectin, tartrates and citrates, were more viscous than sucrose solutions; and cloudy apple, grape and orange juices, which contained a significant amount of pulp and suspended particles, were pseudoplastics (Saravacos, 1970; Moresi and Spinosi, 1980, 1984; Rao et al., 1984). Thus, it appears that Eq. (10) and sugar solutions data can be applied to estimate viscosity only in the case of clarified fruit juices. The presence of suspended material not only increased the viscosity but also changed the rheological behavior of the product so that a different approach must be used in that case.

Riedel (1949) reported practically the same values of thermal conductivities for different sugar solutions and fruit juices. A similar behavior was observed in this work (Table 2). There was a difference at the 1% level between the thermal conductivity data of apple juice and sucrose solutions, which can be attributable also to the effect of malic acid on the refractometric reading and the contribution of minor components. However, as the experimental errors in the thermal conductivity results were estimated on the order of 5%, no conclusion can be drawn out in that sense.

The approach suggested, based only on the contribution of the major sugar components, provides a way of predicting density and thermal conductivity and estimating specific heat and viscosity of fruit juices as a function of concentration, the last property restricted to clarified juices. It is important to note that a similar behavior was observed at other temperatures and that the validity of the expressions proposed is independent of this variable. Thus, thermophysical properties of different juices at other temperatures can be predicted provided the composition of the product is known and experimental data for the basic constituents are available.

CONCLUSIONS

THE EXPERIMENTAL RESULTS of this study showed that temperature and concentration have a strong influence on thermophysical properties of clarified apple juice. Density, viscosity and specific heat of apple juice differ from those of sugar solutions due to the effect of minor components and differences in the measurement of soluble solids by refractometry, mainly attributed to malic acid. Theoretical models have been presented to be used in a predictive sense, i.e: to estimate thermophysical properties of fruit juices of different compositions as a function of concentration and temperature. The results of this work have direct applications to several fruit juices process involving fluid flow and heat transfer.

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Nonenzymic Browning in Kiwifruit Juice Concentrate Systems during Storage

M. WONG and D.W. STANTON

———————— ABSTRACT ————————

The contributions of different nonenzymic browning reactions to color changes in stored kiwifruit juice concentrate were investigated using five synthetic model concentrates. The model systems were stored at 20°C for 10 weeks and analyzed for browning, haze, ascorbic and dehydroascorbic acids, titratable acidity, formol value. In addition the profiles of sugars, nonvolatile acids, and amino acids were determined. Two different classes of nonenzymic browning mechanisms were found to be of significance: the Maillard browning reactions and ascorbic acid browning reactions. Reactions involving ascorbic acid were found to contribute more to overall browning than Maillard browning.

INTRODUCTION

THE GENERAL FIELD of nonenzymic browning has been reviewed by Hodge (1953), Reynolds (1963, 1965), and Eskin et al. (1971). Fruit juice concentrates typically exhibit some degree of nonenzymic browning when stored at ambient temperatures and this may be of commercial significance depending on the severity of the browning. Several different nonenzymic browning reactions are known to occur in juices and their relative importance is determined by the composition of the juice. Maillard browning reactions occur between reducing sugars and the α-amino group of amino acids, peptides, or proteins (Hodge, 1953). Ascorbic acid degradation can occur whereby ascorbic acid at moderate temperatures either alone or in the presence of amino acids will react under both aerobic and anaerobic conditions to produce brown pigments (Wedzicha, 1984). The reaction between α-dicarbonyls and amino acids is known as the Strecker degradation reaction and can lead to pigment formation (Eskin et al., 1971). Caramelisation is the decomposition of sugars at relatively high temperatures, in the absence of amino compounds (Hodge, 1953). The oxidative nonenzymic browning of phenolic juice components is also known to occur.

In apple and pear juice concentrates the main reaction contributing to nonenzymic browning is considered to be the Maillard browning reaction (Cornwell and Wrolstad, 1981; Toribio and Lozano, 1984; O'Beirne, 1986; Babsky et al., 1986). Fruit juices and juice concentrates with a high acid content and a relatively high ascorbic acid content, such as citrus juices,

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exhibit ascorbic acid browning reactions. In studies of citrus juice browning it has been found that the extent of browning increased with ascorbic acid level and that the presence of amino acids. with ascorbic acid, augmented the browning rate (Joslyn, 1957; Clegg, 1964). The amino acids increased the browning potential after the oxidation of ascorbic acid had occurred.

Several workers have used model juice systems to investigate nonenzymic browning reactions (Joslyn 1957; Clegg, 1964; O'Beirne, 1986). Such systems have enabled the effects of the various reactive components to be studied independently, thereby establishing the relative importance of the different reactions. Kiwifruit juice concentrate is a new product in international trade. It browns at a very high rate at room temperature, and as a consequence must be stored at -18°C to reduce this problem. Kiwifruit juice has a high nonvolatile acid and ascorbic acid content, greater than in most citrus juices.

The objective of this work was to determine the contribution of four separate browning reactions to overall nonenzymic browning in kiwifruit juice concentrate using a series of synthetic kiwifruit juice concentrate models formulated, stored and monitored for browning and compositional changes.

MATERIALS & METHODS

Synthetic juice concentrate models

The formulations of the five different model concentrates are given in Table 1. The composition of the model concentrates as formulated is given in Table 2. An invert sugar syrup was used as the source of fructose and glucose. Sorbitol (90 g/L) was used as a non-reactive sugar substitute in the sugar-free model solution. The five major amino acids, which in total account for approximately 80% of the amino acid composition in kiwifruit, were used in the model concentrates.

Three liters of each model solution were prepared at single strength, with pH adjusted to 3.5. They were all then sparged with compressed air for 10 min to oxidise some of the ascorbic acid, present in three of the models, to dehydroascorbic acid. An average of 5% conversion of ascorbic acid to dehydroascorbic acid was obtained. This degree of conversion corresponds to that found in a good quality juice made under conditions of minimal oxidation. The model solutions prepared at single strength were less than 13.5°Brix before concentration. Each model solution was then concentrated to approximately 69°Brix in a rotary evaporator at 40°C. Samples of each model concentrate were then transferred into 25 mL glass sample vials and held at -25°C until the first day of storage, day 0. The model concentrates were then stored at 20°C for a 10-week period, during which the samples were analyzed each week, except week 8.

Analyses

The five model concentrates were analysed together. Each concentrate was diluted to 13.5°Brix, with distilled water, then analysed for

Model Number	Composition of model solutions	Nonenzymic[a] browning reactions	Nonenzymic browning rate $A_{420} \cdot week^{-1}$	Correlation coefficient
1	Sugars + nonvolatile acids	C	0.0007	0.74
2	Sugars + nonvolatile acids + ascorbic acid	C + A	0.0048	0.99
3	Sugars + nonvolatile acids + amino acids	C + M	0.0042	0.96
4	Sorbitol + nonvolatile acids + amino acids + ascorbic acid	A + S	0.0030	0.99
5	Sugars + nonvolatile acids + amino acids + ascorbic acid	C+M+A+S	0.10	0.97

[a] Possible enzymic browning reactions which would occur in each model concentrate; C: caramelization, A: ascorbic acid browning, M: Maillard browning, S: Strecker degradation.

Table 2—*Compositional data for clarified kiwifruit juice and the formulated synthetic model solutions*

Parameter	Published literature[a]		Model solutions formulation
	Range	Mean	
% Soluble solids (°Brix)	12.0 - 14.5	13.7	
Titratable acidity (%W/v as citric acid)	0.95 - 1.50	1.33	
pH	3.20 - 3.40	3.27	3.50
Glucose (g/L)	41.0 - 48.5	44.0	ca 40.0
Fructose (g/L)	42.1 - 44.0	43.0	ca 40.0
Sucrose (g/L)	8.3 - 10.5	9.2	ca 10.0
L-Malic acid (g/L)	1.4 - 2.7	2.3	1.50[c]
Citric acid (g/L)	8.3 - 11.3	10.2	9.1
Quinic acid (g/L)	5.8 - 9.0	7.9	10.0
Ascorbic acid (mg/100 mL)	37 - 85	67	
Dehydroascorbic acid (mg/100 mL)	12 - 61	37	
Total ascorbic acid (mg/100 mL)	97 - 98	98	100.0
Formol index (meq/100 mL)	1.45 - 1.75	1.60	1.65
Major amino acids in kiwifruit[b] (g/L, as free amino acid)			
L-Glutamic acid			0.45
L-Aspartic acid			0.50
L-Glutamine			0.50
L-Asparagine			0.41
L-Arginine			0.27

[a] Summary of data from Heatherbell et al. (1980), Ferguson (1984), Lodge et al. (1986), Tercel (1986); and unpublished data provided by J. Venning, T. Nguyen, and C. Kahukura.

[b] Based on unpublished data provided by R.J. Redgwell

[c] DL-Malic acid was added to the model solutions.
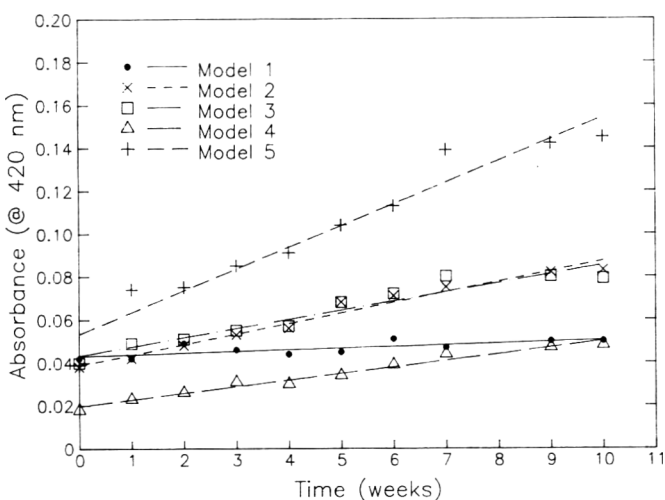
Fig. 1—*Effect of storage time on the browning (measured as Absorbance @ 420 nm) of the synthetic juice concentrate models, stored at 20°C.*

pH, titratable acidity, browning, haze and formol value. A small sample of each concentrate was retained for ascorbic and dehydroascorbic acid determination on the same day. Samples of the model concentrates diluted to 13.5°Brix for weeks 0,3,6, and 10 were frozen at -25°C until analyzed for sugar, nonvolatile acid and amino acid composition.

Haze and browning

The amount of haze present was determined from the absorbance at 700 nm using a Pye Unicam SP8-100 ultraviolet spectrophotometer (Pye Unicam Limited, Cambridge, England). The samples were then filtered through Whatman 542 filter paper. The extent of browning was determined from the absorbance at 420 nm, for the model solutions at 13.5°Brix.

Ascorbic and dehydroascorbic acids by HPLC

To determine the ascorbic acid content, each concentrate sample (200 mg) was dissolved in dilute sulfuric acid (pH 2.5–2.7) and made up to 10.0 mL. At the same time an ascorbic acid standard, 12 mg/100 mL, was prepared in the dilute sulfuric acid. Quantitation was by HPLC on a 'Polypore H' 25 cm x 7.0 mm cation exchange column (Brownlee Laboratories) at 60°C, with sulfuric acid pH 2.1, as eluant at a flow rate of 0.6 mL/min, and UV detection at 256 nm.

To determine the dehydroascorbic acid content the total ascorbic acid content was determined using the method of Dennison et al. (1981). The dehydroascorbic acid present in a second sample (200 mg) was reduced to ascorbic acid by a solution of homocysteine in phosphate buffer, pH 7.5 (Bochringer Mannheim, 1980) and adjusted to a final volume of 10 mL. Total ascorbic acid was measured by HPLC as above and the difference between the total ascorbic acid and the original ascorbic acid was considered to be due to dehydroascorbic acid.

Total and individual amino acid content

Total amino acids, for Models 3,4 and 5, were determined according to the formol index (AOAC, 1984). Samples of the single strength model solutions containing amino acids, from Weeks 0,3,6 and 10 were kept and analysed for amino acid composition. The amino acids were isolated, using ion exchange Sephadex (Redgwell, 1980), and quantified by HPLC using a Waters Associates 'Pico-tag Amino Acid Analysis System'.

Sugars

Sugar compositions were determined for samples (at 13.5°Brix) from weeks 0 and 10. Fructose, glucose and sucrose were determined by HPLC on a 25 cm x 4.9 mm Hichrom Zorbax-NH_2 column, at 40°C, with acetonitrile:water 85:15 as eluant at a flow rate of 2 mL/min. Detection was by a Waters R401 differential refractometer. Samples were diluted approximately 10-fold (w/v) and 20 μL aliquots were injected. Quantification was by comparison of peak heights with those of standards.

Total acidity and nonvolatile acid compositions

Titratable acidity was determined using the method described by the International Federation of Fruit Juice Producers (IFFJP) (1985). The individual nonvolatile acids were determined for samples (at 13.5°Brix) from weeks 0 and 10. For quinic acid the sample was diluted as for the sugar analysis, then injected onto a Hichrom Zorbax-NH_2 column at 35°C with aqueous KH_2PO_4 (10 g/L) at 1.0 mL/min as eluant. Detection was by refractive index. Citric and malic acids were determined with Boehringer Mannheim enzymatic kits. The assays gave values for L-malic acid only, therefore, DL-malic acid was reported as twice the L-malic acid value.

Statistical analysis

Statistica comparisons of difference between Week 0 and 10 values were determined using the Student's t test. The method of least squares was used to determine linear regression equations.

RESULTS & DISCUSSION

Synthetic juice concentrate models

Table 2 gives average values for kiwifruit juice composition from published data. The data are from work with kiwifruit of the Hayward variety (*Actinidia deliciosa* (A.Chev) C.F. Liang and A.R. Ferguson, var. *deliciosa*). The composition of the synthetic juice concentrate models was based on the published data presented.

The reasoning behind the model concentrates' compositions is as follows: Model 1 contained only sugars and nonvolatile acids and therefore could exhibit only caramelisation browning. Mode. 2 contained ascorbic acid and therefore would include browning reactions of this type. Model 3 could exhibit Maillard browning reactions as amino acids were present. Models 2 and 3 would also be susceptible to caramelization. Model 4 which contained ascorbic acid and amino acids, in the absence
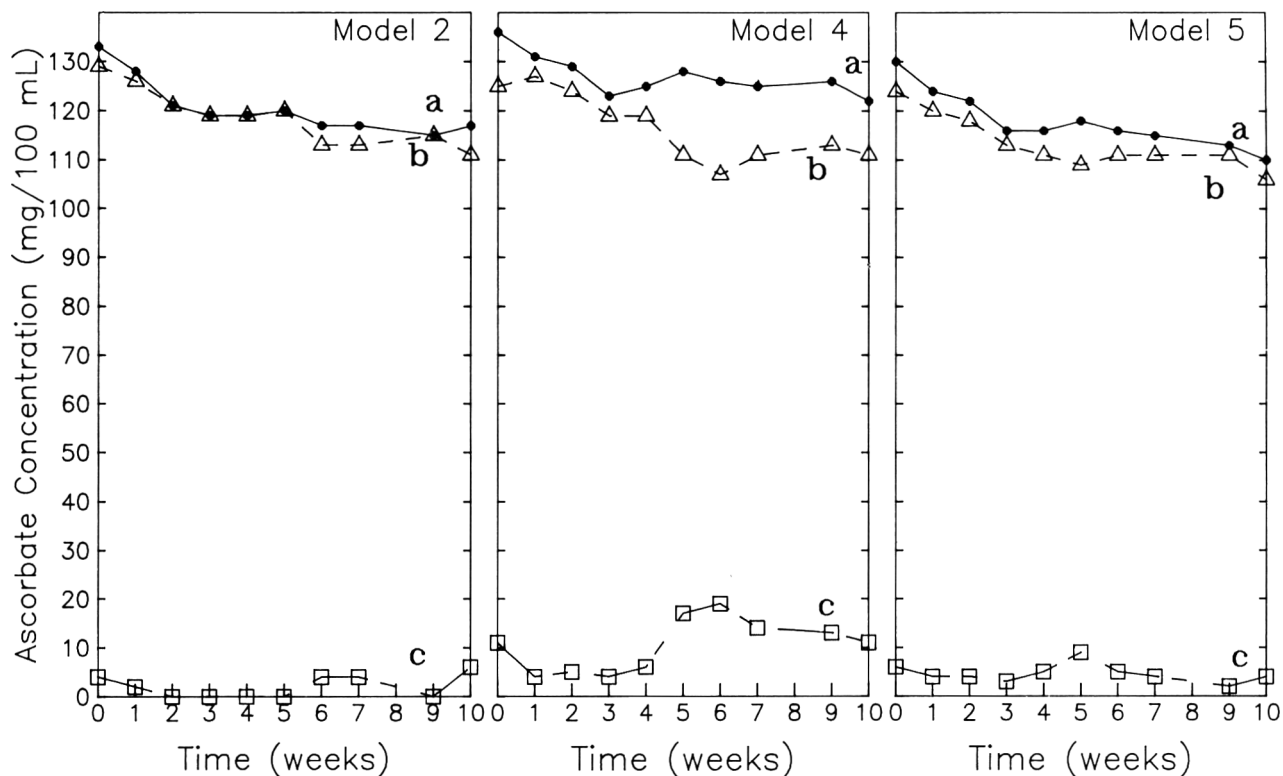
Fig. 2—Effect of storage time on total ascorbic, ascorbic and dehydroascorbic acids (mg/100 mL), for Models 2,4, and 5 (at 13.5°Brix), stored at 20°C. a ●———● Total ascorbic acid; b △ — — △ Ascorbic acid; c □— — —□ Dehydroascorbic acid.

Table 3—Sugar and nonvolatile acid compositions, for Weeks 0 and 10, for the five model solutions, at 13.5°Brix, stored at 20°C

	Model 1		Model 2		Model 3		Model 4		Model 5	
Week	0	10	0	10	0	10	0	10	0	10
Fructose (g/L)	45.7	46.1	46.7	45.4[b]	45.4	45.8	—	—	45.3	43.6[b]
Glucose (g/L)	50.0	50.5	50.2	50.2	49.3	49.6	—	—	49.3	47.1[b]
Sucrose (g/L)	11.1	10.1	11.1	9.6[b]	10.9	9.6	—	—	10.7	10.1
Total sugars (g/L)	106.8	106.7	108.0	105.2	105.6	105.0	—	—	105.3	100.8
Citric acid [a] (g/L)	11.2	10.9	11.3	10.7	11.2	10.6	12.5	11.1	11.0	10.4
DL-Malic acid (g/L)	1.83	1.76	1.85	1.75	1.76	1.64	1.93	1.69	1.69	1.69
Quinic acid (g/L)	11.0	11.0	12.0	11.0	11.0	11.0	13.0	12.0	11.0	11.0

[a] Anhydrous

[b] Value at week 10 is significantly different to the value at week 0, p<0.05.

Table 4—Changes in titratable acidity and formol index, measured over 10 weeks for the five model solutions, at 13.5°Brix, stored at 20°C

	Titratable acid ties (%w/v as citric acid)					Formol index (meq/100mL)		
Week	Model 1	Model 2	Model 3	Model 4	Model 5	Model 3	Model 4	Model 5
0	1.23	1.23	1.24	1.41	1.34	1.42	1.63	1.48
1	1.20	1.22	1.23	1.37	1.25	1.38	1.64	1.43
2	1.20	1.21	1.23	1.38	1.25	1.42	1.68	1.47
3	1.21	1.23	1.24	1.36	1.26	1.40	1.62	1.46
4	1.20	1.21	1.22	1.35	1.25	1.43	1.70	1.44
5	1.21	1.22	1.24	1.37	1.27	1.41	1.64	1.43
6	1.21	1.23	1.24	1.37	1.27	1.35	1.69	1.42
7	1.20	1.22	1.23	1.35	1.26	1.35	1.66	1.41
9	1.18	1.19	1.21	1.32	1.24	1.33	1.68	1.41
10	1.17	1.19[a]	1.20[a]	1.30[a]	1.23	1.31[a]	1.66	1.44[a]

[a] Value at week 10 is significantly different to the value at week 0, p<0.05.

of sugars, would be expected to exhibit browning due to ascorbic acid oxidation and possibly the Strecker degradation reactions. Model 5 contained all the reactive components and this could have all four of the above reactions taking place. Nonvolatile acids were present in all of the model solutions.

Browning rates of the model concentrates

In the model solutions, browning, measured as absorbance at 420 nm, was found to be linear with time (Fig 1). A plot of the linear regression lines fitted to these data is shown and the browning rates for each model solution are given in Table

1. There was no significant increase in haze in any of the model solutions over 10 weeks storage. The pH of the model solutions remained constant throughout the storage period.

Model 5 which was analogous to kiwifruit juice, except that it did not contain any phenolic compounds, showed a browning rate of 0.01 A_{420}·week^{-1}. Model 5 represented the total of all the browning reactions in Models 1,2,3, and 4 (Table 1). As an approximation, the browning rates for the various classes of reactions were considered to be additive. The browning rate in Model 1 was 0.0007 A_{420}·week^{-1}, which represents caramelization. The browning rate in Model 3 was 0.0042 A_{420}·week^{-1} which when corrected for caramelization gives a
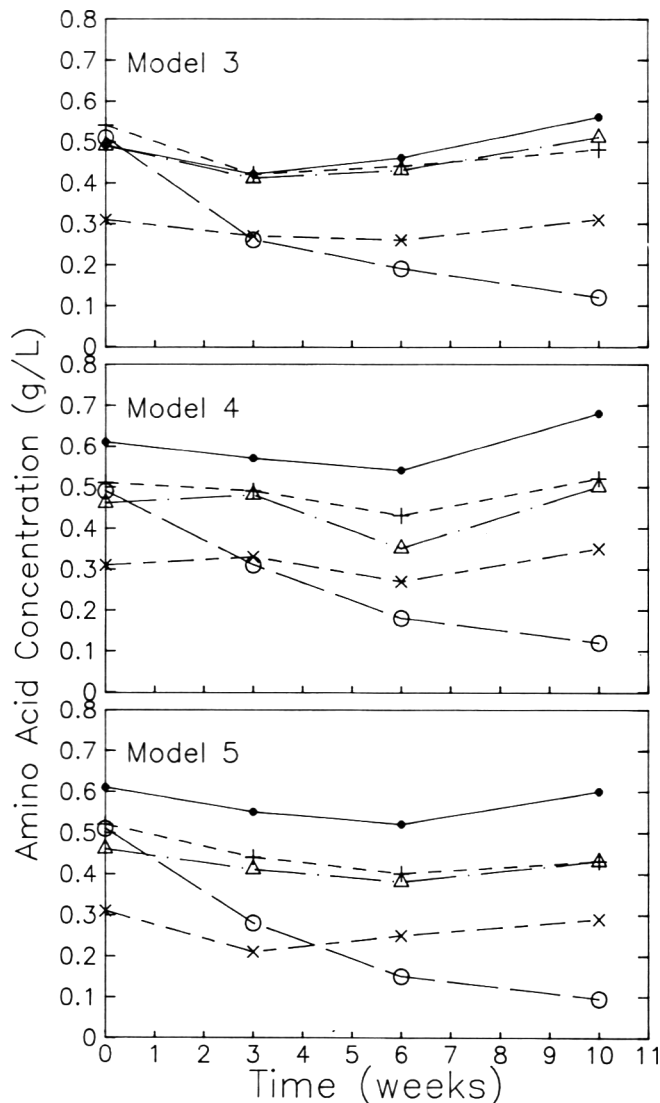
Fig. 3—Effect of storage time on individual amino acids (g/L); aspartic acid, glutamic acid, asparagine, glutamine and arginine, for Models 3, 4, and 5 (at 13.5°Brix), stored at 20°C. ●—— ● Aspartic acid; + - - + Glutamic acid; △—·—△ Asparagine; ○— —△ Glutamine; x— — —X Arginine.

rate of browning due to the Maillard reaction of 0.0035 $A_{420}\cdot week^{-1}$ (0.0042–0.0007 $A_{420}\cdot week^{-1}$.

Models 2 and 4 exhibited browning rates of 0.0048 and 0.0030 $A_{420}\cdot week^{-1}$, respectively. The major contributor to browning in these two model systems is ascorbic acid oxidative browning which occurs via dehydroascorbic acid; this browning pathway can result in the formation of furfural with accompanying loss of carbon dioxide. Furfurals either alone or in the presence of amino compounds undergo condensation and polymerization resulting in the formation of color pigments (Erdman and Klein, 1982). As well as ascorbic acid browning, the Strecker degradation may also occur in Model 4. The reaction between α-dicarbonyl compounds (e.g. dehydroascorbic acid), and amino groups of amino acids, proteins and other amines (Eskin et al., 1971; Tolbert and Ward, 1982), which forms aldehydes, carbon dioxide, and α–amino carbonyl compounds, can lead to the formation of pigments (melanoidins).

The difference between the total browning rate in Model 5 and that of Model 3 is 0.0058 $A_{420}\cdot week^{-1}$, which could be taken as the combined rate for ascorbic acid browning and the Strecker degradation. The observed rate in Model 4 is lower than expected since it was anticipated that ascorbic acid browning would be enhanced by the presence of amino acids.

Ascorbic acid and dehydroascorbic acid

There were 12%, 10% and 15% losses in total ascorbic acid, after 10 weeks storage, in Models 2, 4 and 5, respectively. The changes in total ascorbic, ascorbic and dehydroascorbic acids over the storage period are shown in Fig. 2. The loss of ascorbic acid overall was sufficient to account for nonenzymic browning, which occurred. Joslyn (1957) noted that with only a 10-15% decrease in ascorbic acid content, in the presence of relatively small amounts of oxygen, darkening of an orange juice will occur.

Sugars and nonvolatile acids

Sugar and nonvolatile acid compositions for Weeks 0 and 10 are shown in Table 3. Significant (p<0.05) losses in sugar composition from Week 0 to 10 were found only in Models 2 and 5. Babsky et al. (1986) noted that only small losses in reducing sugars were necessary to produce large changes in browning There was a loss of citric and malic acids, in all the model solutions. All the model solutions showed a drop in titratable acidity (Table 4), with Models 2,3 and 4 showing significant losses (p<0.05). Arkhavan and Wrolstad (1980) also observed a loss in total acid in stored pear juice concentrate. They postulated the loss could be due to polymerisation of acids with browning products of sugars or other compounds present. Babsky et al. (1986) and Bielig and Hofsommer (1984) also observed a decrease in acidity in fruit juices after storage. Clegg (1966) found that the combination of citric and ascorbic acid in orange juice enhanced the level of browning. O'Beirne (1986) observed that malic acid caused a similar effect in apple juice concentrate.

Amino acids

The changes in total amino acids determined by the formol index in Models 3,4 and 5, over the 10-week period, are shown in Table 4. There was a significant decrease in total amino acids in Models 3 and 5 but not in Model 4 as would have been expected. The changes in the individual amino acid compositions in Models 3, 4 and 5 are shown in Fig. 3. Glutamine was the only amino acid of the five amino acids present which was lost to a significant extent over the 10-week period. In Model 5 there was also a slight loss in asparagine and glutamic acid after 10 weeks. Ashoor and Zent (1984) ranked the order of reactivity of common amino acids with reducing sugars according to the intensity of Maillard browning. The order of decreasing reactivity of the five amino acids added to the models is glutamine, asparagine, aspartic acid, arginine and glutamic acid. Although there was no overall change in formol value for Model 4, a loss of glutamine did occur. The Strecker degradation could be yielding a reaction product which is still reactive in the formol titration. The loss of glutamine in Model 4 indicates that some Strecker degradation reactions are taking place.

CONCLUSIONS

IN THE MODEL CONCENTRATES, analogous to kiwifruit concentrate, ascorbic acid was found to have an important contribution to nonenzymic browning. Nonenzymic browning in kiwifruit juice concentrate appears to involve similar mechanisms to other high acid juices, such as citrus juices. Ascorbic acid and dehydroascorbic acid are involved in the formation of brown color compounds in kiwifruit concentrate. Maillard browning contributes less to nonenzymic browning in model kiwifruit juice concentrate than ascorbic acid browning, but is still a significant contributor. The presence of nonvolatile acids also appears to be important. It can be anticipated from the results of this study that kiwifruit juice made under conditions of reduced oxidation will lead to less conversion of ascorbic

acid to dehydroascorbic acid, whereas a highly oxidised juice will leave more reactive products available for further reaction. The effect of the initial level of oxidation in kiwifruit juice is the subject of current study. The contribution of natural kiwifruit juice phenolics to browning in juice concentrate is also under investigation, along with the determination of the nonenzymic browning rate in stored kiwifruit concentrate.

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peratures. The activity was unaffected up to 40°C, then a progressive activity loss was observed up to 90°C, where no activity was detected. The percent residual PME activity as a function of the temperature is shown in Fig. 5.

As far as the heterogeneity of apple PME is concerned, the two forms exhibited different behavior both in their charge and in molecular weight. This is not an apple peculiarity. The presence of multiple forms of PME is common in fruit. Brady (1976) described two forms of the enzyme in banana, both with a molecular weight of 30000 daltons, differing in their isoelectric point. Versteg (1980) reported three forms of PME from orange; two forms had the same molecular weight of 36000 daltons, but differed in their charge, and the third had a molecular weight of 54000 daltons.

CONCLUSION

TWO PME FORMS are present in apple (*Malus communis*). The form purified to homogeneity has a molecular weight of 55000 daltons and exhibits a pH optimum in the range of 6.5-7.5 and a Km of 1.05 mg/mL for citrus pectin. The enzyme activity is completely lost after 1 min of incubation at 90°C.

These results do not differ substantially from those found for PME from other sources. However, the knowledge of the properties of pectin methylesterases could help to clarify the structure-function relationship of these enzymes. This may be important not only for a better understanding of some processes of fruit physiology and pathology, but also to improve the control of some technological processes in which the activity of pectic enzymes is a critical factor.

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Use of Acidification, Low Temperature, and Sorbates for Storage of Orange Juice

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─────────── ABSTRACT ───────────

The effects of acidification, combination of acidification and low temperature and sorbates on the storage of orange juice were investigated. Acidification of orange juice to pH 2.0 and pH 2.5 followed by storage at 5°C resulted in marked reduction in total plate count and yeast and mold population during twelve weeks of storage. Use of 0.03% sorbic acid or potassium sorbate in combination with acidification at pH 2.5 resulted in preservation of orange juice stored at 10°C over 10 wk period; however, vitamin C degradation was enhanced by the presence of the sorbates. The results also indicated that relatively high concentrations of vitamin C were present in orange juice containing high population of microorganisms.

INTRODUCTION

THE MAJOR CAUSE of deterioration or spoilage of oranges during storage is molds (Goepfert, 1980). Certain molds, e.g., *Penicillium, Rhizopus,* and *Alternaria* which occur frequently in orange juice are acid tolerant and prefer lower pH; the pH of orange juice (pH 3.0 - pH 4.0) favors the growth of these microorganisms (Schlegel, 1986; Splittsleesser, 1987). Acid tolerant lactic acid bacteria also contribute to spoilage of orange juice, even at low temperature (Hill et al., 1953). To avoid this kind of deterioration or spoilage and to preserve oranges, processing of oranges into juice followed by (a) long term storage of the juice concentrate in cans or in the frozen state and (b) short-term refrigeration storage is practised widely. In a study on the preservation of tomato juice, the use of acidification was investigated (Sidhu et al., 1984). This study demonstrated that the technique for pressing tomato juice and crushed tomatoes, followed by storage under acid condition, could eliminate the need for the usual heat processing.

Sorbates (potassium sorbate, sorbic acid) are widely used as yeast and mold inhibitors in the preservation of cheese products, baked foods, fruit, fresh fruits and vegetables and soft drinks (Deuel et al., 1954a,b; Sofos and Busta, 1981). Although sorbates are recommended as preservative for fruits, vegetables and juices, there is relatively little published information on their use in the scientific literature. The present study was carried out to investigate the effect of acidification, low temperature and sorbates on the storage of orange juice.

MATERIALS & METHODS

Materials

Appropriate quantities of California (Valencia) orange (*Citrus sinensis* (L.) Osbeck) were purchased in a local store as required for each experiment. The peel and seeds were removed from the fruit and the pulp was blended (30 sec) in a Waring Blendor.

Determination of vitamin C

Vitamin C was determined according to (a) the 2,6-dichloroindophenol method of the AOAC (1984) and (b) the UV spectrophotometric method of Fung and Luk (1985a,b).

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Determination of total plate count

The population of total bacteria (TPC) was determined by the method of Keddie (1962). A quantity (23.5g) of Total Plate Count Agar (DIFCO Laboratories, Detroit) was dissolved in distilled water (1L) with heat. The solution was sterilized, cooled, then poured into petri-dishes (approximately 15 mL agar per plate) containing a sample dilution (1 mL) of orange juice; for each analysis, three appropriate serial dilutions within the range of 10^1–10^6 were prepared from the juice and used for TPC determination. After gelation of the TPC agar, the petri-dishes were stored for 5 days at 30°C. The number of colonies which developed on the plates was counted.

Determination of yeast and mold population

The population of yeasts and molds (Y and M) was determined by the method of Koburger (1968). A quantity (39g) of Potato Dextrose Agar (DIFCO Laboratories, Detroit) was dissolved in distilled water (1L), sterilized and cooled. The serial dilutions prepared for the TPC determination were also used for Y and M determination; however, for Y and M determination, the petri-dishes were stored for 5 days at 25°C. The number of colonies which developed on the plate was counted.

Determination of total soluble sugars

The colorimetric anthrone method of Fairbairn (1953) was used for the determination of total soluble sugars. A quantity (5 g) of the orange juice was diluted (100 mL) with distilled water, mixed, then filtered. The filtrate (10 mL) was diluted (100 mL) with distilled water further and a quantity (2 mL) of this final solution was subjected to the colorimetric anthrone reaction.

Effects of acidification on storage of orange juice

Approximately 1500g of orange juice was acidified to pH 2.0 by addition of concentrated HCl. The acidified juice was placed in 18 previously sterilized bottles (60 mL), each containing 50 mL of the juice. Nine bottles containing the juice were stored at 25°C and the remaining 9 bottles were stored at 5°C. The procedure described above was also performed on additional quantities of orange juice which were (a) acidified to pH 2.5 with concentrated HCl, (b) acidified to pH 3.0 with concentrated HCl and (c) not acidified juice; the nonacidified orange juice served as controls. The juice was stored for a period of 12 weeks. At intervals of 0, 2, 6 and 12 wk, triplicate samples of orange juice were removed and analysed in triplicates for Y and M, TPC, vitamin C and total soluble sugars.

Changes in vitamin C content of orange juice and of standard solutions

Orange juice was stored at 5°C and at 25°C and the vitamin C of the juice was measured at 2-day intervals over a 21-day period. Distilled water was acidified to pH 2.0, pH 2.5 and pH 3.0 with HCl (0.5N) and a solution of vitamin C (100 mg/100 mL) was prepared using the acidified water. The acidified vitamin C solutions were stored at 5°C and 25°C and the vitamin C in the solutions was measured at 7-day intervals during 21-day storage period.

Effects of potassium sorbate, acidification, and storage temperature

Orange juice was acidified to pH 2.5 with concentrated HCl and then treated as follows: (1) 2 kg of acidified juice was treated with potassium sorbate (KS) at the rate of 0.05%, (2) 2 kg of acidified juice was treated with KS at the rate of 0.1% and (3) 2 kg of acidified
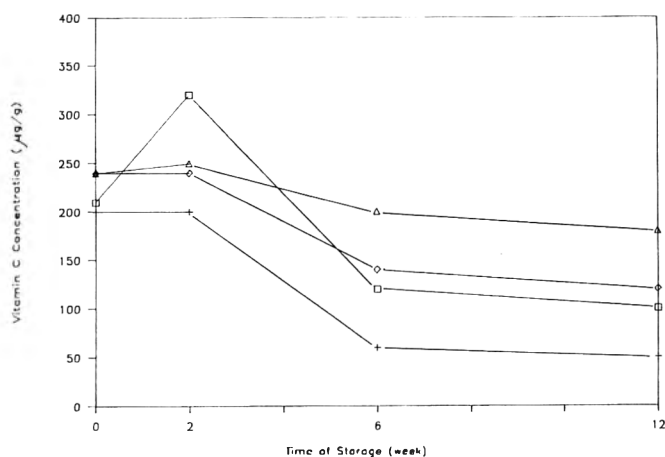
Fig. 1a — Effects of acidification on vitamin C in orange juice during storage at 5°C, pH 2.0 (+ ---- +), pH 2.5 (◇ ---- ◇), pH 3.0 (△ ---- △) and unacidified, pH 4.06 (□ --- □).
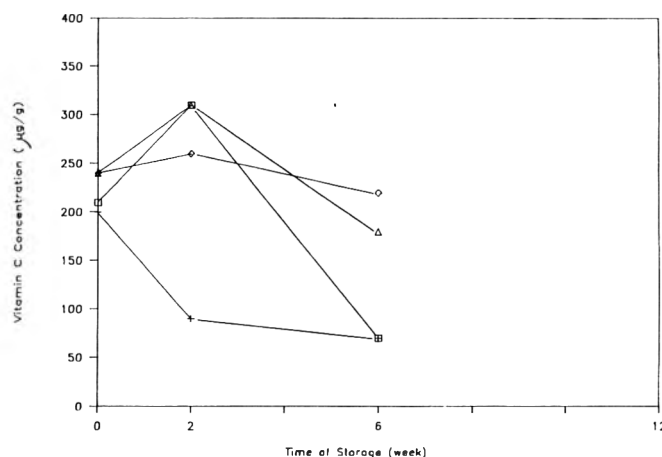
Fig. 1b — Effects of acidification on vitamin C in orange juice during storage at 25°C, pH 2.0 (+ ---- +), pH 2.5 (◇ ---- ◇), pH 3.0 (△ ---- △) and unacidified, pH 4.06 (□ --- □).

juice was not treated; this served as control. The treated and untreated juices were stored at 10°C and at 25°C for a 12 week period. At intervals of 0, 2, 4, 6, 9, and 12 wk, samples of the juice were analyzed in triplicates for Y and M, TPC and vitamin C.

In a second experiment, orange juice was treated as follows: (1) 2 kg of juice was acidified to pH 2.5 and treated with KS at the rate of 0.01%, (2) 2 kg of juice was treated with KS at the rate of 0.01%; this juice was not acidified and had a pH 3.88, (3) 2 kg of juice was acidified to pH 2.5 and treated with KS at the rate of 0.03% and (4) 2 kg of juice was treated with potassium sorbate at the rate of 0.03%; this juice was not acidified and had a pH 3.88. The treated and untreated orange juices were stored at 10°C. At intervals of 0, 2, 4, 7, and 10 wk, samples of juice were analyzed in triplicates for Y and M, TPC, and vitamin C.

Effects of sorbic acid, acidification, and storage temperature

Orange juice was treated as follows: (1) 3 kg of juice was acidified to pH 2.5 and treated with sorbic acid (SA) at the rate of 0.03%, (2) 3 kg of juice was acidified to pH 2.5 and treated with SA at the rate of 0.05% and (3) 3 kg of nonacidified juice (pH 3.88) was treated with SA at the rate of (a) 0.03% and (b) 0.05% acid. The treated and untreated juice were stored at 10°C and at 25°C over a 10 wk period. After intervals of 0, 2, 4, 7, and 10 wk, the samples of juice were analyzed in triplicates for Y and M, TPC and vitamin C.

RESULTS & DISCUSSION

Effects of acidification on vitamin C content

Juices stored at 25°C were analyzed up to the 6 wk period only. Beyond this storage time, the juice was visually unacceptable. In general, there was a decrease in vitamin C of orange juice stored at 5°C regardless of the acidification treatment (Fig. 1a). The smallest decrease was obtained in the juice acidified to pH 3.0 while the greatest decrease in vitamin C was observed in the juice acidified to pH 2.0. The decrease in vitamin C occurred mainly between the second and the sixth week of storage; the decrease after the six week storage period was relatively small. The results suggest that acidification of the juice to pH 2.0 accelerated the decrease in vitamin C during storage at 5°C and 25°C (Fig. 1a,b). Sidhu et al. (1984) found that acidification did not affect the vitamin C content of tomato juice stored at room temperature for 12 wk. At both 5°C and 25°C, there was an increase in vitamin C content during the first two weeks of storage of the juice which was not acidified. Short term (20 days) storage of orange juice resulted in greater deterioration of vitamin C at 5°C than at 25°C (Fig. 2). The higher concentration of vitamin C in juice stored 25°C might be the result of production of vitamin C by microorganisms. It was also considered that increased vitamin C which was
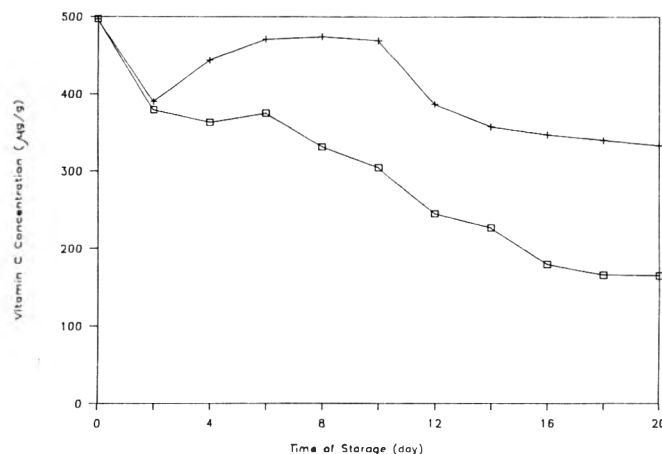
Fig. 2 — Changes in vitamin C of orange juice during storage at 5°C (□ --- □) and 25°C (+ ---- +).

observed during storage might be due to interfering substances such as metabolites produced by microorganisms during the storage period. However, the stored orange juice was also analyzed for vitamin C by the highly specific UV spectrophotometric method of Fung and Luk (1985a,b); use of this analytical procedure confirmed that the increase in vitamin C during storage was not due to interfering substances.

The degradation of vitamin C during storage under acid conditions was confirmed by use of standard solutions of vitamin C. The results (Fig. 3a,b) indicated that regardless of the degree of acidification, considerable degradation of vitamin C occurred at 25°C, while acidification of vitamin C solution to pH 2.0, followed by storage at 5°C, promoted the deterioration of vitamin C.

Microbial population

There was an increase in bacterial populations during the first 2 wk of storage at both 5°C and 25°C. (Fig. 4a,b); however, the bacterial populations in the juice stored at 5°C were lower than those of juice stored at 25°C. The juice acidified to pH 2.0 showed considerably lower TPC than the nonacidified juice or the juice acidified to pH 3.0 or pH 2.5. The results suggested that acidification below pH 2.5 was required to effect a marked reduction in bacterial population. Y and M populations in the juice acidified to pH 2.5 and 2.0 and stored
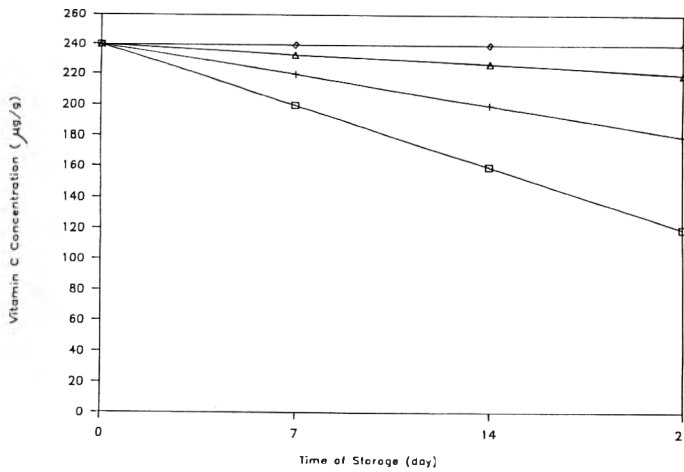
Fig. 3a — Changes in vitamin C of standard vitamin C solution during storage at 5°C, pH 2.0 (+----+), pH 2.5 (◊----◊), pH 3.0 (△----△) and unacidified, pH 3.8 (☐----☐).
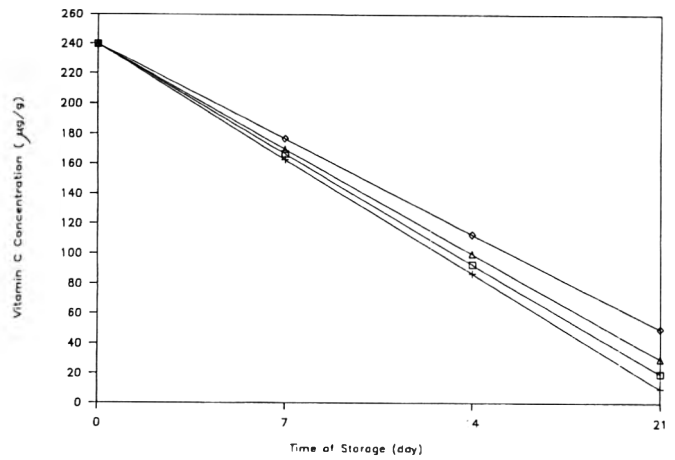
Fig. 3b — Changes in vitamin C of standard vitamin C solution during storage at 25°C, pH 2.0 (+----+), pH 2.5 (◊----◊), pH 3.0 (△----△) and unacidified, pH 3.8 (☐----☐).
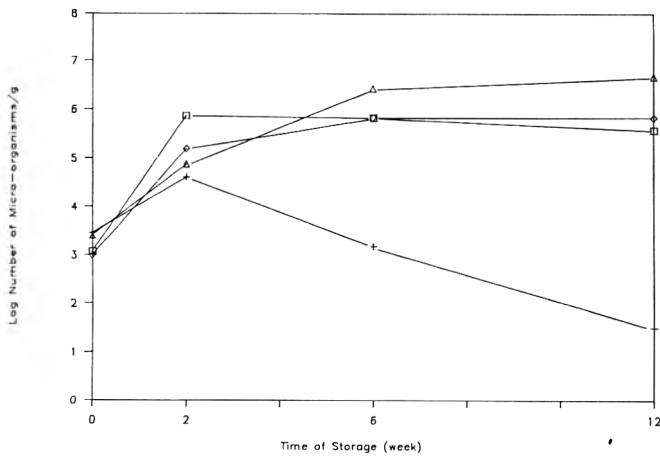
Fig. 4a — Effects of acidification on total plate count (TPC) of orange juice during storage 5°C, pH 2.0 (+----+), pH 2.5 (◊----◊), pH 3.0 (△----△) and unacidified, pH 4.06 (☐----☐).
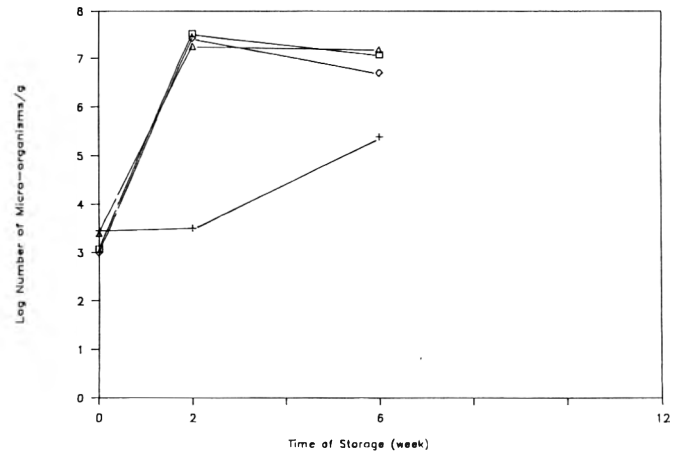
Fig. 4b — Effects of acidification on total plate count (TPC) of orange juice during storage at 25°C, pH 2.0 (+----+), pH 2.5 (◊----◊), pH 3.0 (△----△) and unacidified, ph 4.06 (☐----☐).

Table 1 — Changes in population of yeast and mold in acidified and non-acidified orange juice during storage at 5°C

Storage time (Wk)	Number of microorganisms/g juice			
	pH			
	2.0	2.5	3.0	4.06[a]
0	<10	<10	<10	<10
2	30	15×10^4	<10	<10
6	<10	<10	80×10	80×10^2
12	<10	<10	20×10^2	14×10^4

[a] pH unadjusted (control).

at 5°C were, in general, lower than in juice acidified to pH 3.0 and in non-acidified juice (Table 1). The juice acidified to pH 2.0 showed little increase in Y and M populations during the storage period. Relatively low populations of Y and M were observed by the twelfth week in the juice acidified to pH 2.0 and pH 2.5. Acidification to pH 3.0 resulted only in a slight reduction in Y and M populations when compared with the nonacidified juice.

Total sugar

The effects of acidification of juice to pH 3.0, 2.5 and 2.0 on the total sugar of the juice were similar (Fig. 5a). After the first two weeks of storage, there was a slight decrease in sugar.

This was followed by a slight but gradual increase to the twelfth week. In the case of orange juice stored at 25°C (Fig. 5b), there was a marked reduction in sugar during the first 2 wks of storage of the unacidified juice and the juice acidified to pH 3.0 and to pH 2.5. By comparison, there was a gradual decrease in sugar in the juice acidified to pH 2.0. The higher concentrations of sugars in the orange juice stored at 5°C when compared to the juice stored at 25°C could be related to the lower population of micro-organisms in the juice stored at 5°C (Fig. 4a). The slight increase in sugar observed during the first 2 weeks of storage at 5°C could be the result of polysaccharide degradation by micro-organisms (Ting and Attaway, 1971).

Effects of acidification, sorbates, and storage temperature

The results (Table 2) indicate that regardless of the temperature of storage, KS was effective in reducing the TPC when compared to the control. Both the 0.05% and 0.10% concentrations of KS showed TPC of <20 over most of the 12 week storage period. As would be expected, the TPC of untreated juice stored at 25°C was, by comparison, relatively high. This nontreated juice was visually unacceptable after 2 wk. The results indicate (Table 2) that at the 10°C storage both 0.05% and 0.10% concentrations of KS led to lower populations of
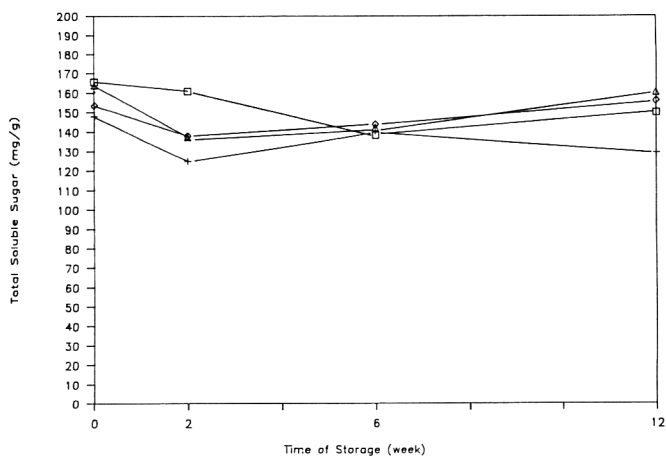
Fig. 5a — Effect of acidification on total soluble sugars in orange juice during storage at 5°C, pH 2.0 (+ ---- +), pH 2.5 (◊ ---- ◊), pH 3.0 (△ ---- △) and unacidified, pH 4.06 (□ ---- □).
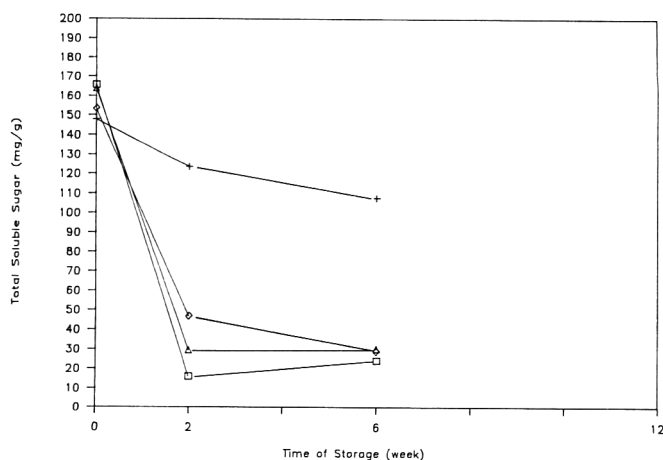
Fig. 5b — Effect of acidification on total soluble sugars in orange juice during storage at 25°C, pH 2.0 (+ ---- +), pH 2.5 (◊ ---- ◊), pH 3.0 (△ ---- △) and, unacidified pH 4.06 (□ ---- □).
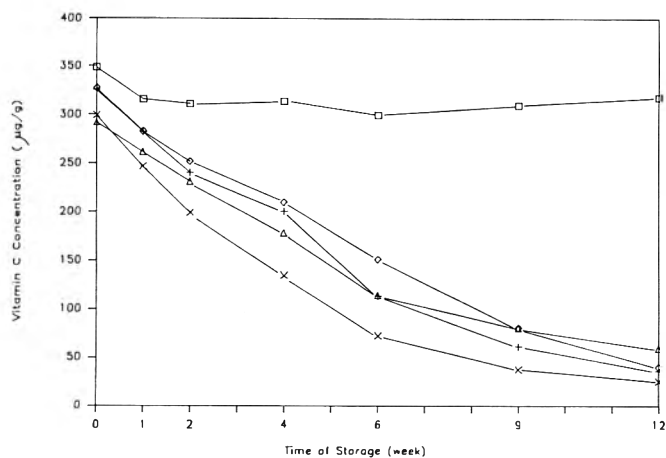
Fig. 6 — Effect of potassium sorbate (KS) on vitamin C of acidified orange juice (pH 2.5) stored at 10°C with addition of 0.05% KS (+ ---- +) and 0.10% KS (◊ ---- ◊) and stored at 25°C with addition of 0.05% KS (△ ---- △) and 0.10% KS (X ---- X) compared to untreated control sample (□ ---- □).
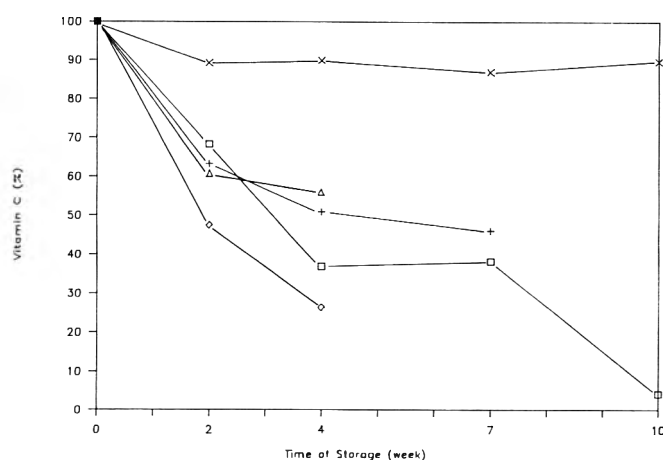
Fig. 7 — Effects of sorbic acid (SA) on vitamin C of acidified orange juice stored at 10°C with addition of 0.03% SA at pH 3.8 (□ ---- □) and at pH 2.5 (◊ ---- ◊) and addition of 0.05% SA at pH 3.8 (+ ---- +) and at pH 2.5 (△ ---- △) and compared to control sample (X ---- X).

Table 2—Effect of potassium sorbate on changes of total plate count (TPC), yeast (Y) and mold (M) populations of acidified orange juice (pH 2.5) during storage at 10°C and 25°C.

Storage time (Wk)		Number of Microorganisms/g Juice					
		Stored at 10°C			Stored at 25°C		
		0.00%[a]	0.05%[a]	0.10%[a]	0.0%[a]	0.05%[a]	0.10%[a]
0	TPC	10×10	18×10	23×10	10×10	43×10	20
	Y&M	40	60	20	40	< 20	< 20
1	TPC	70	< 20	26	17×10^2	< 20	< 20
	Y&M	70	< 20	< 20	52×10	< 20	< 20
2	TPC	31×10^2	20	20	-b	40	< 20
	Y&M	< 20	< 20	< 20	-b	48×10	< 20
4	TPC	73	< 20	< 20	-b	< 20	< 20
	Y&M	29×10^3	< 20	< 20	-b	37×10^2	< 20
6	TPC	30×10^4	< 20	< 20	-b	< 20	< 20
	Y&M	40×10^4	< 20	< 20	-b	27×10^4	< 20
9	TPC	58×10^5	< 20	< 20	-b	< 20	< 20
	Y&M	20×10	< 20	< 20	-b	36×10^5	< 20
12	TPC	64×10^6	< 10	< 10	-b	< 10	< 10
	Y&M	< 20	< 10	< 10	-b	45×10^5	54

[a] Level of potassium sorbate (g/100g juice).
[b] Not analyzed (visually unacceptable).

Table 3—Effects of potassium sorbate and acidification on total plate count (TPC), yeast (Y) and mold (M) populations in orange juice stored at 10°C

Storage time (Wk)		Number of Microorganisms/g Juice			
		pH 2.50[a]		pH 3.88[b]	
		0.03% KS[c]	0.01% KS[c]	0.03% KS[c]	0.01% KS[c]
0	TPC	38×10	55×10	60×10	10×10^2
	Y&M	16×10	18×10	21×10	19×10
2	TPC	< 20	46	20	19×10^2
	Y&M	< 10	< 10	< 10	< 10
4	TPC	30	53×10^6	15×10^2	80×10^4
	Y&M	< 10	< 10	< 10	< 10
7	TPC	42×10	33×10^6	$< 15 \times 10^2$	-d
	Y&M	< 10	52×10	< 10	-d
10	TPC	40	-d	-d	-d
	Y&M	< 10	-d	-d	-d

[a] Acidified by addition of HCl.
[b] Unacidified orange juice.
[c] Potassium sorbate.
[d] Not analyzed (visually unacceptable).

Y and M when compared to the control. At the 25°C storage temperature, relatively high populations of Y and M were observed with the 0.05% KS treatment; however treatment of juice at 0.10% was effective in preventing the proliferation of Y and M during storage. These results suggested that KS used at the level of 0.05% in orange juice stored at 10°C, led to a

Table 4—Effects of sorbic acid, acidification, and storage temperature on total plant count (TPC) of orange juice

	Number of Microorganisms/g Juice							
	pH 2.50[a]				pH 3.88[b]			
	Storage temperature °C							
	10		25		10		25	
Storage time (Wk)	Sorbic acid (%)							
	0.03	0.05	0.03	0.05	0.03	0.05	0.03	0.05
0	15×10	44×10	15×10	44×10	89×10	73×10	89×10	73×10
2	<20	<20	<20	<20	40	<20	35×10^2	<20
4	<10	<10	<10	67×10	37×10^4	20	24×10^3	40×10^4
7	50	50	-c	27×10	73×10^5	20×10^2	-c	17×10^3
10	20	<10	-c	<10	-c	20×10^3	-c	-c

[a] Acidified to pH 2.5 with HCl.
[b] Not acidified
[c] Not analyzed (visually unacceptable).

Table 5—Effects of sorbic acid, acidification, and storage temperature on population of yeast (Y) and mold (M) of orange juice

	Number of Microorganisms/g Juice							
	pH 2.50[a]				pH 3.88[b]			
	Storage temperature °C							
	10		25		10		25	
Storage time (week)	Sorbic acid (%)							
	0.03	0.05	0.03	0.05	0.03	0.05	0.03	0.05
0	17×10	17×10	17×10	17×10	28×10	16×10	28×10	16×10
2	<10	<10	28×10^3	<10	20	<10	<10	<10
4	<10	<10	11×16^6	17×10	<20	<10	<10	<10
7	<10	<10	-c	<20	<20	28×10	-c	15×10^2
10	<10	<10	-c	<10	-c	<10	-c	-c

[a] Acidified to pH 2.5 with HCl.
[b] Not acidified
[c] Not analyzed (visually unacceptable).

marked reduction in the population of micro-organisms during the storage. The use of KS resulted in a continuous decrease in vitamin C of the juice during the 12-wk storage period (Fig. 6). This might be related to the lower level of bacterial population in the KS treated juice. It will be recalled that higher vitamin C was related to higher level of TPC. KS (0.03%) was effective in reducing the TPC and Y and M (Table 3) over the 10 wk storage period; the 0.03% KS was more effective in orange juice acidified to pH 2.5 than at pH 3.88. This suggested that the combination of acidification to pH 2.5 and KS at 0.03% was effective in preventing proliferation of microorganisms during storage of juice at 10°C. This finding agrees with that of Sofos and Busta (1981) who reported that sorbate was more effective in low pH foods. The fact that 98.0% of undissociated form (effective form) of sorbate exists below pH 3.0 could explain the effect of sorbates in low pH foods.

Acidified juice (pH 2.5) treated with 0.03% SA and 0.05% SA and stored at 10°C, showed relatively low TPC (Table 4) during the 10 wk storage period. SA (both 0.03% and 0.05%) was also effective in reducing the population of Y and M when the juice was stored at 10°C, regardless of whether acidification was used (Table 5). In all instances when SA was used there was a marked reduction in vitamin C during storage when compared to the absence of sorbic acid (Fig. 7). This might be related to the lower bacterial population of the SA-treated juice. Use of the sorbates resulted in orange juice which could be preserved for at least 10 weeks without spoilage; however, there was some loss of vitamin C of the juice. The SA and KS were more effective in inhibiting the growth of yeasts and molds than the growth of bacteria. This observation is supported by the findings of other workers (Sofos and Busta, 1981) who showed that sorbates could inhibit growth of yeasts and molds but were less effective against bacteria.

CONCLUSIONS

ACIDIFICATION of orange juice to below pH 2.5 resulted in the reduction in TPC and Y and M populations and a reduction of vitamin C during the storage of the juice at 5°C over 12-wk period. The acidification did not extend the shelf life of orange juice stored at 25°C beyond 6 wk. The combination of acidification (to pH 2.5), use of sorbate (potassium sorbate, sorbic acid; 0.03% concentration) and low temperature storage (10°C) led to reduction of Y and M, TPC and vitamin C during storage.

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Influence of Atmospheric Oxygen Content on Growth and Fumitremorgin Production by a Heat-resistant Mold, *Neosartorya fischeri*

P.V. NIELSEN, L.R. BEUCHAT, and J.C. FRISVAD

─────────────── ABSTRACT ───────────────

Growth and fumitremorgin production by a heat-resistant mold, *Neosartorya fischeri*, in atmospheres containing 0.0095–20.9% O_2 were studied over a 38–day period. The mold was cultured on Czapek Yeast Autolysate agar adjusted to pH 3.5 and 7.0 and incubated at 25°C in darkness. Colonies were examined and extracts were analyzed for fumitremorgin content using high performance liquid chromatography. Growth occurred at O_2 levels as low as 0.10% but not at 0.0095%. Growth was always associated with production of verruculogen and fumitremorgins A and C. Production of these fumitremorgins was essentially the same in atmospheres containing 3.0 and 20.9% O_2, but was greatly reduced in an atmosphere containing 1.0 and 0.10% O_2. Fumitremorgin production was not significantly different at pH 3.5 and 7.0

INTRODUCTION

NEOSARTORYA FISCHERI (anamorph *Aspergillus fischerianus* = *A. fischeri*) is one of the most frequently isolated heat-resistant molds from fruit juices and other heat processed fruit-based products (Hocking and Pitt, 1984). The organism grows at 10–52°C with an optimal temperature range of 26–45°C (Beuchat, 1986; Samson et al., 1984). Ascospores formed by the teleomorph are exceptionally heat resistant compared to those of other molds (Beuchat, 1986; Conner and Beuchat, 1987).

Widely distributed in soil (Samson et al., 1984), *N. fischeri* causes spoilage of products containing fruits harvested from or close to the ground. The organism was first isolated from canned strawberries in 1963 (Kavanagh et al., 1963) and has since been isolated from several spoiled, heat-processed fruit products (Hocking and Pitt, 1984; Scott and Bernard, 1987).

Some strains of *N. fischeri* are capable of producing the mycotoxins terrein (Misawa et al., 1962), fumitremorgins A, B and C (Horie and Yamazaki, 1981; Nielsen et al., 1988 a,b) and verruculogen (Patterson et al., 1981). The latter four mycotoxins are members of the fumitremorgin group that also includes TR-2. Fumitremorgins cause tremors and convulsions, and may cause death in animals as a result of reversible biochemical changes affecting neurotransmission (Perera et al., 1982; Yamazaki et al., 1979).

Byssochlamys nivea, another mold capable of producing heat-resistant ascospores, has been demonstrated to grow at atmospheric O_2 contents as low as 0.27% supplied at a flow rate of 10 L/hr (King et al., 1969). Slight growth was observed after 6 days when the mold was cultured in an atmosphere of 99.999% pure N_2, but not under strict anaerobic conditions. Patulin was produced in apple juice at O_2 levels as low as 0.2%, but growth

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was completely inhibited by pure N_2 (less than 1 ppm O_2) (Orth, 1976). When inoculated into grape juice and incubated in glass jars, *B. nivea* reduced the atmospheric O_2 to 0.5% while growing and producing patulin (Rice, 1980). Growth of field and storage fungi in atmospheres containing 0.14–21% O_2 was studied by Magan and Lacey (1984); the O_2 level required to halve linear growth was 0.4–6.4% for *Aspergillus* spp. (3.4 % for *A. fumigatus*) and from less than 0.14–5.1% for other fungi.

Although it is known that *N. fischeri* is capable of producing fumitremorgins under a wide range of environmental conditions (Nielsen et al., 1988 a, b), no studies have been reported on its ability to produce these mycotoxins at reduced atmospheric O_2 levels. This study was designed to determine if growth and fumitremorgin production by *N. fischeri* cultured on Czapek Yeast Autolyate (CYA) agar at pH 3.5 and 7.0 are affected by reduced atmospheric O_2 content when incubated at 25°C in darkness.

MATERIALS & METHODS

Organism

N. fischeri (IBT3023), obtained from the Department of Biotechnology, Food Technology, The Technical University of Denmark, DK–2800 Lyngby, Denmark, was used in this study.

Preparation of inoculum

The mold was cultured on oatmeal agar (OAT) plates (Beuchat, 1986) at 25°C in darkness. After 30 days of incubation, ascospores were harvested by flooding the surface of the mycelial mats with 9 mL potassium phosphate buffer, pH 7.0 and gently rubbing the mold surface with a sterile bent glass rod. Suspensions of asci were pipetted into a sterile beaker. This procedure was repeated, and the two suspensions combined. In order to free the ascospores from asci and cleistothecia, the suspension was subjected to a 2-min sonication treatment of 60 W at 20 kHz using an ultrasonic homogenizer 4710 series (Cole Parmer Instruments Co., Chicago, IL). Intact asci and cleistothecia were allowed to settle to the bottom of the beaker for 2 to 3 min. Free ascospores in suspension were decanted through sterile glass wool into a sterile test tube, and asci and cleistothecia were resuspended in ca. 30 mL buffer. The sonication procedure was repeated. Aliquots (0.5 mL) of the ascospore suspension were deposited in 20 × 150 mm screw-cap test tubes containing 4.5 mL buffer tempered at 75°C in a water bath shaker. After 30 min at 75°C, the tubes were withdrawn and quickly cooled to 10°C in cold water. This suspension of heat shocked ascospores was used as an inoculum for all experiments.

Substrate

CYA was chosen as culturing medium for *N. fischeri* since previous studies revealed that it was a good substrate for supporting fumitremorgin production (Nielsen et al., 1988 a, b).

To evaluate the combined effect of pH and atmospheric O_2 content on growth and fumitremorgin production, CYA was adjusted to pH 3.5 and 7.0 with sterile HCl and poured into 90-mm Petri plates, ca. 20 mL in each. All plates were stored in plastic bags for 1 wk before inoculation; the plates to be incubated under reduced atmospheric O_2 were stored under an atmosphere of pure N_2 (99.999% N_2, < 10 ppm

Fig. 1—Experimental system used to determine the effect of reduced atmospheric O_2 on growth and fumitermorgin production by N. fischeri. (A) Humidifier (water), (B) desiccator, (C) wire mesh, (D) fan, (E) siphon trap, (F) inoculated Petri disnes with ruler to facilitate measurement of colony diameter through the glass lid, and (G) rubber seal through which the internal gas was removed for analysis.

O_2) for 24 hr immediately prior to inoculation to reduce dissolved O_2 content in the agar medium.

Inoculation procedure

Plates were inoculated at three points using an inoculation loop (3-mm diameter) which had been submerged in the heat-activated ascospore suspension. The viable ascospore population in the inoculum was determined by enumeration on CYA (pH 3.5 and pH 7.0).

Experimental incubation system

Experiments were carried out in desiccators equipped as shown in Fig. 1. Customized gas mixtures or air were first humidified by passing through water in a conical flask (A) and then led through a tube to the center of the desiccator (B) just above a wire mesh (C) on which the Petri dishes (F) were placed. Rulers were placed on the top of each stack of inoculated plates to facilitate measurement of colony diameters through the glass lid without opening the desiccator. Gas was vented out of the top of the desiccator and through a siphon trap (E), allowing unencumbered gas flow through the system. A fan (D) mounted in the center of the desiccator just under the wire mesh ensured a uniform level of O_2 and relative humidity throughout the desiccator. The gas mixture inside the desiccator was monitored by withdrawing samples through a rubber seal (G); analysis was done using gas chromatography.

Gases containing 3.03, 1.01, 0.107, and 0.0095% O_2, and zero grade N_2 (99.999% pure, less than 10 ppm O_2) (Air Products and Chemicals, Inc., Jacksonville, FL) were passed through individual desiccators; air (20.9% O_2) supplied by an aquarium pump was used as a control. The N_2 was either used directly or scrubbed with alkaline pyrogallol (5% solution) to remove traces of O_2.

Incubation

Twenty plates each of CYA at pH 3.5 and 7.0 inoculated with *N. fischeri* were placed in each disiccator. In addition, three batches of 20 plates each of CYA at pH 3.5 and pH 7.0 were incubated inperforated plastic bags outside desiccators.

The O_2 content X_t at a certain time t inside a desiccator with the initial gas composition X_o and volume V that is being flushed with a gas containing X_1% O_2 at a flow rate F, can be calculated by assuming that the gas composition throughout the desiccator is uniform (e.g.,

the gases are completely mixed). The percent O_2 content inside the desiccator can be determined by:

$$dX_t = \frac{X_1 dF + X_t V}{V + dF} - X_t = \frac{F}{V}(X_1 - X_t)dt$$

$$t = \frac{V}{F} \ell n \frac{X_0 - X_1}{X_t - X_1}$$

$$X_t = (X_0 - X_1)/e^{tF/V} + X_1$$

When $X_0 = 20.9\%$, $X_1 \approx 0\%$ (nitrogen atmosphere containing < 10 ppm O_2 and $V = 8$ L. Hence, $X_t = (20.9\% O_2)/e^{F/8}$.

The O_2 level in desiccators was reduced immediately after introducing inoculated plates by flushing for 40 min with nitrogen at a flow rate of 0.60 L/min (ca triple the desiccator volume in 40 min), resulting in ca 1.1% O_2 in the desiccators. The desiccators were then flushed with the respective gas mixtures at a high flow rate (0.60 L/min). After 30 min, the flow rate was reduced to ca 0.07–0.08 L/min (=4–5 L/hr) and maintained at that level throughout the incubation period. Upon removal of plates from desiccators after various incubation times, the desiccators were first flushed with N_2 for 30 min or longer and then with the appropriate gas mixture for 30 min, both at a high flow rate (ca 0.6 L/min) after which the lower flow rate was resumed.

Incubation was carried out at 25°C in complete darkness. After 38 days the experiment was terminated and air was pumped through those desiccators that had an oxygen level preventing germination of *N. fischeri*. This was done to determine if O_2 levels which prohibited colony development were fungistatic or fungicidal.

Analyses

Plates were examined and colony diameters were measured daily. Samples (5 plates at pH 3.5 and pH 7.0) were randomly selected 5, 11, 17 and 25 days after growth occurred at any given O_2 level, colony diameters were measured and conidia and ascospore formation were monitored visually and with a stereo microscope. The contents of the five Petri dishes (agar and mycelium) were extracted as described in Nielsen et al. (1988a).

Chromatographic analysis. Samples were analyzed using a HPLC system consisting of a Shimadzu Solvent Delivery Module LC-6A, a Shimadzu System Controller Module SCL-6A, a Shimadzu Integrator - CR5A Chromatopac, a Micromeritics 787 Variable UV/Visible Detector (Micromeritics, Norcross, GA) and a Rheodyne 7125 Injector (20 μl loop). The column was a reverse phase C18 (5μ Econosphere C18, 150 mm, i.d. 4.6 mm) from Alltech Associates, Deerfield, IL.

The gradient solvent system used by Frisvad and Thrane (1987) was optimized for analysis of fumitremorgins. Solvent A was acetonitrile (Optima, Fisher Scientific, Fair Lawn, NJ), and solvent B was deionized water which had been filtered (0.22 μm). The percent solvent A was raised from the initial 30% to 50% in 18 min, and to 90% during the succeeding 5 min. After 4 min at this level, solvent A was adjusted back to 30% within 1 min, and 2 min later intergration ceased. The flow rate was 2.00 mL/min and the UV/VIS detector was operated at a setting at 225 nm which is near the maximum wavelength for absorption of fumitremorgins.

Gas chromatographic analysis was performed on a Hewlett Packard 5790A gas chromatograph equipped with a thermal conductivity detector. An Alltech CTRI column 6 ft × 1/4 in. o.d. packed with a molecular sieve and propak mixture was used; the carrier gas was helium at 100 mL/min.

Mycotoxin standards. Standards of three mycotoxins (verruculogen and fumitremorgins A and C) were kindly supplied by Dr. R.J. Cole, USDA National Peanut Research Laboratory, Dawson, GA. Bracketed retention indexes and area-concentration factors were determined for each fumitremorgin as described by Hill et al. (1984) and Frisvad and Thrane (1987). Concentrations of the fumitremorgins were calculated by dividing areas of the peaks corresponding to each fumitremorgin by the area-concentration factor for that mycotoxin.

Compounds with the same retention times as the fumitremorgin standards were collected as they eluted from the liquid chromatograph, and their ultraviolet spectra were obtained using a Hewlett packard diode array spectrophotometer (model 8451A) and compared to the standards.

RESULTS & DISCUSSION

THE *N. FISCHERI ASCOSPORES* germinated and grew on CYA when incubated under atmospheric O_2 levels as low as

Table 1 – Length of lag phase and growth rate of N. fischeri cultured on CYA (pH 3.5 and 7.0) under various atmospheric O_2 contents

Atmospheric O_2 content (%)	Lag phase (days) before colonies appeared		Growth rate (mm/day) of colonies	
	pH 3.5	pH 7.0	pH 3.5	pH 7.0
20.9	2	2	6.9	12.0
3.03	2	3	7.0	14.0
1.01	3	3	5.4	13.0
0.107	4	7	1.6	2.5
0.0095	>38	>38		

Fig. 2 – Growth and fumitremorgin production by N. fischeri on CYA (pH 3.5) incubated at 25°C under variuos atmospheric O_2 levels: (●), 20.9% O_2 (air); (■), perforated plastic bags; (○), 3.03% O_2; (□), 1.01% O_2; and (△), 0.107% O_2 in N_2.

0.1%. Growth was always accompanied by production of fumitremorgins A and C and verruculogen. Growth did not occur at 0.0095% O_2 or in pure N_2 (< 10 ppm O_2) within the 38–day incubation period; however, exposure to air after this incubation period resulted in germination of ascospores within 36 hr, indicating that low O_2 levels were not fungicidal to N. fischeri.

The gas composition within desiccators during the study was essentially the same as the composition of the gas mixture entering the system. In desiccators wherein growth occurred, small amounts of CO_2 were produced.

The lag phase for growth of N. fischeri, i.e., the time required before mycelial growth was evident, was somewhat longer on CYA at pH 7.0 compared to pH 3.5 but the growth rate at pH 7.0 was almost twice that at pH 3.5 (Table 1). However, after 18 days of incubation the growth rate at 0.1% O_2 decreased to ca. 1 mm/day at both pH values and remained at that level throughout the study.

Large differences in growth rates resulted in larger colony diameters at pH 7.0 compared to pH 3.5 but did not generally result in a clear disparity in eventual production of fumitremorgins (Fig. 2 and 3). During the first few days of growth, colony diameters were less on CYA, pH 7.0 compared to CYA, pH 3.5, but thereafter colony diameters were significantly (P < 0.05) larger on CYA pH 7.0. Growth was similar on plates incubated in the desiccator flushed with air and in perforated plastic bags, but a lowering of the O_2 level to 3.0, 1.0 and 0.1% in desiccators significantly decreased radial growth. At 0.0095% O_2, N. fischeri ascospores did not germinate.

The production of fumitremorgins on CYA incubated under atmospheric air (20.9% O_2) was lower at pH 3.5 than at pH 7.0 in the beginning of the incubation period (day 7). During the balance of the incubation period, concentrations of the fumitremorgins were similar at the two pH values. The production of fumitremorgins was slightly lower on CYA incubated in plastic bags compared to production in the desiccator with constant air exchange.

Production of fumitremorgins C and A was largely unaffected by lowering the O_2 level to 3.0%. The highest concentrations of these fumitremorgins detected in the study were in plates incubated under 3.0% O_2 after 28 days of incubation. Concentrations were 56 μg/plate (fumitremorgin C) and 562 μg/plate (fumitremorgin A) at pH 3.5 and 7.0, respectively. However, these concentrations were not significantly higher than concentrations reached on CYA incubated in air. Verruculogen production was significantly (P < 0.05) repressed when the O_2 level was lowered to 3.0%.

Production of fumitremorgins under an atmosphere containing 3.0% O_2 was generally higher on CYA, pH 3.5 than on CYA, pH 7.0; only the concentration of fumitremorgin A after 32 days of incubation on CYA, pH 7.0 exceeded the concentration reached on CYA, pH 3.5.

Significantly lower concentration of fumitremorgins were recovered when the O_2 level was reduced from 3.0 to 1.0 and 0.10%. However, on the first 2 days of analysis (day 8 and 14), fumitremorgin C production was similar to production at higher O_2 levels. Least repressed was fumitremorgin A production at 1.0% O_2, pH 3.5 where the maximum reached after 28 days of incubation (302 μg/plate) was just 20% less than
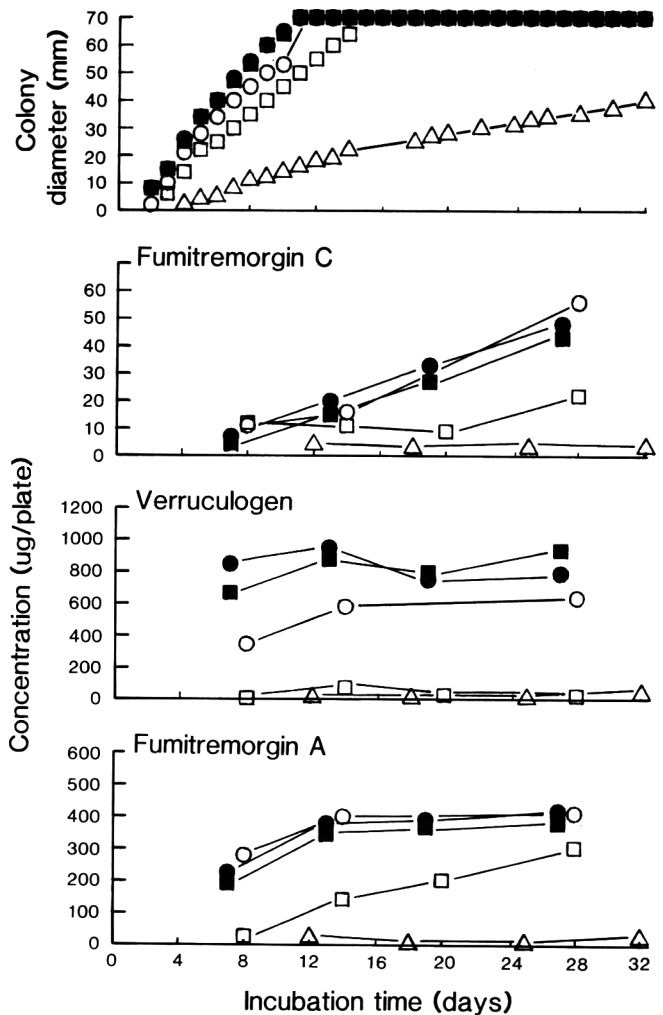
the production reached in perforated plastic bags after 27 days. Fumitremorgin C production was generally lower at pH 3.5 than pH 7.0, while the maximum production of verruculogen and fumitremorgin A was at pH 3.5.

The difference in the length of lag phase for mycelial growth between CYA at pH 3.5 and pH 7.0 may be caused by a more than fivefold increase in recovery of heat-shocked ascospores at pH 3.5 as compared to pH 7.0. Averages of 2.64×10^5 and 4.80×10^4 colony forming units (cfu) per mL were formed on CYA, pH 3.5 and pH 7.0 recovery media, respectively, corresponding to 780 and 138 cfu per inoculum point, respectively. Beuchat (1976) also observed a much higher recovery of heat shocked B. nivea ascospores on potato dextrose agar at pH 3.5 than at pH 5.5. These findings indicate that resuscitation of injured ascospores and/or germination of heat-activated ascospores following heat treatment may be enhanced at pH 3.5 compared to higher pH. Growth rate may be less affected by the size of inoculum than lag phase. Gonzalez et al. (1988) reported that for some Aspergillus, Penicillium and Fusarium species the lag phases increased with decreasing numbers of spores, while the growth rates were unaffected.

Formation of conidia and cleistothecia was also delayed by lowering the O_2 level (Table 2). It should be noted, however, that formation may have occurred somewhat earlier since plates were examined only after removal from desiccators at 6–8 day intervals during incubation. The differences between growth rate and colony diameter at the two pH levels did not result in

Fig. 4—Hypothetical lowest atmospheric O_2 content permitting growth of N. fischeri on CYA at pH 3.5 (o) and 7.0 (□). Values are at the intersection of the abscissa and the line of best fit calculated using least square regression analysis. For CYA (pH 3.5), log_{10} O_2 content is -1.2490 or 0.056% O_2; for CYA (pH 7.0), log_{10} O_2 content is -1.1001 or 0.079% O_2.

Fig. 3— Growth and fumitremorgin production by N. fischeri on CYA (pH 7.0) incubated at 25°C under various atmospheric oxygen levels: (●), 20.9% O_2 (air); (■), perforated plastic bags; (o), 3.03% O_2; (□), 1.01% O_2 and (△), 0.107% O_2 in N_2.

clear differences in appearance of colonies. Conidia and cleistothecia were formed simultaneously at pH 3.5 and 7.0; however, cleistothecia formation was expedited from 28 to 20 days at pH 3.5. Data do not suggest that a correlation exists between sporulation and production of fumitremorgins.

In an attempt to find the hypothetical minmum O_2 level permitting growth of N. fischeri, various relationships between growth and atmospheric O_2 content were analyzed. The reversed square equation $[1/t^2 = b \times log (X/X_{min})]$ gave a good description of the relationship between growth and O_2 level (Fig. 4). In this figure, t is the time required for colonies to reach 15 mm at an O_2 level X. The hypothetical lowest O_2 level permitting growth X_{min} is the value at the intersection of the axis of the abscissa and the line of best fit calculated using least-squares regression analysis. X_{min} values of CYA at pH 3.5 and 7.0 were 0.0056 and 0.079% O_2, respectively, with correlation coefficients of 0.993 and 0.995. Similar values were

noted when the time taken for diameters of colonies to reach 20 mm was used (0.059 and 0.066% O_2, with the correlation coefficients of 0.990 and 0.998 on CYA at pH 3.5 and 7.0, respectively). These results are in agreement with the observation that N. fischeri grew in an atmosphere containing 0.10% but not 0.0095% O_2.

The lower atmospheric O_2 limit for growth and production of fumitremorgins by N. fischeri is slightly lower than the 0.2% O_2 limit for growth and patulin production by the heat resistant mold B. nivea (Orth, 1976). Rice (1980) reported that the headspace O_2 contents in some retail juice products, e.g., grape juice and grape drink in glass containers, were 0.16-0.58%. One could infer from these observations that N. fischeri may grow and produce fumitremorgins in some retail fruit products, especially those packed in containers that are not hermetically sealed, e.g., some cardboard containers.

N. fischeri has been demonstrated to grow and produce fumitremorgins A and C and verruculogen at static O_2 levels as low as 0.1%. The presence of the mold in processed fruit products may represent a public health concern. Additional investigations are warrented to determine growth and fumitremorgin production characteristics of N. fischeri in fruit products stored and commercially processed by various means.

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Table 2—Number of days required for production of conidia and cleistothecia by N. fischeri cultured on CYA (pH 3.0 and 7.0) under various atmospheric O_2 contents

| Atmospheric O_2 content (%) | Days required for production of | | | |
| | Conidia | | Cleistothecia | |
	pH 3.0	pH 7.0	pH 3.0	pH 7.0
20.9	7	7	13	13
3.03	8	8	14	14
1.01	14	14	20	28
0.107	>32	>32	>32	>32

Heat Resistance of *Eurotium herbariorum*, a Xerophilic Mold

D.F. SPLITTSTOESSER, J.M. LAMMERS, D.L. DOWNING, and J.J. CHUREY

―――――――― ABSTRACT ――――――――

Eurotium haerbariorum, isolated from a spoilage outbreak involving grape preserves, was a true osmophile in that optimal growth was obtained in media containing 40–60% sucrose. Survival curves showed logarithmic death of heated spores followed by a tailing. In 5° Brix grape juice D-values at 70°C and z-values were 2.5 min and 9.1°C compared to 5.2 min and 7.1°C in 65°C Brix juice. Low concentrations of sorbic acid and fumaric acid in the heating menstruum had little effect on heat resistance.

INTRODUCTION

INVESTIGATIONS of a spoilage outbreak of grape jams and jellies due to a mold revealed that the organism grew poorly on common media used for fungi such as malt extract and potato dextrose agars. However, better growth was obtained when 10% or more sucrose was added to these media. The mold was identified as a strain of *Eurotium herbariorum* (*E. repens*) based on colonial pigmentation, echinulate conidia, and smooth-walled ascospores (Samson et al., 1984). Fungi in the genus *Eurotium* (Anamorph: *Aspergillus glaucus* group) are recognized as being xerophilic.

Pitt and Christian (1970) did not find the ascospores of this mold to be very heat resistant in that when heated 10 min at 70°C in a 20° Brix plum extract the survivals were only 2–3%. Stadler (1985) obtained a D_{65} of 1.3 min in single strength apple juice and a D_{65} of 17 min in an apple juice concentrate that contained 59% sugar.

The objective of this study was to determine whether our isolate was an especially resistant strain and to measure the effect of different variables on its heat resistance.

MATERIALS & METHODS

Ascospore production

The mold was propagated on Czapek yeast autolysate agar (King et al., 1986) containing 400 g/L sucrose (CY40S). After an incubation of at least 2 wk at 30°C, the plates were flooded with sterile water containing 0.1% Tween-80 and then scraped with a sterile bent glass rod to remove the spores. Free ascospores were obtained by homogenizing the suspension 2 min in a Stomacher (Tekmar Co., Cincinnati) followed by 30 sec bursts in a sonicator (Model W185D, Heat Systems-Ultrasonics, Inc., Plainview, NY) until intact asci were no longer visible under the microscope. The spores were stored frozen to prevent growth of chance contaminants. Repeated freezing and thawing did not affect the viable counts nor the heat resistance.

Heat resistance

The spores were heated in sealed 1.7 × 100 mm capillary tubes. In a typical trial, following centrifugation, the stock spores were resuspended in the test menstruum. A volume of 0.04 ml was injected into each capillary tube from a 1 mL syringe equipped with a Hamilton repeating dispenser. After flame sealing, the tips of the tubes were inserted into a square of 1" styrofoam which served as the holder. They then were then submerged in a constantly mixed water bath that had been equilibrated to the desired temperature. After heating for a given time, the tubes were cooled in an ice water bath. They were

placed in 95% ethanol for 2 min to eliminate most chance contaminants and then blotted dry on sterile filter paper. Five capillaries were transferred to a milk dilution bottle containing 20 mL sterile water which gave a 10^{-2} dilution of the spores after the tubes had been crushed with a sterile glass rod. Further decimal dilutions were made prior to plating on Czapek yeast autolysate agar (CYA) containing 200g/L sucrose (CYA-20). The plates were incubated seven days at 30°C before counting.

RESULTS & DISCUSSION

Recovery media

Initial studies were conducted to determine the influence of the plating medium on growth rates and on the recovery of spores that had been exposed to a lethal temperature.

The influence of sugar concentration on growth rates was determined by measuring the diameter of colonies that developed after heat activated spores had been stabbed into the center of agar plates. The results indicated that Czapek yeast autolysate agar containing 40 to 60% (w/v) sucrose permitted the most rapid growth. After 6 days at 30°C, for example, plates within this range gave an average colony diameter of 40 mm compared to 27 and 28 mm in media containing, respectively, 20% and 80% sucrose.

While the higher sucrose concentrations favored more rapid vegetative growth, they did not improve the recovery of spores that had been exposed to a lethal temperature. Thus spores heated at 70°C for 8 min gave comparable counts when cultured on CYA containing 10, 20, 40, and 60% (w/v) sucrose. CYA-20 was adopted as the standard plating medium because it permitted maximal recoveries in seven days and was easier to prepare than media containing the higher concentration of sucrose.

Rose bengal dye has been incorporated into various agar media to restrict the spreading of fungal colonies and thus facilitate counting. A comparison of counts in CYA-20 containing 0 and 8.4 μg/mL rose bengal dye, an amount recommended for *Byssochlamys* (Splittstoesser and King, 1984), showed that this level of dye did not reduce the recovery of spores that had been heated at 70°C for 12 min. Similar results have been obtained with the ascospores of *Talaromyoes flavus* (King and Halbrook, 1987) in that concentrations of rose bengal dye as high as 15 μm/mL did not affect recoveries. The use of the dye was discontinued after it was observed that excessive spreading of colonies was not a problem with this culture of *Eurotium* when propagated on CYA-20.

Activation

The ascospores of *E. herbariorum* were found to be similar to those of other heat resistant molds (Conner and Beuchat, 1987a; King and Halbrook, 1987; Splittstoesser and Splittstoesser, 1977) in that they possessed a dormancy that required heat activation before most spores would germinate. A heat treatment of 30 to 60 min at 60°C resulted in a 100-fold increase in the viable count which indicates that prior to heating 1% or less of the spores were in an active state (Fig. 1). Dilute grape juice enhanced activation only slightly, if at all, which is different from observations on *Neosartorya fischeri* where activation in grape juice had increased the spore counts by
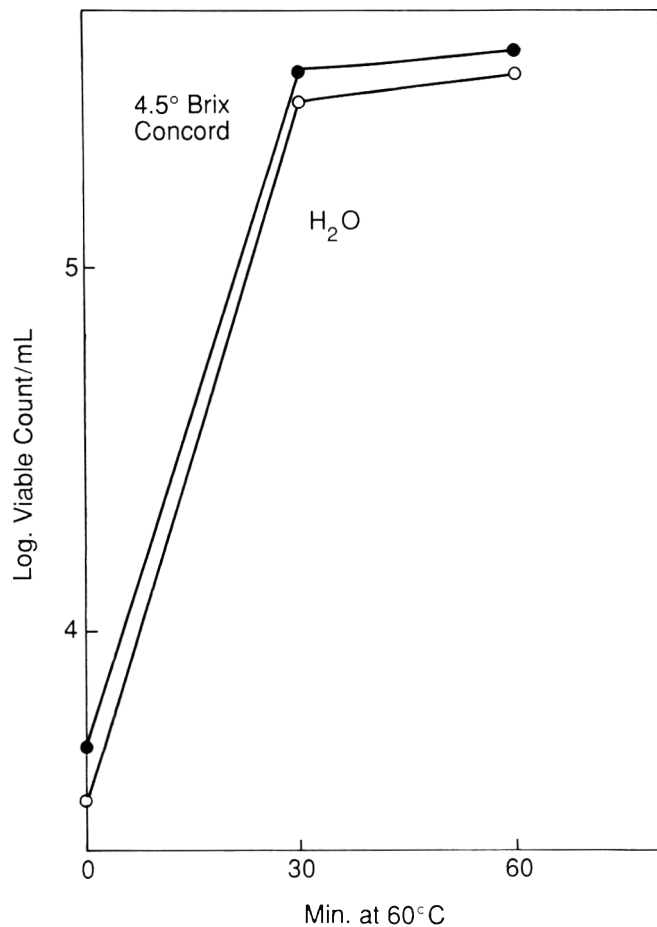
Fig. 1 – Effect of heat activation in water and grape juice on the viable counts of ascospores incubated 7 days on CY20S agar.

Fig. 2 – Heat resistance of ascospores in pH 3.5 Concord grape juice containing different concentrations of sucrose.

several orders of magnitude over that obtained in water (Splittstoesser and Splittstoesser, 1977).

In another study, activation in 5° and 56° Brix Concord grape juice was compared. Since *Eurotium* is xerophilic, it seemed possible that a medium containing a higher level of solubles might stimulate activation as well as growth. However, the recoveries in the two menstrua were comparable (data not shown) so it has been concluded that this is not an important variable.

In the heat activation trials, the spores were first suspended in 76% (final concentration) ethanol to destroy viable conidia and hyphal fragments. This was necessary because viable heat-labile structures would mask the effect of heat on dormant ascospores (Splittstoesser et al., 1970). The fact that following activation ethanol-treated suspensions gave counts comparable to their nontreated counterparts indicates that the ascospores of *E. herbariorum* are resistant to ethanol as are those of *Byssochlamys* and perhaps other heat-resistant molds.

Heat resistance

Survivor curves indicated that most of the spores underwent logarithmic death but that this was followed by a tailing which suggests that a small number of spores, under 0.1%, were of greater heat resistance (Fig. 2). Stadler (1985), observed similar logarithmic death followed by tailing when *E. herbarorium* spores were heated in apple juice. These curves differ from that reported for other heat resistant molds (Beuchat, 1988a; King and Halbrook, 1987; Splittstoesser and Splittstoesser, 1977) in that generally activated spores have exhibited a shoulder followed by an accelerated reduction in the viable count.

Heat resistance in Concord grape juice adjusted to 65° Brix with sucrose was of special interest because grape preserves
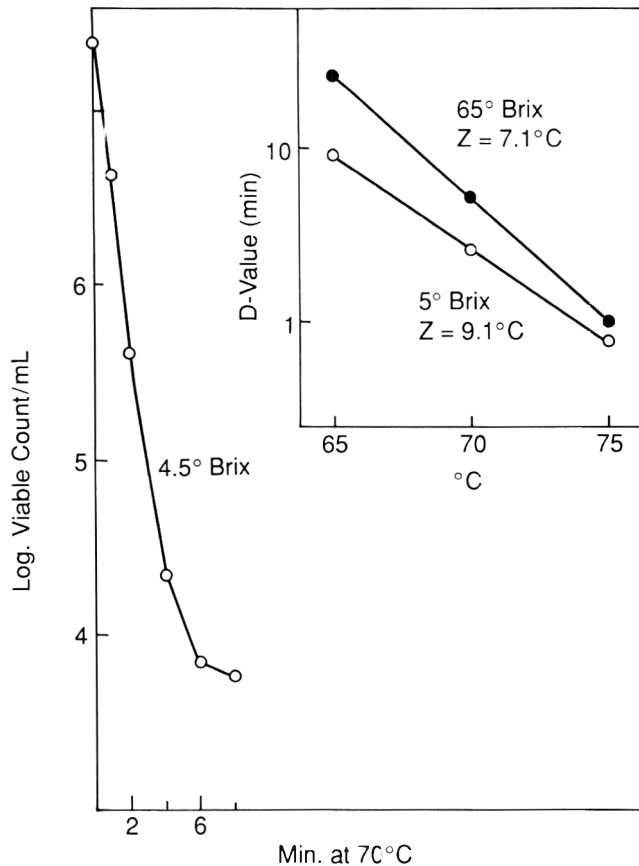
contain at least this amount of sugar and it is well known that the ascospores of many molds are more resistant in media of lower water activity (Corry, 1987). In a typical trial, the D_{70} values were 2.5 min in 5° Brix juice and 5.0 min in the 65° juice, thus about a 2-fold increase in resistance at the higher sugar level (Fig. 2). Because of the difference in z-values in the two juices, the protective effect of sugar was reduced as the temperature was increased.

The z-values reported for *Byssochlamys, Neosartorya fischeri, Talaromyoes flavus,* and other heat resistant molds have ranged from 3.0 to over 12°C. (Beuchat, 1986; Scott and Bernard, 1987; Splittstoesser and King, 1984) and thus our figures of 7.1 and 9.1°C fall within this range. Our finding that the composition of the heating menstruum affected the z-value has also been observed with other molds. The ascospores of *T. flavus,* for example, gave a z-value of 7.8°C in a peach product and a value of 12.9°C in a similar cherry filling (Beuchat, 1986).

Other factors

It is common to harvest ascospores from cultures that had incubated 30 days or longer because as the spores mature they seem to develop greater heat resistance. For example, the ascospores of *T. flavus* increased in resistance up to 30 days incubation (Beuchat, 1988a) while those of *N. fischeri* harvested at 114 days were considerably more resistant than scores from 42 day and younger cultures (Conner and Beuchat, 1987b).

The effect of culture age was less apparent with *E. herbariorum* (Table 1). While the spores harvested at 1 wk appeared to be somewhat less resistant, those harvested later showed little correlation with age. The good agreement between the two sets of D-values, which represent separate tests run 15 days apart, indicate that the differences between spore suspen-

Table 1—Effect of spore age and organic acids on the heat resistance of ascospores in 5° Brix Concord grape juice[a]

Variable		D_{70}	
Spore age at harvest	1 week	2.0	<2.1
	2	4.6	4.1
	6	2.7	2.6
	7	3.1	3.0
Sorbic acid	0 mg/L	1.2	
	200	1.1	
	500	1.1	
Fumaric acid	0 mg/L	1.4	
	0 mg/L, pH 3.0	1.4	
	1000 mg/L, pH 3.0	1.4	

[a] pH 3.5 unless noted otherwise

sions were real but that they related to some factor other than age.

Various organic acids, especially fumaric and sorbic acids, have been shown to sensitize ascospores when included in the heating menstruum (Beuchat, 1988b; Splittstoesser and Splittstoesser, 1974). It is the undissociated acid that appears to be the active form, which means that activity is inversely related to pH.

The maximal level of sorbic acid tested was 500 mg/L, a concentration often used in various high acid fruit products. At a pH of 3.5, the grape juice contained about 475 mg/L undissociated acid, a level which had little effect on the heat resistance of *E. herbariorum* ascospores (Table 1). These results agree with observations made on other species. *Byssochlamys nivea* ascospores, for example, were sensitized very little when heated in the presence of 1000 mg/L potassium sorbate that at pH 4.5 produced about 480 mg/L undissociated sorbic acid (Beuchat, 1981), and with *T. flavus*, concentrations of sorbic acid exceeding 4000 mg/L (5000 mg/L, pH4) had little effect (Beuchat, 1988b).

Studies on fumaric acid showed that 1000 mg/L (520 mg/L undissociated acid at pH 3) failed to affect the heat resistance of *E. herbariorum* ascospores. Again, the results are similar to observations made with *T. falvus* where 484 mg/L undissociated acid (5000 mg/L pH 4) also had little effect (Beuchat, 1988a). With *Byssochlamys fulva*, on the other hand, as little as 260 mg/L undissociated fumaric acid greatly reduced spore resistance to heat. (Splittstoesser and Splittstoesser, 1977). Thus it appears that the spores of different mold species vary in their reaction to this acid.

CONCLUSIONS

WHILE THE SPORES of *E. herbariorum* were not as resistant to heat as those reported of *B. fulva, T. flavus* or *N. fischeri,*

the organism should be added to the list of molds that are potential spoilers of thermally processed foods. Low water activity foods that receive only a mild heat treatment might be especially vulnerable. Fruit preserves normally would receive sufficient heat to destroy the spores. It is believed that the problem with the grape jams and jellies resulted because the hot water sprays used to sterilize the jar lids after filling and closing were malfunctioning.

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Growth Conditions Affecting Proteolytic Enzyme and Extracellular Vesicle Production by *Pseudomonas fragi* ATCC 4973

ROBERT M. MYHARA and BRENT J. SKURA

———————— ABSTRACT ————————

Pseudomonas fragi ATCC 4973 was grown in liquid culture (trypticase soy broth) and on a solid surface (tryticase soy broth + 1.5% agar). *Pseudomonas fragi* grown in liquid culture started extracellular proteolytic enzyme production at 24 hr, during the late exponential early stationary growth phase. *Pseudomonas fragi*, grown on solid surfaces, initiated proteinase production at 4 hr, 20 hr earlier than in liquid medium. Proteinase production was greatest during periods when the cells were not actively growing. Electron micrographs of *P. fragi* grown on solid surfaces revealed extracellular vesicles ca. 20 nm in diameter "blebbing" off the surface of the cells. The greatest number of vesicles coincided with maximum proteinase production. These vesicles were absent from the surface of *P. fragi* cells grown in liquid culture.

INTRODUCTION

AT CHILL TEMPERATURES the bacterial flora present on meat surfaces consist of cold tolerant organisms. *Pseudomonas fragi* is commonly involved in such psychrotrophic spoilage of meat (Shaw and Latty, 1982). Extracellular proteolytic enzymes produced by this bacterium attack the proteinaceous components of the meat and are one of the primary spoilage mechanisms (Yada and Skura, 1981, 1982; Dainty et al., 1975).

Borton et al. (1970) found that *P. fragi* preferentially utilized the low molecular weight, water-soluble meat components first and delayed production of the enzymes. Proteolysis could not be demonstrated until later in the growth cycle.

McKellar (1982), Murray et al. (1983) and Skura et al. (1986) conducted research on the proteolytic enzymes produced by *Pseudomonas* sp. and found extracellular proteinase reached a maximum rate of production during the late exponential, early stationary phase of growth. Similar results were reported by Stepaniak et al. (1987) for *P. fluorescens* P1 and AFT-21 in shaken cultures of whole milk.

Lee Wing et al. (1983) examined the fine structure of *Pseudomonas fragi* during spoilage of meat surfaces. Electron micrographs revealed the presence of extracellular bleb-like structures. These extracellular vesicles appeared to emanate from the surface of the bacterium and accumulated in the supernatant. Thompson et al. (1985) found a close association between these vesicles and proteinase production by *Pseudomonas fragi* in liquid culture.

Previous studies have concentrated on proteinase and vesicle production in liquid culture only. Since *P. fragi* is known primarily as a surface spoilage organism, the appearance of proteinase and the time of maximum enzyme production were determined for *P. fragi* cells grown both in liquid and on solid

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culture. In addition, the fine structure of the cell surfaces during this growth were also examined.

MATERIALS & METHODS

The organism

Pseudomonas fragi ATCC 4973 was purchased from the American Type Culture Collection (Rockville, MD). The organism was grown in trypticase soy broth [TSB] (BBL, Baltimore, MD), harvested by centrifugation (10,000 × g, 20 min), resuspended in TSB and stored frozen in liquid N_2. Cultures were thawed and incubated in TSB at 21°C for 18 hr and streaked onto trypticase soy agar (TSA, BBL) slants. These stock cultures were stored at 4°C and replaced at 4 wk intervals.

Inoculum growth

Pseudomonas fragi was incubated with agitation at 21°C for 24 hr in TSB. After incubation the cells were harvested by centrifugation (10,000 × g, 20 min) and resuspended in 0.1% peptone water.

Incubation on solid medium

An aliquot of inoculum containing ca. 4.8 × 10³ colony forming units (CFU) *P. fragi* was mixed with 100 mL 0.1% peptone water and the suspension drawn through a sterile 47 mm polycarbonate membrane filter (0.45 μm, Nucleopore Corp., Pleasanton, CA). The membrane, together with the collected cells, was placed onto the surface of 15 mL TSB + agar, contained in a 50 mm petri plate and incubated at 21°C at 4 hr time intervals up to 60 hr. Experiments were repeated, in duplicate, three times.

Incubation in liquid medium

Separate 250 mL flasks containing 150 mL TSB were inoculated with ca 1.5 × 10⁶ CFU washed cells and incubated with agitation (150 RPM) at 21°C at 4 hr intervals up to 96 hr. These experiments were also repeated, in duplicate, three times.

Enumeration of cells

After incubation, *P. fragi* cells grown on solid medium along with the membrane filter support were removed together from the agar surface. The cells were dispersed with a Stomacher 400 (Cooke Laboratory Products, Alexander, VA) into 100 mL 0.1% peptone water. Mixing time was 2 min. Cells grown in liquid culture were dispersed, if necessary, into 99.0 mL 0.1% peptone water.

Pseudomonas fragi suspensions were enumerated on TSA plates using the Spiral plating technique (Spiral Systems Inc., Cincinnati, OH). Plates were incubated at 21°C for 24–48 hr. Colony forming units were counted with a Model 500A bacteria colony counter (Spiral Systems Inc., Cincinnati, OH) interfaced with a Spiral Systems Computer Assisted Spiral Bioassay data system.

Proteinase activity

The enzyme substrate was prepared by adding 10.0g Hammersten casein (Chemical Dynamics Corp., NJ) to 187.5 mL 0.4M Na_2HPO_4 followed by boiling for 30 min to solubilize the casein. After cooling,

312.5 mL of 0.4M NaH_2PO_4 and 0.2g NaN_3 were added and the mixture diluted to 1L.

For determination of proteinase activity in liquid medium, 0.2 mL of culture supernatant, clarified by centrifugation (3,000 × g, 20 min) and filter-sterilized, were added to 2.0 mL substrate. Proteinase activity of whole cells grown in liquid medium was determined by adding 0.2 mL unclarified culture medium to 2.0 mL substrate. Proteinase activity in solid medium was determined by adding the agar medium contained within a petri plate, without the membrane filter (15.0 mL agar) to 150.0 mL substrate, followed by blending for 2 min with a Stomacher 400. In all cases, 2.2 mL test mixture aliquots were incubated at 40°C for time periods ranging up to 2 hr. After incubation the reaction was stopped with 2.0 mL 24% trichloroacetic acid (TCA), and the mixture centrifuged (2,000 × g, 10 min). Absorbance at 280 nm of the supernatants was determined with a Shimadzu model UV-160 spectrophotometer (Shimadzu Scientific Instr. Inc., Columbia. MD). One proteinase unit was defined as the enzyme quantity liberating TCA soluble amino acids and peptides equivalent to 0.001 μmole of tyrosine per minute. Tyrosine equivalents of unincubated controls were deducted from incubated samples to compensate for nonproteinaceous U.V. absorbing material present in the substrate.

Electron microscopy

Sample preparation. Cells from liquid medium were centrifuged (2,000 × g, 10 min), washed in 0.05M phosphate buffer (PB) pH 7.0, recentrifuged and fixed with 2.5% glutaraldehyde (J.B. EM Services, Dorval, PQ) in PB at room temperature for 1 hr. Fixed cells were washed in PB and post-fixed with 1% w/v osmium tetroxide (J.B. EM Services, Dorval, PQ) in PB at room temperature for 1 hr. Fixation of *P. fragi* cells grown on solid medium was achieved by placing the entire polycarbonate membrane filter (Nucleopore) into the fixatives.

Scanning electron microscope samples were dehydrated on the surface of polycarbonate membrane filters (Nucleopore) through a graded series of aqueous ethanol solutions from 30 to 80% for 5 min each, two changes of 90% for 10 min each, followed by two changes of 100% for 20 min each. Samples were critical point dried in a Parr-bomb (Parr Instruments Co., Moline, IL) using CO_2 as the transition fluid. Dried membrane filters were mounted on aluminum stubs with silver paste (J.B. EM Services Inc., Dorval, PQ) and sputter-coated with gold (SEMPREP II Sputter Coater, Nanotech Ltd., Prestwich, England).

Fixed cells to be examined by TEM were suspended in a mixture of 1.5% agar in PB. After solidification 1 mm^3 blocks were cut and immersed in a graded series of aqueous ethanol solutions as described for SEM. Samples were infiltrated with EPON 812 (J. B. EM Services Inc., Dorval, PQ) using propylene oxide as a transition solvent and polymerized at 60°C for 36 hr.

Embedded samples were sectioned with a Porter-Blum ultramicrotome (Ivan Sorvall Inc., Norwalk, CT). Sections were mounted on 3 mm copper grids and stained with 2% w/v uranyl acetate and lead citrate.

Microscopy. Scanning electron microscopic examination of cells was done with a Cambridge Stereoscan 250 operated at 40 Kv. Transmission electron microscopy was done with a Zeiss EM-10 at an accelerating voltage of 80 Kv.

Transmission electron micrographs of *P. fragi* cells grown on TSB + agar for 20, 32, 40 and 56 hr were examined. The total of the cell perimeters for each electron micrograph was calculated, in μm. In addition, the total number of extracellular vesicles appearing on those cell perimeters were counted. With this information the number of vesicles per μm of cell perimeter was calculated. At each time interval, four electron micrographs were examined. Mean values and standard deviations were determined.

RESULTS

Growth of *P. fragi*

In liquid medium (Fig. 1a) the *P. fragi* population increased from the initial 2.0 × 10^5 CFU/mL to 2.0 × 10^9 CFU/mL after 90 hr. The bacteria entered an exponential growth rate at 8 hr and continued up to 20 hr, followed by a stationary growth phase. The population began to decline somewhat, after 56 hr, but began to increase again after 76 hr.

On solid medium (Fig. 2a) *P. fragi* increased from an initial population of 2.8 × 10^5 CFU/cm^2 to 8.2 × 10^9 CFU/cm^2

(a)

(b)
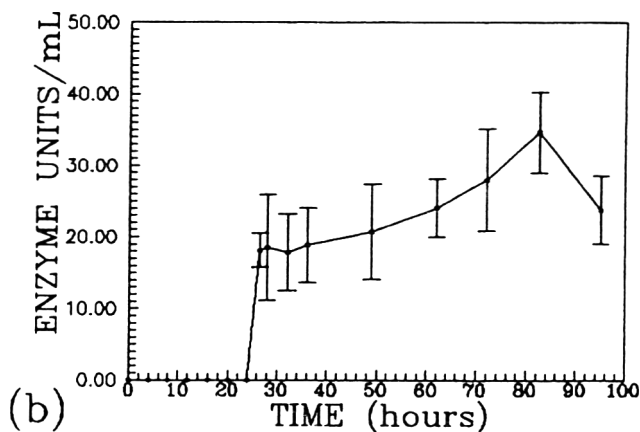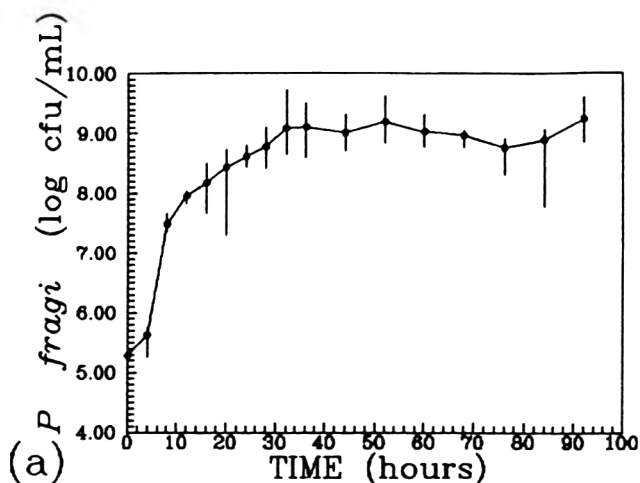
Fig. 1 — P. fragi *growth and proteinase production in liquid medium. (a) log* P. fragi *colony forming units/mL. The data points represent the geometric mean of three separate experiments. Bars represent ranges. (b) proteinase activity (enzyme units/mL). The data points represent the arithmetic mean of three separate experiments. Bars represent the sample standard deviation.*

after 60 hr. The growth rate became exponential at 4 hr but slowed by 16 hr. From 16 to 32 hr a reduced rate of growth, similar to that of a stationary phase, occurred. During this final stationary phase of growth, cell numbers did increase slightly to the end of the experiment. Cell colonies on the membrane surface became visible after 8 hr. By 24 hr a thick mat of cells could be seen growing on the membrane surface. At 60 hr the mat was several millimeters thick.

Proteolytic activity of *P. fragi*

Proteolytic activity in liquid medium (Fig. 1b) was first detected at 26 hr and increased steadily to a maximum at ca. 82 to 88 hr, at which point it began to decline. The amount of proteinase produced per *P. fragi* CFU peaked at two times; one at 26 hr and one at 72 hr. These times corresponded to points in the growth curve where the growth rate of the organism had slowed (Fig. 1a). Proteolytic activity of whole *P. fragi* cells grown in liquid medium could not be demonstrated at time periods before 26 hr.

Proteolytic activity, on solid medium first appeared at 4 hr (Fig. 2b) approximately 22 hr sooner than in liquid culture. The proteinase activity declined somewhat at 8 hr but began to increase quickly after 16 hr. The rate of increase slowed

(a)

(b)

(C)
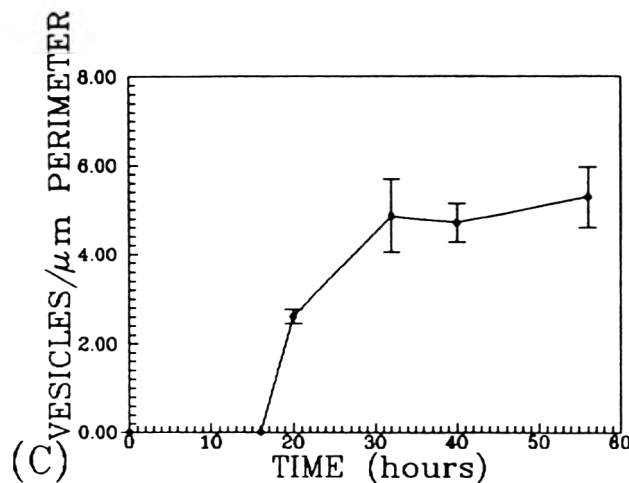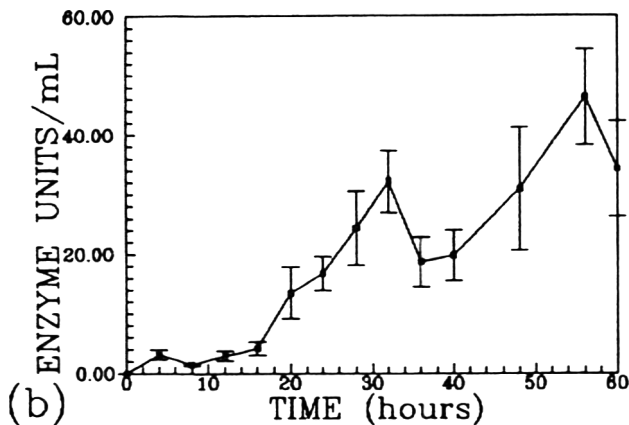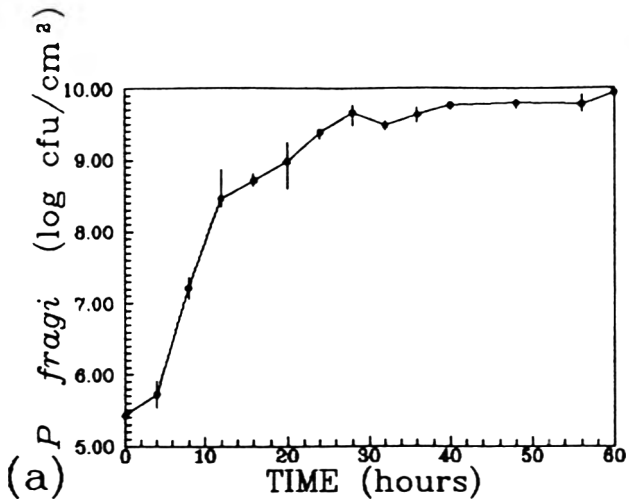
Fig. 2 — P. fragi *growth and proteinase production on solid medium. (a) log* P. fragi *colony forming units/cm². The data points represent the geometric mean of three separate experiments. Bars represent ranges. (b) proteinase activity (enzyme units/mL agar). Data points represent the arithmetic mean of three separate experiments. Error bars represent sample standard deviation. (c) vesicles per unit perimeter. Transmission electron micrographs of* P. fragi *cells at 20, 32, 40, and 56 hr were examined. Cell perimeters were calculated and vesicles appearing on the perimeters were counted. Mean values of vesicles per μm of cell perimeter were plotted as a function of time. Error bars represent sample mean standard deviation.*

somewhat after 20 hr, but total proteolytic activity peaked at 32 hr. At this point, activity declined sharply and did not rise
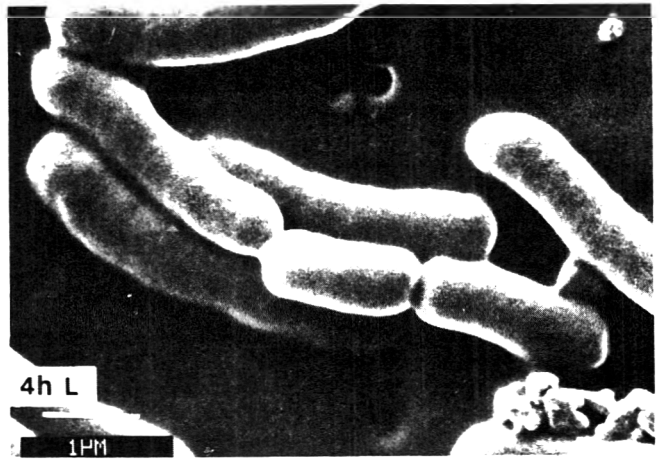
Fig. 3 — Scanning electron micrograph of P. fragi *grown in liquid medium for 4 hr. Cell surfaces appeared smooth, with no evidence of accumulated surface globules. Bar represents 1 μm.*
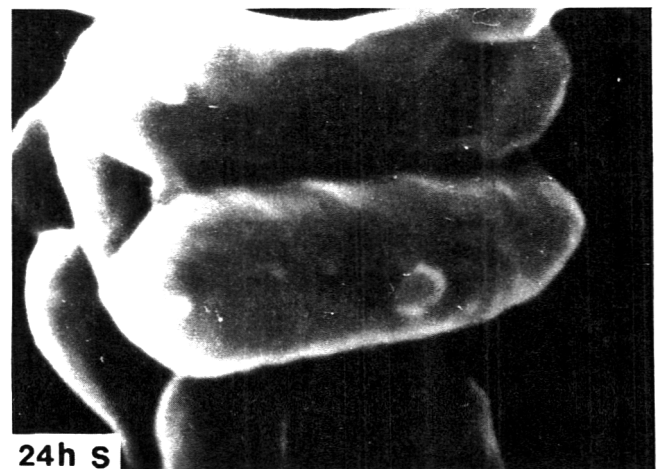
Fig. 4 — Scanning electron micrograph of P. fragi *grown on solid medium for 24 hr. Cell surfaces and accumulation of globules, approximately 40–190 nm in diameter.*

substantially for 8 hr. Proteolytic activity, at 40 hr began to increase steadily for 16 hr to a maximum at the 56 hr mark. Past this point, activity declined to the end of the experiments.

Scanning electron microscopy

At 4 hr, the surface of *P. fragi* cells grown in liquid medium appeared smooth (Fig. 3). The surfaces of *P. fragi* grown on solid surfaces were similar to those cells grown in liquid medium. They did not change appreciably until the end of the late exponential phase and the beginning of the stationary phase (ca 24 hr) at which point the cell surfaces became noticeably rough (Fig. 4) with accumulation of globules ca. 40–190 rm in diameter. This point coincided with a dramatic increase in proteolytic enzyme (Fig. 1b). *Pseudomonas fragi* cells grown in liquid medium did not display such globules until about 76 hr incubation.

Transmission electron microscopy

Liquid medium. Transmission electron micrographs of *P. fragi*, grown in liquid medium for 16 hr, showed the outer cell margin as a complex structure composed of several layers (Fig. 5) with the outer most portion being highly convoluted. The
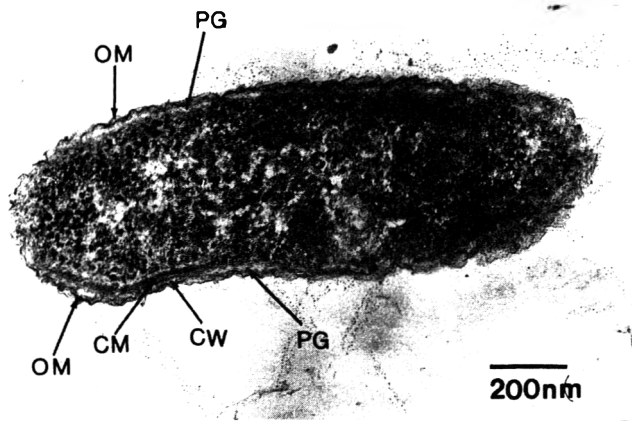
Fig. 5—Transmission micrograph of P. fragi grown in liquid medium for 16 hr: (OM) outer membrane; (PG) periplasmic gap; (CP) cytoplasm; (CM) cytoplasmic membrane; (CW) cell wall.
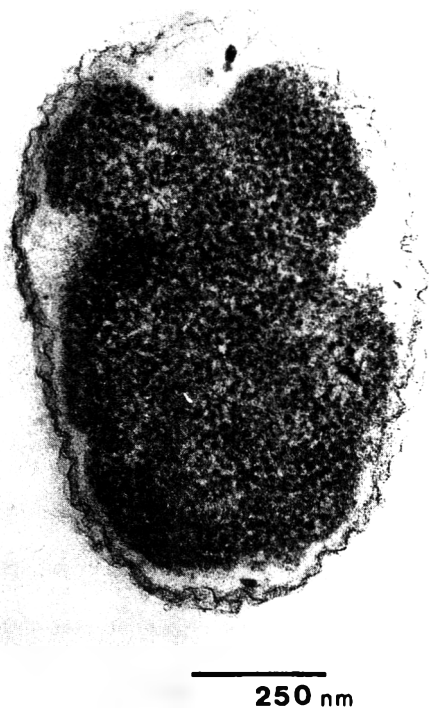
S 4h

Fig. 6—Transmission electron micrograph of P. fragi grown on solid medium for 4 hr. Note the absence of extracellular vesicles.

Fig. 7—Transmission electron micrograph of P. fragi grown on solid medium for 20 hr showing presence of vesicles approximately 20–40 nm in diameter.

cytoplasmic portion (CP) was surrounded by an inner cytoplasmic membrane (CM) which was in turn contained by a cell wall (CW) structure. Together they contain the cytoplasm and define the overall shape of the cell. Beyond these structures the periplasmic gap (PG) separates the cell wall from the outer cell membrane (OM). It was this outer membrane, completely surrounding the cell, which gave the convoluted appearance to the cell outer margin. Although the outer cell membrane was highly convoluted, no evidence of extracellular vesicles could be seen on P. fragi cells grown in liquid medium for time periods of up to 92 hr.

Solid medium. Transmission electron micrographs of P. fragi grown on a solid surface for 4 hr (Fig. 6), again showed the cell margin, similar in form to that of cells grown in liquid

medium. At 20 hr (Fig. 7) most cells were surrounded by many round vesicles more or less bound to the cell surface. All the vesicles, between 20 and 40 nm in diameter, appeared to originate from the outer cell membrane. In some instances the vesicles appeared to be attached to the cell by a stalk, presumably of outer cell membrane material. The individual vesicles, when examined in section, were surrounded with a membrane similar in appearance to that of the outer cell membrane, surrounding intact cells. Those cells actively shedding particles may have had a reduced periplasmic gap, although cell wall-cytoplasmic membrane structures appeared to be intact and did not appear to contribute to vesicle formation.

The process of vesicle shedding began between 4 hr and 20 hr and seemed to increase with time, since the number of vesicles per cell at 32 hr (Fig. 8) was greater than those at 20 hr (Fig. 7).

Vesicle shedding versus enzyme production of P. Fragi

Liquid medium. Scanning electron micrographs revealed globules on P. fragi 40 to 190 nm in diameter visible after 76 hr, approximating the time period when proteolytic activity was at a maximum (Fig. 1b). Transmission electron micrographs of cells grown in liquid medium failed to display evidence of extracellular vesicle shedding at all time periods examined.

Solid medium. Scanning electron micrographs revealed globules on P. fragi cells grown for 24 hr on solid medium (Fig. 4), ca 40 to 190 nm in diameter. This time period co-
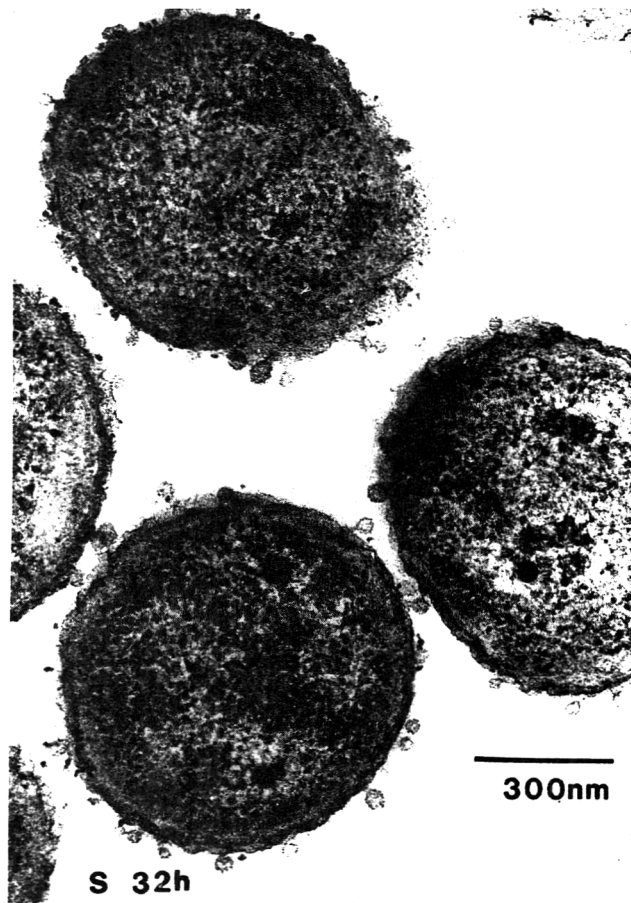
Fig. 8 — Transmission electron micrograph of P. fragi *grown on solid medium for 32 hr. Vesicles continued to be present on the cell surfaces.*

incided with that of increased proteolytic activity by cells grown on the solid surface (Fig. 2b).

TEM revealed that extracellular vesicle shedding commenced by 20 hr (Fig. 7). This point in time also approximated increased proteinase activity (Fig. 2b). A count of the extracellular vesicles per unit perimeter of cells grown on solid medium further supported the association of vesicle appearance and proteinase production (Fig. 2c).

DISCUSSION

Growth of *P. fragi*

Pseudomonas fragi, in general, grew equally well in liquid and on solid culture medium. Medium type had little apparent effect on its overall growth rate (Fig. 1a and 2a). Both cultures entered a phase at 40 hr which was similar to stationary growth.

Since the cell numbers continued to increase to the end of the experiments, a true stationary growth phase might not have been reached. This observation was similar to that of Rowe and Gilmour (1982) who found *P. fluorescens* sp. did not enter a stationary growth phase but rather alternated between periods of slow and more rapid growth. This behavior may have been the result of the organisms shifting from one nutrient source to another, with a slight lag period during which biochemical adjustments were made within the cell.

Enzyme activity

In liquid medium, proteolytic enzyme was first detected at 26 hr during the late exponential, early stationary growth phase (Fig. 1b). This supports the findings of McKellar (1982) and

Stepaniak et al. (1987). Apparently, the cells were not producing proteinase, since proteolytic enzyme activity could not be demonstrated in whole cells grown in liquid medium at 8 and 12 hr. These observation were in agreement with previous research (Thompson et al., 1985). Several explanations regarding this behavior have been postulated including depletion of soluble nitrogenous nutrients (Lee Wing, 1984) or depletion of dissolved oxygen (Rowe and Gilmour, 1982).

On solid medium, proteolytic enzyme first appeared at 4 hr, approximately 22 hr sooner than that of liquid culture (Fig. 2b). Obviously, the type of culture (solid versus liquid) influenced enzyme production. As with those cells grown in liquid culture, some sort of growth factor depletion might have been involved in stimulating enzyme production. To produce this early appearance of proteinase production, each bacterial cell must have increased its enzyme production rate. This increase in enzyme production rate appeared to vary inversely with cell growth rate. When the cells were actively growing, less enzyme was produced. When the cells entered a less active growth stage, an increase in enzyme production rate followed. Whether the cells slowed their growth rate to produce more enzyme or whether the increase in enzyme triggered the cells to slow their growth rate was not known. The former seemed more likely, however, since apparent growth rate reduction preceded enzyme rate increase.

Electron microscopy

Scanning electron micrographs of *P. fragi* cells both in liquid and on solid culture medium, grown to the lag and exponential growth phases, appeared to have smooth surfaces. At ca. 24 hr (Fig. 4), cells grown on solid medium became rough in appearance with accumulation of globules 40–190 nm in diameter.

This change in surface texture coincided with an increase of proteolytic enzyme. A similar surface was observed on cells grown in liquid medium but at 76 hr. This time period approximated the culture's maximum proteinase production, suggesting that cell surface disruption and globule formation were linked to proteolytic enzyme production. Lee Wing (1984) described cell surface globule formation of *P. fragi* cells grown on solid culture medium but did not link these globules to proteinase production. The globules observed here and in other studies were much larger than those extracellular vesicles approximately 20–40 nm in diameter visible on the surface of *P. fragi* cells and described as "blebs" by Lee Wing (1984) and Thompson et al. (1985). It is possible that globules found here and also reported by Lee Wing et al. (1983) were composed of coalesced extracellular vesicles. Further investigation of these globules is required.

Transmission electron microscopy

Transmission electron micrographs of *P. fragi* thin sections (Fig. 5) revealed the classic trilayer typical of gram negative bacteria as described by Costerton et al. (1974) and Michaelis and Beckwith (1982). *Pseudomonas fragi* grown for 20 hr on solid growth medium (Fig 7). displayed many extracellular particles attached to the cell surface. These particles, identical in size to those described by Lee Wing (1984) and Thompson et al. (1985), were present in large quantities only on cells grown on solid surfaces. *Pseudomonas fragi* cells, grown in liquid medium for time periods of up to 92 hr, failed to display such extracellular vesicles.

The largest number of extracellular vesicles appeared on *P. fragi* grown on solid medium at about 32 hr. This coincided with the time period at which maximum proteinase production occurred. This would be expected if these vesicles were the main means of enzyme transport through the outer cell membrane.

Extracellular vesicles were present on the surface of *P. fragi*

Correlation of Proton T_1 with Polymer and Solute Waters in Starch-Sucrose Mixtures

PAVINEE CHINACHOTI and M. P. STEINBERG

ABSTRACT

Proton pulsed NMR methodology was applied to correlate the observed spin-lattice (T_1) and spin-spin (T_2) relaxation times with the amount of water associated to sucrose (SOL) and that to starch (POL) in a sucrose-starch model system containing both. Sucrose, starch and four mixtures were equilibrated to four water activities (a_w, 0.86–0.97). Calculations for T_2 were invalid due to an undeterminable amount of molecular diffusion caused by field inhomogeneity in the magnet. Model equations were developed for calculating SOL and POL from T_1 and a_w. These values showed high correlations ($R^2>0.97$) with SOL and POL as determined from sorption data for sucrose and starch. This validated the use of this instrument with the mathematical models developed.

INTRODUCTION

LANG AND STEINBERG (1981), applying the Smith (1947) linear sorption isotherm, found marked differences in sorption behavior between macromolecules and solutes. They concluded that each sorbed a different state of water; the water sorbed by macromolecules such as starch and casein was termed polymer water and that sorbed by solutes such as sugar and salt was termed solute water. The amount of each state present in a food can profoundly affect the chemical, physical, biological and rheological properties of the food (Lang, 1981; Urbanski et al., 1982, 1983).

Pulsed Nuclear Magnetic Resonance (NMR) spectroscopy is a desirable technique for determining the quantity of water in a biological system because it is noninvasive, i.e., the sample is not changed by the analysis. Hester and Quine (1976) and Miller et al. (1980) demonstrated the versatility of the pulsed NMR technique for water analysis by applying the Praxis PR-103 process analyzer to milk products and grains, respectively. Brosio et al. (1978) determined moisture in starch-rich foods from the FID signal measured twice after the pulse. This shows that the NMR signal is strongly correlated with the amount of water present.

Because ^1H NMR is usually sensitive not only to the amount but also the mobility of water, one might expect that ^1H NMR should be able to detect the amount of free water and solute water by measuring T_1 (spin-lattice) and T_2 (spin-spin) relaxation of the system. This would be of great contribution to this field. However ^1H NMR relaxation measurements of water-solid mixtures are known to show a significant effect of cross-relaxation between protons of water and protons of each solid as shown in case of spin-lattice (T_1) relaxation by Edzes and Samulski (1978), Koenig et al (1978), Shirley and Bryant (1982) and Wise and Pfeffer (1987). This means that T_1 cannot directly determine the amount of bound water since T_1 is not only dependent on protons of bound and free water but also those of polymers and solutes, as well as the cross relaxation rates among them. However, the NMR signals may still be very sensitive to protons in the liquid states, i.e., those of water associated to solutes (SOL). The correlation between T_1 and

SOL (and T_1 and POL) may be significant and this may lead to an empirical NMR method of SOL and POL analysis. Therefore, the objective of this work is to correlate the observed spin-lattice(T_1) relaxation and spin-spin(T_2) relaxation with SOL and POL content calculated from a mass balance equation.

MATERIALS & METHODS

Materials

Corn starch, Argo brand (Best Foods, Inc., Inglewood Cliffs, NJ) and sucrose, analytical grade (Mallinckrodt, Inc., St. Louis, MO) were used. Lots of both solids were adjusted to water activities (a_w) of 0.878, 0.902, 0.925 and 0.973. In case of sucrose, this was done by adding water as determined from sorption data (Chinachoti and Steinberg, 1984). Starch lots were equilibrated against salt slushes at these a_w (Greenspan, 1977; Stokes and Robinson, 1949) in Proximity Equilibration Cells (Lang et al, 1981). Mechanical mixtures of sucrose and starch at the same a_w were prepared to contain 0, 10, 20, 50, 70, and 100% sucrose, dry basis. These were prepared in duplicate to give 48 samples. In addition, samples of sucrose and starch were moistened to a large number of moisture contents in the range 0 to about 75% moisture. These were analyzed for T_1 and T_2.

Pulsed NMR

All samples were analyzed by a 10 MHz Praxis Pulsed NMR Spectrometer (model PR-103, the Praxis Corp., San Antonio, TX) at 25°C. T_1 was determined from the 90°-VTD-90° pulse sequence and T_2 from the 90°-VTD-180° pulse sequence, where VTD (variable time delay) is the time interval between the two pulses, 10 msec for the 90–90° pulse and 0.5 msec for the 90–180° pulse. The signal decay with time was recorded for a large number of iterations and averaged by a built-in computer interface. The slopes of the log amplitude from a 90–90° pulse vs time plots were used to calculate the observed T_1 (Farrer and Becker, 1971). The similar plots for the 90–180° pulse could not provide a means to calculate T_2 due to an undeterminable amount of molecular diffusion which is more obvious when T_2 is short and the magnetic field is inhomogenous. Therefore, further analysis was limited only to T_1.

All measurements were done on duplicate samples; only the averages are presented here. Curve fitting analysis was applied to obtain deterministic equation between T_1 and each water states. This was done by linear and non-linear regression under SAS statistic software (SAS, 1982).

RESULTS & DISCUSSION

T_1 OF STARCH and sucrose at various moisture contents is shown in Fig. 1. Some of these T_1 data together with most of the data from the mixtures are in Fig. 3.

The overall T_1 in Fig. 1 shows a correlation with moisture content for both sucrose and starch, and it was found to be more sensitive to sucrose-water then to starch-water samples. This explains, in sucrose-starch mixtures (Fig. 2), the increased T_1 with increasing a_w and sucrose content.

The observed T_1 reported here is not only a result of the water proton but also a result of the spin-diffusion or more commonly cross-relaxation behavior (Edzes and Samulski, 1978; Koenig et al., 1978; Shirley and Bryant, 1982). These T_1 values, therefore, should not indicate directly the mobility of water protons. However, these may be correlated with the amount of water as clearly shown in Fig. 2, where the observed T_1
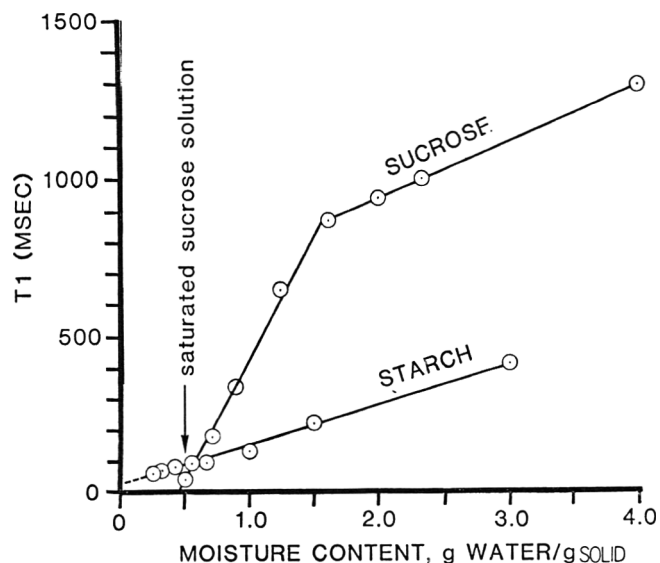
Fig. 1 – Spin-lattice relaxation time (T₁) for sucrose and raw corn starch as a function of moisture content.
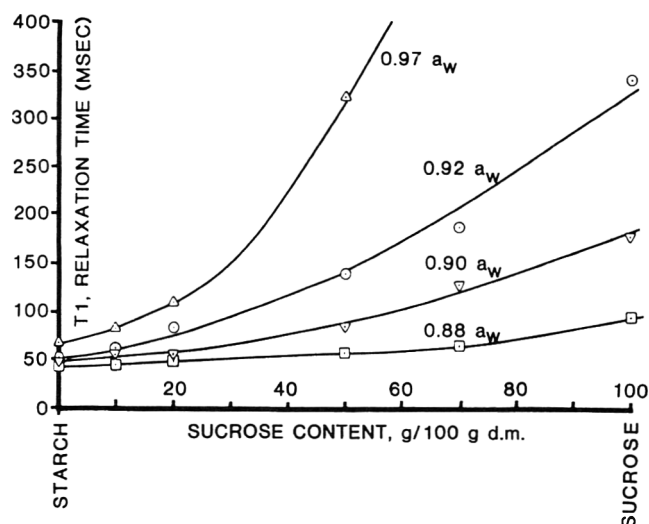
Fig. 2 – Spin-lattice relaxation time (T₁) for starch, sucrose and their mixtures at four aw

Fig. 3 – Amount of solute water (SOL) and polymer water (POL) present in sucrose-starch mixtures at 0.88 a_w as calculated from experimental isotherm data (open symbols), computer fitted curves for each state of water and values calculated from the equation for each curve (closed symbols).

Table 1 – Statistical models between T_1 and water states of starch sucrose mixtures at 0.88 a_w obtained from data in Fig. 3

Models[a]	R²
$T_1 = a_s + b_s SOL$	0.300
$T_1 = a_p + b_p POL$	0.302
$T_1 = a_s + b_s SOL + c_s SOL^2$	0.395
$T_1 = a_p + b_p POL + c_p POL^2$	0.990
$\log T_1 = a_s + b_s SOL$	0.952
$\log T_1 = a_p + b_p POL$	0.954

[a] T_1 is spin-lattice relaxation time, SOL is solute water, POL is polymer water, a, b, and c are any constants.

Table 2 – Variations of constants a, b, and c in quadratic equations[a] relating T_1 and water states in starch-sucrose systems at various a_w

a_w	a_s	b_s	c_s	a_p	b_p	c_p
0.88	48.91	−0.32	0.02	94.25	−4.85	0.12
0.90	48.60	−0.42	0.02	180.53	−9.66	0.17
0.92	57.18	−0.34	0.03	334.62	−20.10	0.36
0.97	78.55	−0.41	0.02	1022.29	−59.98	0.95

[a] $T_1 = a_s + b_s SOL + c_s SOL^2$
$T_1 = a_p + b_p POL + c_p POL^2$

increases with a_w and sucrose content. Because the amount of water sorbed to sucrose (SOL) increased with a_w and sucrose content in a sucrose-starch mixture (Chinachoti and Steinberg, 1984), T_1 and SOL may be very well correlated statistically.

Using our sorption data for pure sucrose and starch previously reported (Chinachoti and Steinberg, 1984) and the mass balance theory (Lang and Steinberg, 1980) which states that each component in a mixture sorbs independently, the amount of water bound by sucrose (SOL) and the amount of water bound by starch (POL) were calculated for each mixture at each water activity. These SOL and POL values for each sample at each a_w were plotted against T_1 for that condition; Fig. 3 is a pair of SOL and POL curves representing all the data at 0.88 a_w.

Statistical correlations were made between T_1 and SOL and between T_1 and POL at each a_w by applying most common statistical models, such as linear, quadratic, cubic and logarithmic equations. Best fitted equations (R² > 0.90) for the case of 0.88 a_w are shown in Table 1. Similar results were found in other a_w (not shown). It was reasonable to choose the equations with the highest R² which, in this case, were the quadratic equations. Constants a, b, and c for the quadratic

equations in Table 1 are shown in Table 2 for all a_w. There is an obvious necessity to interpolate all constants to other a_w to apply to any a_w. However, correlations between these constants and a_w were significant only for the case of POL but not SOL. This was not as expected since plots between T_1 and SOL at all a_w (not shown) showed a family of curves according to a_w; this meant that a, b and c for the equation of each curve must depend on SOL. Therefore, it was concluded that this model was not sensitive to a_w and it was discarded for further investigation.

Analysis was repeated but only this time, with a nonlinear regression by computer interactions and least square analysis. The equation chosen was based on the fact that each curve on Fig. 3 starts from low SOL or POL and approaches a maximum SOL (SOL_{max}) of 55 and a maximum POL (POL_{max}) of 23g water/100g dry mix, respectively. The curves show typical exponential functions approaching asymtodes at SOL_{max} and POL_{max}.

The data for each case were fitted to an exponential expression previously applied to sorption by wheat flour (Udani et al., 1968) and earlier applied to agricultural yield studies by Mitscherlich (1909):

$$SOL = SOL_{MAX}\left[\frac{(T_1^0 - T_1)}{R_s}\right]$$

where: SOL is weight of solute water/100g dry matter, SOL_{max} is water sorbed by sucrose alone at the stated a_w, T_1^0 is T_1 of starch alone at the stated a_w, T_1 is NMR relaxation time for the sample, R_s is a constant, and

$$POL_{MAX}\left[\exp\frac{(T_1^0 - T_1)}{R_p}\right]$$

where POL is weight of polymer water/100 g dry matter, POL_{max} is water sorbed by starch alone at the stated a_w, and R_p is another constant.

As indicated by Fig. 3, the amount of each water state present in a given sample composition, and thus T_1, is strongly dependent on a_w of the material when the NMR measurement is made. One possibility was to work from a family of curves, as in psychrometric charts, for interpolation of a_w. We succeeded in overcoming this problem by expressing each constant, SOL_{max}, T_1^0 and R_s in case of the expression for SOL and POL_{max}, T_1^0 and R_p in case of the expression for POL, in terms of a_w. Additional data points were generated by interpolation in Fig. 3 and the corresponding graphs at the other three a_w. The data at each a_w were fitted to the exponential expressions above by computer iteration to obtain the three constants at each a_w. Each constant was then correlated with water activity by linear and non-linear regression computer programs; the resulting expressions with their statistical validations are in Table 3.

The relationships in Table 3 allow us to calculate SOL and POL at any a_w within the range covered. This was done to test applicability of the mathematical models. First, each constant was calculated at each of the four experimental a_w. Assuming SOL and POL of the samples were not known, T_1 for each composition at each a_w was then substituted into the master equations to calculate SOL and POL. SOL calculated from NMR data was then plotted against SOL calculated from sorption data in Fig. 4. The same comparison was made for POL in Fig. 5.

The SOL data in Fig. 4 were evaluated showing a high correlation coefficient of 0.986. The POL comparison in Fig. 5 also shows a high correlation coefficient of 0.972.

These figures validated use of this instrument with the math-

Fig. 4—Solute water in six sucrose-starch mixtures at four water activities as determined from the NMR model correlated with that calculated from sorption data (A is one obs., B is 2 obs., C is 3 obs., and G is 7 obs.).
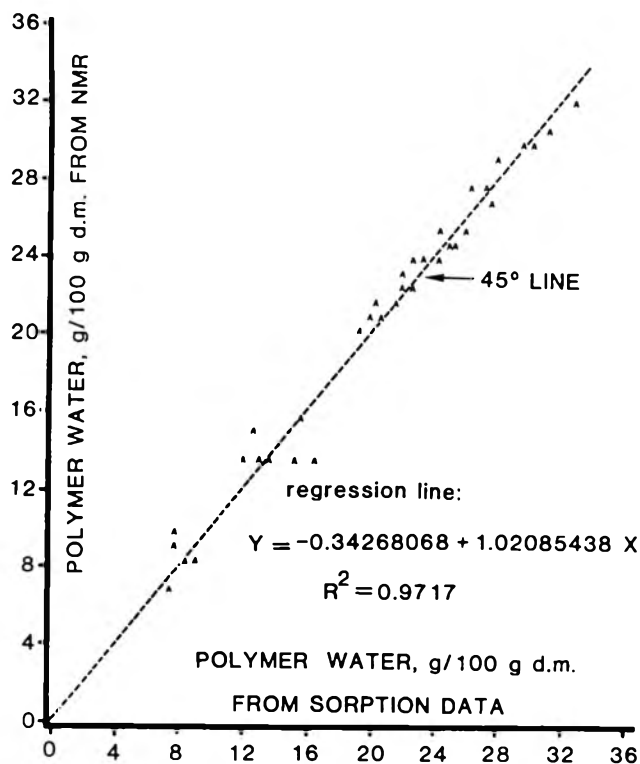
Fig. 5—Polymer water in six sucrose-starch mixtures at four water activities as determined from the NMR model correlated with that calculated from sorption data.

Table 3—Relationship between equation constants and water activity obtained from computer iteration

Equations[a]	a	b	c	R²
$SOL_{max} = a.a_w b + c$	349.82	18.43	25.10	—
$T_1^0 = a-b \log (1-a_w)$	21.26	− 28.38	—	0.968
$R_s = a.a_w \cdot b$	3797.26	− 3347.47	—	.955
$POL_{max} = a-b \log (1-a_w)$	9.99	− 14.02	—	.983
$R_p = a.a_w \cdot b$	3389.42	− 2995.81	—	.957

[a] All constants are related to T_1 as follows.

$$SOL = SOL_{MAX}\left[1 - \exp\frac{(T_1^0 - T_1)}{R_s}\right]$$

$$POL = POL_{MAX}\left[\exp\frac{(T_1^0 - T_1)}{R_p}\right]$$

ematical models developed here for the quantitative calibration of observed T_1 and calculated values of SOL and POL in a

mixture of the two. However, this was true only in case of the sucrose-starch-water system studied here. Further work is needed to determine how much these equations are effected by proton exchange, types of solutes and polymers and the complexity of the system. Positive answers to these questions will lead to a NMR approach to determining moisture content of samples of unknown composition. The exciting prospect is that here we have the possibility of obviating the vacuum oven moisture determination with all its ambiguities and problems. Thus, by NMR determination of SOL and POL content of a food product, one would have both a knowledge of the states of water with implications to processing, stability and a total water measure for economic and engineering considerations.

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cells grown on solid surfaces but were not seen on cells grown in liquid medium. Several reasons for this difference might exist. The vesicles may have been removed by the continual washing action of the liquid medium. Vesicles were isolated from the supernatant and were found to contain proteinase (Myhara and Skura, 1988). The characteristics and structure of vesicles isolated from liquid and solid media will be the subject of another manuscript. On the other hand, since the nature of the culture environment (i.e., liquid vs. solid medium) had such a profound influence on production of proteinase, it is possible some culture condition related to nutrient or oxygen depletion may be involved.

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Effect of Phytate on Solubility, Activity and Conformation of Trypsin and Chymotrypsin

S.S. DESHPANDE and SRINIVASAN DAMODARAN

─────────── ABSTRACT ───────────

Phytate:proteinase interactions and their effects on the solubility, activity, and conformation of trypsin and chymotrypsin were investigated in model systems. At pH 3.0, phytate at 0.167–0.250 (w/w) ratios formed insoluble complexes with both the enzymes, while at pH 7.8, Ca^{++} was required for the complex formation. At pH 7.8, phytate increased trypsin and chymotrypsin activity by 5–7%. The mean chymotrypsin activity was increased by about 18% when interactions were carried out at pH 3.0, whereas under similar conditions, trypsin activity was strongly inhibited. Circular dichroism spectroscopic studies revealed conformational changes in the enzyme secondary structure that seemed to have influenced their activity. The role of phytate in protein metabolism and the possible ramifications of present findings in human nutrition were addressed.

INTRODUCTION

CHELATES have long attracted the attention of nutritionists because of their ability to complex dietary nutrients and lower their bioavailability. Phytic acid (*Myo*-inositol 1,2,3,5/4,6 - hexakis dihydrogen phosphate), one such widely studied chelating compound, is an ubiquitous seed constituent comprizing 1-3% of all nuts, cereals, legumes and oilseeds (Cheryan, 1980; Reddy et al., 1982). Its ability to chelate polyvalent trace elements is attributed to the presence of six ortho-phosphate moieties in the molecule that provide this compound with a potential of 12 coordinate ligands for complexing metal ions. Although most studies show an inverse relationship between phytic acid and mineral bioavailability, there are great differences in the behavior of individual minerals, the latter is seemingly influenced by the dietary calcium concentrations (Morris, 1986). Also, many of the mineral bioavailability studies utilize either wheat bran or sodium phytate as the source. The results in the former case are often confounded by the presence of dietary fiber which, although considered as a 'good, essential component' of the diet, is also a strong metal chelator, while in the normal diets, phytate may seldom be expected to be present in the 'free' form.

The negative bias generated by the antinutritional chelation properties of phytate has largely neglected some of its possible beneficial effects in human nutrition. In the recent past, one such positive influence of phytate in the better management of chronic diseases such as diabetes has been shown (Thompson and Yoon, 1984; Thompson et al., 1987; Thompson, 1988). Such beneficial effects are largely attributed to the phytate interactions with dietary proteins, carbohydrates and enzymes of the digestive system. Phytate can strongly interact with proteins in a pH-dependent manner. However, in several studies concerning the effect of phytate on both the *in vitro* and *in vivo* digestibility of complexed proteins and absorption of amino acids, phytate had little, if any, adverse influence (Serraino et al., 1985; Thompson and Serraino, 1986; Reddy et al., 1988). Any deleterious effects of phytate on protein metabolism thus seem to be related to its ability to inhibit digestive enzymes.

That phytate should inhibit proteolytic enzymes such as pep-

sin (Camus and LaPorte, 1976; Knuckles et al., 1985) is not suprising, since the acidic pH at which it is active can promote strong electrostatic linkages between phytate and the positively charged groups of protein. Indeed, the same can be said of enzymes whose pH optima is on the acid side of the scale. In an earlier study, phytate inhibition of α-amylase decreased with increasing pH and the inhibitor constant K_I was almost doubled as the pH was raised from 4.0 to 7.0 (Deshpande and Cheryan, 1984). The more negative charges on proteins at neutral to alkaline pH may prevent formation of phytate:protein binary complexes unless an external polyvalent cation (such as Ca^{++} or Mg^{++}) is present. In a study on the inhibition of trypsin activity *in vitro* by phytate, Singh and Krikorian (1982) reported 20% enzyme inhibition at 90 mM phytate in the assay system. Based on the data provided by these authors, this turns out to be almost 3000 : 1 phytate : enzyme ratio. Not only are such ratios of free phytate difficult to encounter under *in vivo* situations, but also because of their high ionic strength, they may effectively salt out the enzyme from the assay system. Any such study of phytate on proteinase inhibition should, therefore, use ratios that are more likely to occur under normal dietary conditions. The present study was undertaken to investigate such effects of phytate on the solubility, activity and conformational changes in trypsin and chymotrypsin.

MATERIALS & METHODS

THE FOLLOWING were the sources of chemicals: Phytic acid (dodecasodium salt from corn, 97% purity containing 9 mole H_2O/mole, lot 97F-0376, used without further purification) and trypsin (Type III, from bovine pancreas, lot 17F-0188) from Sigma Chemical Co., St. Louis, MO; α-chymotrypsin (3X crystallized, from bovine pancreas, lot 8999) from ICN Pharmaceuticals, Inc., Cleveland, OH; Hammarsten casein from EM Science, West Germany. All other chemicals used were of reagent grade.

Protein concentrations of the enzymes were routinely determined using $E^{1\%}$ values of 16.1 for trypsin and 18.0 for chymotrypsin at 280 nm in a 1 cm light path cell (Yang et al., 1985). All experiments (except the enzyme activity studies) were performed at room temperature (24–26°C) using either 1 mM HCl (pH 3.0) or 20 mM phosphate buffer (pH 7.8) systems. The phytate : enzyme ratios used for activity assays were selected based on preliminary experiments.

Solubility of phytate:enzyme complexes

The enzyme and phytate stock solutions were prepared using appropriate buffers. The phytate stock solutions were readjusted to the desired pH by titrating against 2N HCl. Aliquots were then mixed to yield the appropriate phytate : enzyme ratios and left standing for 2 hr with intermittant mixing. The enzyme concentrations were 0.575 mg/mL and 1.0 mg/mL, respectively, for trypsin and chymotrypsin. The samples were then centrifuged, and the absorbances of the supernatants were read at 280 nm using appropriate blanks to correct for the phytate concentration. Since no visible precipitation was observed at pH 7.8, calcium was used to investigate the possibility of ternary complex formation. When added, the enzyme: Ca^{++} ratios were either 5:1 or 10:1 (w/w). All experiments were carried out in duplicate at room temperature (24–26°C), and the data were expressed relative to the solubility of the enzyme in the absence of phytate.

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Effect on enzyme activity

The effect of phytate : enzyme interactions on enzyme activity was studied using a modification of the method described by Kunitz (1947) using Hammarsten casein as the substrate. The interactions were carried out at both pH 3.0 and 7.8. In the former case, the pH of the incubation mixture was readjusted prior to the assay to 7.8 by adding phosphate buffer. In all the experiments, the enzymes were incubated with phytate for 15 min prior to the addition of the substrate. The reaction was carried out for 30 min at 37°C, and was stopped by the addition of TCA to a final concentration of 5%. Controls were run including the appropriate amounts of phytate wherever necessary, and the enzyme was added after the addition of TCA. The samples were centrifuged, and the absorbances of the supernatants were read at 280 nm. The data presented here are means of triplicate determinations each of six different runs.

Effect on enzyme secondary structure

The influence of phytate on the enzyme secondary structure was studied using circular dichroism (CD) spectroscopy. The CD measurements were made using a 0.1 cm cell in a modified and computerized CARY Model 60 spectropolarimeter (On-Line Instrument Systems, Inc., Jefferson, GA). The instrument was calibrated using d(+)-10-camphorsulfonic acid. The scan rate, time constant and the sensitivity of the instrument were all set so as to obtain the best signal-to-noise ratio and reproducibility of the spectrum. Ten scans each of duplicate samples (0.03-0.06 mg/mL protein concentration) were averaged and the mean residue ellipticity [Θ], expressed as degree-cm^2/dmole, was calculated using the following equation:

$$[\theta] = 100 d \overline{M}/C$$

where d is the rotation (deg/cm path length), $\overline{M}$ is the average molecular weight of the amino acid residues in the protein, and C is the protein content (mg/mL). Mean residue molecular weights of 108 and 104, respectively, were used for trypsin and chymotrypsin (Yang et al., 1985). All spectra were corrected for the appropriate baselines. The secondary structure estimates were calculated as described by Chang et al. (1978).

Statistical analyses

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

RESULTS & DISCUSSION

THE INFLUENCE of phytate : enzyme interactions on protein solubility at both acidic and alkaline pH is shown in Fig. 1 and Fig. 2. As expected, stronger interactions were observed at acidic pH. Although the solubility profiles in the presence of phytate for both trypsin and chymotrypsin (Fig. 1A and 1B) were nearly identical, the ratios at which minimum solubility was observed were somewhat different. For example, at 0.5 phytate : trypsin ratio, nearly 60% of the enzyme was precipitated, while chymotrypsin was completely soluble. Maximum precipitation (over 70-80%) for both the enzymes was observed at phytate : enzyme ratios of 0.167-0.250 (w/w). Total precipitation was not observed for either enzymes at the various ratios studied. On the contrary, no insoluble complexes were formed at the alkaline pH used in this study up to a phytate concentration of 5% (data not shown). Some binding, however, did occur as was evidenced by the CD spectroscopy studies, but any complexes that may have formed at pH 7.8 were completely soluble.

The influence of Ca^{++} concentration on the formation of ternary complexes between phytate-Ca^{++}-protein was further studied using 5:1 and 10:1 enzyme : Ca^{++} ratios. At higher enzyme : Ca^{++} ratios, trypsin was still completely soluble, while increasing the relative Ca^{++} concentration further (i.e., 5:1 ratio), had only marginal influence on its solubility (Fig. 2). Compared to this, chymotrypsin was much more susceptible to the formation of ternary complexes involving phytate and Ca^{++}, with stronger interactions being observed at higher
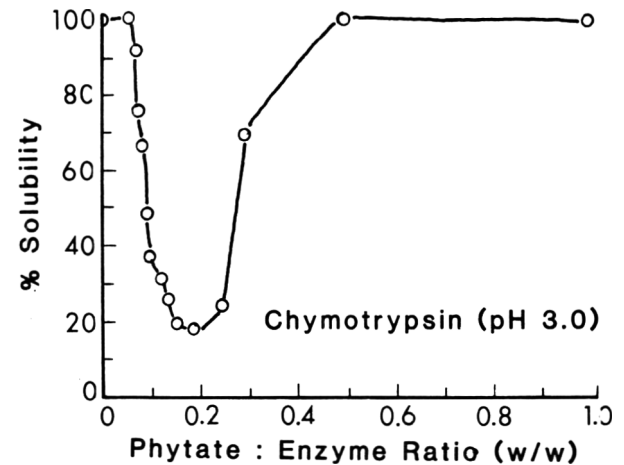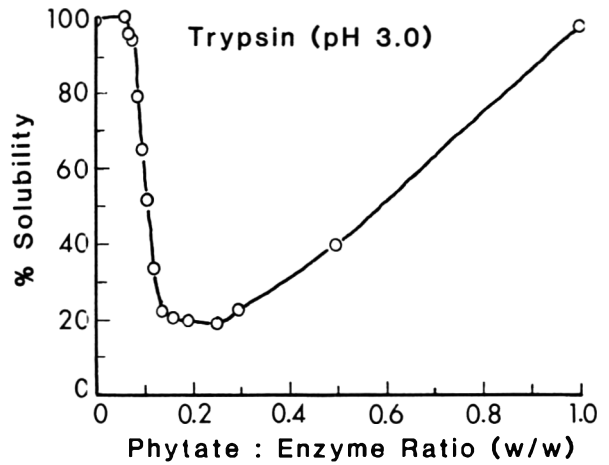
Fig. 1—Solubility profiles of trypsin (0.575 mg/mL) and chymotrypsin (1 mg/mL) at pH 3.0 in the presence of phytate.

Ca^{++} concentrations (Fig. 2). However, the overall solubility of the enzyme was still 60% or higher under the experimental conditions used in this study.

The precipitation of trypsin and chymotrypsin by phytate at pH 3.0, which is below their isoelectric pH, might be due to the formation of unionized salts. At acidic pH, phytate can form strong electrostatic linkages with the basic lysine, arginine and histidine residues. Such interactions may cause a modification in structure brought about by close packing of protein molecules around the relatively small and highly charged phytate anion, leading to the formation of an insoluble complex (Courtois and Lino, 1961). Such insoluble complexes are believed to be soluble only below pH 3.0. The results obtained in this study suggested the formation of such insoluble complexes to be dependent upon phytate : protein ratios and in turn on the number of positively charged and amino terminal groups that were able to react with the negatively charged phytate molecule. At low phytate-enzyme ratios, because of the insufficient screening of the positive charges on the enzyme, the phytate-enzyme complex had a net positive charge which made it soluble. On the other hand, at higher phytate concentrations, the nonspecific binding of phytate to the positively charged side chains of amino acids will confer a greater electronegativity than that required for the formation of insoluble complexes. The molar ratios of phytate : protein to form insoluble complexes will also vary depending upon the type
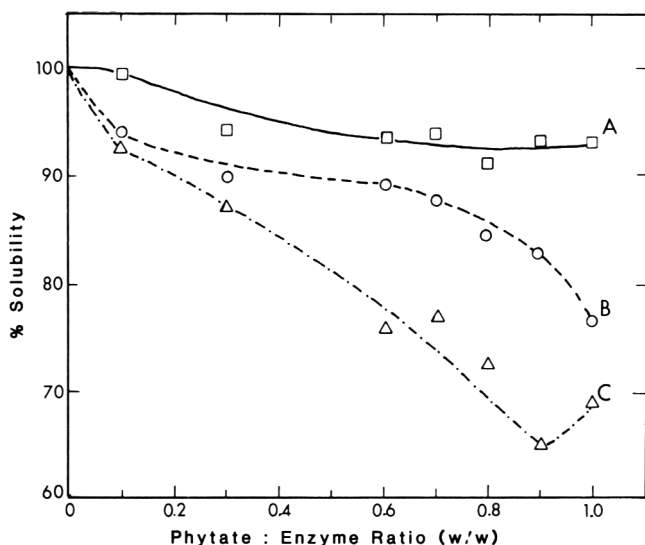
Fig. 2—Solubility profiles of trypsin (0.575 mg/mL, curve A) and chymotrypsin (1 mg/mL, curves B and C) at pH 7.8 in the presence of phytate and Ca⁺⁺ ions. A: Trypsin:Ca⁺⁺ ratio 5 : 1 (w/w), B: Chymotrypsin: Ca⁺⁺ ratio 10: 1 (w/w), C: Chymotrypsin: Ca⁺⁺ ratio 5 : 1 (w/w).

Table 1 — Trypsin activity as influenced by phytate interactions

Phytate:Trypsin ratio (w/w)	% Change in enzyme activity	
	pH 3.0ᵃ	pH 7.8ᵇ
0	0ᶜ	0
0.26	+ 3.7	+ 1.5
0.52	+ 1.8	3.3
1.04	+ 2.3	+ 2.6
1.56	+ 2.6	+ 3.4
2.08	+ 2.0	+ 7.6
2.60	+ 2.8	+ 6.9
5.20	+ 0.3	+ 6.4
10.40	− 2.8	+ 3.6
15.60	− 2.5	+ 7.0
20.80	− 6.3	+ 7.7
26.00	− 10.9	+ 7.4
39.00	− 17.2	+ 2.4
52.00	− 24.3	+ 2.2
LSDᵈ (p = 0.05)	1.78	1.35

ᵃ Enzyme (0.575 mg/mL) and phytate incubated for 15 min at pH 3.0 prior to assay at pH 7.8.
ᵇ Enzyme (0.575 mg/mL) and phytate incubated for 15 min at pH 7.8 prior to assay.
ᶜ +/− = Increase/decrease in activity as compared to the respective controls
ᵈ Least significant difference at 5% level. Differences of two means exceeding this value are significant.

Table 2 — Chymotrypsin activity as influenced by phytate interactions

Phytate:Chymotrypsin ratio (w/w)	% Change in enzyme activity	
	pH 3.0ᵃ	pH 7.8ᵇ
0	0ᶜ	0
0.14	+ 16.8	+ 7.6
0.28	+ 18.6	+ 6.7
0.56	+ 19.2	+ 4.3
0.84	+ 21.8	+ 6.6
1.12	+ 25.8	+ 5.2
1.40	+ 24.7	+ 6.3
2.80	+ 27.9	+ 5.2
5.60	+ 25.5	+ 4.0
8.40	+ 3.8	+ 3.9
11.20	+ 2.2	+ 3.8
14.0	− 1.2	− 1.3
21.0	− 3.8	− 4.1
28.0	− 7.9	− 7.0
LSDᵈ (p = 0.05)	2.11	1.63

ᵃ Enzyme (1 mg/mL) and phytate incubated for 15 min at pH 3.0 prior to assay at pH 7.8.
ᵇ Enzyme (1 mg/mL) and phytate incubated for 15 min at pH 7.8 prior to assay.
ᶜ +/− = Increase/decrease in activity as compared to the respective controls.
ᵈ Least significant difference at 5% level. Differences of two means exceeding this value are significant.

and conformation of the protein. Barre and Nguyen-van Huot (1965a) reported the formation of salt-like linkages between phytate and human serum albumin first with the terminal α-NH₂ group and ε-NH₂ groups of lysine, followed by the histidine and guanido groups of arginine. In hen albumin, however, phytic acid first binds with the arginyl residues, then lysyl and finally histidyl residues (Barre and Nguyen-van Huout, 1965b). The relative position of the positively charged side chain on the protein as well as its accessibility to solvent thus seemed to influence phytate : protein interactions.

That no insoluble complexes between phytate and either enzymes were formed at pH 7.8 was not surprising. Both protein and phytate were negatively charged at this pH and the electrostatic repulsion might not allow direct binding except perhaps in minor quantities with a few terminal and ε-NH₂ groups of lysine and the arginine side chain. These groups would still be protonated at this pH. Although both enzymes formed Ca⁺⁺ mediated ternary complexes with phytate, such interactions were much stronger for chymotrypsin than for trypsin. At comparable phytate ratios, trypsin not only required the presence of higher Ca⁺⁺ concentrations, but also was still quite soluble. Overall, at pH 7.8, the enzymes were over 60% soluble even in the presence of Ca⁺⁺ ions compared to the 18-20% solubility at pH 3.0 at equivalent phytate concentrations. The common binding site for the ternary complex appears to be the ionized carboxyl groups as well as the unprotonated imidazole groups of histidine, the latter being the major site (Martell and Calvin, 1952).

The influence of phytate on enzyme activity was strongly influenced by the pH at which the interactions were carried out (Table 1 and 2). At pH 7.8 and the ratios used, trypsin activity increased by 1.5-7.7%, the mean average increase in enzyme activity being 4.8% over the entire range (Table 1). Changes in trypsin activity were marginal and statistically insignificant at both very low and very high phytate concentrations. Maximum increase in enzyme activity was observed at phytate : trypsin ratios of 2-21 (w/w). The extent of increase for chymotrypsin activity was similar, although at almost half the phytate concentrations than those used for trypsin (Table 2). Unlike trypsin, maximum increase in chymotrypsin activity was observed at lower phytate : enzyme ratios. Little differences were observed in the enzyme activities within these ratios, although these values were significant at 5% probability when compared to the control values obtained in the absence

of phytate. The mean increase in activity when observed was about 5.4% over the respective controls. At ratios 14 and above, chymotrypsin activity was actually inhibited. Based on the data presented in Table 1 for trypsin activity at pH 7.8, it would be appropriate to conclude that similar inhibition perhaps would have occurred at phytate : trypsin ratios above 60:1. Nonetheless, trypsin seemed to be twice as stable to phytate as chymotrypsin when the interactions were carried out at pH 7.8.

Greater differences were observed in the activity of both the enzymes when the interactions were carried out at pH 3.0. Trypsin activity did increase at 0.26-2.6 phytate : enzyme ratios, although the mean increase observed (2.2%) was only half as much as was observed at alkaline pH (Table 1). Unlike at pH 7.8, trypsin activity was significantly inhibited at higher phytate concentrations in the acidic medium. The enzyme activity decreased by about 2.5-24% at 10-52 phytate ratios. On the other hand, chymotrypsin activity increased by over three fold at 0-5.6 phytate ratios, while comparable enzyme inhibition was observed for similar phytate ratios (14-28, w/w) irrespective of the incubation pH (Table 2). It was interesting to note that at the phytate ratios where an increase in activity was observed, both enzymes precipitated out of solution (Fig.1). Such complexes, however, were soluble when the pH was adjusted to 7.8 prior to the assay, although no attempts were made to quantitate this parameter. It was quite likely that the

active site for both the enzymes was little affected during such interactions and that the nature of the interactions which might have taken place, at least for chymotrypsin, greatly increased its affinity towards substrate.

The phytate-proteinase interactions were further investigated
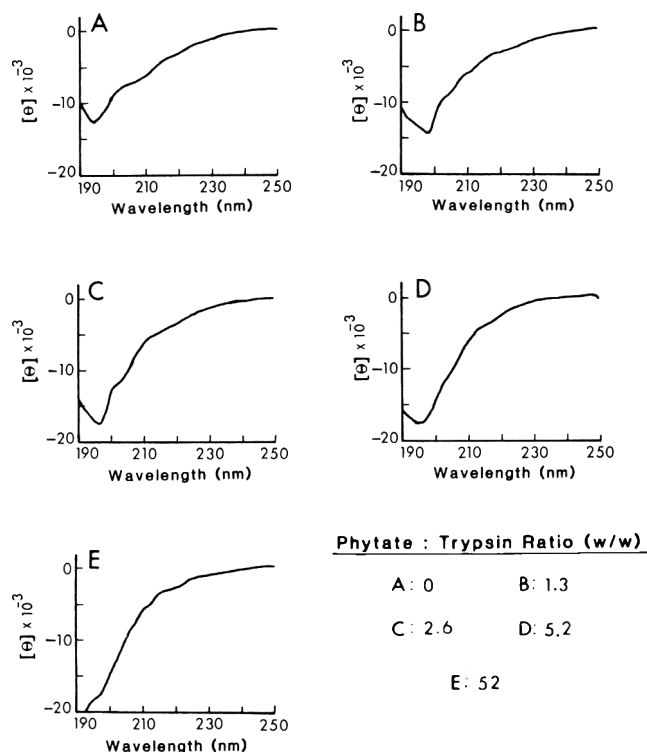
Fig. 3—Far-UV CD spectra of trypsin (0.575 mg/mL, pH 7.8) in the presence of phytate. [θ] represents mean residue ellipticity as degree-cm²/dmole.

Phytate : Trypsin Ratio (w/w)

A: 0   B: 1.3

C: 2.6   D: 5.2

E: 52

Phytate : Chymotrypsin Ratio (w/w)

A: 0   B: 0.7

C: 1.4   D: 2.8

E: 28
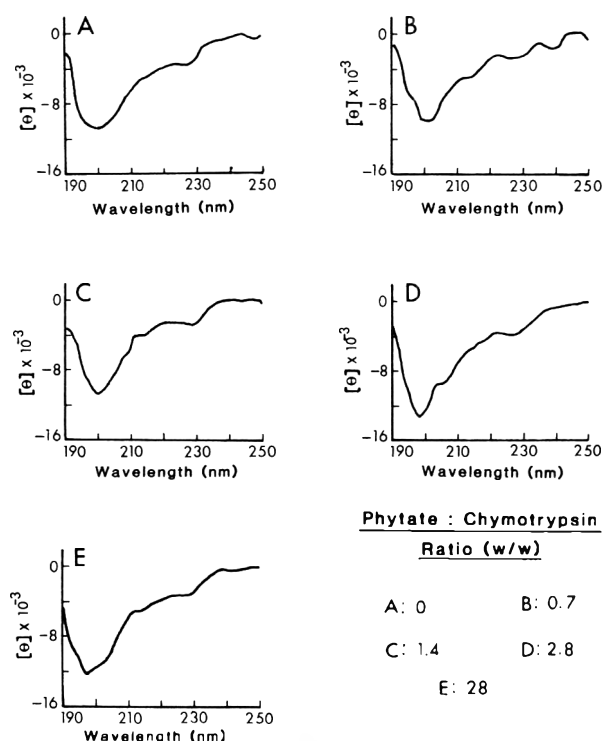
Fig. 4—Far-UV CD spectra of chymotrypsin (1 mg/mL, pH 7.8) in the presence of phytate. [θ] represents mean residue ellipticity as degree-cm²/dmole.

by following their effects on enzyme conformation using CD spectroscopy. Both enzymes showed typical CD specta for predominantly β-sheet proteins (Fig. 3 and 4). Significant changes were observed in the β-sheet conformation of trypsin on interactions with phytate. The β-sheet content decreased progressively with increasing phytate concentrations and was lowered by over 50% at 52:1 phytate ratio (Table 3). This was accompanied by an increase in the random structure of the enzyme. The shoulder at 216 nm (Fig. 3A) disappeared gradually (Fig. 3B-3E), while the negative trough at 197 nm increased correspondingly. This trough arose due to π-π* transition characteristic of the random coil structure.

The CD spectrum of native chymotrypsin showed its characteristic trough at 228 nm with a broad negative peak at 200 nm (Fig. 4A) (Simons, 1981). At 0.7 phytate ratio, the negative ellipticity of this trough decreased by about 25%, while a new trough was observed at 238 nm. The significance of this trough at present is unknown. The negative ellipticity of the 228 nm trough which is primarily associated with the n-π* transition in α-helical structures (but not solely due to it), gradually decreased with increasing phytate concentration (Fig. 4B-4E), whereas a new shoulder at 208 nm due to π-π* transition of the helical structure parallel to the peptide chain appeared. The non-specific nature of phytate-protein interactions at various ratios was quite evident from the quantitation of secondary structure parameters for chymostrypsin (Table 3). Although such quantitation differs greatly based on the method used, predominant changes due to phytate-chymotrypsin interactions seemed to have occurred in the helical region of the enzyme. The CD spectroscopy studies clearly indicated that phytate induced changes in the conformational characteristics of the enzymes. Given the nature of phytate-protein interactions at pH 7.8 (i.e., only lysyl and arginyl side chains are available in the absence of polyvalent cations), it is also quite likely that the observed effects might be a direct measure of a local perturbation in the protein conformation.

To our knowledge, there is only one other report on the positive influence of phytate on enzyme activity. Altschuler and Schwartz (1984) reported an enhancement of both mutant and wild type enzyme activity of alcohol dehydrogenase from corn in the presence of phytate. While the negative influence of phytate on the activity of acid proteases (such as pepsin) is clearly due to the pH at which such enzymes are active, the observations of Singh and Krikorian (1982) on trypsin activity might be cue to their use of high phytate : enzyme ratios. The excessive changes in ionic strength as a result of high phytate concentrations seemingly gave higher inhibition values in their study. Although in the present study, trypsin activity was strongly inhibited when interactions were carried out at acidic pH, conditions for such interactions are difficult to encounter under in vivo conditions.

Both trypsin and chymotrypsin belong to the serine proteinase family in which the active site involves the catalytic triad of seryl, histidyl and aspartyl residues. Based on our current

Table 3—Effect of phytate on the secondary structure of trypsin and chymotrypsin[a]

Phytate:Enzyme ratio (w/w)	% Secondary structure			
	Helix	β-Sheet	Turns	Random
Trypsin				
0	0	45.0	13.0	42.0
1.3	0	34.0	16.0	50.0
2.6	0	32.5	15.0	52.5
5.2	0	21.5	21.5	57.5
52.0	0	21.0	21.5	57.5
Chymotrypsin				
0	1.0	35.0	12.5	51.5
0.7	3.5	25.0	21.5	50.0
1.4	2.5	31.5	18.0	48.0
2.8	4.5	25.0	17.0	53.5
28.0	1.0	39.5	11.0	48.5

[a] Interactions were carried out at pH 7.8.

understanding of phytate : protein interactions at alkaline pH, none of these three groups will interact with phytate in the absence of Ca⁺⁺ or other divalent cations. Indeed any inhibition that may be observed at high phytate concentrations may be due to its interactions with positively charged amino side chains that in turn induce sufficient conformational changes so as to disrupt the conformational proximity of these three residues involved at the active site of the enzyme. Although the beneficial effects of phytate at low concentrations do not seem to be limited to the serine proteinases alone, as is evidenced by Altshuler and Shwartz (1984) study on alcohol dehydrogenases, an understanding of the apparent changes in K_m of a wide variety of enzymes from different classes must await basic enzymological studies followed by a more detailed analysis of the effect of phytate on the enzyme's kinetic parameters.

The present study raises several questions regarding the actual role of phytate in protein metabolism. Several studies have clearly shown that although the digestibility of protein and the subsequent rate of release and absorption of amino acids is only marginally higher in low-phytate diets, that from phytate-complexed proteins is not significantly different (Thompson and Serraino, 1986; Serraino et al., 1985; Thompson, 1988). Then there is also the question of how much 'free' phytate would be available to inhibit the digestive enzymes. Also most *in vitro* enzyme inhibition studies (including the present one) use sodium phytate whose solubility behavior is completely different from that observed for phytate from plant sources (Cheryan, 1980). Phytic acid is a highly unstable molecule and in nature is always present as a salt of Ca^{++}, Mg^{++} or K^+. When one considers its strong affinity to various cations and the type of interactions involved in its association with dietary protein as well as factors such as food processing, thermal degradation of inositol esters and pH, little free phytate would be expected to be available to interact with enzymes in the digestive system so as to cause any significant influence. Also, a previous study has clearly shown that in the presence of minerals such as Ca^{++} or Mg^{++}, the actual *in vitro* inhibition of enzymes would be much lower (Deshpande and Cheryan, 1984). Similarly, a host of different enzymes are involved in the digestion process. Proteins in which some lysyl or arginyl side chains are complexed with phytate may not be effectively hydrolyzed by trypsin, while under these conditions, enzymes such as chymotrypsin would prove more effective, since the latter shows specificity for large hydrophobic side chains. Until several questions regarding the phytate-protein interactions under a wide variety of conditions and its influence on protein digestibility under simulated *in vivo* conditions are addressed and more definitive answers found, the adverse effects of phytate in human nutrition, given its nature and chemistry, should

be strictly addressed from the mineral bioavailability point of view.

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Processing Parameters of Chymosin Extraction by Ultrasound

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—————————— ABSTRACT ——————————

Conventional chymosin (rennin) production requires a prolonged extraction. An ultrasound method of chymosin extraction was developed for increased efficiency. Ultrasound extraction was carried out with Tekmar sonic disruptor (TK 1000) and Lauda circulator. Control extraction was carried out by shaking. The use of ultrasound resulted in a significant reduction of extraction time with increases in activity and yield of chymosin. The optimal parameters for ultrasound extraction were: specific intensity, 36 W/cm^2 at 20 kHz frequency; time of exposure, 80 min; temperature, 25°C; ratio of abomasa to extraction solution, 1:25; and activation time of prochymosin, 13.0–13 5 hr.

INTRODUCTION

CHYMOSIN (rennin) is the predominant milk-clotting enzyme secreted in the fourth stomach of milk-fed calves. Like other proteinases obtained from the digestive tract, chymosin is secreted as an inactive precursor, which is generally called prochymosin (prorennin). The prochymosin is activated in an acid environment.

In commercial production, chymosin is traditionally extracted from abomasa with an aqueous salt solution and acid. The chymosin is activated in the extract by adjusting pH and salt content. However, this traditional process of chymosin production has two drawbacks. First, the process requires a long extraction time, which is detrimental to the quality of chymosin. Second, the process does not allow full extraction of chymosin from abomasa.

Ultrasound has a unique effect on extraction processes, causing destruction of the cellular structure by a cavitation effect (Hughes and Nyborg, 1962) and increasing the activity of substances that are bound in cell structures (Fry, 1978). Ultrasound cavitation is the formation of bubbles or cavities in liquids. Collapse of the bubbles results in intense shock waves that can cause considerable damage to surrounding material. Ultrasound had selective action on animal tissues, destroying some tissues without damaging others. During tissue disruption, there was migration of proteins, minerals, and other substances into the solution (Zayas, 1985).

The chymosin used in the commercial manufacture of cheese in the United States is a liquid extract. Approximately 3 million calves have been slaughtered annually for chymosin production. It has been a great problem to maintain an adequate supply of abomasa.

The objective of this research was to establish the influence of ultrasound on the efficiency of chymosin extraction and to develop optimal processing parameters for ultrasound extraction.

MATERIALS & METHODS

Material

Abomasa were obtained from Chr. Hansen's Laboratory, Inc. and stored at −25°C until used. Frozen abomasa were cut into pieces of approximately 7 × 15 × 7 mm and thoroughly mixed before using.

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Control method for chymosin production

Production of the control chymosin from abomasa was carried out according to the procedure utilized in the United States (Placek, 1960). Flow chart of control and experimental chymosin production is shown in Fig. 1.

A 30-g sample of frozen abomasa was placed in a 1000–1500 mL beaker containing the required amount of 10% NaCl solution previously adjusted to pH 5.9 with HCl at various abomasa:NaCl solution ratios. The slurry was agitated at 60 times/min in a Dunnoff metabolic shaking incubator (GCA Co., Chicago, IL). After maximum activity was obtained, the supernatant was filtered and activated at 3°C.

Chymosin extraction by ultrasound

Chymosin extractions by ultrasound were carried out with a Tekmar sonic disruptor, TK 1000 (Tekmar, Cincinnati, OH), and a Lauda circulator, K-2/R (Brinkmann, Westbury, NY), which was used for cooling the extraction mixture. Frequency of ultrasound generated was 20 kHz, and specific ultrasound intensity was controlled in the range from 20 to 41 W/cm^2. Other parameters of ultrasound extraction were the same as those of the control method.

Chymosin activity

Chymosin activity was determined according to methods of McMahon and Brown (1985) and Ernstrom (1958). Milk substrate was prepared by dispersing 12g low heat, nonfat dry milk (NFDM) in 100 mL 0.01M $CaCl_2$ solution. The substrate solution was then refrigerated for 20 hr to allow time for complete hydration of the NFDM. Prior to analysis (30 min), the substrate was warmed to 35°C and maintained

Frozen abomasa stored at -25°C
↓
Cutting
↓
Extraction with 10% NaCl solution, pH 5.9 with HCl
↙   ↘
Control   Ultrasound
↘   ↙
Filtration
↓
Activation of prochymosin at 3°C (Adjustment of pH to 4.6 with HCl)
↓
Adjustment of pH to 5.7 with sodium bicarbonate
↓
Preservation of chymosin
(Addition of 5% propylene glycol. 2% sodium propionate and 0.1% sodium benzoate)
↓
Packaging
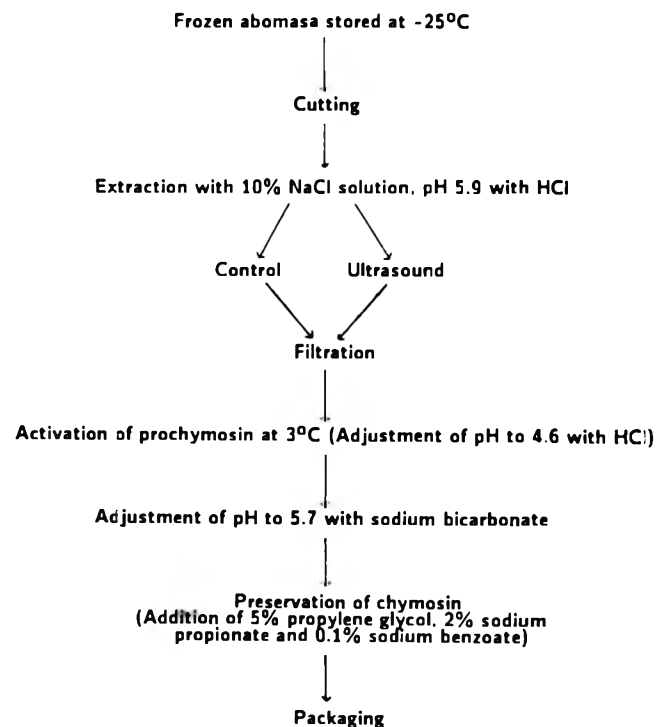
Fig. 1—Flow chart for chymosin production.

at that temperature throughout the analysis. One mL chymosin extract was added to 100 mL substrate. Milk clotting time was measured visually, and chymosin activities were calculated as:

$$Cu/mL = \frac{100Ts}{Ta} \times \frac{Cs}{Ca}$$

where Cu = chymosin units; Ts = Coagulation time of standard chymosin; Ta = Coagulation time of unknown chymosin; Cs = Concentration of standard chymosin; Ca = concentration of unknown chymosin.

Standard chymosin was obtained from Chr. Hansen's Laboratory, Inc. and kept in the refrigerator at 4°C until used. Chymosin yield (Cu/g) was defined as:

$$Chymosin \; yield = \frac{A \times B}{C}$$

where, A = chymosin units/mL; B = amount of chymosin extract, mL; C = weight of abomasa, g.

During conventional chymosin extraction, a certain amount of active chymosin as well as prochymosin was obtained from abomasa (Rand and Ernstrom, 1964). In the present study, the extracted enzyme before activation was described as prochymosin and after activation as chymosin and its activity was expressed as chymosin activity (Cu) in figures and tables.

Statistical analysis

The ranges and intervals of experimental parameters for response surface methodology (RSM) followed the designs of Box and Behnken (1960) and Box and Wilson (1951). Data analysis and graphic plotting were done with SAS programs (SAS, 1982). Quadratic models were used to create the 3-dimensional response surfaces. In response surfaces, independent variables are located along the traditional X and Y-axes, respectively, whereas the response variable is at the Z-axis perpendicular to the X-Y axes.

Analysis of variance and least significant differences were used to ascertain significant effects at the 5% level. Regression analysis was conducted by the method of Steel and Torrie (1980) for simple regression models.

RESULTS & DISCUSSION

Control method of prochymosin extraction

An optimal extraction time of 51.6 hr and a ratio of abomasa to NaCl solution of 1:42 were established in the control experiments carried out at 3°C (Fig. 2). Long extraction time had a negative effect on the activity and yield of prochymosin. According to Mickelsen and Ernstrom (1967[7]), during extraction, chymosin was partially inactivated because of its low stability in an aqueous medium.

Prochymosin extraction by the control method at 3°C with the different abomasa:NaCl solution ratios showed that there was no further increase in prochymosin yield after about 51.6 hr of extraction (Fig. 2). An extraction time of 51.6 hr for control samples of chymosin was significantly shorter than for the counter-current extraction system used in industrial conditions (72 hr). The time of 11 hrs for prochymosin activation obtained for control samples in our experiments (Fig. 3) was shorter than the time under industrial conditions (14–36 hr) (Placek, 1960).

The quadratic model for prochymosin extraction by the control method at 3°C was:

$$Z = -513.235 + 19.065X + 61.738Y - 0.198X^2 + 0.033XY - 0.756Y^2$$

$$(R^2 = 0.880, P < 0.005)$$

where Z = prochymosin yield, Cu/g; X = extraction time, hrs; Y = abomasa:NaCl solution ratio.

Prochymosin extraction by ultrasound

The effectiveness of prochymosin extraction with combinations of specific intensity, time of ultrasound treatment, and
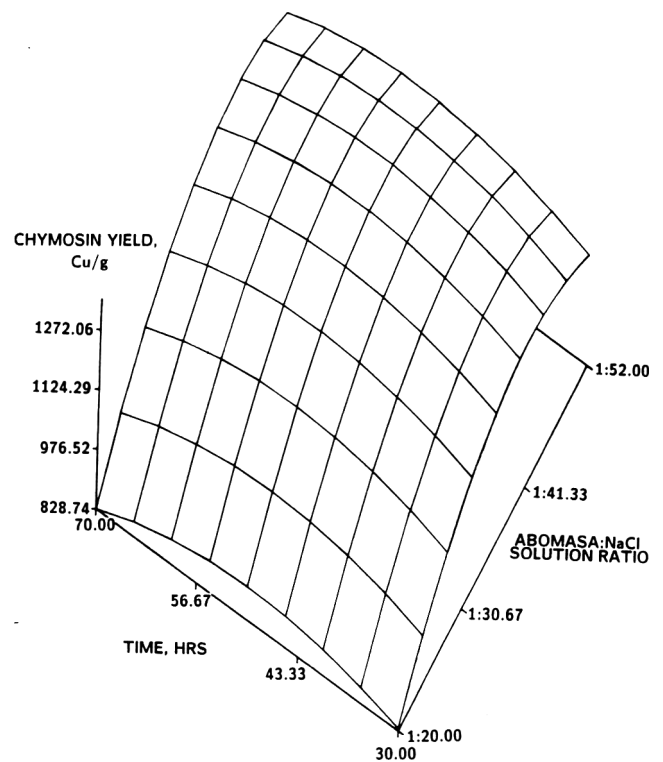
Fig. 2—Prochymosin extraction by control method at 3°C as a function of extraction time and abomasa:NaCl solution ratio.
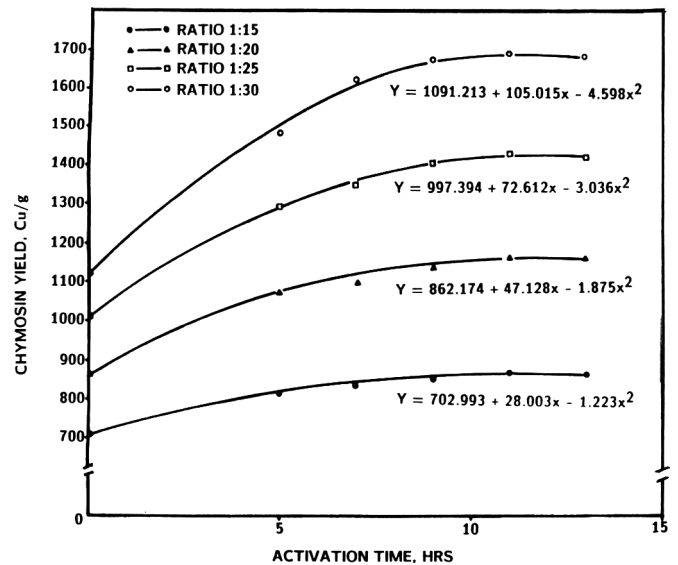
Fig. 3—Activation of prochymosin extracted by control method at the different abomasa:NaCl solution ratios.

temperature of extraction medium was determined to find the optimal parameters. The intervals of variation for each parameter were chosen on the basis of preliminary experiments.

Response surface analysis showed that specific intensity of ultrasound influenced the extraction process (Fig. 4 and 6). The yield of prochymosin significantly increased up to a specific ultrasound intensity of 36 W/cm² (Fig. 4 and 6) and a temperature of 25°C (Fig. 4 and 5) of the extraction mixture and then slightly decreased. This effect occurred at the abomasa:NaCl solution ratio of 1:25. Consequently, the recommended maximum temperature for the ultrasound treatment will be 25°C. Obviously, at the higher temperatures, there is the possibility of local overheating of the extraction mixture and enzyme inactivation. Specific intensity of ultrasound ap-
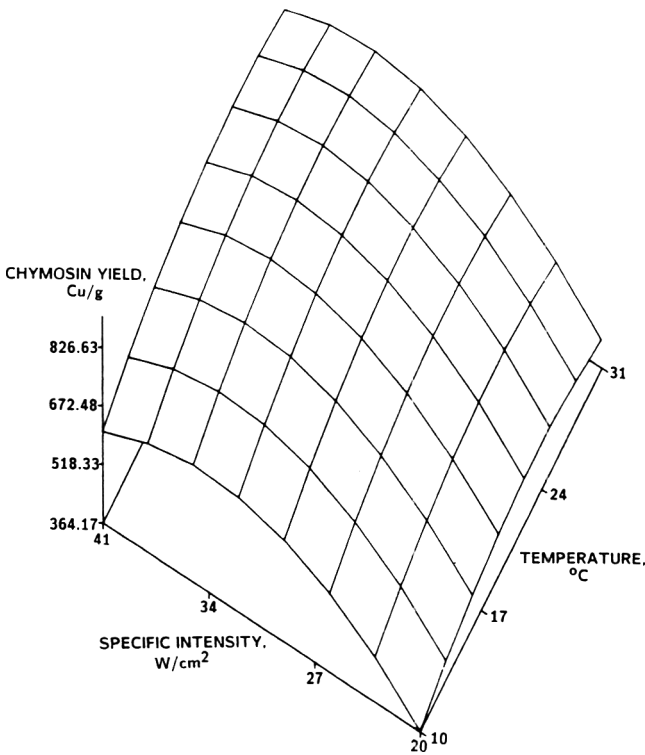
Fig. 4—Prochymosin extraction by ultrasound at 1:25 abomasa: NaCl solution ratio as a function of ultrasound specific intensity and extraction temperature.
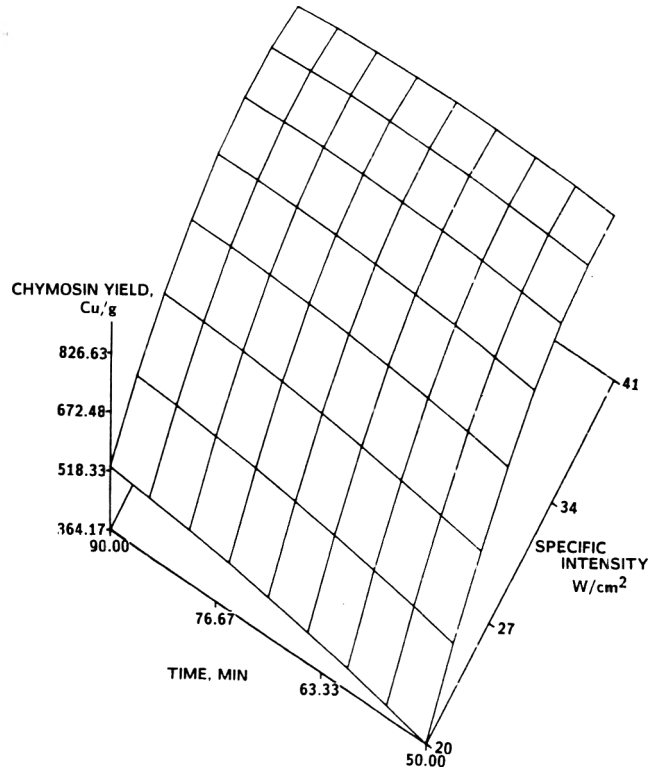
Fig. 6—Prochymosin extraction by ultrasound at 1:25 abomasa: NaCl solution ratio as a function of treatment time and ultrasound specific intensity.
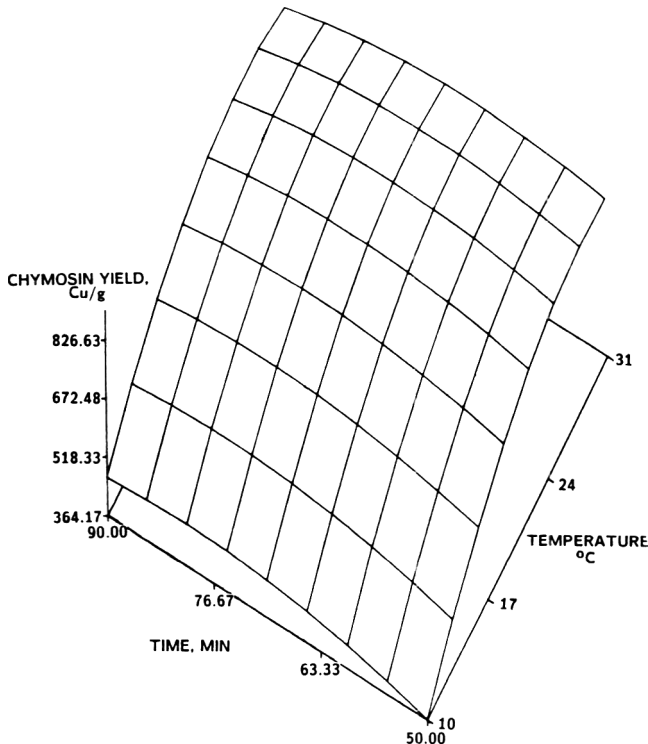
Fig. 5—Prochymosin extraction by ultrasound at 1:25 abomasa: NaCl solution ratio as a function of treatment time and extraction temperature.

plied in this study was significantly higher than in a previous study (Zayas, 1986).

Prochymosin yield increased with increasing extraction time up to 80 min (Fig. 5 and 6) and temperature of the extraction mixture up to 25°C (Fig. 4 and 5). Increasing treatment time

and specific intensity of ultrasound also resulted in an increase of prochymosin yield (Fig. 6). Therefore, extraction time, specific intensity of ultrasound, and temperature of extraction medium may be considered major parameters affecting prochymosin extraction by ultrasound treatment. These parameters were different than recommended in the previous study (Zayas, 1986).

The development of cavitation and an increase in the diffusional osmotic process during extraction depend on the amount of ultrasound energy generated in the liquid medium. Intensity of ultrasound determines not only the rupture of cell structures, but also the state and activity of biologically active substances.

Chymosin is a very heat stable enzyme at pH values between 5.3 and 6.3 (Foltmann, 1959; Mickelsen and Ernstrom, 1967). Generally, during extraction of biologically active substances, yield increases with increasing temperature because of the increasing diffusion rate. But in ultrasound treatment, a temperature rise will increase vapor pressure inside of the cavitation bubbles. This will reduce the violence of the bubbles' collapse, because gas molecules within the bubbles provide a cushion (Sirotyuk, 1966).

The destructive effect of ultrasound increased the permeability of the extraction solution into abomasa, ruptured the cellular structure, and gave correspondingly higher activity and yield of chymosin (Zayas, 1986).

There was a significant increase in the yield of prochymosin and chymosin with increasing the abomasa: extraction solution ratios (Table 1). However, based on relative costs of chymosin production, an abomasa:NaCl solution ratio of 1:25 was the best condition for extraction (Table 1).

The quadratic model for prochymosin extraction by ultrasound was:

$$Z = -1443.85 + 14.06X + 78.53Y + 23.45T - 0.04X^2 - 0.17XY - 0.94Y^2 - 0.06XT + 0.10YT - 0.44T^2$$

$$(R^2 = 0.934, P < 0.02)$$

where Z = prochymosin yield, Cu/g; X = ultrasound treat-

Table 1—Prochymosin and chymosin yield and costs of chymosin extraction by ultrasound at the different abomasa:NaCl solution ratios[e]

Abomasa : NaCl solution ratio	Yield, Cu/g		Total cost of chymosin/g abomasa[f], cent	Total cost per 100 unit chymosin, cent	Relative cost,[g] cent
	Prochymosin	Chymosin			
1 : 15	621.12[a]	1039.02[a]	1.8665	0.1796	1.011
1 : 20	699.07[a]	1312.00[b]	2.3406	0.1783	1.003
1 : 25	786.80[b]	1589.15[c]	2.8246	0.1777	1.000
1 : 30	795.17[b]	1750.82[d]	3.2888	0.1878	1.057

[a,b,c,d] Means in the same column with different superscripts are significantly different ($P < 0.05$).
[e] Mean values obtained from three replications.
[f] Total cost per g abomasa was calculated.
[g] Relative cost was obtained by taking the total cost per 100 unit of chymosin production at the ratio of 1 : 25 abomasa: NaCl solution as 1.000

ment time, min; Y = ultrasound intensity, W/cm^2; T = extraction temperature, °C.

Optimal parameters for prochymosin extraction by ultrasound treatment were: specific ultrasound intensity, 36 W/cm^2 at 20 kHz frequency; extraction temperature, 25°C; time of ultrasound treatment, 80 min; and ratio of abomasa to NaCl solution, 1:25. Optimal time of prochymosin activation was 13.0–13.5 hr, which was longer than the 11 hrs required for the control samples (Fig. 7). Yield of chymosin extracted by control procedure was higher immediately after extraction but lower after activation than that extracted by ultrasound. Consequently, prochymosin was considered to be activated slowly during control extraction process. The activity and yield of the chymosin extracted by ultrasound and control methods were compared (Table 2). Yield of chymosin was somewhat higher than that for control procedure. Yield of ultrasound extracted chymosin was 10.67% and 16.03% higher than for control procedure at 25°C and 3°C, respectively. The activity of experimental chymosin was also somewhat higher than that of control samples.
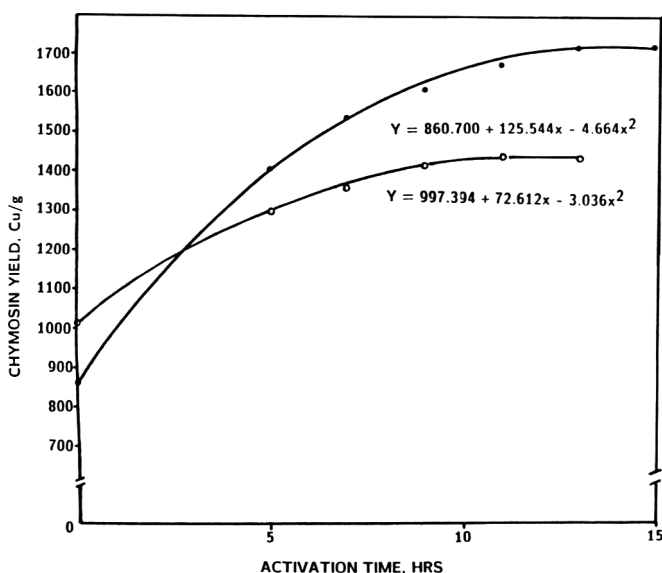
Fig. 7—Activation of prochymosin extracted by ultrasound method (●) and control method (○).

Table 2—Activity and yield of chymosins extracted by ultrasound and control methods[c]

Method	Activity, Cu/mL	Yield, Cu/g	Chymosin yield,[d] %
Ultrasound at 25°C	68.14[a]	1610.67[a]	116.03
Control at 25°C	63.09[b]	1462.60[b]	105.36
Control at 3°C	59.88[b]	1388.20[b]	100.00

[a,b] Means in the same column with different superscipts are significantly different ($P < 0.05$).
[c] Mean values obtained from three replications.
[d] % increased chymosin yield obtained by taking control at 3°C as 100.

The chymosin extracted by the control method had more viscous, mucinous precipitates, which inhibited the filtration process. The chymosin extracted by ultrasound treatment had more fine, white precipitates.

Experimental and control procedures utilized in this study for chymosin extraction were different from the previous study (Zayas, 1986), in which dry abomasa was used for extraction. Chymosin can be partially inactivated as the result of overheating during drying of abomasa. The use of frozen abomasa prevented chymosin inactivation and eliminated expensive drying process and subsequent swelling before extraction. Influence of the freezing on the structure of abomasa increased the rate of enzyme withdrawing during extraction. In the previous study (Zayas, 1986), solution of chymosin obtained after extraction was exposed to prolonged drying and chymosin was obtained as a dry substance.

CONCLUSIONS

AN ULTRASOUND METHOD of chymosin extraction was developed. Ultrasound extraction resulted in an increase in yield and activity of chymosin, while significantly decreasing the extraction time. Optimal extraction parameters for ultrasound extraction were: specific intensity, 36 W/cm^2 at 20 kHz frequency; extraction temperature, 25°C; treatment time, 80 min; and ratio of abomasa to NaCl solution, 1:25. Optimal prochymosin activation time was 13.0–13.5 hr.

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Pulse NMR of Casein Dispersions

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──────────── ABSTRACT ────────────

The spin-spin relaxation time, T_2, of skimmilk, sodium caseinate dispersions and milk ultrafiltrate was measured as a function of pH and temperature using pulse proton NMR. The T_2 shows a maximum around pH = 5.2 for skimmilk, decreases with decreasing pH for sodium caseinate dispersions and is independent of pH for milk ultrafiltrate. For all systems studied T_2 increases with temperature, but, the extent of increase depends on the system. It is concluded that the T_2 of casein dispersions is mainly determined by factors determining the state of aggregation of the caseins. Apart from affecting the extent of aggregation of the casein particles, micellar calcium phosphate probably contributes also directly to the measured T_2.

INTRODUCTION

NUCLEAR MAGNETIC RESONANCE (NMR) spectroscopy is a widely used noninvasive, relatively rapid, spectroscopic technique. In NMR measurements one makes use of the specific magnetic properties of certain nuclei, especially ^1H and ^{13}C. A treatment of the basic principles of NMR has been given e.g. by Dwek (1973) and Farrar and Becker (1971). This study is limited to pulse NMR applied to hydrogen nuclei. Pulse NMR can be a very suitable technique for studying the physical state of water in complicated biological systems such as protein solutions, cellular suspensions and tissues (e.g. Packer, 1977, Lynch, 1983, Fullerton et al., 1982, Belton and Ratcliffe, 1985), but also in rennet-treated milk (Lelievre and Creamer, 1978), powdered milk and dried milk protein species (Brosio et al., 1983, 1984; Leung et al., 1976).

In pulse NMR two characteristic relaxation times, which depend on the environment and mobility of the observed nuclei, may be determined: the spin-spin relaxation time, T_2, and the spin-lattice relaxation time, T_1. T_2 is a parameter, that may be determined relatively simply. Its value (ms to s) strongly depends on the mobility of the hydrogen nuclei. Even in dilute solutions the T_2 of the hydrogen nuclei of water molecules is greatly affected by the interactions of (only a small fraction of) the water molecules with a macromolecular species. The precise character of these interactions very often is not known. However, it is clear that water molecules in close contact with a macromolecular surface will largely lose their mobility and, thus, their T_2 may be decreased by several orders of magnitude. Via rapid (spin) exchange processes on a time scale shorter than the NMR measuring time scale (μs to ms) this will affect the T_2 of the aqueous bulk phase. It is questionable whether one should interpret these features in terms of 'bound' and 'free' water, as they generally are denoted. Anyhow, T_2 is very sensitive to changes in the interactions between water molecules and macromolecules.

In this study the spin-spin relaxation time (T_2) of casein dispersions and skimmilk was determined. Under conditions as in skimmilk up to thousands of casein molecules form aggregates of colloidal size (diameter may vary from 20 to 300

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nm), the so called casein micelles (Walstra and Jenness, 1984). They contain a considerable amount of colloidal calcium phosphate (CCP), which is indispensable for the micellar structure. Upon acidification the structure and composition of these casein particles markedly alters, partly because the CCP goes into solution with decreasing pH. It is completely dissolved at a pH below about 5.2 (Van Hooijdonk et al., 1986). Below pH = 5.0 the particles become unstable and may coagulate to form a gel (Roefs, 1986). In a sodium caseinate dispersion of proper ionic strength a similar acid casein gel is formed after acidification. Here, acidification causes the casein molecules first to aggregate into casein particles which subsequently form a gel (Roefs, 1986). The purpose of this study was to determine the effect of pH and temperature on the spin-spin relaxation time (T_2) of hydrogen nuclei of the water molecules of casein dispersions and skimmilk.

MATERIALS & METHODS

Protein solutions

Reconstituted skimmilk was made from a commercial low-heat skimmilk powder by dissolving 12g powder in 100g demineralized water. Sodium caseinate dispersions contained 3g sodium caseinate powder per 100g solution and about 0.013 mol NaCl per 100g dispersion. The complete composition of the powders has been given elsewhere (Roefs, 1986). For acidification, the caseinate and skimmilk dispersions were cooled in ice water and subsequently acidified (using 0.5N HCl and 3.0N HCl for sodium caseinate and skimmilk, respectively) to pHs varying from 6.7 to 4.6. Milk ultrafiltrate was obtained from skimmilk or acidified skimmilk by means of an Amico concentrator model CH3, whereby constituents with a molecular mass above 10,000 were separated.

NMR measurements

NMR tubes were filled immediately after acidification and, for any particular solution or gel, the same tube was used at all temperatures. Samples were first equilibrated for at least 1 hr at the measuring temperature. Experiments were done in sequence of increasing temperature. If not used for some time (1–48 hr) they were stored at 4°C. Measurements of the spin-spin relaxation time, T_2, were carried out in a 20 MHz pulse NMR, consisting of modified Bruker Minispec PC 20 electronics and a Newport 7-in electromagnet. For thermostatting, a Varian ESR Dewar flask, coupled with a Varian temperature controller was used. Temperature inaccuracy was about 0.5°C. A Bruker Aspect 2000 computer and a Z17C pulse programmer were used for regulation of the measurements and storage and processing of experimental data. The Carr-Purcell-Meiboom-Gill pulse sequence was applied. The length of the 90° and 180° pulses were 12.5 and 25 μs, respectively. The time between the 180° pulses was 1.0 ms (for skimmilk and sodium caseinate) or 3.0 ms (for milk ultrafiltrate). The accumulated exponential decay curve of the nuclear magnetization was fitted with one single exponential, according to the method of McLachlan (1977). When extensive curve-fitting calculations based on nonlinear least squares fit (Provencher, 1976) were used, experiments with skimmilk (pH = 6.7) and acid skimmilk gels (pH = 4.6) revealed only one dominant exponential (> 96% of total amplitude), except when a small overlying layer of condensed water or syneresis fluid was present. Experimental deviations were within a few per cent.

RESULTS

THE T_2 OF SKIMMILK as a function of pH measured at 4, 10.5, 20, 30, 40, and 49.5°C, respectively, is shown in Fig.
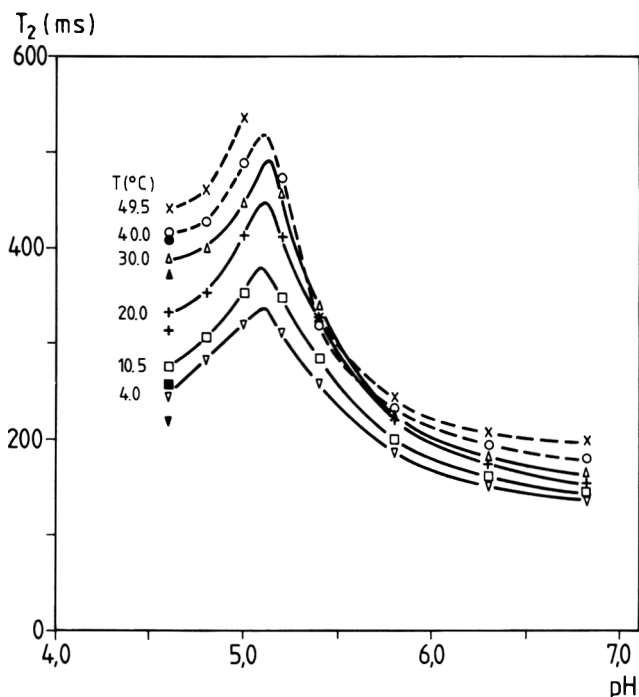
Fig. 1. — The T_2 (ms) of acidified skimmilk as a function of pH. The temperature was varied as indicated. The filled symbols at pH 4.6 belong to a skimmilk solution that was aged 10 hrs at 30 °C before the first measurement and the other symbols to samples prepared via the standard procedure.

Fig. 2. — The T_2 (ms) as a function of pH for milk ultrafiltrate obtained from standard skimmilk and subsequently acidified (o) and for milk ultrafiltrate, obtained from skimmilk acidified to pH 5.2 ($\triangle$) and pH 5.0 ($\square$). Temperature was varied as indicated. For the sample with pH = 6.76 at 49.5 °C, where T_2 strongly decreased during measurement, the highest value obtained is given (see text).

1. In the sample of pH 5.2 at 49.5°C the milk protein had flocculated and too much syneresis fluid was formed to give a reliable value for T_2.

A very strong effect of pH on T_2 was found at every temperature investigated. A maximum in T_2 was observed between pH 5.0 and pH 5.2. These pH values may be somewhat uncertain, because they were measured just after acidification at 2°C. Because of the dissolution of colloidal calcium phosphate (CCP), which is a time consuming process at pHs above 5.0, the actual pH may have been shifted upwards in the course of time by approximately 0.1 to 0.3 units, depending on temperature.

An increase of T_2 with temperature is to be expected as the mobility of the water molecules and, thus, of the hydrogen nuclei, increases with temperature.

The maximum found in the T_2 value when pH of the skimmilk was varied (Fig. 1), must be ascribed to the skimmilk proteins and possibly the CCP, but not to the salt solution itself. This follows from a comparison of the results shown in Fig. 1 for skimmilk with those of Fig. 2 for milk ultrafiltrate.

The milk ultrafiltrate, obtained at room temperature from standard skimmilk, was acidified at 0–2°C to pHs varying from 6.7 to 4.6. As a check, we also used ultrafiltrate obtained at 4°C from skimmilk which had been acidified first to the range of pH 5.0 to 5.2. The T_2 values for the ultrafiltrates were about five times as high as those of the corresponding skimmilk and are very close to the T_2 values of pure water. No clear influence of pH on T_2 was found. The T_2 values of ultrafiltrate obtained from skimmilk acidified to pH 5.2 and 5.0 agreed well with those of ultrafiltrate made from skimmilk of pH 6.8 and subsequently acidified to the same pH (Fig. 2). The temperature dependence was almost the same at all pHs.

The ultrafiltrate sample at pH 6.8 showed a remarkable decrease in T_2 during the measurement at 49.5°C. This must have been due to the formation of a calcium phosphate precipitate. Prolonged heating at 50°C enlarged the decrease, which, after an extended waiting time, led to a T_2 value of about 1.6 s.
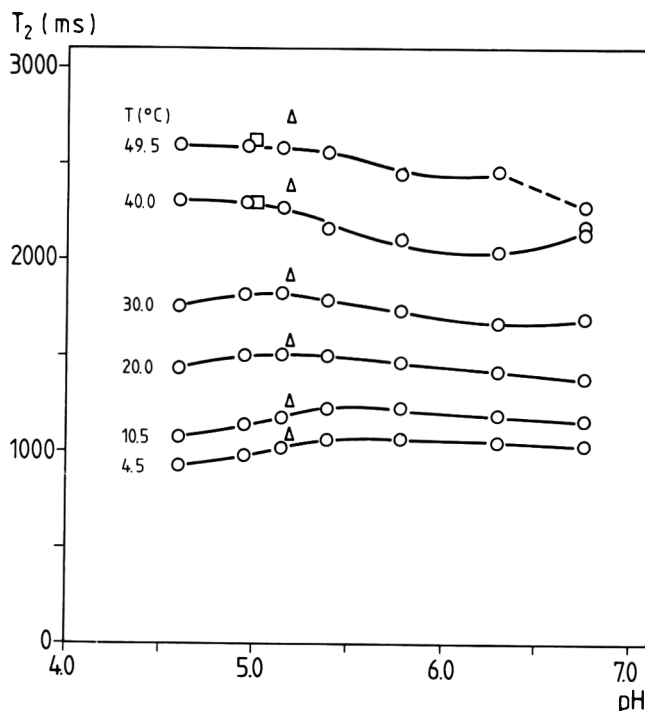
At pH 4.6 gelation at 30°C followed by ageing of the gel over 10 hrs tended to decrease the T_2 at a not-too-high temperature compared to the nongelled or nonaged sample (Fig. 1). Especially at low temperature a significantly shorter T_2 was found for the gel compared to a solution of skimmilk at pH 4.6. The difference vanished as the solution became a gel during the measurements at 20°C and higher (Roefs 1986). At 49.5°C there was no longer a difference.

In order to study the influence of the casein on the T_2 values in the absence of colloidal calcium phosphate and serum proteins the same experiments were repeated with solutions of sodium caseinate in water.

The results for sodium caseinate dissolved in a solution of 0.13 mol NaCl/kg dispersion are shown in Fig. 3. The T_2 value diminished with decreasing pH. There was no optimum pH unlike the behaviour of skimmilk. The drop in T_2 with decreasing pH became more pronounced at higher temperatures, especially in the pH region between 5.0 to 6.0. At low pH, the T_2 was only slightly longer than for skimmilk, while at a higher pH the T_2 of sodium caseinate solutions was much longer. Part of the difference, especially at low pH, must have been due to the absence of serum proteins and the other soluble milk components.

The temperature dependence of T_2 is shown explicitly in Fig. 4 and 5 where log T_2 is depicted as a function of the inverse temperature for sodium caseinate and skimmilk, respectively. Except at pH 5.4 for skimmilk T_2 always increased with temperature. At low pH (<5.1) the increase observed for sodium caseinate was similar to that shown by skimmilk. (Note that the scale of the vertical axis in Fig. 4 differs from that in Fig. 5). However, for the sodium caseinate dispersions, at higher pHs the rise in T_2 as a function of temperature was much greater than at low pHs, in contrast to the behaviour of skimmilk solutions.

Upon acidification at 0–2°C, the appearance of the sodium caseinate solutions changed from a rather clear, greyish color
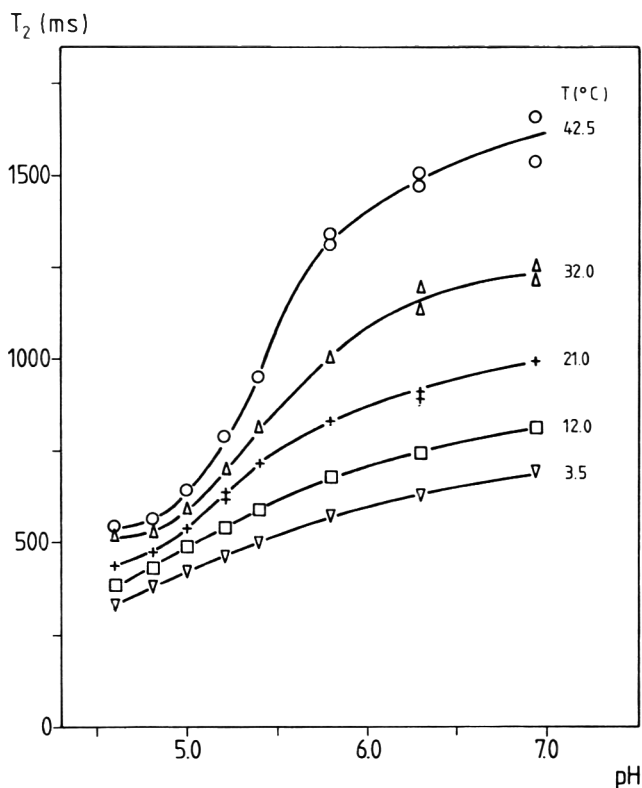
T_2 (ms)

Fig. 3.— The effect of pH on the T_2 (ms) of acidified sodium caseinate solutions. Temperature was varied as indicated. The casein concentration was 28 — 29 g/kg dispersion. The caseinate was dissolved in a solution of approximately 0.13 mol NaCl/ kg dispersion.

T_2 (ms)

Fig. 4.— The T_2 (ms) of sodium caseinate solutions depicted on a logarithmic scale as a function of $1/T$ (K^{-1}). The values are derived from the data of Fig. 3.

to colloidal white and turbid (like milk) at pH 4.6. This must have been caused by the aggregation of casein, as was also observed on electron micrographs (Roefs, 1986). The transition from smaller to larger casein aggregates, which took place between pH = 5.8 and 5.2, is paralleled only at higher temperatures by a steep decline in T_2.

In a separate experiment about 50% of the NaCl was replaced by $CaCl_2$ keeping the ionic strength at the same level. Then at pH values > 5.9 T_2 levelled off and at T = 32°C even became shorter with increasing pH (e.g. T_2 at pH 6.6 and T = 32°C was 1000 ms), reflecting the casein aggregation caused by the $CaCl_2$ addition.

DISCUSSION

THE LARGE EFFECT of pH on the T_2 of skimmilk and sodium caseinate dispersions proves that the T_2 of hydrogen nuclei of water is a very sensitive parameter for monitoring the protein-water interactions in these systems. Rapid or, at least, moderate exchange between bulk water molecules and those close to the surface of a protein must be responsible for the T_2 values obtained, because the data could be fitted with only one dominant exponential. Then the T_2 measured is a weighted average of relative spin populations P_i in the ith phase and relaxation times T_{2i} of the ith phase according to:

$$\frac{1}{T_2} = \sum_i \frac{P_i}{T_{2i}} \tag{1}$$

The large difference in T_2 between milk ultrafiltrate and skimmilk or sodium caseinate dispersions shows that the macromolecules and or macromolecular aggregates present strongly affect the overall T_2 of the aqueous dispersions.

Relation of T_2 to state of aggregation

Comparing Fig. 1, 2 and 3 it is clear that mainly the individually dispersed casein molecules and/or casein molecules aggregated into casein particles determine the T_2. The contribution of the dissolved salt ions by itself is negligible since the T_2 of milk ultrafiltrate hardly differs from that of pure water. The contribution of serum proteins is only small, as follows from a comparison of the T_2 at pH 6.7 and T = 34°C of whey (serum proteins present) (1.3 s according to Lelievre and Creamer, 1978), milk ultrafiltrate (no protein present) (1.9 s) and skimmilk (0.17 s). The low T_2 of skimmilk and of sodium caseinate dispersions at low pH implies that a considerable part of the water inside the casein particles, which are known to be strongly 'hydrated' (water content roughly 2 to 3 times its protein content) is to some extent immobilized. What remains unclear is the relative contribution of colloidal calcium phosphate (CCP), which certainly affects T_2 indirectly, since it plays a crucial part in the structure of casein particles at pH above 5.2. However, the lowering of T_2 due to calcium phosphate precipitation in the milk ultrafiltrate sample at pH 6.8 and T 49.5°C, indicates a possible direct contribution of CCP inside the casein particles.

The observed pH dependency of the T_2 of skimmilk must be related to a change in structure of the casein particles upon acidification, whereas the observed pH dependence of sodium caseinate seems to be related to the formation of casein particles upon acidification. Since at low pH almost the same T_2 is found for skimmilk and sodium caseinate dispersions, the structure of casein particles is then probably also similar. A
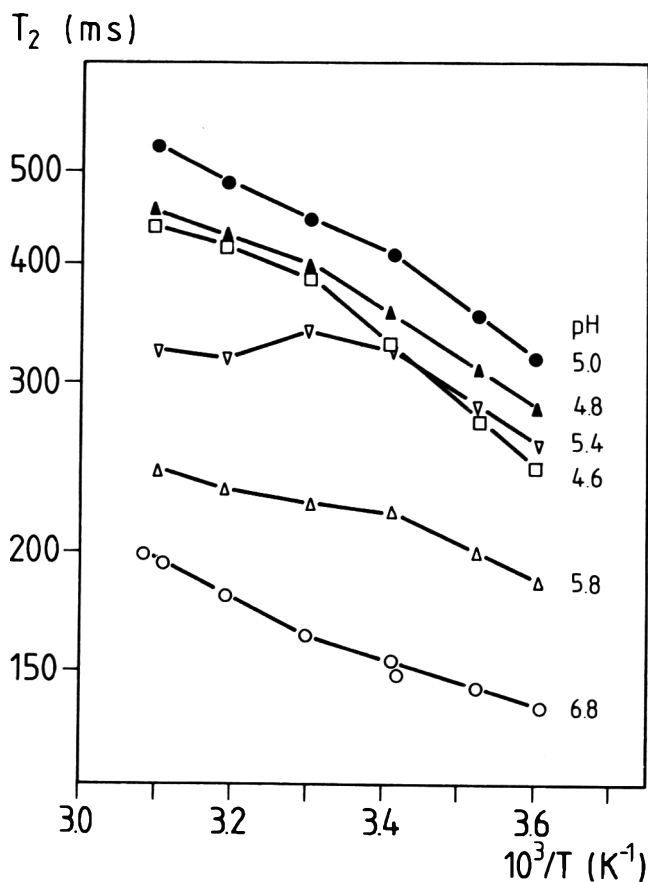
T_2 (ms)

Fig. 5. — The T_2 (ms) of skimmilk depicted on a logarithmic scale as a function of 1/T (K⁻¹). The values are derived from the data of Fig. 1.

change in structure of the casein particles during acidification from pH 6.7 to 4.6 has been shown before by means of e.g. rheological measurements and electron microscopy (Roefs, 1986; Roefs et al., 1985; Heertje et al., 1985). In skimmilk above pH 5.2 the casein is present in the form of casein micelles, or the relics of casein micelles, consisting of aggregated casein molecules and amorphous CCP. Below pH 5.2 the particles no longer contain CCP. At pH 6.8 casein in sodium caseinate dispersions will be present as small aggregates containing e.g. 20 protein molecules (Schmidt, 1982; Roefs, 1986). During acidification casein molecules will start to aggregate below pH 5.5 to form at first particles of increasing size and subsequently (below pH 5.0) a coagulum or a gel.

The strong increase of T_2 with decreasing pH for skimmilk at pH > 5.2 (Fig. 1) runs parallel to the dissolution of CCP from the casein micelles. At low temperature (~4°C) this is paralleled by a strong increase in dissolved casein. However, such dissolution does not occur at temperatures above about 35°C and this suggests that the rise in T_2 with decreasing pH at pH > 5.2 is not primarily caused by dissolution of casein, but is due to changes in the state of aggregation of the caseins. Of course, interpretation of T_2 of casein dispersions in terms related to the state of aggregation of the protein may only be done if its concentration is constant (e.g. Belton et al., 1988).

Experimental evidence that the internal structure of the casein particles rather than coagulation or gel formation determines T_2 has also been given by Lelievre and Creamer (1978). They found almost the same value for T_2 of skimmilk at natural pH, whether or not it had been gelled by rennet action, as long as no syneresis had occurred. In our experiments T_2 of skimmilk acidified to pH 4.6 and measured at 4°C was significantly higher than that of the corresponding skimmilk gel aged first for 10 hrs at 30°C and then measured at 4'C. The difference,

however, is probably caused by irreversible changes in the internal structure of the casein particles caused by heating and subsequent gel formation rather than by gelation as such.

It remains unclear if the increase in T_2 with decreasing pH in skimmilk at pH > 5.2 (Fig. 1) mainly stem from a change in the interactions between amorphous CCP and water, or from a change in protein conformation. Probably both mechanisms play a part.

Interpretation of T_2

Roughly speaking, the T_2 in a casein dispersion depends on the fraction and T_2 of the hydrogen nuclei of the aqueous bulk phase and of the water molecules in the vicinity of the large (aggregated) protein molecules (Eq. 1). Next, a distinction is often made in two phases: the free bulk water and the slowed down water molecules close to a macromolecular surface which are (strictly speaking, incorrectly) called 'bound' water. Since only one dominant T_2 was measured the exchange rate is assumed to be very fast. Thus, the amount of 'bound' water which is assumed to be related to the state of aggregation of the molecular species and the small T_{2b} of the 'bound' water will determine the measured T_2. However, it is still unkown whether there is a direct relation between the state of aggregation and the amount of 'bound' water and the T_{2b} of it and so with the measured T_2.

A more general model than the two-site exchange model to describe relaxation in heterogeneous systems (e.g. cells) was introduced by Brownstein and Tarr (1979) and extended by Reinders (1987). For a review see Belton and Ratcliffe (1985). This model is based on the following assumptions: (1) there is bulk relaxation; (2) in addition to this, magnetization also decays at a surface containing relaxation sinks characterized by a parameter representing their efficiency; (3) the probability that a water molecule reaches the wall is determined by diffusion. Depending on geometry, dimensions, surface relaxation and diffusion coefficient one or more T_2's are found describing the relaxation behavior (Reinders, 1987, Belton et al., 1988). A change in the state of aggregation of the casein molecules will immediately influence the geometry and the dimensions of the cavities in the aggragated casein particles wherein water molecules may be more or less trapped. This effect may explain the close correlation between the T_2 and the state of aggregation of casein in skimmilk at pH < 5.1 and the sodium caseinate dispersions at pH < 5.8.

This correlation is further illustrated by the fact that parallel to the maximum in T_2 around pH 5.2 for skimmilk also a maximum in the voluminosity of casein micelles is found around pH 5.4 at temperatures below 30°C (Darling, 1987; Tarodo de la Fuente, 1975; Snoeren et al., 1984; Van Hooijdonk et al., 1986). Both parameters probably reflect a minimum in the state of association of the casein molecules in skimmilk in the pH range of 5.1 - 5.4. However, in contrast to the maximum in T_2, which became more distinct as the temperature was raised, the maximum in voluminosity disappeared at temperatures above 30°C indicating that T_2 does not depend only on the total amount of water included in casein particle as measured by voluminosity.

Temperature dependence of T_2

For fast moving molecules, such as water molecules in the bulk water phase, T_2 will be linear to η/T (Carrington and McLachlan, 1969), where η is the viscosity of the medium and T absolute temperature. However, for water molecules close to a surface, molecular motion will be constrained. In that case, if there is a fast exchange between bulk water and that close to a surface log T_2 will decrease linearly with 1/T (Dwek, 1973; Lynch, 1983). For that reason T_2 of biological systems (Lynch, 1983; Bryant and Shirley, 1980) is generally depicted on a logarithmic scale as function of 1/T on a linear

scale. The linearity of log T_2 with 1/T may be lost when an intermediate exchange rate exists or the conditions for the water trapped in cavities change.

Except for the sample at pH 6.76 (particularly at 49.5 °C) straight lines (not shown) were found for milk ultrafiltrate if T_2 was depicted on a linear scale as a function of η/T. The T_2 values were close to those of pure, free water.

This was, as expected, not the case for sodium caseinate solutions and skimmilk because several phenomena will be involved when the temperature is raised. Firstly, the mobility of the hydrogen nuclei will increase. Secondly, the calcium phosphate equilibria between dissolved and undissolved CCP will shift towards the undissolved species, resulting, directly or indirectly, in a change in protein aggregation. Thirdly, the association of casein molecules will increase with temperature leading to a less open internal structure of the casein particles. This may lead to a decrease in T_2 (see above). The latter effect was especially seen with sodium caseinate, when pH was varied at constant temperature (Fig. 3). The relative change in T_2 with decreasing pH increased with T.

Since in sodium caseinate dispersions no calcium phosphate was present only the first and third effect play a part. A more or less linear decrease of log T_2 with 1/T was found for the samples of pH 5.4 and above (Fig. 4). At lower pH values a linear decrease is seen only over a part of the curves. Here, the downward deviation from linearity may be due to an increased aggregation of casein molecules inside the casein particles as the temperature is raised.

Except at pH 5.4 roughly parallel curves were found for log T_2 of skimmilk plotted as a function of the inverse temperature (Fig. 5) with T_2 decreasing with 1/T. For all samples, the dependence is non-linear and at pH > 5.0 the slope of the curves is much smaller than in case of the sodium caseinate dispersions. At pH < 5.0, where CCP is no longer present, the curves of skimmilk and sodium caseinate have almost identical slopes. At pH 5.4, which is close to the pH limit for the dissolution of CCP, the curve of T_2 versus 1/T levels off above 20 °C. At this, and higher pH values, not only increased casein aggregation, but also reformation of amorphous CCP may be involved on raising the temperature, resulting in a large deviation from linearity and less steep curves. At all pHs, a change with temperature in the extent of casein aggregation inside the casein particles may be expected. The voluminosity of the casein particles decreases with temperature for all pH. In as far as this decrease reflects an increase in casein aggregation inside the casein particles it will cause a reduction in the increase of T_2 with temperature, as is indeed found for skimmilk as compared to sodium caseinate dispersions.

Thus, the variation of T_2 with temperature is greatly influenced by temperature induced variation in the state of association of the protein molecules.

CONCLUSIONS

THE SPIN-SPIN RELAXATION TIME, T_2, of hydrogen nuclei of the water molecules is a sensitive parameter for monitoring water-protein interactions. Since these interactions are strongly influenced by the intra- and intermolecular protein-protein interactions, T_2 is correlated with the state of aggregation of the protein molecules in the casein particles. Increased aggregation normally leads to a lower T_2. Coagulation of casein particles followed by gel formation hardly effects T_2.

Interpretation of T_2 in terms of amounts of 'bound' and 'free' water is not possible. With a change in protein conformation the T_2 of individual hydrogen nuclei may alter as well and water molecules may be more or less captured in cavities in and between the casein molecules. Therefore, a more general theory seems to be more appropriate.

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Analysis of Pressure Drop in Extruder Dies

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ABSTRACT

Entrance pressure drop of extruder dies increased with a decrease in the ratio of barrel-to-die diameter during the extrusion of corn. For the low Reynolds number flow studied ($1.77 \times 10^{-3} \leq Rc \leq 9 \times 10^{-5}$) the viscous component, obtained from Weissberg's analysis, contributed 2% to 40% of the entrance pressure drop and increased as the barrel-to-die diameter ratio decreased. The friction factor for laminar fully developed flow through the die followed the expression $f = 16/Re'$. Fully developed flow within the die was achieved at a die length-to-diameter ratio of 9.

INTRODUCTION

EXTRUDERS are widely used in the food industry for production of snack foods such as ready-to-eat (RTE) cereals and breading. During extrusion processing, food materials undergo physiochemical changes such as starch gelatinization and protein denaturation (Harper, 1986). These reactions alter dough structure and rheological properties.

The die is a major component of the extruder set-up. Understanding the material properties and the nature of the flow in the extruder die is of primary importance in controlling the extruder performance and extrudate quality. The die can also be used as a capillary rheometer to monitor continuously the rheological properties of the dough, as most rheometers cannot simulate the flow in the extruder.

When dough enters a die from an extruder barrel, a velocity profile starts to develop and continues to change until it reaches a specific distance beyond which flow is fully developed. The pressure drop at the die entrance, ΔP_{ENT}, for a viscoelastic fluid has been observed to be far greater than the ΔP_{ENT} for a Newtonian fluid of nearly the same viscosity (Han, 1976). The reason for the large ΔP_{ENT} may be attributed to the elastic component of the viscoelastic fluid. For viscoelastic polymer melts flowing from a reservoir into a circular tube, the pressure drop at the entrance is around 25 per cent of the total pressure drop across the capillary (Han, 1971). The entrance pressure drop for food doughs can be as high as 50 per cent of the total pressure drop across the die (Hawkins et al., 1986). Jao et al. (1978) investigated the effect of process and product conditions on the entrance pressure drop. Their results indicate that entrance pressure drop is affected by product moisture content, temperature and shear rate.

This study was undertaken to investigate the pressure drop at the entrance and in the die during extrusion cooking of corn grits. Specifically the study had the following objectives: (1) evaluate the dependence of entrance pressure drop on the barrel-to-die diameter ratio, (2) relate appropriate dimensionless pressure drop to the Reynolds number to determine the effect of flow on the pressure drop, (3) analyze the contribution of viscous and elastic effects to the entrance pressure drop, and (4) develop a relationship between friction factor and Reynolds number for doughs.

Theory

LaNieve and Bogue (1968) have theoretically analyzed the pressure loss in a capillary rheometer. Neglecting kinetic energy (Bernoulli) effect because of low flow rates, the total pressure drop, ΔP_{TOT}, from the die entrance to die exit can be written as follows:

$$\Delta P_{TOT} = \Delta P_{ENT} + \Delta P_{CAP} + P_{EXIT} \qquad (1)$$

where ΔP_{ENT} is the entrance pressure drop, ΔP_{CAP} is the pressure drop in the capillary and P_{EXIT} is the pressure at the exit. Han (1976) hypothesized that ΔP_{ENT} is composed of two parts: viscous and elastic. Thus,

$$\Delta P_{ENT} = \Delta P_{VIS} + \Delta P_{ELASTIC} \qquad (2)$$

where ΔP_{VIS} is the viscous component and $\Delta P_{ELASTIC}$ is the elastic component of entrance pressure.

According to LaNieve and Bogue (1968), the elastic component can be further subdivided into two parts: the dissipative loss caused by irregular flow patterns related to elasticity and the elastic internal energy of the fluid. Metzner and White (1965) concluded that the elastic effects due to profile development are a small part of the elastic component of the pressure drop and hence negligible.

For creeping flows (low Reynolds number flow) Weissberg's (1962) analysis for Newtonian flow can also be applied to non-Newtonian fluids to obtain,

$$\Delta P_{VIS} = \frac{3.47\eta Q}{2R^3} \qquad (3)$$

where R is the radius of the die, Q is the volume flow rate and η is the apparent viscosity corresponding to each value of flow rate at a particular temperature and moisture content. Boles et al. (1970) give an empirical equation for a power-law fluid,

$$\Delta P_{vis} = \frac{1.18\,m}{n^{0.7}} \left[\left(\frac{4\,Q}{\pi\,R^3} \right) \left(\frac{3\,n + 1}{4\,n} \right) \right]^n \qquad -(3b)$$

The viscosity can be obtained (Harper et al., 1971) as

$$\eta = \eta_0\, \dot{\gamma}^{n-1} \exp\left[\frac{\Delta E}{RT} + b \cdot MC \right] \qquad (4)$$

where $\dot{\gamma}$ is the shear rate (s^{-1}), T is the absolute temperature (K), R is the universal gas constant, MC is the moisture content (wet basis), n is the flow behavior index and ΔE, b and η_0 are material constants.

MATERIALS & METHOD

Sample preparation

Corn meal was obtained from Gooch Mills, Inc. (Lincoln, NE). The proximate analysis (dry basis) indicated in %: ash-0.39, protein-9.23 and fat-0.65. Moisture contents of the milled samples were determined and the amount of water to be added to bring the samples to the required moisture contents of 15, 18, 21, 25, and 30% wet basis was calculated. Water was added slowly to the samples while being stirred in a Hobart mixer. The samples were sealed in plastic containers and allowed to equilibrate at room temperature for 6-8 hr.

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They were then stored in a freezer. Prior to extrusion, the samples were removed from the freezer and allowed to reach room temperature.

Extrusion

A Brabender laboratory food extruder (Model #2003-G8R) with a 1.9 cm diameter barrel (D_b) and a 20:1 barrel length-to-diameter ratio was used in this study. Four dies with diameters (D_d) of 0.95, 0.64, 0.48, and 0.32 cm and length (L) of 11.11 cm each were used to generate D_b/D_d ratios of 2, 3, 4, and 6 and L/D_d ratios of 11.67, 17.50, 23.33, and 34.50, respectively. A schematic diagram of the die is shown in Fig. 1. Heating of the extruder-die setup was accomplished by dividing it into three zones. The first heating zone included the feed zone and part of the compression zone of the extruder. The second heating zone included the melting zone and the remaining part of the compression zone of the extruder. The third heating zone was for the entire die. The first heating zone was always maintained at a temperature of 80°C while the second and third heating zones were maintained at 110, 130, 160 or 180°C. Screw compression ratio used was 3:1 and for each combination of moisture content and barrel temperature, the screw speeds were maintained at 100, 140, and 180 rpm.

The experimental design is given in Table 1. Selection of the experimental point was governed by: (1) the amperage of the motor (if too high, the operation was shut off) or (2) the magnitude of pressure developed (if too low, it could not be measured accurately with the transducers used).

Before extrusion, the extruder was equilibrated with 22% moisture content corn meal. Flights at the feed port were kept full throughout the extrusion run. For each run measurements were taken after the extruder reached steady state as indicated by constant amperage and constant pressure.

Four melt pressure transducers (PTE 462, Dynisco, Inc., Norwood, MA) were used to measure pressure data. One transducer (range: 0 to 52 MPa) was located in the barrel, close to the barrel exit, and the remaining three transducers (ranges: 0 to 35 MPa, 0 to 21 MPa and 0 to 21 MPa) were located along the length of the die as shown in Table 2. The transducers were connected to the data acquisition system via a signal conditioner (Dynisco Model #EC 100-5, Norwood, MA). The data acquisition system consisted of a multiplexer board (Action Instruments, San Diego, CA) that received the signals from all four transducers and transmitted them to an IBM AT personal computer where the data was recorded in data files. Data was recorded at one second intervals after steady-state was reached in the die. Entrance pressure drop was calculated as the difference between barrel

exit pressure and die entrance pressure which was estimated by extrapolating the pressures in the die to the die entrance (Fig. 2).

Calculation of viscosity

Apparent shear rate (γ_{ap}) for the flow of a fluid through a cylindrical tube is given by (Wilkinson, 1960):

$$\gamma_{ap} = 4/\pi R^3 \qquad (5)$$

Wall shear stress was calculated using the equation

$$\tau_w = \left(-\frac{dP}{dx} \right) \frac{R}{2} \qquad (6)$$

where the pressure gradient (-dP/dx) was obtained from the slope of the axial pressure profile. The slope of the plot of log τ_w vs log γ_{ap} will give the flow behavior index (n). Wall shear rate can then be calculated using Rabinowitsch-Mooney equation as:

$$\gamma_w = \left(\frac{3n + 1}{4n} \right) \gamma_{ap} \qquad (7)$$

Apparent viscosity was calculated using $\eta = \tau_w/\gamma_w$ and the constants η_o, b and ΔE of equation (4) were calculated using regression analysis.

In order to calculate shear rate, the volumetric flow rate (Q) in the die must be known. The mass and moisture content of the extrudate sample collected was determined to obtain the dry mass of the sample. The mass flow rate of the dough in the die was calculated using a mass balance as:

$$M_1 = M_2 (1 - MC_e)/(1 - MC_d) \qquad (8)$$

where M_1 is the mass flow rate in the die (kg/s), M_2 is the mass flow rate of the extrudate (kg/s), MC_e is the moisture content of extrudate sample (w.b.), MC_d is the moisture content of the dough (w.b.).

The density of the dough was measured for the experimental conditions used and was found to vary between 1150 to 1250 kg/m³ with no specific trend. Hence, a constant value of 1200 kg/m³ was assumed which was in agreement with that used by Alvarez Martinez et al. (1988).

Data analysis

All data analysis was accomplished on a VAX/8600 computer using SAS software (Barr et al., 1979). The effect of product and process variables on pressure drop and constants in the viscosity model were calculated using the General Linear Model procedure.

RESULTS & DISCUSSION

THE VISCOSITY DATA indicated that the corn dough was pseudoplastic with the following values for the material constant: $\eta_o = 1480.3$ Pa-s^n, n = 0.26, $\Delta E/R = 1919.08$ K and b = -8.285. A plot of viscosity versus shear rate for the entire ranges of moisture contents and temperatures is shown in Fig. 3. As shear rate increased viscosity decreased, indicating shear thinning behavior. Increasing moisture content and temperature caused a decrease in viscosity (Fig. 4 and 5). The value of n agrees closely with that of Cervone and Harper (1978) and Bhattacharya and Hanna (1987). The coefficient of determination (R^2) obtained was 0.96.
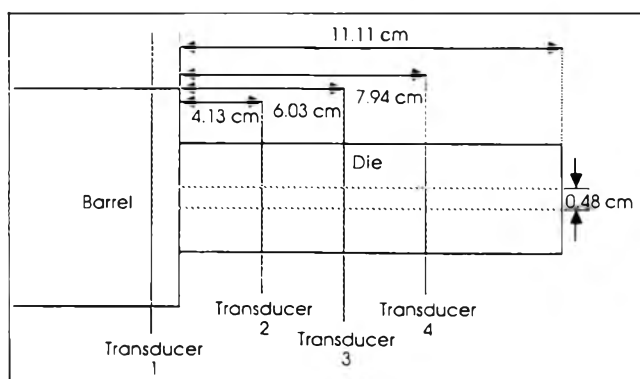
Fig. 1.—Dimensions of die and location of transducers on the die for measurement of pressure.

Table 1 — Experimental design used in the study and diameters of the dies used for the different die temperatures and product moisture content

Moisture content (% wb)	Temperature (°C)[a]			
	110	130	160	180
15			0.48	0.48
18	0.95[b]	0.95	0.32, 0.48, 0.64	0.32, 0.48, 0.64
21	0.95	0.48, 0.64, 0.95	0.32, 0.48, 0.64	0.32, 0.48, 0.64
25	0.95	0.48, 0.64, 0.95	0.32, 0.48, 0.64	0.32, 0.48, 0.64
30		0.48, 0.64, 0.95	0.32, 0.48, 0.64	0.32, 0.48, 0.64

[a] Each combination of temperature and moisture content shown above was operated at the three screw speeds of 100, 140, and 180 rpm.
[b] Indicates die diameter in cm.

Table 2—L/D_d ratios at the various transducer locations for the four dies used in the study

Die diameter cm	L/D^d at various transducer locations		
	Transducer 2	Transducer 3	Transducer 4
0.3175	12.50	18.50	24.50
0.4763	8.67	12.67	16.67
0.6350	6.50	9.50	15.50
0.9525	5.67	7.00	8.33
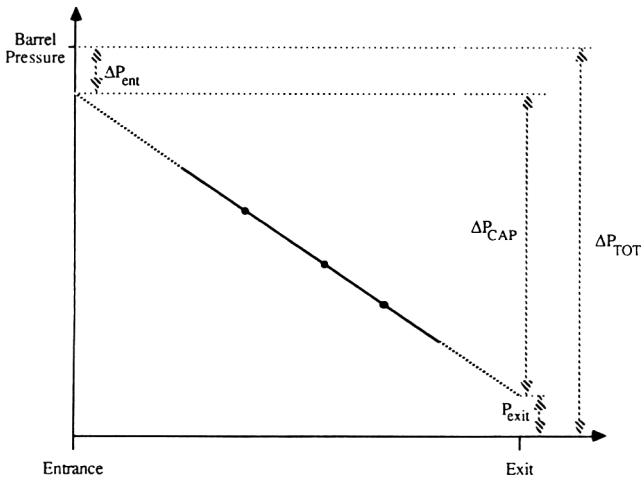
Fig. 2.—Typical pressure profile in the die and the calculation of the various pressure drops along the length of the die.
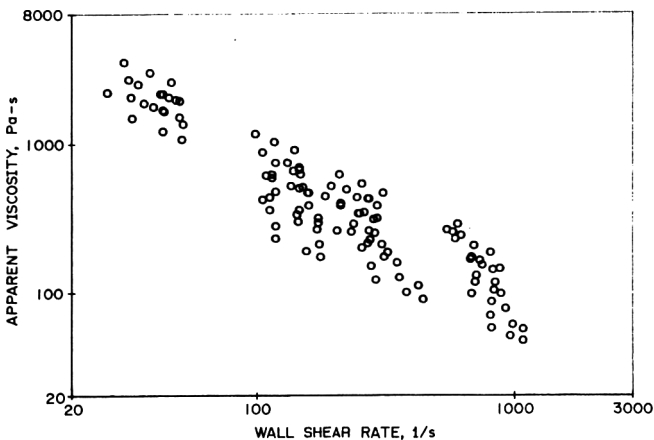
Fig. 3.—Apparent viscosity and wall shear rate of the given ranges of moisture content and temperature.

Fig. 4.—Apparent viscosity and wall shear rate for various moisture contents (M.C) studied at die temperature of 160°C and die diameter of 0.32 cm.
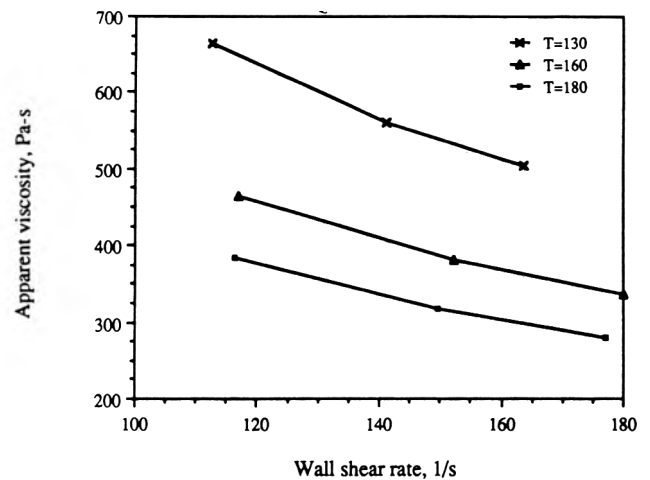
Fig. 5.—Apparent viscosity and wall shear rate of various temperature (T) studied at product moisture content of 25% (w.b.) and die diameter of 0.64 cm.

It should be noted that even though it is difficult to perfectly flush-mount pressure transducers, the error incurred in measurement of viscosity is minimal (Boger and Denn, 1980). The pressure measured by the pressure transducer at any point along the length of the die is the sum of the isotropic pressure and the radial component of normal stress. The isotropic pressure is independent of radius whereas the normal stress component depends on the radial location of the transducers. Thus:

$$S_{rr}(r,x) = -P(x) + \tau_{rr}(r) \qquad (9)$$

where s_{rr} is the reading of the transducer, P is the isotropic pressure and τ_{rr} is the radial normal stress. Since at any location along the axis of the die, P is independent of the radial position while τ_{rr} is a function of the radial position, if transducers mounted at various positions along the axis of the die are at the same radial distance, then the difference between their readings will give the isotropic pressure drop, ΔP. When threads

for mounting the transducers in the die were machined and tapped, care was taken to ensure that the number of threads tapped were the same for all the transducer locations on each die, thereby minimizing the error due to a lack of flush mounted transducers. The transducers were mounted such that the diaphragm tips were tangent to the die hole and no deeper. Hence the transducers did not seriously affect or alter the flow lines of the material in the die.

The range of parameters obtained are shown in Table 3. The Reynolds number was calculated using the expression Re = $\rho D_d V/\eta$ where V is the average velocity of the dough in the die and D_d is the die diameter. Apparent viscosity was calculated for a given wall shear rate, moisture content and temperature. A representative axial pressure profile is shown in Fig. 2. The entrance pressure drop (ΔP_{ENT}) varied with shear rate, dough moisture content and die temperature. The pressure drop at the entrance increased as shear rate increased, but it decreased as dough moisture content and die temperature increased. The entrance pressure drop ranged from 2.5 to about 37 percent of the total pressure drop. Under the experimental conditions used in this study, the median ratio for the entrance pressure drop was between 10–20%.

The viscous component of entrance pressure drop was calculated using Eq. 3a and 3b. Weissberg's equation (Eq. 3a) was found to predict reasonable values, while the empirical equation given by Boles et al. (1970) predicted erroneous values and hence the latter was not considered further. The vis-

Table 3—Range of parameters obtained at different die temperatures, screw speed and product moisture content in the study

Die diam (cm)	Reynolds No. ($\times 10^3$)	Friction factor	$\Delta P_{VIS}/\Delta P_{ENT}$[a] %	$\Delta P_{ENT}/\Delta P_{TOT}$[b] %	γ_w[c] (s^{-1})	τ_w[d] (kPa)	η[e] (Pa-s)
0.3175	1.77–20.67	1463.4–17452.8	2.05– 6.33	10.35–22.18	566–1125	48.2–218	48– 310
0.4763	0.64– 9.87	3000–56433.6	3.27–20.21	4.59–20.13	201– 457	40.7–192.5	92– 675
0.6350	0.29– 3.60	9210–136281	4.96–40.28	2.53–15.24	103– 190	27 –186.5	180–1250
0.9525	0.09– 0.29	54885–220285	2.68–35.51	6.40–37.09	27– 55	24.3–152.25	1150–3500

[a] Ratio of viscous to entrance pressure drop
[b] Ratio of entrance to total pressure dropt
[c] Wall shear rate
[d] Wall shear stress
[e] Apparent viscosity

cous component of the entrance pressure drop (from Eq. 3a) was found to increase with increasing shear rate, decreasing moisture content and decreasing temperature.

In the case of inelastic or purely viscous fluids, the entrance length required to attain fully developed flow is given by (Darby, 1976)

$$L_e = C D_d \, Re \qquad (10)$$

where D_d is the tube diameter, L_e is the length required to achieve fully developed flow from the entrance to the tube and C is a constant that ranges from 0.02 to 0.06. For polymer melts, Han and Charles (1970) and Bagley et al. (1963) indicated that the L_e/D_d ratio could be as high as 15 to 20. The criterion for achieving fully developed flow in viscoelastic fluids is not fully understood. The generally accepted conventional notion of constant pressure gradient in the tube for achieving fully developed flow in Newtonian fluids may not be sufficient for viscoelastic fluids (Han, 1971). Since there has been no prior work done on fully developed flow with food doughs, we selected four dies: two with L/D_d greater than 20, one with L/D_d between 15 and 20 and one with L/D_d less than 15. As die diameter increased, thereby decreasing L/D_d and D_B/D_d, the spread in the data increased (Fig. 6 and 7). As entrance pressure drop was calculated by linear extrapolation of pressure readings in the die, any deviation from constant pressure profile would lead to spread in the data.

Sylvester and Rosen (1970a, b) proposed the following equation for an inelastic power-law fluid:

$$\frac{\Delta P_{ENT}}{\dfrac{\rho V^2}{2}} = f\,(Re, \alpha, n) \qquad (11)$$

where $\alpha = D_B/D_d$. They also recognized the dependence of the dimensionless pressure drop on a additional dimensionless number (not specifically identified) characterizing elasticity for viscoelastic fluids. For creeping flow, Eq. 11 can be written
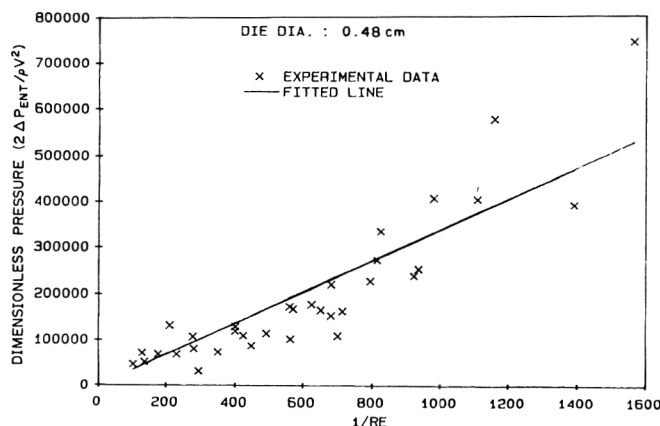
Fig. 6.—Dimensionless entrance pressure drop and Reynolds number (Re) for die diameter of 0.48 cm.
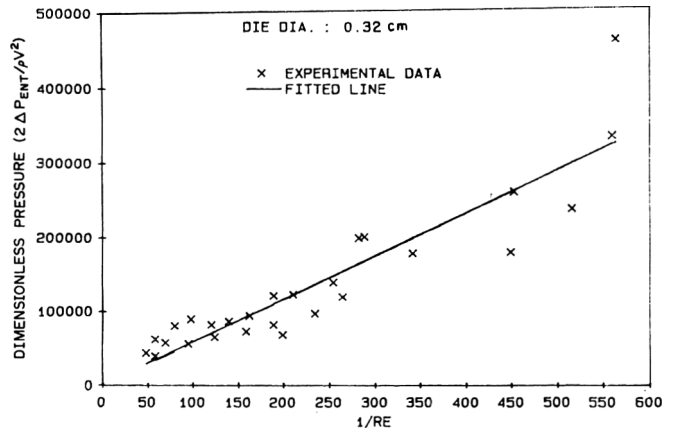
Fig. 7.—Dimensionless entrance pressure drop and Reynolds number (Pe) for die diameter of 0.32 cm.

Table 4—Evaluation of constant K in equation $2\Delta P_{ENT}/\rho V^2 = K/Re$

α	K	R^2
6.00	585	0.94
4.00	339	0.91
3.00	143	0.80
2.00	153	0.82

as (Sylvester and Rosen, 1970b),

$$\frac{\Delta P_{ENT}}{\rho V^2/2} = \frac{K}{Re} \qquad (12)$$

where K is a function of α, n (for power-law fluids) and elasticity (for viscoelastic fluids). Equation 12 was used to represent data obtained in this experiment. K was a function of α as expected (Table 4). Decreasing α causes a decrease in K values except for the die with a diameter of 0.9525 cm (largest diameter die). The reason for an inconsistent value of K for the largest diameter die is believed to be due to the flow having not fully developed throughout the entire length of the die. As α approached unity ΔP_{ENT} approached zero and, from Eq. 10, K would also approach zero.

The amount of scatter in the data increased with decreasing Re (or increasing 1/Re) (Fig. 6 and 7). The major cause for the increasing scatter could be experimental error in calculating the velocity. It may be recalled that flow rate (and hence velocity) was calculated from dry mass flow rate. Thus, at low Re, a small error in velocity is significant and is further amplified due to a quadratic velocity term in the denominator of the ordinate and linear term in the numerator of the abscissa.

A plot of the friction factor versus Reynolds number is shown in Fig. 8. Friction factor (f) was calculated using the equation

$$f = \frac{\left(-\dfrac{dP}{dx}\right)\dfrac{R}{2}}{\dfrac{\rho V^2}{2}} \qquad (13)$$
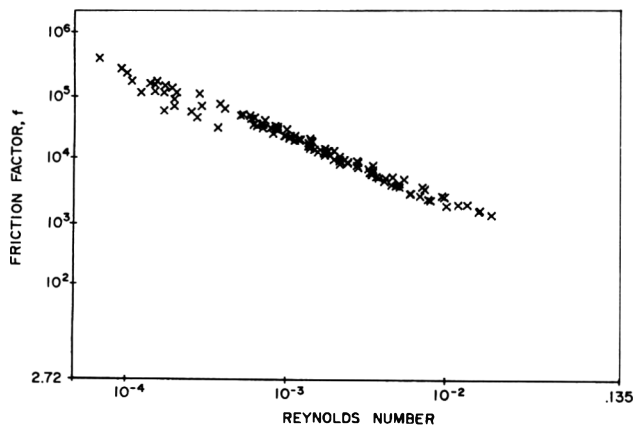
Fig. 8.—Friction factor and Reynolds number (Re) data for different dies.

Results indicate that the friction factor and Reynolds number can be correlated by the expression:

$$f = \frac{27.3}{Re} \qquad (14)$$

with a coefficient of determination of 0.98. This expression is similar to:

$$f = \frac{16}{Re'} \qquad (15)$$

where Re' is the generalized Reynolds number and can be related to Re by (Wilkinson, 1960):

$$Re' = \frac{(3n + 1)}{4n} Re \qquad (16)$$

Agreement to Eq. 15 has been reported by Sylvester and Rosen (1970b) for dilute polymer solutions and by Han (1971) for polymer melts. The good correlation of the data (Fig. 8) in obtaining Eq. 15 (or Eq. 14) indicates that flow in the fully developed region of the die is independent of fluid elasticity.

The data points can be observed to exhibit very little scatter except at low Re (Fig. 8). The data points obtained for the four dies fell along various ranges of Re (Table 3) with the values at low Re being obtained for the largest diameter die (diameter = 0.95 cm). The large scatter for the largest diameter die clearly indicated a marked departure from fully developed flow. The L/D_d ratios for the transducers in the four dies are shown in Table 2. The die with a diameter of 0.64 cm has predominatly a fully developed flow (Fig. 8) and hence it can be concluded that for corn melt, within the experimental conditions studied, fully developed flow was achieved at L/D_d ratio or 9. This value is very much·larger than that for Newtonian fluids which have a value close to 0.5. Whereas in polymer melts wall slip may be a problem in achieving fully developed flow, this should not be the case for starchy foods because they are more adhesive by nature in the molten state. The good satisfaction of the relation f = 16/Re' (Fig. 8), which is obtained from an assumption of no-slip wall boundary condition for a laminar fully developed flow, clearly indicates the absence of slip at the walls.

CONCLUSIONS

THE ENTRANCE PRESSURE drop varied directly with the barrel-to-die diameter ratio. Entrance pressure ranged from 2.5 to 37% of the total pressure drop. The entrance pressure drop was believed to consist of viscous and elastic components only due to the low Reynolds number flow. Viscous pressure drop, evaluated using Weissberg's analysis, accounted for 2 to 40% of the entrance pressure drop. The entrance pressure drop and its viscous component increased with increasing shear rate, decreasing moisture content and decreasing temperature. The friction factor varied with the generalized Reynolds number Re' following the relation f = 16/Re'. Fully developed flow within the die was achieved at an L/D_d of 9 for the experimental conditions studied.

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Optimization of Carrot Dehydration Process using Response Surface Methodology

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——————————— ABSTRACT ———————————

The effect of temperature and time in a fluidized bed dryer, the concentration of biopolymer treatment and blanching time on quality attributes of dehydrated carrots was evaluated by response surface methodology. Optimization maximized rehydration ratio and minimized bulk density and carotene loss. Based on surface responses and contour plots, optimum conditions were: drying temperature of 150°C, exposure time of 12.5 minutes, biopolymer concentration of 1.40%, and blanching time of 12 minutes. Experimentally determined values for rehydration ratio, bulk density, and carotene loss in product processed under the optimum conditions were very close to the predicted values of 9.5, 0.05, and 15.7%, respectively.

INTRODUCTION

CARROTS, one of the most important vegetable crops, is a good dietary source of vitamin A. Carrots have been preserved by canning, freezing and dehydration. Among these processes, dehydration offers many advantages, such as reduced weight, inexpensive packaging, dry shelf stability and negligible deterioration in quality due to enzymic changes.

Although several processes are available for carrot dehydration, these processes result in considerable loss of carotene during processing and poor product rehydration. Maximum retention of carotene is necessary to preserve the attractive appearance and dietary value of the product. Carotene is a precursor of vitamin A. Arya et al. (1982) reported 28.7% loss in total carotene during the process of dehydration. According to Baloch et al. (1977), carotene oxidation in dehydrated carrots followed a first order reaction up to 60% destruction. Degradation of carotene has been reported to be related to overall storage life of the dehydrated carrots. Tomkins et al. (1944) reported that off flavor became noticeable when about 20–45% of the total carotenoids had been destroyed. They also reported that considerable shrinkage occurred during the de-

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hydration process due to cell collapse following the loss of water, which leads to poor rehydration. Hass et al. (1974) found that blanching improved the rehydration properties of carrots and suggested that certain additives can improve the rehydration ratio of the carrots. Horn and Sterling (1982) and Mazza (1983) reported rehydration ratio in the range of 5–7 for pretreated dehydrated carrots. Grishin and Marinpolskaya (1973) studied the kinetics of carrot dehydration and recommended a drying temperature of 160°C for carrot dehydration in a fluidized bed drier. Darmanyan and Grishin (1983) studied the effect of different drying conditions on the quality of dried carrots in a fluidized bed drier and suggested a drying temperature of 100°C for 55 minutes.

Response Surface Methodology (RSM) has been successfully applied in optimizing food processing operations by several investigators (Smith et al. 1977; Lah et al. 1980; Floros and Chinnan 1987), but so far it has not been used in optimizing a dehydration process.

An expanding interest currently exists for dehydrated carrots in the domestic and world markets. This investigation was undertaken to develop and optimize a process for carrot dehydration which would result in superior color retention, maximum rehydration ratio and maximum puffing.

MATERIALS & METHODS

Experimental design

Response surface methodology was employed to optimize the High Temperature Fluidized Bed (HTFB) drying process and a three level four factor design was adopted (Box and Behnken, 1960). The basic theoretical and fundamental aspects of RSM have been discussed elsewhere (Davies, 1954; Cochran and Cox, 1957; Johnson and Leone, 1964; Hill and Hunter, 1966; Myers, 1971; Box et al. 1978; Thompson, 1982). It was assumed that three mathematical functions, f_k ($k = 1$, 2, 3), exist for each response variable Y_k (Rehydration Ratio, RR; Carotene Loss, CL; Bulk Density, BD) in terms of four independent processing factors.

$$Y_k = f_k (T, t, c, b) \qquad (1)$$

Where: T = drying temperature in HTFB; t = exposure time in HTFB; c = concentration of biopolymers; and b = blanching time.

Table 1 — Coding of levels of independent variables used in developing experimental data for optimization of the process for dehydration of carrot dice in a high temperature fluidized bed (HTFB) drier.

Independent variables	Symbols			Levels		
	Uncoded	Coded	Transformed	Uncoded	Coded	Transformed
Drying temp. in HTFB, (°C)	T	X1	z1	155	1	2.178
				145	0	0.000
				135	−1	0.368
Exposure time in HTFB, (min)	t	X2	z2	15	1	2.178
				10	0	0.000
				5	−1	0.368
Concentration of biopolymers, (%)	c	X3	z3	1.5	1	2.178
				1.0	0	0.000
				0.5	−1	0.368
Blanching time, (min)	b	X4	z4	15	1	2.178
				10	0	0.000
				5	−1	0.368

Table 2—Experimental data for the rehydration ratio (RR), bulk density (BD) and carotene loss (CL) under different treatment conditions (coded, refer to Table I for coding) of drying temperature in the fluidized bed, drying time, blanch time and concentration of biopolymer in the blanch solution

Treatment[a] no.	Drying temp T	Expos. time t	Conc c	Blanc time b	RR	BD	CL %
1	+1	+1	0	0	8.55	0.131	25.81
2	+1	−1	0	0	7.90	0.196	15.71
3	−1	+1	0	0	7.58	0.170	17.04
4	−1	−1	0	0	6.82	0.294	17.27
5	0	0	+1	+1	8.69	0.135	13.01
6	0	0	+1	−1	8.37	0.139	24.05
7	0	0	−1	+1	7.83	0.137	21.31
8	0	0	−1	−1	8.28	0.143	30.89
9	0	0	0	0	8.50	0.135	20.71
10	+1	0	0	+1	8.76	0.145	29.70
11	+1	0	0	−1	8.40	0.145	26.23
12	−1	0	0	+1	7.83	0.243	12.78
13	−1	0	0	−1	7.51	0.260	12.65
14	0	+1	+1	0	8.33	0.117	14.87
15	0	+1	−1	0	8.06	0.137	24.56
16	0	−1	+1	0	8.09	0.229	13.22
17	0	−1	−1	0	7.77	0.242	23.97
18	0	0	0	0	8.87	0.141	16.48
19	+1	0	+1	0	8.36	0.124	16.91
20	+1	0	−1	0	8.32	0.136	29.91
21	−1	0	+1	0	7.35	0.207	17.84
22	−1	0	−1	0	7.27	0.245	25.83
23	0	+1	0	+1	8.98	0.119	20.34
24	0	+1	0	−1	7.77	0.128	24.74
25	0	−1	0	+1	7.91	0.241	19.05
26	0	−1	0	−1	7.73	0.236	25.59
27	0	0	0	0	8.64	0.139	20.12

[a] Experimental runs were performed in random order.

Table 3—Values of the regression coefficients of the second order polynomials[a] representing the relationship between the indicated response variables (Y_k) and the independent variables of drying time in the fluidized bed (i or j = 1), exposure time (i or j = 2), concentration of biopolymers in the blanch solution (i or j = 3), and blanch time (i or j = 4)

Coefficients	Rehydration ratio RR k = 1	Bulk density BD k = 2	Carotene loss CL k = 3
bk_0	4.50	0.50	33.79
bk_1	2.27	−0.22	3.12
bk_2	2.07	−0.22	1.04
bk_3	0.71	−0.02	−12.41
bk_4	0.85	−0.04	−13.12
bk_{11}	−0.62	0.05	−1.10
bk_{21}	0.05	0.01	1.46
bk_{22}	−0.57	0.05	−0.62
bk_{31}	−0.04	0.00	−1.06
bk_{32}	0.02	0.00	−0.26
bk_{33}	−0.23	0.00	3.51
bk_{41}	0.05	0.00	2.26
bk_{42}	−0.27	0.00	0.31
bk_{43}	0.11	0.00	−0.68
bk_{44}	−0.24	0.00	2.89

$$^aY_k = B_{ko} + \sum_{i=1}^{4} B_{ki}x_i + \sum_{i=1}^{4} B_{kii}x_i^2 + \sum_{i=1}^{3}\sum_{j=i+1}^{4} B_{kij}x_ix_j$$

To approximate the function f_k, second degree polynomial equations were used:

$$Y_k = B_{ko} + \sum_{i=1}^{4} B_{ki}x_i + \sum_{i=1}^{4} B_{kii}x_i^2 + \sum_{i=1}^{3}\sum_{j=i+1}^{4} B_{kij}x_ix_j \qquad (2)$$

Where B_{ko}, B_{ki}, B_{kii}, and B_{kij} are constant coefficients and x_i the coded independent variables, linearly related to T, t, c, and b.

Statistical analysis

Statistical Analysis System (SAS, 1985) was used to fit the second order polynomial equation to the experimental data shown in Table 2.

Table 4—Analysis of variance table showing the effect of treatment variables as a linear term, quadratic term and interactions (cross product) on the response variables, rehydration ratio, bulk density, and carotene loss

Source	DF	Sum of Squares		
		Rehydration ratio RR	Bulk density BD	Carotene loss CL
Model	14	6.450**	0.072**	595.924*
Linear	4	2.307**	0.042**	359.958**
Quadratic	4	3.172**	0.029**	146.539
Cross Product	6	0.971	0.001	89.427
Residual	12	1.008	0.003	191.255
Lack of fit	10	1.018	0.003	180.759
Pure error	2	0.070	0.000	10.496
% Variability explained (R^2)		85.57	96.03	75.70

* Significant at 5% level; ** Significant at 1% level

Table 5—Analysis of variance table showing the significance of the effect of the processing variables on each of the indicated reponse variables

Process variables	DF	Sum of squares		
		Rehydration ratio, RR	Bulk density BD	Carotene loss CL
Drying temp (T)	5	4.062***	0.033***	217.822*
Exposure time (t)	5	2.618***	0.040***	35.827
Conc of biopolymers (c)	5	0.550	0.001	289.542**
Blanching time (b)	5	1.249	0.001	144.587

Significant at *10% level; **5% level; ***1% level

Table 6—Predicted levels of process variables yielding optimum response of rehydration ratio (RR), bulk density (BD) and carotene loss (CR)

Process variables		Levels for optimum response			Graphical optimum
		RR	BD	CL	
Drying temp	(T)	0.64	0.58	1.47	0.50
Exposure time	(t)	0.44	0.65	1.70	0.50
Conc of biopolymers	(c)	0.61	0.75	0.99	0.80
Blanching time	(b)	0.44	0.49	−0.51	0.40
Morphology		Max	Min	S.P.[a]	

[a] S.P. = Saddle point

Carrot processing

The carrots (*Daucus carota*) of a hybrid cultivar were purchased locally. After washing, the top and narrow bottom part were removed before dicing to 3/8 × 3/8 × 3/8 inch in a dicer (Dito Dean model TR-22). One kg carrot dices were blanched in 3 kg water containing a mixture of biopolymers (maltodextrins, pectins and others) at concentrations shown in Table 1, for each treatment. After blanching, the carrot dices were dried in HTFB drier at temperature and exposure time given in Table 1. The drying in HTFB drier was done in a batch system using 250 g blanched carrots at one time. After removing the carrots from HTFB, dehydration was completed in a tunnel drier at 70°C and 4 m/sec air velocity to a final moisture level of 3–5%. The drying temperature (T), exposure time in HTFB (t) and blanching time (b) were maintained within ±1°C, ±5 sec and ±5 sec, respectively.

Analysis

The dried carrots were stored in opaque, air tight plastic bottles until the analyses were performed. Rehydration Ratio was measured as the total mass of rehydrated carrots per unit weight of dry matter after rehydration. It was calculated after boiling 2g dried carrots in 100 mL distilled water for 30 min. Bulk density is an indication of puffing. It was determined as weight per volume basis (Kim and Toledo, 1987). The total carotenoids were determined by the AOAC (1984) method. The carotene was extracted with acetone-hexane mixture (40:60), and the absorbance was measured at 449 nm. The total carotenoids were calculated as β-carotene and are reported on wet basis in rehydrated carrots by using the following expression:

$$E_{1cm}^{1\%} \times 2500$$
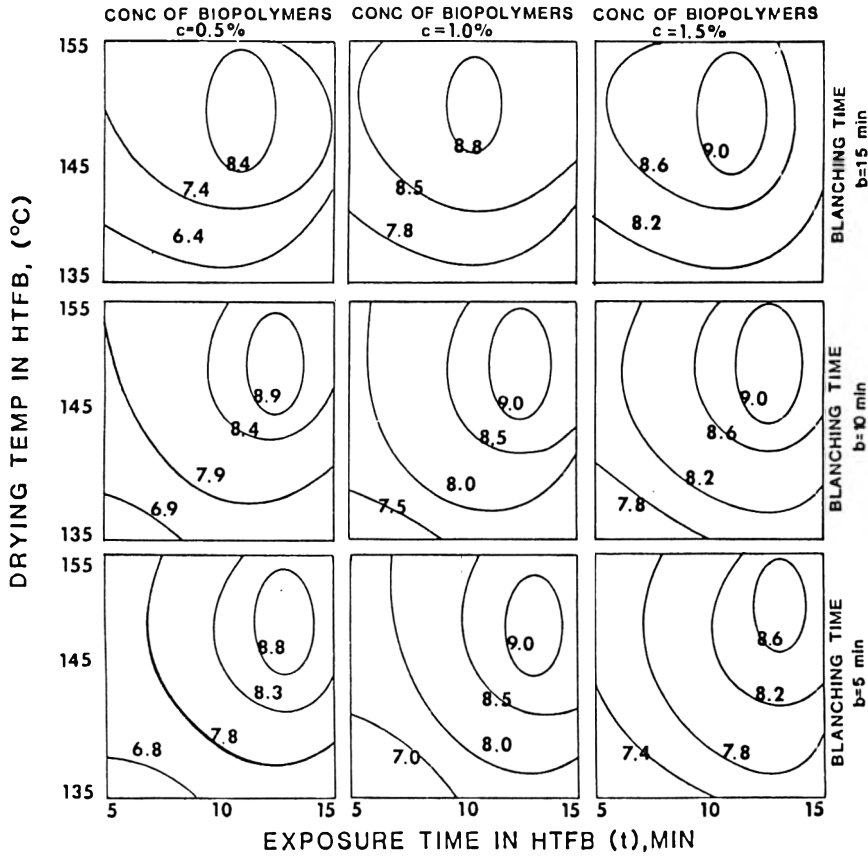
Fig. 1 — Contour plots of rehydration ratio at different levels of independent variables during dehydration in high temperature fluidized bed (HTFB).
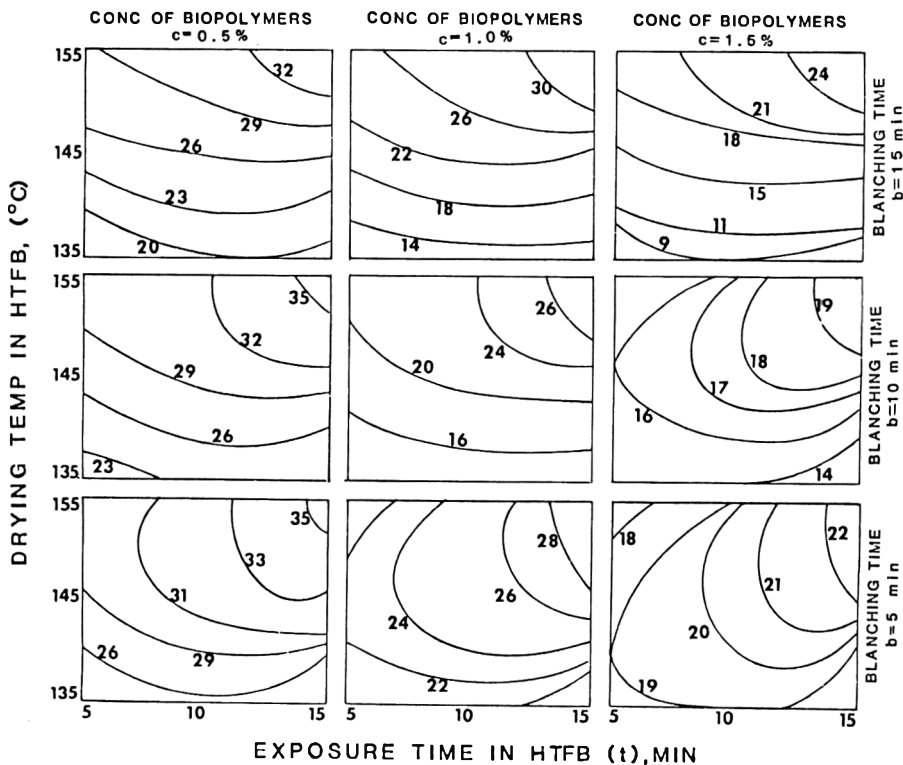
Fig. 2 — Contour plots of carotene loss (%) at different levels of the independent variables during dehydration in a high temperature fluidized bed (HTFB).
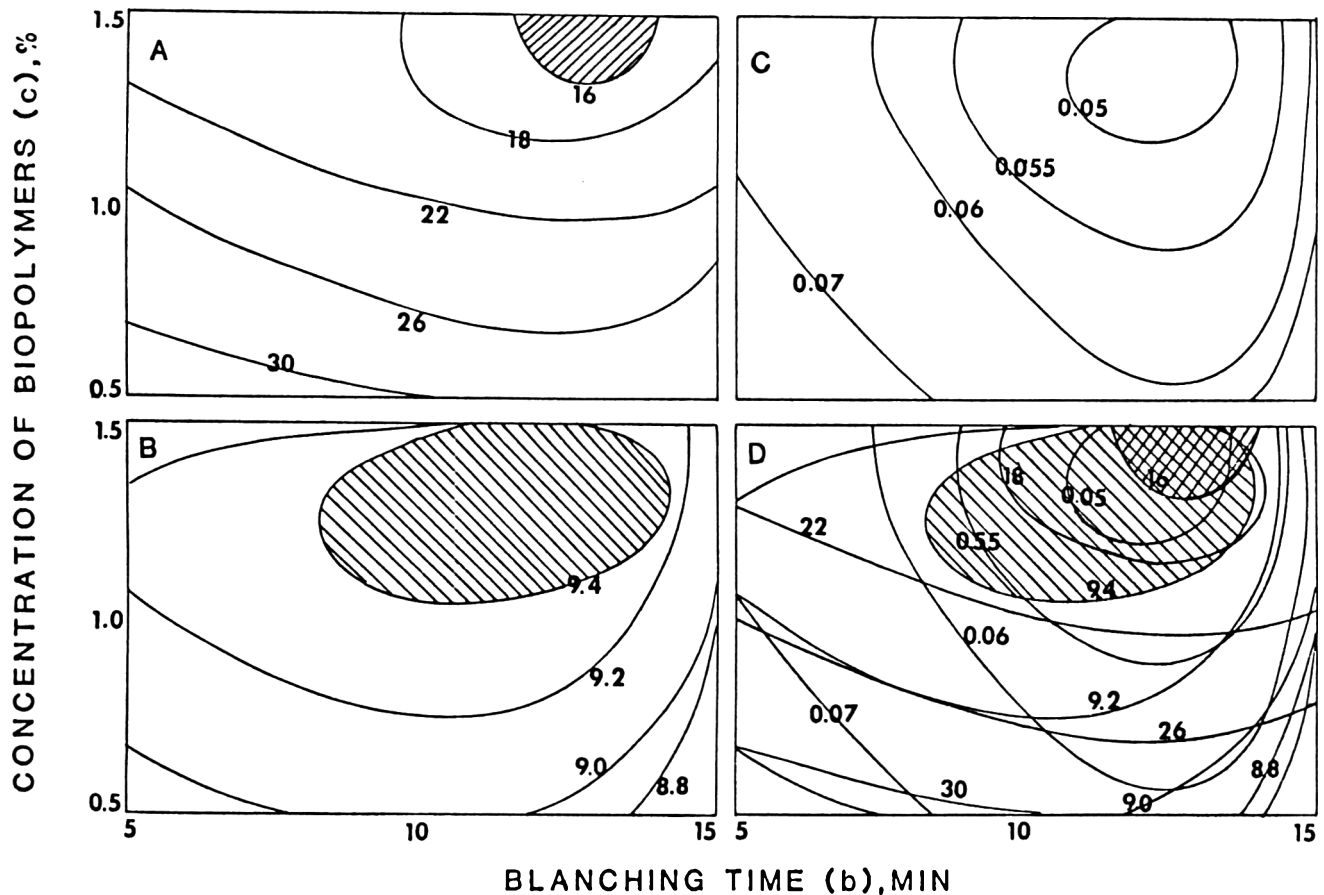
Fig. 3 — Contour plots at T = 150°C, t = 12.5 min for (A) carotene loss, CL; (B) rehydration ratio, RR; and (C) bulk density, BD. Shaded area indicated CL<16% in (A) and RR>9.4 in (B). (D) is superimposed plots of (A), (B) and (C).

The carotene loss was calculated on the basis of raw carrots by a formula suggested by Murphy et al. (1975) as given below:

$$\% \text{ Carotene loss} = \left[1 - \frac{\text{mg/g Carotene in rehydrated sample} \times \text{RR}}{\text{mg/g Carotene in raw carrot} \times \text{dehydration ratio}} \right] \times 100$$

Dehydration ratio is the ratio of weights of raw carrots before dehydration and of the dried product. The calculated carotene loss includes losses during blanching, dehydration and rehydration.

Verification

After collecting experimental data and determining the optimum processing conditions using RSM, the optimum responses were verified by pilot plant processing under the optimum conditions. The response RR, BD and CL at the optimum processing conditions were compared to values predicted by the model.

RESULTS & DISCUSSION

TO OBTAIN the proper experimental region, the transformation of x_i into z_i was made by the following equation:

$$Z_i = e^{x_i} \qquad (3)$$

The regression coefficients (B_{ki}) are presented in Table 3. The analysis of variance for the three response variables (Table 4), indicates that the model developed for rehydration ratio, bulk density and carotene loss appeared to be very adequate, possessing no significant lack of fit, and with satisfactory values of R^2.

From the statistical analysis (Table 5), the drying temperature (T) was shown to be the most important factor among the four factors affecting the response variables. Exposure time (t) in HTFB had a significant effect on rehydration ratio and bulk

density but it did not affect the carotene loss significantly. Concentration of biopolymers (c) was a significant factor for carotene loss only. Arya et al. (1979), Baloch et al. (1977) and Sullivan et al. (1981) also reported that drying temperature and exposure time during the drying process affect the color, rehydration and shape of the product. At low drying temperatures, puffing of carrots did not take place and rehydration was poor. At very high drying temperatures (above 150°C) and long exposure time in fluidized bed, the product rehydrated poorly and non-enzymic browning occurred. Blanching caused sterioisomerization of major carotenoids and also promoted disintegration of chromoplasts enhancing leaching of carotene (Ogunlesi and Lee, 1978). Feinberg et al. (1964) reported that blanching before dehydration improved the stability of carotenoids during dehydration and storage. Baloch et al. (1981), Speck et al. (1977), Arya et al. (1979) and Mazza (1983) found that some pretreatments such as dipping in solution of sodium bisulfite, sodium chloride and starch improved the color and reconstitution of the dried carrots.

Using the procedure described by Myers (1971) and Draper (1963), the stationary points (Table 6) were located in four dimensional space. The first series of plots (9 for each response, Fig. 1 and 2) was generated by holding concentration of biopolymers and blanching time constant. These variables had the least influence on the response variables, RR and CL (Table 5). BD was found to be affected only slightly by b and c (Table 5). Minimum values of BD were obtained at a drying temperature in the HTFB of 150°C and an exposure time of 10 min or greater (Coded T of 0 and coded t of 0 and +1 in Table 2).

Since the optimum response for each variable did not fall in exactly the same region in the four dimensional space formed by the processing variables, constraints were set such that the
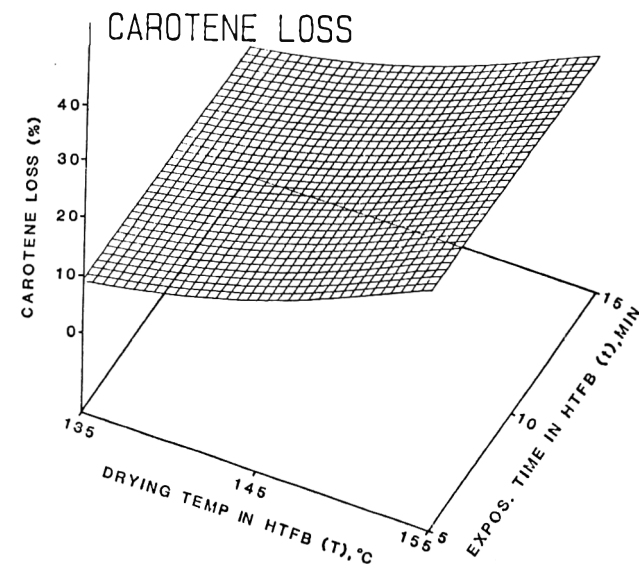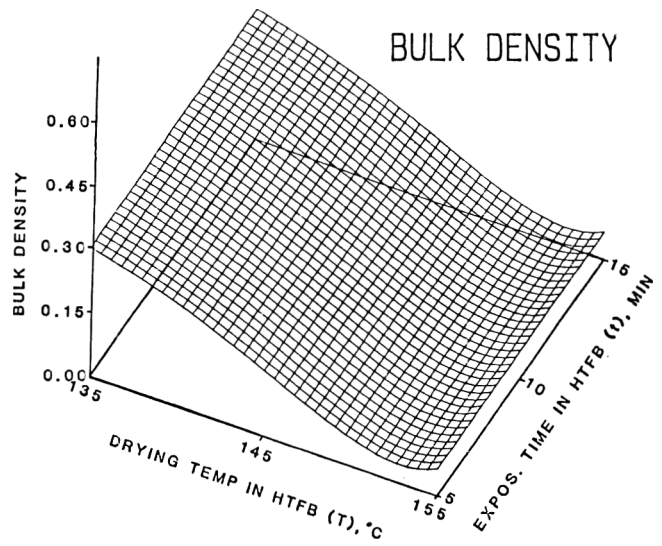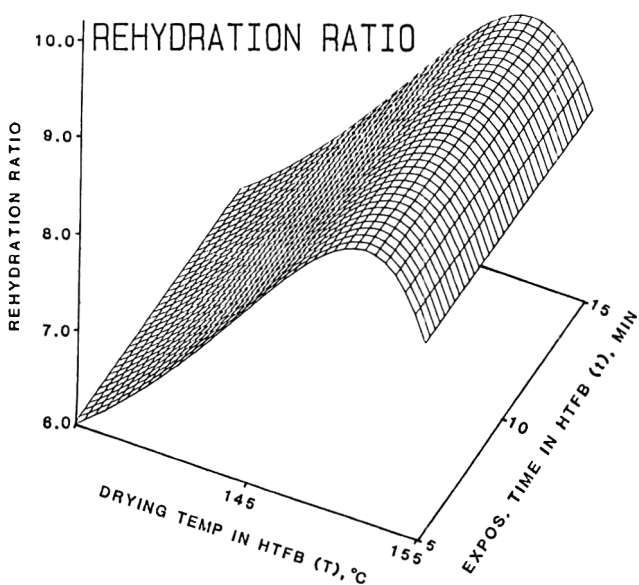
Fig. 4 — Response surfaces of rehydration ratio, bulk density, and carotene loss at constant biopolymer concentration of 1.40% and 12 min. blanching time.

Table 7 — Predicted and experimental values of the responses at optimum conditions

Response	Predicted value	Experimental value[a]	
		Mean	Range
Rehydration ratio, RR	9.51	9.29	8.93 - 9.73
Bulk density, BD	0.05	0.110	0.103 - 0.116
Carotene loss, CL	15.70	17.05	15.90 - 18.39

[a] Results are of five replications. Optimum conditions are: drying temperature in the high temperature fluidized bed (HTFB) of 150°C, exposure time in the HTFB of 12.5 min, biopolymer concentration in the blanch solution of 1.4% and blanch time of 12 min.

selected temperature and drying time would be optimum for the most important product attribute and close to the optimum for the others. RR, the most important quality attribute, was maximized, BD was set at 0.07 and CL was set at 16%. A BD of 0.07 is much lower than any reported for an explosion-puffed product (Sullivan et al., 1981), and a CL of 16% did not impair the appearance of the dry or rehydrated product. These constraints were met in the region where the drying temperature was 150°C and the drying time was 12.5 min. Contour plots for each of the response variables at a T of 150°C and a t of 12.5 min (Fig. 3) show dependence on biopolymer concentration(c) and blanching time(b). Again, the optimum for each of the variables did not fall in the same region in the two dimensional space formed by c and b. The selected optimum conditions for the process will be based on the region

which will satisfy the stated constraints. Superimposing the individual contour plots for the response variables, CL (Fig. 3A), RR (Fig. 3B) and BD (Fig. 3C), results in the identification of a region which satisfied all constraints as shown in Fig. 3D. The optimum region for the optimum response was in the vicinity of a biopolymer concentration of 1.4% and a blanching time of 12 min. Values of the response variables within this optimum region are (Table 7) CL = 15.7%, BD = 0.05 and RR = 9.51). Computer generated response surfaces, were obtained using predictive models of RR, CL and BD (Table 3) and are shown in Fig. 4.

Adequacy of the model equations for predicting optimum response values was tested in the pilot plant using a drying temperature of 150°C, exposure time in HTFB (t) of 12.5 min, concentration of biopolymers (c) of 1.40% and blanching time (b) of 12 min. This set of conditions was determined to be optimum by the RSM optimization procedure. The predicted and experimental values for RR, BD and CL at these conditions are given in Table 7. The experimental values were very close to the predicted values for RR and CL but not for BD. Thus the model is a good predictor of rehydration ratio and carotene loss, but is a poor predictor for bulk density.

CONCLUSIONS

RSM is a powerful tool for optimization of a process involving several processing conditions and several response variables.

The results shown for high temperature fluidized bed dehydration of carrot dice identified the optimum processing parameters which were a drying temperature of 150°C for 12.5 min, a blanching time of 12.5 min in a solution containing 1.4% biopolymer. The model equation for the response variables predicted values under the identified optimum conditions which were experimentally verified to be in general agreement with the model.

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An Experimental Design for Determination of D-values Describing Inactivation Kinetics of Bacterial Spores; Design Parameters Selected Using Computer Simulation

JACK HACHIGIAN

────────── ABSTRACT ──────────

An improved experimental design is proposed for studies of bacterial extinction. The new design may be used for either inoculated pack or model system studies. The design uses variable spore loads, and judiciously spaced time points (or doses) with a variable number of replicates over a selected range. A computer simulation of the process simplifies parameter selection in an economic way. The improved design results in more useable data points over an increased range leading to more accurate estimates of the D-value with the same level of experimental effort used in current experiments.

INTRODUCTION

STUDIES in food science are frequently concerned with the death, or inactivation kinetics of microbiological contaminants such as *C. botulinum* or PA3679 (Hachigian, 1979). Inoculated pack studies, or model system studies are intended to result in data necessary for the determination of estimates for the death rate constant or its reciprocal "D" (Stumbo, 1966, 1973; Pflug and Holcomb, 1983). These experiments are expensive, time consuming, and not easily reproduced. Data from these studies have been analyzed using a method proposed by Stumbo (1948) and Stumbo et. al. (1950). The use of the Stumbo method of analysis was extended to radiosterilization data by Schmidt and Nank (1960).

Inoculated pack studies, where replicates are sterile or have at least one remaining spore are known as quantal response experiments sometimes referred to as "most probable number" (MPN) methods. They differ significantly from direct enumeration experiments where counts are possible.

Other works [Hachigian, 1979, 1982] provide additional means of understanding inoculated pack studies whether the sterilizing agent is heat or radiation. Computer simulations found in these papers are designed to support experimenters by providing an additional tool with which to analyze such studies. The simulations result in reproducible data that are both quick and inexpensive to obtain. In both papers the underlying model of bacterial death is based on the statistical nature of the processes involved, and both followed the classical experimental design for such studies.

The purpose of this paper is to provide an improved experimental design for inoculated pack and model system studies which results in more useable data for the determination of a D-value.

THE CLASSICAL EXPERIMENTAL MODEL

TWO MAJOR STATISTICAL PROCEDURES are in current use for estimating bacterial densities: the colony count method (direct enumeration), and the quantal-response method (Fin-

ney, 1964). Quantal-response experiments are those in which the remaining number of replicates containing viable microorganisms are counted from among the N inoculated replicates that are subjected to a lethal environment. This type of experimental design has been used for example, in determining the death kinetics of *C. botulinum* spores (e.g., Anellis et. al., 1967, and Hachigian, 1979). Quantal-response type studies, whether a model system or inoculated pack, are conducted over a number of preselected treatment levels chosen as a result of past experience. The range is typically from a low treatment level, where all replicates are viable, to a higher treatment level where all replicates are nonviable.

In the classical design N cans are prepared for each level, each can containing an average number of spores, say **LAMBDA**. The preparation of the inoculate is assumed to be in accordance with good laboratory technique (that is, the inoculate was distributed at random without clumping, and therefore following a Poisson distribution with mean **LAMBDA** in a small unit of volume, an aliquot). The N inoculated replicates are exposed to a treatment level equal to **T**. The experiment is conducted at several treatment levels T_1, T_2, ...,T_S.

The N cans at each treatment level are then tested to determine how many of the cans contain surviving bacteria; π_1 — the proportion of cans with surviving bacteria at level T — is recorded. Assuming a logarithmic order of death, Mather (1949), Stumbo (1966, 1973), and Schmidt (1963), used the proportion of cans (π_T) to calculate estimates of extinction rates of the organism being investigated (where π_T = k/N, k is the number of non-sterile cans at T, and N is the number of replicates). Epstein (1967), and Hachigian (1971) provided theoretical justification to the Mather approach.

Another possibility exists, included for completeness. Following exposure each of the N cans at each treatment level is serially diluted so as to increase the number of data points on which to base the analysis. The statistical analysis of this method is described by Koch and Tolley, (1975). Their method may have some utility in food science, however in hazardous situations serial dilution may not be prudent, as when dealing with *C. botulinum*. The analysis is also cumbersome for common use.

When only the ratios k/N are available at each T, Cornell and Speckman (1967) indicated that a weighted least squares analysis was likely to result in incorrect estimates of the death rate constant $B = 2.3026/D$. These incorrect results were most likely to occur when the number of replicates at each level was small, or when either all or none of the cans were sterile. Conditions such as these occur at the extremes of the range at which the experiment is conducted, and occur frequently in food sterilization experiments. Consequently, for accurate determination of D-values it is important to guard against these occurrences. Further, compounding these concerns, it is commonly understood (Olkin et al., 1980) that the difference between two competing models describing death kinetics is best observed at the extremes of the range over which the experi-

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ment is conducted—precisely where the incorrect results are most likely to occur.

Data from Anellis et al. (1967) (Table 1.) on radiation sterilization using Co[60] illustrates these points and the need for more carefully designed dose spacings, spore loads, and replicate numbers so as to obtain sufficient data with which to conduct statistical analyses.

Apart from the control at 0.0 Mrads the experiment was conducted at nine different doses. Only *two* of the nine resulted in data points which can be used for determining a "D" value. Those at 1.5 and 2.0 Mrads. Data obtained at 1.0 Mrad (all viable), and that from doses of 2.5, 3.5, 4.0, 4.5 Mrads (all nonviable) do not provide useful statistical information. The reason is that the ratio k/N is either equal to 1 or zero at these extremes. Data at 0.5 and 3.0 Mrads should be treated as outliers for the purposes of calculating D. At these doses we observe directional changes in the data which are in part, a result of the governing probability mechanism of the experiment, and in part due to the possibilities mentioned before, namely activation, injury, etc. (Hachigian, 1979; Cerf, 1977).

These observations in quantal response (MPN) type experiments lead to the recognition that data of greatest interest and utility are frequently masked at the extremes of the dose range. It is desirous, therefore, to have a method of obtaining data at these extremes. The experimental design proposed below is intended to achieve as its goal a larger number of valid data points while insuring that valid data is obtained over a larger range of treatment levels than in constant spore load designs.

PROPOSED EXPERIMENTAL DESIGN

TO PROVIDE a consistent narrative hereafter radiosterilization of an inoculated pack is used as a descriptive model. The methods however, apply whatever the sterilizing conditions.

In the past selection of replicate numbers, doses at which to place those replicates, and the spore load per can was largely based on past experience. Since these experiments were typically conducted using fixed spore loads the other parameters were chosen as a matter of economics and convenience.

In the conduct of an actual experiment doses ideally should be spaced over as large a range as possible without causing all replicates to remain viable at one end or all nonviable at the other. This ideal necessitates different spore loadings, different replicate numbers, and possibly varied dose spacings.

To approximate this ideal, a computer simulation of a fictitious experiment is performed to assist in the selection of the experimental parameters. In this fictitious experiment an *array* of cans [N × (SD + 1)] is exposed to each dose T rather than the customary N replicates at each dose. The array is formed by choosing the number of replicates N, a spore load **LAMBDA**, a dilution factor **DF**, and the number of serial dilutions **SD**.

Dilutions are carried out by the computer in accordance with the chosen dilution factor, **DF**. For purposes of illustration let us suppose that the dilution factor is 10. Each can in the first row of the array (corresponding to zero dilutions, **SD = 0**) receives a spore load equal to the stock **LAMBDA** on average

per aliquot. Each replicate in the second row (**SD = 1**) receives 1/10th **LAMBDA** on average per aliquot. And, in general each replicate in the jth row receives **LAMBDA** × 1/10^j spores per aliquot, on average. This array is "prepared" and "exposed" at increasing doses with the results printed out for analysis and observation.

The parameters of the proposed design will be recursively selected from the results of the simulation. The selection of these parameters is best demonstrated by tabulating and analyzing the results of the computer "run". An example of such a tabulation is found in Table 2. This run was conducted using 10 cans with inoculant count equal to 2000/can, 10 cans with 200/can, etc., being exposed at various doses. At each dose there are *four* ratios of the proportion of cans with surviving bacteria, rather than one. The stock spore load is chosen by the user of the simulation. In this example 2000/can was chosen for purposes of illustration only.

For a given constant spore load (2000, 200, or 20) the number of useful data points is limited (those printed in bold face in Table 2). The first column in Table 2 has at most five points; the second column, six useful points; and the third, five. Most experiments in food science are conducted using constant spore loads and consequently have the same limitations since the dose range from which useful data is obtained is limited and, moreover, differs for each constant spore load level. The proposed experimental design uses all three spore loadings to obtain data from a much wider range of doses. That is, a variable spore load is used. Hence all the ratios indicated by bold faced type will be available for analysis in determining a D-value.

To accomplish this efficiently it is necessary to select judiciously the variable spore load across all the doses. For example, instead of 2000, 200, and 20 a spore loading of 3000, 1500, 750 and 400 would more likely extend the range of useful data. It is also apparent, when viewing data in Table 2, that dose spacings should be adjusted so as to obtain more useful information over critical dose ranges. For example, it would be advantageous to include an additional point or two between 4.5 and 5.0 when the spore load is 2000/can since the fraction positive has had a substantive change from 8/10 to 1/10 in that span. This implies a need to explore this range more carefully. Similarly, when the spore load is 200 there is a substantive change from 4/10 to 1/10 at doses 2.5 to 3.0, respectively.

With these adjustments in both spore load and dose as a result of a computer run the simulation can be run again adjusting the dose spacings ΔT, the number of serial dilutions SD, the dilution factor **DF**, and N to obtain the desired result: as many valid, useable data point as possible.

An actual experiment can be performed, using the dose spacings, spore loads and replicate numbers as gleaned from the simulation. This will be done only when the experimenter is

Table 1—Destruction of Clostridium botulinum *(Strain 62A)[a] by exposure to Co[60]*

Spores/Can	Strain	Radition MRAD	No. viable cans/No. replicates
6.9 × 10^{6}	62A	0.0	16/20 (control)
		0.5	18/20
		1.0	20/20
		1.5	4/20
		2.0	1/20
		2.5	0/100
		3.0	1/100
		3.5	0/100
		4.0	0/100
		4.5	0/100

[a] Destruction data from Anellis et al. (1967).

Table 2—Computer simulated data of a quantal response (MPN) experiment with serial dilution prior to exposure to a lethal agent

Dose Mrads	Spore load/Can			
	2000	200	20	2
1.0	10/10[a]	10/10	10/10	0/10
1.5	10/10	10/10	**6/10**	1/10
2.0	10/10	10/10	**5/10**	0/10
2.5	10/10	**9/10**	**4/10**	2/10
3.0	10/10	**4/10**	**1/10**	0/10
3.5	**9/10**[b]	**3/10**	**1/10**	0/10
4.0	**9/10**	**3/10**	0/10	0/10
4.5	**8/10**	**1/10**	1/10	0/10
5.0	**1/10**	**1/10**	0/10	0/10
5.5	2/10	0/10	0/10	0/10
6.0	2/10	0/10	0/10	0/10
6.5	1/10	0/10	0/10	0/10
7.0	0/10	0/10	0/10	0/10

[a] Ratios in the body of the table represent the results of "exposing" 10 replicates at each dose-load combination.
[b] Bold faced ratios are the statistically useable fraction positives after "exposure" to the lethal agent at each dose-load.

satisfied with the results of the simulation. Although the parameter selection process could be accomplished by experimentation, it is suggested that it would be more costly and time consuming, without the ease of refinement offered by the simulation.

As an example of the proposed design, the spore loading, the replicate numbers, and the dose spacing shown in Table 3 is a more efficient experimental design, providing a larger number of useable data points than one using a constant spore load. The numbers in Table 3 refer to the number of replicates at each spore loading to be placed at each dose. The design is illustrated using a fixed number of replicates. However, it should be apparent that a larger number of replicates at 1.5, 2.0 with 20 spores/can, 3.0, 3.5 with 200 spores/can, 4.5 and 4.75 with 2000 spores/can would result in increased accuracy. The other doses (2.5, 2.75, and 4.0) have two data points as a result of the variable spore load and have increased statistical accuracy. The illustration further maintains the original spore loadings so as to make the point of the new design rather than complicate its description.

The total number of replicates used is 130, the same as would have been used in a constant spore load experiment. The total number of spores required for a constant spore load experiment (Table 2) is 260,000 using 130 cans with a load of 2000/can; the total is 26,000 when the load is 200/can; and 2,600 when it is 20/can. For the variable load experiment (Table 3) the total required would be 90,800, a clearly favorable comparison especially when considering the resulting quality of data, i.e., 13 fraction positives versus at most five for fixed spore loads.

Epstein (1967) provided an estimate of the modal dose (the most probable dose for bacterial extinction) for quantal experiments. It is given by $T = \ln(L)/B$, where L is the spore load per can and B is the death-rate constant (i.e. $B = 2.3026/D$, where D is the dose necessary to reduce the population by 90%). The modal dose is that value where 37% of the replicates are expected to be sterile. The modal values for this example are indicated in Table 3 in bold type. Since the modal value differs for different spore loads, spore loads can be seen to influence where replicates should be placed across the dose range. The simulation provides a visual (Table 2), and therefore a good judgmental basis for placing the replicates; Table 3 is but one recommended placement. An array could, of course, have been chosen to be placed at each dose as in the simulation procedure but that would be substantially more expensive and cumbersome.

The description of bacterial destruction above refers to the case when serial dilution was performed *before* exposure. The data resulting from this type of experiment will be referred to as "H-type" data. However, when $SD = 0$ is chosen, the

destruction is carried out only in each of the N replicates, resulting in a single partial spoilage data point at each dose level T (rather than a vector of points) being printed out. This single data point at each dose is the classical and most common type of data used in food science. We designate it as "Q-type" data.

In some experimental settings serial dilution is performed *after* the N replicates are exposed. In those cases the exposed cans at each T are serially diluted after having been exposed. They are then incubated. After incubation fraction positive data is collected. The simulation output in this case will be referred to as "KT-type" (Koch and Tolley, 1975) data. This isn't customarily the type of data that food scientists encounter when dealing with C. botulinum. However, it is a possible experimental method when dealing with other contaminants. The KT-type simulation has been included for completeness.

Example

An example of the simulation output is given in Table 4 demonstrating an H-type simulation. A constant dose increment was chosen (0.5 Mrads) producing the output found in Table 2. The tables labeled "Initial cells" are the array of "cans" prior to exposure, where the spore load per can is displayed inside the array. The row labeled "0" ($SD = 0$) has 10 cans with an inoculum generated using a Poisson average of 2000 spores/can. The row labeled $SD = 1$, has an average spore load of 200/can since the dilution factor chosen was 10.

The table labeled Surviving cells is the consequence of "exposing" each spore in the first array to the lethal environment. The remaining cells in each "can" are enumerated and the column at the extreme right of Surviving cells indicates the fraction positive after exposure.

The complete output is not reproduced here for reasons of economy. They can be obtained from the author upon request.

DISCUSSION

AN EXPERIMENTAL DESIGN is proposed for inoculated pack studies with the aid of a computer simulation. The simulation extends previously described simulations (Hachigian, 1979; 1982) in three different directions which may be used independently or in combinations. The extensions allow: (a) serial dilution after exposure to a lethal environment; (b) serial dilution before exposure; and (c) incubation after exposure. The simulation is not intended for production purposes. Its primary purpose is to assist in the design of actual experiments used for determining the inactivation kinetics of bacterial spores. Ross (1977) suggested a variable spore load approach but did not give specific methods for developing and testing a design. Moreover, he did not indicate how one should select the doses over which to conduct the experiment, nor that certain doses should have larger numbers of replicates and how one is to select these doses. The use of the computer simulation takes a large amount of guess work out of the process of designing and conducting an experiment when all of these parameters are simultaneously adjusted.

The methods and design described are equally applicable to sterilizing heat, sterilizing radiation, or combinations of these methods with or without the use of chemical additives.

From a statistical perspective inoculated pack and model system studies based on classical designs provide too few valid data points from which to obtain consistently accurate estimates of D-values. A variable spore load, variable replicate numbers, plus judicious spacing of doses at which to conduct the experiment is essential to achieve the desirable goal of more statistically valid data points over an extended range which can be consistently reproduced.

The Stumbo (1973) estimate for the D-value (labeled D_s) and the Hachigian (1971) estimate (labeled D_H) in Table 5 indicate the results for constant spore loads, 2000/can, 200/

Table 3—Dose-load combinations recommended for an actual experiment to insure a larger number of statistically valid data points when it is anticipated that D = 1.32

Dose Mrads	Spore load/Can		
	2000	200	20
1.5	—	—	10
1.71[a]	—	—	
2.0	—	—	10
2.5	—	10	10
2.75	—	10	10
3.0	—	10	—
3.03	—		—
3.5	—	10	—
4.0	10[b]	10	—
4.25	10	—	—
4.34		—	—
4.5	10	—	—
4.75	10	—	—

[a] Bold face doses indicate the calculated modal dose for bacterial extinction at the indicated spore load.

[b] Entries indicates the number of replicates recommended for each dose-load combination.

```
        RUN²
ENTER INITIAL TIMEᵇ: 1.0
ENTER LENGTH OF TIME INCREMENT: 0.5
DO YOU WANT EQUAL LOGARITHMIC SPACING? Y/N: N
ENTER NUMBER OF REPLICATES: 10
DO YOU WANT TO SERIALLY DILUTE BEFORE OR AFTER EXPOSURE? (B/A): B
ENTER NUMBER OF SERIAL DILUTIONS: 5
ENTER THE DESIRED DILUTION FACTOR: 10
ENTER AVERAGE SAMPLE SIZE: 2000
ENTER WEIBULL PARAMETERS (A/B): 1/1.75
DO YOU WANT OT INCUBATE? Y/N: N
ENTER CONSECUTIVE VOIDS:
DO YOU WANT TO PRINT THE DETAILS? Y/N: Y
NUMBER OF REPETITIONS FOR THIS RUN:
*** TIME =    1.00 ***
INITIAL CELLSᶜ:
```

	1	2	3	4	5	6	7	8	9	10ᵉ
0|	1910ᵈ	1964	1908	1951	1923	1984	1982	1999	2029	1953
1|	186	206	195	190	184	210	205	190	191	199
2|	13	22	17	28	20	22	27	25	18	18
3|	0	2	0	2	4	1	4	2	0	0
4|	0	0	0	0	0	0	0	0	0	0
5|	0	0	0	0	0	0	0	0	0	0

```
AVERAGE INOCULANT COUNT: 1960.3 195.6 21 1.5  0  0
DESTROY PROBABILITYᶠ = 0.8262260565
SURVIVING CELLS:ᵍ
```

	1	2	3	4	5	6	7	8	9	10	
0|	304	332	340	330	313	350	371	358	335	361	10/10ʰ
1|	33	33	35	32	27	31	37	42	32	36	10/10
2|	2	7	4	3	6	5	6	8	1	4	10/10
3|	0	0	0	0	0	0	0	0	0	0	0/10
4|	0	0	0	0	0	0	0	0	0	0	0/10
5|	0	0	0	0	0	0	0	0	0	0	0/10

```
AVERAGE RESIDUAL COUNT: 339.4 33.8  4.6  0  0  0
*** TIME =    1.50 ***
INITIAL CELLS:
```

	1	2	3	4	5	6	7	8	9	10
0|	1946	1963	2064	2004	1975	2005	1983	1995	2026	2031
1|	213	188	216	218	210	217	182	179	228	225
2|	14	12	30	17	22	26	15	20	29	20
3|	0	0	3	0	2	0	0	1	1	2
4|	0	0	0	0	0	0	0	0	0	0
5|	0	0	0	0	0	0	0	0	0	0

```
AVERAGE INOCULANT COUNT: 1999.2  207.6  2C.5  0.9  0  0
DESTROY PROBABILITY = 0.927560243
SURVIVING CELLS:
```

	1	2	3	4	5	6	7	8	9	10	
0|	138	114	150	172	127	156	130	163	149	133	10/10
1|	14	15	21	11	12	16	13	13	15	18	10/10
2|	0	1	0	1	0	3	0	1	2	2	6/10
3|	0	0	0	0	1	0	0	0	0	0	1/10
4|	0	0	0	0	0	0	0	0	0	0	0/10
5|	0	0	0	0	0	0	0	0	0	0	0/10

```
AVERAGE RESIDUAL COUNT: 143.2  14.8  1  0.1  0  0
*** TIME =    2.00 ***
INITIAL CELLS:
```

	1	2	3	4	5	6	7	8	9	10
0|	1983	2012	1898	1998	2025	1949	2070	1955	1931	1973
1|	205	196	191	207	227	229	190	202	193	200
2|	22	16	18	18	29	23	17	18	17	20
3|	3	0	0	0	1	4	0	0	0	5
4|	0	0	0	0	0	0	0	0	0	0
5|	0	0	0	0	0	0	0	0	0	0

```
AVERAGE INOCULANT COUNT: 1979.4  204  19.3  1.3  0  0
DESTROY PROBABILITY = 0.9698026166
SURVIVING CELLS:
```

	1	2	3	4	5	6	7	8	9	10	
0|	53	49	55	54	63	65	65	65	49	51	10/10
1|	6	6	3	7	8	7	3	5	5	5	10/10
2|	0	0	1	0	2	1	1	0	2	0	5/10
3|	0	0	0	0	0	0	0	0	0	0	0/10
4|	0	0	0	0	0	0	0	0	0	0	0/10
5|	0	0	0	0	0	0	0	0	0	0	0/10

```
AVERAGE RESIDUAL COUNT: 56.9  5.5  0.7  0  0  0
```

ᵃ RUN is entered to begin the simulation whereupon a series of initializing prompts are presented requiring the user to respond. These prompts are used to define the mode in which the simulation is to be run.

ᵇ TIME should be read as DOSE in the case of radiosterilization.

ᶜ INITIAL CELLS represents the array of replicates prior to "exposure" at any given dose. Each entry either in INITIAL CELLS or SURVIVING CELLS represents one replicate.

ᵈ The number in each entry is the spore load of that replicate whether before or after "exposure". A zero entry means the replicate has no (viable) spores.

ᵉ The number of replicates chosen are the columns in the table. Each row consists of the chosen number of replicates at a different level of spore load.

ᶠ The probability computed at each dose using the formula $F(T) = 1 - e^{-1.75T}$. Here TIME = 1.0 and therefore computes out to be 0,8262260565.

ᵍ This is the array of replicates after "exposure" wherein the entries are the number of "surviving" cells. A replicate with a zero entry is considered sterile.

ʰ The ratio positive is printed out at each dilution level. It is obtained by enumerating the number of nonzero entries in a row divided by the number of replicates chosen.

Table 5—D-values calculated using the Stumbo D_s formula and the D_H formula

Spore load	D_H	D_S
2000[a]	1.36[b]	1.27[b]
200[a]	1.33[b]	1.27[b]
20[a]	1.40[b]	1.28[b]
Variable Load[c]	1.32[c]	1.21[c]

[a] Rows represent results from clasical experiments at constant spore loads.

[b] The D-values are calculated here are simple averages of D-values calculated for each dose at constant spore loads.

[c] These D-values are the simple averages of D-values calculated using all the dose-load combinations.

can and 20/can, and for a **H**-type experiment. The D-value used in the simulation was **D = 1.32**.

Epstein (1967) and Hachigian (1971) derived a formula for the proportion of replicates expected *not* to have survivors after exposure to dose **T**. It is given by:

$$p(t) = e^{-Le^{-BT}}$$

where **L** = LAMBDA the spore load, and **B** = the death rate constant. The maximum likelihood unbiased estimate of the proportion p(t) of replicates not having survivors is indeed the proportion (N − k)/N at each dose **T**. It follows then that **B** is estimated by simply solving the above equation after having replaced p(t) by its estimated value for each **T**. Namely,

$$\hat{B} = -\frac{1}{T}\ln(-\frac{1}{L}\ln\frac{N-k}{N})$$ Since D = 2.3026/B the estimate for D is given by:

$$D_H = -2.3026\,T/\ln(-\frac{1}{L}\ln\frac{N-k}{N})$$

where ln represents the natural logarithm.

The Stumbo estimate is given by:

$$D_S = t/(\log A - \log B)$$

where A is the total number of spores, i.e. the number of replicates N times the micro-organisms L per replicate, and B is the number of remaining nonsterile replicates assuming one spore remains per replicate.

Both D_S and D_H are slopes in the respective straight line graphs: one on semi-log paper (Stumbo) and the other on log-log paper. This fact makes it possible to make direct comparison of the estimates.

The desirability of using the D_H formula for calculating D from quantal data is shown in Table 5. It also demonstrates that the Stumbo estimate underestimates D-values in *low* dose ranges. It can be shown that the two estimates agree for higher doses, and the Stumbo estimate asymptotically approaches D_H. However, the conditions under which the two estimates agree vary with D itself as well as with all the other parameters we are concerned with here, that is, spore load, number of replicates, and dose.

Since inoculated pack studies, by their nature, are not often reproduced, it is important these studies be designed to provide the best possible data upon which to base analysis of the process. Computer simulation of an efficient experimental design for inoculated pack studies appears to offer a way by which to accomplish this goal. Although actual data were not produced using the proposed design, data were generated using the simulation as shown in the Example (Table 4). Analysis of the data in Table 4 is evidence of the validity of the approach.

The true effectiveness of the proposed design can only be demonstrated by the conduct of laboratory experiments. To that end, it is the author's hope that comparative experiments will be conducted using the classical and the proposed design with the results published for evaluation by the scientific community.

Implementation of the computer program requires the selec-

tion of a random number generator. Pseudo-random number generators should be chosen carefully so that the output of the simulation can be relied upon. An excellent reference for this purpose is Dudewitz and Ralley (1981).

A complete listing of the computer code for the simulation may be obtained from the author.

APPENDIX

THE ALGORITHM for the computer simulation is listed below for those who may wish to implement the program at their facility.

STEP 1: THE INITIAL DOSE AT WHICH TO EXPOSE THE ARRAY OF CANS IS SELECTED. THIS DOSE IS DENOTED AS INITIALTIME (T).

To begin the simulation a user selects the initial dose **T**. In the computer code provided this selection requires a response to a prompt. The choice is made initially by taking into account various judgmental factors based on actual experimental results, or from previous runs of the simulation. One of the purposes of the methodology proposed is to assist in the selection of the INITIALTIME to obtain statistically useful data. Since running the simulation is inexpensive, what one chooses at first is unimportant, the results will quickly show whether a change is necessary given that other parameters are held constant.

STEP 2: SELECT A DOSE INCREMENT. DENOTE THIS BY THE VARIABLE NAME TIMEINCREMENT, (ΔT).

Incremental changes in dose over the range of interest is governed by the selection of ΔT. Selection of $\Delta T = 0$ results in the experiment being conducted at a single time point. This is a useful option designed to conserve computer time when a repetition may be necessary at just one dose for evaluation purposes.

Unless there is a specific reason, the selection of very small values of $\Delta T > 0$ (e.g. $\Delta T = 0.001$) only increases the run time without a commensurate increase in statistically useful data. Selection of ΔT is again judgmental based on analysis of previous runs and actual experimental results. Too large an increment will skip over the relevant range of interest, and too small an increment will be nearly repetitious of the points near by and most likely difficult to accomplish in actuality.

A single dose run can be used in another interesting way. Suppose the results of an actual experiment are suspect at a particular dose and the data are inconsistent with the rest of the experiment. The single dose option can then be used with estimates of the parameters derived from the other points to see if comparable data can be produced. If it can, then the data can be reasonably assumed to be correct. If not, then the simulation should be run using estimates derived from all the data including the suspect point. If the run gives results comparable to the ones obtained from the actual experiment, then it is possible that the experimental results were correct. If the two separate attempts to validate the experimental results using the simulation do not produce comparable results then the inconsistent data may be suspect (Koch and Tolley, 1975). Of course, before resorting to the use of this simulation in a situation as just described, it is incumbent upon the experimenter to carefully analyze the experiment and the procedures to decide what, if anything, extraneous or procedural could have caused or produced the inconsistent data.

As noted by Cornell and Speckman [1967], there are experimental investigations where it is convenient for a microbiologist to space the doses equally on a logarithmic scale. This option is provided at the initialization of the simulation with a prompt requesting the user select either logarithmic (natural) or arithmetic spacing. The choice of arithmetic spacing leads to the simulation being conducted at **T, T + ΔT, T + 2ΔT, T + 3ΔT**, ..., etc until the simulation ends. The choice of logarithmic spacing results in the following doses being used for the simulation: **T, $Te^{\Delta T}$, $Te^{2\Delta T}$, ...,** etc. until

termination. How the simulation ends is discussed in the last step of the algorithm.

STEP 3: THE NUMBER OF REPLICATES N, TO BE EXPOSED AT EACH DOSE T, IS CHOSEN

The simulation is designed for a constant number of replicates to be exposed at each of the doses. However, it is occasionally the case that the number of replicates at each dose is different, as in the one by Anellis (Table 1) where 20 cans were exposed at each dose to 4.0 Mrads and 100 cans at the last. With a slight inconvenience it is possible to accomplish the same thing by selecting $\Delta T = 0$, and running the simulation with different replicate numbers at selected doses.

STEP 4: CHOOSE WHETHER SERIAL DILUTION IS DESIRED BEFORE OR AFTER EXPOSURE

Serial dilution can be performed *before or after* exposure to the lethal agent. The utility of serial dilution *before* exposure was explained, wherein the array of cans to be exposed is obtained by serial dilution prior to exposure.

Serial dilution after exposure is useful in another context (Koch and Tolley, 1975).

STEP 5: THE NUMBER OF SERIAL DILUTIONS (SD), IS SELECTED WITH THE SELECTION OF THE DILUTION FACTOR (DF) OF THE INNOCULATOR

The number of serial dilutions SD may be chosen to be 0, 1, 2... . If SD is chosen to be zero, the resulting simulation is of classical experimental design with N replicates exposed at each dose T rather than an array. In this situation quantal data results (Mather type) as found for example in Anellis, et. al. (1965, 1979).

The dilution factor DF may be chosen as any real number greater than zero. The "dilution" is carried out by the computer according to the selected factor.

STEP 6: SELECT LAMBDA, THE AVERAGE INOCULUM LEVEL

LAMBDA is chosen as equal to the average stock spore inoculum level before dilution (i.e. SD = 0). Inoculation at all levels of the selected dilution factor (DF) is carried out automatically. The simulation was designed under the assumption that inoculation is performed under conditions where clumping and/or aggregation is not possible. Hence the number of spores in a small unit volume (aliquot) follows the Poisson distribution.

The inoculation procedure with serial dilution before exposure is described as follows: In STEP 5 a value for SD, e.g. $q \neq 0$, and a dilution factor DF, e.g. 10 are selected. In STEP 6 LAMBDA is chosen equal to, e.g. L. With these selections made, the N replicates at level SD = 0 are inoculated with an average number of spores equal to L. At the next level SD = 1 the N replicates are inoculated with an average of L/10 spores, and at SD = 2 the N replicates are inoculated with an average of $L/10^2$, and so on until at SD = q, the N replicates are inoculated with an average of $L/10^q$ spores per replicate. In this way an array with $(q + 1) \times N$ cans is prepared for exposure at each dose T.

For each dilution level, SD, a uniform random number U[J,SD] is generated for the Jth replicate at dilution level SD. The product $\pi^r_{j=1}U[J,SD]$ of these random numbers is computed until the following inequality is satisfied for the first time:

$$\pi^{K+1}_{j=1}U[J,SD] < exp\{-L/10^{SD}\}.$$

Then K[1,SD] = K is the inoculum level put in the first replicate of the first dilution level SD. The procedure continues in this way to inoculate each of the N tubes at the dilution level SD, where SD = 0, 1, 2, 3, ..., q, and J = 1, 2, ..., N.

STEP 7: EXPOSE THE BACTERIA TO A RADIATION DOSE OR LETHAL TEMPERATURE EQUAL TO T (TIMELENGTH)

Cornell and Speckman (1967) have discussed a number of experimental situations for which the governing probability

distribution for decay or destruction is the simple exponential (logarithmic order of death) model. This certainly is the case in many types of experiments conducted by food scientists. However, since the exponential distribution (i.e. the logarithmic order of death) is a special case of the Weibull distribution, we have encoded the Weibull distribution to allow for wider applicability.

Simulated destruction of bacteria is performed at each dose probabilistically using the Weibull in the form:

$$= 1 - exp\{-BT^A\}$$

where A and B are parameters selected by the user at the outset of the computer run. A choice of A = 1 results in the simulation being run using the logarithmic order of death. B is related to the D-value by the formula B = 2.3026/D. The exponential distribution is used to describe spore destruction by the computer.

Designate by (I,J,K) the array of cans to be exposed for a dose equal to T. The I index runs through the number of serial dilutions selected, I = 0, 1, ..., SD. The J index is for the number of replicates, J = 1, 2, ..., N. And, the K index is for the inoculate in each can (I,J), that is K = 1, 2, 3, . . ., K(I,J), where K(I,J) is the number of spores before exposure in can (I,J). For example, at dilution level I = 1, the first can (J = 1) of the N replicates, is inoculated with K(1,1) spores; at the second dilution level I = 2, the third can of the replicates at that level (J = 3) is inoculated with K(2,3) spores. A uniform random number R(0,1,1) is generated between (0,1). If R(0,1,1) is less than F(T) then K(0,1) is diminished by one; otherwise it is left unchanged. F(T) is the calculated value of the exponential at the chosen B and at dose T. A second uniform random number is then generated, R(0,1,2), and if R(0,1,2), < F(T) the remaining number of spores from the previous step is reduced by one. If R(0,1,2) > F(T), the result of the previous step is left unchanged. One continues in this fashion for K = 1,2, ..., K(0,1). In this way each of the spores in the first can of the N replicates at dilution level zero will have been "exposed".

The second can is "exposed" in the same way, i.e. R(0,2,1) is generated and compared to F(T), reducing the spore level K(0,2) by one or leaving it unchanged depending on whether R(0,2,1) < F(T) or whether R(0,2,1) > F(T), respectively. This is repeated until all the bacteria in the second replicate of the zero level of dilution have been "exposed". The procedure is continued through all N replicates at that level of dilution.

When all the replicates are completed in the first row (the undiluted row) the second row of the array, corresponding to the first level of dilution of the requested serial dilution, is exposed. The program continues in this manner until each can in the SD × N array is exposed at dose level T. Sequencing through the array after "exposure" in essentially the same way, each can is checked to determine whether there are any remaining viable spores. A can having one or more remaining spores is considered fertile. The number of fertile cans, FERT(I), at each dilution level I is divided by the number of replicates N and is printed out as LIVE/N at dose level T. Thus, there will be more than one ratio of partial spoilage data at each dose T.

The description of bacterial destruction above refers to the case when serial dilution was performed *before* exposure. The data resulting from this type of experiment will be referred to as H-type data. However, when SD—0 is chosen, the destruction is carried out only in each of the N replicates, resulting in a single partial spoilage data point being printed out at each dose level T. This is the type of data is the classical and most common type in food science. We designate it as "Q-type" data.

Some experimental settings perform serial dilution *after* the N replicates are exposed. In those cases the exposed cans at each T are serially diluted after having been exposed. They are then incubated. After incubation partial spoilage data is

collected. The simulation output in this case will be referred to as KT-type (Koch and Tolley, 1976) data. This is *not* customarily the type of data that food scientists encounter when dealing with *C. botulinum*. However, it is a possible experimental method when dealing with other contaminants. The KT-type simulation has been included for completeness.

Incubation can be chosen as an option at the outset of the computer run for any of the three types of experiments. KT-type data would only make sense if incubation is selected as an option.

Incubation is performed by the computer using the residual spores in each of the replicates, at each of the dilution levels, at each **T**. That is, under the assumptions stated in the section entitled EXPERIMENTAL MODEL, the probability that an inoculated can is sterile is given by:

$$\exp\left[-\textbf{LAMBDA (I,J)}\right]$$

where LAMBDA (I,J) is the remaining number of spores in the can at the Ith dilution level for the Jth can among the **N** replicate cans.

In the Koch and Tolley (1975) situation, "incubation" is performed as follows. After exposure to **T** there are **N** replicates at the zero dilution level which have LAMBDA (0,J) surviving bacteria in each can J = 1, 2, ..., **N**. Then each can is serially diluted as described above, except that in this case the dilution begins using as stock the contents of the can *after* dose **T**, namely LAMBDA(0,J). This results in an array of cans having **SD** rows and **N** columns which is to be incubated. Each can in this array now has on average LAMBDA(I,J) spores, where I ranges through the dilution levels and J through the replicates. To test for sterility a uniform random number is generated and compared to the computed $e^{-\text{LAMBDA(I,J)}}$. If the random number is less than the computed value then the can is said to show no growth and the value of LAMBDA for that (I,J) is set to zero. Otherwise it is left unchanged. The entire array is tested in this way for growth after incubation. For each dilution level I = 0,1,2, ..., **SD**, the number of fertile cans (i.e. those with zeros) are counted divided and printed out as LIVE/N for that dose **T**.

STEP 8: WITH ALL INITIALIZED SELECTIONS HELD FIXED THE ALGORITHM IS AUTOMATICALLY REPEATED AT DOSE **T + ΔT**

STEP 9: THE ALGORITHM STOPS

The program will stop, in the case of Q-type data, when all the cans are sterile for a preselected number of successive doses. This number is chosen at the option of the user and is entered in response to a prompt. (See the section on EXAMPLE to see how these appear.) When serial dilution is performed before exposing the cans (i.e. an array of cans is exposed at each **T** resulting in H-type data), the program will stop when the replicates at the undiluted level (i.e. **SD** = 0) are all sterile for a preselected number of successive doses.

In the situation when KT-type data are desired (i.e., when serial dilution is performed after exposure) the program stops when all the undiluted replicates are sterile for the preselected number of successive doses. However, in this case serial dilution can be carried out only for those dilution levels, such that

$$\frac{\textbf{LAMBDA (I, J)}}{\textbf{DF}^I} > 1$$

for all I = 1,2, ..., **SD**, and **DF** ≠ 0. This condition assures us that the output of the simulation is accurately generated by the computer. The stopping procedures detailed above are based on those described in Hachigian (1979). An example of the simulation output is given for an H-type experiment in the body of the paper. A constant dose increment (0.5 Mrads) was used and produced the output found in Table 2 in the section entitled PROPOSED EXPERIMENTAL DESIGN.

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Formulation and Sensory Evaluation of a Low Calorie Fiber Bar

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ABSTRACT

Three levels of fiber were incorporated, and five combinations of wheat and corn bran were evaluated in formulating the fiber bars. A total of 15 fiber-bar variations were tested for physical characteristics and subjected to sensory evaluation by trained taste panelists. The three fiber-bar variations which were ranked highest by the sensory panel were also tested by consumer sensory evaluation. Findings indicated that the optimum level of fiber replacement of flour were 20% by weight. At this level of replacement, either all wheat bran or a mixture containing a maximum of half corn and half wheat was found by consumer sensory evaluation to produce acceptable fiber bars.

INTRODUCTION

OVERNUTRITION is a major concern in the United States today. The American diet is high in energy-dense foods such as fats and sugars. In fact, obesity is the most prevalent nutritional problem in the United States (Kreutler, 1980). Haber et al. (1977) reported that removal of fiber from foods may result in effects that lead to overnutrition.

Recently, the value of incorporating higher levels of dietary fiber into the diet has been the focus of much research. Dietary fiber may help protect individuals against constipation, diverticulosis, obesity, diabetes, cancer and heart disease (Stephenson, 1985). The significant role of dietary fiber in the diet is related to its bulk density, hydration capacity, binding properties and fermentability (Van Soest, 1978). Fiber consumption can be increased by consuming more whole grains, fruits and vegetables and/or by incorporating fiber-enriched products into the diet (Vetter, 1984).

The purpose of this study was to develop a high-fiber, low-calorie product which had acceptable physical and sensory characteristics. The fiber-bar product which was developed was intended to be a snack-food item which would increase fiber intake, help produce satiety and be low in caloric value for dieters and diabetics.

MATERIALS & METHODS

Materials

The basic fiber-bar formulation was developed during a preliminary pilot study following basic concepts from the "Food Products Formulary" (Inglett and Inglett, 1982). The fiber-bar formulation was used as a standard formulation. All ingredients and preparation procedures were held constant except for the partial substitution of bran combinations for flour.

Miller's bulk wheat bran, Ener-G corn bran (batch #9-k-19) and bulk lecithin were obtained from a health food store in Costa Mesa, CA. Coarse-ground guar gum (Nutriloid Guar Coarse lot #T2703.A, TCI Gums, Inc., NY), crystalline fructose (Lot #317013 Hoffman-LaRoche, Inc.), corn oil, instant nonfat dry milk, flour and salt were also used.

The three levels of fiber to be incorporated to replace flour by weight were set at 10, 20 and 30% of the formulation. Five combinations of wheat and corn bran were established (Tables 1 and 2).

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Table 1 — Basic fiber bar formulation[a]

Ingredients	% By Weight
Fructose	15.0
Corn oil	9.5
Water	33.0
Lecithin	1.0
Nonfat dry milk powder	9.0
Salt	0.5
Gum	2.0
	100.0

	Variations		
	1	2	3
Flour %	20.0	10.0	0.0
Bran (fiber or mixture) %	10.0	20.0	30.0

[a] Cookie sheets were sprayed with nonsticking cooking spray (Vegalene edible oil). Dispensing nozzle delivers 1.3 mL/cookie sheet (enough for baking 90 bars).

Table 2 — Fiber ingredient mixtures by level and combination

Combination of brans	Level of fiber in formulation		
	10%	20%	30%
0% wheat	0.0	0	0.0
100% corn	10.0	20	30.0
25% wheat	2.5	5	7.5
75% corn	7.5	15	22.5
50% wheat	5.0	10	15.0
50% corn	5.0	10	15.0
75% wheat	7.5	15	22.5
25% corn	2.5	5	7.5
100% wheat	10.0	20	30.0
0% corn	0.0	0	0.0

Table 3 — Results of objective fiber analysis

Analysis	Wheat bran	Corn bran	t-Value
Moisture %	10.59 ± 0.21	6.34 ± 0.14	32.91*
Water-holding capacity	3.77 ± 0.24	3.48 ± 0.09	3.32**
Fat absorption %	2.61 ± 0.11	1.54 ± 0.03	25.01*
Density (wt/vol)	0.24 ± 0.01	0.34 ± 0.01	−13.93*
Hydrated density	1.26 ± 0.16	1.45 ± 0.21	−1.22

[a] Results are the mean and standard deviation of the analysis in triplicate.
* Significant at the 0.001 level.
** Significant at the 0.01 level.

Preparation

Fiber bars were prepared from preweighed bags of raw ingredients which were kept under refrigeration and allowed to attain room temperature prior to preparation. All bags of ingredients were thoroughly mixed, individually, for 60 sec to ensure homogeneity of ingredients. The ingredients were combined following a standardized procedure, then the mixture was shaped and placed on a aluminum cookie sheet. The cookie sheet was sprayed with a nonstick cooking spray (Vegalene--a product of Tryson Co., Costa Mesa, CA--blend of oils, lecithin, beta-carotene, imitation butter flavor and propellent contributes less than 0.1 cal/bar) prior to each use. This was done to prevent the fiber mixture from adhering to materials. The cookie sheet was then placed in a preheated oven set at 350°F (177°C) and baked for 10 min. Upon removal from the oven, the fiber mixture was allowed to cool for 30 min prior to being cut. The fiber bars (size 2' long × 1' width × 1/2" thickness) were then sealed in 2.7 mil Ziplock freezer bags and placed under refrigeration until required for testing, 24 to 48 hrs later.

Table 4—Results of objective analysis of fiber bars[a]

Bar #	% Wheat bran	% Corn bran	Density[b]	Moisture %	Water-holding capacity
1	0.0	10.0	0.909 ± 0.078	31.61 ± 1.37	0.35 ± 0.06
2	0.0	20.0	0.958 ± 0.133	30.12 ± 1.72	0.39 ± 0.13
3	0.0	30.0	0.706 ± 0.069	31.62 ± 2.24	0.70 ± 0.06
4	2.5	7.5	0.878 ± 0.07ʹ	33.38 ± 1.25	0.25 ± 0.07
5	5.0	15.0	0.924 ± 0.10ʹ	32.95 ± 1.12	0.34 ± 0.12
6	7.5	22.5	0.677 ± 0.176	30.06 ± 4.16	0.77 ± 0.10
7	5.0	5.0	1.102 ± 0.098	31.93 ± 1.45	0.32 ± 0.07
8	10.0	10.0	0.948 ± 0.085	32.80 ± 1.39	0.27 ± 0.07
9	15.0	15.0	0.732 ± 0.063	31.82 ± 1.67	0.78 ± 0.07
10	7.5	2.5	0.945 ± 0.138	32.64 ± 0.81	0.28 ± 0.05
11	15.0	5.0	0.929 ± 0.075	32.53 ± 1.82	0.25 ± 0.04
12	22.5	7.5	0.813 ± 0.087	28.40 ± 1.36	0.75 ± 0.13
13	10.0	0.0	0.988 ± 0.099	31.24 ± 1.32	0.28 ± 0.03
14	20.0	0.0	1.013 ± 0.114	32.69 ± 1.40	0.33 ± 0.14
15	30.0	0.0	0.852 ± 0.058	31.87 ± 1.19	0.82 ± 0.08

[a] Results are reported as means and standard deviations in triplicate.
[b] Density was calculated from the results of volume analysis and the mass of the samples.

Table 5—F Values of combined fiber-bar variations and correlation coefficients to chemical characteristics

Independent variables	Dependent variables		
	Moisture	Density[a]	Water-holding capacity
Level of fiber incorporated	9.72*	60.51*	400.51*
Combination of fiber ingredients	2.27	6.69*	1.81
Level x combination	5.11*	2.91**	3.07**
Amount wheat bran	−0.09	−0.04	0.39**
Amount corn bran	−0.17	−0.49**	0.38**
Total bran	−0.27**	−0.57**	0.81**

[a] Based on calculated results using bread volumeter.
* Significant at the 0.001 level.
** Significant at the 0.01 level.

Chemical Analysis

The individual fiber materials used in the study were tested to evaluate their physical characteristics. Moisture was determined by air oven drying (AOAC, 1984). Water-holding capacity (WHC) was determined according to modified methodology from Collins and Post (1981), Holloway and Greig (1984), Longe (1984), McConnell et al. (1974) and Robertson and Eastwood (1981). Fat absorption was measured by centrifuge according to modified methodology of Collins and Post (1981) and Sosulski et al. (1976). Density and hydrated density were determined according to the method described by Toma et al. (1979).

Analyses of fiber materials were done in triplicate. Mean score and standard deviation of the three replicas for each test were computed. Unpaired t-tests were computed to determine any significant differences between the bran materials for the physical characteristics which were evaluated. The level of significance used was 0.05.

Analysis of Fiber Bars

The fiber bars were tested for volume (density) using rape seed displacement, moisture content, water-holding capacity and sensory evaluation.

Analyses of fiber bars were done in triplicate. Mean score and standard deviation of the three replicates for each test (for all 15 variations) were computed. An analysis of variance (F test) was computed to determine any significant differences among fiber-bar variations. Correlations were also performed to determine the degree of relationships between level of wheat, level of corn, amount of bran and the analyses of the fiber bars.

Sensory evaluation was conducted and performed according to the methodology of Amerine et al. (1965) and Larmond (1977) where comfortable environments (adequate light, odor free room, 23°C) were provided to panelists. It consisted of two phases. The first phase utilized a trained taste panel who evaluated three replications of all 15 variations of fiber bars. A total of 15 college students participated in the study. Trained taste panel members were chosen on the basis of availability and interest in the project. Each panelist had to complete at least two replications of all 15 fiber-bar variations for the panelist's evaluations to be included in data analyses. All trained taste panel sessions were conducted at 2 pm on Wednesdays. A total of 10 sessions were held and a maximum of two sample sets were presented at each session.

The second phase was consumer testing of the top three fiber-bar variations as determined by the trained taste panel. Consumer sensory evaluation was conducted using 75 untrained panelists, both males and females. The panelists evaluated the fiber bars using a nine-point hedonic scale, rating the overall quality of the fiber bars (Amerine et al., 1965; Larmond, 1977). Mean scores, standard deviations and analysis of variance were reported.

RESULTS & DISCUSSION

ANALYSES of the fiber ingredients, wheat and corn bran are presented in Table 3. It was found that wheat bran was significantly ($p < 0.001$) less dense than corn bran and corn bran was significantly lower in moisture, water holding capacity and fat absorption than wheat bran. No significant difference was found in hydrated density between the wheat and corn brans. Therefore, these differences reflected greater variation in the final product.

Objective analyses of the 15 fiber-bar variations are presented in Table 4. Fiber bar combination 4 (2.5% wheat bran and 7.5% corn bran replacing flour) was found to have the highest moisture, while fiber bar 6 (7.5% and 22.5% wheat and corn brans, respectively) was the least dense, and fiber bar 15 (30% wheat bran) had the highest water holding capacity. In examining the physical characteristics of all fiber bars, favorable low-calorie products should contain high-moisture, high water holding capacity and lower density. Thus, fiber bar numbers 6, 3, and 9 were favorable for their physical characteristics.

Significant differences were found for moisture, density and water holding capacity at each level of fiber incorporated (Table 5); significant differences from combination were found only for density. Also, significant differences from the interactions of level and combination were found for all physical characteristics evaluated. The results are in agreement with Brockmole and Zabik (1976) who found no significant effect on product moisture due to bran substitution in cake formulas. Results indicated that bran content strongly correlated with water holding capacity and weakly with the moisture content. Also results were in agreement with Shafer and Zabik (1978) who found that substitution of corn bran resulted in highest volume of cakes and, therefore, decreased product density.

Sensory evaluation

Sensory evaluation by trained taste panelists (Table 6) identified fiber bar 14 (20% wheat bran) to possess the highest flavor, appearance and texture scores, followed by fiber bar 11 (15% wheat bran and 5% corn bran) with the second highest flavor and texture scores and the third highest appearance score.

Table 6 – Sensory analysis of fiber bars[a]

Bar	% Wheat bran	% Corn bran	Flavor	Appearance	Texture
1	0.0	10.0	3.78 ± 1.66	2.60 ± 1.44	3.87 ± 1.69
2	0.0	20.0	3.21 ± 1.41	2.59 ± 0.91	3.33 ± 1.05
3	0.0	30.0	1.74 ± 0.95	2.23 ± 1.18	1.32 ± 0.49
4	2.5	7.5	4.76 ± 1.28	3.60 ± 1.25	4.60 ± 1.38
5	5.0	15.0	4.39 ± 0.95	4.33 ± 1.12	4.78 ± 1.29
6	7.5	22.5	2.90 ± 1.18	3.58 ± 1.41	1.90 ± 0.66
7	5.0	5.0	4.74 ± 1.51	4.37 ± 1.68	4.87 ± 1.48
8	10.0	10.0	5.00 ± 1.44	5.81 ± 1.60	5.62 ± 1.36
9	15.0	15.0	3.38 ± 1.47	4.31 ± 1.93	2.74 ± 1.24
10	7.5	2.5	4.72 ± 1.72	4.39 ± 1.66	4.77 ± 1.75
11	15.0	5.0	5.99 ± 1.44	6.34 ± 1.44	6.24 ± 1.22
12	22.5	7.5	4.99 ± 1.69	5.93 ± 1.34	4.07 ± 1.53
13	10.0	0.0	4.82 ± 1.79	4.31 ± 1.81	4.73 ± 1.88
14	20.0	0.0	6.00 ± 1.33	6.74 ± 1.02	6.49 ± 1.26
15	30.0	0.0	4.67 ± 1.50	6.36 ± 1.72	4.68 ± 1.46

[a] Results are reported as means and standard deviations in triplicate and based on hedonic scale 1-9 (extremely dislike to extremely like. One score being poor or unacceptable, 5 intermediate "neither/nor" and 9 as extremely like "optimum" for product)

Table 7 – Consumer sensory evaluation of top three fiber-bar variations

Bar #	Bran composition		Mean ± SD[a]	F Value
	Wheat	Corn		
14	20%	0%	5.48 ± 1.67	1.05
11	15%	5%	5.40 ± 1.63	1.05
8	10%	10%	5.11 ± 1.88	1.05

[a] Mean scores were the results of consumer panelists rating fiber-bars using a hedonic rating scale. A 9-point scale was used, from 1 "dislike extremely" to 9 "like extremely."

Fiber bar 8 (10% wheat and corn bran each) was found to have the third highest flavor and texture scores and the fifth highest appearance score.

It was noted that as the amount of wheat bran in the fiber bars increased, the sensory scores increased. Conversely, as the amount of corn increased, the sensory scores decreased. No significant correlation was found between the total amount of bran in the fiber bars in the mean appearance score. A possible explanation could be that wheat bran content correlated positively with appearance scores at approximately the same strength that corn bran content correlated negatively, thus producing a cancellation of effects.

Weak positive correlations were found between the percentage of wheat in the fiber bar formulations and the flavor and texture scores. Stronger negative correlations were found between the amount of corn bran in fiber bars and the flavor and texture scores. Negative correlations were found for the total bran content of fiber bars and the flavor and texture scores. Thus, it appeared that the negative effect of corn bran on sensory scores overpowered the positive effect of wheat bran. Therefore, to produce a fiber bar with acceptable sensory qualities, a maximum level of corn bran which can be incorporated into the formulation existed.

Bars number 14, 11, and 8 were ranked highest by the trained taste panel. These bars were further rated by the consumer taste panel. These scores are shown in Table 7. No significant difference (0.05 level) was found between the bars.

The mean consumer ratings were all found to be above a score of 5.0. The scale used for evaluation by the consumers went from a score of 1 for "dislike extremely" to a score of 9 for "like extremely." Polizzoto et al. (1983) used a 9-point line scale for sensory evaluation. A score of greater than or equal to 5.0 was arbitrarily set to indicate an acceptable product. Using the criteria of Polizzoto et al. (1983) to evaluate acceptability, all three variations would be judged as being

acceptable products by the consumer panel. Brockmole and Zabik (1976) found that consumer panelists had good acceptance of products that contained wheat bran. Sensory acceptance of wheat and corn bran in bakery products was also found by Shafer and Zabik (1979) and Polizzoto et al. (1983).

Formulation of low or reduced calorie foods such as these high in fiber content are growing in demand by consumers, especially among dieters and diabetics alike. The results of the study indicated that a fiber-bar product should be made with a level of bran incorporation of no more than 20%. This level of bran ingredients could be either all wheat or a mixture of 10% wheat and 10% corn.

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Fractionation of Rice Glutelin Polypeptides using Gel Filtration Chromatography

S.D. SNOW and J.R. BROOKS

──────── ABSTRACT ────────

Preparative gel filtration chromatography using Sepharose CL-6B was utilized to obtain working quantities of dissociated rice glutelin polypeptides. Rice flour samples were extracted and fractionated using dilute Tris buffer containing either 0.5% SDS, 6M urea or 8M urea. Gel filtration under each set of extraction conditions yielded fractions containing dissociated acidic and basic glutelin polypeptides, but acidic polypeptides were more effectively separated from basic polypeptides in urea than in SDS. The presence of glutelin polypeptides in higher molecular weight aggregates under each set of buffer conditions indicated that the dissociation of these components during extraction was not complete.

INTRODUCTION

GLUTELIN, the major rice storage protein, comprises approximately 80% of the milled rice endosperm protein (Juliano, 1972). Glutelin subunits have an approximate molecular weight of 60 kilodaltons (KD) and consist of a heterogeneous collection of disulfide linked polypeptides (Yamagata et al., 1982: Zhao et al., 1983). Upon reduction and under denaturing conditions, glutelin subunits can be dissociated into two major fractions, i.e. the acidic or α-polypeptides and the basic or β-polypeptides. The acidic and basic polypeptides have isoelectric points betwen pH 6.5-7.5 and pH 9.4-10.3 and molecular weights ranging from 28.5—39KD and 20—23KD, respectively (Juliano and Boulter, 1976; Villareal and Juliano, 1978; Yamagata et al., 1982; Luthe, 1983; Zhao et al., 1983; Robert et al., 1985; Wen and Luthe, 1985). Glutelin shares a number of similarities with the principal, salt-soluble globulins of oat, pea, and soybean including subunit biosynthesis, molecular weight, amino acid composition and immunological properties (Zhao et al., 1983; Robert et al., 1985; Wen and Luthe, 1985; Takaiwa et al., 1986). Despite these similarities with the 'legumin-like' proteins, post-translational modification of the salt-soluble precursor polypeptides results in mature subunits that are largely insoluble in salt solutions and classified as glutelins (Yamagata et al., 1982). The reasons for this insolubility are unknown, although its low salt-solubility has been attributed to such factors as extensive subunit aggregation (Palmiano et al., 1968; Robert et al., 1985; Sugimoto et al., 1986) and glycosylation (Wen and Luthe, 1985). Current efforts are directed toward a more thorough characterization of the rice glutelin polypeptides and the reasons for insolubility. Glutelin polypeptides, extracted under denaturing and reducing conditions, are typically fractionated using ion exchange chromatography or chromatofocusing (Juliano and Boulter, 1976; Zhao et al., 1983; Wen and Luthe, 1985; Sarker et al., 1986). These studies, however, do not consider the degree of polypeptide dissociation following extraction. Preparative sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) provides dissociated polypeptide samples (Krishnan and Okita,

1986), but the method is tedious and sample quantities are small. In addition, removal of SDS following fractionation is difficult and could interfere with further efforts to characterize the polypeptides. Attempts to fractionate glutelin polypeptides using gel filtration chromatography appear promising but have met with limited success (Juliano and Boulter, 1976; Villareal and Juliano, 1978; Chen and Cheng, 1986). Fractions containing dissociated acidic and basic polypeptides can be recovered but are found to be poorly separated from one another even when column lengths are extended to 100 cm. Further, because each of these studies use sample buffers containing 0.5% SDS, the difficulties associated with the removal of the detergent from the samples still remain.

In contrast to SDS, urea has little effect on methods to further characterize or fractionate the polypeptides, including isoelectric focusing, ion exchange and chromatofocusing. If necessary, urea can be readily removed from sample preparations using dialysis or desalting columns. This study was conducted to further evaluate the use of preparative gel filtration chromatography for obtaining working quantities of dissociated acidic and basic rice glutelin polypeptides. Samples
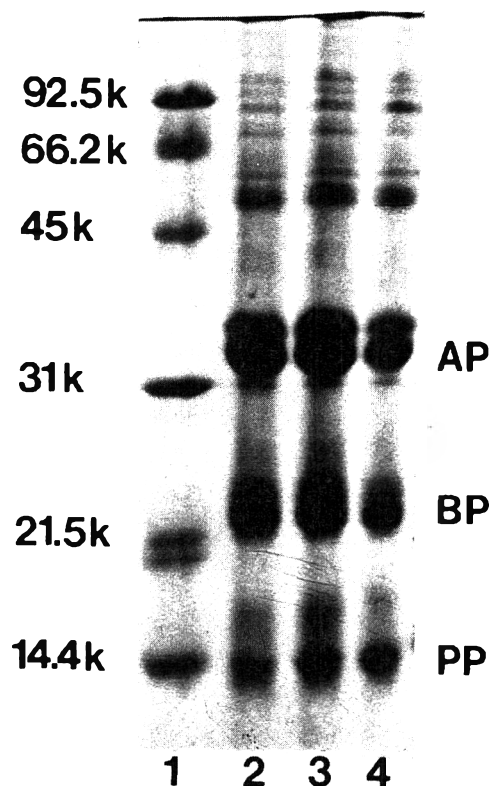
Fig. 1 – SDS-PAGE patterns for glutelin extracts: (lane 1) Bio-Rad low molecular weight standards (in kilodaltons); (lane 2) 6M urea extract, 15ug protein; (lane 3) 8M urea extract, 15µg protein; (lane 4) 0.5% SDS extract, 15µg protein. The letters AP and BP refer to the acidic and basic polypeptides of glutelin, respectively. PP refers to the prolamin polypeptide.

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were extracted and fractionated in 6M and 3M urea and compared with those extracted in 0.5% SDS.

MATERIALS & METHODS

General methods

Reagents and molecular weight standards for electrophoresis were purchased from Bio-Rad Laboratories, Richmond, CA., and gel filtration media was obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Molecular weight standards for gel filtration chromatography and all other chemicals and reagents were purchased from Sigma Chemical Company (St. Louis, MO). Dialysis tubing was Spectropor

Fig. 2—Sepharose CL-6B elution patterns for glutelin extracted in (A) 0.5% SDS, (B) 6M urea and (C) 8M urea.

1 with a 32mm flat diameter and 6-8KD molecular weight cutoff (Fisher Scientific, Dallas, TX).

Short grain rice (var. Nortai) was obtained from the 1984 crop at the University of Arkansas Experiment Station. Prior to use, it was dehulled, milled, and ground to a fine flour (200 mesh). Next, the flour was defatted by stirring with ten volumes of petroleum ether for 6 hrs at 21°C, decanted and allowed to air dry overnight. The defatted flour was stored at -20°C.

Protein contents were assayed using the Coomassie Blue dye-binding method (Anonymous, 1976) for samples containing urea or the Biuret method (Gornall et al., 1949) for samples containing SDS. Bovine serum albumin was used as the reference protein.

The standard buffer used throughout this study was 0.01M Tris-HCl adjusted to pH 8.0 and contained 0.05% sodium azide as a preservative. Immediately prior to use, 0.001M dithioerythritol (DTE) and 0.0002M phenylmethylsulfonyl fluoride (PMSF) were added as a disulfide bond reducing agent and protease inhibitor, respectively.

Crude glutelin extraction procedure

Defatted rice flour was extracted with ten volumes (w/v) (this volume was adequate in removal of water-and salt-soluble proteins) of standard buffer containing 0.4M NaCl for 1 hr at room temperature (21°C) to solubilize salt-soluble albumins and globulins. Following centrifugation at 35,000 × g for 20 min at 20°C, the supernatant was discarded. The residue was reextracted using standard buffer containing either 0.5% SDS, 6M urea or 8M urea for 3 hr at 21°C. The flour-to-solvent ratios (w/v) used for these extractions were 1:10, 1:15, and 1:60, respectively. A crude glutelin supernatant was obtained following centrifugation as above. Each sample was assayed for protein and immediately applied to the gel filtration column.

Gel filtration chromatography

Twenty-four mL aliquots of each crude glutelin solution were fractionated on a 2.5cm i.d. × 110cm Sepharose CL-6B gel filtration column (Pharmacia Fine Chemicals, Piscataway, NJ) previously equilibrated with the standard buffer containing either 0.5% SDS, 6M urea or 8M urea. The column was eluted at a flow rate of 13 mL/hr, and absorbance was continuously monitored at 280 nm using a Type 6 optical head connected to a UA-5 Absorbance/Fluorescence monitor (Instrumentation Specialities Co., Lincoln, NE). Effluent was collected in 4 mL portions using a Frac-100 fraction collector (Pharmacia Fine Chemicals, Piscataway, NJ). Column effluent for each fraction was combined and concentrated by ultrafiltration using a 50 mL stirred cell equipped with a YM-10 membrane (Amicon Corp., Danvers, MA) prior to examination by SDS-PAGE. Elution volumes were determined to the mid-point of each fraction. They were compared to the elution volumes of bovine serum albumin (66 KD) and carbonic anhydrase (29 KD) standards, which were solubilized and passed individually through the column under similar buffer conditions.

Gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 12% or 13% separating gels with 4% stacking gels according to a modification of the Laemmli (1970) method and using the Protean II Slab Cell (Bio-Rad Laboratories, Richmond, CA) vertical unit. Samples were diluted in buffer in which the SDS concentrations were increased to 0.2% (w/v), and 0.001M DTE was used in place of mercaptoethanol as the reducing agent (Brooks and Morr, 1984). The 0.75 mm thick gels were electrophoresed at a constant current of 13 milliamps (mA) in the stacking gel and increased to 18 mA for migration through the separating gel. Low molecular weight protein standards (Bio-Rad Laboratories, Richmond, CA) consisting of phosphorylase B (92.5KD), bovine serum albumin (66.2KD), ovalbumin (45KD), carbonic anhydrase (31KD), soybean trypsin inhibitor (21.5KD), and lysozyme (14.4KD) were simultaneously electrophoresed as reference markers. Gels were stained overnight with 0.1% Coomassie Brilliant Blue R-250 (w/v), 40% ethanol (v/v) and 10% acetic acid (v/v) in water and destained in the same solution excluding the dye.

RESULTS & DISCUSSION

THE RICE FLOUR SAMPLES formed very viscous solutions when extracted with urea. This was due to interactions of the
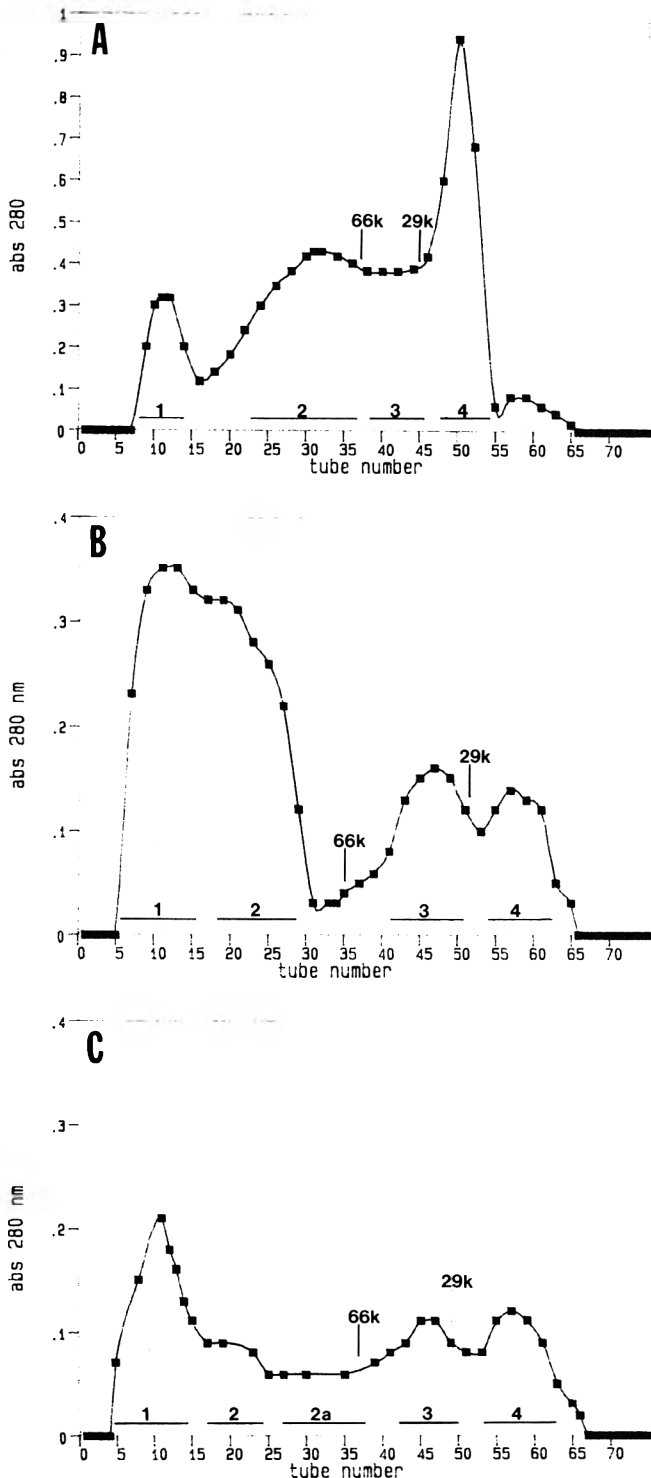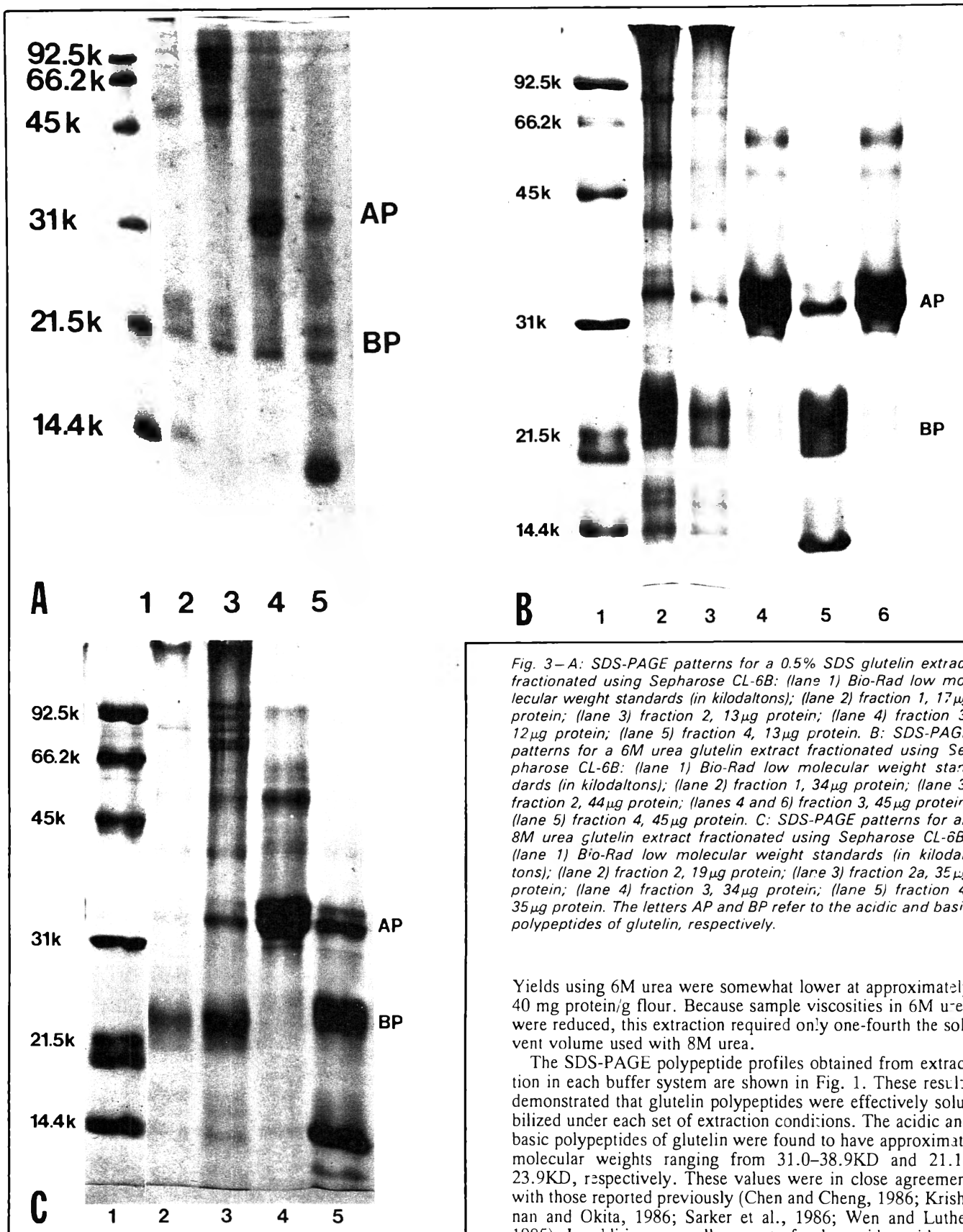
Fig. 3—A: SDS-PAGE patterns for a 0.5% SDS glutelin extract fractionated using Sepharose CL-6B: (lane 1) Bio-Rad low molecular weight standards (in kilodaltons); (lane 2) fraction 1, 17μg protein; (lane 3) fraction 2, 13μg protein; (lane 4) fraction 3, 12μg protein; (lane 5) fraction 4, 13μg protein. B: SDS-PAGE patterns for a 6M urea glutelin extract fractionated using Sepharose CL-6B: (lane 1) Bio-Rad low molecular weight standards (in kilodaltons); (lane 3) fraction 1, 34μg protein; (lane 3) fraction 2, 44μg protein; (lanes 4 and 6) fraction 3, 45μg protein, (lane 5) fraction 4, 45μg protein. C: SDS-PAGE patterns for an 8M urea glutelin extract fractionated using Sepharose CL-6B: (lane 1) Bio-Rad low molecular weight standards (in kilodaltons); (lane 2) fraction 2, 19μg protein; (lane 3) fraction 2a, 35μg protein; (lane 4) fraction 3, 34μg protein; (lane 5) fraction 4, 35μg protein. The letters AP and BP refer to the acidic and basic polypeptides of glutelin, respectively.

Yields using 6M urea were somewhat lower at approximately 40 mg protein/g flour. Because sample viscosities in 6M urea were reduced, this extraction required only one-fourth the solvent volume used with 8M urea.

The SDS-PAGE polypeptide profiles obtained from extraction in each buffer system are shown in Fig. 1. These results demonstrated that glutelin polypeptides were effectively solubilized under each set of extraction conditions. The acidic and basic polypeptides of glutelin were found to have approximate molecular weights ranging from 31.0–38.9KD and 21.1–23.9KD, respectively. These values were in close agreement with those reported previously (Chen and Cheng, 1986; Krishnan and Okita, 1986; Sarker et al., 1986; Wen and Luthe, 1985). In addition, a small amount of polypeptides with molecular weights in excess of 58KD and a major band, at approximately 14KD, were also evident. The high molecular weight components were likely comprised of residual albumins and globulins. The low molecular weight fraction was most probably a prolamin polypeptide that is typically reported as a principal contaminant of glutelin preparations (Krishnan and Okita, 1986; Wen and Luthe, 1985). Sepharose CL-6B gel

starch fraction with urea and was especially evident in 8M urea extracts. As a result, increased solvent extraction ratios were necessary when using high concentrations of urea. The total protein yields for samples extracted in 0.5% SDS and 8M urea were comparable with values ranging from 75–80 mg/g flour.

filtration chromatography using each of the denaturing buffers resulted in the separation of the crude rice protein extracts into approximately 4 broad and incompletely resolved fractions that eluted over a wide molecular weight range (Fig. 2A,B and C). The elution volumes of the principal fractions from the urea extracts were similar, indicating generally similar molecular weight distributions. The overall elution profile obtained in the presence of 0.5% SDS was somewhat different from those obtained in urea extracts. This is likely due to differences in shape effects or protein solubility resulting from extraction in SDS versus urea.

Each fraction from Sepharose CL-6B chromatography was recovered, concentrated and analyzed by SDS-Page (Fig. 3A, B and C). In the 0.5% SDS extract, the basic polypeptides of glutelin were found distributed throughout each of the fractions, and the acidic polypeptides were found primarily in fraction 3 and, to a lesser extent, fraction 4 (Fig. 3A). In the 6M urea extract, the basic polypeptides were again found in the higher molecular weight regions, i.e., fractions 1 and 2, as well as in fraction 4, the smallest molecular weight region. The acidic polypeptides were primarily in fraction 3, although small amounts of these polypeptides were dispersed throughout (Fig. 3B). When the urea concentration was increased from 6M to 8M, there was an apparent decrease in the contribution of fraction 2 combined with an increase in the region labeled 2a (Fig. 2C). This most likely indicates a possible shift toward decreased polypeptide aggregation. Fractions, 2, 2a and 4 contained the basic polypeptides, and the acidics were again predominant in fraction 3 with minor amounts present in other fractions as was found in 6M urea (Fig. 3C). In this sample, fraction 1 yielded almost no protein upon concentration and was omitted from SDS-PAGE.

As indicated in Fig. 2, the majority of the acidic polypeptides were recovered betwen the 66KD and 29KD molecular weight markers under each set of extraction conditions, and a significant portion of the basic polypeptides eluted after the 29KD marker. Many of the fractions collected for subsequent SDS-PAGE in this study were closely spaced, and, therefore, a certain degree of component overlap was expected. However, the elution patterns from gel filtration in 0.5% SDS showed a less defined separation of the dissociated acidic polypeptides from the basic polypeptides. These findings were in general agreement with earlier studies (Chen and Cheng, 1986; Villareal and Juliano, 1978; Juliano and Boulter, 1976). Fractionation in 6M and 8M urea resulted in the recovery of an enriched acidic polypeptide fraction (fraction 3) that was largely separated from the basic and prolamin polypeptides. Because these components eluted just prior to the 29KD standard, they appeared present as free polypeptides and, as such, would be suitable for further characterization and study. Similarly, fraction 4 most likely contained free basic polypeptides, but this fraction was heavily contaminated with the prolamin fraction as well as acidic polypeptides. This was not surprising, because the low molecular weight cutoff for Sepharose CL-6B was very close to that of the basic polypeptides.

The additional presence of glutelin polypeptides eluting from the gel filtration column at molecular weights in excess of the 66KD standard indicated that a portion of these components was not completely dissociated by the extraction conditions employed or was able to reaggregate following solubilization. Heating in the presence of SDS and DTT, as was done in the preparation of samples for SDS-PAGE, provided further dissociation to free polypeptides. Although the results were not quantitated, SDS-PAGE for each set of buffer conditions appeared to show a larger percentage of the basic polypeptides eluting in the higher molecular weight regions (>66KD) than was found for the acidic polypeptides.

Efforts to more fully characterize the dissociated glutelin polypeptides, obtained from gel filtration chromatography in 6M urea, are currently in progress.

CONCLUSIONS

THIS STUDY demonstrated that glutelin extraction, under denaturing and reducing conditions, did not necessarily result in the complete dissociation of the protein into free polypeptides. The presence of heterogeneous polypeptide aggregates would likely confound attempts to further characterize or fractionate the individual polypeptides. Gel filtration chromatography, using Sepharose CL-6B, was effective for partially purifying crude glutelin extracts and obtaining preparative samples of dissociated glutelin polypeptides suitable for further characterization and study. Urea was the preferred extractant, because it would be less likely to interfere with further attempts at characterization and, if required, could be readily removed. In addition, a highly enriched fraction of dissociated acidic polypeptides was obtained using either 6M or 8M urea. Extraction in 6M urea yielded less total protein than 8M urea, but the overall polypeptide profiles, as indicated by SDS-PAGE, were similar. Decreased sample viscosities during extraction with 6M urea permitted the use of decreased solvent ratios. This, in turn, resulted in higher protein concentrations, which permitted a larger protein load, per fixed volume of extract, on the gel filtration column.

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An Integral Method Analysis of Steam Infusion Heating of a Free Falling Film

K.L. McCARTHY and R.L. MERSON

ABSTRACT

An analytical and experimental study of direct contact condensation to a free falling film of liquid was modeled. The thermal energy equation governing the steady state heat transfer was solved by an integral technique. The predictive model for velocity and temperature profiles was verified experimentally. Water was used as the test fluid in a steam infusion unit at flow rates of 1.6-2.3 L/min. The water fell in intact sheets 7.5 × 10⁻² m wide and at an initial thickness of 8.9 × 10⁻⁴ m. Fluid velocity and average fluid temperatures compared well with model predictions within the experimental range.

INTRODUCTION

HEAT TRANSFER in food processing applications can be either by direct or indirect heating. However, direct contact between the heating medium and product allows higher heat transfer rates than indirect heating. Steam infusion methods of direct heating disperse the liquid as falling drops or films (Rosenau and Chen, 1983; Murty and Sastri, 1976). In a falling film sterilizer, thin films of homogeneous fluid food are introduced into a pressurized steam chamber (McCarthy, 1987; Slater, 1973). Due to the high ratio of heat transfer area to volume, heating is fast, efficient, and uniform. The product leaves the sterilizer, flows through a constant temperature holding tube to complete sterilization and is then flash-cooled to its initial temperature and solids concentrations (Nahra and Westhoff, 1980).

Murty and Sastri (1973) studied the velocity profile of a falling liquid film analytically, assuming no heat or mass transfer at the interface. The liquid emerged from a long thin slit. The surrounding gas near the issuing liquid was forced to move along with the liquid due to the momentum transfer at the gas-liquid interface. Integrated mass and momentum equations represented the liquid as it fell through the gas without surface contact. In subsequent work, the same authors added heat transfer to the integral analysis and predicted film velocity and temperature profiles in the transverse and axial directions (Murty and Sastri, 1974, 1976). The steady state two dimensional boundary layer equations for mass, momentum and thermal energy were written for a liquid falling in a saturated vapor environment. The point equations were then integrated across the film thickness. The analysis was divided into two regions: a thermally developing region before the center line temperature was affected by heat transfer and a thermally developed region thereafter. Murty and Sastri (1976) concluded that direct contact condensation for the accelerating liquid film was controlled mainly by the Prandtl number, the ratio of Reynolds number to Froude number and the Jakob number. In addition, the effect of interfacial shear between the liquid and vapor was negligible for the range of parameters considered. However, no experimental work was done in their studies. Earlier work on this problem described the falling film sterilizer using a fine element heat transfer analysis (McCarthy and Merson, 1988). It did not take into account the effect of condensate.

The purpose of the present paper was to extend the integral

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analysis approach and compare analytical results with experimental measurements of temperature and velocity profiles.

Theory

The film, whose thickness is measured in the y-direction, is thinning due to the acceleration of gravity as it falls in the x-direction steam (Fig. 1). The integral analysis provides an approximate solution for the heat transfer equations for this situation. The technique is to assume appropriate expressions for the velocity and temperature profiles, substitute them into the thermal energy equation and integrate with respect to y across the film thickness to give one dimensional axial equations that can be easily solved numerically. The method described here is similar to the two-film approach used by Rao and Sarma (1984). The free falling film is viewed as two layers (Fig. 1), the original liquid with local half film thickness, δ, and the condensate layer of thickness, δ_c. The thermal energy equation is divided into two stages: the developing region with heat penetration thickness, δ_t, and the developed region beyond the
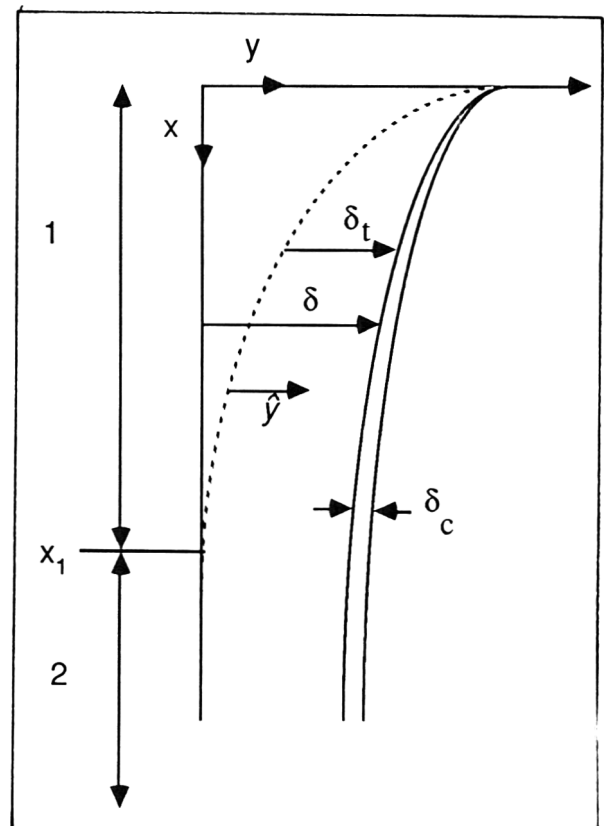
Fig. 1. — Coordinate system. y is measured from the center line of the film. Thicknesses are designated as: half film thickness, δ, heat penetration thickness, δ_t, and condensate layer, δ_c. Region 1 is the thermally developing region and region 2 is the thermally developed region. Film width extends in the z-direction (not shown).

point x_1 where the film center line experiences a temperature rise.

The analysis is simplified by the following assumptions: (1) laminar flow, (2) steady state, (3) Newtonian fluid with constant physical properties, (4) negligible entrance effects, so that the initial velocity of the fluid, u_o, is independent of position in the y-direction, (5) no heat transfer in the z-direction (perpendicular to the x-y plane, (6) no axial heat conduction, and (7) negligible momentum transfer between liquid and vapor. Due to assumptions (4) and (7), both the fluid exiting the slit and the condensate forming on the surface are assumed to be in free fall with a velocity given by Brown, (1961) and Lin and Roberts, (1981):

$$u(x) = (u_o^2 + 2gx)^{1/2} \qquad (1)$$

The thermal energy equation for the free falling film is (McCarthy, 1985):

$$\rho\, c_p\, u(x)\, \partial T/\partial x = k\, \partial^2 T/\partial y^2 \qquad (2)$$

Approximate expressions for the temperature profiles of the fluid and condensate are now assumed. Due to the high heat transfer coefficient for steam, the temperature at the liquid/gas interface is approximated by the saturated vapor temperature, T_{st}. The temperature at the fluid/condensate interface is the interfacial temperature, T_i. The temperature profile through the condensate layer, T_c, is assumed to be linear. The gradient for the condensate temperature is, therefore,

$$dT_c/dy = (T_{st} - T_i)/\delta_c \qquad (3)$$

Quadratic expressions are used to described the temperature profiles for the original fluid. The dimensionless expression for the developing region ($x < x_1$) is assumed to be of the form

$$\emptyset = A + B[\hat{y}/\delta(x)] + C[\hat{y}/\delta(x)]^2 \qquad (4)$$

where $\emptyset$ is equal to $(T - T_o)/(T_{st} - T_o)$, A,B,C are constants and $\hat{y}$ is measured from the heat penetration depth (see Fig. 1) and is related to y by the coordinate transformation, $\hat{y} = y - [\delta(x) - \delta_t(x)]$.

The constants A, B, and C are determined with the aid of the boundary conditions.

$$\hat{y} = 0 \qquad \emptyset = 0 \qquad (5)$$
$$\hat{y} = 0 \qquad \partial\emptyset/\partial\hat{y} = 0 \qquad (6)$$
$$\hat{y} = \delta_t \qquad \emptyset = \emptyset_i \qquad (7)$$

where $\emptyset_i$ is equal to the dimensionless interfacial temperature, $(T_i - T_o)/(T_{st} - T_o)$. Equations (5) and (6) state that at the edge of the heated region $T = T_o$ and the temperature gradient is zero.

The resulting expression for temperature as a function of y and x is

$$\emptyset = \emptyset_i(x)\, [\hat{y}/\delta_t(x)]^2 \qquad (8)$$

For the thermally developed region ($x > x_1$), the assumed quadratic form for the temperature profile is

$$\emptyset = A + B\, [y/\delta(x)] + C[y/\delta(x)]^2 \qquad (9)$$

subject to the boundary conditions

$$y = 0 \qquad \partial\emptyset/\partial y = 0 \qquad (10)$$
$$y = \delta(x) \qquad \emptyset = \emptyset_i \qquad (11)$$

The resulting expression is

$$\emptyset = \emptyset_i(x) - C(x)\, \{1 - [y/\delta(x)]^2\} \qquad (12)$$

The variable C(x) allows matching the temperature profiles at the transition between the thermally developing and thermally

developed regions where $\delta_t = \delta$. By setting $\emptyset$(developing) = $\emptyset$(developed) at $x = x_1$, $C = \emptyset_i$.

The temperature profile for the developing region [Eqn (8)] is substituted into the thermal energy equation [Eq. (2)] and integrated from $y = 0$ to $y = \delta_t$. The resulting ordinary differential equation (ODE) is

$$d\delta_t/dx = -(\delta_t/\emptyset_i)\, d\emptyset_i/dx + 3d\delta/dx + 6\alpha/(u\delta_t) \qquad (13)$$

where α is the thermal diffusivity, $k/\rho c_p$. The expression for the rate of change of the local half film thickness for the original fluid is derived from the continuity equation, i.e.,

$$d\delta/dx = -u_o h_o g/u(x)^3 \qquad (14)$$

The interfacial temperature and the condensate layer thickness are determined from the boundary conditions at the interfaces. The expression for $\emptyset_i$ is obtained from the interfacial flux condition between liquid and condensate:

$$-k\partial T/\partial y|_{y=\delta} = -k_c \partial T_c/\partial y|_{y=\delta} \qquad (15)$$

For the thermally developing region, the dimensionless interfacial temperature is

$$\emptyset_i = k_c\delta_t/(2k\delta_c + k_c\delta_t) \qquad (16)$$

The mass transfer boundary condition yields the derivative of the condensate layer thickness with respect to position.

$$q = -k_c dT_c/dy|_{y=\delta+\delta_c} = -m\lambda \qquad (17)$$

where λ = latent heat of vaporization and m = the local condensate rate,

$$d\left[\int_{\delta}^{\delta+\delta_c} \rho_c\, u\, dy\right]/dx \qquad (18)$$

The ODE resulting from Eq. (17) is

$$d\delta_c/dx = k_c(T_{st} - T_o)(1 - \emptyset_i)/(\rho_c\lambda\delta_c u) - (\delta_c/u)du/dx \qquad (19)$$

Similarly, the temperature expression for the thermally developed region is substituted into the thermal energy equation. The integration is taken over the domain from $y = 0$ to $y = \delta(x)$. The resulting ODE for C(x) is

$$dC/dx = (3/2)d\emptyset_i/dx - (C/\delta)d\delta/dx - 3\alpha C/(u\delta^2) \qquad (20)$$

The flux condition between the original fluid and condensate yields the interfacial temperature:

$$\emptyset_i = 1 - 2C(x)k\delta_c/(k_c\delta) \qquad (21)$$

The expression for $d\delta_c/dx$ in terms of δ_c for the thermally developed region is identical to Eq. (19).

The numerical solution proceeds by solving the coupled ordinary differntial equations by fourth order Runge-Kutta (Hornbeck, 1975). The gradient of the interfacial temperature with respect to x is calculated by a forward difference approximation of the derivative. The initial conditions for the developing region [Eq. (13), (16), (19)] are

$$x = 0 \qquad \delta_t = 0,\ \emptyset_i = 1,\ \delta_c = 0. \qquad (22)$$

At $x = x_1$, the ordinary differential equations [Eq. (19), (20), (21)] are solved for δ_c, C, and $\emptyset_i$ subject to $C = \emptyset_i$ and the current values of δ_c and $\emptyset_i$. These values are then substituted into the appropriate expressions for temperature profiles and local condensate rate.

MATERIALS & METHODS

Experimental apparatus

The experimental apparatus (Fig. 2) was that used by McCarthy and Merson (1988). It consisted of a steam chamber which housed a
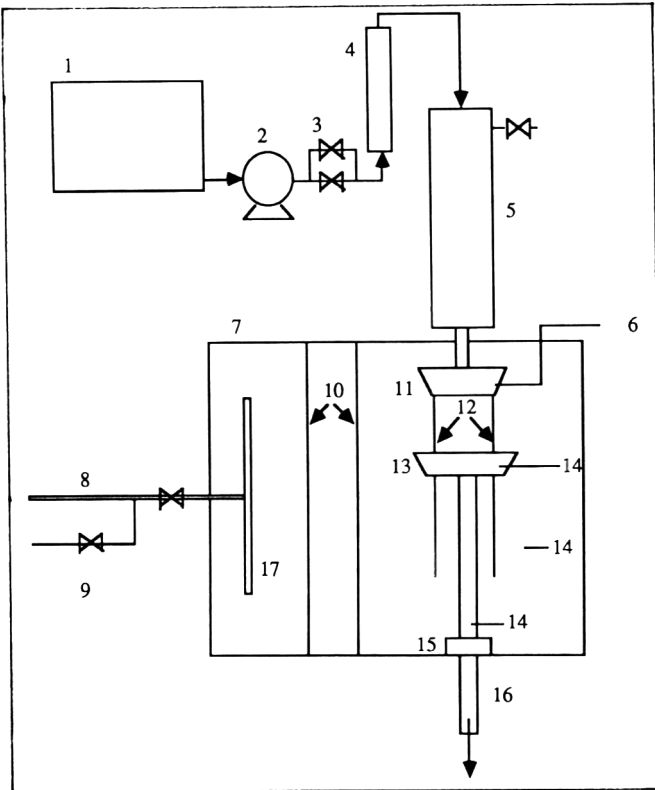
Fig. 2.—Experimental apparatus:

1 Liquid reservoir with level control	6 Manometer	13 Adjustable height trough
2 Centrifugal pump with speed controller	7 Steam chamber	14 Thermocopules to datalogger
3 Bypass and valves	8 Steam sparger	15 O-ring seals
4 Rotameter	9 Condensate drain	16 To drain
5 Constant head reservoir	10 Baffles	17 Steam spreader
	11 Trough with narrows lit	
	12 Guides wires	

falling film. Atmospheric pressure steam entered the chamber through a vertical spreader and was evenly dispersed as it passed through two perforated baffles. Continuous venting through loose fittings proved adequate to maintain atmospheric pressure and to prevent buildup of noncondensible gases.

Water at 303K, used as the test fluid, was pumped through a rotameter to the constant-head cylindrical reservoir at flow rates from 1.6 L/min to 2.3 L/min. The fluid entered the steam chamber through a slit 7.6×10^{-2} m in width and 8.9×10^{-4} m thick. As the fluid fell, the sheet remained intact with the aid of two guide wires (1.0×10^{-3} m diameter) set 7.6×10^{-2} m apart. Before flowing to the drain, the fluid was detained temporarily in a variable-height trough in which temperature measurements were taken. The collection trough position was adjustable from film lengths of 1.2×10^{-2} to 1.0×10^{-1} m.

Temperature measurements were recorded as a function of film length with 30 gauge copper-constantan thermocouples and a recorder/datalogger (Molytec Recorder/Datalogger, Pittsburg, PA). The temperature of the steam chamber was constant and uniform, and one thermocouple measurement proved adequate. Four thermocouples were secured in the adjustable collection trough. Due to the mixing in the trough, these measurements were considered the average temperature at a given film length. The estimated uncertainty in the experimental temperature measurements was $\pm 1°C$ according to procedures found in Holman (1978). As the fluid exited the steam chamber, a thermocouple recorded the temperature. No increase in fluid temperature due to residence time in the trough and drain tube was measurable.

The velocity profile of the fluid was determined by streak photography. A 35 mm camera captured the trajectory of small particles (neutrally buoyant ground pepper) as they fell in the film. The shutter speed was set at 1/125 sec. The vertical streaks were measured; the distance travelled by a particle was plotted against its position in the film.

Experimental design

The parameters varied experimentally were the flow rate and the position of the collection trough. Initial velocities ranged from 0.39–0.57 m/sec. Intact laminar film lengths up to 7.5 cm were attained in the saturated steam environment.

Physical properties of water from published literature were used to predict the temperature profiles: $c_p = 4174$ J/kg K, $k = 0.626$ J/s m K, $\mu = 1.67 \times 10^{-4}$ kg/m sec, and $\rho = 995$ kg/m^3 (McCabe and Smith, 1976). Values were taken at 311 K, the average temperature of the fluid in the steam chamber. The initial half thickness of the film, h_o, was 4.44×10^{-4} m for all experimental and theoretical calculations. The initial temperature of the water, T_o, was 303 K and the steam temperature, T_{st}, was 373 K.

RESULTS & DISCUSSION

Velocity profile

The experimental data obtained from streak photography at an initial fluid velocity of 0.36 m/sec is illustrated in Fig. 3; also plotted is the particle travel expected for free fall. Free fall was verified for initial velocities in the range of 0.36 to 0.60 m/sec. The most plausible explanation for the characteristic discrepancy between the data and predicted profile near $x = 0$ is that the fluid may not be in free fall. Shear stresses created by the solid surfaces in the trough relaxed as the fluid exited the slit. Relaxation lengths have been studied for circular jets (Scriven and Pigford, 1959; Haldar and Mitra, 1971). Similar forces affect the free falling film. By analogy, relaxation lengths for the film are expected to be on the order of one centimer for water.

Temperature profile

The theoretical prediction and experimental measurement of the average temperature as a function of position for initial velocities in the experimental range of 0.39–0.57 m/sec is typified in Fig. 4. The average dimensionless film temperature was plotted against the dimensional distance in the x-direction. The initial velocity at the slit for this experimental run was 0.39 m/sec. The data points were taken at film lengths from 1.2×10^{-2} to 6.0×10^{-2} m. The experimental values compared well with the model-predicted curve within the experimental range. For lengths greater than 6.0×10^{-2} m, the film did not remain intact and no data were collected.

Effect of condensate

The integral method quantified the resistance to heat transfer and dilution from condensed steam during direct heating. The penetration thickness, as well as the local half film thickness, and condensate layer forming on its surface are shown in Fig. 5 for the initial fluid velocity of 0.39 m/sec. The thicknesses
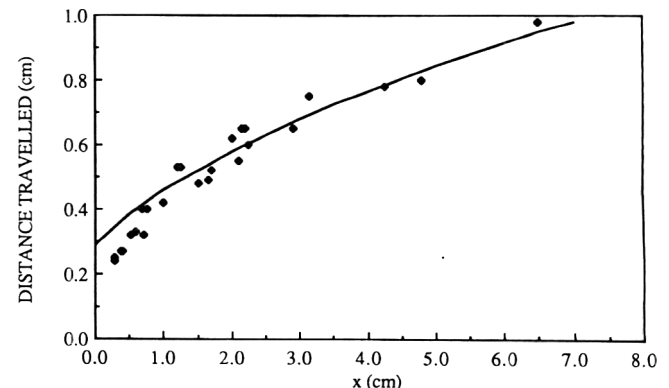
Fig. 3.—Streak photography for $u_o = 0.36$ m/sec. Distance travelled by particles in 1/125 sec. —— $u_o = 0.36$ m/sec predicted; ● $u_o = 0.36$ m/sec experimental.
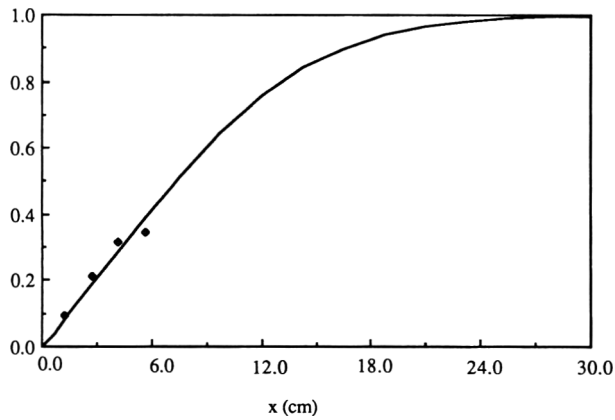
Fig. 4. — Temperature profile for water at u_c = 0.39 m/sec. —— predicted; ● experimental.
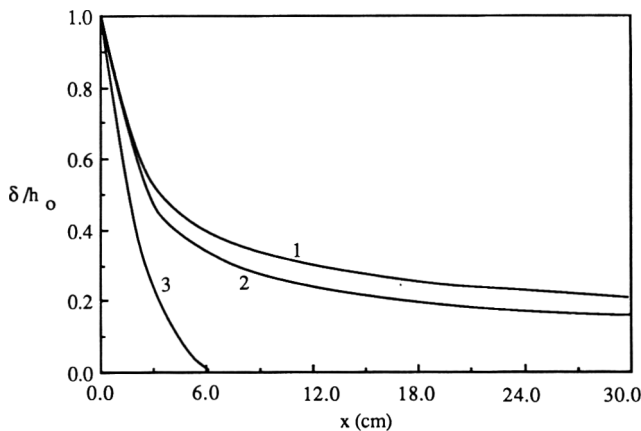
Fig. 5. — Film thickness for u_o = 0.39 m/sec. (1) $(\delta + \delta_c)/h_o$; (2) δ/h_o; (3) $(\delta - \delta_t)/h_o$.

Fig. 6. — Average temperatures for u_o = 0.39 m/sec with and without condensate addition. (1) Average temperature without condensate; (2) Average temperature with condensate.

fluid foods such as milk, mild products, and fruit and vegetable juices to achieve commercial sterility.

SUMMARY & CONCLUSIONS

This work extended the integral approach and compared analytical results with experimental measurements of temperature and velocity profiles for a free falling film of fluid. The model predicted the length of fall required to bring the average film temperature to within a specified percentage of the steam temperature for a given flow rate and initial film thickness. It also accounted for the insulating effect of the condensate and predicted that the surface temperature of the original film liquid would decrease initially before being restored to the steam temperature.

were made dimensionless by the initial half film thickness, h_o, and were plotted against distance in the direction of flow. The effect of the steam environment reached the center line of the film at a length of approximately 20% of the distance required to achieve the steam temperature. From an overall energy balance, the original fluid was diluted by 13% as it approached the steam temperature at the experimentally specified steam temperature and initial fluid temperature. If the flow rate was increased, the length of the developing region and the length required to approach the steam temperature increased.

The interfacial temperature between the original fluid and the condensate was also approximated by the integral approach. The interfacial temperature was initially equal to the steam temperature at x=0. At that point, the fluid was in contact with the saturated steam. As steam condensed on the film surface, the interfacial temperature rapidly dropped to a minimum of approximately 70% of the temperature difference, ΔT. It then approached the steam temperature as the film temperature increased.

The effect of the condensate as resistance to heat transfer is apparent in Fig. 6. The predicted average dimensionless temperature of the film was plotted against distance for the initial fluid velocity of 0.39 m/sec. Assuming no resistance to heat transfer (no condensate), the film attained a higher predicted temperature at a given position compared to the temperature profile for the film with the condensate layer. The temperatures obtained experimentally were in the range in which the condensate provided negligible resistance to heat transfer.

Future work will apply the model to the thermal process design of a commercial-type free falling film sterilizer. The model will predict the time/temperature history required for

NOMENCLATURE

Roman symbols

c_p	heat capacity, J/kg K
g	gravitational constant, m/sec^2
h	half thickness of film, m
k	thermal conductivity, J/m sec K
L	film length, m
m	local condensate rate, kg/m^2 sec
q	heat flux, J/m^2 sec
T	temperature, K
u	velocity in x-direction, m/sec
x	direction of flow, m
y	direction perpendicular to flow, m
ŷ	transformed coordinate in direction perpendicular to flow

Greek symbols

α	thermal diffusivity, m^2/sec
ΔT	temperature difference, T_{st}-T_o in K
ρ	density, kg/m^3
δ	half thickness of the film, m
λ	latent heat of vaporization, 2256 kJ/kg at 373 K
μ	viscosity, kg/m sec
ν	kinematic viscosity, m^2/sec
$\emptyset$	dimensionles temperature, $(T - T_o)/(T_{st}-T_o)$

Subscripts

c	condensate
i	interfacial
o	initial
st	steam
t	penetration

A New Water Sorption Equilibrium Expression for Solid Foods based on Thermodynamic Considerations

C. RATTI, G.H. CRAPISTE, and E. ROTSTEIN

──────────── ABSTRACT ────────────

Solution thermodynamics was used to derive a new semitheoretical expression to represent sorptional equilibrium in food products having both temperature and water content as independent variables. A number of correlations have been studied and compared with the proposed equation using different requirements, necessary for their application in mathematical modeling and engineering calculations. These criteria were their capabilities to fit the experimental data, to evaluate the derivatives and the heat of sorption, and to predict the behavior at high moistures. On this basis, it was concluded that the present expression provided an accurate description of the sorptional behavior for many food systems.

INTRODUCTION

SORPTIONAL EQUILIBRIUM DATA for water vapor-foodstuff systems are required in a number of food processes. In particular, when there is a need to design, simulate or model a process, it becomes important to have a suitable correlation. This is because the calculations involved in these activities require data input and calculations which need a continuous rather than a discrete representation.

A large number of correlations have been proposed to represent the isothermal case. They originated in the field of surface science, such as the classical BET equation (Brunauer et al., 1938) or they were proposed for food systems, such as the Henderson (1952) correlation. Because of the need to have a continuous $X = X(\emptyset, T)$ representation indicated above, it is more useful to have correlations where temperature is a variable. The number of correlations which provide this feature is much smaller. Some of the better known are the Thompson (1972) modification of the Henderson (1952) correlation, the Pfost et al. (1976) modification of Chung and Pfost's (1967) equation, and the Iglesias and Chirife (1976) modification of Halsey's (1948) equation. A very popular one is the Guggenheim-Anderson-deBoer (GAB) equation (Van den Berg, 1984).

The purpose of this work was to develop a new correlation which accounted for temperature dependence. The aim in this case was to start from basic thermodynamic considerations, as in Crapiste and Rotstein (1986), to obtain a semi-theoretical expression. This time the starting point was solution thermodynamics.

MATERIALS & METHODS

EXPERIMENTAL DESORPTION EQUILIBRIUM DATA, produced by some of the authors or retrieved from the literature, were used to put together a representative data bank. Foods considered and the corresponding data sources were: almonds (Pixton and Henderson, 1979), apples (Nunes, 1984), corn meal (Wolf et al., 1973; Labuza et al., 1985), potatoes (Crapiste and Rotstein, 1986), rice (Wolf et al., 1973; Aguerre et al., 1983) and wheat (Pixton and Henderson, 1981). The data bank was used to evaluate the predictive quality of the correlations presented in this work as compared with other cor-

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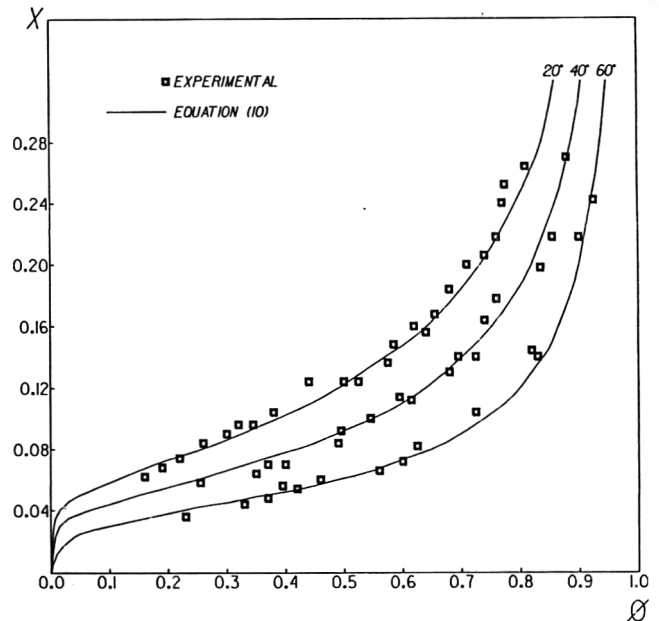
Fig. 1.—Experimental and predicted equilibrium values for potatoes. X, moisture content; $\emptyset$, relative humidity

relations. All constants were obtained using the Levenberg-Marquardt procedure for non-linear least squares problems, based on Levenberg (1944) and Marquardt (1963), as implemented in subroutine ZXSSQ/IMSL (International Mathematics and Statistical Library).

Theory

A solution thermodynamics approach was used to develop the proposed correlation. Consider the adsorbent as a solid phase made of the non-aqueous foodstuff materials; this phase is in equilibrium with a water-only gas phase made of N_{wg} moles of water vapor. As a result of this equilibrium there is a condensed phase of N_{wc} moles of water and N_d moles of absorbent. Equality of water chemical potential requires:

$$-\bar{S}_{wc}\, dT + \bar{V}_{wc}\, dp + (\partial \mu_{wc}/\partial \Gamma)_{T,p}\, d\Gamma = -\bar{S}_{wg}\, dT + \bar{V}_{wg}\, dp \quad (1)$$

where the molar coverage Γ is defined as:

$$\Gamma = N_{wc}/N_d \quad (2)$$

The above molar description of coverage makes the development independent of shrinkage. A process at constant Γ results in Eq. (1) yielding:

$$(\partial p/\partial T)_\Gamma = (\bar{S}_{wg} - \bar{S}_{wc})/(\bar{V}_{wg} - \bar{V}_{wc}) \quad (3)$$

At the low pressures associated with typical water-foodstuff equilibrium, it is acceptable to consider an ideal gas; also $\bar{V}_{wg} \gg \bar{V}_{wc}$. Using this in Eq. (3) and taking into account that at equilibrium

$$\bar{S}_{wg} - \bar{S}_{wc} = (\bar{H}_{wg} - \bar{H}_{wc})/T \quad (4)$$

Table 1 — Sorption equilibrium with temperature dependence

Key	Source	Equation
CR	Crapiste and Rotstein (1986)	$\ln \phi = -k_1 (1/T - 1/k_2)W^{-k_3} \exp(-k_4 W)$
GAB	Guggenhein et al. (1984)	$X/X_m = ck\phi / (1-k\phi)(1-k\phi + ck\phi)$
IC	Iglesias and Chirife (1976)	$\ln \phi = -\exp(k_1 + k_2 T) X^{k_3}$
PMCM	Pfost et al. (1976)	$\ln \phi = -[k_1/R(T+k_2)] \exp(-k_3 X)$
SY	Strohman and Yoerger (1967)	$\ln \phi = -k_1 \exp(k_2 X) + k_3 \exp(k_4 X) \ln p^\circ$
T	Thompson (1972)	$\ln(1-\phi) = -k_1 (T+k_2) X^{k_3}$

Table 2 — Constants for the proposed correlation

Foodstuff	c_1	c_2	c_3	q_1	q_2	q_3
			$\ln\phi = c_1 \exp(-c_2 X)X^{c3} + q_1 \exp(-q_2 X)X^{q3} \ln p^\circ$			
Almond	−0.00215	−	−1.842	1,311.52	87.473	1.925
Apple	−0.182	−	−0.696	0.232	43.949	0.0411
Potato	−0.0267	−	−1.656	0.0107	1.287	−1.513
Corn meal	−7.703	19.787	−	147.122	36.604	1.317
Rice	−8.811	17.134	−	2.621	16.720	0.110
Wheat	−9.049	21.065	−	24.868	27.994	1.022
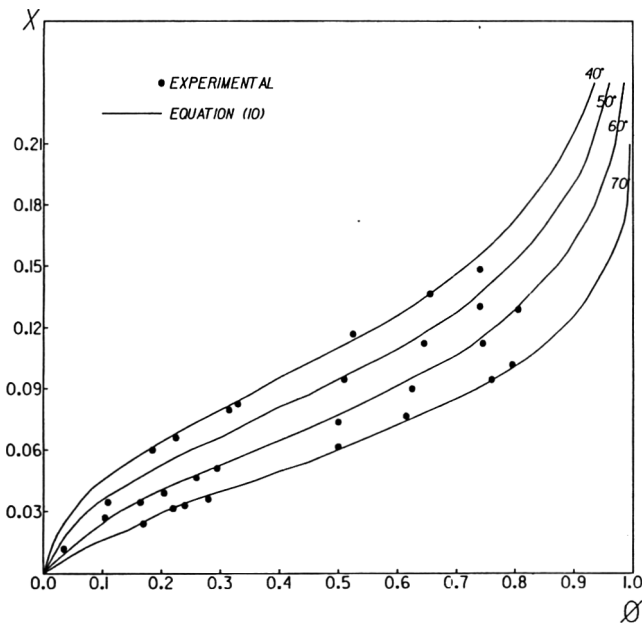
Fig. 2. — Experimental and predicted equilibrium values for rice. X, moisture content; ϕ, relative humidity

Eq. (3) results in:

$$(\partial \ln p / \partial T)_\Gamma = (\bar{H}_{wg} - \bar{H}_{wc}) / RT^2 \qquad (5)$$

In turn, for pure water, the Clapeyron Clausius equation states:

$$d \ln p^\circ / dT = (\bar{H}_{wg} - \bar{H}_{wl}) / RT^2 \qquad (6)$$

Equations (5) and (6) are similar in shape, the difference being that while $(\bar{H}_{wg} - \bar{H}_{wl})$ depends only on temperature, $(\bar{H}_{wg} - \bar{H}_{wc})$ is a function of both temperature and moisture content. To develop the correlation it is assumed that:

$$\bar{H}_{wg} - \bar{H}_{wc} = (\bar{H}_{wg} - \bar{H}_{wl}) \cdot Q'(X) \qquad (7)$$

i.e., the effect of T and X is separable and the T dependence is analogous to that of pure water. Using Eq. (7) in Eq. (5):

$$(\partial \ln p/\partial T)_\Gamma = \frac{\bar{H}_{wg} - \bar{H}_{wl}}{RT^2} Q'(X) \qquad (8)$$

which, because of Eq. (6), results in:

$$\left(\frac{\partial \ln p}{\partial T}\right)_\Gamma = \frac{d \ln p^\circ}{dT} Q'(X) \qquad (9)$$

Integrating Eq. (9) and subtracting $\ln p^\circ$ from both sides of the result, it follows:

$$\ln \phi = Q(X) \ln p^\circ + C(X) \qquad (10)$$

Equation (10) is the proposed correlation. To use it, it is suggested that:

$$Q(X) = q_1 \exp(-q_2 X) X^{q3} \qquad (11)$$

which is consistent with the exponential decrease of adsorption energy as more layers are successively adsorbed (Brunauer et al., 1938). In a similar manner:

$$C(X) = c_1 \exp(-c_2 X) X^{c3} \qquad (12)$$

The constants q_i and c_i must be obtained from experimental data.

Prediction evaluation

A number of tests were designed to evaluate the predictive capability of this correlation. These were: (a) statistical tests; (b) behavior of derivatives; (c) behavior at high moisture contents and (d) estimation of heat of sorption.

The statistical procedures were "t" test, and average residual and the percentage standard error calculations. In the first:

$$t = Y_{mean} / \left[\sum_{i=1}^{n} (Y_i - Y_{mean})^2 / (n-1)n\right]^{0.5} \qquad (13)$$

Table 3 — T-values for the different correlations and foodstuffs[a]

Foodstuff	CR	GAB	IC	PMCM	RCR[b]	SY	T
Almond	−0.093	0.117	−0.911	−1.386	−0.526	−0.899	−1.028
Apple	0.027	0.562	0.007	−0.186	−0.031	−0.014	−0.253
Potato	−0.161	0.389	−0.240	−0.519	−0.186	−0.854	−0.626
Corn meal	0.093	2.263	−0.195	0.242	0.171	0.252	−0.379
Rice	0.743	1.626	0.866	0.474	−0.046	−0.164	−0.373
Wheat	−0.203	0.945	0.851	−0.004	0.021	−0.050	−1.056

[a] Keys in Table 1.
[b] This work.

Table 4—Average residue for all correlations and foodstuffs[a]

Foodstuff[b]		CR	GAB	IC	PMCM	RCR	SY	T
Almond	Y	0.36	1.28	0.62	3.79	0.41	2.93	3.59
	E	0.44[c]	1.66	0.73	4.03	0.50[d]	3.42	3.95
Apple	Y	0.53	0.31	0.46	1.24	0.46	0.69	0.70
	E	0.67	0.36[c]	0.53[d]	1.42	0.53[d]	0.88	0.87
Potato	Y	0.57	2.22	˙.15	3.92	0.61	3.53	3.77
	E	0.74[c]	2.72	˙.45	4.92	0.77[d]	4.08	5.21
Corn meal	Y	0.86	1.04	˙.13	1.10	0.59	1.01	2.33
	E	1.06[d]	1.27	˙.38	1.25	0.71[c]	1.32	2.50
Rice	Y	1.45	1.20	2.33	2.01	0.64	0.92	1.98
	E	1.66	1.46	2.72	2.22	0.85[c]	1.03[d]	2.30
Wheat	Y	0.62	1.60	2.06	0.71	0.67	0.68	1.23
	E	0.78[c]	1.95	2.46	0.86	0.83[d]	0.83[d]	1.56
Average	Y	0.73	1.28	1.29	2.13	0.56	1.63	2.27
	E	0.89[d]	1.56	1.54	2.45	0.70[c]	1.97	2.73

[a] Keys in Table 1

[b] Y = average residue × 100; E = percentage standard error

[c] = best correlation

[d] = second best correlations on the basis of the above.
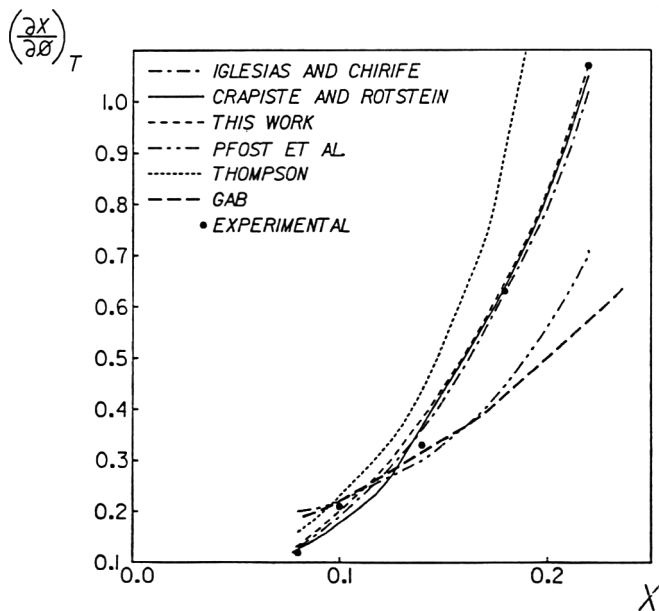
Fig. 3.— The derivative of moisture content with respect to relative humidity for potatoes as a function of moisture content (40°C)
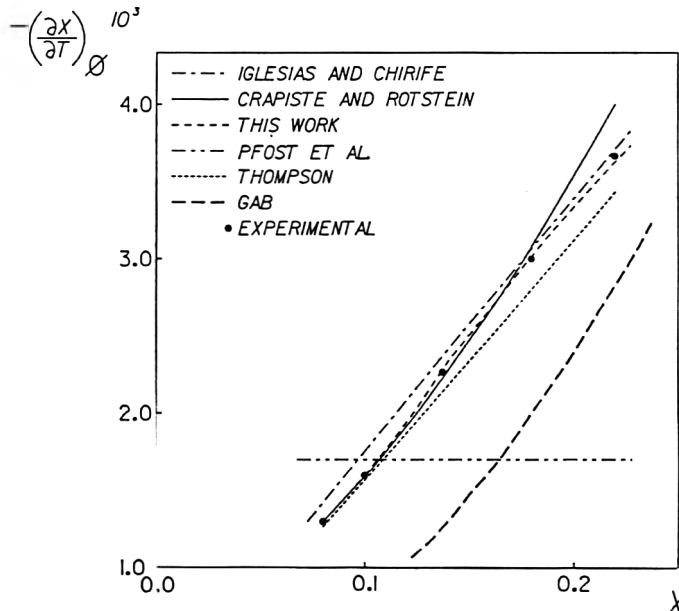
Fig. 4.— The derivative of moisture content with respect to temperature for potatoes at varying moisture (40°C)

where n is the number of observations, Y_i are the residuals obtained subtracting calculated values y_{ic} from experimental values y_i:

$$Y_i = y_i - y_{ic} \qquad (14)$$

and:

$$Y_{mean} = \sum_{i=1}^{n} Y_i / n \qquad (15)$$

The average residual and percentage standard errors were calculated using:

$$Y_{avg} = \sum_{i=1}^{n} |Y_i| / n \qquad (16)$$

$$E = 100 \left(\sum_{i=1}^{n} Y_i^2 / n \right)^{0.5} \qquad (17)$$

The proposed correlation predictions were contrasted, using the above procedures, with a number of existing correlations which explicitly account for temperature dependence. They are summarized in Table 1. The corresponding constants were obtained using the same non-linear least squares procedure as above.

RESULTS & DISCUSSION

THE c_i AND q_i CONSTANTS for almonds, apples, corn meal, potatoes, rice and wheat are shown in Table 2. It was observed that for these products the temperature dependence of the equilibrium vapor pressure followed approximately the same behavior as that of pure water. Two behaviors were identified: In one group of foods, it is acceptable at the 1% level to put $c_2 = 0$, and in another group, $c_3 = 0$, in a similar manner.

As an example Fig. 1 and 2 show experimental isotherms for potato and rice, and the corresponding predicted values obtained using Eq. (10). In the case of rice the c_i and q_i values were obtained from data at 40°C and 60°C. The values at 50°C and 70°C were obtained with those constants, thus showing that the equation can be safely used for interpolation or extrapolation of data. In a similar manner the constants for potatoes were obtained from data at 20°C and 60°C, the values at 40°C being interpolations using those constants.

Statistical tests

For the Student's t a value $\alpha = 0.5$ was selected. This resulted in $t_{n-1,\alpha} = 0.687$ for all foodstuffs but corn meal (n = 21) and $T_{n-1,\alpha} = 0.689$ for corn meal (n = 18). Table 3
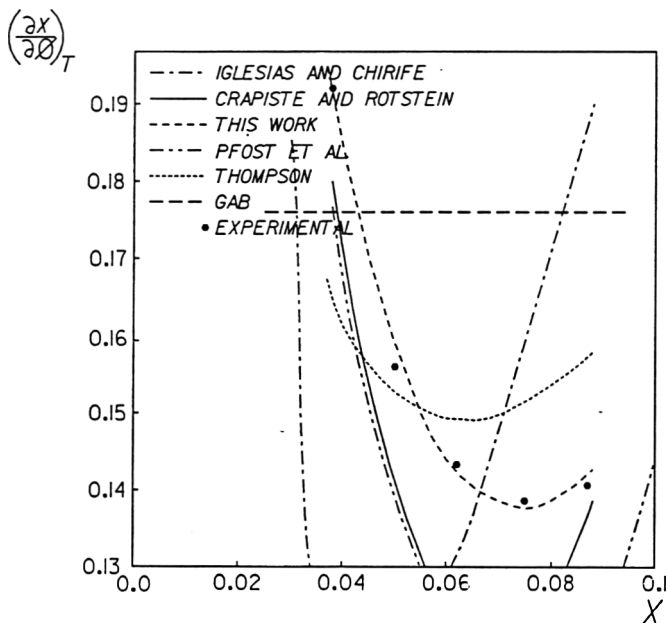
Fig. 5.—The derivative of moisture content with respect to relative humidity for rice at varying moisture (50°C)
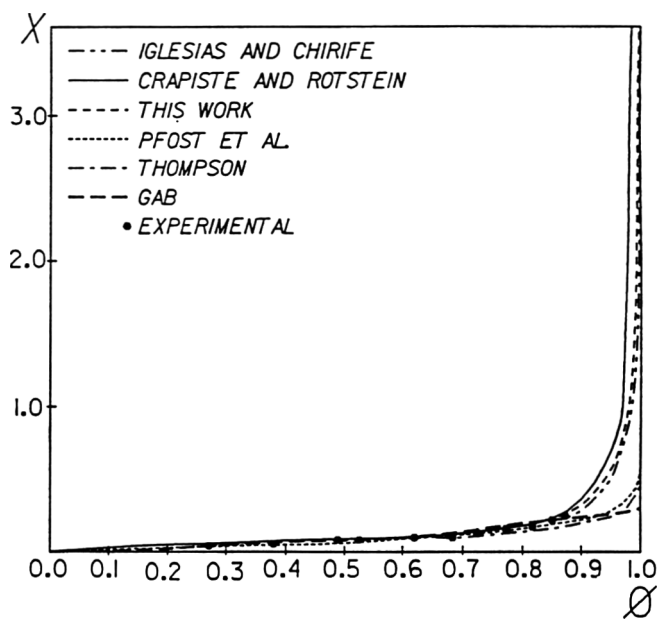
Fig. 7—Predictions of moisture at high moisture contents for potatoes at 40°C
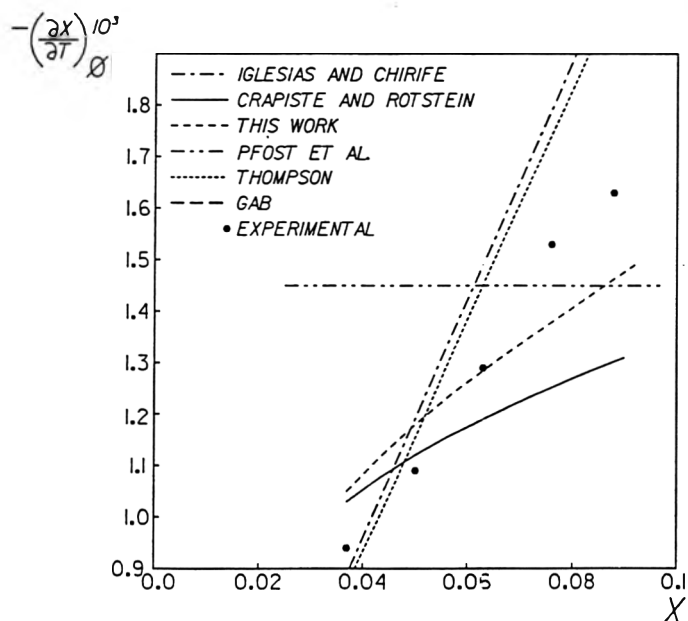
Fig. 6—The derivative of moisture content with respect to temperature for rice at varying moisture (50°C)
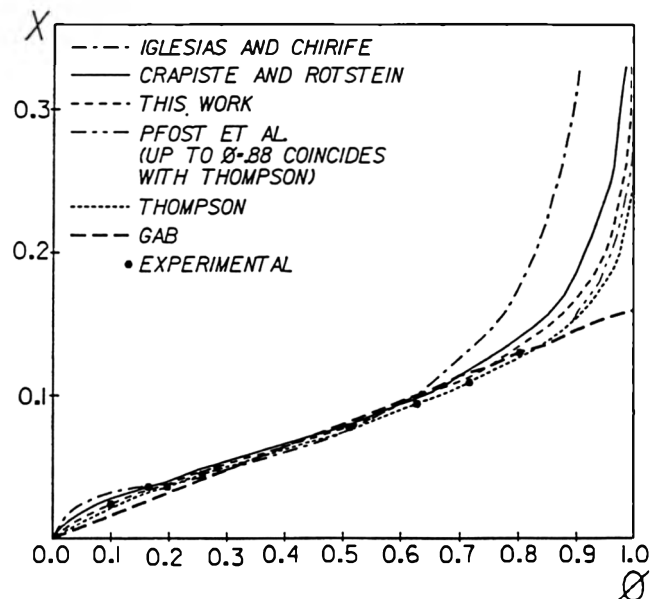
Fig. 8—Predictions of moisture at high moisture contents for rice at 60°C.

shows the values of t for the different correlations, the expectation being that $t \leqslant t_{n-1,\alpha}$. The proposed correlation met this constraint in all cases, Pfost et al. (1976) and Crapiste and Rotstein (1986) correlations in five of the six cases, Strohman and Yoerger's (1967) in four and Iglesias and Chirife's (1976), Thompson's (1972), and the GAB equation only in three cases.

The average residual and percentage standard errors are shown in Table 4. It can be seen that from both standpoints Eq. (10) provides the best prediction in terms of minimum average residual and standard errors, with Crapiste and Rotstein (1986) a close second. The worst estimates were given by Thompson (1972), Strohman and Yoerger (1976) and Pfost et al. (1976). The results show that some correlations perform better than others for a given foodstuff. The correlations of Strohman and Yoerger (1967) and Pfost et al. (1976) give better predictions

for grains, the products for which they were intended. Thompson (1972) gives the worst results when applied to products of high moisture content. The first and second best correlations in terms of the statistical evaluation given above are shown in Table 4. Equation (10) appears in this table in almost all entries.

The behavior of derivatives

Many processes of practical importance involve upsetting the water-foodstuff equilibrium and moving the system towards a new equilibrium situation. Examples are drying, conventional or controlled atmosphere storaging, packaging, refrigerating and many others. Modeling, simulating or designing these processes require posing mass transport equations of the
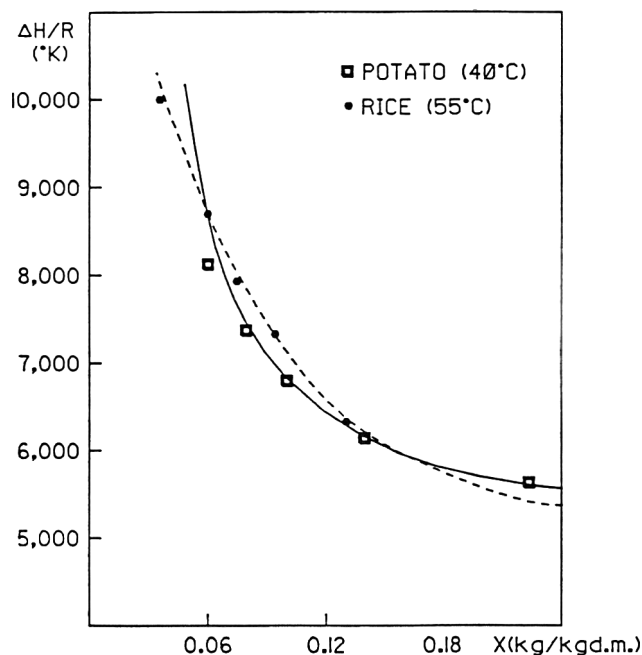
Fig. 9.—Heats of sorption as a function of moisture content.

type of (Crapiste et al., 1988);

$$\rho_s \, \partial X/\partial t + \rho_s \, w \, \partial X/\partial z = \partial(\rho_s D_{eff} \, \partial X/\partial z)/\partial z \quad (18)$$

and its analogous energy counterpart. To solve Eq. (18), local equilibrium is assumed and an equilibrium expression is required. Whatever is the expression, its functionality will be:

$$X = X(\phi, T) \quad (19)$$

This equation is then used to write $\partial X/\partial z$ in terms of ϕ and T:

$$\partial X/\partial z = (\partial X/\partial \phi)_T \, \partial \phi/\partial z + (\partial X/\partial T)_\phi \, \partial T/\partial z \quad (20)$$

To solve the partial non-linear differential equations, use is made of numerical procedures which involve discretizing the t, T, ϕ, and z differentials. It is apparent from Eq. (20) that in trying to calculate $\partial \phi$'s and ∂T's, if the derivatives do not have the proper values, there will be either undershooting or overshooting at each time step. Thus, analyzing the prediction of derivative values is an essential part of the evaluation of an equilibrium expression. The experimental behavior of $(\partial X/\partial \phi)_T$ and $(\partial X/\partial T)_\phi$ for potatoes at 40°C and the values predicted by the equations which best approximate this behavior are shown in Fig. 3 and 4. It can be seen that the $(\partial X/\partial \phi)_T$ derivative is well predicted by Crapiste and Rotstein (1986), Iglesias and Chirife (1976) and Eq. (10), while Thompson overpredicts and GAB and Pfost et al. (1976) underpredict. In turn, Iglesias and Chirife (1976) and Eq. (10) provide a good estimate of $(\partial X/\partial T)_\phi$, Crapiste and Rotstein (1986) begin to over predict as X increases, GAB underpredicts and Pfost et al. (1976) show an almost constant low value. Figures 5 and 6 provide analogous data for rice at 50°C. The only good estimate of $(\partial X/\partial \phi)_T$ is given by Eq. (10), while no correlation provides good approximation of $(\partial X/\partial T)_\phi$.

High moisture content

The behavior for potatoes and rice of different equilibrium expressions over the entire range of moistures is shown in Fig. 7 and 8. It is well known that experimental data as ϕ tends to 1 is very hard to obtain. On the other hand, for application involving the transport processes discussed earlier, an equation will be acceptable in principle if it converges to reasonable values of X_o. From Fig. 7, it can be seen that GAB, Thompson (1967) or Strohman-Yoerger and Pfost et al. (1976) converge to low values of Xo. Figure 8 indicates that, while GAB con-

verges to a low X_o, Iglesias and Chirife (1976) overpredict. The other equations provide estimates which in principle look reasonable.

Heat of sorption

An additional criterion to evaluate moisture sorption correlations for foods is given by their capacity to predict heat of sorption, which usually should decrease as the water content and temperature increase. Recently, Balaban et al. (1987) posed these facts in the following inequalities:

$$\frac{\partial}{\partial T}(\bar{H}_{wg} - \bar{H}_{wc}) = \frac{\partial}{\partial T}[RT^2 \frac{\partial}{\partial T}(\ln\phi)$$
$$+ (\bar{H}_{wg} - \bar{H}_{wl})] < 0 \quad (21)$$

$$\frac{\partial}{\partial X}(\bar{H}_{wg} - \bar{H}_{wc}) = \frac{\partial}{\partial X}[RT^2 \frac{\partial}{\partial T}(\ln\phi)] < 0 \quad (22)$$

using them to investigate the validity of a number of correlations. The proposed equation not only met the above criteria but also gives accurate estimations of the heat of sorption (Fig. 9).

CONCLUSIONS

A NEW NONISOTHERMAL equilibrium expression for water sorption in food materials was presented, and several criteria to evaluate the applicability of different equilibrium correlations were proposed. It was shown that, in average and from a statistical point of view, the proposed equation gave the best representation of the experimental data. Furthermore, this equation provided acceptable estimates of the partial derivatives of moisture content with respect to temperature and relative humidity and of the heat of sorption for different materials. In addition, the expression can be used to predict the sorptional behavior in the entire range of moistures and at different temperatures.

NOTATION

c_i	coefficients in Eq. (11)
$C(X)$	integration coefficient
D_{eff}	effective diffusivity, m²/sec
E	percentage standard error
$\bar{H}_{wg}$	molar entalphy of water vapor, J/mole
$\bar{H}_{wl}$	molar entalphy of liquid water, J/mole
$\bar{H}_{wc}$	partial molar entalphy of condensed water, J/mole
k_i	constants of correlations
n	number of observations
N	number of moles
p	vapor pressure, kPa
p^o	saturated water vapor pressure, kPa
q_i	coefficients in Eq. (12)
$Q'(X)$	function defined in Eq. (7)
R	universal gas constant, J/mole K
$\bar{S}_{wg}$	molar entropy of water vapor, J/mole K
$\bar{S}_{wc}$	partial molar entropy of condensed water, J/mole K
t	time, sec
T	temperature K
$\bar{V}_{wg}$	molar volume of water vapor, m³/mole
$\bar{V}_{wc}$	partial molar volume of condensed water, m³/mole
w	shrinkage velocity, m/sec
X	moisture content, kg water/kg dry matter
y_i, y_{ic}	experimental and calculated values
Y_i	residuals
Y_{mean}	mean residual

Effects of Lysozyme, Clupeine, and Sucrose on the Foaming Properties of Whey Protein Isolate and β-Lactoglobulin

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———————— ABSTRACT ————————

Lysozyme and clupeine interacted with β-lactoglobulin to form aggregates. Sucrose reduced the aggregation. The addition of lysozyme (0.5%) to β-lactoglobulin (2.5%) reduced the time required to reach an overrun maximum and increased foam stability and heat stability by 124% and 377%, respectively. Lysozyme (0.5%) also improved overrun (98%), foam stability (114%) and heat stability of the foams (12%) made with whey protein isolate (WPI, 5%). Lysozyme and sucrose further improved the foaming properties of β-lactoglobulin and WPI. The addition of clupeine and sucrose gave similar results. The foaming properties of β-lactoglobulin and WPI with the inclusion of sucrose and lysozyme were superior to those of egg white.

INTRODUCTION

PROTEINS enhance film formation in foams by concentrating at the interface, reducing interfacial tension and partially unfolding and associating with neighboring protein molecules to form continuous cohesive films (Cumper, 1953; Halling, 1981; Kinsella, 1981; Graham and Phillips, 1976). In homogenous protein systems, the predominant attractive forces between contiguous proteins are short-range hydrogen bonding, hydrophobic, electrostatic and Van der Waals interactions (Kinsella, 1984; Poole et al., 1984). The role of electrostatic interactions have not been fully characterized though the net negative charge on the outer film lamella helps stabilize foams via net repulsion of adjacent films (Aubert et al., 1986); however, electrostatic interactions within the film may also be important (Kinsella and Phillips, 1988).

In heterogenous protein systems, such as egg white, the constituent proteins have different isoelectric points and carry different net charges. In egg white, electrostatic interactions contribute to the excellent foaming and heat stability characteristics of egg white (Johnson and Zabik, 1981). At the natural pH of fresh egg white (pH 7-8), the basic protein lysozyme (pI 10.7) is positively charged and can interact electrostatically with the other negatively charged proteins.

Poole et al. (1984) demonstrated that the addition of low concentrations (0.01-0.1% w/v) of clupeine (pI 12) and lysozyme (pI 10.7) to solutions of acidic proteins (0.50%) improved their foam volume and stability by as much as 260% and 600%, respectively, at pH 8.0. They suggested that electrostatic interactions between basic and acidic proteins in the film enhanced foam volume and stability. The addition of sucrose together with lysozyme and clupeine enhanced the foam volume and stability by as much as 420% and 700%, respectively. They suggested that sucrose decreased the hydration of the proteins facilitating movement to the interface (Poole et al., 1984).

The objectives of this study were to determine if different combinations of lysozyme, clupeine, and sucrose could enhance the foaming properties of β-lactoglobulin, (β-Lg) or whey protein isolate (WPI) and to determine the extent of electrostatic interaction of whey proteins or β-Lg with clupeine and lysozyme (with and without added sucrose).

MATERIALS & METHODS

Proteins

The studies were conducted with the following proteins: lysozyme (14600, pI 10.7; lot # 65f-8215) and clupeine (4700, pI 12; lot # 55f-0746) purchased from Sigma, whey protein isolate (WPI) (Mitchelstown Isolates, Cork, Ireland) and β-lactoglobulin (>92% purity, 18400, pI 5.2) purified from whey protein isolate (Aschaffenburg and Drewery, 1957).

Turbidity

Before whipping, the aggregation of β-Lg in the presence of clupeine or lysozyme, turbidity was determined by transmittance at 500 nm using a Spectronic 700 spectrometer (Bausch and Lomb, Rochester, NY). The values were expressed as $Tb = (1-T_{500}) \times 100$. A value of 100 corresponds to a completely turbid solution (0 transmittance).

Viscosity

Viscosity measurements were done with a Cannon Fenske viscometer having a flow time of 249.0 sec with distilled water at 25°C. Flow times of protein solutions were measured to within ± 0.2 sec at 25 ± 0.1°C. Specific viscosity was reported.

Foaming properties

For measurement of all foaming properties, 75 mL of either 2.5% β-Lg or 5% whey protein isolate (WPI) solution were used containing 0.5% or 0.25% of lysozyme or 0.25% or 0.125% clupeine (with or without 10% sucrose). For overrun measurements this solution was whipped, the amount of air incorporated measured at 5 min intervals and recorded as percent overrun (Phillips et al., 1987).

Foam stability was measured after whipping for 15 min as described by Phillips et al. (1987). The weight of the liquid separating from the foam was continuously recorded using a Sartorius balance (model 1212MP Brinkman Instruments Co., Westbury, NY) connected to an Apple IIe computer using an interface board (IMI State College, PA). The time to 50% drainage was reported as an index of instability. The drained liquid was collected in a tared container on the balance pan.

The heat stability of the foams was measured after whipping for 5 min intervals. A petri dish (107.5 mL) was filled with the protein foam and placed in an oven for 30 min at 90°C. Constant relative humidity was maintained in the oven. The petri dish containing the foam was removed after 30 min and allowed to cool to 25°C. The free liquid in the dish was removed and the height and diameter of the foam remaining were measured. The reduction in volume was calculated using the equation for a right circular cylinder [Volume (mL) $= \pi \times$ (diam (cm)/2)$^2 \times$ height (cm)].

Data were analyzed using the statistical analysis package, release 82.3, computer program with the general linear models option (SAS, 1985). Comparison of means for treatment effects was based on Duncan's multiple-range test.

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RESULTS & DISCUSSION

Turbidity and viscosity

The development of turbidity was used as an index of protein-protein interaction. The interaction of β-Lg (2.5%) and lysozyme (0.5%) was substantial as indicated by the increase in turbidity of the solution which ranged from 3.7 for the β-Lg to 99.9 for the β-Lg, lysozyme solution, respectively. This was accompanied by a 25% increase in viscosity of the solutions (Table 1). The addition of sucrose (10%) to the β-Lg solution caused a small reduction in turbidity and a 358% increase in viscosity (Table 1). The addition of both lysozyme and sucrose to the β-Lg solutions decreased the turbidity by 55% compared to the β-Lg, lysozyme solution and increased the viscosity by 382%. The sucrose apparently reduced the extent of interaction between β-Lg and lysozyme probably by altering the structure of water around the proteins as proposed by Arakawa and Timasheff (1982).

The addition of clupein (0.25%) to β-Lg caused the formation of a very turbid solution, indicating that aggregation was occurring following interaction of the β-Lg and clupeine. The inclusion of sucrose (10%) limited the aggregation of the clupeine and β-Lg as indicated by a 52% reduction in turbidity compared to the clupeine, β-Lg solutions. The viscosity of the β-Lg, clupeine solution was increased by 42% with the addition of sucrose (Table 1).

Foaming studies

Lysozyme. The overrun obtained with β-Lg (2.5%) progressively increased with time of whipping attaining a maximum after 20 min whipping (Fig. 1). The addition of lysozyme (0.25%) did not improve the overrun maximum but decreased the time required to reach a maximum. This behavior resembled that of egg white in which overrun after reaching an optimal whiptime is decreased by continual whipping, i.e., overwhipping (Phillips et al., 1987). Overwhipping egg white protein disrupts film formation and reduces overrun because of the formation of an insoluble lysozyme/ovomucin complex

(Johnson and Zabik, 1981). A similar interaction may be occurring between β-Lg and lysozyme. However, by increasing the lysozyme concentration to 0.5% the overrun changed little after 5 min whipping. Evidently, increasing the lysozyme content negated the overwhipping effect (Fig. 1).

The stability of the β-Lg foam was increased 124% by 0.5% lysozyme while 0.25% lysozyme had no significant effect (p < 0.05) (Fig. 2). Thus, the lysozyme concentration affected the foam stability and overrun maximum of β-Lg in the same way (Fig. 1 and 2). Whipping egg white for 15 min also causes a reduction in foam stability which implies that a similar mechanism of protein/protein interaction may be involved (Phillips et al., 1987).

Foams made with β-Lg alone showed very poor heat stability which decreased further with increasing whiptime (Fig. 3). However, increasing the concentration of lysozyme increased the heat stability of the foam with an optimum improvement of 377% for 0.5% lysozyme (Fig. 3). This may be the result of the formation of a stronger, more elastic film

Table 1 — Effect of lysozyme and sucrose on the turbidity and specific viscosity of β-lactoglobulin

Protein	Turbidity[a]	Specific viscosity
2.5% β-lactoglobulin, pH 8.0	3.7	0.0865
β-lactoglobulin & 0.50% lysozyme	99.9	0.1085
β-lactoglobulin & 10% sucrose	2.4	0.3959
β-lactoglobulin, 10% sucrose & 0.50% lysozyme	44.9	0.4173
β-lactoglobulin & 0.25% clupeine	98.3	0.0727
β-lactoglobulin, 10% sucrose & 0.25% clupeine	40.4	0.4286

[a] Turbidity = $(1 - T_{500}) \times 100$

Fig. 2 — Effects of lysozyme on the foam stability of β-lactoglobulin (pH 8.0, 2.5% conc) whipped for 15 min (with 95% confidence intervals).

Fig. 1 — Effects of lysozyme on the overrun of β-lactoglobulin (pH 8.0, 2.5% conc).
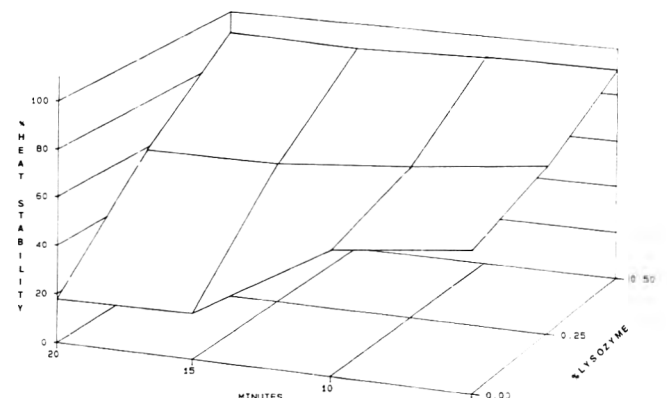
Fig. 3 — Effects of lysozyme on the heat stability of β-lactoglobulin foams (pH 8.0, 2.5% conc).

which was capable of holding the expanding air and resisting denaturation during heating.

Sucrose. The addition of sucrose reduced the maximum overrun of β-Lg (Fig. 4). This may reflect the knowledge that sucrose limits protein unfolding and protein/protein interaction (Poole et al., 1987). Sucrose and lysozyme acted synergistically to increase the maximum overrun of β-Lg and the duration of whipping had a more important effect in the presence of sucrose (Fig. 4). The overrun of β-Lg increased twice as fast after whipping 5 min with lysozyme than when both sucrose and lysozyme were added (Fig. 1 and 4). After 10 min whipping, the effect was reversed with the sample containing sucrose having three times the overrun increase compared to the sample without sucrose (Fig. 1 and 4). The higher overrun after 10 min whipping with added sucrose suggested that more energy, i.e., longer whipping time, was required to cause unfolding of the β-Lg and to allow for more cohesive film formation. The reduction in turbidity of the β-Lg solution containing lysozyme and added sucrose prior to whipping suggested that less protein was involved in protein aggregation; hence more protein was available for foam formation (Table 1).

Sucrose did not improve the foam stability but lysozyme and sucrose together improved the overrun and the stability of β-Lg foams (Fig. 4 and 5). Less stable foams were obtained at higher overruns since more gas was trapped in smaller bubbles with less protein per unit area to retain the air. Thus, the similar stability of foams made from the β-Lg containing lysozyme and sucrose indicates that the system was further stabilized by the presence of sucrose. The greatest improvement in foam stability from added sucrose was obtained with 0.25%
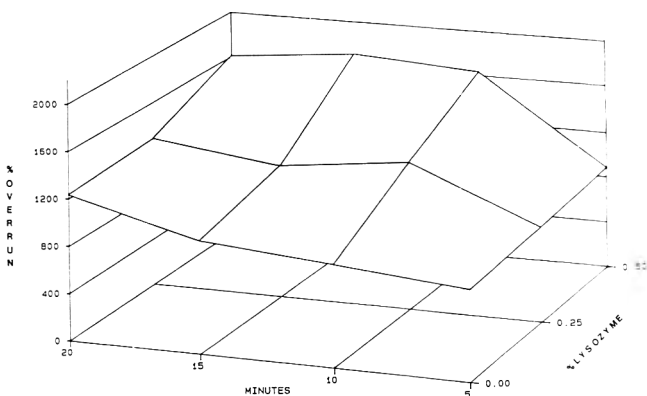
Fig. 4 — Effects of lysozyme and sucrose (10%) on the overrun of β-lactoglobulin (pH 8.0, 2.5% conc).
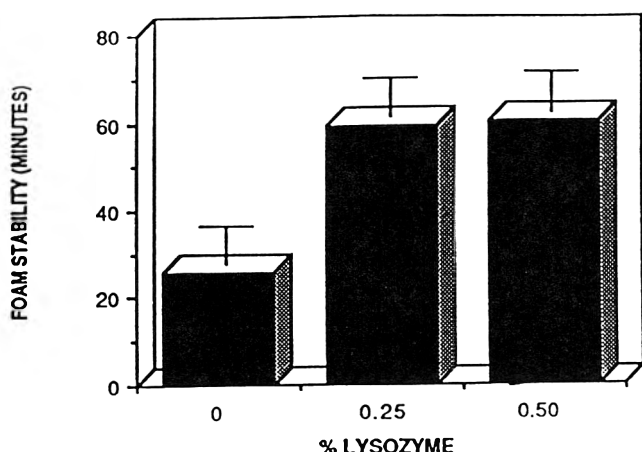
lysozyme (Fig. 2 and 5). As described above, lysozyme alone did not improve foam stability because after 15 min whipping the formation of β-Lg, lysozyme aggregates reduced the amount of protein available for film formation (Fig. 2). This is, perhaps, analogous to overwhipping. However, the addition of 0.25% lysozyme and sucrose brought about a 91% increase in the foam stability of β-Lg (Fig. 5). The higher overrun obtained with the addition of sucrose suggests that sucrose decreased the overwhipping effect (Fig. 4). The reduction in foam stability for β-Lg with sucrose suggests that sucrose retards the interaction of β-Lg molecules and thus decreases the stability of the films surrounding the air bubbles (Fig. 5).

Adding sucrose to the β-Lg solutions improved the heat stability of the foams by 292% (Fig. 6). Poole et al. (1987) determined that added sucrose retards the gelation process such that more orderly matrix formation can occur which allows the films to remain elastic and intact during the critical stages of heating the foams. Adding lysozyme and sucrose increased the heat stability of β-Lg foams by 335% and 429% for 0.25% and 0.5% lysozyme, respectively (Fig. 6).

The effects of clupeine were similar to those obtained with lysozyme except that clupeine was bound more tenaciously to the β-Lg which restricted overrun development (Table 2). Sucrose improved the overrun of the β-Lg clupeine mixture. The overwhipping effect was observed for 0.25% clupeine (Table 2). Clupeine improved foam stability and the heat stability of foams. The addition of sucrose further improved both foam and heat stability (Table 2).

Whey protein isolate

The effects of lysozyme, clupeine and sucrose on whey protein isolate (WPI) were investigated because purified proteins are rarely used in foods. The addition of lysozyme (0.5%) improved overrun (98%), foam stability (114%) and the heat stability of the foams (12%) made with WPI (Fig. 7a, b and c, respectively). Lysozyme and sucrose further improved the foam stability of WPI (Fig. 7b). Noteworthy, the addition of sucrose and lysozyme made the foaming properties of β-Lg and WPI superior to egg white (Table 3).

The addition of clupeine and sucrose to WPI gave similar results as those observed with lysozyme and sucrose (Table 2). The synergism between sucrose and clupeine was more evident than with lysozyme. The addition of sucrose and clupeine to WPI improved overrun, foam stability and the heat stability of the foam by 55%, 300%, and 38%, respectively.

In conclusion, clupeine and lysozyme improved the foaming properties of β-Lg and WPI. Sucrose acted synergistically to

Fig. 5 — Effects of lysozyme and sucrose '10%) on the foam stability of β-lactoglobulin (pH 8.0, 2.5% conc) whipped for 15 min (with 95% confidence intervals).
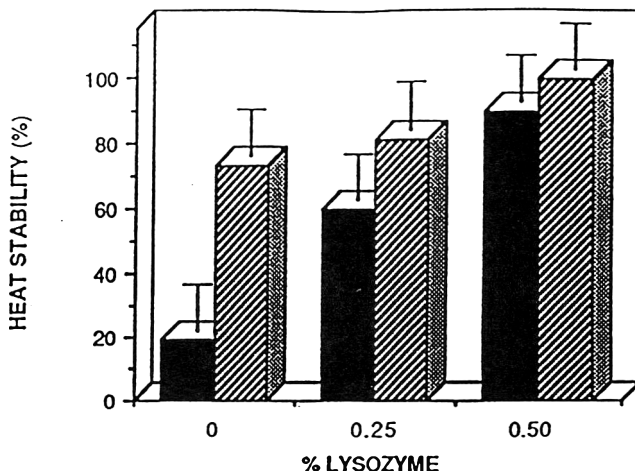
Fig. 6 — Effects of lysozyme and sucrose (10%) on the heat stability of β-lactoglobulin foams (pH 8.0, 5% conc) (with 95% confidence intervals). ■ No sucrose; □ 10% Sucrose.

Table 2—Effect of clupeine and lysozyme on the foaming properties of β-Lg (2.5%) and WPI (5%), pH 8.0 using 75 mL of protein solution

Treatment[a]	Overrun (%)				Foam Stability (MIN)	Heat stability of the foam (%)			
	5	10	15	20		5	10	15	20
β-Lg	875	1010	1346	1583	30.9	57.3	50.9	18.7	17.8
β-Lg & 0.25% lys	999	1201	939	1123	35.3	70.0	63.6	58.9	58.9
β-Lg & 0.50% lys	1205	1251	1282	1374	69.3	90.7	90.7	89.1	90.7
β-Lg & 10% sucrose	757	858	951	1234	25.9	92.3	82.7	73.2	70.0
β-Lg, suc & 0.25% lys	813	1267	1140	1284	59.0	89.1	81.1	81.1	85.9
β-Lg, suc & 0.50% lys	882	1633	1706	1603	60.5	100.2	100.2	98.6	98.6
β-Lg & 0.125% clu	967	1055	942	881	42.0	90.7	87.5	87.5	87.5
β-Lg & 0.25% clu	649	559	540	530	43.8	79.5	75.8	51.9	47.6
β-Lg, suc & 0.125% clu	705	802	913	1008	86.9	97.0	95.4	95.4	93.8
β-Lg, suc & 0.25% clu	722	1328	1100	967	63.9	100.2	85.5	85.1	86.7
WPI	608	616	640	663	26.2	76.3	76.3	76.3	76.3
WPI & 0.25% lys	752	833	1041	1250	45.0	84.3	81.1	77.9	54.1
WPI & 0.50% lys	724	867	1266	1357	56.0	98.6	93.8	85.9	71.6
WPI & 10% sucrose	495	549	578	626	31.1	95.4	97.0	98.6	98.6
WPI, suc & 0.25% lys	658	760	923	1113	56.6	101.8	100.2	98.6	100.2
WPI, suc & 0.50% lys	691	832	1192	1240	67.3	98.6	98.6	98.6	100.2
WPI & 0.125% clu	691	829	1133	1197	72.3	93.8	93.8	92.3	89.1
WPI & 0.25% clu	865	978	1124	1249	58.5	95.4	95.4	95.4	93.8
WPI, suc & 0.125% clu	621	835	896	1025	80.5	101.8	101.8	101.8	101.8
WPI, suc & 0.25% clu	640	826	994	1013	105.0	101.8	104.8	104.9	101.8

[a] β-lg = β-lactoglobulin; WPI = whey protein isolate; lys = lysozyme; clu = clupeine, suc = sucrose.
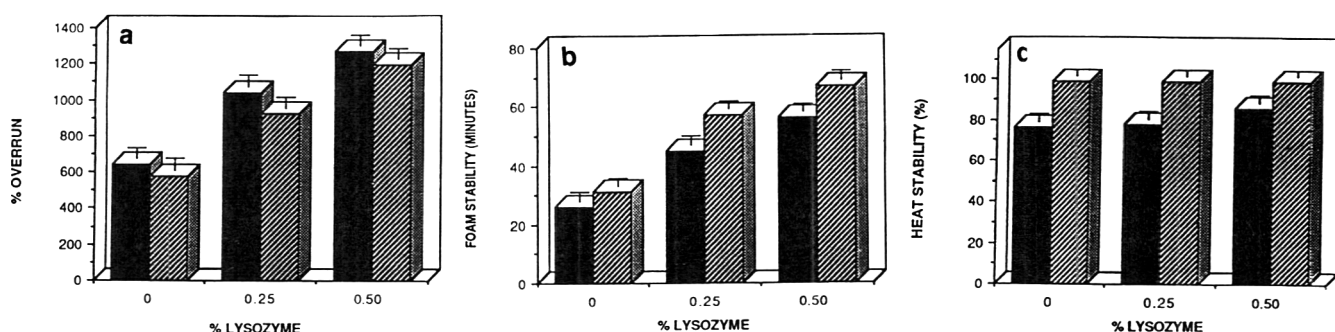
Fig. 7 — Effects of lysozyme on the overrun (a), foam stability (b), and heat stability (c) of WPI (pH 8.0, 5% conc) (with 95% confidence intervals). ■ No sucrose; □ 10% Sucrose.

Table 3—Foaming properties of whey protein isolate, beta-lactoglobulin and egg white[a]

Protein[c]	Overrun (%)	Foam stability (Min)	Foam heat stability (%)	Remaining overrun[b] (%)
β-Lg	1346	31	19	251
β-Lg, lysozyme & sucrose	1706	61	99	1689
WPI	640	26	76	488
WPI, lysozyme & sucrose	1192	67	99	1180
EWP	1162	42	57	667

[a] Each was whipped for 15 min using 5%, pH 8.00 solutions for whey protein and egg white and 2.5%, pH 8.00 solutions for beta-lactoglobulin.
[b] A remaining overrun = % overrun × % foam heat stability
[c] β-Lg = β-lactoglobulin; WPI = whey protein isolate; EWP = egg white protein

further improve the foaming properties of β-Lg and WPI. The resultant foams were in many ways superior to egg white foams.

Lee and Timasheff (1981) showed that the stabilizing effect of sucrose originates from the preferential hydration of proteins in solution. Arakawa and Timasheff (1982) demonstrated that sugars can strongly enhance the self-association of β-Lg in solution. This association might better explain the synergistic effect of sucrose on β-Lg, lysozyme and β-Lg, and clupeine foams by the reduction of aggregation. This reduction in aggregation allowed more protein to be involved in film formation and stabilization. The sucrose might have limited the interaction of the basic proteins, clupeine and lysozyme with β-Lg and hence reduced the characteristic aggregation that occurred. Furthermore, the protein aggregates formed in the pres-

ence of sucrose, may have been easier to disrupt during whipping and thereby, formed films more easily.

Sucrose improved the heat stability of the β-Lg and WPI foams. The sucrose may have allowed formation of a better network in the film instead of random aggregation during heating which would disrupt the film (Poole et al. 1987). Lysozyme and clupeine also improved the heat stability of the β-Lg and WPI foams. Basic proteins such as clupeine may alter the structure of flexible acidic proteins at a pH between the pIs of the two proteins (Poole et al., 1987). This interaction may lead to the formation of an elastic film which is disrupted less rapidly during heating. Clupeine appeared to be more tightly associated with β-Lg than lysozyme as observed by a decrease in overrun. Molecular size appeared to be an important variable in electrostatic interactions. Poole et al. (1984) reported that the basic amino acid, arginine, did not enhance foaming and clupeine lost its capacity to enhance ovalbumin foams after 18.4% hydrolysis (Poole et al., 1987). These studies suggested that a minimum size for the basic protein might be required for electrostatic interaction and enhancement of foaming properties. The use of basic proteins in conjunction with acidic proteins such as whey protein isolate might prove very useful for stabilizing food foams. Further studies are needed to investigate the interaction between basic and acidic proteins in films and to determine the mechanism of foam stabilization.

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WATER SORPTION EQUILIBRIUM FOR SOLID FOODS. . .From page 742

Y_{avg}	average residual
z	spatial coordinate
Γ	molar coverage, mol water/mol absorbent
μ_{wc}	water chemical potential, J/mole
ρ_s	solid density, kg/m^3
φ	relative humidity

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A Research Note

Postmortem Degradation of Titin and Nebulin of Beef Steaks Varying in Tenderness

T.J. ANDERSON and F.C. PARRISH JR.

———————— ABSTRACT ————————

Purified myofibrils were isolated from "tender" and "less-tender" bovine *longissimus* muscle at death and at 1, 3, 7, and 14 days of postmortem storage (4°C). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to detect changes in the my-ofibrillar/cytoskeletal proteins, titin and nebulin. Titin and nebulin bands were observed to be less intense on gels from "tender" than from "less-tender" steaks. These results suggest that titin and nebulin were more rapidly degraded in "tender" than in "less-tender" steaks, and that the extent of beef loin steak tenderness may be dependent upon the postmortem degradation of titin and nebulin.

INTRODUCTION

TWO large-molecular-weight proteins having a cytoskeletal function, titin and nebulin (Wang et al., 1979), have been shown to undergo degradation in beef longissimus muscle during postmortem storage (Lusby et al., 1983). Very recently, titin and nebulin have also been shown to be susceptible to degradation by the natural muscle protease, calcium-activated factor (Zeece et al., 1986). Wang et al. (1984) demonstrated that titin is a closely spaced doublet (T_1 and T_2) and suggested that titin may be the major protein composing a set of very thin, longitudinally running elastic filaments in the sarcomere with the function of providing structural integrity to the muscle fiber. Indeed, we have demonstrated that gap filaments in beef muscle were composed of titin (LaSalle et al., 1983). Wang and Williamson (1980) reported that nebulin was a component of the N_2-line of skeletal muscle and that it may function in the three-dimensional lattice of thin filaments in the I-band.

Locker and Leet (1976a, b) discovered a third set of filaments in skeletal muscle, termed gap filaments. Locker et al. (1977) has also proposed a theory of meat tenderness based on the disassembly and/or degradation of gap filaments.

The objective of this research was to determine the difference in postmortem degradation of titin and nebulin from tender and less-tender bovine longissimus muscles.

MATERIALS & METHODS

MYOFIBRILS were prepared from longissimus muscle of two "tender" and two "less-tender" loin steaks within 45 min of exsanguination, and at 1, 3, 7, and 14 days of postmortem storage at 4°C. "Tender" and "less-tender" steaks were characterized by sensory panel evaluation and Warner-Bratzler Shear force values. "Tender" steaks were scored moderately to very tender and "less-tender" steaks were scored moderately tough to slightly tender. Highly purified myofibrils were prepared from minced muscle (trimmed free of fat and connective tissue) by the method of Goll et al. (1974). Protein concentration of purified myofibrils was then determined by the biuret method (Robson et al., 1968). Gel samples were prepared by a modified method of Lusby et al. (1983). Two hundred microliters tracking dye [20% SDS; 40% sucrose; 1M morpholinoethanesulfonic acid (MES) in NaOH, pH 6.5; bromophenol blue] and 100 μL mercaptoethanol were added to 1 mL sample myofibril (diluted to a protein concentration of 2 mg/

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3.2%

0 Day
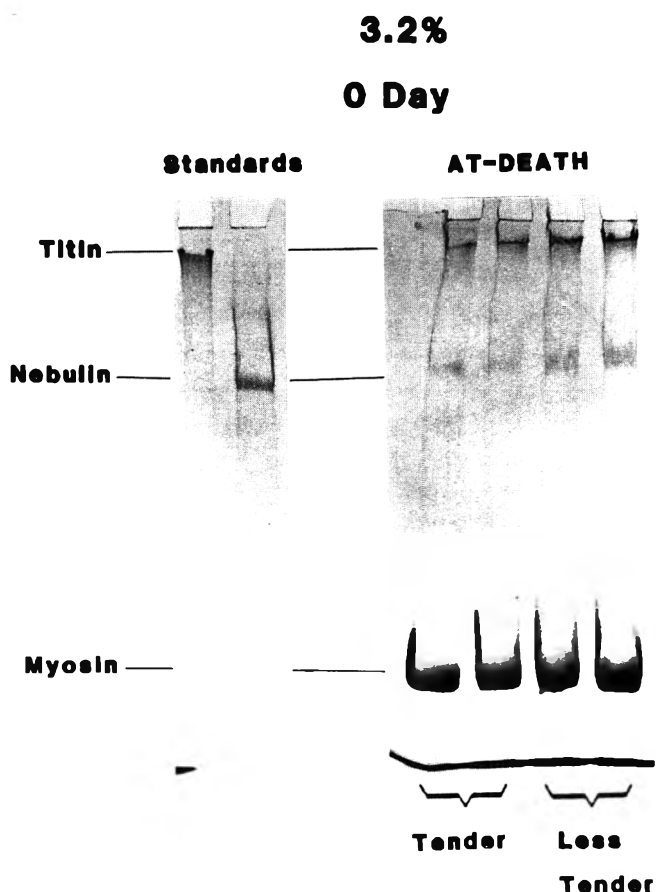
Fig. 1.—3 2% SDS-polyacrylamide gel electrophoretogram of myofibrils from two tender and two less-tender at-death bovine longissimus samples.

mL). Gel samples were than heated for 20 min at 50°C and frozen at −29°C for subsequent gel electrophoresis.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 3.2% polyacrylamide [acrylamide/bisacrylamide, 30:0.8 (w/w), Tris-HCl (pH 8.9)] and 5.0% polyacrylamide [acrylamide/bisacrylamide, 100:1 (w/w), Tris-HCl (pH 8.0)] slab gels for the detection of nebulin and titin; 10% polyacrylamide [acrylamide/bisacrylamide, 30:0.8 (w/w), Tris-HCl (pH 8.9)] with a 5% polyacrylamide stacking gel was used to detect lower-molecular-weight myofibrillar proteins, especially the 30,000-dalton component. Sixty micrograms protein were loaded on each gel lane. Gels were stained according to the method of Lusby et al (1983) except that only 0.1% (w/v) Coomassie brilliant blue was used. Finally, after the SDS-PAGE was completed and gels photographed, densitometer scans of gels were made by using the Hoefer Scientific Instruments GS 300 Transmittance/Reflectance Scanning Densitometer.

RESULTS & DISCUSSION

DIFFERENCES IN AMOUNTS of titin and nebulin between "tender" and "less-tender" steaks seem to exist already in the at-death samples (taken within 45 min of exsanguination)

5%

DAYS POSTMORTEM

Fig. 2.—5.0% SDS-polyacrylamide gel electrophoretogram of myofibrils from tender and less-tender bovine longissimus samples at 1, 3, 7, and 14 days postmortem.

(MacBride and Parrish, 1977) earlier in "tender" than in "less-tender" loin steaks. These results are supported also by the work of Paterson and Parrish (1986) showing that less titin was present in a tender muscle, the Infraspinatus, than in a less-tender muscle, the Rhomboideus. Moreover, our results support the theory of meat tenderness based on gap filaments (Locker et al., 1977; Locker, 1984), especially in view of our work showing that titin is a protein of the gap filament (LaSalle et al., 1983), the degradation of titin and nebulin in postmortem muscle (Lusby et al., 1983) and their degradation is caused by the natural muscle protease, CAF (Zeece et al., 1986).

In summary, the results of this study demonstrate that titin and nebulin are more rapidly and extensively degraded in "tender" than in "less-tender" loin steaks. Thus, tenderization of beef loin steaks may be dependent on, or at least in part due to, the degradation of these two large cytoskeletal proteins. Use of a rapid, accurate method of analysis to determine differences in the degradation of titin and nebulin of "tender" and "less-tender" beef would seem to have potential market value. Early and accurate identification of titin and nebulin, or their breakdown products, could prove to be useful as an objective method of quality grading fresh beef.

(Fig. 1). The more prominent titin and nebulin bands in the "less-tender" steaks suggest that muscle of "less-tender" steaks contains larger amounts of titin and nebulin than muscle from more tender steaks, or more significant degradation of titin and nebulin occurs in muscle of "tender" steaks.

In "tender" steaks, titin was degraded to a mixture of T_1 and T_2 within 3 days postmortem, then to predominantly a T_2 band at 7 days postmortem (4°C), and finally to smaller polypeptides at 14 days of postmortem storage (4°C). On the other hand, in "less-tender" steaks, degradation of titin was slower and the T_2 fragment of titin was still prominent at 14 days postmortem (Fig. 2).

In "tender" steaks, nebulin was more rapidly degraded (less intense or no bands) compared with "less-tender" steaks at 3 days postmortem storage (4°C) (Fig. 2).

Results from densitometer scans substantiated the titin and nebulin gel patterns observed on SDS-PAGE of "tender" steaks and "less-tender" steaks. Titin from "tender" steaks was about one half as much as for "less-tender" steaks at 3 and 7 days postmortem. Indeed, the densitometer scan changed very little for titin from the "less-tender" steaks at 1, 3 and 7 days postmortem. Correspondingly, nebulin from "tender" steaks had much lower densitometer readings than nebulin from the "less-tender" steaks at 3 and 7 days postmortem (about 50% less at 7 days). Furthermore, 10% SDS-PAG electrophoretograms showed the presence of the 30,000-dalton component

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A Research Note

Resistance to Tear: An Instrumental Measure of the Cohesive Properties of Muscle Food Products

J.H. MacNEIL and M.G. MAST

ABSTRACT

An instrument was developed to measure cohesive properties of re-structured muscle foods. The device, an attachment for the Texture Test System, consists of a spiked split-platform designed to hold a meat slice in place as the two sections of the platform are pulled apart. The force [referred to as resistance to tear (RTT)] required to tear a sample is transmitted via a transducer located at the stationary end of the split-platform. Experiments in which fish, broiler or turkey meat patties were formulated to provide differences in cohesive properties, showed that RTT measurements were responsive to the effects of formulation manipulations and corresponded well with sensory evaluations.

INTRODUCTION

THE MUSCLE FOOD INDUSTRY is currently marketing products, such as nuggets, tenders, strips, cutlets, and patties. While some of these are made of intact muscle, others are fragmented and restructured, and contain ingredients designed to provide required flavor, texture, and color. Since cohesiveness is an important physical property of restructured products, there is a need to quantify this parameter by instrumental methods, as well as by sensory methods. The purpose of this study was to develop and test an apparatus designed to measure the binding or cohesive properties of restructured muscle foods.

MATERIALS & METHODS

Instrument development

In previous tenderness/texture studies on poultry and fish products conducted at Penn State University, a Texture Test System (Food Tech. Corp., Rockville, MD) and an Instron Universal Testing Machine were utilized. The attachments available for these instruments, however, were not effective in obtaining appropriate data on cohesiveness of patty products. It was determined that a device that could pull a patty apart on a horizontal plane without appreciable friction would be more effective. A collaborative effort was established with Food Technology Corporation of Rockville, MD, manufacturers of the Texture Test System, which resulted in the development of an instrument to measure cohesive properties of patty-type products. The instrument (Fig. 1) was developed as an attachment to the TP-2 Texture Press and consisted of a spiked split-platform with a force transducer at the static end.

This split platform holds the meat patty in place during separation as the two sections of the platform are pulled apart; the product being evaluated usually breaks in the area of platform separation. The force

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used in the separation process is delivered to the platform by a rod and chain device attached to the texture press ram. The resulting "resistance-to-tear" (RTT) measurement is recorded on a FTC-TR-5 Texturecorder (Food Technology/Corp., Rockville, MD) in the same manner as that used in obtaining shear values. Results are reported as N/cm^2.

The apparatus reported here is similar in appearance to the slice-holding accessary (SHA) developed by Union Carbide and described by Gillett et al. (1978). However, the two systems differ in the manner in which tensile strength is transmitted. In the SHA, the cable transmitting the force required to tear the sample was attached to the moving portion of the slice holder, which travelled along parallel rods; according to Gillett et al. (1978), frictional forces were inherent in the apparatus and had to be accounted for. In the RTT apparatus, the force is transmitted via a transducer, located at the pivoting "stationary" end of the split-platform, thereby eliminating the frictional forces present with the SHA.

Preparation of test products

Test products were obtained from a series of experiments in which meat from fish, broilers, spent layers or turkeys was used to prepare patties, using texture modifying additives and procedures. Ingredients, shown in Table 1 as "treatments," were mechanically separated poultry (MSP), granulated fish muscle (GFM, fine particles produced during the sawing of frozen fish block), spent layer meat (various combinations of breast and leg, and types of fragmentation), and turkey meat with NaCl and/or phosphates.

Sensory tests

A panel of 10–12 graduate students, consisting of both males and females, was used to evaluate binding strength in poultry and fish patties. Training sessions were held prior to test sessions to develop terminology that panel members felt reflected a measurement of the test characteristic, i.e., chewiness or cohesiveness. Chewiness measurements were determined using magnitude estimation (Moskowitz, 1977), while cohesiveness measurements were made using a 10 cm line, anchored with the terms "less cohesive; crumbly" and "more cohesive; bound."

Data were subjected to analysis of variance, using the general linear models (GLM) procedure (SAS, 1985). Significant means were separated by the Student-Newman-Keuls test.

RESULTS & DISCUSSION

THE SENSORY and mechanical measurements obtained from the poultry and fish test products are summarized in Table 1. With broiler and fish patties, when either MSP or GFM replaced ground broiler meat or ground fish fillet, sensory measurements for chewiness decreased significantly ($P < 0.05$). In these particular experiments, both shear values and RTT values
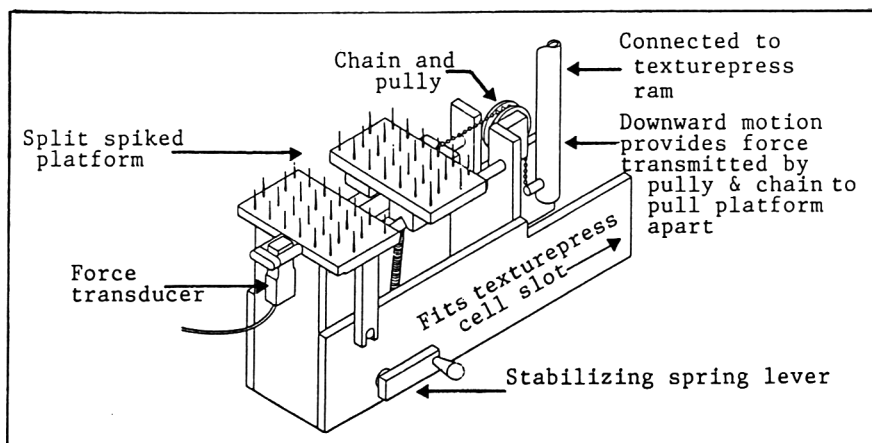
Fig. 1 — Resistance to tear apparatus (RTT)

Table 1 — Mechanical and sensory measurements of fish and poultry meat patties made with various ingredients

Meat source	Treatment	Sensory measurements[a]		Mechanical measurements N/cm²	
		Cohesiveness	Chewiness	Shear	Resistance to tear (RTT)
Fish[b]					
mince & GFM	0% GFM	---	1.10a[c]	6.1a	2.0a
	50% GFM	---	0.85ab	5.0b	1.5a
	100% GFM	---	0.81b	4.7b	0.4b
Chicken broiler[b]					
flaked & MSP	0% MSP	---	1.01a	11.7a	4.3a
	50% MSP	---	0.76a	10.4a	4.4a
	100% MSP	---	0.35b	3.5b	1.5b
Chicken spent layer[d]					
flaked & ground	100% Breast	---	1.10a	15.6b	1.9a
	50-50% Breat-leg	---	1.10a	17.0a	1.6b
	100% Leg	---	1.06a	17.1a	1.2c
Chicken spent layer[d]					
breast & leg	Flaked-Comitrol	---	0.98a	14.1c	1.9a
	Finely-ground (0.64 cm)	---	1.04b	16.5b	1.5b
	Coarsely ground (2.54 cm)	---	1.27a	19.0a	1.3c
Turkey[e]					
flaked	No NaCl or STPP	4.1bc	1.00ab	14.3a	2.0c
	1.2 % NaCl	5.0b	0.96ab	10.7c	3.3b
	0.38% STPP	3.6c	0.91b	12.5b	1.8c
	1.2 % NaCl and 0.38% STPP	7.1a	1.07a	11.7b	4.8a

[a] Chewiness: ratio of number of chews required to swallow treated sample divided by number of chews required to swallow control sample. Cohesiveness: 0 = least cohesive, 10 = most cohesive.

[b] All samples were deep-fat-fried; shear samples were trimmed to 7.2 cm × 6.4 cm × 1 cm and measured using a single blade Model CA-1 test cell; RTT samples were trimmed to 7.2 cm × 6.4 cm × 1 cm and measured with a Model MS-1 test cell. GFM = granulated fish muscle, MSP = mechanically separated poultry. For mechanical measurements, n = 8.

[c] Means in a column within each group having the same letter are not significantly different (P > 0.05). Within each variable a higher value indicates higher levels of cohesiveness, chewiness, shear and RTT.

[d] All samples were deep-fat-fried; shear samples were trimmed to 1 cm × 6 cm × 1 cm and measured using a multiple blade Model CS-1 test cell; RTT samples were trimmed to 10 cm × 10 cm × 1 cm and measured with a Model MS-1 test cell. For mechanical measurements, n = 48.

[e] All cooked patties were thawed 12-15 hr at 4°C; shear samples were trimmed to 5 cm × 5 cm × 0.85 cm and measured using a multiple blade Model CS-1 test cell; RTT values were obtained on whole patties using a Model MS-1 test cell. STPP, sodium tripolyphosphate. For mechanical measurements, n = 10.

also reflected the lack of cohesiveness in patties made with 100% MSP or GFM.

There were no significant differences in chewiness, as perceived by the sensory panel, among spent layer patties made with breast meat, breast-leg (50-50 combination) or 100% leg meat. However, the presence of leg meat significantly (P < 0.05) increased the shear values and decreased the RTT scores of these patties. These RTT values support results reported by Froning and Norman (1966), in which the tear strength or binding of white meat was significantly (P < 0.05) greater than that observed in dark meat.

When spent layer patties were produced using flaked (Urschel Comitrol, Model 3600, Urschel Inc., Valparaiso, IN), finely ground (0.64 cm) or coarsely ground (2.54 cm) meat, panelists could not tell the difference between a product prepared from flaked meat or finely ground meat; however, patties made with the coarsely ground meat were more chewy, possibly a reflection of the large particle size in the formulation. Shear values on these same products increased from 14.1 to 19.0 N/cm² as the type of fragmentation method changed from flaked meat to finely ground to coarsely ground. When RTT

values were examined, there also was a significant (P < 0.05) decrease as fragmentation changed from a flaked meat to finely ground to coarsely ground product.

In the above experiments, chewiness was the only texture-related sensory attribute used in the training of the panel. For the turkey breast meat experiments, both chewiness and cohesiveness variables were introduced in the training program. In the previous experiments, a ratio scaling technique was used to measure chewiness; in this experiment a 10 cm scale was used to measure cohesiveness. Using sensory responses only, the addition of both 1.2% NaCl and 0.38% STPP (sodium tripolyphosphate) resulted in turkey patties with the highest cohesiveness and chewiness characteristics; lowest scores were observed for patties with phosphate only. Shear values of patties were lowered by adding NaCl and/or phosphate. The RTT values were consistent with both sensory measurements. This was particularly evident for the sensory cohesiveness scores and the RTT scores, which had a correlation coefficient of 0.98.

The resistance to tear (RTT) procedure offers an alternative measurement to conventional shearing tests. In place of

A Research Note

Stability of Residual Acid Phosphatase Activity in Cured/ Canned Picnic Samples Stored at −34°C for 15 and 36 Months

W. E. TOWNSEND

ABSTRACT

Calculated internal temperatures (based on residual acid phosphatase activity values) were determined for samples obtained from domestically prepared cured/canned picnics which previously had been heat-processed to 61°, 62.7°, 65.5°, 68.3°, and 70.9°C, respectively, and stored at −34°C for up to 36 months. There was a greater loss in residual acid phosphatase activity (based on an increase in calculated internal temperature) during the first 15 months of storage, with the least loss occurring during the next 21 months of storage. The greatest loss of activity occurred in those samples heat-processed to 61°C and 62.7°C which also contained the greatest amount of residual acid phosphatase activity.

INTRODUCTION

THE CODE OF FEDERAL REGULATIONS, Title 9, Chapter I, Animal and Plant Health Inspection Service/Veterinary Services (APHIS/VS) Regulations, Sections 94.4(b)(2); 94.8(a)(3)(iii); 94.9(a)(ii)(b), and 94.12(ii)(B) establishes prescribed thermal treatment for imported pork and pork products (USDA-APHIS, 1988). The Animal and Plant Health Inspection Service/Veterinary Services (APHIS/VS) requires that imported pork and pork products (canned hams, canned picnics and canned luncheon meat) be heat-processed to an internal temperature of 69°C (156°F). These requirements by APHIS/VS were instituted to ensure the destruction of viruses and other microorganisms that cause exotic diseases in animals and man. To determine the adequacy of heat treatment of imported canned hams, canned picnics and canned luncheon meat, the Food Safety and Inspection Service (USDA-FSIS, 1986) is using a procedure developed by Lind (1965) for determining the amount of residual acid phosphatase activity remaining in the sample after heat treatment.

At times, it becomes necessary to either refrigerate or freeze samples of meat until they can be analyzed. Lind (1965) found no statistical difference between the residual acid phosphatase activity of minced samples stored for 5 days at 4°C and that determined immediately after mincing of the sample or in canned ham samples analyzed immediately after heat processing and those held for 1 month at 4°C. However, no information is available concerning the effect of extended storage at subfreezing temperatures on the stability of residual acid phosphatase activity in cured/canned picnics. Therefore, in concert with some other enzyme activity tests conducted on the samples of picnics used in this study, the purpose of this study was to determine the stability of the acid phosphatase activity during storage for 36 months at −34°C.

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MATERIALS & METHODS

THE PICNIC CORE SAMPLES used in this study were obtained from the geometric center of cured/canned pork picnics that were prepared under contract by the American Bacteriological and Chemical Research Corporation (ABC) (Gainesville, FL) for FSIS. Picnics were cured and canned at a commercial USDA-inspected pork processing plant and then heat-processed by ABC Research Corporation to varying internal temperatures of 61.0°C (141.8°F), 62.7°C (144.8°F), 65.5°C (150°F), 68.3°C (155°F), and 70.9°C (159.9°F), respectively. Core samples were ground three times through a 3-mm grinder plate, placed in Ziplock bags and stored at −34°C. Representative samples of each temperature group were removed from the freezer at 15 and 36 months storage and allowed to thaw overnight at 4°C.

The FSIS procedure (USDA-FSIS, 1986) was used for determining the amount of residual acid phosphatase activity in the samples of picnics. In this procedure, 2.5g of sample (in triplicate — two for analysis and one to serve as a control) are used. The activity of the acid phosphatase left after heat processing is expressed as the micromoles (µM) of phenol formed per 1000g of sample when the sample is allowed to react with the substrate disodium phenylphosphate (0.01M) for 60 min, at 37°C and pH 6.5. The phenol produced is reacted with 2,6-dibromoquinone chlorimide in the dark to allow color development. Absorbance of the blue color formed is determined spectrophotometrically at 610 nm using 1 cm spectrophotometric cells with distilled water serving as a reference sample for setting the spectrophotometer at zero absorbance. Through a series of calculations, a phosphatase activity value (EF') is obtained which is then converted to the log of the EF' value. Lind (1965) reported that the log of the EF' value correlates linearly with the internal cooked temperature of canned hams. The log of the EF' value is then used in the formula [77.3985-(5 7109)(Log EF')] for calculating the internal temperature of canned hams and canned picnics which can be converted to °F, if desired. The calculated internal temperature value was used as a criterion for determining the stability of residual acid phosphatase activity. A marked increase in calculated internal temperature would indicate that some denaturation (loss of activity) of the acid phosphatase enzyme had taken place during freezer storage.

Since Lind (1965) showed that a 1% increase in salt content over the average salt content of 3.55% means that a decrease of 0.55°C has to be taken into account when calculating the final center temperature, salt content was determined on all picnic samples according to the Volhard Method (USDA-FSIS, 1987).

Lind (1965) reported that the addition of ascorbic acid and phosphates to cured hams had no or negligible effect on acid phosphatase activity, therefore, these analyses were not conducted on the picnic samples. In addition, the effect of storage for 2–4 weeks (to simulate transit from Europe to the U.S.) at refrigerated temperatures was not investigated because Lind (1965) found no statistical difference between the residual acid phosphatase activity of ham samples analyzed immediately after heat processing and after 1 month storage at 4°C.

RESULTS & DISCUSSION

PERCENT SALT CONTENT for each temperature group of picnic samples is shown in Table 1. Salt contents were below the average level of 3.55% reported by Lind (1965), therefore, no adjustment had to be included in the calculations involved in calculating the final center temperature.

The effect of frozen storage time on residual phosphatase activity (based on calculated internal temperature) of cured/

Table 1—Percent salt content and stability of residual acid phosphatase activity in samples from domestically prepared cured/canned picnics stored for 15 and 36 months at −34°C

Internal temperature of cooked product[a]	Salt content (%)	Calculated internal temperature °C[b]		
		15 Months of storage	36 Months of storage	Total change in degrees C
61.0°C (141.8°F) [2][c]	2.50	63.3	64.7	3.7
62.7°C (144.8°F) [4]	2.46	63.8	64.7	2.0
65.5°C (150°F) [8]	2.43	66.3	67.0	1.5
68.3°C (155°F) [12]	2.45	68.8	69.6	1.1
70.9°C (159.9°F) [9]	2.37	70.8	70.8	0.0

[a] Internal temperature to which cured/canned picnics were heat-processed.
[b] Calculated internal temperature in (°C) using the formula [77.3985-(5.7109)(Log EF')] as described by FSIS for cured/canned picnics.
[c] Numbers in brackets refer to number of samples of cured/canned picnics analyzed within each product temperature group.

canned picnics stored for 15 and 36 months at −34°C is presented in Table 1. The data indicated that the greatest loss in residual acid phosphatase activity, as evidenced by an increase in calculated internal temperature, occurred during the first 15 months of storage, with the least loss occurring during the next 21 months of storage. The greatest loss of acid phosphatase activity occurred in those samples heat-processed to internal temperatures of 62.7°C (145°F) and below. Within an internal temperature group (Table 1), residual acid phosphatase activity was less stable in those samples from picnics heat-processed to 61°C (141.8°F) and 62.7°C (144.8°F) than in those picnics heat-processed to 65.5° (150°F), 68.3° (155°F) and 70.9°C (159.9°F), respectively. The increase in calculated internal temperature indicated that some denaturation of the acid phosphatase enzyme occurred during storage. The increase in calculated temperature could also be explained by the fact that there was more residual acid phosphatase enzyme (higher Log of EF' values, Table 2) available for denaturation in those

samples from picnics heat-processed to internal temperatures of 61°C and 62.7°C than in those samples from picnics heat-processed to higher internal temperatures.

The results of this study could be important to APHIS/VS and FSIS, especially for imported product, where laboratory findings showed that product internal temperatures from a specific production run were below the required temperature of 68.8° (156°F) and must be held frozen for extended periods of time until APHIS/VS and FSIS determined that a reasonable history of compliance had been reestablished.

Table 2—Change in Log of EF values obtained from samples of domestically prepared cured/canned picnics stored for 36 months at −34°C

Internal temperature of cooked product[a]	Log of EF' values[b]		Difference
	0 Months	36 Months	
61.0°C (141.8°F) [2][c]	2.56	2.23	−0.33
62.7°C (144.8°F) [4]	2.43	2.23	−0.20
65.5°C (150°F) [8]	1.98	1.80	−0.18
68.3°C (155°F) [12]	1.59	1.44	−0.15
70.9°C (159.9°F) [9]	1.24	1.02	−0.22

[a] Internal temperature to which cured/canned picnics were heat-processed.
[b] Log of EF' values calculated from residual acid phosphatase activity values based on micromoles of phenol formed per 1000g of sample.
[c] Numbers in brackets refer to number of samples of cured/canned picnics analyzed within each product temperature group.

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the cutting action used in shear tests, a tearing action is used. This provides useful information on cohesive properties of a food system, especially one involving restructured muscle foods.

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A Research Note
Changes in the Total Oxalate Content in the Fermentation of Fish Paste Hentak

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ABSTRACT

Total oxalate of the sliced petioles of *Alocasia macrorhiza* was reduced by 41.3% on incubation for 7 days at room temperature (18–23°C). When incubated after mixing with an equal weight of powered sundried fish *Esomus danricus* for the preparation of a fermented fish paste hentak, oxalate was decreased by 84.4%. This loss was inhibited to a great extent by treatment with antibiotics. Some specific microorganisms grown in this particular medium might play an important role in the destruction of oxalate in the paste.

INTRODUCTION

Consumption of fermented foods has been an age old practice in rice-eating countries of the Orient (Van Veen, 1962). This is either used as a condiment or as main meal (Wood and Min, 1975). In Manipur, India people also use a fermented fish paste named 'hentak' as a flavoring agent in curry preparations. It is regarded as nutritious and thus given to convalescing patients and mothers in confinement. The flavor becomes more appetizing with longer preservation time. Hentak as old as 3 years may also be used as antiseptic for minor wounds in cattles.

The interesting feature in the preparation of hentak is the inclusion of fresh petioles of *Alocasia macrorhiza* (fam: Araceae) as a compulsory component. The plant is not suitable for human consumption since it contains calcium oxalate in the form of needle-like raphide crystals that cause irritation. The fermented paste does not cause irritation to consumers. Instead, oral administration of roasted paste relieves the irritation caused by accidental consumption of *Alocasia*.

Preliminary studies on the composition of hentak were carried out by Sarojnalini and Vishwanath (1985, 1987). In the present paper, changes in the total oxalate of the paste during fermentation and the possible role of bacteria and fungi in the process were studied.

MATERIALS & METHODS

Preparation of hentak

Sundried fish (*Esomus danricus*) (4–6 cm length) was purchased from the Imphal market. It was spread on a tray and exposed to sunlight until crisp and then powdered using a mortar and pestle. Fresh petioles of *Alocasia macrorhiza* were washed briefly with water, cut into slices and then drained for 1 hr. Equal weights of the fish powder and the plant material were mixed and ground to form a paste. It was then incubated in earthen pots with lids on for 7 days at room temperature (18–23°C). Fungal growth was observed at this time. The paste was then thoroughly ground. Sliced *Alocasia* were also incubated in the same way for determination of oxalate.

Treatment with antibiotics

An antifungal antibiotic, nystatin, and a broad spectrum antibiotic, cephalexin (supplied by BDH chemicals Ltd., India), were mixed with

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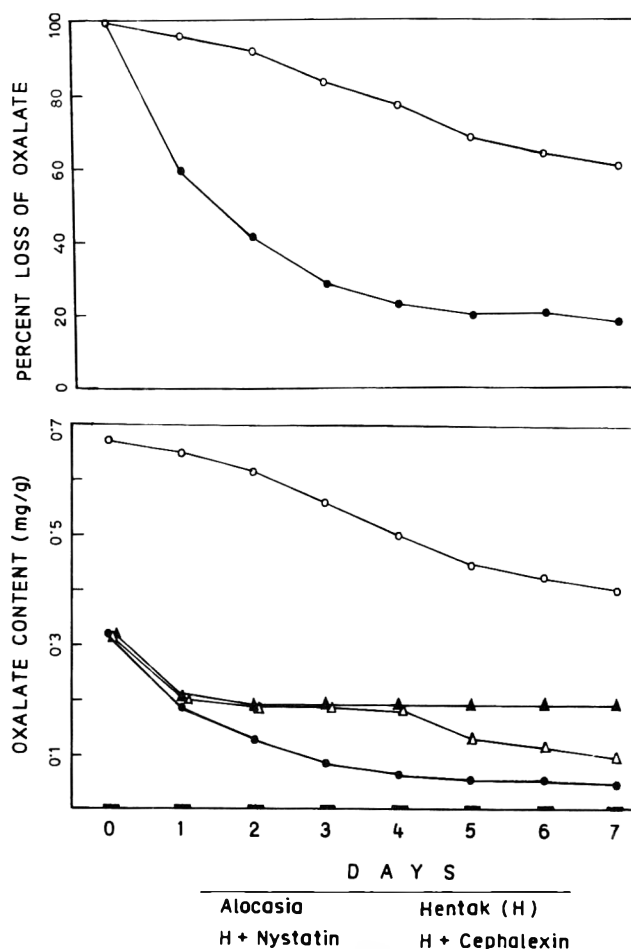
Fig. 1 – Changes in total oxalate content in Alocasia and hentak during incubation. o–o, Alocasia; •–•, Hentak; ▲–▲, Hentak + nystatin; △–△, Hentak + cephalexin.

hentak separately at 50 μg/g and 100 μg/g, respectively; the pastes were then incubated for 7 days at room temperature (18–23°C) as described above.

Oxalate content

Total oxalate of the pastes and *Alocasia* was estimated every 24 hr for 7 days following the method of Baker (1952).

Identification of fungi

Diluted suspensions of the paste before and after fermentation were separately inoculated in potato dextrose agar (PDA) media and then incubated at 27 ± 2°C for 5 days. The total number of colonies was counted, and the species were identified based on Gilman (1957).

Before incubation			After incubation		
Species	No./g	%	Species	No./g	%
Aspergillus candidus	1.0×10^5	2.17	*Aspergillus fumigatus*	6.0×10^5	31.58
A. janus	2.0×10^5	4.34	*A. niger*	2.0×10^5	10.53
A. niger	1.0×10^5	2.17	*Cladosporium* sp.	2.0×10^5	10.53
Chaetomium sp.	2.4×10^6	52.17	*Geotrichum* sp.	1.0×10^5	5.26
Cladosporium herbarum	3.0×10^5	6.52	*Myrothecium striatisporum*	1.0×10^5	5.26
Geotrichum candidum	2.0×10^5	4.34	*Penicillium rubrum*	2.0×10^5	10.53
Helminthosporium nodulosum	1.0×10^5	2.17	*P. rugulosum*	1.0×10^5	5.26
Nigrospora sphaerica	1.0×10^5	2.17	*Rhizopus nigricans*	1.0×10^5	5.26
Penicillium lanosum	1.0×10^5	2.17	White sterile mycelium	2.0×10^5	10.53
Rhizoetonia sp.	1.0×10^5	2.17	Black sterile mycelium	1.0×10^5	5.26
Torula lucifuga	1.0×10^5	2.17			
White sterile mycelium	7.0×10^5	15.21			
Yellow sterile mycelium	1.0×10^5	2.17			
Total	4.6×10^6	99.94	Total	1.9×10^6	100.00

Total count of bacteria

The samples before and after incubation were taken separately and inoculated in nutrient agar medium containing tryptone (ISI, 1980). The total number of colonies was counted using a colony counter.

RESULTS

TOTAL OXALATE changed from 0.68 to 0.40 mg/g in *Alocasia* and from 0.32 to 0.05 mg/g in hentak in 7 days of incubation (Fig. 1). When treated with nystatin and cephalexin, oxalate was reduced to 0.19 and 0.09 mg/g, respectively. The species of fungi, their number per gram weight of hentak before and after incubation are shown in Table 1. The total numbers and types of fungi were greatly different. From the paste before incubation, 11 species and 2 types of sterile mycelia were identified in PDA culture. The total number was 4.6×10^6 per g of which, *Chaetomium* sp. constituted as much as 52.17%. The paste after 7 days of incubation contained only 1.9×10^6 fungi/g. Out of the identifiable 8 species and 2 types of sterile mycelia, *Aspergillus fumigatus* was maximum (28.57%). In both cases, *Aspergillus niger* and *Cladosporium* sp. were present. Total viable bacteria were 6.5×10^5 per g before incubation and 4.2×10^6 per g after incubation.

DISCUSSION

THE CHANGES in the oxalate of the paste seemed to be affected by the growth of certain fungi on it. The loss in hentak was found to be much higher than that of *Alocasia* alone. The prevention of loss of oxalate by treatment with nystatin also suggested that fungi directly or indirectly acted on the oxalates. Bacterial action might be negligible as treatment with cephalexin did not affect the change greatly.

Metabolism of oxalates in fungi may be discussed from the viewpoints of Wehmer's contribution as explained by Foster (1949). Though light decomposes oxalate spontaneously, especially in presence of metals, such as iron, neither light nor iron affects the formation or destruction of oxalate by fungus. Since incubation took place in the dark, the possibility of the loss due to light was ruled out. Though fungi are known to

accumulate oxalate, the critical factor is the presence of free base in the medium. The alkaline neutralizing agents conducive to maximum yields of oxalate might be absent. Oxalate is attacked by mold only when it is in the undissociated state, i.e., in the form of free acid. The extracellular fungal enzymes probably produced free oxalic acid, which in turn was absorbed by fungi. *Penicillium glaucum* is reported to highly consume oxalate at 37°C. It was also noted that when furnished oxalate, some fungi converted it to formic acid (Foster, 1949). From the standpoint of energy and cell synthesis, it means little to the organism since oxalic acid is so near the endpoint of complete oxidation that its further utilization by the organism entails little advantage. Though oxalic acid has an energy value of 60 cal/g Mol, compared to 705 cals/g Mol glucose, the absence of ample quantity of sugar might lead to the utilization of oxalic acid for its energy requirements. However, the real mechanism of oxalate metabolism is still unknown.

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A Research Note

Analysis of Amino Acids in Soy Isolates and Navy Beans Using Precolumn Derivatization with Phenylisothiocyanate and Reversed-Phase High Performance Liquid Chromatography

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ABSTRACT

Amino acid analysis of three legume samples using precolumn phenylisothiocyanate (PITC) derivatization and reversed-phase high performance liquid chromatography (RP-HPLC) was compared with literature data or that obtained by the conventional ion-exchange chromatographic (IEC) method. The results indicated that the amino acid profiles obtained by PITC derivatization and RP-HPLC compared fairly well with literature data for soy isolates, and fairly with that obtained by the IEC method for navy beans. Average coefficient of variability of all amino acids determined by the PITC method was 6%. It was not essential to remove lipids (2.1%) in navy beans prior to acid hydrolysis and PITC derivatization for the analysis of amino acids.

INTRODUCTION

SINCE THE REPORT of automatic separation and quantitation of amino acids by Spackman et al. (1958), ion-exchange chromatography (IEC) and postcolumn derivatization with ninhydrin using amino acid analyzers (IEC method) has become a standard procedure for analyzing amino acids in food protein hydrolyzates (Satterlee et al., 1982; Sarwar et al., 1983). Recently, more rapid and sensitive methods using precolumn derivatization followed by reversed-phase high performance liquid chromatography (RP-HPLC) have become increasingly popular. Analysis of amino acid derivatives of o-phthalaldehyde (OPA) has been reported to be rapid, sensitive and quantitative (Hill et al., 1979; Fernstrom and Fernstrom, 1981; Larsen and West, 1981; Schuster, 1984). However, OPA does not react with secondary amines such as proline and hydroxyproline.

More recently, a rapid, sensitive, and quantitative method for analyzing phenylthiocarbamyl amino acid derivatives (PTC-amon acids) using RP-HPLC has been described to measure all amino acids in acid hydrolyzates of purified proteins and peptides (PITC method) (Heinrikson and Meredith, 1980; Bidlingmeyer et al., 1984). However, there has been only one report in the application of this procedure to analyze amino acids in acid hydrolyzates of cereal foodstuffs (Elkin ad Wasynczuk, 1987).

The objective of this study was to determine the suitability of the PITC method for analyzing amino acids in legume proteins by comparing the PTC-amino acid composition with literature data or the data obtained by the conventional IEC method.

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MATERIALS & METHODS

Materials

Soy isolates Supro 620 and 710 (Ralston Purina Co., St. Louis, MO) and navy beans (_Phaseolus vulgaris_) were used. The bean sample was ground to pass through a 100-mesh screen. Nitrogen content was determined by the Kjeldahl method (AOAC Method 2.057, 1984), and crude protein content calculated using conversion factor 6.25. Lipid in the navy beans was determined by AOAC Method 7.060 (1984). Amino acid standards and phenylisothiocyante were obtained from Pierce Chemical Co. (Rockford, IL). Alpha-amino butyric acid was obtained from the Sigma Chemical Co. (St. Louis, MO).

Acid hydrolysis and derivatization - PITC method

Samples (20-50 mg crude protein) containing internal standard α-amino butyric acid were hydrolyzed in 10 mL 6N HCl under vacuum (50 μg Hg) at 110°C for 24 hg. The hydrolyzates were filtered, rotary-evaporated to dryness at 60°C, and dissolved in 10 mL 50% acetonitrile. Five microliters were derivatized with PITC using Waters Workstation equipped with a vacuum pump (Bidlingmeyer et al., 1984).

Chromatography

The PTC-amino acids were analyzed by the method of Bidlingmeyer et al. (1984) using a Waters HPLC system equipped with a Model 730 Data Module and Model 720 System Controller.

Cysteine determination

Cysteine in navy beans was determined colorimetrically using 5, 5'-dithiobis (2-nitrobenzoic acid) as a color reagent after reduction with sodium borohydride in 8M urea (Chang et al., 1982).

Ion-exchange chromatography method

Samples of ground navy beans were hydrolyzed in the same manner as described in the PITC method. The hydrolyzates were treated and analyzed by ion-exchange chromatographic method using the method of Satterlee et al. (1982).

Coefficient of variation

Coefficient of variation or of variability (CV) was calculated according to the procedure described by Steel and Torrie, 1960.

RESULTS & DISCUSSION

Soy isolate

Amino acids in acid hydrolyzates of both soy isolate 620 and 710 compared fairly well with the data determined by the IEC method by the manufacturer (Ralston Purina Company, 1987) (Table 1). The average coefficient of variation for all

Table 1 — Amino acid profiles of soy isolate.

Amino acid	Literature data IEC method[b]	PITC method[a] Supro 620 Mean ± SD	CV,%	Supro 710 Mean ± SD	CV,%
ASP	11.6	9.73 ± 0.44	4.5	10.54 ± 0.75	7.1
Glu	19.1	17.32 ± 0.52	3.0	17.08 ± 0.95	5.6
Ser	5.2	4.72 ± 0.26	5.5	4.69 ± 0.15	3.2
Gly	4.2	4.16 ± 0.16	3.8	4.14 ± 0.12	2.9
His	2.6	2.61 ± 0.33	12.6	2.56 ± 0.08	3.1
Arg	7.6	8.50 ± 0.27	3.2	7.89 ± 0.58	7.4
Thr	3.8	3.81 ± 0.22	5.8	4.25 ± 0.54	12.7
Ala	4.3	4.27 ± 0.11	2.6	4.25 ± 0.08	1.8
Pro	5.1	5.42 ± 0.33	6.0	5.29 ± 0.27	5.1
Tyr	3.8	4.03 ± 0.10	2.5	3.95 ± 0.15	3.8
Val	5.0	5.18 ± 0.04	0.8	5.17 ± 0.31	6.0
Met	1.3	1.47 ± 0.22	15.0	1.31 ± 0.24	18.3
Cys	1.3	1.3[c]	—	1.3[c]	—
Ile	4.9	5.11 ± 0.13	2.5	5.03 ± 0.16	3.2
Leu	8.2	8.04 ± 0.26	3.2	7.75 ± 0.22	2.8
Phe	5.2	6.05 ± 0.47	7.8	6.68 ± 0.69	10.3
Trp	1.4	1.4[c]	—	1.4[c]	—
Lys	6.3	6.90 ± 0.43	6.2	6.70 ± 0.31	4.6
Average	---	----	5.3	----	6.1

[a] Data are g amino acid/100 g protein, and are average of 6 derivatizations from 6 separate hydrolyzates. Amino acid recovery ranged from 95-100%. Data were adjusted to 100% amino acid recovery.

[b] Data furnished by Ralston Purina Co. (1987), same composition for both Supro 620 and Supro 710.

[c] Literature data (Ralston Purina Co., 1987) of Cys and Trp were used for calculation of amino acid composition.

Table 2 — Amino acid profiles of navy beans[a]

Amino acid	Method IEC Mean ± SD	CV,%	PITC Mean ± SD	CV,%	CV,% between IEC and PITC
Asp	10.79 ± 0.43	4.0	9.31 ± 1.00	10.7	11.4
Glu	13.49 ± 0.24	1.7	13.32 ± 0.26	1.9	1.0
Ser	5.57 ± 0.18	3.2	5.47 ± 0.10	1.8	1.6
Gly	4.29 ± 0.11	2.5	4.06 ± 0.08	2.0	3.9
His	3.44 ± 0.14	4.0	3.00 ± 0.26	8.6	9.7
Arg	7.73 ± 0.04	0.5	9.17 ± 0.36	3.9	12.0
Thr	4.79 ± 0.08	1.6	4.29 ± 0.26	6.2	7.8
Ala	4.60 ± 0.09	1.9	4.34 ± 0.15	3.4	4.1
Pro	4.05 ± 0.15	3.6	5.60 ± 0.84	15.0	22.7
Tyr	3.96 ± 0.06	1.5	5.40 ± 0.22	4.0	21.8
Val	6.18 ± 0.01	0.1	5.75 ± 0.40	7.0	5.1
Met	1.14 ± 0.08	6.9	1.05 ± 0.18	18.0	5.8
Cys[b]	1.08[b]	—	1.08[b]	—	—
Ile	5.43 ± 0.41	7.6	4.77 ± 0.14	3.0	9.2
Leu	8.13 ± 0.13	1.6	7.86 ± 0.38	4.8	2.4
Phe	6.21 ± 0.23	3.6	7.39 ± 0.22	3.0	12.2
Trp[c]	1.10[c]	—	1.10[c]	—	—
Lys	7.42 ± 0.08	1.0	7.10 ± 0.39	5.5	3.1
Average	----	2.9	----	6.2	8.4

[a] Data are g amino acid/100 g protein, and are average of three derivatizations from three separate hydrolyzates. Amino acid recovery ranged from 88-105%. Data were adjusted to 100% amino acid recovery.

[b] Cys was determined colorimetrically.

[c] Various dry beans contained approximately 1.1 g tryptophan/100 g protein (Tobin and Carpenter, 1978). Trp data 1.1 was used for calculation.

amino acids for the two soy isolate samples determined by the PITC method was 5.7%, which was greater than the average 3.3% CV in the analysis of feedstuffs by Elkin and Wasynczuk (1987). However, the results still indicate good accuracy and reproducibility of the PITC method for the analysis of isolated soy proteins. The report of Elkin and Wasynczuk (1987) did not indicate if the six derivatizations were from separate hydrolyzates. It could be anticipated that more variation would result from different hydrolyzates.

Navy beans

The amino acid composition of navy beans obtained by the PITC method also compared fairly with that obtained by the IEC method (Table 2). The average 6.2% CV of all amino acids determined by the PITC method was similar to that of soy isolate samples, and was slightly greater than that by the

IEC method. When data obtained by the two methods were compared, proline and tyrosine had the greatest variation (22.7 and 21.8% CV) among all amino acids. The variation in this study could be partly attributed to interlaboratory differences since the PITC method and IEC method were carried out in different laboratories (IEC method was carried out at the Food Science Dept., Pennsylvania State Univ.). Inter- and intra-laboratory variation in amino acid composition of food proteins analyzed by the standard IEC method had been reported by Sarwar et al. (1983). Interlaboratory variation (% CV) of tryptophan was as high as 24% (Sarwar et al., 1983). Interlaboratory variation for amino acids in soy isolate determined by the IEC method was up to 25.7% as reported by Cavins et al. (1982). The average variability (8.4% CV) for all amino acids in navy beans between the IEC and PITC method compared fairly well to the average interlaboratory CV (6 to 9.9%) for all amino acids (excluding ammonia) in seven food proteins as reported by Sarwar et al. (1983).

In the examination of the overall data for the three samples, twenty of the 32 amino acid values determined by the PITC method were greater than the IEC literature values for the two soy isolates, while only four of the 16 values by the PITC method were greater than the IEC values for the navy beans (Tables 1 and 2). This type of variation has been reported in the comparison of the PITC method with the IEC method for analyzing cereal products (Elkin and Wasynczuk, 1987). Irregular interlaboratory variations for individual amino acids determined by the IEC method have also been reported to differ in different food protein sources (Sarwar et al., 1983). For the same protein source (for instance, the two soy isolates), the PITC values for individual amino acids were consistently greater or lower than the literature values (Table 1). Hence, the irregular pattern of the variation of the individual amino acid values between the two types of protein samples by the two methods used could be partly dependent upon food protein sources. It is not well understood what effects food components other than amino acids have on PITC derivatization and quantification.

Soy isolate contained 0.5% lipid (Ralston Purina, 1987), and navy beans contained 2.1% lipid. The separation of peaks of PTC-amino acids from navy beans was as good as that of soy isolate. Undesirable peak interference from lipid components was not observed. It is, therefore, not essential to defat dry beans of low lipid content prior to hydrolysis and derivatization for PTC-amino acid analyses.

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Effect of Pretreatment on the Cooking Time of the African Yam Bean (*Sphenostylis sternocarpa*)

H. O. NJOKU, I. ELI, AND C. O. OFUYA

ABSTRACT

The effect of soaking in tap water, 1% potash, and 4% sodium chloride for different periods on the cooking time of African Yam Bean was studied. The results showed that 12 hr soaking was adequate for the beans to reach maximum hydration, and that pretreatment of the beans before cooking appreciably influenced the overall cooking time. Cooking after pretreatment for 12 hr in 1% potash or in 4% sodium chloride reduced the normal cooking time by 50%.

INTRODUCTION

LEGUMES are a major source of protein in developing countries (Akroyd and Doughty, 1969; Siegel and Fawcett, 1976). In Nigeria the African Yam Bean (AYB), is consumed within a small geographical region. Consumption is low mainly because of the length of time it takes to cook. The seed is hard and by traditional processing methods, it takes about 24 hr to prepare a meal of AYB. This is time and energy consuming and does not favor general acceptability. The protein of AYB is 21–29% with a lysine and methionine composition that is comparable to those of soybean (Anonymous, 1979). It is believed that if new processing methods are developed to improve the cooking time, the beans could become more readily acceptable to a wider group of consumers.

This paper describes preliminary work on studies directed at enhanced utilization of the pulse. It describes the effects of different treatments on the cookability of African Yam Bean (*Sphenostylis sternocarpa*).

MATERIALS & METHODS

Bean samples

African Yam Bean (*Sphenostylis sternocarpa*) "marble" cultivar was obtained from a food market in Etiti, Imo State Nigeria. The beans were transported to the laboratory in sealed polythene bags and stored at ambient temperature ($28 \pm 2°C$) until required. The bean samples were analyzed for moisture according to the method described by Jacobs (1951). The hydration experiment was done by determining the effect of tap water, 1% w/v potash and 4% w/v sodium chloride on the water absorbing capacity of the beans at $28 \pm 2°C$.

Ten grams of AYB were soaked in 50 mL of each soaking medium contained in a 250 mL flask. At the end of the soaking period (1–24 hr), the beans were blotted dry with filter paper (Whatman No 1) to remove surface water and weighed. The water absorbing capacity was expressed as hydration coefficient as reported by Abou-Samaha et al. (1985). The experiments were carried out in triplicates. The pH of the soaking media was determined during the first 10 hr soaking using a Philips digital pH meter (PW 9409).

Traditional cooking

Ten grams beans soaked in the different soak media for 8, 12, 18, and 24 hr, respectively, were cooked to tenderness using an electric cooker (Tricity contessa) at 160°C. Tenderness was determined subjectively by feeling between the fingers as done traditionally. The

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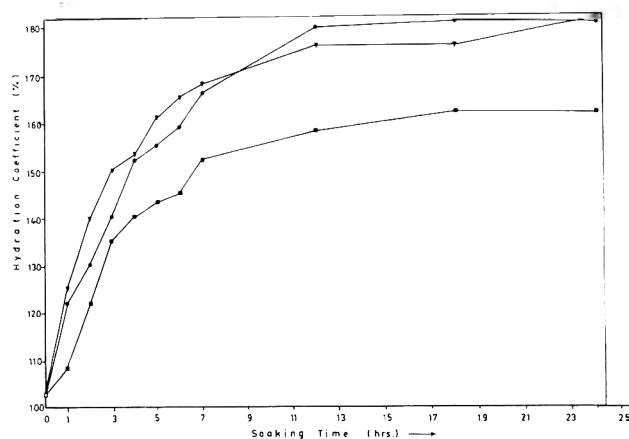
Fig. 1 — Effect of soaking time on the hydration coefficient of African Yam Beans. ▼ 1% potash; ■ 4% NaCl; ● Tap water
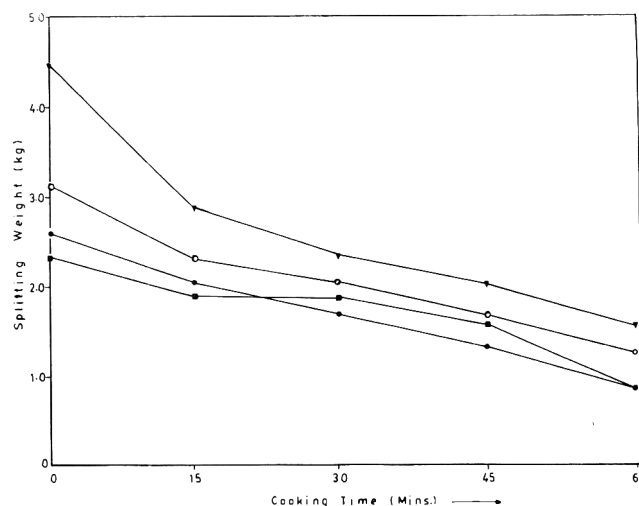
Fig. 2 — Effect of time of cooking on the tenderness of the African Yam Beans. ▼ Unsoaked beans; ○ Tap water; ■ 1% potash; ● 4% NaCl.

degree of tenderness was scored on a 0 to 5 scale, with a zero rating for very hard beans and a rating of 5 for very soft beans. The time required to achieve a rating of 4 (soft) was recorded as the cooking time. Tenderness was determined on individual beans that made up the sample lot. Unsoaked AYB and cowpea were subjected to the same treatment as a control and for comparison, respectively.

In a separate experiment, 30g each of the AYB were soaked in 150 mL of the different soak media for 12 hr, after which the soak medium was drained off. Five grams each of the soaked beans were added to boiling water at 100°C, and the beans were simmered at this temperature for various periods of time. Five grams of the unsoaked beans were used as control. The change in bean texture with cooking was determined on individual beans of the sample lot, according to the method described by Okafor et al. (1984) for studies on the retting of cassava. The hardness of the texture was expressed as the average

maximum force in kilograms which caused the split of the individual beans.

RESULTS & DISCUSSION

THE RESULTS showed that the hydration coefficient of the seeds increased with the duration of soaking (Fig. 1). However, in the three soak media that were used, by 12 hr soaking, over 95% of the total water absorbing capacity of the beans had been attained. The seeds soaked in tap water and potash solution showed a higher rate of water uptake than those soaked in the sodium chloride solution. Similar observations such as these have been reported for lentil seeds by Abou-Samaha et al. (1985).

The pH of the soak solution decreased from about neutral (7.3) to acidic pH (4.3) for tap water; for salt medium, there was a decrease of about 3 log units (7.1–4.4). There was a decrease of 2 log units of pH for the potash soak solution (10.9–8.7). This reduction in pH might be due to the nature of the leachates or to bacterial growth. Kialasapathy et al. (1985) noted that it was difficult to soak beans for more than a few hours without bacterial growth.

The duration of soaking and the type of soak medium influenced cooking time. Unsoaked African Yam Beans required 90 min to cook under the conditions examined compared to 60 min for unsoaked cowpeas. An increase in the soaking time, irrespective of the soaking solution reduced cooking time For the potash and sodium chloride solutions a 50% reduction in cooking time was achieved after 12 hrs soaking. A further 5% reduction was achieved by soaking for another 12 hr in this solution. Beans soaked in tap water achieved a 50% reduction in cooking time after 24 hr soaking.

These results showed that after 12 hr soaking in the salt solution, further hydration did not cause any appreciable reduction in cooking time. Quast and Dasilva (1977) and Silva et al. (1981) found that after a minimum water uptake, further hydration cause no appreciable effect on the reduction in bean cooking time. The greater reduction in cooking time observed by pretreatment in sodium chloride and potash solution could be explained by the fact that the presence of sodium salts in the soaked medium and high pH increases the softening rate (Silva et al., 1981). The mechanism of softening as affected by salts was explained by Varriano-Marston and De-Omana (1979) as due to ion exchange and probably also by chelation of ions responsible for cellular firmness. The net effect was the solubilization of pectic substances.

Texture measurements (Fig. 2) also indicated that soaking solution influenced the cooking time. After 45 min cooking, beans soaked in sodium chloride solution had attained an eating tenderness. Beans pretreated in the other solutions reached this degree of tenderness after 60 min cooking. For the unsoaked beans, even after 60 min cooking, this level of tenderness was not achieved. Soaking in either sodium chloride or potash was preferable than soaking in tap water, because under such conditions 45 min was found to be adequate for cooking after 12 hr soaking.

Based on these observations, it was inferred that soaking for 12 hr in any of the three soak media could be used as an initial processing step, since the beans were almost fully rehydrated by this time. This shorter time would minimize the deleterious effect of bacterial growth and loss of beneficial solids which has been shown by Lo et al. (1968) to increase with increasing soaking time.

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A Research Note

Peroxidase of Kiwifruit

GUADALUPE PRÉSTAMO

———————— ABSTRACT ————————

Peroxidase from kiwifruit (*Actinidia chinensis*) was extracted, precipitated with ammonium sulfate, and purified by DEAE-cellulose chromatography. Only one band with peroxidase activity with an estimated molecular weight of 40,00–42,000 daltons was found by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

INTRODUCTION

KIWIFRUIT (*Actinidia chinensis*) is native to China. In 1906 Alexander Allison (Zuccherelli, 1987) imported seeds from China to New Zealand and the plants produced their first fruits in 1910. In 1940 a crop was started in the Tauranga area, where today 90% of the New Zealand production is grown.

Beutel et al. (1976) have determined the composition of kiwifruit including Brix, minerals, vitamins, ash, and proteins among other parameters. Kiwifruit is important in nutrition due to the high ascorbic acid and fiber levels. The former could explain, as Okuse et al. (1981) point out, why kiwifruit undergoes less browning than other typical fruits.

Further studies of Kiwifruit characteristics have been done by Park and Luh (1981) who found four catecholase isoenzymes of 15000, 20000, 25000, and 45000 daltons molecular weight and confirmed that the active site was histidine. Grison and Pilet (1985) found that there was a relationship between peroxidase (POD) and polyphenoloxidase (PPO) and that the PPO activity could be due to POD activity.

The purpose of this study was to purify and characterize the peroxidases in kiwifruit.

MATERIALS & METHODS

THE KIWIFRUIT used in this experiment was a Hayward cultivar from New Zealand. The flesh was blended (1:1 w/v) in an omni-mixer for 30 sec with 50 mM Tris-HCl, pH 7.8 buffer, and the extract centrifuged at $20000 \times g$ for 10 min. The supernatant (200 mL) was treated with ammonium sulfate (351g/L) and the resulting precipitate dissolved in small portions of 50 mM Tris-HCL, pH 7.8 buffer and dialyzed against the same buffer. The extract was partially purified with a DEAE-cellulose column using 50 mM Tris-HCl, pH 7.8 buffer, in a gradient from 0 to 1M NaCl and the fractions with peroxidase activity were pooled. All steps were carried out at 4°C.

Peroxidase activity

(POD) was determined spectrophotometrically at 460 nm using o-dianisidine as chromogenic indicator (Srivastava et al., 1983). The total reaction volume was 3 mL containing: 2.7 mL 50 mM sodium

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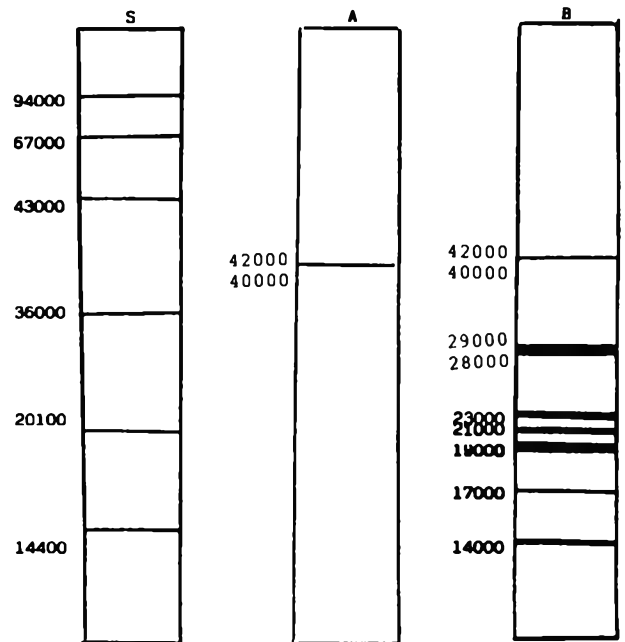
Fig. 1 — Electrophoresis of crude protein extract and purified peroxidase of kiwifruit. Molecular weights of: A-kiwifruit PO isoenzyme; B-kiwifruit proteins from crude extract; S-standard proteins.

acetate, pH 6 buffer; 0.1 mL 0.5% hydrogen peroxide; 0.1 mL enzyme extract and the addition of 0.1 mL 0.25% O-dianisidine (w/v). The POD activity was expressed in units of peroxidase per mg protein. A unit of peroxidase was defined as an increase of 0.001 unit of absorbance per min.

Protein content

Protein was determined using the Bio-Rad protein assay method (Bradford, 1976) and bovine serum albumin as standard.

Electrophoresis

SDS-PAGE (sodium dodecyl sulfate, polyacrylamide gel electrophoresis) was performed according to Laemmli (1970). Ten percent acrylamide/bisacrylamide was used in a slab gel system. Electrophoresis was carried out at 4°C using 25 mA/gel current for 4–5 hr running time. The electrode buffer was glycine-Tris pH 8.3. Samples of 100 µL (7.15 µg protein, with activity of 24.88 units POD/mg protein) were layered on the top of the gel. For activity staining, the gels were soaked for 30 min at 20°C in 50 mM sodium acetate, pH 6 buffer. They were then treated with 0.1% hydrogen peroxide and 0.25% o-dianisidine. Appearance of a dark brown band indicated peroxidase activity. For protein staining, the gels were fixed in a solution of 0.1% Coomasie Blue R-250, in 10% acetic acid and 25% isopropanol and 65% water. The gels were destained in a solution of 7% acetic acid and 10% methanol in water.

Molecular weight

SDS-PAGE was performed using the following molecular weight standards: phosphorylaseb (94,000 daltons): albumin (67,000 dal-

Germination of Amaranth Seeds: Effects on Nutrient Composition and Color

O. PAREDES-LÓPEZ and R. MORA-ESCOBEDO

———————— ABSTRACT ————————

Amaranth seeds were germinated at a water activity of about 0.92 for up to 72 hr. Crude protein, true protein and crude fiber were found to increase and fat content to decrease. For 48 hr of germination reactive lysine values did not change. At 72 hr, a slight decrease was observed in lysine and *in vitro* protein digestibility was similar to the control. During germination Hunter color parameters L were lowered and a and b were enhanced. Germinated seeds showed a pinky color which appeared very attractive for various food uses.

INTRODUCTION

AMARANTH PLANT has some remarkable agronomic traits, quite important for the agro-food sector of developing countries. It is relatively tolerant to drought, high temperatures and pests and produces good yields of seeds (Saunders and Becker, 1984; Paredes-López et al., 1989). This plant also grows in soils of lower quality as compared to most commercial crops. Moreover, the seed proteins have some outstanding nutritional and physicochemical properties (Pedersen et al., 1987; Paredes-López et al., 1988). In spite of these potentialities, the present consumption of amaranth seeds, all over the world, is practically negligible.

Germination appears to be an inexpensive, effective method, to provide desirable changes to nutritious crops. Germinated seeds have become a widely accepted food item. Alfalfa sprouts have been quite popular in some regions of Mexico for many years. It has long been claimed that germination, an ancient technology, generally enhances the nutritive value of cereals and legumes (Everson et al., 1944; Fernández and Berry, 1988; Price, 1988). Germination may also improve functional characteristics of some cereals (Lukow and Bushuk, 1984) and oilseeds (Canella et al., 1985). The development of processing techniques to generate amaranth based products, with desirable nutritional and sensory properties, could provide incentives to increase production and consumption of this crop. Thus, the purposes of this preliminary study were to examine the effects of germination on composition and on some nutritional properties of amaranth seeds, and to assess, color changes during germination.

MATERIALS & METHODS

Amaranth samples and germination of seeds

Seeds of *Amaranthus hypochondriacus*, Mercado type, were harvested in the experimental farm of INIFAP-Chapingo, México. This is a new, high yielding variety with light-brown seed coat. After harvest, seeds were cleaned and stored at 4°C in sealed containers until tested.

Seeds were surface-sterilized by soaking for 10 min in distilled water containing 0.1% sodium hypochlorite at room temperature. The soaked seeds were then washed with distilled water for 10 min, drained, and spread evenly in a single layer on pieces of dampened cloth which were placed in plastic trays. Seeds were incubated at 35°C for up to

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72 hr, sampling at various times during germination. After incubation, samples were dried in an oven with forced air at 45°C and milled in Cyclone Sample Mill (UD Corp., Ft. Collins, CO); before the analysis, flour was passed through an 80-mesh sieve.

Analytical methods

Water activity (a_w) of germinating seeds was measured in a Rotronic Hygroscop DT apparatus (Rotronic Instr. Co., Huntington, NY) at 25°C. Samples of 2 g reached equilibrium at about 30 min. Percent germination and hypocotyl length were determined on 50 randomly selected seeds. Moisture, Kjeldahl crude protein (N% × 5.85), fat, crude fiber and ash were determined according to AOAC (1984) procedures. True protein was estimated as follows: nonprotein nitrogen (NPN) was quantitated as the nitrogen in the supernatant recovered after having precipitated the protein from solution by means of trichloroacetic acid (24% w/w), followed by filtration. True protein was the difference between crude nitrogen by Kjeldahl and NPN, times 5.85. Reactive lysine (available lysine) was assessed by the colorimetric method of Hurrel et al. (1979). *In vitro* protein digestibility was estimated by the multienzymatic method of Hsu et al. (1977); for comparison purposes, casein (Sigma de Mexico, Mexico, D.F.) was used as reference for this determination.

Surface color of samples was measured using a Hunter-Lab D25-2 Color Difference Meter (Hunter Associates, Inc., Reston, VA). L (0 = black, 100 = white), a (+ values = red, − values = green), and b (+ values = yellow, − values = blue) were recorded. Total color difference (ΔE) was calculated from the previous Hunter parameters.

The data are means of triplicate determinations and were analyzed for statistical significance by the least significant difference (LSD) test at 5% level of probability.

RESULTS & DISCUSSION

DURING GERMINATION, a_w was kept around 0.92 (Table 1). After 12 hr, more than 89% of seeds were germinated. Thereafter, this percentage ranged from 94–95% (results not shown). Hypocotyl length attained very high values, especially as compared to the seed diameter of about 1 mm. Crude protein, true protein and crude fiber increased during germination. The rate of increase was higher for crude protein than for true protein. In other words, the NPN also was enhanced during sprouting, in agreement with reported results for winged bean germination (King and Puwastien, (1987). These NPN changes might be attributed to an increase of the proteolytic activity, consequently increasing the free amino acid content, and/or to an enhancement of nucleic acid content (Lorenz, 1980). Fat content decreased significantly (P < 0.05) and ash content remained very close to the control. Fat might have been used as an important energy source for the developing embryo (King and Puwastien, 1987). Reactive lysine values did not change statistically (P > 0.05) up to 48 hr of incubation, which is in agreement with the trend followed by total lysine during legume sprouting as reported by Hsu et al. (1980). However, with further germination reactive lysine decreased. In relation to the control, *in vitro* protein digestibility was not affected significantly (P > 0.05) at 48 and 72 hr of germination. Tests on *in vivo* digestibility need to be performed in future studies.

In general, germination resulted in lower L and higher a and b values compared to the control (Table 2). This led to an

Table 1 —Effect of germination on hypocotyl length, composition, reactive lysine and in vitro protein digestibility of amaranth seeds[a]

Time of incubation (hr)	Water activity (a_w)	Hypocotyl length (cm)	Crude protein (%)	True protein (%)	Fat (%)	Crude fiber (%)	Ash (%)	Reactive lysine (g/100g protein)	In vitro protein digestibility[b] (%)
0	0.92[c]		15.5	14.4	8.0	3.9	3.4	5.8	80.6
12	0.92	0.3	16.2	13.6	7.1	3.9	3.4	5.5	78.0
24	0.93	0.6	16.2	13.6	5.3	4.2	–	5.6	77.0
36	0.93	1.4	20.0	15.8	4.9	4.1	–	5.7	74.9
48	0.93	2.6	20.8	16.8	4.5	4.6	3.2	5.7	79.2
72	0.93	3.1	21.9	17.6	3.3	4.7	3.2	4.9	79.2
LSD (0.05)	0.00	–	1.4	0.8	0.5	0.4	0.2	0.4	0.4

[a] Values of composition expressed on a dry weight basis.
[b] Casein in vitro digestibility was 91.4%.
[c] Soaked sample.

Table 2 —Changes in Hunter color during germination of amaranth seeds

Time of incubation (hr)	Hunter parameters			
	L	a	b	ΔE
0	63.0	0.8	10.3	30.6
12	62.4	0.7	11.2	31.6
24	60.5	0.9	11.2	33.4
36	54.8	1.6	12.5	39.2
48	53.1	5.2	12.7	40.6
72	48.5	3.7	12.7	45.2
LSD (0.05)	5.9	1.2	0.7	0.7

increase in ΔE. In other words, sprouting decreased lightness of samples and enhanced red and, to a lesser degree, yellow colors. Interestingly, this suggests that the amaranth variety used here synthesized amaranthin pigments. The germinated flour showed a pinky color, resembling strawberry-based products, which could be attractive for food formulations.

In summary, a high percentage of amaranth germination was attained in a short time. Germinated samples showed high contents of true protein and available lysine. Also, protein digestibility was relatively high. The final color appeared adequate for various food applications.

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tons); ovalbumin (43,000 daltons); carbonic anhydrase (30,000 daltons); trypsin inhibitor (20,000 daltons); and α-lactalbumin (14,400 daltons).

RESULTS

THE RESULTS of kiwifruit POD activity in the crude extract and after purification with a DEAE-cellulose column are shown in Table 1.

Before determining POD activity it was necessary to dialyze the extract against pH 7,8 Tris-HCl buffer to remove the high ascorbic acid, which interfered with the peroxidase activity. Determination after purification of the crude extract by dialysis showed that the POD activity had increased over four-fold.

The results of electrophoresis are shown in Fig. 1. Only one band of 40,000–42,000 M.W. obtained by staining the separated band from the crude and purified extracts was found (column A).

In column B, (Fig. 1) the most representative kiwifruit protein bands from the crude extract are shown. These protein bands can characterize the kiwifruit. More studies are needed to identify these proteins.

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A Research Note

Odor Components of Human Breath After the Ingestion of Grated Raw Garlic

TAKESHI MINAMI, TAKASHI BOKU, KATSUHIRO INADA, MASANORI MORITA and YUKO OKAZAKI

——————————— ABSTRACT ———————————

The major odor components of finely grated, raw garlic in the air of the human mouth have been studied. Immediately after garlic ingestion, tests of the mouth air of six subjects revealed two major peaks that were positively identified as being allyl mercaptan and diallyl disulfide; these findings are based on an evaluation of the fragment patterns of a GC-MS analysis and from the actual retention times of these authentic compounds. The allyl mercaptan level was higher than the level of diallyl disulfide, although both compounds smell like garlic. Our results suggest that after garlic ingestion, allyl mercaptan is the major garlic-smelling compound in human mouth air, and diallyl disulfide is secondary.

INTRODUCTION

THE IDENTITY OF odor compounds in human mouth air after garlic ingestion have yet to be established, even though a number of volatile compounds of garlic have been determined (Akashi et al., 1975; Freeman, 1975; Malpathak and David, 1986). In other studies on garlic, Brodnitz et al. (1971) reported that diallyl thiosulfinate is a major odor compound and Nishimura et al. (1971) claimed that methyl allyl disulfide, diallyl disulfide, and methyl allyl trisulfide are the major odor compounds. These determinations, however, were done under *in vitro* conditions.

Thus, the purpose of this study was to identify the odor components of garlic in the air of the human mouth immediately after the ingestion of finely grated garlic.

MATERIALS & METHODS

DOMESTIC GARLIC CLOVES were purchased locally. Allyl mercaptan was obtained from Nakarai Chemicals Co. (Kyoto) and diallyl disulfide was a gift from Dr. K. Yamaji, Kanebo Co. Ltd. (Osaka).

The mouth air evaluations were performed in six subjects, 20 to 30 years old, who were randomly selected from a nonsmoking group.

Raw garlic was finely grated and 5.0g were consumed between two slices of bread. One side of a glass microhematocrit capillary tube (Drummond Sci. Co., USA) was sealed with clay and the needle of a 5.0 mL Shimadzu gas tight syringe (MS-GAN 500) was inserted into the sealed side. At the time of consumption, and at 1, 2, and 3 hr after eating, the open side of the tube was placed between the subject's teeth and 5.0 mL mouth air was collected. The air was injected into a Shimadzu GC-9A gas chromatograph (Shimadzu Co., Japan) and on to a 2m × 2mm i.d. glass column packed with 25% 1,2,3-Tris (2-cyanoethoxy) propane and an 80 to 100 linear inch mesh coated with Shimalite (AW-DMCS) (Shimadzu Co.). The column oven temperature was programmed from 60°C to 130°C at 2.5°C per min. Injection and detection port temperatures were maintained at 150°C. The separated components were detected with a flame photometric detector (FPD) and recorded with a Shimadzu C-R3A Chromatopac.

One gram of finely grated garlic was put into a 30 mL glass vial to which 1.0 mL distilled water was added; and, for a separate test,

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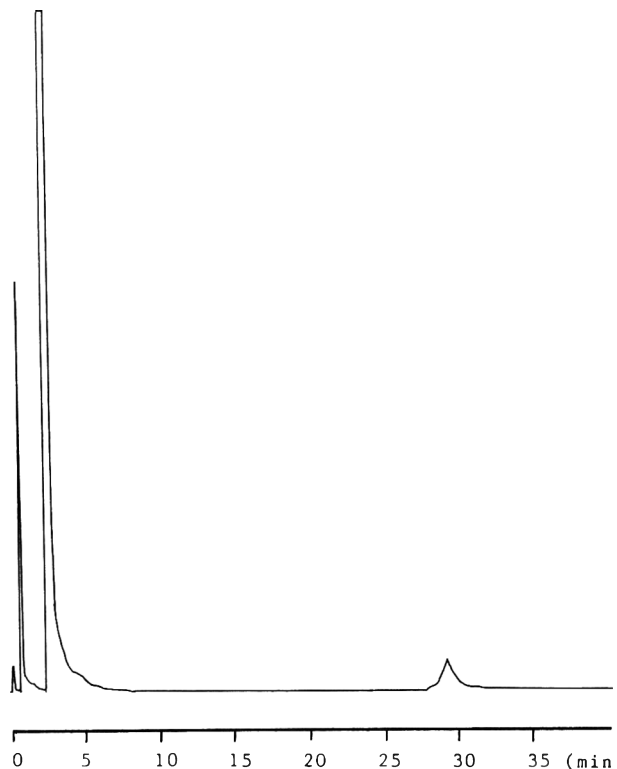
Fig. 1—Typical gas chromatographic pattern of human mouth air after eating grated garlic.

0.5g sliced garlic was put into a second 30 mL glass vial. These vials were then sealed with silicon caps, incubated at 37°C for 15 min, after which 0.5 mL of the head space vapor from each vial was analyzed by GC.

Mass spectral analyses of the head space vapor were carried out on a JEOL JMS-HX 100 and a JEOL-DA 5000 (JEOL Japan). Five grams sliced garlic were put into a 30 mL vial and incubated for 1 hr at 37°C, after which 2.0 mL head space vapor was injected directly into the GC-MS column.

RESULTS & DISCUSSION

WHEN THE MOUTH AIR was analyzed by GC after ingestion of the grated garlic, four peaks were detected with retention times of 0.26 min, 1.4 min, 2.6 min, and 29.0 min (Fig. 1). The head space vapor of the grated garlic also had four peaks with retention times of 1.5 min, 18.3 min, 27.8 min, and 29.0 min, though no comparable peak at 2.6 min was seen. With regard to the sliced garlic, however, four peaks with retention times of 2.6 min, 18.3 min, 27.8 min, and 29 min were observed in the head space vapor, and the peak at 2.6 min was even greater when the incubation time was prolonged. The head space vapor of the sliced garlic that had been incubated for 1 hr at 37°C was analyzed by GC-MS and six peaks were detected.

Characterization of Water Soluble Egg Yolk Proteins with Isoelectric Focusing

WALDEMAR TERNES

———————— ABSTRACT ————————

Separation and characterization of some water-soluble egg yolk proteins such as livetins and phosvitins were carried out by isoelectric focusing (IEF). The isoelectric points (IP) of phosvitins were in the pH-range of around 4, those of the livetins were in the range of 4.3 - 7.6. There are occurring differences between the bands of α, β- and γ-livetins. While the first mentioned had an amount of bands in the pH range 4.3–4.7, the γ-livetins indicated only a few. Above that, a large number of bands can be noted in the range 5.3 to 5.5 referring to the α, β-livetins whereas none can be found when regarding the γ-livetins.

INTRODUCTION

THE EGG YOLK is a complex food system of particles called granules and the soluble phase, the plasma. These phases can be separated easily by high speed centrifugation (Bernardi and Cook, 1960; Saito et al., 1965). The granules can be disrupted to release lipoproteins and water soluble phosvitins by means of sodium chloride. The plasma consists of low density lipoproteins and livetins.

Several apoproteins in the individual egg yolk fractions have been separated by column chromatography (Tsutsui and Obara, 1982; Kocal et al., 1980) and preparative sodium dodecylsulfate gel electrophoresis (SDS) techniques (Itoh et al., 1986). Chang et al. (1970) and Itoh et al. (1986) described the overlapping problems of the individual bands of the egg yolk proteins with the SDS-method. Abe et al. (1982), isolated two components (α- and β-phosvitins) by gel filtration. By SDS-electrophoresis the α-phosvitin fraction can be separated into three bands and the β-phosvitin into one. With disk-gel-electrophoresis, Powrie (1977) isolated fifteen protein bands from the livetins. In the present study, isoelectric focusing (IEF) was used to determine the separation efficiency for water-soluble yolk proteins.

MATERIAL & METHODS

THE YOLK OF EGGS (approx. 1 wk old) was separated into granules and the plasma fraction by ultracentrifugation according to the method of Saito et al. (1965). The yolk was diluted with a 0.02 M NaF and 0.05M NaCl solution at the rate 1:1 and centrifuged at 4,000 × g for 1 hr. The separated plasma and granules were lyophilized, prior to the isolation of livetins and phosvitins according to the method of Bernardi and Cook (1960). In addition, phosvitin was produced from Serva for comparison. A further separation into α, β- and γ-livetins was effected with a (NH$_4$)$_2$SO$_4$ solution of 37.5% with the γ-livetin fractions removed as a precipitate. The protein fractions, obtained in this way, were dialyzed against distilled water in order to remove the (NH$_4$)$_2$SO$_4$ and then lyophilized.

The isolated protein samples were stirred with water and centrifuged

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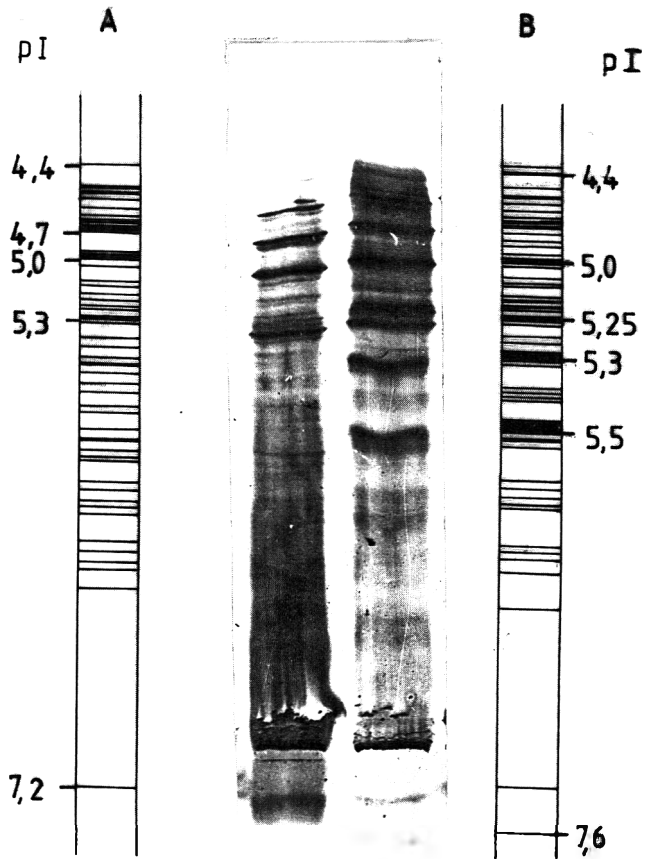
Fig. 1. — Isoelectric focusing of the livetins. A: γ-livetin; B: α-and β-livetin.

at 30,000 × g for 5 min. Referring to the aqueous phase, 10 μL of the sample solution was applied to the gel with the aid of an application stripe. Precotes of polyacrylamide gel for isoelectric focusing from Serva with varying pH range (3–10) were used as gels. The electrophoresis was conducted with a potential difference of 2,000 volts for 1 hr. The gel was fixed by a Coomassie blue solution of 0.05% in a mixture of water/methanol/acetic acid (9:9:2 v/v/v) and destained with a mixture of water/methanol/acetic acid (6:3:1 v/v/v). The pH gradient was measured with standard proteins (from Serva) of which the individual IP values were known.

RESULTS

BY MEANS OF THE IEF approx. 25 bands are certainly occurring in the pH-range 4.3 to 7.6. (Fig. 1) While bands of α- and β-livetins can be observed in the pH range 4.3 to 4.6, 5.3, and 5.5, and γ-livetin bands can not be found in these areas.

pI

Fig. 2.—Isoelectric focusing of phosvitin from Serva.
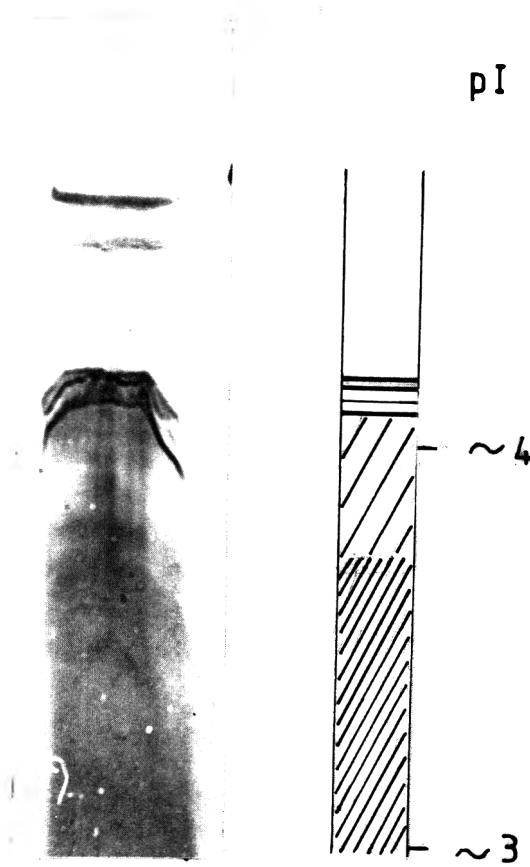
Within the pH-ranges of 4.5, 4.7 and 5.0, clearly recognizable bands of α-, β- and γ-livetins were found. When a 10% egg-yolk water solution was applied to a gel for the IEF, only the bands of the livetins were formed since the lipoproteins did not move into the gel under the conditions given. Only at high concentrations phosvitin fractions were visible (Fig. 2). Three near-located bands were found in the pH-range of 4.0. The IEF of phosvitins leads to the supposition that their IPs are situated within low pH-values.

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GARLIC ODOR COMPONENT OF HUMAN BREATH. . .From page 763
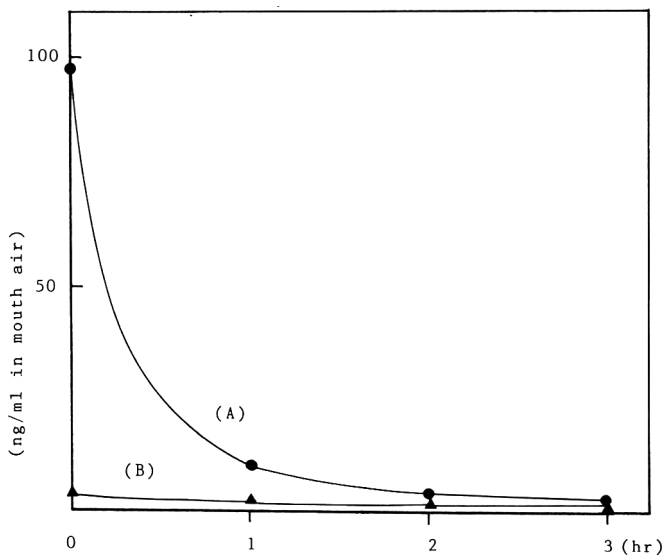
Fig. 2—The time course of the allyl mercaptan and diallyl disulfide concentrations in mouth air after ingestion of grated garlic. Allyl mercaptan (A) and diallyl disulfide (B) concentrations were calculated from their standard curves. Results are expressed as the average obtained from six samples.

From the published data and the standard fragment patterns, dimethyl disulfide, dipropyl disulfide, allyl mercaptan, methyl allyl disulfide, and diallyl disulfide were identified; one peak is still unknown. From the fragment patterns obtained by GC-MS analysis and from the retention times of the authentic compounds, the compound with a retention time of 2.6 min was allyl mercaptan, and the compound with a retention time of 29.0 min was diallyl disulfide.

Many reports suggest that diallyl disulfide is one of the main odor components of garlic (Akashi et al., 1975; Nishimura et al., 1971). Yet allyl mercaptan was detected in the mouth air after eating grated garlic, and when the allyl mercaptan and diallyl disulfide concentrations in mouth air were calculated from their standard curves, the allyl mercaptan level was greater (Fig. 2). Therefore, though both the standard substances of allyl mercaptan and diallyl disulfide smell like garlic, our results suggest that allyl mercaptan is the major garlic-odor compound in human breath after the ingestion of grated garlic.

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A Research Note

Soy Proteins in Milk Replacers Identified by Immunoblotting

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——————————— ABSTRACT ———————————

A method was developed for detecting soy proteins in milk replacers and infant formulas. The method is based upon polyacrylamide gel electrophoresis of proteins, electroblotting the proteins to nitrocellulose, and detection of specific proteins by immunoblotting. As little as 25 ng of total soy protein per gel lane can be detected with this method. Proteins from other plant products were detected by the method but care must be used in comparing milk replacer proteins directly to soy proteins. This method should be of value to the animal and food industries that manufacture and use milk replacers.

INTRODUCTION

PROTEIN is a major cost ingredient in milk replacers and the demand for and availability of milk protein often leads to use of alternative protein sources. Soybean protein has been used as an alternated protein source in milk replacers and infant formulas (Dawson et al., 1988; May et al., 1982; Ramsey and Willard, 1975; Silva et al., 1986a). However, the presence of soy protein in milk replacers can be detrimental to the growth performance of young animals (Dawson et al., 1988; Flavin, 1982; Silva et al., 1986a), resulting in gastrointestinal allergic responses (Barrett et al., 1979; Kilshaw and Sissons, 1979), and altered intestinal morphology (Seegraber and Morrill, 1986; Silva et al., 1986b). The increasing use of alternative nonmilk protein sources in milk replacers must be accompanied by methodologies for the detection of those proteins in milk replacer products. A sensitive method for detection of soy proteins in milk replacers is described in this paper.

MATERIALS & METHODS

Sample preparation

Soy flour, soy protein concentrate, soy isolate and calf milk replacers were supplied by Milk Specialties Co. (Dundee, IL). Milk replacer protein contained skim milk protein with soy isolate added at 0% (all milk), 2.5%, 5%, and 10% by weight, resulting in soy protein accounting for 0%, 10.9%, 21.8%, and 43.6% of the total protein, respectively. Skim milk used as a control in the immunoblotting was obtained by centrifuging fresh whole milk at 10,000 × g for 30 min at 4°C. Commercially available infant formulas tested included an all-milk whey protein-based formula and two soy protein-based formulas.

All samples were diluted to 1 mg/mL with water (total protein concentrations determined using a dye-binding assay, BioRad, Richmond, CA). Twenty microliters of sample were mixed with 4 µL sample buffer [25% 2-mercaptoethanol, 14.5% sodium dodecyl sulfate (SDS) and 0.28 M Tris, pH 6.8] and 4 µL dye solution (70% glycerol with 0.12% bromophenol blue) in preparation for gel electrophoresis. Samples were heated to 90°C for 10 to 15 min just prior to gel electrophoresis. Unless otherwise indicated, 5 µg of sample protein (7 µL) were loaded per lane.

Gel electrophoresis and protein blotting

Samples were electrophoresed on 15% SDS-polyacrylamide slab gels (8 × 10 cm, 0.8 mm thick, Idea Scientific, Corvallis, OR), as described by Laemmli (1970). Proteins were transferred to nitrocel-

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lulose (BA-85, Schleicher and Schuell, Keene, NH) by electroblotting (Transblot. BioRad, Richmond, CA) at 0.06 A for 12 to 14 hr at 22°C, in 24 mM Tris, pH 8.3, 192 mM glycine, and 20% methanol (v/v).

Immunoblotting

Protein blots were blocked with 5% bovine serum albumin (BSA) in TBS/NF-40 (0.1 M NaCl, 0.05 M TRIS, pH 7.6, 0.05% Nonidet P-40) for 20 min at 22°C. Primary antiserum (rabbit anti-soy protein serum was graciously supplied by Dr. A.G. Hunter, Univ. of Minnesota) was diluted (1:500) in 5 mL of 1% BSA TBS/NP-40 and applied to the blots for 2 hr at 22°C with constant agitation. After blots were washed three times (5 min each wash) in TBS/NP-40, affinity purified goat anti-rabbit IgG (H & L) conjugated to alkaline phosphatase (Bio-Rad Laboratories, Richmond, CA) was diluted (1:10,000) in 5 mL 1% BSA TBS/NP-40 and applied to the blots. Blots were incubated with second antibody for 2 hr at 22°C with constant agitation. Blots were washed as before and detected using the alkaline phosphatase color substrate solution freshly prepared as follows: 66 µL of NBT (nitroblue tetrazolium, 50 mg/mL in 70% dimethylformamide) was added to 10 mL of buffer (0.1M TRIS, pH 9.5, 0.1M NaCl, 5 mM $MgCl_2$) and mixed, followed by addition of 33 µL of BCIP (5-bromo-4-chloro-3 indolyl phosphate, 50 mg/mL in dimethylformamide) and mixed again. The solution was protected from light until use (within 1 hr). Developed blots were rinsed in stop solution (0 02M TRIS, pH 8.0, 5 mM EDTA) and dried for 10 to 15 min at 75°C.
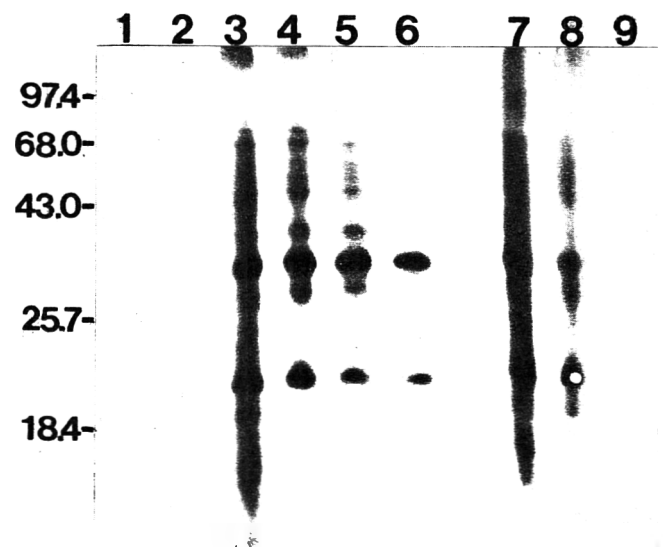
Fig. 1 — Immunoblot of soy proteins in milk replacers. Lanes are [1] skim milk, [2] milk replacer containing no soy protein, [3] soy isolate, [4] milk replacer containing 10% soy isolate, [5] milk replacer containing 5% soy isolate, [6] milk replacer containing 2.5% soy isolate, [7] infant formula containing 3.8% soy isolate, [8] infant formula containing 4.5% soy isolate, and [9] infant formula containing no soy isolate. Molecular weight markers are in kilodaltons (left side of the blot).
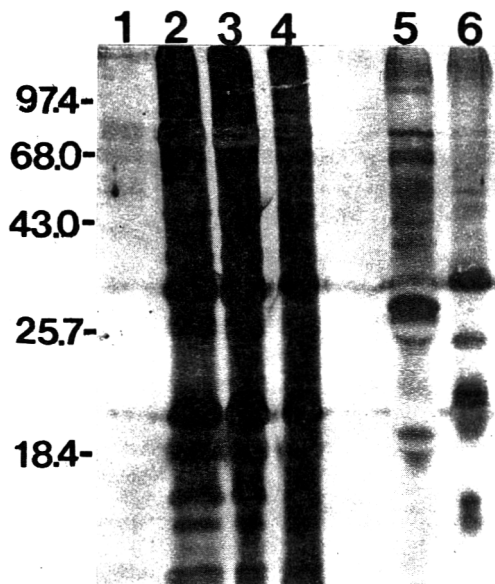
Fig. 2—Immunoblot of proteins. Lanes are [1] milk replacer containing no soy protein, [2] soy flour, [3] soy protein concentrate, [4] soy isolate, [5] sesame seed, and [6] sunflower seed. Molecular weight markers are in kilodaltons (left side of the blot).

RESULTS & DISCUSSION

NUMEROUS PROTEIN BANDS were detectable in milk replacers containing 10%, 5% or 2.5% (w/w) of soy isolate, corresponding respectively to 43.6%, 21.8%, and 10.9% of the total protein in the milk replacers (Fig. 1, lanes 4, 5, and 6, respectively). No proteins were detected in either skim milk or a milk replacer based only on cow milk protein (Fig. 1, lanes 1 and 2, respectively). The pattern of bands in the milk replacers containing soy protein were similar to that of soy isolate (Fig. 1, lane 3). Immunoblotting infant formulas that contained soy protein concentrate resulted in a protein band pattern similar to that in soy protein-containing milk replacers (Fig. 1, lanes 7 and 8). An infant formula based only on cow milk protein was negative for soy proteins (Fig. 1, lane 9).

Proteins in soy flour, soy protein concentrate, and soy isolate were compared by immunoblotting. The overall protein band patterns were similar, but there was greater proportion of immunostaining at the higher molecular weight regions of the soy flour or soy protein concentrate lanes than in the soy isolate lane (Fig. 2, lanes 2, 3 and 4, respectively). Protein bands were detectable by immunoblotting when as little as 25 ng soy isolate protein was loaded per lane (not shown).

Immunoblotting methods have been used to detect several non-meat proteins in heat-processed meat products (Janssen et al., 1987). Enzyme-linked immunosorbant assays (ELISA) have been used to quantitate soy protein in meat products (Olsman et al., 1985) and a dot blotting method has been used for screening meat products for non-meat proteins (Janssen et al., 1987). However, the ELISA and dot blot assays do not necessarily account for cross-reactivity of antiserum with other plant antigens. Dot blot immunoassays for zein proteins have been found to cross-react with proteins from other cereal species (Esen et al., 1983).

To evaluate whether other plant protein sources might be detected by this anti-soy protein serum, proteins from sesame seeds and sunflower seeds were examined by the immunoblotting procedure. Proteins were detected in both the sesame and the sunflower seed samples (Fig. 2, lanes 5 and 6, respectively). However, the protein patterns detected in those samples did not correspond to protein patterns in the soy protein preparations (Fig. 2, lanes 2, 3, and 4). The immunoblotting procedure, although not quantitative, provides the advantage over many immunoassays of distinguishing between cross-reaction of the antiserum by comparing protein band patterns with controls.

This immunoblotting technique provides a sensitive and specific method of detecting soy proteins in milk replacers or infant formulas. The method should be of value to the food industry in quality control of milk replacer products. The method could be modified to detect other nonmilk proteins in milk replacer products and could be used in conjunction with quantitative immunoassays.

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A Research Note
Compositional Analysis of Powdered Cocoa Products by Near Infrared Reflectance Spectroscopy

J. J. PERMANYER and M. L. FEREZ

ABSTRACT

Near infrared reflectance (NIR) spectroscopy was used to measure moisture, fat, and sucrose in powdered cocoa products. Spectra of a series of known samples were recorded and multiple linear regression techniques were used to relate the concentrations of each parameter to reflectance measurements at selected wavelengths. Precision and accuracy were estimated to evaluate the potential application of the NIR spectroscopy in the quality control of powdered cocoa products. Results showed that moisture, fat, and sucrose could be analyzed in powdered cocoa products by near infrared reflectance spectroscopy because good correlation coefficients and low standard errors were achieved in prediction study.

INTRODUCTION

NEAR INFRARED REFLECTANCE (NIR) spectroscopy is receiving widespread attention as a rapid method for determining the composition of many foods. It has been applied to the rapid analysis of food and agricultural products including cereals and cereal products, feedstuffs, forages, milk, meat, seeds, legumes and confectionery (Giangiacomo et al., 1981; Osborne, 1981, 1986; Baer et al., 1983; Suzuki et al., 1986). Only a few studies, however, have been reported for the analysis of cocoa products (Kaffka et al., 1982).

Powdered cocoa products added to milk are commonly consumed for breakfast by children. They mostly consist of sugar and cocoa powder. Therefore, the quality control for such products focuses on their compositional analysis. To this end, the traditional wet chemical methods are involved and time-consuming.

The objective of this study was to investigate the use of NIR spectroscopy as a rapid analytical method for determining moisture, fat, and sucrose of the powdered cocoa products which would aid their quality control.

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MATERIALS & METHODS

SINCE THE NIR technique is based on the correlation of spectroscopic response with the constituent concentration, calibration of the NIR instrument was carried out on a set of known samples in which moisture, fat, and sucrose varied. Fifty-six samples were directly taken from production, and nine samples were specially formulated in the pilot plant by mixing the raw materials (sugar and cocoa powder containing 10–12% fat) in different ratios (Table 1). Another set of 10 different samples from production was used as a prediction set to test the performance of the calibration equations.

The reference chemical methods were: for moisture and fat, the official methods of the International Office of Cocoa, Chocolate and Sugar Confectionery (IOCCC, 1952a, 1952b) and for sucrose, the method from Boehringer Mannheim (1974).

For the NIR analysis, no sample preparation was required because of the homogeneous particle size and the fineness of the product. Samples were scanned from 1900 to 2320 nm using a single beam NIR spectrophotometer with three tilting filters (Pacific Scientific, Gardner Neotec Division, Model Compscan 3000). It had a baseline correction to correct the sample curves for system response. The detection system was based on lead sulfide detectors mounted at a 45° angle to the surface of the sample. The reflectance data were the average of 32 scans per sample. For the development of calibration equations a mathematical transformation of the raw optical data was made to obtain the first and second derivative spectra (Fig. 1). Reference chemical values of the 65 samples were entered into the computer and were correlated with the reflectance energies. Step-forward linear least square regression yielded an equation of the form:

$$C = K_0 + \sum_{i=1}^{P} K_i L_i$$

where, C is the concentration of a given constituent in the product, p is the number of terms in the equation, K_0 and K_i are the calibration constants, and L_i, the mathematical transformation of the raw optical data measured at the wavelength λ_i, i.e., first or second derivatives.

The performance of the NIR procedure was checked by calculating the standard error of calibration (SEC):

$$SEC = \sqrt{\frac{\sum_{i=1}^{n} (Y_i - y_i)^2}{n-1}}$$

where, Y_i is the reference chemical value, y_i the NIR values, and n

Table 1 —Development and performance of NIR spectroscopy calibration equations for the analysis of powdered cocoa products

Parameter	Range (%)	Calibration constants		Wavelengths (nm)	Mathematical treatment	Correlation coefficient (r)	Standard error of calibration (SEC)	Range (%)	Correlation coefficient (r)	Standard error of prediction (SEP)
						Calibration			Prediction	
Moisture	1.5 - 4.0	K0 =	3.592		1st derivative	0.978	0.157	1.6- 2.1	0.955	0.034
		K1 =	1.306	1939						
Fat	1.5 - 3.5	K0 =	3.640		2nd derivative	0.987	0.100	2.1- 2.7	0.842	0.051
		K1 =	−10.407	2296						
		K2 =	− 2.109	1959						
Sucrose	51.5 - 81.5	K0 =	69.726		1st derivative	0.998	0.526	68.0-74.5	0.962	0.680
		K1 =	−6.505	2080						
		K2 =	36.671	2285						
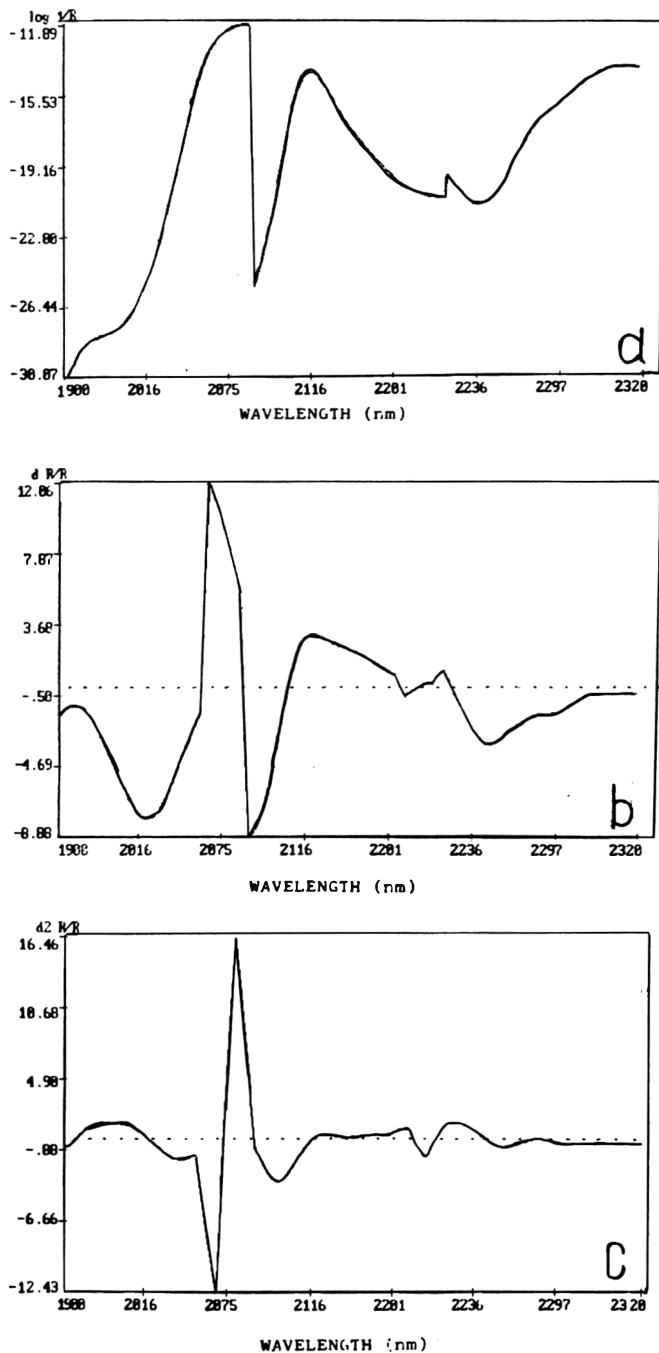
Fig. 1—Near infrared spectra of powdered cocoa product: (a) original spectrum; (b) 1st derivative-spectrum; (c) 2nd derivative-spectrum.

is the number of samples. Accuracy and precision of calibration were evaluated by the standard error of prediction (SEP):

$$SEP = \sqrt{\frac{\sum_{i=1}^{n} di^2}{n}}$$

where, di is the difference between reference and NIR values and n is the number of samples.

RESULTS & DISCUSSION

THE COMPUTER PROGRAM determined the characteristic wavelengths, calibration constants and mathematical transfor-

mations that gave the best fit. Results are summarized in Table 1. Only two term equations were used because the addition of other wavelength provided no improvement in calibration results.

High correlation coefficients and low standard errors of calibration indicated that the calibration equations were satisfactory and that good prediction of unknown samples could be achieved. This was demonstrated by results of the prediction study (Table 1) which contains multiple correlation coefficients and standard errors of prediction.

No significant bias was found calculating the t-value for each constituent by the equation,

$$t = \frac{\sqrt{n}\ (\overline{LAB} - \overline{NIR})}{\sigma b}$$

where n is the number of samples and σb = $\sqrt{1/2\ (\sigma lab^2 + \sigma nir^2)}$ and comparing with the Student's t-distribution values with n-1 degrees of freedom and a two-sided alternative hypothesis at $\alpha = 0.05$.

The precision of the NIR method was measured by recording ten spectral scans on the same sample and calculating the standard deviations of the prediction data for each constituent. The coefficients of variation were 0.86% for moisture, 1.49% for fat and 0.61% for sucrose.

Accuracy, compared with reference methods, was measured by comparing the root mean square prediction error or standard error of prediction (SEP) with the standard error of calibration (SEC) (Moen, 1976; Miner et al., 1978; Osborne and Fearn, 1986) and by the analysis of variance (ANOVA). The ANOVA test showed no significant difference between the results obtained by NIR and by chemical analysis (p<0.05), meaning that the NIR method was as accurate as wet chemistry methods for moisture, fat and sucrose of the powdered cocoa products examined.

In summary, a NIR spectroscopy method was developed to provide a routine, fast and efficient method for determining moisture, fat and sucrose in powdered cocoa products. Calibration equations were checked for precision and accuracy and good prediction results were achieved. Therefore, the NIR spectroscopy technique could be used in quality control of powdered cocoa products, for moisture, fat, and sucrose.

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A Research Note

Isolation and Identification of Volatile Compounds from Glassine Packages

H. KIM and S. G. GILBERT

──────────── ABSTRACT ────────────

The volatile flavor compounds in glassine packages were characterized using headspace concentration technique and GC/MS. The major compounds identified were 2,2-dimethyl-1,3-propanediol, 3,3-dimethyl-4-hydroxybutanal, and 2,2-dimethyl-1,3-propanediol diacetate. Compounds representing hydrocarbons, alcohols, and phthalates were also identified. The majority of these compounds could be attributed to residues from the lacquer applied to the glassine paper as a curable coating.

INTRODUCTION

PAPER is one of the most important packaging materials. The weight of paper and board used in packaging is approximately equal to the weight of all other packaging materials combined. Paper products are modified for particular uses in packaging by three types of operations in converting paper for package use: coating, impregnation, and lamination.

One practical concern in polymeric packaging is the presence of compounds in amounts accepted by FDA as toxicologically insignificant (e.g., 1×10^{-4} g/g for toluene) but at concentrations affecting taste and/or odor quality in the packaged food (Wilks and Gilbert, 1972; Gilbert, 1985). A frequent source for those compounds has been residual solvents from printing and laminating inks and adhesives (Kumai et al., 1983; Heydanek et al., 1979). Styrene, a residual monomer in polymeric materials, was also shown to create undesirable taste when it migrated into food simulants (Miltz et al., 1980).

Compounds formed through thermal degradation of additives and/or polymers during processing also can migrate into package contents and create off-flavors (Kim et al., 1988; Fernandes et al., 1986). Morano (1974) found that certain batches of glassine candy wraps created an undesirable flavor in the chocolate candy.

The objectives of this study were: (a) to isolate and identify the major volatile compounds released from glassine packages and (b) to determine the possible source for those compounds in the package structure.

MATERIALS & METHODS

A ROLL SAMPLE which had induced deleterious effects on product flavor was received from the manufacturer. The paper was constructed of wax-coated glassine base with a printed color system and an overprint lacquer. The cure coat lacquer used during manufacturing the packages was also obtained. The authentic compound such as 2,2-dimethyl-1,3-propanediol used during the analyses was obtained from Fisher Scientific Co. (Fair Lawn, NJ).

Headspace volatiles were concentrated by a polymer trap made by packing a glass tube (0.4 cm × 12 cm) with 80 mg of Tenax GC and 80 mg of Carbopack B. A total of 1.3g of paper was cut into small pieces and placed into a 1.2 cm i.d. × 17.5 cm L stainless steel tube. The sample was kept at 70°C with the headspace continuously purged for 2 hr onto the polymer trap at the flow rate of 25 mL/min. The

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Table 1 — Volatile compounds identified in a glassine package

Peak no.[a]	Scan no.[b]	Compound
1	74	pentane
2	108	heptane
3	112	isopropanol
4	131	toluene
5	154	3,3-dimethyl-4-hydroxybutanal
6	322	2,2-dimethylpropane-1,3-diol
7	509	2,2-dimethyl-1,3-propanediol diacetate
8	587	2-methyl-2-heptanol
9	613	2,4-dimethyl hexanol
10	647	undecane
11	671	2,2-dimethyl undecanol
12	680	tridecane
13	701	3-cyclohexen-1-yl benzene
14	771	2,7,10-trimethyl dodecane
15	849	2,6-bis-(1,1-dimethylethyl)-2,5-cyclo-hexadiene-1,4-dione
16	897	BHT (butylatedhydroxytoluene)
17	906	pentadecane
18	943	4-ethyl-2,6-di-tert-butyl phenol
19	969	propanoic acid, 2-methyl-, 1-(1,1-dimethyl-ethyl)-2-methyl-1,3-propanediyl ester
20	983	hexadecane
21	1043	heptadecane
22	1072	tetradecanoic acid
23	1094	octadecane
24	1118	pentadecanoic acid
25	1148	dibutyl phthalate (DBP)
26	1161	palmitic acid
27	1225	oleic acid
28	1284	n-butyl benzyl phthalate
29	1304	stearic acid
30	1346	bis-(2-ethylhexyl) phthalate (DOP)

[a] Represents the peak number in Fig. 1.
[b] Represents the scan number of each peak in Fig. 1.

trap was then inserted into a heating unit connected to the injection port of the gas chromatograph (Varian 3400, Varian, Palo Alto, CA) for desorption. The heating unit used for desorption and the desorption procedure were described in a previous paper (Kim et al., 1988). Splitless on-column injection was used during desorption. The cooling bath was then removed, and the temperature was programmed from 50°C to 340°C at the rate of 5°C/min. The gas chromatograph was interfaced to a Finnigan Mat mass spectrometer model 8230 (Finnigan Mat, Germany), linked on-line to a SS-Finnigan data processing system. The ionization voltage of the mass spectrometer was 70 eV and scan speed was 1.0 sec/dec. A 60m L × 0.25 mm ID fused silica capillary column with bonded phase DB-1, 0.25 μm thickness (J & W Scientific, Rancho Cordova, CA) was used. The mass spectrum of each peak was obtained and compared to the published data (NBS, 1986) stored in the data processing system for tentative identification. The same procedure was followed with the same sample using chemical ionization mass spectrometry.

Gas chromatographic profiles were obtained from 0.5g of glassine packages and 4g of cure coat lacquer using static headspace analyses. A 60 mL glass vial was used to prepare each sample, sealed with a silicon rubber septum and an aluminum crimp cap (Fisher Scientific Co, Fair Lawn, NJ) and flushed with nitrogen for 30 min before being heated in the oven at 110°C. After one hour heating, headspace was taken from each vial using a gas-tight syringe and injected into a gas chromatograph (Varian 3700, Varian, Palo Alto, CA) equipped with flame ionization detector. The column used was a 15 m × 0.53 mm fused silica megabore column

with bonded phase DB-17, 1μm thickness. The column temperature was programmed from 50°C to 210°C at the rate of 10°C/min with an initial hold period of 5 min. Helium was used as carrier gas at the flow rate of 8 mL/min. The injection port was kept at 250°C, and the detector was kept at 270°C with hydrogen flow rate of 30 mL/min and air flow rate of 240 mL/min.

A total of 5g of sample was extracted with methylene chloride for 48 hr using a Soxhlet apparatus to isolate and identify high boiling point compounds. The extract was concentrated to 4 mL using a 375 mm L Snyder distilling column (Kontes, Vineland, NJ). One microliter of the concentrate was injected on-column to GC/MS, and mass spectrum of each peak was obtained. The same condition for the mass spectrometer as described above was used except the column was a 60m L × 0.25 mm i.d. fused silica column with bonded phase DB-5, 0.25 μm thickness. The oven temperature was programmed from 50°C to 320°C at the rate of 10°C/min. The injection port temperature was maintained at 250°C.

RESULTS & DISCUSSION

THE RECONSTRUCTED ion chromatogram obtained with concentrated headspace volatiles is shown in Fig. 1. The list of compounds identified by GC/MS using both EI and CI are shown in Table 1. The largest peak at scan #322 in Fig. 1 was confirmed as 2,2-dimethyl-1,3-propanediol by matching the mass spectrum of the authentic compound to that of the unknown obtained at the same condition. The characteristic odor of the authentic compound was also very similar to the overall odor of the glassine package as judged by a small panel. However, quantitative sensory evaluation was not performed. The compound is used as a bifunctional monomer for increasing molecular weight of base polymer—polyester—in cure coat lacquer. The bulky dimethyl group could have decreased the crystallinity of base polymers. Cure coat lacquers are usually applied to impart gloss and stain resistance to the packaging material (Wolper and Goodwin,

1968). When the gas chromatographic profiles obtained from glassine packages and cure coat lacquer using static headspace analysis were compared to each other, the major volatile compounds released from the sample paper were found to be derived from cure coat lacquer.

Most of the long-chain hydrocarbons and branched alcohols were likely derived from the wax during hot melt coating of the glassine base. BHT, a commonly used antioxidant, and BHT related compounds such as 2,6-bis-(1,1-dimethylethyl)-2,5-cyclohexadiene-1,4-dione and 4-ethyl-2,6-di-t-butyl-phenol were also identified. Morano (1974) previously reported that p-tertiary butyl phenol (PTBP) could create bitter and burnt and old rubber-like aftertaste in packaged contents. Another compound identified in the headspace volatiles of glassine packages, 2,2-dimethyl-1,3-propanediol diacetate, is also considered to be a residual compound formed during polyester manufacturing. Propanoic acid, 2-methyl-, 1-(1,1-dimethylethyl)-2-methyl-1,3-propanediyl ester has been identified in the volatiles of PVC film (Kim et al., 1988) and is considered to be intentionally added during manufacturing as a plasticizer,

Dibutyl phthalate (DBP) and bis-(2-ethylhexyl) phthalate (DOP) were the major compounds identified in the Soxhlet extract of the glassine packages. Although DBP has bland oily odor, DOP has a characteristic undesirable odor which could considerably contribute to the potential off-odor of the the glassine packages. DOP has been reported as one of major precursors in the formation of volatile compounds in PVC films (Kim et al., 1988).

In summary, the major components of the volatile flavor in the lacquered glassine package were 2,2-dimtheyly-1,3-propanediol and its reaction products such as 2,2-dimethyl-1,3-propanediol diacetate and 3,3-dimethyl-4-hydroxybutanal. The major source for these compounds was confirmed as the cure coat lacquer.
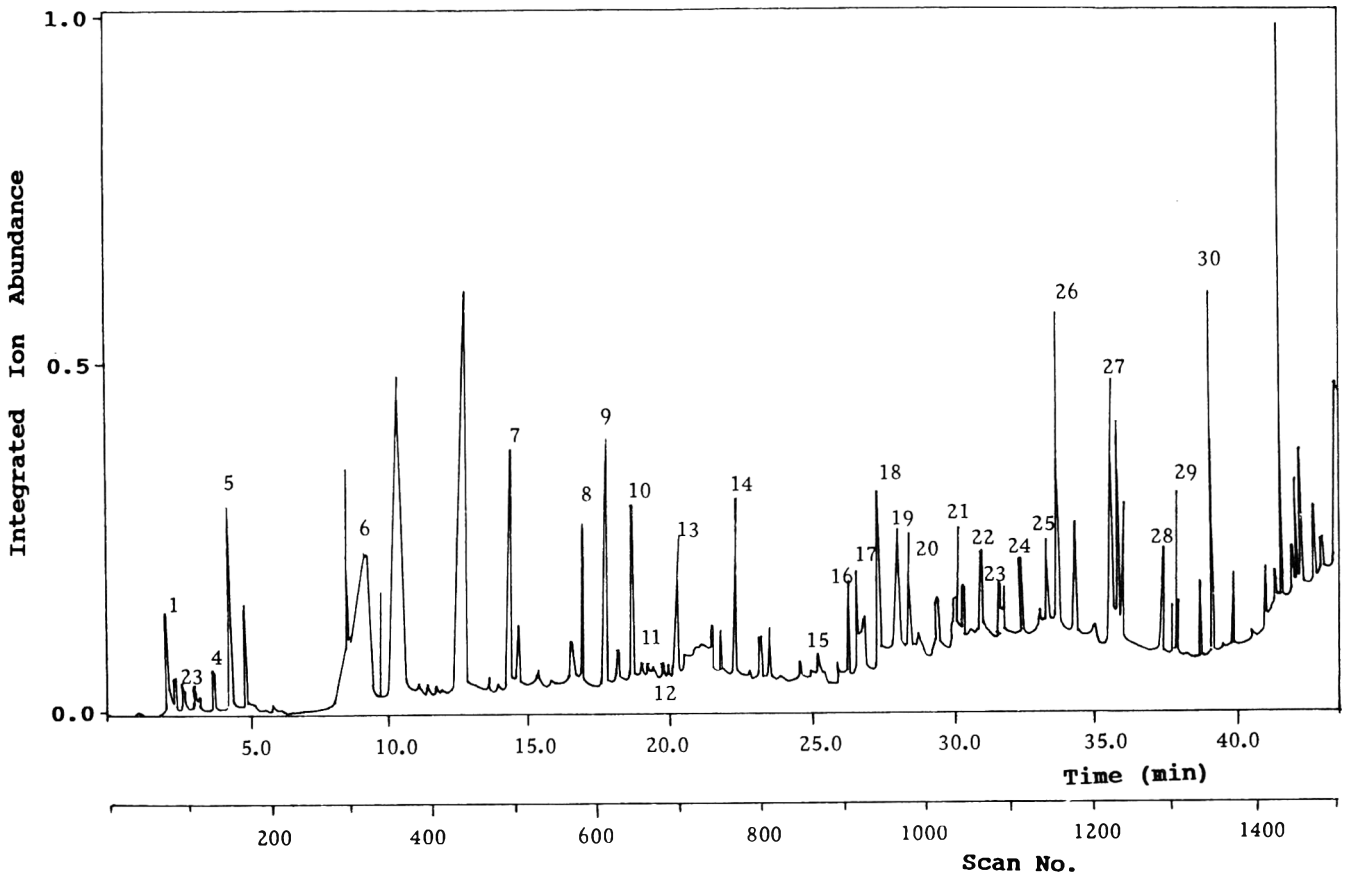
Fig. 1—Reconstructed ion chromatogram obtained with concentrated headspace volatiles of a glassine package.

A Research Note

Explosion Puffing of Fruits and Vegetables

M.F. KOZEMPEL, J.F. SULLIVAN, J.C. CRAIG, JR., and R.P. KONSTANCE

———————— ABSTRACT ————————

Explosion puffing is a processing system which facilitates hot air drying of fruits and vegetables. Explosion puffed foods are easily rehydrated and have excellent sensory properties. The process costs are similar to the cost of conventional hot air drying. Previous publications have reported the results relevant to carrots, potatoes, apples, and blueberries. Sufficient research has been done on other fruits and vegetables to establish that they, too, can be satisfactorily explosion puffed and dried to yield a good product. The processing conditions for explosion puffing of mushrooms, celery, onions, peppers, rutabagas, beets, yams, pears, pineapples, strawberries, and cranberries are presented.

INTRODUCTION

DRYING is an ancient, yet still excellent method for preserving fruits and vegetables. Conventional hot air drying is very practical during the early stages of drying; but, when drying passes from the first falling rate period to the second falling rate period, drying becomes difficult (Van Arsdel et al., 1973). The product to be dried shrivels and case hardens (Van Arsdel, 1963). The drying slows dramatically in the second falling rate period, and much more energy is consumed than in the first period. Conventionally hot air dried products are difficult to rehydrate, frequently requiring more than 20 min in boiling water to fully rehydrate (Luh and Woodroof, 1988).

Freeze drying yields an excellent product (Luh and Woodroof, 1988). Freeze-dried products rehydrate rapidly and retain high quality; but, its expense, resulting from high energy and capital costs, prohibit its use for most commodities.

Explosion puffing of fruits and vegetables is a relatively new process, investigated for some years at the Eastern Regional

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Research Center (Sullivan and Craig, 1984). Early batch work used a modified cereal puffing gun. After years of research and modification of a series of puffing guns, the continuous explosion-puffing system (CEPS) evolved.

Sullivan and Craig, (1984) have summarized explosion puffing research, both batch and continuous, and Heiland et al. (1977) have described the CEPS. Logically, most of the research centered on commodities that would command a large portion of the dried food market—potatoes and carrots for vegetables (Sullivan et al., 1977, 1980, 1983) and apples for fruit (Sullivan et al., 1981). In 1984 the Georgia Blueberry Association built a plant to explosion puff blueberries (Anon, 1984 and Sullivan et al., 1982).

During the research on both batch and continous puffing, various other commodities have been studied on a limited basis. Usually the work was done as the result of a request for a particular product. The objectives of this research were to determine whether the commodities studied could be explosion-puffed and to establish acceptable processing conditions for those which could be puffed.

MATERIAL & METHODS

THE EQUIPMENT and process have been fully described in the earlier publications (Sullivan et al., 1977, 1984; Heiland et al., 1977).

The raw material was peeled, trimmed, washed, cut, and sized as appropriate. Celery, rutabagas, sweet potatoes, beets, peppers, and onions were cooked or blanched before drying to the proper puffing moisture; whereas, mushrooms, cranberries, strawberries, pineapples, pears, apples, and blueberries were dried without cooking. The preferred processing conditions for all products successfully puffed are listed on Table 1.

Because the puffed commodity was porous and easy to dry, finish drying to a safe moisture level (generally from 2–10% depending on the commodity) was done at 54–66°C to avoid scorching the product. Products were rehydrated by placing in boiling water for the required time—typically about 5 min.

Table 1 — Processing conditions in preparation for explosion puffing

Commodity	Steam blanch, min	NaHSO₃ %	NaHSO₃ min	Dry bulb temp	Moisture %	Size mm	Varieties
Celery	6	1/2	1/2	66	25	12.7	Calif. Paschal
Mushrooms	--	1	5	79	20	8.0 (slices)	*Agaricus Lampestris*
Onions[a]	--	--	--	--	15	slices	Calif. White Globe/Crecle
Peppers	2	1/2	2	77	19	12.7 × 12.7 × 19.1	Calif. Wonder
Beets	50	--	--	77	20-26	9.5	Detroit Dark Red
Rutabaga	6	1/2	6	82	25	9.5	—
Yams	8	1/2	1	82	25	12.7 × 12.7 × 19.1	Centennial
Pears	--	1	2	77	18	9.5	Anjou; Bartlett
Pineapple[a]	--	--	--	66	18	(chunks)	—
Strawberries	--	--	--	66	25	whole	Cardinal
Cranberries	--	--	--	77	17-26	whole	Champion/Early Black
Cranberries	--	--	--	77	24	sliced	Champion/Early Black
Potatoes[b]	15	1/2	1/2	93	25	9.5	Any variety
Blueberries	--	--	--	88	18	whole	Any variety
Apples[c]	--	1	5	82	15	wedges	Any variety
Carrots	14	--	--	95	25	9.5	Imperator

[a] Preprocessing done by supplier.
[b] Precooked @ 71°C for 15 min.
[c] Add 0.75% (dry basis) granulated monoglyceride to prevent sticking.

RESULTS & DISCUSSION

ONIONS, peppers, rutabagas, beets, yams, and pears were successfully explosion puffed in the batch process. Pineapples, strawberries, and cranberries were successfully explosion-puffed in the CEPS. With only minor adjustments in the conditions used in the successful batch processing, celery puffed satisfactorily in the CEPS. The explosion puffing processing conditions for these commodities as well as those previously reported (potatoes, apples, carrots, blueberries, and mushrooms) are listed in Table 2.

In previous studies, Rome Beauty and Winesap apples as well as Rabbiteye blueberries were puffed. In this study, Granny Smith apples and New Jersey Bluecrop blueberries were used. There was virtually no difference in the processing conditions used. In fact, no fruit or vegetable successfully puffed in the batch system has subsequently failed in the CEPS.

Not all foods were successfully explosion-puffed. Legumes, such as beans, with a tough outer skin failed. The skin separated from the bean without puffing. Raisins scorched without puffing. Raspberries blew apart. Attempts at puffing peanuts

and coconut also failed. Grains, such as wheat, rye, amaranth, and rice, require pressure (>700kPa) beyond the system capacity. Meat could not be explosion-puffed.

Most of the products rehydrate in boiling water in 5 min. The three notable exceptions are yams, peppers, and pineapples. Yams take up to 10 min whereas peppers take 2 min and pineapple only 1 min. In fact, pineapple rehydrates in about 15 min in cold water. The rehydration times are included in Table 2.

CONCLUSION

CELERY, onions, peppers, rutabagas, beets, yams, pears, pineapples, strawberries, and cranberries can be successfully explosion-puffed in addition to potatoes, carrots, apples, mushrooms, and blueberries. Processing conditions to achieve a good product are listed. Only minor process adjustments are needed to account for natural raw produce variability.

Table 2 – Explosion puffing conditions for fruits and vegetables

Commodity	Type	Steam pressure kPa	Temp °C	Dwell time sec	Rehydration min
Onions	Batch	414	154	30	5
Peppers ·	Batch	207	· 149	45	2
Beets	Batch	276	163	120	5
Rutabagas	Batch	241	160	60	6
Yams	Batch	241	160	75	10
Pears	Batch	228	154	60	5
Pineapple	CEPS[a]	83	166	60	1
Strawberries	CEPS	90	177	–	3
Celery	CEPS	275	149	39	5
Carrots	CEPS	275	149	49	5
Apples	CEPS	117	121	35	5
Blueberries	CEPS	138	204	39	4
Mushrooms	CEPS	193	121	39	5
Potatoes	CEPS	414	176	60	5
Cranberries	CEPS	138	163	64	3

[a] CEPS = continuous explosion puffing system

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A Research Note
Determination of Carotenoids in Fruits of *Rosa sp. (Rosa Canina* and *Rosa Rugosa)* and of Chokeberry *(Aronia Melanocarpa)*

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————————— ABSTRACT —————————

Carotenoid composition of fruits of two Rosa species (*Rosa canina* and *Rosa rugosa*) and of chokeberry (*Aronia melanocarpa*) was studied by high performance liquid chromatography. Nine carotenoids were determined: three carotenes (lycopene, ζ-carotene, β-carotene) and six xanthophylls (neoxanthin, trans-violaxanthin, cis-violaxanthin, 5,6-epoxylutein, lutein, β-cryptoxanthin). This high number of compounds classified these fruits among those with the greatest variety of carotenoid pigments. Quantitatively large differences occurred in the carotenoid composition of the three fruits. Rosa hips contained the highest concentrations of total carotenoids, which were mainly comprised of lycopene and β-carotene. Conversely, total xanthophylls were low. In contrast to Rosa, fruits of Aronia were lower in total carotenoids while xanthophylls composed a higher proportion.

INTRODUCTION

FRUITS of wild and cultivated roses (*Rosa canina* and *Rosa rugosa*) and of chokeberry (*Aronia melanocarpa*) have a high phenolic content (Oszmianski et al., 1986; Wilska-Jeszka et al., 1986; Oszmianski and Sapis, 1988), and some are used as raw material for food products (Rosa and Krugly, 1987; Plocharski and Smolarz, 1987). Furthermore, the high phenolic content can be of pharmacological interest as in grapes and wines (Masquelier, 1982; Bourzeix et al., 1986; Boukharta, 1988; Fallet, 1988). With regard to the latter point, the fruits of Rosa species and of chokeberry also contain carotenoids, some of which are considered as precursors of vitamin A. As listed by Goodwin (1980), the studies on these carotenoids are old, not very numerous and were carried out with old fractionation methods. The purpose of this study was to determine the qualitative and quantitative composition of carotenoids in two species of roses and one of chokeberry using high performance liquid chromatography.

MATERIALS & METHODS

Extraction from fruits

One hundred grams of berries of *Rosa canina, Rosa rugosa*, and *Aronia melanocarpa* were picked at random in the botanical garden of the University of Wroclaw (Poland). The fruits of Rosa species were deseeded as seeds form a large proportion of the weight of the berries. As this operation was not possible in *Aronia* fruits, these were used whole with the seeds.

Fruits were crushed in liquid nitrogen in a Dangoumau type ball grinder to obtain a very fine, homogenous powder. Ten grams of this powder were picked for analysis. Carotenoids were extracted in different steps which have been described in detail in previous work (Razungles, 1985; Razungles et al., 1987). All the operations were carried out at 0°C and in the dark to prevent oxidation and isomerization of the compounds. The final carotenoid extract was dissolved in 2 mL acetone to which was added 1 mL 0.01% sodium picrate solution as internal standard. An aliquot of 20 μL was injected in the chromatograph.

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HPLC of carotenoids

Fractionation and determination of carotenoids were carried out by HPLC (Baranyai et al., 1982; Razungles et al., 1987), using a Varian 5500 liquid chromatograph fitted with a Varian UV-200 detector set at 450 nm and connected to a Varian Vista 401 computer. An analytical RP-18 column (L = 250 mm, ID = 4 mm, 5 μm particles, Brownlee labs. Inc. Santa Clara, CA) was used, protected with a guard cartridge of the same packing. Elution was effected with a gently increasing gradient of acetone mixed in water run as follows: 0–20 min = from 70% acetone - 30% water to 100% acetone; 20–30 min = 100% acetone. The flow rate was 1 mL/min.

Carotenoids were identified with reference to control compounds prepared in the laboratory from plant extracts and purified according to previously described techniques (Davies, 1976; Camara, 1981; Razungles, 1985; Razungles et al., 1987). For quantitation, the accuracy of the figures measured on five replications was between 5% and 8% according to the different carotenoids.

RESULTS & DISCUSSION

From a qualitative point of view, chromatographic patterns of the extracts of fruits of roses and chokeberry were similar. Nine carotenoids were fractionated (Fig. 1) : three carotenes (lycopene, ζ-carotene, β-carotene) and six xanthophylls (neoxanthin, trans-violaxanthin, cis-violaxanthin, 5.6-epoxylutein, lutein, β-cryptoxanthin). This high number of compounds classifies these fruits among those with the greatest variety of carotenoid pigments.

Quantitative determinations (Table 1) indicated large differences between the carotenoid composition of the different fruits. Total carotenoids were high in Rosa extracts and similar to those of mangoes and oranges (Thomas, 1975; Goodwin, 1980; Gross, 1982) and some red peppers (Camara and Moneger, 1978; Goodwin, 1980). These concentrations were also considerably higher than in grapes (Razungles et al., 1987). Individual high concentrations were observed in particular for carotenes (lycopene and β-carotene) mainly in *Rosa canina*. This high concentration of lycopene appeared to be a characteristic of the *Rosa* species studied here. In other fruits, this carotenoid is generally considered as a minor compound. β-Carotene, considered as an important carotenoid in numerous small berry fruits, was also present at high concentrations in the Rosa extracts. α-Carotene, often found in particular in Prunus and Pyrus species (Goodwin, 1980), was not identified here. Total xanthophylls were low, the highest being β-cryptoxanthin which was the major xanthophyll in both rose hips.

Total carotenoids of *Aronia melanocarpa* were considerably lower than those in *Rosa* as results were based on non-deseeded fruits. Concentrations were half those of *Rosa rugosa* and less than a quarter of those of *Rosa canina*. Similar concentrations occur in tomatoes and apricots (Goodwin, 1980). With regard to carotenes, lycopene was particularly low. Xanthophylls formed 64% of the total carotenoids in *Aronia* while the proportion was considerably lower in *Rosa*. The β-carotene was high in the three fruits, Rosa canina being by far the highest. Since this compound is considered to be a precursor of vitamin A, products made with them would have a high vitamin supplement value if they were prepared without too much cooking (e.g., home made jams), since β-carotene is sensitive to thermal degradation (Onyewu et al., 1982). This also makes *Rosa*
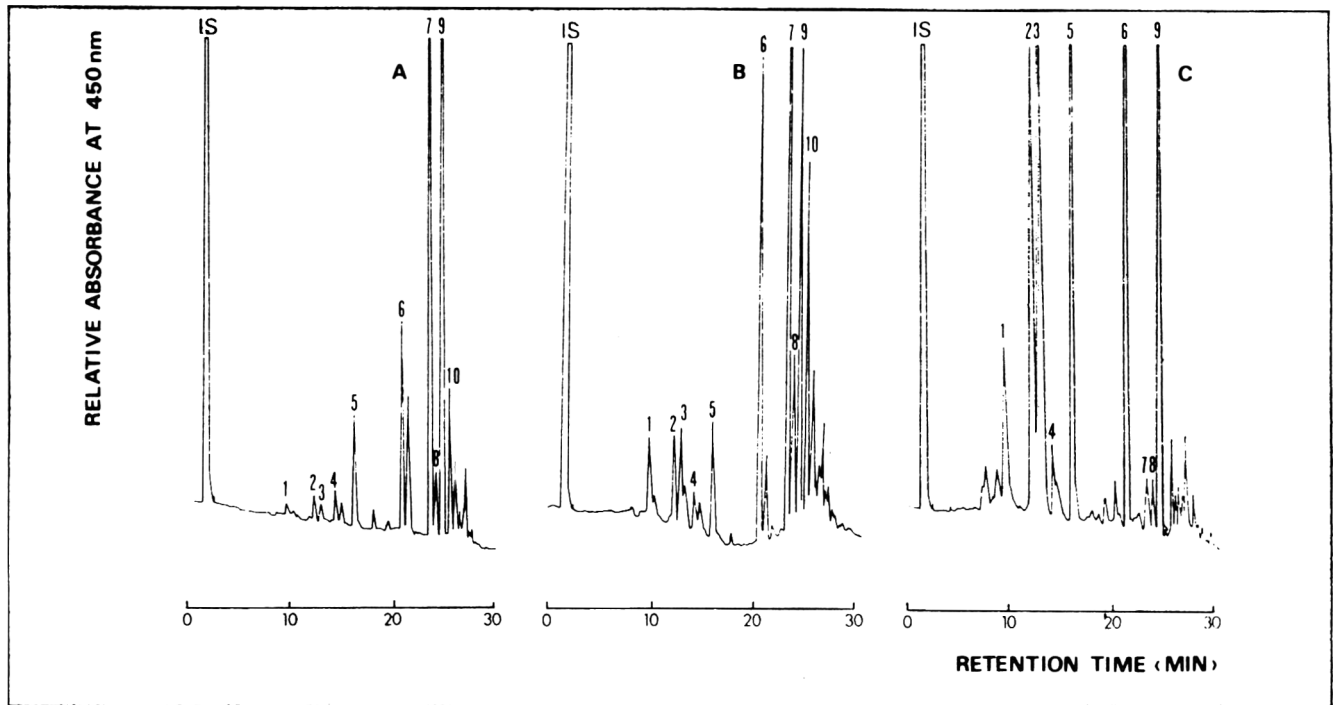
Fig. 1—HPLC separation of carotenoids of *Rosa canina* (A), *Rosa rugosa* (B) and *Aronia melanocarpa* (C) fruits: (1) neoxanthin, R_T 9.82 min; (2) trans-violaxanthin, R_T 12.24 min; (3) cis-violaxanthin, R_T 12.97 min; (4) 5,6-epoxylutein, R_T 14.33 min; (5) lutein, R_T 16.14 min; (6) β-cryptoxanthin, R_T 21.50 min; (7) lycopene, R_T 23.66 min; (8) ζ-carotene, R_T 24.15 min; (9) β-carotene, R_T 24.77 min; (10) unknown. IS : internal standard (sodium picrate).

Table 1—Carotenoid composition of fruits of Rosa canina, Rosa rugosa, and Aronia melanocarpa

Carotenoid	Rosa canina[a]	SD[c]	Rosa rugosa[a]	SD[c]	Aronia melanocarpa[b]	SD[c]
Lycopene	111.2	5.4	43.3	2.2	0.6	0.05
β-Carotene	72.1	3.6	17.6	1.1	16.7	1.1
ζ-Carotene	5.6	0.4	6.5	0.5	0.3	0.03
β-Cryptoxanthin	17.5	1.1	19.1	1.2	12.2	0.8
Lutein	9.2	0.6	4.3	0.3	3.4	0.2
5,6-Epoxylutein	2.6	0.2	1.5	0.1	0.4	0.03
trans-Violaxanthin	2.5	0.2	3.4	0.2	4.5	0.3
cis-Violaxanthin	1.8	0.1	4.4	0.3	8.5	0.6
Neoxanthin	1.5	0.1	6.0	0.4	2.0	0.1
Total carotenoids	224.0	10.7	106.1	5.3	48.6	2.5

[a] Mean of five replications; mg/kg of deseeded berries.
[b] Mean of five replications; mg/kg of nondeseeded berries.
[c] SD = Standard deviation.

canina fruits excellent natural raw material for the pharmaceutical industry.

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A Research Note

Kinetics of Color Change of Grape Juice Generated using Linearly Increasing Temperature

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ABSTRACT

Kinetics of the color change of grape juice was studied using linearly increasing temperature for heating Noble grape juice in a stainless steel reactor. The juice was heated from 60° to 95°C in 9 hr. Kinetic parameters for the color change were determined using Hunter L,a,b-values, chroma values, and Total Color Difference (TCD) values. The reaction of the color change of grape juice measured by L- and a-values, and Chroma value followed first order kinetics with activation energies of 114.75, 131.80, and 121.21 kJ/mol and frequency factors of 1.30×10^{12}, 8.95×10^{14} and 1.93×10^{13} sec^{-1}, respectively. However, the reaction measured by TCD followed zero order kinetics with the activation energy and the frequency factor of 92.81 kJ/mol and 4.80×10^{10} mol/l/sec, respectively.

INTRODUCTION

PRODUCT COLOR is one of the most important quality factors of grape juice. Color in grape juice is composed of a complex mixture of anthocyanins which are mainly water-soluble diglucosides such as the 3,5-diglucosides of malvidin, peonidin, petunidin, cyanidin, and delphinidin (Ballinger et al., 1973; Francis et al., 1966). Ponting et al. (1960) reported that browning is an important deteriorative process which proceeds independently of loss of anthocyanin pigment and is reported to proceed or to follow the destruction of anthocyanins. Browning of fruit juice is frequently investigated by measuring the ratio of absorbances at two wavelengths (Lukton et al., 1956; Ponting et al., 1960; Flora, 1976; Skrede, 1985). This is based on the fact that thermal treatment causes fading of red color with a resultant decrease in absorbance in the region of 500–535 nm and the development of brown color which is associated with an increase in absorbance in the region of 400–440 nm.

Flora (1976) also used a color difference meter and showed that the browning index was highly correlated with panel scores. Although color change has been used for quality control of grape juice, kinetic data for Hunter L, a, b-values are not available. The linearly increasing temperature method of kinetic data generation proposed by Rhim et al. (1989) offers a simple, rapid method filling this void. This method consists of a single experiment in which temperature is increased at a constant rate and samples are withdrawn at intervals and analyzed.

The objective of this study was to generate kinetic data and to estimate parameters associated with color change of grape juice using the linearly increasing temperature method.

MATERIALS & METHODS

FRESH *VITIS ROTUNDIFOLIA* BERRIES of the Noble cultivar, a commercially important grape variety in the southeast U.S., harvested from experimental vineyards at Clayton, NC, were washed and stored at 4°C until used. They were then crushed, treated with Klerzyme 200 (Gist Brocades USA Inc., Charlotte, NC) for 30 min at 60°C, pressed

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using cheese cloth and then filtered (Whatman No. 1 paper). The juice was held overnight at 4°C to allow tartrates to form and settle and filtered as before. Soluble solids, pH and total titratable acidity were determined for the juice by the method described by Flora (1977). Thermal treatments were performed aerobically in a stainless steel vessel as described by Rhim et al. (1989). Temperature was increased linearly from 60° to 95°C in 9 hr. The initial temperature (60°C) was based on no appreciable loss in pigment content in strawberry jellies (Decareau et al., 1956). Sample (300 mL) was introduced in the prewarmed reactor; heating medium was circulated until the sample reached 60°C (approximately 4 min); and the temperature programmer was started. The juice was stirred continuously. Samples (20 mL) were taken when juice reached 60°C and at 1 hr intervals, thereafter. The samples were cooled immediately in an ice water bath and analyzed. The light transmitted through samples in a 1 cm glass cuvette was measured with the Spectrogard Color System (Gardner/Neotec Instrument Division, Pacific Scientific, Silver Spring, MD) standardized to distilled water. Hunter L, a, b-values for the samples were measured using a quartz halogen lamp filtered to simulate CIE source C (average daylight) and a 10° standard observer. Chroma and total color difference (TCD) were calculated from the Hunter L,a,b-values according to the formulas:

$$Chroma = (a^2 + b^2)^{1/2}$$

$$TCD = \{(L_o - L)^2 + (a_o - a)^2 + (b_o - b)^2\}^{1/2},$$

where the subscript "o" refers to the value for the nonheated grape juice. These color data were analyzed using the method of Rhim et al. (1989).

RESULTS & DISCUSSION

THE FRESH GRAPE JUICE was clear and a bright purple-red color. Soluble solids was 11.3 °Brix; pH, 3.3; and titratable acidity, 0.43% as tartaric acid. This juice was typical of commercial grape juice (Flora, 1977).

Due to the slow heating rate (3.86 °C/hr), temperature lag between heating medium and grape juice was not appreciable.
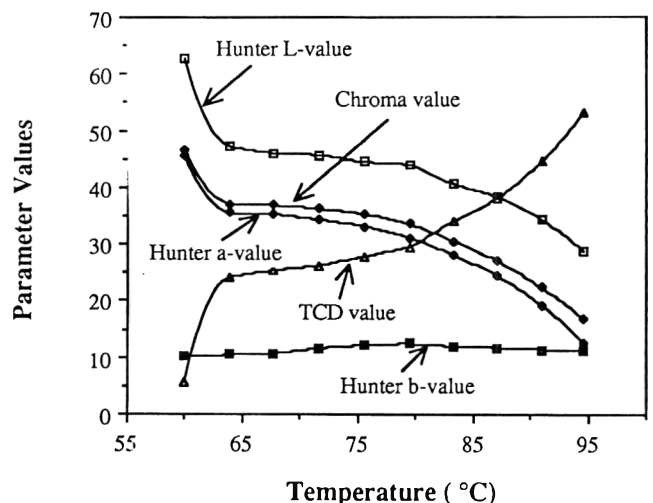
Fig. 1.—Change of each parameter value of grape juice with linearly increasing temperature from 60° to 95°C in 9 hr (heating rate, 3.86°C/hr).
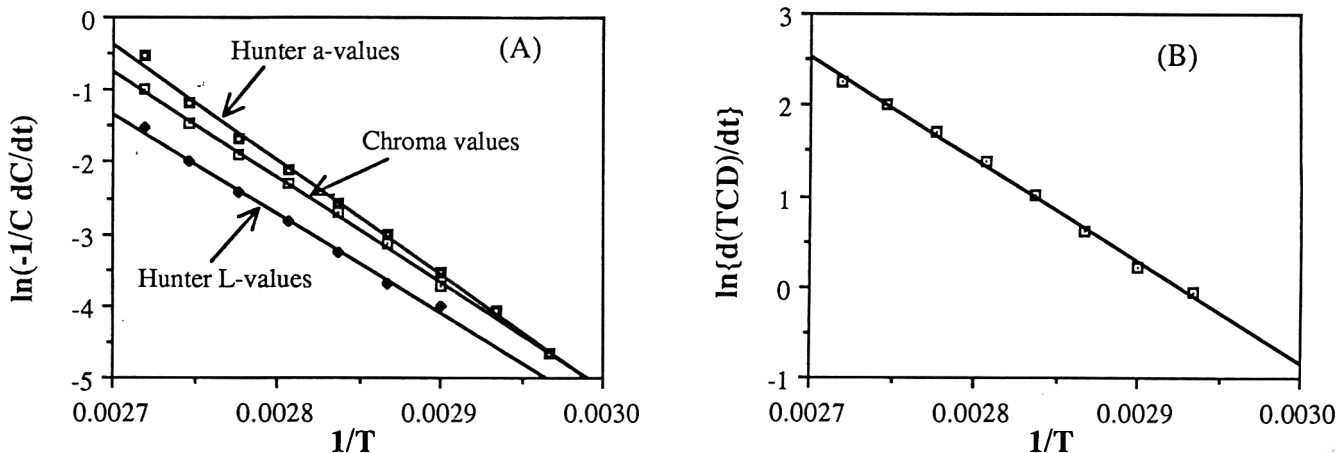
Fig. 2.—Arrhenius type plot for the change of each parameter value of grape juice fitted to first (Hunter L-,a-values and chroma values) and zero order (TCD values) kinetics (C represents the intensity of each parameter value).

The change of each parameter as a function of temperature is shown in Fig. 1. The Hunter L-value indicates the degree of lightness or darkness and the a-value indicates the degree of redness or greenness of the sample with a positive a-value indicating redness. The sharp initial decrease of the Hunter L- and a-values was due to fading of the red color as heat destroyed anthocyanin pigments. Anthocyanin pigments are unstable in fruit juices and loss of color in grape juice is influenced by time-temperature relationships during processing and storage, exposure to air and light, the presence of metal ions and enzymes, and many other factors (Simard et al., 1982; Flora, 1976; Cash et al., 1976; Skalski and Sistrunk, 1973; Ponting et al., 1960). The decrease in the values following the initial sharp reduction was due to the development of a brown color. This color change is typical of heated grape juice (Flora, 1976; Ponting et al., 1960). Chroma values closely followed the a-values since b-values were relatively small and constant. The chroma value indicates the degree of saturation of color and is proportional to the strength of the color.

Since changes in each parameter during the first hour were due to anthocyanin destruction rather than browning, only the values for 1 hr and greater were used for the kinetic analysis of browning. The Hunter L- and a-values fitted well to a first order kinetic model as shown in Fig. 2. Activation energies of 114.75 and 131.80 kJ/mole and frequency factors of 1.30×10^{12} and 8.95×10^{14} sec^{-1} were calculated for Hunter L- and a-calues, respectively. Chroma values also followed first order kinetics with the activation energy and the frequeny factor values of 121.21 kJ/mole and 1.93×10^{13} sec^{-1}, respectively (Fig. 2).

Similar first order reactions have been reported by Sastry and Tischer (1952) for absorbance of grape juice; Tanchev (1985a, b) for anthocyanins in fruit juices; Decareau et al. (1956) for pigments in strawberry products; and Palamidis and Markakis (1975) for anthocyanins in a nonalcoholic carbonated beverage. Palamidis and Markakis (1975) also reported activation energies of 57.25 and 61.38 kJ/mol for anthocyanin pigments extracted with hot water and SO_2, respectively. Their values for anthocyanins should not be compared with values for browning from this investigation, since the initial rapid change in anthocyanin was intentionally neglected.

The TCD showed a reversed pattern from the Hunter L-, a-, and Chroma values (Fig. 1). Flora (1976) found very low correlation between TCD value and panel scores of grape juice. Ponting et al. (1960) suggested that a 10% loss in red color measured by TCD was easily perceptible in grape juices. In this experiment, TCD proved to be a good indicator of color change of heated grape juice. The change in TCD values followed zero order kinetics (Fig. 2). The activation energy and the frequency factor of color change of grape juice evaluated by TCD was 92.81 kJ/mole and 4.80×10^{10} sec^{-1}, respectively.

This study demonstrated the use of the linearly increasing temperature method to successfully generate kinetic parameters for describing color changes in grape juice. These results can be utilized in predicting color changes in juice during processing and storage and can also be utilized as a quality control tool for monitoring processing and storage effects on grape juice.

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A Rapid Method for Determining Allylisothiocyanate in Horseradish-Containing Products

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--------- ABSTRACT ---------

A rapid method for determining allylisothiocyanate (AITC) in horse-radish-containing products was developed. Headspace samples were analyzed by gas chromatography and the AITC peak identity was confirmed by mass spectrometry. The coefficient of variation was less than 4%. Recovery of added AITC from commercial ground horse-radish products averaged 91% for six different samples. Recovery of added AITC from a tomato-based sauce containing horseradish oil averaged 94% for seven samples. The slopes of the standard curves for AITC were dependent upon the sample matrix with the slope for AITC in oil less than 5% of that for AITC in water.

INTRODUCTION

THE PRINCIPAL volatile flavor component of horseradish is allylisothiocyanate (AITC) (Gilbert and Nursten, 1972). This compound is responsible for the characteristic pungent flavor of horseradish. Isothiocyanates are released from glucosinolates by the action of thioglucosidases (Josefsson, 1967; Fenwick et al., 1983) which are trivially known as myrosinase. In horseradish this is a desirable phenomenon, but it is undesirable in cabbage used for coleslaw. The "hotness" of these products is related to the type and content of glucosinolates present and the ability of the enzymes present to release the isothiocyanates. The glucosinolate content varies from variety to variety and from species to species and is dependent upon growing conditions as well (Fenwick et al., 1983). Glucosinolate content can be measured directly by HPLC (Olsen and Sorensen, 1981) or by GLC (Heaney and Fenwick, 1980; Sosulski and Dabrowski, 1983) or indirectly by measuring glucose (Van Etten et al., 1974) or sulfate (McGhee et al., 1965) released. Released isothiocyanates have been measured and identified by GLC-MS (Gilbert and Nursten, 1972; Daxenbichler and Van Etten, 1977; Daxenbichler et al., 1977), but these methods are not suitable for routine analysis. The purpose of this investigation was to develop a rapid, quantitative method for determining the AITC content of some horseradish-containing products.

MATERIALS & METHODS

ALLYLISOTHIOCYANATE was obtained from Aldrich Chemical Co. (Milwaukee, WI). Horseradish oil was obtained from T.J. Marzetti Co. (Columbus, OH). The tomato base used was StarCross brand catsup. Commercial samples of prepared ground horseradish and tomato-based cocktail sauce containing horseradish were purchased in supermarkets in Columbus, OH.

Samples were prepared for analysis by pipetting 10 mL or weighing the appropriate amount of sample to give a volume of 10 mL into a 30 mL serum bottle and sealing the bottle with a rubber septum and metal crimp cap. The samples were equilibrated for 30 min at 40°C in a circulating water bath before analysis. Note that it is critical that the temperature of the samples is controlled if reproducible data are

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to be obtained. Headspace samples (0.7 mL) were removed from the serum bottles using a disposable plastic syringe (1 mL, 26 gauge needle). A new syringe was used for each sample.

Gas chromatography was conducted using a Hewlett-Packard 5890A gas chromatograph equipped with a flame ionization detector and an electronic integrator. A 183 × 0.32 cm (i.d.) stainless steel column was packed with 80/100 mesh TenaxGC and used for all the analyses. The colum, injector, and detector temperatures were 190°C, 220°C, and 240°C, respectively, and the flow rate of the N_2 carrier gas was 35.5 mL/min. Gas chromatography-mass spectrometry was accomplished using the same column and a Finnigan Model 9610 gas chromatograph coupled to a Finnigan Model 402 mass spectrometer via an all glass inlet and jet separator. Helium was used as the carrier gas. Data were collected and analyzed using a Data General DS 50 S/NOVA 4 computer.

AITC standards were prepared by diluting a stock solution in water, vegetable oil, or catsup. For the recovery studies, 12.0g of the ground horseradish samples were weighed into the serum bottles and 1.0mL 0.01% AITC was added. Additional recovery studies used known concentrations of horseradish oil in catsup which had either 1.0 mL water or 1.0 mL 0.015% AITC added.

RESULTS & DISCUSSION

THE MAJOR PEAK from headspace samples of AITC in water, horseradish oil, ground horseradish, and tomato base (catsup) eluted at 2.48 min. The identity of the peak was confirmed by GC-MS as AITC (data not shown). No coeluting compounds were detected in the tomato base. The coefficient of variation for the peak areas of the AITC peak for seven samples of horseradish oil in tomato base was less than 4%.

Standard curves for AITC in water, in oil and in tomato base are shown in Fig. 1. The slopes of these curves are quite different due to the differences in solubility of AITC in these matrices. The ratios of the slopes of the curves are 44:3:1 respectively. The low solubility of AITC in water requires that care be taken when preparing AITC standards in water. The impact on AITC in the headspace of adding sugar to water was investigated. As expected the concentration of AITC in the headspace increased with increasing sugar content. This is due to the ordering of the water by the added sugar which reduces the solubility of AITC. This data taken alone suggest that the slope of the standard curve for AITC in tomato base should be steeper than that for water because of the increased sugar content of the tomato base. As seen in Fig. 1, this was clearly not the case. The reduced (as compared to water) slope of the standard curve for AITC in tomato base must be due to increased solubility of AITC in components of the tomato base or to adsorption of AITC to components of the tomato base. Because the lipid content of the tomato base is very low, adsorption to components in the catsup appeared to be the more likely mechanism.

The recovery of added AITC to commercial samples of prepared ground horseradish is shown in Table 1. The mean recovery for the 6 samples which span the range from "medium" to "hot" was 91%. The mean recovery of added AITC from samples containing from 0.075 to 0.55% horseradish oil in the tomato base was 94% (Table 1). These results suggest that it should be possible to quantitate AITC in unknown matrices by
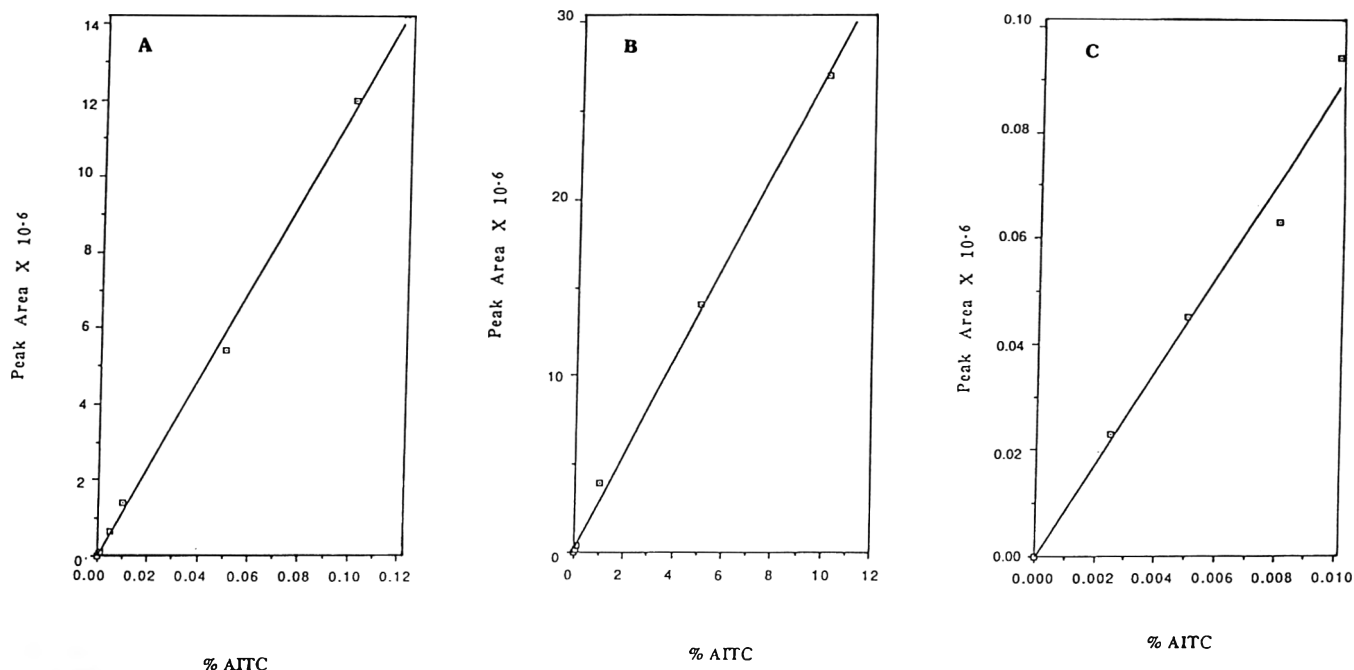
Fig. 1 — Standard curves for allylisothiocyanate in water (A), vegetable oil (B), and tomato base (C).

Table 1 – Recovery of AITC[a] added to prepared horseradish and tomato base containing horseradish oil

Sample	AITC Peak Area	Percent Recovery
Horseradish		
1	1.72×10^5	
1 + AITC	2.59×10^5	92
2	3.92×10^5	
2 + AITC	4.69×10^5	82
3	3.72×10^5	
3 + AITC	4.62×10^5	95
4	4.80×10^5	
4 + AITC	5.61×10^5	85
5	4.50×10^5	
5 + AITC	5.41×10^5	96
6	5.10×10^5	
6 + AITC	6.00×10^5	94
Tomato base with HRO[b]		
1 (0.075% HRO)	8.86×10^3	
+ AITC	2.22×10^4	100
2 (0.10% HRO)	1.12×10^4	
+ AITC	2.23×10^4	85
3 (0.15% HRO)	1.61×10^4	
+ AITC	2.75×10^4	88
4 (0.25% HRO)	2.57×10^4	
+ AITC	3.94×10^4	103
5 (0.35% HRO)	3.53×10^4	
+ AITC	4.78×10^4	96
6 (0.45% HRO)	4.49×10^4	
+ AITC	5.67×10^4	91
7 (0.55% HRO)	7.38×10^4	
+ AITC	8.63×10^4	97

[a] AITC = allylisothiocyanate
[b] HRO = horseradish oil

adding a known amount of AITC and comparing the values obtained with and without the added spike. This would not be necessary for a quality assurance procedure if the matrix was constant, but as seen in Fig. 1, matrix effects can be quite large. Adding ground horseradish to the tomato base gave similar recoveries to those obtained for AITC in water (data not shown).

This rapid method for determining AITC in horseradish-containing products is suitable for quality assurance purposes for standardizing the horseradish flavor. This use would require substantial taste panel work to establish acceptable values for AITC.

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A Research Note

Effect of Fermentation by Pure Cultures of Yeasts and Lactobacilli on Phytic Acid and Polyphenol Content of Pearl Millet

NEELAM KHETARPAUL and B.M. CHAUHAN

ABSTRACT

Single and mixed pure culture fermentations of pearl millet flour with yeasts and lactobacilli, namely, *Saccharomyces diastaticus*, *Saccharomyces cerevisiae*, *Lactobacillus brevis* and *Lactobacillus fermentum*, at 30°C for 72 hr brought about a significant reduction in phytic acid and polyphenols which were present in considerable amounts in pearl millet flour. Reduction in phytic acid was more pronounced than that in the polyphenols.

INTRODUCTION

ANTINUTRIENTS like phytic acid or inositol 1, 2, 3, 4, 5, 6-hexaphosphate and polyphenolic compounds that are interchangeably called "tannins" by numerous investigators are widely distributed in higher plants used as human food. In plant foods, particularly cereals and legumes, phytic acid is the principal storage form of phosphorus.

Phytic acid and polyphenols present in considerable amounts in pearl millet (Chauhan et al., 1986; Mahajan and Chauhan, 1987) may affect the mineral availability (Oberleas et al., 1966; Maga, 1982; Reddy et al., 1982; Bressani and Elias, 1980) and digestibility of carbohydrates (Yoon et al., 1983; Deshpande and Salunkhe, 1982; Thompson and Yoon, 1984) and protein (Serraino et al., 1985; Knuckles et al., 1985; Aw and Swanson, 1985) in pearl millet.

For more effective utilization of pearl millet, it is desirable to lower the concentration of these antinutrients. Previous work showed that the natural fermentation lowered the concentrations of phytic acid (Mahajan and Chauhan, 1987) and polyphenols and improved the availability of minerals from pearl millet flour. This paper reports the effect of fermentation by pure cultures of lactobacilli and yeasts on phytic acid and polyphenols of pearl millet flour.

MATERIALS & METHODS

PEARL MILLET GRAINS procured from the local market were coarsely ground (1.5mm sieve) using an electric grinder. The coarsely ground pearl millet flour (100g) was mixed with distilled water (900mL), autoclaved (15psi for 15 min), inoculated with *Saccharomyces diastaticus*, *Saccharomyces cerevisiae*, *Lactobacillus brevis* and *Lactobacillus fermentum* and incubated at 30°C for 72 hr. Each of the microorganisms supplied 10^5 cells/mL in single culture fermentation whereas in mixed culture fermentation, a combination of yeast (10^5 cells/mL) and lactobacillus (10^5 cells/mL) was employed (Khetarpaul and Chauhan, 1989). Four different types of mixed fermentations included *S. diastaticus* and *L. brevis* (SdLb), *S. diastaticus* and *L. fermentum* (SdLf), *S. cerevisiae* and *L. brevis* (ScLb), and *S. cerevisiae* and *L. fermentum* (ScLf). The autoclaved unfermented pearl millet and raw pearl millet served as the controls. The fermented and unfermented autoclaved

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samples were dried for 48 hr at 65°C to constant weight and finely ground in cyclone mill (Cyclotec, M/s Tecator, Sweden) using 0.5 mm sieve.

Phytic acid and polyphenols extracted in 0.2N HCl and 1% HCl in methanol, respectively, were determined colorimetrically (Haug and Lantzsch, 1983; Swain and Hills, 1959). The data were subjected to analysis of variance in a completely randomized design (Panse and Sukhatme, 1961).

RESULTS & DISCUSSION

Phytic acid

As a result of the pure culture fermentations by yeasts and lactobacilli, a significant (P<0.05) reduction in phytic acid of pearl millet flour was noticed (Table 1). Autoclaving lowered the phytic acid of the pearl millet flour and when this autoclaved flour was fermented with single and mixed pure cultures of yeasts and lactobacilli, phytate was further reduced (P<0.05).

Among the single culture fermentations, lactobacilli lowered the phytic acid to a greater extent compared to the yeasts; *L. fermentum* had the most pronounced phytate lowering effect followed by *L. brevis*, *S. cerevisiae* and *S. diastaticus*. When the pearl millet flour was fermented by mixed pure cultures of yeasts and lactobacilli, further significant (P<0.05) reduction in phytic acid was noticed in all the combinations except ScLb. Lactobacilli in combination with *S. diastaticus* significantly decreased the phytic acid to a greater extent than their combinations with *S. cerevisiae*. SdLf fermented flour had the lowest amount of phytic acid.

Microbial phytase, as reported in several microorganisms (Daniels and Fisher, 1981; Lopez et al., 1983), may hydrolyze phytic acid during fermentation and account for the reduction in phytic acid in the fermented product. Acidic pH of the fermented product and the temperature of fermentation may

Table 1 — Changes in phytic acid and polyphenols during pure culture fermentations of pearl millet flour[a]

Fermentation	Phytic acid	Polyphenols
Single fermentation		
S. diastaticus	585 ± 0.7	641 ± 16.9
S. cerevisiae	570 ± 1.4	719 ± 4.7
L. brevis	555 ± 0.0	634 ± 4.4
L. fermentum	525 ± 3.5	561 ± 3.3
Mixed fermentation		
S. diastaticus + L. brevis	488 ± 2.6	678 ± 3.2
S. diastaticus + L. fermentum	480 ± 1.5	618 ± 5.6
S. cerevisiae + L. brevis	580 ± 2.9	644 ± 2.1
S. cerevisiae + L. fermentum	510 ± 3.5	648 ± 7.5
Control		
Raw pearl millet	990 ± 3.5	761 ± 3.2
Autoclaved unfermented pearl millet	630 ± 10.6	693 ± 2.4
SE(m)	± 2.12	± 4.14
CD[b] (P < 0.05)	3.36	12.4

[a] Values are means ±SD of four replicates (mg/100g, on dry matter basis).
[b] Critical difference determined by ANOVA (P < 0.05). Differences of two means within/between the fermentation treatments exceeding this value are significant (P < 0.05).

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