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CONTENTS CONTINUED (on the inside of the front cover) . . .

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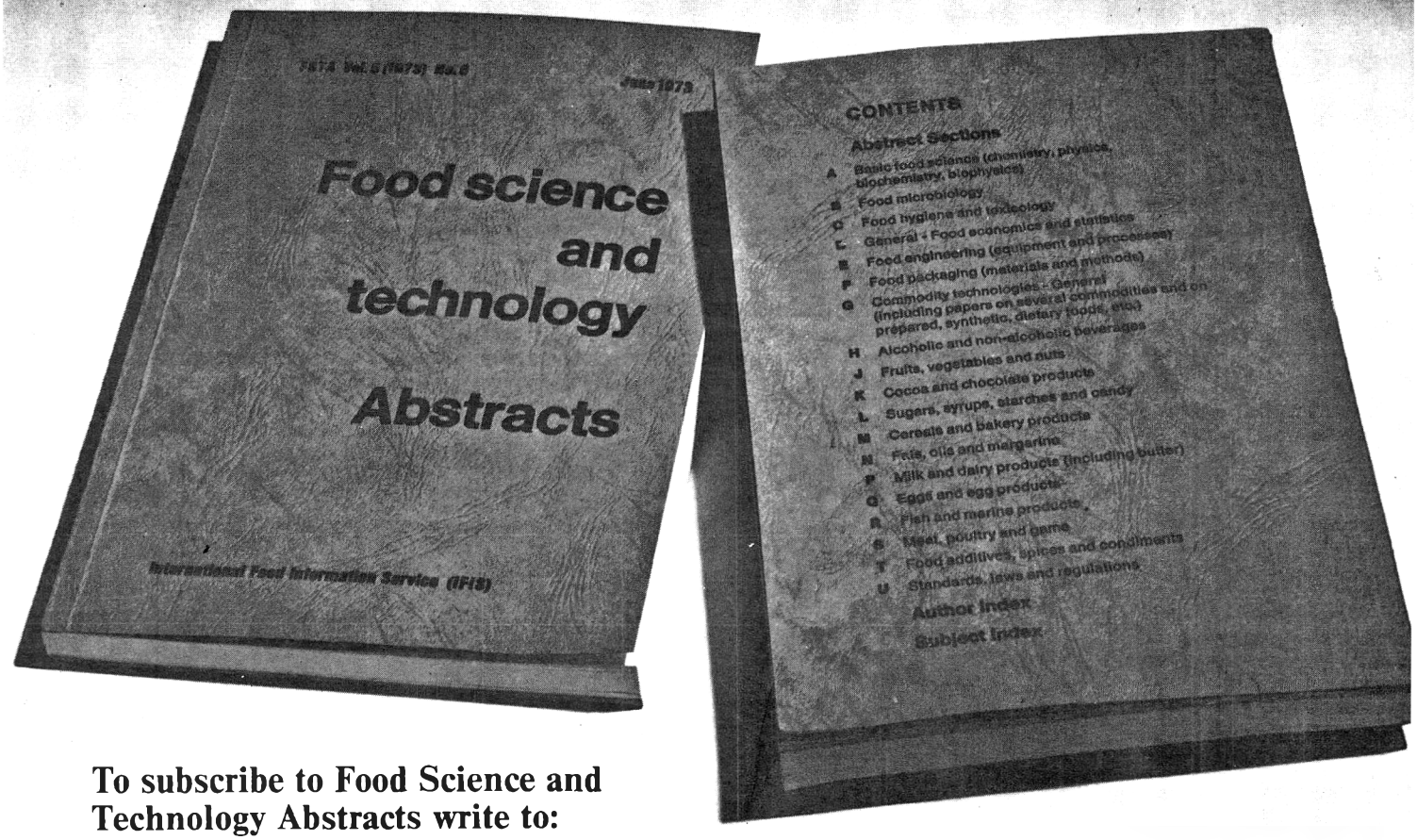

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CHEMICAL, PALATABILITY AND COOKING CHARACTERISTICS OF NORMAL AND LOW QUALITY PORK LOINS AS AFFECTED BY FREEZER STORAGE

ABSTRACT

Loins of normal (Wisc. score 3) and low (Wisc. score 1) quality were compared for pH (fresh only), thiamine (fresh and 120 days), organoleptic and tenderness scores and cooking losses while fresh and after 60 and 120 days' freezer storage. pH was significantly lower for PSE than for normal loins. Thiamine content also was lower for the PSE loins and decreased for both quality groups during storage. TBA values indicated a greater degree of rancidity for PSE loins, but contrary to expected outcome they tended to decrease with freezer storage. Normal loins received significantly higher organoleptic evaluation scores for flavor, juiciness and overall satisfaction. On the other hand, PSE loins tended to be slightly more tender. Tenderness increased with freezer storage time. There were no differences in flavor, juiciness or overall satisfaction scores due to freezer storage. PSE loins had significantly more total cooking and evaporative loss than normal loins. There was a significant increase in evaporative loss as storage time increased.

INTRODUCTION

WITH THE NUMBER of home freezers on the increase, a timely question in line with the study of consumer satisfaction with pork products is: How does pale, soft, exudative (PSE) pork compare with normal pork after normal freezing intervals? Sayre et al. (1964) reported PSE pork frozen 8 days to have higher evaporative cooking losses, slower cooking rates and higher mechanical shear values than dark, firm and dry (DFD) pork. Merkel (1971) reported higher freezer moisture loss and rancidity for PSE than for normal pork loins after 3–4 months' freezer storage. Kemp et al. (1968) reported higher overall satisfaction sensory evaluation scores for DFD than PSE hams.

This study was designed to determine the effect of normal freezer storage on normal and PSE pork loins. Of primary concern were the changes in thiamine content, rancidity, shrink, drip loss, evaporative loss, tenderness, flavor, juiciness and overall satisfaction.

EXPERIMENTAL

12 LOW QUALITY PORK LOINS (Wisc. score 1) and 12 normal loins (Wisc. score 3) were selected for study from carcasses exhibited at the 1974 Louisville Barrow Show. The loins were then divided into blade, center and sirloin roasts. Of the 72 roasts available, 12 of each quality were selected for immediate cooking and chemical analysis. Selection for blade, center and sirloin roasts were made so as to balance location between quality groups. Two samples from each of the 24 fresh roasts were analyzed for pH, two for rancidity by the 2-thiobarbituric acid test (TBA) (Turner et al., 1954) and four for thiamine content (Association of Vitamin Chemist, Inc., 1966). Roasts were weighed before cooking, oven-cooked at 163°C to an internal temperature of 77°C. then re-weighed to obtain weight loss. The drippings from each roast were weighed, and the difference between shrink and drip loss was assumed to be evaporative loss. Two or three 2.5 cm core samples approximately 3 cm long were taken from the longissimus of each roast, cooled to room temperature, and shear values obtained using an Instron universal testing device equipped with a Warner-Bratzler shear attachment. Each core was sheared three times and a mean shear value was calculated for each.

Samples from the remaining portion of the longissimus were served to an eight-member sensory evaluation panel. The panel evaluated each roast for flavor, juiciness, tenderness and overall satisfaction using a 9-point hedonic scale with 9 equaling "like extremely" and 1 equaling "dislike extremely." The remaining 48 roasts were wrapped in freezer paper, frozen at a temperature of −16°C and held at that temperature

Table 1—Comparison of tissue from PSE and normal pork loins

Quality[a]	pH	TBA	Thiamine[b]
PSE (1)	5.88***	0.30**	11.24**
Normal (3)	6.17	0.14	14.65

[a] Univ. of Wisc. (1963)
[b] μ thiamine/g
** Sig. at 0.01 level
*** Sig. at 0.05 level

Table 2—Effect of fresh quality and freezer storage time on TBA values and thiamine content

	Quality 1				Quality 3			
Trait	Fresh	60 days	120 days	Avg	60 days	Fresh	120 days	Avg
TBA[z]	0.30	0.20	0.13	0.21***	0.14	0.10	0.07	0.10***
Thiamine[a,z]	11.2	–	8.8	10.0***	14.6	–	11.4	13.0***

[a] μ thiamine/g
[z] Sig. at 0.01 level for time
*** Sig. at 0.01 level for quality

Table 3—Effect of fresh quality and freezer storage time on organoleptic traits, tenderness and cooking losses of pork loin roasts

Trait	Quality 1				Quality 3			
	Fresh	60 days	120 days	Avg	Fresh	60 days	120 days	Avg
Tenderness[a,y]	7.53	7.24	7.96	7.58	7.26	7.43	7.56	7.42
Flavor[a]	7.29	7.06	7.21	7.18***	7.34	7.46	7.48	7.43***
Juiciness[a]	6.54	6.39	6.48	6.47***	7.05	7.15	6.90	7.03***
Overall satisfaction[a]	6.79	6.64	6.72	6.72***	6.98	7.18	7.11	7.11***
W.B. shear, kg[b,z]	7.91	9.50	7.27	8.23*	8.95	10.40	7.70	9.02*
Total cooking loss, %	21.9	23.9	22.7	22.9***	18.9	20.7	20.0	19.9***
Drip loss, %	3.9	4.1	2.0	3.8	4.3	4.2	3.2	4.2
Evaporative loss, %[x]	18.0	19.8	20.7	19.1***	14.6	16.5	16.8	15.7***

[a] Based on 9-point hedonic scale with 9 = like extremely and 1 = dislike extremely
[b] Force required to shear a 2.5 cm core
[x] Sig. at 0.10 level for time
[y] Sig. at 0.05 level for time
[z] Sig. at 0.01 level for time
* Sig. at 0.10 level for quality
*** Sig. at 0.01 level for quality

until thawed for cooking. After 60 days' storage, 12 roasts of each quality were selected, thawed overnight at 10°C and subjected to the same testing procedure. Selection for location was random. After 120 days' storage, the remaining 24 roasts were thawed and tested similar to the preceding groups. Data were analyzed using the SAS program of Barr and Goodnight (1972).

RESULTS & DISCUSSION

RESULTS of chemical analysis of the two quality groups are shown in Table 1.

pH values were significantly ($P < 0.01$) lower for the PSE loins than for the normal loins. This association of low pH to PSE has been confirmed in the results of Wismer-Pederson (1960), Sayre et al. (1964) and Kemp et al. (1971). pH was measured on fresh samples only since it is assumed that no further change occurs after the termination of muscle glycolysis. The PSE roasts were found to have significantly higher ($P < 0.01$) TBA values than the normal roasts. Merkel (1971) found similar results after freezing normal and PSE center roasts for 3–4 months. Thiamine values were significantly ($P < 0.05$) higher in normal pork. This agrees with Meyer et al. (1963).

TBA values were lower after freezer storage, as shown in Table 2. There is no apparent explanation for the almost linear decrease in TBA values with storage time other than possible procedural errors. If there were no procedural errors a possible change in the malonaldehyde reactant of the thiobarbituric acid test might be indicated. TBA values remained lower in normal loins at each sampling period. Thiamine content decreased significantly ($P < 0.01$) with time in storage in both quality groups.

The means show an initial difference in thiamine content between normal and PSE, a decrease for both normal and PSE thiamine content with time but no advantage in thiamine retention for normal over PSE pork.

Organoleptic evaluations (Table 3) indicated significantly lower ($P < 0.01$) flavor, juiciness and overall satisfaction for the PSE than for normal loins. No significant difference was found in tenderness, but shear values were lower for PSE loins ($P < 0.10$). Fox et al. (1970) reported similar panel evaluations for flavor and overall satisfaction between hams of high and low quality. Flavor differences could be due to the presence of protein breakdown products but probably reflect the higher degree of rancidity indicated by the TBA values. Merkel (1971) reported sensory panel juiciness scores lower for PSE loins than for normal loins.

Table 4—Correlations among selected traits

Trait	Thiamine	Tenderness	Flavor	Juiciness	Overall satisfaction	W.B. shear	Total cooking loss	Drip loss	Evap loss
TBA	0.01	0.14	−0.30**	−0.30**	−0.22*	−0.14	0.27**	0.21*	0.21*
Thiamine[a]		−0.26	−0.11	0.14	−0.07	0.31	−0.34	0.05	−0.36
Tenderness			0.04	0.07	0.28**	−0.69***	0.06	−0.14	0.13
Flavor				0.71***	0.81***	0.02	−0.17	−0.18	−0.11
Juiciness					0.85***	−0.08	−0.49***	−0.37**	−0.37**
Overall satisfaction						−0.07	−0.29**	−0.35**	−0.18
W.B. shear							−0.17	0.04	−0.20
Total cooking loss								0.43***	0.92***
Drip loss									0.05

[a] Thiamine correlations are based on fresh and 120 days only
* Sig. at 0.10 level
** Sig. at 0.05 level
*** Sig. at 0.01 level

Tenderness was affected ($P < 0.05$) by freezer storage as shown by higher panel scores after 120 days' storage. Shear values also were lower ($P < 0.01$), indicating improved tenderness. Flavor, juiciness and overall satisfaction scores were not affected by storage.

Cooking losses mirrored previous findings of high total loss and evaporative loss for PSE pork compared with higher quality pork (Merkel, 1971). Both total cooking loss and evaporative loss were significantly higher ($P < 0.01$) for PSE than for normal roasts. Drip losses, however, remained about the same for the two quality groups. Since drip loss is primarily loss of fat, this result is in agreement with previous findings (Sink et al., 1967) that no difference exists between the composition or quantity of lipids in PSE and normal pork. Overall difference in evaporative loss between freezing periods was ($P < 0.01$) as storage time increased.

Correlations among various traits are shown in Table 4. TBA values were negatively correlated ($P < 0.05$) with flavor and juiciness. Since TBA values were lower in the high quality roasts, and high quality roasts had higher flavor and juiciness scores, it is logical that this negative relationship existed. Thiamine content was negatively correlated ($P < 0.10$) with total weight loss and evaporative loss. The high quality roasts, which were higher in thiamine, lost less during cooking and resulted in a significant negative correlation between thiamine and cooking loss. Panel tenderness was related ($P < 0.05$) with overall satisfaction and (<0.01) with shear values. Flavor, juiciness and overall satisfaction were highly related ($P < 0.01$). Juiciness was negatively related to total cooking loss ($P < 0.01$) and drip and evaporative loss ($P < 0.05$). Overall satisfaction, also, was related to total loss and drip loss. These data further show that quality is important for both organoleptic and cooking loss standpoints.

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MICROWAVE AND CONVENTIONAL COOKING IN RELATION TO QUALITY AND NUTRITIVE VALUE OF BEEF AND BEEF-SOY LOAVES

ABSTRACT

Beef or soy flavor of meat loaves containing 0, 15% soy flour or 15% soy concentrate were evaluated after cooking in microwave or in conventional ovens. Also, cooking time, cooking losses, fat and moisture content and thiamine retention were determined. Beef or soy flavor was significantly affected ($P < 0.01$) by loaf type, but not by oven treatment. Loaves (960g) cooked electronically reached 74°C in 19 min and had consistently higher cooking losses than those cooked conventionally for 78 min. Substitution of 15% soy reduced cooking losses more in loaves cooked in electric than in microwave ovens. Soy had no effect on the fat content, but cooking had a significant ($P < 0.01$) effect; i.e., 11.5% fat in electronically and 9.6% in conventionally cooked loaves. Thiamine retention was not affected by 15% soy substitution. The average thiamine content, regardless of treatment, was 0.09 mg/100g of the cooked loaves.

INTRODUCTION

IN CONVENTIONAL OVENS, the temperature of the environment is raised and roasting is accomplished by conduction of heat from the outside to the interior of the meat. This results in a higher temperature at the surface of the meat than in the interior, and under these conditions, browning occurs and characteristic color and flavors are developed. In microwave cooking, the electromagnetic waves interact with specific components throughout the meat, instantly producing heat and resulting in rapid cooking. A shorter cooking time, however, may result also in a lack of "normal" color and flavor that the consumer expects to find in cooked meats. Thus it is not surprising that results of studies have indicated that roasts cooked in the electronic oven differed in appearance and had significantly lower mean total palatability scores than those cooked in the conventional oven.

Kylen et al. (1964) reported that the chief disadvantages of microwave cooking of beef and pork roasts were the adverse effects on color, tenderness and flavor.

Recently, the energy crisis has created interest in microwave cooking as a possible method of conserving energy. In 1955, in a report on browning methods in microwave cooking, Copson et al. stated, "Economy of energy is inherent in the microwave method compared with the conventional situation, in which the temperature of the environment is raised, in order to heat and cook through the mass of food by conduction." In 1967, Power conducted time, cost and efficiency studies of electronic and conventional cookery. From the results obtained for oven cookery, the electronic range was superior in time of operation, cost and fuel utilization efficiency. But the meat loaves were more evenly done and were more acceptable in appearance when cooked conventionally. No differences in moistness and flavor were found between the two methods of cooking loaves.

Soy flour has been used extensively in the U.S. for ca. 50 yr in food products and on a relatively limited scale for ca. 40 yr in meat products. The finely ground soy flour, permitted at a level not to exceed 3½%, has its primary purpose to extend meat and is an inexpensive and nutritious protein. It was recognized that soy flour has the advantage of holding meat juices and the fat (Rakosky, 1974). Thus, soy protein-meat mixtures are not new, but textured soy flours or concentrates used at levels up to 30% are new. Since 1959, much attention has been focused on commercial development of textured vegetable protein products designed as meat extenders or spun soy isolates used in meat analogs.

Palatability, nutritional quality and safety are the major concerns of consumers as well as of food processors when a food product is introduced into the market. Textured soy proteins with structural integrity and identifiable texture can be produced from soy flour or soy concentrate by thermoplastic extrusion processes. The textured soy, if made from soy flour, will have 50% protein, or if soy concentrate is used, 70% protein. Textured soy proteins retain their shape and chewable texture on rehydration.

The main interest in soy products is their nutritive value, specifically high-protein content. When either textured soy flour or concentrate is hydrated, the appropriate amount of water is added so that the final product has approximately 18% protein or the amount of protein in the meat it replaces. Thus, soy proteins can be used to substitute for some of the meat without sacrificing nutritional quality. Replacement of up to 30% meat by textured soy protein is permitted by the USDA in the Type A School Lunch Program. Even at this high level of substitution, the Protein Efficiency Ratio (PER) of the meat-soy blend was not decreased (Wilding, 1974; Rakosky, 1974).

The typical flavors (beany, cereal or bitter) of soy products might be one of the main objections to a more extensive use of soy proteins as food. According to Wolf (1970), soy concentrates have reduced flavor levels compared with flours because some of the flavor constituents are removed by the concentration process. Hence, there is need to compare the flavor of meat products containing textured soy flour versus soy concentrate.

It would be a major error to conclude that all soy flours or all soy concentrates are alike (Mattil, 1974). When one considers the natural variability of biological materials, the number of different manufacturers involved and the differences in processing that are employed, the similarities in these commercial products are more impressive than their differences. Each manufacturer strives to produce the most bland product and one designed to fulfill a variety of product needs. For example, some manufacturers produce concentrates with high or with low protein solubility.

Investigators are in agreement that the use of soy in beef loaves reduces total cooking losses. An exact comparison of the results by different research workers is difficult because different soy products were used. Also ingredients, size and treatment of loaf (i.e., fresh or frozen, cooking temperature and final internal temperature) varied from study to study.

Carlin and Nielsen (1974) reported that 0, 10, 20 and 30% soy-substitution in loaves cooked at 163°C to 74°C resulted in total cooking losses of 14.3, 12.5, 9.7 and 7.6%, respectively.

Similar results of progressive decreases in total cooking losses as the amount of soy substitution was increased were reported by Yoon et al. (1974); namely, 24.2, 17.2 and 13.6% for 0, 15 and 30% soy, respectively, in loaves cooked at 177°C. In a comparison of frozen precooked loaves, Nielsen and Carlin (1974) found that, during precooking to 74°C, freezing and then reheating to 54°C, total losses were 18% for 100% beef loaves and only 8% for 70% beef plus 30% textured soy concentrate loaves. Recently, Williams and Zabik (1975) reported total cooking losses of 13.3% and 9.8% for 0 and 30% soy-substituted beef loaves (respectively) that were frozen, thawed and cooked at 177°C to 77°C.

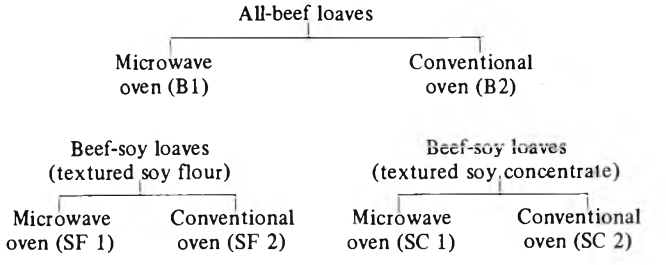
At present, there are no physical or chemical tests that will predict how soy proteins will affect actual food products. Hence, the only way to determine their effect is to incorporate the soy into a formulation (Wolf, 1970). Our main objective was to investigate the eating quality and nutritive value of beef-soy loaves and also to compare the effect of substituting 15% textured soy flour versus 15% textured soy concentrate on these characteristics. Because the use of soy has been recommended to prevent shrinkage in meat products, another of the purposes was to determine cooking losses, as well as fat, moisture and thiamine content of beef-soy loaves. In addition, the effect of cooking in microwave ovens was investigated on these physical, chemical and sensory characteristics of all-beef and 15% soy-substituted meat loaves.

MATERIALS & METHODS

A COMPLETELY RANDOMIZED design was used. The study was replicated on four consecutive days. Analysis of variance was used to determine the effect of oven treatment and loaf type on the parameters measured. When the effect of loaf type was significant, a "t" test was used to compare the individual means (Snedecor and Cochran, 1967).

Preparation of loaves

On each test day, six meat loaves were made, one for each of the variables as follows:



Freshly ground chuck was purchased on alternate days from two supermarkets in Ames. Before the loaves were made, two 50-g samples of ground chuck were removed, wrapped in polyethylene and foil and stored in glass jars at −20°C for fat, moisture and thiamine analyses at a later date. Ingredients and procedures were established in preliminary studies. The formula used is given in Table 1. The textured soy was hydrated with sufficient tap water to obtain 18% protein; thus, less water was added to the textured soy flour (53% protein) than to the soy concentrate (70% protein).

A meat loaf mixture that weighed 2020g was prepared in one batch. This mixture was sufficient for two 960-g meat loaves, plus two 50-g samples for chemical analyses. The ground chuck or ground chuck plus hydrated soy were weighed and then ground with a Kitchen Aid Mixer, model K45, with a breaker plate having 0.4-cm openings. Ingredients were combined by manual and mechanical mixing to insure uniform distribution while avoiding excessive manipulation or compaction of the mixture. All ingredients were placed in a bowl of a Hobart mixer, model C100, and combined with five revolutions on the lowest setting and then mixed 10 times with a wooden spoon. This procedure was repeated plus five more revolutions of the mixer.

Each meat loaf (960g) was baked in a Pyrex glass baking dish (21.5 × 11.3 × 6.3 cm). Approximately 480g of the meat loaf mixture were weighed into the pan and spread evenly by using 10 light strokes with the back of a spoon. The remaining 480g of meat mixture were added and patted 25 times so that no open spaces remained and the same degree of compactness was obtained. The two loaves from each batch were randomly assigned to the two oven treatments.

Cooking of loaves

Aroma and flavor must be evaluated on hot samples and within a short time after cooking is completed. Thus, the time that each loaf was put in the oven was scheduled so that it was removed approximately 10 min before it was sliced and evaluated by the panel. The ovens used were two electric ovens in General Electric Ranges (Model J 390003WH) and three microwave ovens, 2450 MHz. The heating capacity of the Tappan oven (220v) and two Amana ovens (115v) Models R-1H and RR-3H was tested by heating 1 liter of distilled water for 1 min. For the three ovens, the initial temperature of the water was 25°C and the final temperature after 1 min was 42°C, indicating that the heating capacity of the ovens was similar.

The three loaves, B2, SF2 and SC2, were cooked to a final internal temperature of 74°C in preheated conventional ovens set at 163°C. Since only two electric ovens were used, the placement of the three loaves was planned; for example, on day one the beef loaf was cooked alone and on the other days it was cooked with either the beef-soy flour or the beef-soy concentrate loaf. A thermocouple (J-type, iron constantan encased in a stainless steel hypodermic needle) was placed in the geometric center of each loaf. The initial temperature of the loaves and the internal temperatures of the loaves and the ovens throughout the cooking period were recorded on a recording potentiometer (Honeywell, Electronik 16).

The internal temperature of the three loaves cooked electronically, B1, SF1 and SC1, could not be obtained during cooking. But just before and just after cooking, thermocouples were inserted at the geometric center, and the inital and final temperatures of each loaf were recorded. The time required to reach 74°C as determined in preliminary studies was 19 min. In loaves that had a final internal temperature below 66°C, the desired final internal temperature of 74°C was not reached during the postcooking temperature rise, and the loaf had to be returned to the oven to be cooked for 30–85 sec. But if the temperature were above 66°C and below 74°C, it was not necessary to put the loaf back in the oven since the temperature continued to rise to at least 74°C.

Because three microwave ovens were used, cooking was planned so that each of the three types of loaves was cooked in each of the ovens during the experiment. The loaf was cooked for 9.5 min, turned 90 degrees and cooked another 9.5 min. Immediately after 19 min of cooking, the temperature was taken. Eight of the 12 loaves had to be returned to the oven after 19 min of cooking for additional cooking for approximately 30–85 sec.

Table 1—Ingredients for meat loaves

Ingredient	Beef (%)	Beef-soy flour[a] (%)	Beef-soy conc[b] (%)
Ground chuck	73.3	62.3	62.3
Hydrated soy flour dry soy 33.3% water 66.7%	—	11.0	—
Hydrated soy conc dry soy 25.0% water 75.0%	—	—	11.0
Reconstituted milk	12.1	12.1	12.1
Whole eggs (slightly beaten)	8.3	8.3	8.3
Salt	0.8	0.8	0.8
Bread crumbs	5.4	5.4	5.4

[a] Texgran, fortified soy flour (53% protein), Swift and Co., Oak Brook, Ill.
[b] GL-219, mechanical mix of Patti-Pro (granular soy protein concentrate, 70%) with a vitamin-mineral premix, Griffith Laboratories, Chicago, Ill.

After the final temperature was taken, each loaf was weighed. The loaves were removed from the pans onto warm steak plates, covered with aluminum foil and kept warm in an oven set at 93°C until evaluation time (a max of 10 min). After the drip was weighed, it was poured into a graduated cylinder, and the volume of the fat and nonfat fractions was determined.

Sensory evaluation

Each judge evaluated a slice of hot meat loaf from each of the six loaves, B1, B2, SF1, SF2, SC1 and SC2, for intensity of soy aroma, soy flavor and beef flavor. A 0–6 scale was used: 0 = lacking, and 6 = intense. Preliminary training sessions were held for 12 persons. Loaves used were similar to those in the study. Then on the basis of acuity and consistency in scoring, a nine-member panel was chosen. Individual booths with red fluorescent lights were used by the panel during judging. To minimize carry-over of flavor, water at room temperature was provided for the judges to rinse their mouths between samples.

Just before serving, a section was cut lengthwise, 1 in. from the side of the meat loaf. This portion was removed for chemical analyses. Then, the loaf was cut crosswise into 12, 1/2-in. slices. Each slice was served in a warm (93°C) coded white porcelain saucer. The type of loaf that was served first was changed on each day of the experiment. The position of the slice assigned to a judge remained the same for all six types of loaves on any day, but was rotated so that each judge received slices from positions near the center or near the end of the loaf during the experiment.

Chemical analysis

On the day of the experiment, two 50-g samples were obtained from (1) the raw ground chuck, (2) each of the three raw loaf mixtures and (3) each of the six cooked loaves. The samples were immediately wrapped, frozen and stored for fat and moisture or for thiamine analyses.

The ten 50-g samples from each replicate to be analyzed for fat and moisture were thawed in a refrigerator overnight. Then they were ground in a Kitchen Aid grinder by using a breaker plate with 0.4-cm openings. The Brabender semiautomatic moisture tester was used to determine moisture content. Moisture determinations were made on three 5-g samples from each treatment. Then the analysis of fat content (ether extractable material) was made on the three moisture-free samples from each treatment by using the Goldfisch extraction apparatus. Petroleum ether (boiling range 30–60°C) was used as a solvent. The methods followed for both moisture and fat determinations are described by Cash and Carlin (1968).

Later, the 10 frozen 50-g samples to be assayed for thiamine were thawed in a refrigerator overnight. The sample extract was prepared as described by Association of Vitamin Chemists (1966). A microbiological assay was used to determine the thiamine content of the samples (Diebel et al., 1957). A stock culture of *Lactobacillus virdescens* (ATCC 12706, Strain S38A) was maintained in Lactobacillus agar (Difco) stabs in the refrigerator and transferred every 2 wk. Just before the assay, three consecutive transfers of the inoculum were made in All-Purpose media with Tween (APT) broth (Difco). Growth response was determined turbidimetrically at a wavelength of 660 nm in a Bausch and Lomb Spectronic 20 Colorimeter.

RESULTS & DISCUSSION

ALL THE LOAVES weighed 960g, and the internal temperature, taken just before cooking, was similar, averaging 3.9°C. Average cooking time of 78 min for loaves cooked in a conventional oven at 163°C to 74°C was significantly longer than the 19 min for loaves cooked in a microwave oven. Analysis of variance revealed that cooking time was significantly ($P < 0.01$) affected by oven treatment, but not by loaf type.

The internal temperature of loaves cooked conventionally was recorded throughout cooking. For loaves cooked electronically, only the temperature immediately before and after cooking could be obtained. The data plotted in Figure 1 illustrated the similarity in cooking time for the three types of loaves within an oven treatment, also the 77% reduction in cooking time resulting from the use of the microwave ovens.

Rate of heat penetration between 4.0° and 74°C was 3.7°C/min for loaves cooked in the microwave oven and 0.9°C/min for those cooked in the conventional oven. Thus, heat penetration was four times faster in the microwave than in the electric oven.

Heat penetration was noticeably uneven in loaves cooked in the microwave ovens. At the corners of the loaves, the meat was overcooked, dry and hard. These areas were not used in subjective tests or chemical analyses.

Cooking losses

Data on total, drip and volatile losses, plotted in Figure 2, revealed that cooking in the microwave ovens increased total cooking losses regardless of type of loaf, and most of the losses

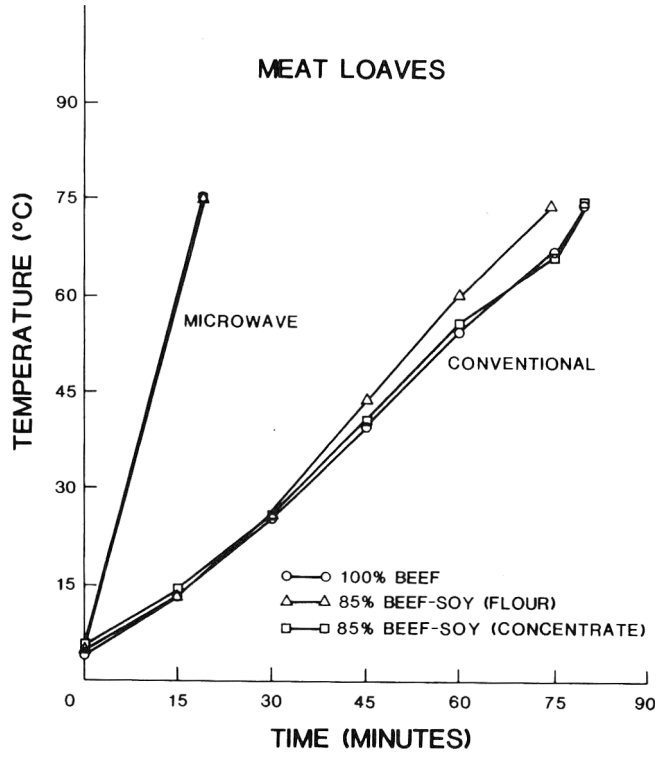


Fig. 1–Rise in temperature in 100% beef and 85% beef-15% soy loaves cooked to a final internal temperature of 74°C in microwave or in conventional ovens.

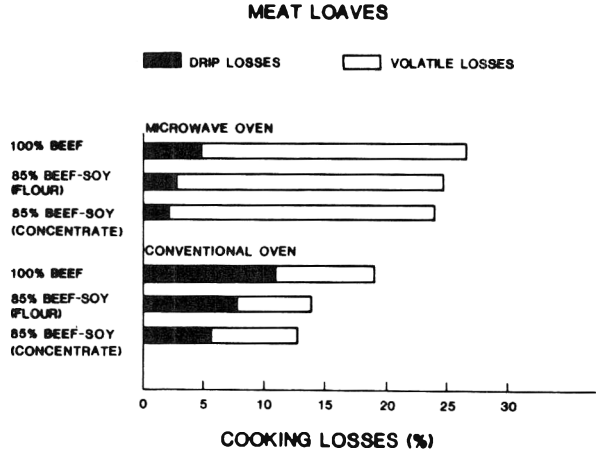


Fig. 2–Total cooking losses, drip losses and volatile losses in 100% beef and 85% beef-15% soy loaves cooked to a final internal temperature of 74°C in mivrowave or in conventional ovens.

Table 2–Average scores for sensory evaluation[a] of beef or beef-soy loaves cooked in microwave or in conventional ovens

Loaves		Beef flavor		Soy aroma		Soy flavor	
Beef (%)	Soy (%)	M[b]	C[b]	M	C	M	C
100	0	4.2 ± 0.1[c]	4.2 ± 0.2	0	0	0	0
85	15[d]	2.7 ± 0.1	2.4 ± 0.4	1.3 ± 0.4	1.8 ± 0.6	2.0 ± 0.2	2.4 ± 0.6
85	15[e]	2.1 ± 0.2	2.0 ± 0.3	2.2 ± 0.3	2.4 ± 0.3	2.8 ± 0.4	3.0 ± 0.4

[a] Average for 9 judges: 0 = lacking; 6 = intense
[b] Ovens: M, microwave; C, conventional
[c] Standard deviation
[d] Textured soy flour hydrated to 18% protein
[e] Textured soy conc hydrated to 18% protein

were caused by evaporation. Cooking losses for loaves cooked conventionally were approximately 50% volatile losses and 50% drip.

Analysis of variance indicated that total and drip losses were significantly ($P < 0.01$) affected by both oven treatment and loaf type, whereas volatile losses were significantly ($P < 0.01$) affected only by oven treatment. The "t" tests on the total cooking losses indicated that the significant difference was between the all-beef and beef-soy loaves cooked in either of the two types of ovens. For the loaves containing textured soy flour or soy concentrate, drip losses were not significantly different when loaves were cooked electronically, but were significantly different for loaves cooked in the conventional oven.

Substitution of 15% textured soy flour or soy concentrate reduced total cooking losses to 24% from 27% in loaves cooked in the microwave oven and to 14% from 19% in loaves cooked in the electric ovens. Drip losses were 5% for all-beef loaves and 2% for either of the beef-soy loaves cooked electronically. During cooking in conventional ovens, drip losses were 12% for B2 loaves, 8% for SF2 loaves and 6% for SC2 loaves. Volatile losses of 22% for loaves cooked in the microwave ovens were significantly greater than the 7% volatile losses for loaves cooked in the conventional ovens.

Our results confirm the results of Kylen et al. (1964), who reported that total cooking losses and evaporation losses were significantly greater during microwave than during conventional cooking of beef loaves. Also our conclusion that the substitution of 15% soy in beef loaves reduced cooking losses by 28% agreed with results of Yoon et al. (1974), who reported a reduction of 29% in cooking losses.

In our study, the hot drippings were poured into graduated cylinders. The results indicated that, for loaves cooked electronically, fat constituted between 40–50% of the drip loss and, for those cooked conventionally, between 24–30%.

Sensory evaluation

Sensory evaluation scores for the 100% beef or the beef-soy loaves cooked in the microwave or in the conventional ovens are presented in Table 2. Analysis of variance indicated that beef flavor, soy aroma and soy flavor were significantly ($P < 0.01$) affected by loaf type, but not by oven treatment. The similarity in beef flavor scores for the B1 and B2 loaves are in agreement with results reported by Kylen et al. (1964). The lower beef flavor scores for the SF1, SC1, SF2 and SC2 loaves were expected because these loaves had 15% less beef; also, the soy flavor masked some of the beef flavor. This is emphasized by the observation that the SC1 and SC2 loaves received higher soy aroma and flavor scores and lower beef flavor scores than the SF1 and SF2 loaves. The higher soy flavor in SC loaves indicated that the additional refinement required to produce soy concentrate does not always lessen the soy flavor as suggested by Wolf (1970). Our results are similar to those of Kalbrener et al. (1971) and Yasumatsu et al. (1972), who reported the similarity in intensity of soy flavor for water suspensions of soy flour, soy cencentrates or soy isolates. Although the manufacturers are striving to eliminate the typical beany soy flavor, scores of 2.2 (weak) for SF loaves and 2.9 (moderate on a 0–6 scale) for SC loaves indicated that the product can be improved. Spices or onions were not used, and it is possible that such ingredients could mask the soy flavor.

Moisture and fat content

The moisture content of the raw and cooked loaves is presented in Table 3. The moisture content of the raw ground chuck ranged from 63.8–71.3% and averaged 68.0% for the four replicates. The addition of eggs, milk and bread crumbs or

Table 3–Average[a] moisture and fat content of raw and cooked[b] all-beef and beef-soy loaves

Sample	Microwave			Conventional		
	B[c] (%)	SF[c] (%)	SC[c] (%)	B (%)	SF (%)	SC (%)
			Moisture			
Raw loaves	67.8 ± 2.2[d]	67.7 ± 1.6	67.8 ± 1.6	67.8 ± 2.2	67.8 ± 1.6	67.9 ± 1.7
Cooked loaves	58.6 ± 2.9	58.0 ± 2.5	58.0 ± 1.9	64.2 ± 2.4	64.4 ± 2.0	64.2 ± 1.8
			Fat			
Raw loaves	10.8 ± 2.8	9.8 ± 2.3	10.5 ± 2.1	10.8 ± 2.8	9.8 ± 2.3	10.5 ± 2.1
Cooked loaves	11.7 ± 2.4	11.4 ± 2.7	11.4 ± 2;2	10.0 ± 2.4	9.5 ± 2.2	9.2 ± 2.2

[a] Average of 12 determinations (four loaves X three samples per loaf)
[b] Cooked to a final internal temperature of 74°C in microwave or in conventional ovens
[c] B, 100% beef; SF, 85% beef and 15% textured soy flour; SC, 85% beef and 15% soy concentrate.
[d] Standard deviation

Table 4—Average[a] thiamine content of raw and of cooked all-beef and beef-soy loaves

Loaves		Raw loaves	Cooked loaves	Thiamine retention
Beef (%)	Soy (%)	Moist basis (mg/100g)	Moist basis (mg/100g)	Dry basis (%)
		Microwave		
100	0	0.09 ± 0[b]	0.08 ± 0	69.1 ± 4.3
85	15[c]	0.12 ± 0.01	0.10 ± 0	64.6 ± 6.9
85	15[d]	0.11 ± 0.01	0.09 ± 0	66.7 ± 3.6
		Conventional		
100	0	0.09 ± 0	0.09 ± 0.01	82.5 ± 4.9
85	15[c]	0.12 ± 0.01	0.10 ± 0	76.0 ± 3.4
85	15[d]	0.11 ± 0.01	0.09 ± 0	74.6 ± 4.7

[a] Average of 12 determinations (four replicates X three assays)
[b] Standard deviation
[c] Textured soy flour hydrated to 18% protein
[d] Textured soy concentrate hydrated to 18% protein

the hydrated soy did not appreciably change the moisture content of the raw loaves compared with the raw chuck (67.8 vs 68.0%). Moisture content of all loaves decreased during cooking, but microwave cooking had a significantly greater ($P < 0.01$) effect than conventional cooking. The use of soy did not affect the moisture content of the cooked loaves within each of the oven treatments. Microwave cooking reduced the moisture content to 58%, a reduction of 14% compared with a 5% reduction (to 64% moisture) for loaves cooked in the conventional oven (Table 3).

The fat content of the raw chuck ranged from 9.6–17.3% and averaged 12.8%. The fat content of the 15% soy-substituted raw loaves was not appreciably different from the fat in all-beef raw loaves. Analysis of variance revealed that fat content of cooked loaves was significantly affected by oven treatment, but not by loaf type. All loaves cooked in the microwave oven had an average fat content of 11.5%, which was slightly greater than the 9.6% fat content of all loaves cooked conventionally.

The thiamine content as reported by the manufacturers on the dry basis was 0.57 mg/100g for the textured soy flour and 0.30 mg/100g for the textured soy concentrate. The substitution of 15% hydrated soy increased the thiamine content (wet basis) from 0.09 mg/100g in raw all-beef loaves to 0.11 or 0.12 mg/100g for raw 15% soy-substituted loaves (Table 4).

Calculation (on the dry basis) revealed that cooking reduced thiamine content from approximately 0.33 mg/100g in raw loaves to approximately 0.22 mg/100g in loaves cooked in microwave ovens and to 0.26 mg/100g in loaves cooked in electric ovens. Thiamine retention (shown in Table 4; calculated on the dry basis) was 65–69% for loaves cooked electronically and 75–82% for loaves cooked conventionally. But thiamine content of all loaves on a moist basis was very similar and averaged 0.09 mg/100g of the cooked loaves.

In conclusion, cooking meat loaves in microwave ovens compared with cooking in conventional ovens at 163°C to a final internal temperature of 74°C reduces cooking time drastically, but increases cooking and volatile losses and decreases moisture content and thiamine retention; flavor of all loaves is not affected. Substitution of 15% soy decreases cooking losses, but is much more effective in reducing losses in loaves cooked in conventional than in those cooked in microwave ovens. No differences exist in the moisture or fat content of the cooked 0 or 15% soy-substituted loaves, and thiamine retention is similar. The use of 15% soy decreases the beef flavor scores compared with all-beef loaves. Soy flavor scores of 2.3 for 15% soy-flour-substituted loaves and 2.9 for 15% soy-concentrate-substituted loaves indicate that there is an opportunity for manufacturers to decrease the soy flavor of textured soy and thus improve the acceptance of soy-substituted meat products.

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Use of a product does not imply an endorsement or recommendation over similar products.

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EFFECT OF QUALITY GRADE AND CUT FORMULATION ON THE PALATABILITY OF GROUND BEEF PATTIES

ABSTRACT

Chucks and short plates were selected from 25 carcasses representing the middle third of each of five U.S. quality grades. Beef patties were prepared from chucks and short plates individually and in combination. Taste tests were conducted on the samples in order to study the effect of quality grade and cut formulation on palatability of the cooked product. Over the range of carcasses graded from Prime to Cutter, taste panel subjective evaluations of tenderness, connective tissue amount and overall acceptability decreased significantly. Patties from Prime, Choice and Good carcasses were rated as acceptable in all palatability traits (5.0 or above on a 9-point scale) whereas patties from Utility and Cutter grades were rated 4.0 or less in tenderness, connective tissue amount and overall acceptability. Differences in juiciness and flavor were not substantially affected by quality grade. Patties formulated from chucks were rated more desirable in tenderness, flavor, connective tissue amount and overall acceptability than patties from short plates or short plate-chuck combinations. Differences in palatability due to quality grade were larger than those due to cuts.

INTRODUCTION

SINCE 1970, the U.S. Department of Agriculture has purchased over 415 million pounds of ground beef for distribution to schools. This product was purchased under the Schedule AA–USDA Specification for Frozen Ground Beef. This specification requires that beef and calf be the only ingredients and these shall be derived from fresh chilled beef or calf carcasses, sides and/or cuts of the U.S. Utility grade or higher for beef and U.S. Standard or higher for calf. The specification also states that at least 50% of the total weight of the beef (or calf) shall be from any one or any combination of primal cuts (major) and the remaining 50% or less may be from any one or any combination of rough (minor) cuts. Limited research information is available as to the acceptability of ground beef from various U.S. quality grades and to the effect of major versus minor cuts on palatability. Fruin and Van Duyne (1961) reported palatability differences in ground beef prepared from chucks and rounds from U.S. Commercial or Standard carcasses. Quality grade had no significant effect on palatability whereas panelists preferred ground beef from chucks to that from rounds. Information is needed as to the effect of wide ranges in quality grade and various combinations of major and minor cuts on palatability of cooked ground beef. Perhaps beef of a lower U.S. quality grade could be utilized to produce acceptable ground beef. Likewise it may be possible to use a higher proportion of rough cuts in the formulation thus producing a more economical product. Therefore, the study objective was to determine how ground beef formulations of varying quality grades and cuts would affect cooked ground beef palatability.

EXPERIMENTAL

FIVE CARCASSES were selected to represent the middle third of each of five USDA quality grades (Prime, Choice, Good, Utility and Cutter). Each carcass was selected from the previous day's slaughter. From each carcass one chuck and both short plates were removed, boned and shipped about 10 miles in a refrigerated truck to the commercial Research Laboratory. To meat of the Utility and Cutter grades enough subcutaneous and kidney fat from other carcasses of those grades was added to approximate 24% fat content in the ground beef.

Sixteen combinations of ground beef were prepared as outlined in Table 1. The lean and fat were ground through a 0.64 cm plate, mechanically mixed for 2 min and ground through a 0.32 cm plate for the final grind. Prior to the final grind, the mixture was sampled for fat analysis by the Modified-Babcock procedure. Fat content was standardized at 24 ± 2% by the addition of appropriate amounts of fat or lean. Fat content was controlled, as much as possible, since the aim of the project was to study the effect of differences in grade and cut on palatability. In preparing the major vs. minor cut combinations for treatments 11–16 (Table 1) each chuck and plate was ground separately through the 0.64 cm plate, combined in equal amounts of fat or lean from chucks and plates, mixed and finally ground through a 0.32 cm plate. The ground meat was formed into 75g patties 10 cm in diameter using a Hollymatic Patty Machine. During processing, the product internal temperature did not exceed 10°C. All patties were placed in boxes (4.5 kg to a box), frozen in a blast freezer (−30°C), and shipped to Beltsville, Md., via air freight.

Cooking

Patties were roasted from the frozen state in a 200°C oven for 9 min, quartered, and served as hot as possible to the panelists. A subsample of 10 patties per treatment was cooked to obtain weights for drip, evaporative and total cooking loss.

Panel selection and training

Male and female panelists were selected from the scientific and ancillary staff of the Agricultural Research Center. Selection was based on assessments of repeatability on duplicate samples and consistency of

Table 1—Sample designation

Sample no.	Grade/Cut	
	Name	Symbol
1	Prime/chuck	P/C
2	Choice/chuck	C/C
3	Good/chuck	G/C
4	Utility/chuck	U/C
5	Cutter/chuck	Cu/C
6	Prime/plate	P/P
7	Choice/plate	C/P
8	Good/plate	G/P
9	Utility/plate	U/P
10	Cutter/plate	Cu/P
11	Prime/combination[a]	P/CP
12	Choice/combination	C/CP
13	Good/combination	G/CP
14	Utility/combination	U/CP
15	Cutter/combination	Cu/CP
16	Choice and Cutter/ combination	C/Cu/CP

[a] Combination consisted of chuck and plate in equal proportions

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ratings in relation to the group. Triangle tests were conducted over a period of 3 wk (total = 15) and 16 panelists were selected on the basis of these data. After each training session, discussions were held with the panelists in order to achieve unanimity in the interpretation of the scoring system. Panelists evaluated the samples under red lights in booths. If each panelist had been present for every session, there would have been an equal number (9) of evaluations by each panelist on every ground beef sample combination. The "ideal" panel situation would have resulted in each sample combination receiving 144 evaluations. In actuality, panelist attendance was not perfect. Three panelists had fewer than 50 evaluations while the remaining 13 averaged 120 evaluations out of a possible 144. To avoid problems in statistical analysis created by a wide disparity between the number of evaluations made by the panelists, the data from those three members were eliminated.

A trained 13-member panel rated each pattie for tenderness and juiciness by using a 9-point scale (9 = extremely tender or juicy and 1 = extremely tough or dry). Tenderness and juiciness were evaluated during the first 5–10 chews. The amount of connective tissue residue remaining at the end of mastication was rated by using a 9-point scale (9 = none and 1 = very abundant amount). Due to the study design, flavor was rated on the basis of "desirability" rather than "intensity." Since off-flavors might be rated as "intense" an "intensity" scoring system could not differentiate between "intense-desirable" and "intense-undesirable" flavors. The taste panel evaluations were conducted over a time span of eight 3-day periods. Panelists evaluated six ground beef samples each day so that during each three-day period all sixteen sample combinations were tested. In addition, two sample combinations were repeated during each session. The daily assignment of sample combinations was presented to the panel nine times during the course of the study.

Statistical techniques

Panel palatability evaluations are discrete in nature (i.e., integer valued from 1 to 9). Nonparametric statistical methods are the most appropriate type of statistical technique to use on taste panel data. These methods do not assume that the data have any specific type of distribution. In particular, nonparametric methods do not require that the data have a normal distribution. On the other hand, it is reasonable to assume that results of cooking loss analyses are normally distributed. That assumption, if not entirely true, is at least sufficiently true to be satisfactory for most practical applications.

Friedman's Test (1937) which was used to analyze the taste panel data, is analogous to the two-way analysis of variance procedure. It is used to test the null hypothesis that all combinations have the same mean effects against the alternative hypothesis, that the combination mean effects are not all equal. The method of McDonald and Thompson (1967) uses the rank sums computed from Friedman's Test for the nonparametric multiple comparison of the combination means. "Experimentwise error rates" are used rather than "significance levels" when discussing this multiple comparison procedure. The "experimentwise error rate" is defined as "the ratio of the number of experiments in which at least one difference between means is falsely declared significant to the total number of experiments conducted over a long period time."

RESULTS & DISCUSSION

RESULTS of Friedman's Test indicated that the mean effects of the 16 sample combinations were significantly different at the 5% level of significance for each of the five palatability characteristics. Table 2 shows the results of nonparametric multiple comparisons of the 16 sample combinations for all five palatability characteristics. The combinations are arranged in order of decreasing values of the rank sums, and their means followed approximately the same order.

The nonparametric multiple comparisons shown in Table 2 enable the use of statistical techniques to objectively investigate the "closeness" of the mean values of the individual sample combinations. Ground beef formulated from Prime or Choice carcasses was more acceptable in all palatability traits (except juiciness) than ground beef prepared from Utility or Cutter carcasses. If a taste panel rating of 5.0 or above is arbitrarily designated as acceptable and 4.9 and below is unacceptable in palatability, then ground beef prepared from Prime, Choice and Good chucks or Prime and Choice plates was rated as "acceptable" in all five taste panel traits. Ground beef prepared from Good plates and Utility and Cutter chucks and plates was usually below 5.0 (unacceptable).

Lean and fat from Choice and Cutter chucks and plates were combined to determine whether the addition of Choice meat would offset the low palatability ratings of ground beef

Table 2—Nonparametric multiple comparisons for palatability traits

Tenderness			Juiciness			Flavor			Connective tissue amount			Overall acceptability		
Combination	Mean	Rank[a,b] sum	Combination	Mean	Rank[a,b] sum	Combination	Mean	Rank[a,b] sum	Combination	Mean	Rank[a,b] sum	Combination	Mean	Rank[a,b] sum
C/C	6.6	196	C/C	5.5	154.5	P/CP	5.9	165.0	C/C	6.7	198.0	C/C	6.4	191.5
P/C	6.3	189.5	G/C	5.4	141.5	C/C	6.0	163.5	P/C	6.6	197.0	P/C	5.7	167.0
G/C	6.0	171.5	P/P	5.3	140.0	P/P	5.8	160.0	P/P	6.2	173.0	P/CP	5.8	167.0
P/P	5.9	169.0	C/P	5.2	132.0	C/CP	5.8	152.0	G/C	6.1	164.0	P/P	5.7	161.5
P/CP	5.6	148.5	G/CP	5.2	130.0	G/C	5.6	146.5	P/CP	5.8	152.0	G/C	5.8	159.5
C/CP	5.2	133.0	Cu/P	5.2	125.0	C/P	5.7	146.5	C/P	5.6	138.5	C/P	5.7	154.0
C/P	5.1	124.5	C/CP	5.2	122.0	P/C	5.5	138.0	C/CP	5.5	134.0	C/CP	5.4	149.0
G/CP	4.8	114.5	P/CP	5.1	115.5	G/CP	5.1	104.5	G/P	5.3	125.0	G/CP	4.9	113.0
G/P	4.6	99.5	C&Cu/CP	5.0	108.5	U/P	4.9	104.0	G/CP	5.0	109.0	G/P	4.5	94.5
C&Cu/CP	4.3	80.5	P/C	5.0	107.5	C&Cu/CP	5.2	98.0	U/C	4.3	83.0	U/P	4.3	92.0
U/C	4.2	77.5	G/P	5.1	104.5	U/C	4.8	91.5	U/P	4.0	78.5	C&Cu/CP	4.4	82.5
U/P	4.4	74.5	U/P	5.0	99.0	Cu/C	4.6	78.0	C&Cu/CP	4.0	73.0	U/C	4.1	74.5
Cu/CP	3.7	62.5	Cu/C	4.9	89.0	U/CP	4.5	73.5	Cu/C	3.2	50.0	Cu/C	3.6	55.0
Cu/C	3.9	58.0	Cu/CP	4.9	81.0	G/P	4.6	60.5	U/CP	3.1	42.5	U/CP	3.4	41.5
Cu/P	3.4	41.5	U/C	4.8	80.0	Cu/CP	4.1	48.5	Cu/CP	2.6	27.5	Cu/CP	3.0	39.5
U/CP	3.4	27.0	U/CP	4.3	38.0	Cu/P	4.1	38.0	Cu/P	2.5	23.0	Cu/P	3.0	26.0

[a] Vertical lines connect all sample combinations which are NOT significantly different for an experimentwise error rate of 5%.
[b] A rank sum difference of at least 83 is required for significance for an experimentwise error rate of 5%.

Table 3—Nonparametric multiple comparisons for palatability traits by overall quality grade and cut

Tenderness			Juiciness			Flavor			Connective tissue amount			Overall acceptability		
		Rank[a,b]			Rank[a,b]			Rank[a,b]			Rank[a,b]			Rank[a,b]
Grade	Mean	sum	Grade	Mean	sum	Grade	Mean	sum	Grade	Mean	sum	Grade	Mean	sum
Prime	5.9	61	Choice	5.3	53	Prime	5.7	56	Prime	6.2	62	Prime	5.7	60
Choice	5.6	54	Good	5.2	47	Choice	5.8	54	Choice	5.9	53	Choice	5.8	57
Good	5.1	41	Prime	5.1	42	Good	5.1	33	Good	5.5	41	Good	5.0	37
Utility	4.0	21	Cutter	5.0	37	Utility	4.7	31.5	Utility	3.8	26	Utility	3.9	26
Cutter	3.7	18	Utility	4.7	16	Cutter	4.3	20.5	Cutter	2.8	13	Cutter	3.2	15

[a] Vertical lines connect all sample combinations which are NOT significantly different for an experimentwise error rate of 5%.
[b] A rank sum difference of at least 23 is required for significance for an experimentwise error rate of 5%.

Table 4—Nonparametric multiple comparisons for palatability traits by cut

Tenderness			Juiciness			Flavor			Connective tissue amount			Overall acceptability		
		Rank[a,b]			Rank[a,b]			Rank[a,b]			Rank[a,b]			Rank[a,b]
Cut	Mean	sum	Cut	Mean	sum	Cut	Mean	sum	Cut	Mean	sum	Cut	Mean	sum
Chuck	5.4	39	Plate	5.2	30.5	Chuck	5.3	35	Chuck	5.4	38	Chuck	5.1	37
Plate	4.7	21	Chuck	5.1	25.5	Plate	5.0	22	Plate	4.8	26	Plate	4.7	23
Combination	4.5	18	Combination	4.9	22.0	Combination	5.1	21	Combination	4.4	14	Combination	4.5	18

[a] Vertical lines connect all sample combinations which are NOT significantly different for an experimentwise error rate of 5%.
[b] A rank sum difference of at least 12 is required for significance for an experimentwise error rate of 5%.

from Cutter carcasses. The addition of Choice grade meat (C&Cu/CP) increased the palatability ratings in all five traits; however, all mean ratings remained below 5.0 except those for flavor and juiciness. Panelists rated tenderness and connective tissue amount lowest of all the palatability traits. Probably the amount of connective tissue from lower grade carcasses significantly influenced the rating for tenderness and overall acceptability. This hypothesis is supported by the magnitude of the simple correlation coefficients between tenderness and connective tissue amount ($r = 0.74$) and overall acceptability and connective tissue amount ($r = 0.71$).

For chuck, mean scores for all palatability traits, except juiciness, were similar for Prime, Choice and Good meat. Choice, however, consistently scored highest. In contrast, the palatability ratings for plates or any combination containing plates tended to steadily decrease as quality grade decreased.

Nonparametric multiple comparisons by overall grade for all five palatability characteristics are presented in Table 3. Except for juiciness, the panelists' evaluations coincide with our "a priori" knowledge of the palatability characteristics of quality grades; that is, the mean values for Prime and Choice were very similar and consistently higher than those for Utility and Cutter. The mean values for the Good grade were usually intermediate. Differences in mean values between grades were not as pronounced for juiciness as for other palatability traits. In general, however, these results tend to confirm that USDA quality grades provide meaningful assessments of the quality of ground beef as measured by the other four palatability characteristics. Palatability ratings for the amount of connective tissue were less than 4.0 for Utility and Cutter ground beef. Possibly that trait accounts for the low scores in overall acceptability.

Nonparametric multiple comparisons for cuts are presented in Table 4. Except for juiciness, the mean palatability values were consistently and significantly higher for chuck than for plate. The mean value for the combination was consistently lower than the mean values for chuck or plate (except for flavor). Apparently the addition of chuck to the formulation did not offset the adverse palatability of the plate. Juiciness did not differ significantly between means of cuts. Analysis of variance for drip loss, evaporation loss and total loss (not shown in table) indicated that the mean values of the sixteen combinations were not significantly different.

CONCLUSIONS

DIFFERENCES in palatability of ground beef patties between grades were significant and large enough to be important. Ground beef from Prime and Choice carcasses was consistently rated higher than that from Utility and Cutter; Good grade ground beef was usually intermediate. Ground beef from chucks was rated higher in palatability, except for juiciness, than that from plates or chuck-plate combinations. Apparently the addition of chuck in equal proportion with plate was not sufficient to bring the "combination" palatability up to an "acceptable" level in the lower grades. These data support the USDA's regulations requiring minimum quality grades. Further work is needed to evaluate additional quality grade and cut combinations to determine the most economical formulation that would yield an acceptable product.

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MICROBIOLOGY AND TECHNOLOGY OF THE PEPPERONI PROCESS

ABSTRACT

A pilot plant process was developed for the production of pepperoni as a fully dry, fermented sausage. The process included: (a) aging in which salted (3% NaCl) meat was held for 10 days at 5°C to encourage the growth of micrococci and lactobacilli; (b) fermentation at 35°C and 85% relative humidity (RH) during which the lactobacilli fermented the sugar and lowered the pH, and the micrococci reduced nitrate to nitrite; and (c) drying at 12°C and 65% RH to about 50% of starting weight. Chemical analyses of the commercial pepperoni yielded the following data: (1) pH ranged from 6.1–4.7; (2) moisture, from 17.0–30.9%; (3) fat, from 38.1–52.5%; (4) water activities ranged from 0.87–0.80; and (5) all had moisture/protein (M/P) ratios < 1.6/1.0, the maximum recommended for pepperoni. The microflora of commercial samples varied, both in bacterial count and type. Pilot plant products had lower moisture and fat contents than commercial products, pH values of 4.7–4.9, and viable microflora almost exclusively lactobacilli. The M/P ratios of the pilot plant products were also < 1.6/1.0.

INTRODUCTION

PEPPERONI is a highly spiced (with or without paprika), fermented, fully-dry sausage prepared from pork or a mixture of pork and beef. Except for the drying step, its processing should be similar to that of other fermented sausages such as Lebanon bologna or summer sausage. However, neither details of the process nor the chemical and microbiological changes which occur during the various steps have been reported in the literature. We wanted to develop a process which would yield sausages with characteristics similar to those of commercial pepperoni and to follow the chemical and microbiological changes throughout processing. We also analyzed commercial pepperoni chemically and microbiologically.

MATERIALS & METHODS

Sugars-spices-cure

Based on published formulae (Kramlich et al., 1973; Komarik et al., 1974; National Provisioner, 1938), the following sugar/spice mixture was developed for our pepperoni:

Sugar/spice	g/kg meat mix
sucrose	10
glucose	10
ground cayenne pepper	2
crushed red pepper	2
pimento	5
whole anise seed	2-1/2
garlic powder	0.1

The curing agent, $NaNO_3$, was added at 1.2 g/kg of meat. This level was a compromise between the 0.6 g/kg recommended by Kramlich et al. (1973) and the ca 1.7 g/kg permitted by Meat Inspection Regulations (Meat and Poultry Inspection Program, APHIS, USDA, 1973, Washington, D.C.).

Meat

Boneless pork shoulders or picnics were ground through a 3/4-in. plate and frozen at –27°C for at least 1 month to inactivate the trichinae. Whole, good-grade beef chuck was removed from the carcass, ground through a 3/4-in. plate, frozen at –27°C and held until needed. Prior to use, meats were thawed at 10°C.

Fermentation

Pilot plant pepperoni was fermented either by the natural lactic microflora of the meat encouraged through aging of salted (3% NaCl) meat at 5°C for 10 days (Palumbo et al., 1973; Smith and Palumbo, 1973) or in one experiment, by the addition of lactic acid starter culture (Lactacel MC, Merck & Co., Rahway, N.J.).

Compositional and chemical analyses

Moisture, ash, fat and protein contents of the various pepperoni were determined on twice-ground (1/8-in. plate) samples by standard AOAC procedures (AOAC, 1965). The ground pepperoni samples were analyzed for nitrosamines by methods described previously (Palumbo et al., 1974). The pH and titratable acidity were measured by a modification of the technique of Kempton and Bobier (1970); 50g of the salted meat sample or of intact sausage were aseptically weighed into a sterile Waring Blendor jar; 200 ml of sterile 0.1% peptone (Difco) water were added, and the mixture was blended for 1 min at high speed. The resulting slurry (1:5 dilution) was first sampled for microbiological analyses, then centrifuged at 5°C for 10 min at 16,500 × G. The pH of the clear extract was measured on a Corning model 10 pH meter using a single, combination electrode. The acid present in the extract (generally a 50 ml aliquot) was titrated to pH 7.0 by using the pH meter and standard base; percent acid was calculated as lactic. Water activity (A_w) was determined on diced slices of pepperoni by use of an Electric Hygrometer-Indicator (model 15-3001, with gray sensor; Hydrodynamics Inc., Silver Spring, Md.).

Microbiology

Microbiological analyses of commercial pepperoni were performed; the microflora of our own pepperoni was determined during aging of the meat and during and after processing of the sausages. The peptone water slurry was used for microbial analyses as follows (Smith and Palumbo, 1973): total count on APT agar (Difco) incubated for 3–4 days at 25°C; micrococci (gram-positive, catalase-positive cocci) on Mannitol salt agar (MSA, Difco) incubated for 3 days at 32°C; lactobacilli (gram-positive, catalase-negative rods) on Rogosa SL agar (Rog, Difco) incubated for 3–5 days at 25°C; yeast on acidified potato dextrose agar (PD, Difco) incubated for 3 days at 25°C; and gram-negative rods (coliforms) on Eosin Methylene Blue agar (EMB, Difco) incubated for 1 day at 37°C. Gram stain and catalase test were performed on all colony types found on the various media.

Processing

The following general procedure was developed in our pilot plant for pepperoni: The frozen meat was thawed and mixed with 3% salt (NaCl); the salted meat was then aged for ca 10 days (the aging step was omitted when starter culture was used). After aging, the sugar-spice mixture and $NaNO_3$ were added to the meat, which consisted of either pork or beef or a 1:1 mixture of the two, and mixed. This mixture was then ground through a 3/16-in. plate, stuffed into 55 mm clear fibrous casings (Union Carbide), coated with paraffin (MP, 52°C), and hung at 35°C and 85% RH for 1–3 days to allow fermentation. The paraffin which was used to prevent mold growth and excessive moisture loss during fermentation was removed after fermentation and the sausages were dried for 6 wk (40–42 days) at 12°C and 65% RH.

RESULTS & DISCUSSION

SEVERAL DIFFERENT commercial pepperoni were purchased and analyzed chemically and microbiologically. All were prepared from a pork and beef mixture, most with a mixed (nitrate/nitrite) cure; ca 1/2 contained paprika, and ca 1/3 were fermented with starter culture (Table 1).

Compositional and chemical evaluations of commercial and

pilot plant pepperoni are given in Table 2. The fat content of commercial products ranged from 38.1–52.8%. The protein varied from 17.9–24.8%, ash from 5.1–6.4 %, and moisture from 17.0–31.5%. For all commercial and pilot plant pepperoni, moisture/protein ratios (M/P) were <1.6/1.0, the maximum permitted for pepperoni in the Laboratory Guide Book (USDA, CMS, Laboratory Services Div.).

The pH values for commercial pepperoni (Table 2) varied widely, ranging from 6.1–4.8. Usually commercial products with low pH had high titratable acidity. With one exception, from company D, only commercial products prepared with starter culture had pH values <5.0 (Tables 1 and 2). Though Lechowich (1971) stated that pH 4.5–5.0 is desirable in fermented sausages, six commercial pepperoni had pH > 5.0 and five, <5.0. Apparently other commercial fermented sausages also exceed pH 5.0. Ostlund and Regner (1968) recorded a pH range of 5.7–4.6 for commercial samples of "Isterband," a Swedish fermented sausage. Deibel et al. (1961) found a pH range of 5.3–4.6 for commercial summer sausage, but a very narrow range of 4.9–5.1 for thuringer. In contrast, commercial Lebanon bologna had a narrow, desirable pH range of 4.6–4.9 (Palumbo et al., 1973). After drying, our pepperoni had pH values of 4.7–4.9 and titratable acidities of 1.14–1.54% (Table 2).

In general, agreement was good among moisture contents, M/P ratios, and A_w values (Table 2). Commercial pepperoni having high moisture contents tended to have high M/P ratios and A_w values.

The microbiological analyses of commercial pepperoni are given in Tables 3 and 4. Except for the total count (APT agar), the counts on the other media varied (Table 3), from undetectable (<1 X 10^2/g) to substantial. For example, on Rog, the counts ranged from 1 X 10^2/g for company E to 8.5 X 10^7/g for company F (loc 1). Data in Table 4 indicated that the selective agars used to examine samples of commercial pepperoni supported the growth of several types of microorganisms in addition to those the agars are supposedly designed to detect and distinguish. Virtually all organisms isolated from these sausages grew on MSA (Table 4). Thus, our

Table 1—Ingredients of commercial pepperoni[a]

Company[c]	Components added to meat cure[b]			
	Paprika	Nitrate	Nitrite	Added starter culture
A	–	+	–	–
B	–	1	2	–
C	–	1	2	–
D	–	1	2	–
E	+	1	2	–
F(loc 1)[c]	+	–	+	+
F(loc 2)[c]	+	1	2	+
F(loc 2)[c] sandwich style	+	1	2	+
G	+	1	2	–
H	–	2	1	+
Imported	–	–	+	–

[a] From their respective lists of ingredients. All commercial products were prepared from a mixture of pork and beef.
[b] A + indicates the presence of that particular curing agent; where both were employed, the one listed first on the ingredient list is indicated by a 1 and the one listed second is indicated by a 2.
[c] The products of company F are manufactured at two plants, designated loc 1 and loc 2. Sandwich style designates a product produced in a wide (ca 45 mm finished size) casing; all other products were of the stick variety, finished diameter ca 25 mm.

Table 2—Compositional analyses and chemical measurements of commercial and pilot plant pepperoni

Company	Moisture (M), (%)	Ash (%)	Fat (%)	Protein (P), (%)	M/P Ratio	A_w	pH	% Acid as lactic
				Commercial pepperoni				
A	30.9	5.4	39.2	20.2	1.48	0.87	5.5	0.49
B	17.0	5.6	50.0	21.2	0.80	0.80	5.2	0.71
C	17.0	5.3	52.8	20.1	0.85	0.81	6.1	0.29
D	31.5	6.4	38.1	20.5	1.54	0.83	4.8	0.84
E	22.6	6.1	44.1	21.5	1.05	0.84	5.8	0.40
F(loc 1)[a]	30.8	5.5	41.7	21.1	1.46	0.87	4.9	0.56
F(loc 2)[a]	27.1	5.8	43.7	24.8	1.09	0.84	4.9	0.61
F(loc 2)[a] sandwich style	26.3	5.4	47.7	20.4	1.29	0.85	4.8	0.65
G	25.4	5.6	42.2	20.8	1.22	0.86	5.1	0.55
H	18.4	5.4	52.5	21.9	0.84	0.81	4.7	0.73
Imported	21.1	5.1	51.0	17.9	1.18	0.83	5.3	0.37
				Pilot plant pepperoni				
Expt. I (all pork)	24.1	6.3	37.5	28.3	0.85	0.81	4.7	not done
Expt. II (all pork)	20.8	6.7	40.6	28.6	0.73	0.80	4.8	1.14
Expt. III								
all beef	28.9	7.9	18.1	41.3	0.70	0.82	4.9	1.54
all pork	27.0	7.1	32.2	32.2	0.84	0.83	4.8	1.42
pork-beef	28.6	7.6	23.0	37.7	0.76	0.82	4.8	1.33

[a] The products of company F are manufactured at two plants, designated loc 1 and loc 2. Sandwich style designates a product produced in a wide (ca 45 mm finished size) casing; all other products were of the stick variety, finished diameter ca 25 mm.

Table 3—Number of viable microorganisms present in selected commercial pepperoni

Company	Viable counts per gram of pepperoni plated on[a]				
	APT	PD	Rog	EMB	MSA
A	1.3×10^8	3.3×10^3	3.7×10^7	1.0×10^5	1.2×10^7
B	1.0×10^4	$< 1 \times 10^2$	1.0×10^2	3.0×10^2	$< 1 \times 10^2$
C	2.5×10^7	9.6×10^3	1.4×10^5	3.0×10^5	1.1×10^7
D	2.3×10^7	1.0×10^5	4.0×10^4	$< 1 \times 10^2$	5.0×10^4
E	6.0×10^6	1.0×10^2	$< 1 \times 10^2$	3.5×10^2	1.5×10^5
F(loc 1)[b]	1.0×10^8	$< 1 \times 10^2$	8.5×10^7	$< 1 \times 10^2$	2.7×10^3
F(loc 2)[b]	3.3×10^6	1.0×10^2	1.6×10^6	$< 1 \times 10^2$	2.0×10^3
F(loc 2)[b] sandwich style	1.0×10^4	$< 1 \times 10^2$	2.7×10^3	3.9×10^3	1.1×10^4
G	7.0×10^6	1.1×10^5	6.9×10^5	3.0×10^4	1.3×10^6
H	1.2×10^7	1.6×10^4	6.0×10^6	1.8×10^3	7.0×10^3
Imported	2.5×10^6	$< 1 \times 10^2$	1.1×10^6	1.0×10^4	1.7×10^6

[a] APT, APT agar; PD, acidified potato dextrose agar; Rog, Rogosa SL agar; EMB, Eosin Methylene Blue agar; MSA, mannitol salt agar.
[b] The products of company F are manufactured at two plants, designated loc 1 and loc 2. Sandwich style designates a product produced in a wide (ca 45 mm finished size) casing; all other products were of the stick variety, finished diameter ca 25 mm.

practice of examining the gram and catalase reaction of all colony types is an effective and necessary method of studying the microflora of commercial sausages.

Process

After examination of commercial pepperoni, we sought to produce a fully-dry sausage with characteristics similar to those of the commercial products. Using the microbiology and technology for Lebanon bologna (Palumbo et al., 1973; Smith and Palumbo, 1973) and some published recipes (Kramlich et al., 1973; Komarik et al., 1974; National Provisioner, 1938), we devised a process consisting of periods of aging, fermentation, and drying. The microbiological and chemical changes were determined during the three periods. Although the data of single experiments are reported in Figures 1–5, all experiments were repeated at least twice and patterns were similar in all instances.

Table 4—Cellular types, and gram and catalase reactions of the microflora found in commercial pepperoni

Company	Microbial types[a] found on the following media				
	APT	PD	Rog	EMB	MSA
A	a	e	a	f	c
B	b,d	–	f	c	–
C	c	e	a	c	c
D	a	e	e	–	e
E	c,b	e	–	d	c
F(loc 1)[b]	f	–	f	–	f
F(loc 2)[b]	c	e	c	–	b
F(loc 2)[b] sandwich style	b	–	a	b	b,d
G	a,c	e	a	f	c
H	f	e	f	b	b,e
Imported	a	–	a	f	c

[a] a = lactobacilli (catalase-negative, gram-positive rods).
b = bacilli (catalase-positive, gram-positive sporeforming rods).
c = micrococci (catalase-positive, gram-positive cocci).
d = catalase-positive, gram-negative rods (not typical coliforms).
e = yeast.
f = catalase-negative, gram-positive cocci.
[b] The products of company F are manufactured at two plants, designated loc 1 and loc 2. Sandwich style designates a product produced in a wide (ca 45 mm finished size) casing; all other products were of the stick variety, finished diameter ca 25 mm.

Microflora during aging

We had examined the microflora of beef during aging (Smith and Palumbo, 1973), but we repeated that earlier study. Pork was included because of its high thiamine content (0.76 mg/100g pork vs 0.06 mg/100g beef; Rice, 1971) and thus might support more rapid development of lactobacilli than beef. Changes in the microflora of aging beef and pork are presented in Figure 1. The selective agars (PD, Rog and MSA) were extremely useful in defining the microbial types developing during aging of meats for our pilot plant pepperoni. This usefulness may be attributed to the fact that we have meats with low background flora and few contaminating bacteria. During aging, PD supported only yeasts, Rog only lactobacilli, and MSA only micrococci. However, gram-positive, catalase-positive rods (bacilli) were recovered on EMB which was used to detect the presence of coliforms. No coliforms were detected in any pepperoni or meat sample, and therefore, EMB counts have not been given. Unexpectedly, the Rog count increased faster in beef than in pork, while the MSA count was just the opposite. The basis for this is unknown, but it could be theorized that the growth of lactobacilli in pork was limited by some component other than thiamine and that specific nutrient content of beef favored lactobacilli and that of pork micrococci. The number of lactobacilli which developed naturally during aging of salted beef and pork were sufficient to carry out the fermentation and the reduction of nitrate to nitrite (Smith and Palumbo, 1973).

Fermentation

The changes in the microflora during fermentation of pilot plant pepperoni are given in Figure 2. Again the selective agars were extremely useful in defining the microbial sequence during the fermentation: the yeasts died off and were not detected after the first day of fermentation; only lactobacilli were found on Rog, and micrococci on MSA. In general, the counts for an all-pork and a pork-beef pepperoni were similar. Data were also similar for an all-beef pepperoni.

The most important change during the fermentation was the conversion of the sugars to lactic acid. During the 3-day fermentation of a pork-beef pepperoni, pH decreased from 6.3 to 4.7, while titratable acidity increased from 0.23 to 0.63%.

Data were similar for all-beef and all-pork pepperoni. Our pilot plant pepperoni contained as much acid after fermentation as the samples of commercial product did after drying (compare Fig. 3 with Table 2).

Drying

During drying for 6 wk (ca 40–42 days), the pepperoni lost ca 50% of their "green" (starting) weight and took on the characteristic appearance of this sausage type; their A_w values decreased to 0.82 (Fig. 4). The 50% weight loss classed these products as fully dry sausage (MacKenzie, 1966); M/P ratios <1.6/1.0 also define the pilot plant pepperoni and commercial products as fully dry. Fermented sausages may also be classed according to their final moisture content; a final moisture content of 35% or less designates all pepperoni examined as fully dry (Kramlich, 1971). The decreases in A_w and weight for a pork-beef pepperoni were similar to those for the all-pork and the all-beef pepperoni.

Figure 4 shows that the sausages lost moisture rapidly early in the drying period, but the A_w did not change much. This suggested that early in drying, large amounts of moisture must be lost to yield small changes in A_w. Townsend and Davis (1972) found that Genoa salami weight losses were greatest early in drying, and decreased as the 21-day drying period ended. Skjelkvole et al. (1974) observed that A_w in Norwegian salami changed little during the early part of the ripening (drying), and decreased substantially only toward the end. Wardlaw et al. (1973), who followed moisture content of summer sausage during 60 days' drying, found a pattern similar to that in Figure 4. Thus, the moisture changes in our pepperoni were similar to those reported for other sausage types during drying.

During drying of the pepperoni, some portions of the microbial population remained constant while others decreased (Fig. 5). For both the pork-beef and the all-pork pepperoni, the total (APT agar) and Rog counts remained constant during the 40-day drying period. On Rog and APT only lactobacilli were detected. The extended viability of lactobacilli observed in our pilot plant pepperoni is consistent with the high lactobacilli counts (APT and Rog) we found in samples of commercial pepperoni (Tables 3 and 4).

The count observed on MSA showed little change during drying. However, at 19 days (see arrow on Fig. 5), the organisms found on MSA changed from micrococci to bacilli (gram-positive, catalase-positive sporeforming rods) and no further micrococci could be detected during the rest of the drying period. Micrococci were detected in only 6 of the commercial products, generally in those with pH values above 5.0. This decline of micrococci to undetectable levels was also observed in Lebanon bologna (Smith and Palumbo, 1973). After the 4-day Lebanon bologna fermentation, no micrococci ($<1 \times 10^2$/g) were detected. Smith and Palumbo (1973) theorized that the acid content of Lebanon bologna was responsible for the decline of viable micrococci. Further support of their acid sensitivity is now offered. On the 19th day of drying, when the acid content of the pepperoni described in Figure 5 reached 1%, the micrococci had become undetectable. In another experiment (data not presented), all-beef pepperoni containing different levels of fat were fermented for only 2 days, and then dried. Because of the short fermentation time, less

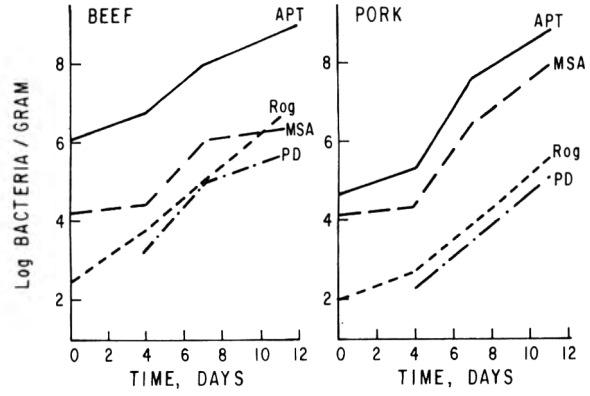

Fig. 1—Changes in microflora plated on various media during aging of salted (3% NaCl) beef and pork at 5°C (see Materials & Methods for media designations).

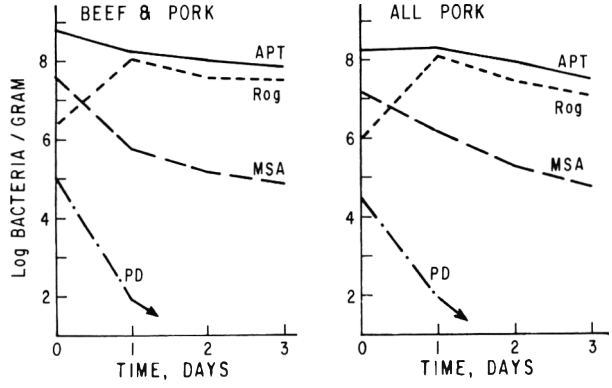

Fig. 2—Changes in microflora plated on various media during the fermentation of a pork-beef and an all-pork pepperoni at 35°C (see Materials & Methods for media designations).

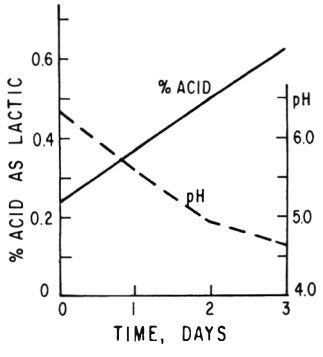

Fig. 3—Changes in pH and % acid (as lactic) during the fermentation of a pork-beef pepperoni at 35°C.

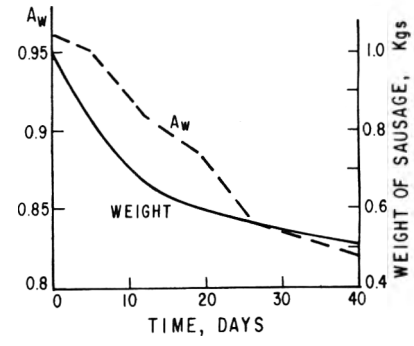

Fig. 4—Changes in moisture relationships of a pork-beef pepperoni during drying at 12°C and 65% relative humidity.

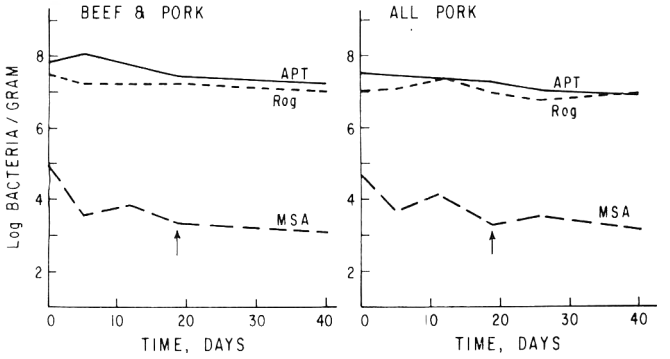

Fig. 5—Changes in microflora during drying of a pork-beef and all-pork pepperoni at 12°C and 65% relative humidity (see Materials & Methods for media designations).

acid was produced so that, during the drying period, the acid content of these all-beef pepperoni never reached the 1% level, and micrococci could be detected throughout the entire drying period. Furthermore, Lebanon bologna produced in our pilot plant had final acid contents substantially above 1% (Palumbo et al., 1973) and no micrococci were detected after the 4th day of fermentation (Smith and Palumbo, 1973). Among commercial Lebanon bolognas, those with higher acid contents generally did not have detectable levels ($<1.0 \times 10^2$/g) of micrococci (Palumbo et al., 1973; Smith and Palumbo, 1973).

Lactobacilli and micrococci have remained viable in other dry sausages as reported by Skjelkvole et al. (1974) during the ripening of Norwegian salami, and by De Ketelaere (1974) during the ripening of a dry sausage. However, the pH of their experimental sausages was low enough that the colonies they counted on S110 agar might have been bacilli. They apparently did not use Gram stains which would have clarified this point.

During drying, the pH of our pepperoni remained constant or changed no more than a tenth of a pH unit. However, since the sausages were dehydrated to 50% of their green weight, the final titratable acidity was double the initial.

To complete our study of pepperoni processing, we sought to determine the factor(s) responsible for development of a firm textured sausage during drying. During other studies, we observed that sausage which was not fermented did not yield firm textured products upon drying. However, many commercial products are barely fermented (pH > 5.0, Table 2) and yet have a firm texture. We theorized that possibly a mild heat treatment in the form of a trichina cook (internal temperature of 137–140°F) would permit development of a firm texture during drying of a nonfermented sausage; we designed an experiment to test this hypothesis using pork-beef (1:1) pepperoni. The variables included: nonfermented, pH 5.6; natural flora-fermented, pH 4.8 (aged meat); and starter culture-fermented, pH 4.6. There were both heated and nonheated

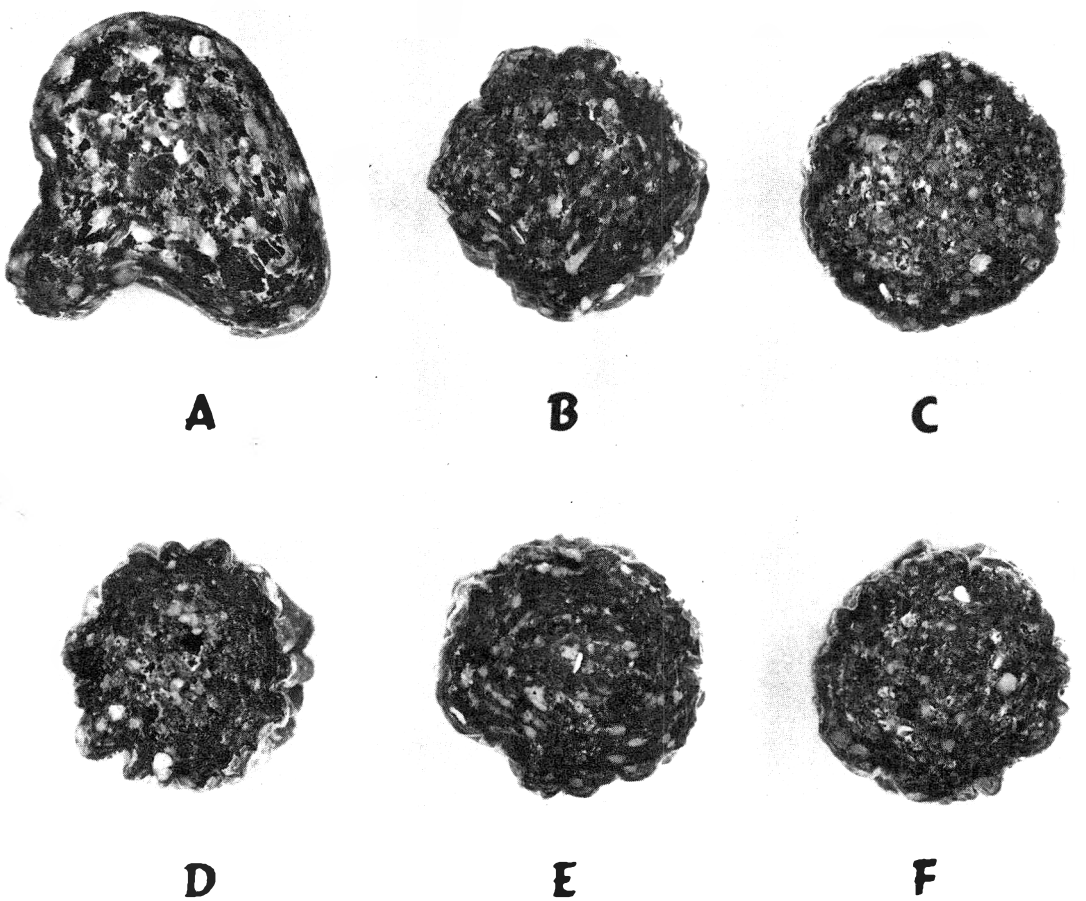

Fig. 6—Photograph of pepperoni slices approx. 1x. A, B, and C were nonheated and D, E, and F were heated to 140°F. A and D were nonfermented (pH 5.6); B and E were natural flora-fermented (pH 4.8); and C and F were starter culture-fermented (pH 4.6).

sausages for each fermentation. All heated sausages, even the nonfermented ones, had firm texture upon drying; with the nonheated sausages, only the fermented ones (natural flora or starter culture) had firm texture upon drying (Fig. 6). The nonfermented, nonheated sausages were misshapen and had hollow centers with poor, grainy texture (Fig. 6). All sausages lost moisture at ca the same rate and had similar final moisture contents. We can conclude from this last experiment that acid formation (fermentation) or heating will yield a firm textured product upon drying.

In a previous study (Palumbo et al., 1974), we investigated the potential for nitrosamine formation during processing of Lebanon bologna, a fermented sausage. Since pepperoni possesses many of the same product characteristics as Lebanon bologna, we investigated the potential for nitrosamine formation during processing of pepperoni. We examined natural flora fermented pork-beef pepperoni, prepared with and without paprika, and found no detectable levels of the 6 volatile nitrosamines for which we tested. One commercial sample examined was also negative for these same six nitrosamines.

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Ms received 6/11/75; revised 7/28/75; accepted 8/4/75.

Reference to a brand or firm name does not constitute endorsement by the U.S. Dept. of Agriculture over others of a similar nature not mentioned.

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QUALITY CHARACTERISTICS OF CONVENIENCE CHICKEN PRODUCTS AS RELATED TO PACKAGING AND STORAGE

ABSTRACT

Precooked and prefried chicken products were prepared and packaged by three methods using commercial polyester/polyethylene laminate pouches and oil resistant papers. The effects of frozen storage on weight losses, TBA value, peroxide value, acid value and organoleptic qualities of the prepared products were studied. Vacuum packaged samples had the lowest weight losses, TBA and peroxide values, and the best preference scores as compared to the heat sealed and the paper wrapped products. Acid values were higher for breast portions than those of the thigh parts. A sharp decline in acid values during storage was observed for the paper wrapped samples. Taste panels preferred the products which were fried just before serving, rather than the prefried ones.

INTRODUCTION

PRECOOKING and freezing of a food product for consumer convenience has become an accepted procedure in recent years. One of the main problems with precooked chicken parts has been the development of "off flavor" associated with rancidity during frozen storage. Hanson et al. (1959) reported the change of flavor in frozen fried chicken as being a "warmed over" flavor and eventually a rancid flavor. In an earlier report, Hanson (1954) stated that off-flavors developed more rapidly in frozen fried chicken than in uncooked frozen fryers. The flavor change of fried samples occurred in the meat, rather than in the skin; therefore, this change in flavor was not related to rancidity development in the cooking fat during frying. Tims and Watts (1958) found that the organoleptical quality of cooked poultry products deteriorated rapidly after a short period of frozen storage, and this loss of flavor paralleled an increase in 2-thiobarbituric acid (TBA) values.

In order to reduce the quality deterioration of food products, various packaging techniques have been employed by the food industry. Sacharow (1969) found that low storage temperature and vacuum packaging extended shelf life of fish 100% but did not stop lipid oxidation. Shank and Lundquist (1963) stated that the value of the vacuum package for cured meat is in its ability to maintain the flavor and the color of the product by preventing the oxidation of fats. In a study on the effect of vacuum and nonvacuum packaging on the quality of chicken frankfurters, Baker et al. (1972) found that the vacuum packaged product showed little change in flavor, juiciness and overall acceptability during storage, while the nonvacuum packaging resulted in faster deterioration in quality. In an evaluation of pretreatment effect and type of packaging materials on the quality of frozen fried chickens, Carlin et al. (1959) stored chicken parts at 0°F in evacuated Cryovac packages, polyethylene packages and aluminum foil and found no significant differences in flavor score and rancidity tests that could be attributed to the effect of packaging materials and conditions up to 6 months of storage. Fried chicken wrapped in foil showed practically no change in weight throughout the 15 wk of storage at 0°F and the weight changes were minimal during storage for chickens packaged in polyethylene or evacuated Cryovac bags. They further indicated that different packaging materials and conditions did not affect the flavor score of frozen fried chicken. Effect of packaging and frozen storage on organoleptic acceptability of microwave reheated chicken pieces has been studied by Sison et al. (1973). Precooked chicken pieces packaged individually in Saran-Mylar laminated pouches were equally acceptable as those bulk-packed in polyethylene bags. Little or no information was published concerning the effect of vacuum packaging on rancidity development and quality of precooked chicken products in commercial polyester/polyethylene laminate pouches.

EXPERIMENTAL

Sample preparation

Commercially dressed broilers were obtained directly from a poultry processing plant. Carcasses were cut up into eight parts by cutting each half into front and hind quarters and further dividing them into breast, wing, thigh and drumstick portions. Only the thigh and breast portions were used in this study.

All parts were precooked in hot water by suspending them in a water-filled steam-jacketed kettle at 87.8°C for 20 min after they were placed in net stockings (Culotta and Chen, 1973). The cooked breast and thigh portions were given one of the following treatments prior to packaging and freezing: (1) Battered and breaded with a commercial product (Golden Dipt. Co., Millstadt, Ill.); (2) battered and breaded with a commercial product and deep-fat fried at 176.6°C for 3 min in a General Electric Mark 313 Deep-Fat Fryer. After frying, samples were allowed to cool for 20 min before packaging. Precooked and prefried parts were separated into two main lots with the same number of thighs and breasts. Parts were further divided randomly into three additional groups. Within each lot, distribution of thigh and breast portions was made so that each sample in each group contained an equal number of breasts and thighs. Packaging variables were assigned randomly to the groups in each lot. Three packaging variables were used: (1) 3.0 mil polyester/polyethylene laminate pouches (All-Vak #13, International Kenfield Distributing Co., Broadview, Ill.) with 25-in. vacuum developed and heat sealed, using Kenfield Vacuum Packaging Sealer (International Kenfield Distributing Co.); (2) 3.0 mil polyester/polyethylene laminate pouches heat sealed, but no vacuum was developed; and (3) wrapped around with two layers of a commercial meat packaging oil resistant paper (2 mil thickness and 31 lb basis weight) and tied with rubber bands. All packaged samples were immediately weighed and frozen at −26°C. After 24 hr, samples were moved into a commercial type freezer with a temperature of −18°C.

Chemical analysis

TBA value, acid value, and peroxide value were determined monthly for a period of 6 months. Samples used for the chemical analysis were removed from the freezer and thawed at 4°C for 12 hr. The breasts and thighs of each sample were hand deboned and the meat was separated from the coating and skin in the prefried parts, while no separation was made for the precooked parts. The deboned portions were ground through a Sears Kenmore meat grinder plate with 2.4 mm diameter holes. TBA values were determined by TBA test as described by Tarladgis et al. (1960). Peroxide and acid values were determined as described by Jacobs (1959).

Organoleptic evaluation

Palatability of both white and dark meat of each cooking treatment and all packaging variables was determined by taste panel evaluation. The taste panels were conducted according to the methods described by

Stone and May (1969). In all tests, a hedonic scale ranging from "extremely tender" to "extremely tough" was used for judging tenderness. Possible scores ranged from 1 to 9 with lower scores indicating greater acceptability. Flavor and juiciness were rated on a similar basis. Samples were taken from the freezer and the packaging materials were removed. The prefried portions were heated for 30 min at 218°C in an oven. Precooked samples were deep-fat fried at 176.6°C for 6 min in a General Electric Mark 313 Deep-Fat Fryer. The heated samples were allowed to cool for 3 min and then coded. The coded breast and thigh samples were served to the taste panelists. A total of six experienced panelists evaluated the samples for flavor, juiciness and tenderness. Taste panel evaluation was made at 0, 3 and 6 months. The same panel was employed at each evaluation period.

Weight loss

Weight loss was determined monthly for 6 months. Samples used were removed from the freezer and the weight was measured immediately with wrap on. At each weighing period, two wrappers of each type were checked for the possible weight losses.

Statistical analysis

Statistical analysis of the data was made according to those procedures described by Steel and Torrie (1960) using the MSU UNIVAC Model 1106 computer. Comparison of the means when required were made using the Duncan New Multiple Range Test (1955).

RESULTS & DISCUSSION

Vacuum packaging and weight losses upon frozen storage

Frozen precooked and frozen prefried poultry products are susceptible to severe weight losses upon storage unless the proper packaging materials and conditions are applied. A drastic weight loss was observed in samples wrapped in paper after 6 months of frozen storage at −18°C (Table 1). Some portions lost as much as 62% of their original weight at the six months of storage. The percent weight losses in vacuum packaged samples were the lowest among all packaging variables. At the end of the six months of frozen storage, vacuum packaged samples lost 5.0% to 8.7% of the original weight, although the water vapor permeability of the polyester/polyethylene laminate used in this study was considered to be one of the lowest (0.03 g/100 in^2/24 hr 72°F–95% RH) as compared to other packaging films. Practically no weight loss was found from the packaging materials. The major changes in weight of the vacuum packaged parts occurred at the first month of frozen storage. It was also noticed that the percent weight loss for breast samples was consistently higher than that of thigh samples, regardless of the type of cooking treatment or packaging variables.

Analysis of variance for percent weight losses showed a highly significant interaction among packaging variables, storage time, meat type (breast or thigh) and the cooking treatments; therefore, no conclusions concerning the main effect could be considered.

Vacuum packaging and rancidity of products upon frozen storage

TBA values for both types of meat and all packaging variables increased during the first 3–4 months of frozen storage. The vacuum packaged samples maintained the lowest TBA values throughout the entire storage period (Fig. 1). These data suggested that frozen precooked and frozen prefried chicken parts were the least susceptible to rancid deterioration when they were vacuum packaged. These results do not agree with Shank and Lundquist (1963) and Baker et al. (1972) who reported that there is no significant difference in flavor score and rancidity tests that could be attributed to the effect of packaging materials and conditions up to 6 months of frozen storage. Samples wrapped in oil resistant paper had the highest TBA values upon frozen storage. In general, TBA values for heat sealed samples were lower than those of the paper wrapped but higher than those of the vacuum packaged. As expected, the TBA values were higher for dark meat than for white meat samples.

In determining the TBA values for the frozen fried chicken parts, skin and coating portions were evaluated separately in order to eliminate the interference of brown pigments, resulting from the frying process. The frozen precooked parts in all packaging variables reached the highest TBA values at the fourth month of storage (Fig. 1). In the frozen prefried parts, however, the maximum TBA values were observed at the third month. These data indicated that peak TBA values can be expected earlier for prefried parts than precooked parts. TBA values for skin and coating also reached a maximum value and then decreased before leveling off (Fig. 1). The decline of TBA values was observed by Tarladgis and Watts (1960) in cooked meat and fishery products. Chang et al. (1961) suggested that the formation of the carbonyl addition products could possibly count for the loss of malonaldehyde during frozen storage. Another explanation was offered by Buttkus (1967) who postulated that a reaction of myosin and malonaldehyde might take place during storage and cause this drop in TBA value.

Explanation for the increasing of TBA values after declining is unknown. In fact, the determination of malonaldehyde can be used to follow oxidative deterioration of food and food products, but the relationship between oxidative food deterioration and malonaldehyde production is not simple. Since foods are complex systems, the oxidation of fat in food is expected to be a complex process in which food constituents play an unclear role. Despite this fact, the quantitative determination of malonaldehyde offers at the present time the only available measure of the oxidation of tissue lipids without ordinary fat-solvent extraction. The quantitative production of malonaldehyde during oxidation of fat in food correlated with off-flavors, rancidity and flavor deterioration of food and food products (Klose et al., 1959, 1960).

Vacuum packaged samples had the lowest peroxide values (Fig. 2). In general, the highest peroxide values of vacuum packaged products were found at the first month and then remained essentially constant throughout the entire observation period. Peroxide values for samples wrapped in paper were consistently the highest as compared to the other packaging variables.

Peroxide values of dark meat were always higher than those of white meat. This agreed with the TBA values and can be

Table 1—The mean percentage weight losses of frozen chicken parts from three packaging variables[a]

Packaging variables	Vacuum packaged	Heat sealed	Oil resistant paper wrapped
Type of cooking treatment			
Prefried	6.4f	10.1d	36.5b
Precooked	7.6e	11.4c	50.8a
Type of meat			
Breast	7.2e	10.4c	47.4a
Thigh	6.9e	9.2d	40.5b
Storage time (Mo at −18° C)			
1	5.1h	7.5f	22.1i
2	6.0gh	9.4de	36.7j
3	7.1fg	10.5cd	44.7k
4	7.6f	11.6bc	49.9l
5	8.1df	12.6b	54.1a
6	8.3df	13.1b	55.8a

[a] For each grouping, means within a row or a column not followed by the same letter differ significantly from one another ($P < 0.01$). Each mean represents the average of eight determinations.

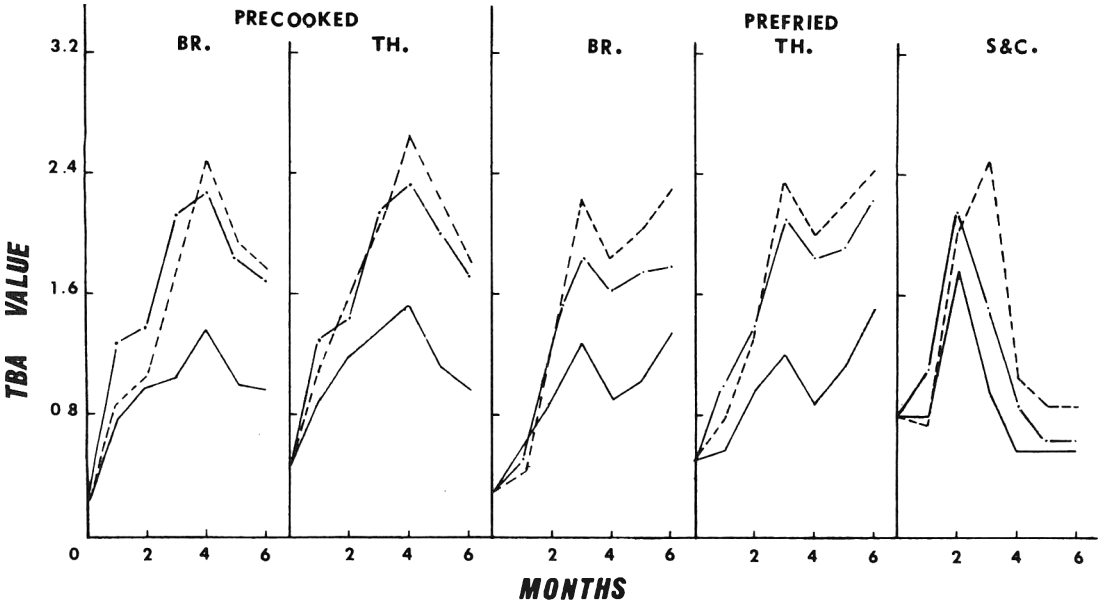

Fig. 1—The TBA values for precooked and prefried breast and thigh pieces of chicken as related to months of storage. ——, vacuum packaged; —·—, heat sealed; ·····, oil resistant paper wrapped; BR, breast part; TH, thigh part; and S&C, skin and coating.

explained by the fact that dark meat contains more lipid and hematin compounds than white meat. The peroxide value for skin and coating of the fried portions increased steadily for all samples up to the fourth month of storage at −18°C. The general trend of increasing peroxide values with storage time agreed with the increase in TBA values and flavor scores in the first three months of frozen storage. These results agreed with Hanson et al. (1959) that the peroxide development paralleled with flavor deterioration of frozen fried chicken.

A peroxide value of 1 me/kg was considered by Weiss (1970) to be the borderline in terms of rancidity. However, data obtained in this study showed that samples with peroxide values as high as 74.0 me/kg (Fig. 2) did not have detectable rancid odors. Peroxides are intermediates in the oxidation of fat, and they can vary considerably as the oxidation proceeds, but their use as a reliable index for rancidity measurement still has not been established.

In contrast to the results previously described for TBA and

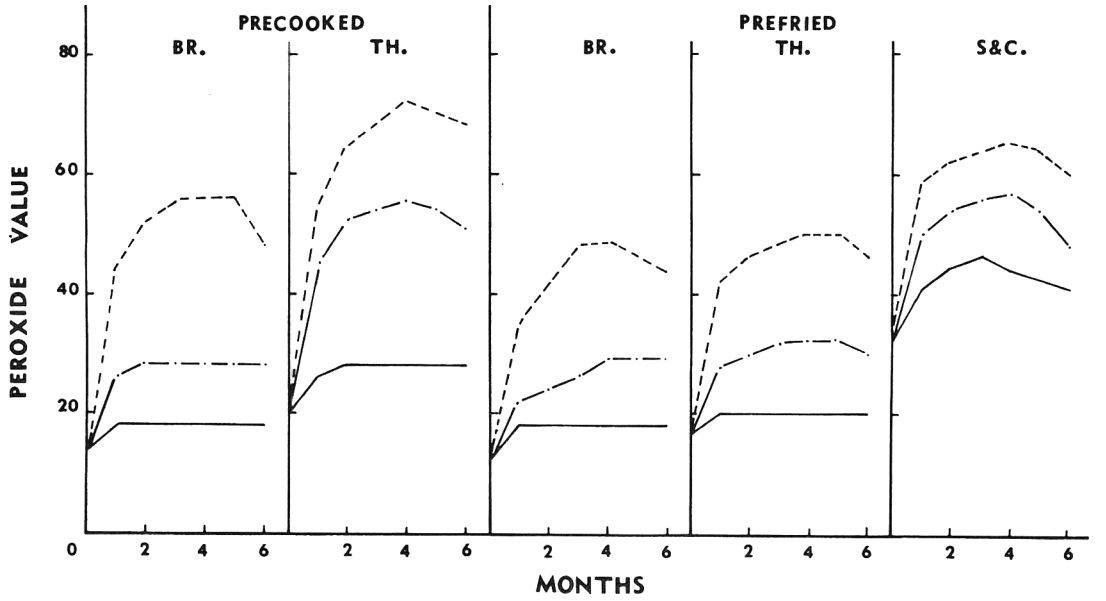

Fig. 2—The peroxide values for precooked and prefried breast and thigh pieces of chicken as related to months of storage. ——, vacuum packaged; —·—, heat sealed; ·····, oil resistant paper wrapped; BR, breast part; TH, thigh part; and S&C, skin and coating.

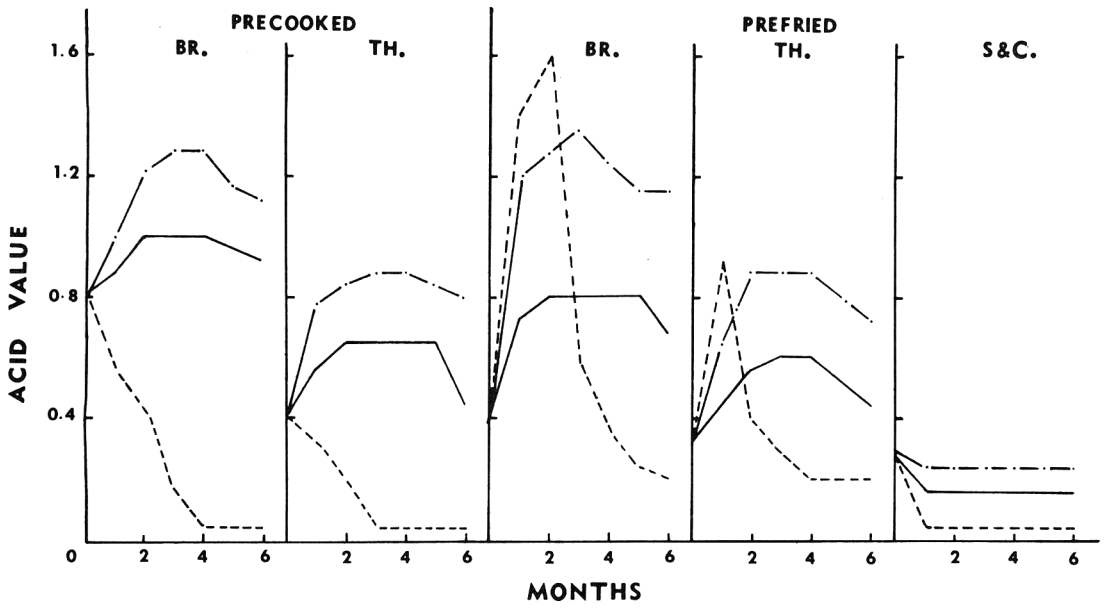

Fig. 3–The acid values for precooked and prefried breast and thigh pieces of chicken as related to months of storage. ——, vacuum packaged; —·—, heat sealed; ·····, oil resistant paper wrapped; BR, breast part; TH, thigh part; and S&C, skin and coating.

peroxide values, the acid values for samples wrapped in oil resistant paper had the lowest reading among all other packaging variables (Fig. 3). In the frozen prefried portions, however, these values were the highest only for the first 2 months, then dropped to the lowest level, as compared to other packaging variables. Acid values of the skin and coating portions dropped at the first month and remained essentially constant for the rest of the frozen storage period (Fig. 3). Vacuum packaged and heat sealed samples showed an increase in acid value for the first 3 months of frozen storage then remained constant before decreasing again. Acid values for heat sealed parts were always higher than those parts packaged under vacuum. Acid values for the breast meat were consistently higher as compared to those of the thigh meat (Fig. 3). Higher lactic acid content in the breast muscle might be the reason. The statistical analyses have shown a significant ($P < 0.01$) correlation between the percent weight losses and rancidity parameter readings (Table 2-B).

Table 2–Correlation between major tested quality factors of convenience chicken product stored at –18° C for 6 months

(A) Organoleptical factors

	Juiciness	Tenderness	Flavor	Overall satisfaction
Juiciness	1.000	0.939**	0.918**	0.974**
Tenderness		1.000	0.945**	0.984**
Flavor			1.000	0.976**
Overall satisfaction				1.000

(B) Chemical and physical factors

	TBA values	Peroxide values	Acid values	% weight loss
TBA values	1.000	0.685**	–0.185*	0.559**
Peroxide values		1.000	–0.377**	0.658**
Acid values			1.000	–0.656**
% Weight loss				1.000

* Significant at the 0.05 level of probability
** Significant at the 0.01 level of probability

Table 3–The mean[a] taste panel scores[b] of the prefried and precooked products of three packaging variables

Time of frozen storage (Mo at –18° C)		Packaging variables: Vacuum packaged	Heat sealed	Oil resistant paper wrapped
Flavor	0	2.002d	2.002d	2.002d
	3	2.252d	3.382bc	8.500a
	6	2.900c	3.941b	9.000a
Tenderness	0	1.652c	1.652c	1.652c
	3	1.849c	2.622b	9.000a
	6	2.483b	3.352d	9.000a
Juiciness	0	1.887d	1.887d	1.887d
	3	2.216cd	2.745bc	8.625a
	6	2.910b	3.756e	9.000a
Type of cooking				
Prefried		2.797b	3.555d	6.034a
Precooked		1.972c	2.660b	6.366a

[a] For each grouping, means within a row or a column not followed by the same letter differ significantly from one another ($P < 0.01$). Each mean represents the average of six observations.
[b] Possible scores ranged from 1–9 with lower scores indicating greater acceptability.

Vacuum packaging and the organoleptic quality of the products

Statistical analysis of the taste panel scores for juiciness, tenderness and overall satisfaction for frozen precooked and *frozen prefried* poultry meats packaged in vacuum, heat sealed and oil resistant paper showed that packaging X month was the only significant interaction in the analysis of variance. However, the analysis of variance for flavor showed significant ($P < 0.05$) interaction among tissues X packs, tissues X months, cook X packs, and packs X months. Duncan's Multiple Range Test for the mean flavor, tenderness and juiciness scores of the tested products is shown in Table 3. Vacuum packaged samples received the best scores among the three packaging variables throughout the entire storage period. After 3 months of frozen storage, samples packaged in oil resistant paper lost about 44.7% of moisture (Table 1). Products with such high percent weight losses were leathery in texture.

Dark meat received the best scores for flavor, juiciness, tenderness and overall satisfaction in comparison to white meat. Results are in agreement with Cash and Carlin (1968) and Culotta and Chen (1973). Flavor scores were associated with tenderness and juiciness in both white and dark meat regardless of the packaging variables or the cooking treatment. Panelists often commented that flavor was the most difficult factor to judge. Those findings are simialr to those of Baker and Darfler (1968). Higher scores indicated less preference as storage time increased. Weight loss of chicken parts wrapped in oil resistant paper was extremely high. The panelists gave those samples the lowest preference scores. In general, samples which were precooked and fried before serving received higher preference scores as compared to prefried samples (Table 3), and similar results were reported by Carlin et al. (1959), Hanson et al. (1959) and Hale (1969). Statistical analysis showed a highly significant ($P < 0.05$) correlation between juiciness, tenderness, flavor and overall satisfaction (Table 2-A).

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UTILIZATION OF LUTEIN AND LUTEIN-FATTY ACID ESTERS BY LAYING HENS

ABSTRACT

The utilization, by laying hens, of lutein-fatty acid esters from alfalfa, marigold petals, and synthetic lutein dipalmitate was compared with crystalline lutein. The lutein-fatty acid esters were better utilized by laying hens than crystalline lutein and the higher solubility of lutein-fatty acid esters in lipids compared to lutein may be responsible for their increased utilization. The egg yolk xanthophyll from different diets containing lutein-fatty acid esters was free lutein indicating that laying hens deacylated the lutein-fatty acid esters prior to deposition into developing ova. A simple procedure for the color measurement of egg yolks was also developed.

INTRODUCTION

THE COLOR OF EGG YOLK is the most important quality factor in eggs (Groote, 1970) and year round constancy of egg yolk color is an important criterion in quality control (Vuilleumier, 1968). Smith and Perdue (1966) compared the xanthophylls in the diet with those in the egg yolk and found the distribution of xanthophylls in the yolk and diet quite similar.

Marigold petals are the most concentrated xanthophyll sources (Scott et al., 1968), and marigold meals are suitable pigment sources for poultry (Alam et al., 1968 a, b). The xanthophylls of marigold petals occur acylated with fatty acids (Alam et al., 1968a, b; Philip and Berry, 1975).

The predominant xanthophyll in alfalfa and marigold petals is lutein. Recently Guenthner et al. (1973) found that crystalline lutein is less efficiently utilized by laying hens than xanthophylls from alfalfa and marigold and this difference in utilization is attributed to unknown factors in alfalfa and marigold petals.

This investigation was initiated to determine whether lutein acylation increases the utilization of lutein by laying hens.

MATERIALS & METHODS

Poultry experiments

Shaver-Babcock Cross laying hens were used in this study. Individually caged hens (five birds per treatment in one replication) were fed a standard laying diet containing milo with or without added xanthophylls. The diet used in this experiment consisted of 69.00% ground milo, 14.26% soybean meal, 5.00% meat and bone scraps, 1.00% hydrolyzed animal and vegetable fat (HEF), 7.00% calcium carbonate, 1.00% dicalcium phosphate, 0.50% salt, 0.20% trace mineral mix, 2.00% vitamin mix, and 0.05% DL-methionine. The standard laying diet (20 lb) was mixed separately for 15 min in a Hobart mixer with known amounts of xanthophylls as follows: alfalfa, 5 mg; marigold petals, 5 and 10 mg; crystalline lutein, 5 and 10 mg; purified lutein esters, 5 and 10 mg; and lutein dipalmitate, 3 and 6 mg per lb of feed. The feeds after mixing were analyzed for xanthophyll content, and adjustments, if any, were made by adding xanthophylls or feed to obtain desired xanthophyll levels.

The feeding experiments were conducted for a period of 14 days. The eggs were collected daily, and eggs from the 7th to the 14th day were used for analyses. Five days' eggs were analyzed objectively, two days' eggs were analyzed subjectively and one day's eggs were analyzed chemically. The average egg production was 80%.

Color measurements

A cover glass (Corning No. 1, 0.13–0.16 mm thick) was taped to the viewing area of the Hunter D25 Color Difference Meter and the instrument calibrated against a white standard (L = 95.3, a = 0.4, b = +1.8). The eggs were broken one by one and the contents poured into a petri dish (100 × 15 mm). The dish was placed over a jack, and the yolk was positioned underneath the viewing area. The jack was then raised slowly until the rim of the petri disk just pressed against the instrument. The color measurement was recorded as L, a, b values. The "Total Color Difference" was calculated based on the equation $(\Delta L^2 + \Delta a^2 + \Delta b^2)^{1/2}$

The subjective color measurements were done using a Roche Color Fan. The eggs were broken, and the contents poured onto a glass plate lined underneath with a cardboard of neutral color (grey). The egg yolk colors were compared with the Roche Color Fan under diffuse day light. The visual color difference was expressed as the arithmetic difference between two samples.

Preparation of lutein derivatives

Dried marigold petals (1 kg) were packed in a column (1200 × 6.5 cm) and extracted with petroleum ether (30–60°C, 3 liters) at room temperature. The extract was evaporated to dryness under vacuum at 40°C (yield 65g). The crude extract (40g) was dissolved in boiling isopropanol (1 liter) and the solution cooled to 15°C. The precipitated lutein-fatty esters were filtered through a sintered funnel and the filtrate concentrated to one-fourth its volume. Lutein-fatty acid esters were again precipitated by cooling to 15°C and filtered. The combined precipitate was dried in a vacuum oven at 30°C (yield 13g). The dried precipitate contained 51.3% lutein-fatty acid esters (a mixture of dipalmitate and dimyristate in the ratio of 5:3) (Philip and Berry, 1975).

Crystalline lutein was prepared by saponifying (Quackenbush, 1973) crude lutein-fatty acid esters (10g). The lutein was crystallized from methanol (yield 2g).

Lutein dipalmitate was prepared by acylating crystalline lutein (1g) with palmitoyl chloride (Philip and Berry, 1975). The crude lutein palmitate (1g) was dissolved in petroleum ether and partitioned with an equal volume of 95% aqueous methanol in a separatory funnel. The methanol layer was drained, and the petroleum ether layer was evaporated to dryness (yield 0.8g). This step removed unreacted lutein and partially acylated lutein monopalmitate.

Carotenoid analysis

The feed (10.0g) was blended with acetone (100 ml) in a Waring Blendor for 1 min and filtered through sintered glass funnel. The residue was washed twice with acetone (100 ml each). The combined filtrate was evaporated to dryness under vacuum at 40°C. The residue was saponified (Quackenbush, 1973) and quantitated, based on the absorbance at the wavelength of maximum absorption in carbon disulfide (474 nm).

The egg yolks were separated from the whites and analyzed in the same way feed was analyzed. The unsaponified egg yolk pigments were used for TLC studies to determine the nature of modification of dietary lutein.

Crystalline lutein, lutein-fatty acid esters, and lutein dipalmitate were assayed by dissolving a weighed amount in carbon disulfide and measuring the absorbance at the wavelength of maximum absorption (474 nm).

The xanthophylls were calculated as lutein based on an $E^{1\%}_{1cm}$ value of 2160 at 474 nm in carbon disulfide (Davis, 1965). The spectra were also recorded in hexane for identification.

Instrumental methods

Visible absorption spectra. Perkin-Elmer 137 UV-Visible recording spectophotometer.

Table 1—Physico-chemical properties of synthetic zeaxanthin, lutein, lutein esters, lutein dipalmitate and saponified carotenoids of milo, *alfalfa* and marigold petals

	Visible absorption maxima, nm		R_f values of major xanthophylls		
	Hexane	Carbon disulfide	Solvent 1	Solvent 2	Major xanthophylls
Milo	428 449 476	–	–	0.29	Zeaxanthin
Alfalfa	429 448 473	453 478 506	–	0.30	Lutein
				0.29	Zeaxanthin
Marigold	421 442 471	448 472 504	–	0.30	Lutein
Lutein esters	421 443 473	449 474 505	0.45		Lutein dipalmitate and
	421 444 474	449 474 504	0.45	–	Lutein dimyristate
Lutein dipalmitate	421 444 474	449 474 504	0.45		
Synthetic zeaxanthin	430 451 479	–	–	0.28	–

[a] Solvent 1 = acetone + petroleum ether (5 + 95)
Solvent 2 = acetone + benzene + petroleum ether (20 + 10 + 70)

Color measurements. Hunter D25 Color and Color Difference Meter with spot lenses and 1/2 in. viewing area.

Thin-layer chromatography

Adsorbant: Silica gel G, 0.250 mm thick and activated at 110°C for 30 min. Solvent 1 for esters: acetone + petroleum ether (5 + 95); Solvent 2 for xanthophylls: acetone + benzene + petroleum ether (20 + 10 + 70).

RESULTS

THE PHYSICO-CHEMICAL properties of lutein, lutein derivatives and xanthophylls of milo (control), alfalfa and marigold diets are given in Table 1. The standard milo diet contained 1 mg/lb xanthophylls which is mostly zeaxanthin. Alfalfa xanthophylls are mostly lutein and zeaxanthin, and the major xanthophyll in marigold petal is lutein. The xanthophyll concentrations of different diets determined by analysis are given in Table 2. There are only slight differences between calculated values of xanthophylls prior to mixing and assayed values after mixing indicating that mixing was adequate to distribute the xanthophylls uniformly.

The color measurement data for egg yolks from different dietary treatments are given in Table 2, and the effect of various lutein derivatives on egg yolk color are tabulated in Table 3. Diets containing lutein-fatty acid esters, lutein dipalmitate, alfalfa and marigold petals gave positive color difference values for egg yolk compared to crystalline lutein (a positive color difference means that hue changes in the direction from green to red when a and b values are plotted in the Hunter α,β chromaticity diagram). The alfalfa and marigold diets gave lower color difference values compared to lutein-fatty acid ester and palmitate diets, presumably due to the presence of other carotenoids that lower the total xanthophyll concentration. The visual color difference between egg yolks from crystalline lutein diets and diets containing lutein derivatives showed the same positive trend except for the alfalfa diet.

The unsaponified egg yolk xanthophylls from different diets containing lutein derivatives were hypophasic when partitioned between petroleum ether and 95% aqueous methanol. On TLC using solvent 2, the egg yolk xanthophylls had low R_f-values typical of free xanthophylls. The R_f-value (0.30 in solvent 2) and visible spectrum λ_{max} in hexane 421, 442 and 471 nm) of unsaponified egg yolk xanthophylls from diets containing lutein derivatives were identical to those of crystalline lutein. These evidences indicated that egg yolks contain free lutein and that laying hens deacylate lutein-fatty acid esters prior to deposition into developing ova.

DISCUSSION

THE XANTHOPHYLLS of alfalfa and lutein are acylated with fatty acids and lutein-fatty acid esters are better utilized by laying hens than crystalline lutein. Solubility of xanthophylls in lipids is a prerequisite for their absorption, and subsequent deposition into the developing ova and acylation of lutein increases its solubility in lipids, therby enhancing its utilization

Table 2—Hunter L, a, b values of egg yolks

Treatment	Pigment conc mg/lb feed	L		a		b		Visual scores (Roche color fan)
		Mean	Std dev	Mean	Std dev	Mean	Std dev	
Milo (control)	1.0	52.8	1.55	−5.2	0.36	23.1	0.89	2.0
Alfalfa	5.7	49.2	1.18	−1.7	0.68	29.4	0.70	6.0
Lutein	4.7	48.9	2.40	−2.2	0.78	28.9	1.70	5.5
Lutein	9.8	48.4	1.63	−0.4	0.40	29.5	1.37	7.2
Lutein esters	5.0	47.0	1.60	−1.0	1.02	28.3	1.38	7.0
Lutein esters	10.4	48.5	1.70	+1.4	1.20	30.2	1.21	8.2
Lutein dipalmitate	3.3	50.9	1.47	−3.5	0.62	28.9	1.53	6.0
Lutein dipalmitate	6.0	49.6	1.99	−2.1	0.90	29.3	1.40	7.7
Marigold petals	5.0	49.8	1.61	−2.1	0.84	29.5	0.99	7.0
Marigold petals	10.0	47.7	1.95	+1.0	0.63	29.0	1.27	9.2

Table 3–The effect of various lutein compounds as compared to crystalline lutein on egg yolk color

Lutein level	Treatment	Total color difference $(\Delta L^2 + \Delta a^2 + \Delta b^2)^{1/2}$	Visual difference Roche color fan units
5 mg/lb	Alfalfa	2.4	−0.5
	Lutein esters	2.3	+1.5
	Lutein dipalmitate	2.4	+2.2
	Marigold	1.1	+1.5
10 mg/lb feed	Lutein esters	1.9	+1.0
	Marigold	1.7	+2.0

by laying hens. The laying hens deacylate the lutein derivatives prior to deposition into the developing ova. This observation is reported for the first time.

A simple method of color measurement of egg yolks is reported. The reproducibility of this procedure depends on strict adherence to sample presentation methods described. The smaller viewing area (1/2 in.) of the Hunter D25 Color Difference meter is suited for color measurement of egg yolks. The taping of the viewing area produces a flat and uniform colored surface. The flattened surface of the egg yolks is about 11–15 times the area of viewing surface. The sample presentation in a petri dish (100 × 15 mm) permits application of uniform pressure to the egg yolk. The L values are affected significantly by this variation in pillowing. A reproducible and reliable method of color measurement of egg yolks is under development in this laboratory and will be the subject of another publication (Philip et al., 1975).

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EXAMINATION OF SWISS CHEESE FOR INCIDENCE OF MYCOTOXIN PRODUCING MOLDS

ABSTRACT

Samples of 11 brands of Swiss cheese were analyzed by plate count for molds capable of growing at 5 and 21°C. Portions of each sample were stored at 5°C and observed for visible mold development throughout a 6-wk storage period. Representative molds were isolated from counted plates and moldy samples, and identified to genus. Isolates were grown in yeast extract sucrose broth and rice powder corn steep agar cultures at 12°C for 2 wk, and then extracted with chloroform. The extracts were tested for toxicity to 7-day-old chicken embryos, and analyzed for the presence of known mycotoxins using TLC. Cheese samples that developed visible mold growth during storage were extracted with acetonitrile. The extracts were examined for the presence of known mycotoxins using TLC. Mold counts ranged <10 to 1580 colonies/g of surface cheese at 5°C and from <10 to 5700 colonies/g of surface cheese at 21°C. All cheese samples stored at 5°C developed visible mold growth within 6 wk. Of the 183 molds isolated, 87% were *Penicillium* species; 93% of the isolates that grew at 5°C were penicillia. Toxicological screening of the mold isolates showed extracts of 34% of all isolates, and 35% of *Penicillium* isolates were toxic to chicken embryos. Chemical analyses of the culture extracts detected known mycotoxins in 5.5%. Toxins detected were penicillic acid, patulin and aflatoxins. Analyses of moldy cheese stored at 5°C for 6 wk for known mycotoxins showed penicillic acid in 4 of 33 samples. The isolated penicillic acid was comparable to standard penicillic acid by TLC in three different solvent systems using three different derivatization procedures. UV and IR absorption spectral data supported the TLC data.

INTRODUCTION

CONSUMPTION of natural cheeses has steadily increased in the U.S. in recent years. Mold growth on these products is a common occurrence during aging and storage at low temperatures (Foster et al., 1957; Frazier, 1967). Since certain molds are capable of producing toxic and carcinogenic metabolites, proliferation of these organisms on foods must be regarded as a potential health hazard.

Recently it was reported that 82.2% of the molds found on refrigerated Cheddar cheese belonged to the genus *Penicillium*, 6.6% were *Aspergillus* species and 1.1% were *Fusarium* species (Bullerman and Olivigni, 1974). A number of species of these genera are capable of mycotoxin production (Bullerman and Olivigni, 1974; van Walbeek, 1973). Toxicological screening of isolates from Cheddar cheese indicated that chloroform extracts of yeast extract-sucrose broth cultures of 19.8% of the isolates were toxic to chicken embryos, causing 50% mortality or more (Bullerman and Olivigni, 1974). Thin-layer chromatographic (TLC) examination of the extracts of the mold cultures showed the presence of known mycotoxins in 7.2% of the culture extracts. The mycotoxins detected were patulin, penicillic acid, ochratoxin A and aflatoxins (Bullerman and Olivigni, 1974). Presence of toxins in the cheese was not demonstrated.

While the majority of the molds isolated from Cheddar cheese were not toxic, further studies on the mold profiles of additional products are warranted to define further the extent of the potential hazard that may be associated with mold growth on cheeses. Additional information about the numbers, kinds and toxin producing potential of molds found on cheeses is needed to assess fully the dangers to human health associated with the presence of these organisms.

MATERIALS & METHODS

Cheese samples

Cheese samples were obtained from all major retail outlets in Lincoln, Nebr. Eleven brands of Swiss cheese, not visibly moldy, were randomly selected for plate count examination. This represented all major brands being offered for sale in local markets. The survey was done in three replicates over a sampling period of 6 months. In addition, samples of Swiss cheese showing visible mold while in display cases were also selected for direct isolation of molds.

Microbiological analyses

Cheese samples without visible mold were removed from the package and prepared for plating in a bacteriological glove box, the inside of which had been previously sanitized with 2.5% solution of sodium hypochlorite and then exposed to germicidal UV light for 1 hr before use. Each sample was divided equally: one-half was rewrapped in a common household film wrap (Saran, polyvinylidene chloride, 1/2 mil, Dow Chemical Co., Midland, Mich.) to simulate partial usage and then stored at 5°C for 6 wk. These samples were observed weekly for mold growth. Material for plate counts was obtained from the remaining half of each sample by trimming 2–3 mm slices from all of the outer surfaces of the sample, or in the case of slices, using the outer slices. This material was grated in a sterile blender and used for plate counts according to the standard method of Reinbold et al. (1972), except that potato dextrose agar (Difco) plus 30 ppm of tetracycline was used in place of acidified potato dextrose agar. Duplicate plates were incubated at 21°C for counting at 3 and 5 days, and at 5°C for counting at 6 wk. Mold counts were reported as number of mold colony forming units per gram of surface cheese.

Representative colonies of each type of mold present were isolated from the counted plates and stored on potato dextrose agar at 5°C. Mold cultures were also isolated from the moldy cheese samples and stored in the same manner. All mold isolates were classified to genus using the keys of Gilman (1957), Raper and Fennell (1965) and Raper and Thom (1968).

Screening of mold isolates for toxin production

All of the mold isolates were prepared for screening for toxin production by growing them in 100 ml of yeast-extract sucrose (YES) broth at 12°C for 14 days (Davis et al., 1966). Each culture was blended, filtered through Whatman No. 4 filter paper to remove particulate matter and the filtrate was extracted with two 100 ml volumes of chloroform.

The cultures were also grown on rice powder corn steep agar (RPCS) and extracted with chloroform as described by Bullerman (1974). The extracts were concentrated on a steam bath and analyzed for the presence of known mycotoxins using TLC plates (20 × 20 cm, 0.25 mm thick Silica Gel G-HR, Brinkmann Instruments, Inc.). Standard mycotoxins used included aflatoxins, ochratoxin A, sterigmatocystin, penicillic acid, patulin, citrinin, luteoskyrin and zearalenone. Aflatoxin standard was obtained from the USDA Southern Utilization R&D Div. (New Orleans, La.); sterigmatocystin from Calbiochem (La Jolla, Calif.); patulin was produced in our laboratory and the remaining toxins were obtained from research workers in the mycotoxin field. The TLC plates were developed in toluene/ethyl acetate/90% formic acid (60/30/10) and treated according to the method of Scott et al. (1970).

The extracts were also injected into the air cell of 7-day-old chicken

embryos according to the method of Verrett et al. (1964). A total of 20 μl of each extract, in chloroform was injected into each of 10 eggs, and the developing embryos were observed for toxic effects during incubation. Control eggs, injected with 20 μl chloroform, were also included. Mortality to the developing embryos in excess of control background mortality was regarded as toxicity.

Examination of cheese for known mycotoxins

All of the cheese which was stored at 5°C for 6 wk and which developed visible mold growth was extracted and analyzed for known mycotoxins. The outer 2–3 mm of these samples, including the mold were trimmed. The trimmings were extracted according to the method of Pohland and Allen (1970), except that 50 ml of acetonitrile plus 3% formic acid, added to improve recovery of penicillic acid, was used. The samples were mixed with the solvent for 15 min. The samples were defatted, as described in the method, concentrated on a flash evaporator, transferred to a small vial and stored under nitrogen at –20°C. Recoveries of various known mycotoxins added to Swiss cheese ranged from 60–100%. The cheese extracts were examined by TLC using the same method as described for culture extracts. When known mycotoxins were found, verification of toxin identity was done using TLC with internal and external standards in several solvents, and ultraviolet (Beckman Model 25) and infrared (in chloroform with reference cell; Beckman Model IR-5A) absorption spectra.

RESULTS & DISCUSSION

Mycological evaluation

Mold counts of the nonvisibly molded cheese were somewhat variable (Table 1). The counts ranged from <10 to 1580 colonies/g of surface cheese at 5°C incubation and <10 to 5700 colonies/g of surface cheese at 21°C. In general, counts were slightly higher at 21° than at 5°C incubation, but the differences were slight except in one case where the difference was over 4000. Most of the molds detected in this study were capable of growing at 5°C in 6 wk (Table 1). The amount of visible mold that developed varied between replicates of a given brand but the average amount of mold growth found ranged from slight (1 or 2 colonies) to extensive (covering 1 or 2 sides) coverage of the samples.

The majority of the molds isolated were classified as *Penicillium* species. A total of 87% of all of the isolates were *Penicillium*, and 13% were other genera, which included one isolate of *Aspergillus flavus* (Table 2). The penicillia were the predominant genus found growing at 5°C, and constituted 93% of the flora. At 21°C, *Penicillium* species accounted for 79% of the isolates. These findings support those of our earlier study with Cheddar cheese which indicated the penicillia are the molds of major concern in refrigerated cheeses.

Toxicological evaluation of mold isolates

Injection of culture extracts into embryos showed that 34% of all isolates were toxic to the embryos, including 35% of the penicillia and 1.3% of the other genera. The single *Aspergillus flavus* isolate produced aflatoxins B_1 and B_2 and was very toxic.

In addition to aflatoxins, the only other known mycotoxins identified in culture extracts were penicillic acid and patulin. The number of isolates that produced known mycotoxins was 10/183 or 5.5%. This is compared to 25/349 or 7.2% of the isolates from Cheddar cheese reported in our earlier study. Again this indicates that the incidence in Cheddar and Swiss cheeses of molds capable of producing known mycotoxins is very low, and that these organisms constitute a minor part of the total mold flora.

The isolates that produced known mycotoxins also caused high embryo mortality (Table 3). This is in agreement with studies of isolates from Cheddar cheese. However, of the isolates that produced unidentified toxic factors, 28% were toxic to embryos and caused embryo mortality of 60% or greater. This is considerably higher than found with isolates from Cheddar cheese, where isolates producing 60% mortality or more comprised only 10% of the total mold flora. In this study 66% of the isolates from Swiss cheese were nontoxic to chicken embryos compared to 80% of the isolates from Cheddar cheese. The chicken embryo is susceptible to many compounds which may not have any effect on older animals or man. Thus it is difficult to interpret data in which unidentified compounds have caused embryo mortality in terms of hazards

Table 1–Total viable mold counts/g of surface cheese in 11 brands of Swiss cheese at 5° and 21°C and the development of visible mold during storage for 6 wk at 5°C

Sample No.	Viable mold counts 5°C	Viable mold counts 21°C	Visible mold[a]
1	<10	<10	+
2	1200	280	++
3	220	400	++
4	1580	5700	++
5	34	830	++
6	<10	25	++
7	26	<10	++
8	10	38	++
9	13	<10	+++
10	10	14	++
11	530	550	++

[a] – No growth; + slight (1 or 2 colonies); ++ moderate (more than 2 colonies but not covering one side); +++ extensive (covering one or two sides); ++–+ Very extensive (almost complete coverage).

Table 2–Source and type of mold isolates from Swiss cheese classified by genus and temperature at which isolated

Genus	Total isolated	Total isolated by temp 5°C	Total isolated by temp 21°C
All Genera	183	102	81
Penicillium	159	95	64
Aspergillus	1	–	1
Other genera	23	7	16

Table 3–Known mycotoxins and chick embryo toxicity found in culture extracts of molds isolated from Swiss cheese and grown on yeast extract sucrose (YES) broth or rice powder corn steep agar (RPCS)

Toxin	Number of isolates + by TLC YES	Number of isolates + by TLC RCPS	Toxicity to chicken embryos (% mortality)
Aflatoxins	1	1	100%
Ochratoxin A	ND[a]	ND	–
Sterigmatocystin	ND	ND	–
Patulin	4	ND	90–100%
Penicillic Acid	5	3	40–60%
Citrinin	ND	ND	–
Luteoskyrin	ND	ND	–
Zearalenone	ND	ND	–
Undetermined	3		100%
Undetermined	7		90%
Undetermined	17		80%
Undetermined	13		70%
Undetermined	12		60%

[a] ND = None detected.

to humans. Since the nature of the compounds are not known, the effect on humans cannot be predicted. The presence of known mycotoxins in culture extracts of molds isolated from cheese can be evaluated with somewhat more confidence. In this study the number of known mycotoxin producing organisms was very low.

Examination of cheese for toxins

When the cheese that had developed visible mold growth after 6 wk at 5°C was extracted and examined for known mycotoxins, penicillic acid appeared to be present in 4 of 33 of the samples. It was first observed by TLC examination of cheese extracts, and further study showed that the compound matched both internal and external standards of penicillic acid when chromatographed by TLC and derivatized with ammonia, phenylhydrazine and p-anisaldehyde. This behavior pattern was the same in three different developing solvents, toluene/ethyl acetate/10% formic acid (60/30/10); chloroform/ethyl acetate/10% formic acid (60/30/10) and benzene/methanol/acetic acid (90/5/5). To characterize the compound further, the extract was purified by preparative TLC and the separated band of penicillic acid was collected. The compound was eluted from the silica gel with chloroform, and the UV and IR absorption spectra determined. The UV spectrum showed a peak at 241 nm which was the same as that for standard penicillic acid. The IR spectrum had an absorption band in the carbonyl region (5.7μ) that matched a similar band in standard penicillic acid; however two subsequent bands at 6.1 and 6.6μ were absent. The IR absorption spectrum for the compound was not completely typical of penicillic acid, but this could be due to the molecular configuration of the compound, since penicillic acid can exist as a five-membered lactone ring, or as a straight-chain structure. The last band at 6.7μ is characteristic of the penicillic acid molecule when it is existing as the lactone ring. Since the penicillic acid obtained from Swiss cheese had been extracted with acetonitrile + 3% formic acid, it is possible that the low pH caused the compound to be in the straight-chain form which would account for the absence of the band at 6.7μ. To prove this, crystallized standard penicillic acid was dissolved in acetonitrile + 3% formic acid, after standing for 2 hr the solvent was removed under a stream of nitrogen and the residue was redissolved in chloroform. This solution was subjected to preparative TLC in the same manner as the cheese extract. The IR spectrum of the purified substance was determined and found to be lacking the absorption band at 6.7μ. Thus, the differences in the two spectra were most likely due to the molecular configuration of the compound, although this does not explain the absence of the second band at 6.1μ. However, from the combination of spectral and TLC data, it appears likely that the compound observed in the extracts of Swiss cheese was penicillic acid. After chemical testing, an insufficient amount of the extracts remained for biological testing, so biological toxicity data are not available. The quantity of penicillic acid in the cheese trimming was estimated by visual comparison to quantitative penicillic acid standards on TLC plates to be 0.5 μg penicillic acid/g cheese.

This study suggests that under certain conditions of storage, penicillic acid may be produced on Swiss cheese. At present, the stability of this compound in cheese and the conditions affecting its production are not known. Further studies in this area are underway.

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EFFECT OF ICE STORAGE ON MICROBIOLOGICAL AND CHEMICAL CHANGES IN SHRIMP AND MELTING ICE IN A MODEL SYSTEM

ABSTRACT

In ice stored shrimp, *Vibrio*, *Pseudomonas* and/or *Moraxella-Acinetobacter* species were initially dominant. After 12–15 days, *Vibrio* sp. had disappeared and *Pseudomonas* sp. predominated followed by *Moraxella-Acinetobacter* sp. The mean increase in aerobic plate count over this period was 2.2–2.4 log. In the melting ice (drip) *Moraxella-Acinetobacter*, *Pseudomonas*, *Flavobacterium*, *Micrococcus*, *Vibrio*, *Corynebacterium*, *Staphylococcus*, *Alcaligenes* and yeasts were present initially. After 9–13 days, *Vibrio*, *Flavobacterium* and *Micrococcus* sp. could not be detected in the drip, whereas *Pseudomonas* or *Moraxella-Acinetobacter* sp. remained present or in some cases increased. The mean increase in bacterial count of the drip was 3.4 log. Considering the loss in TVN in the drip, there was a net increase in TVN ranging from 6.7–60.1 mg N/100g. AA-N levels in shrimp decreased during storage. The TVN-AA-N ratio of drip did not increase significantly until bacterial levels reached 10^7 per ml drip.

INTRODUCTION

MICROBIAL ACTIVITY is one of the main causes of quality deterioration of shrimp. Information on the changes in level and type of microbial population and concomitant chemical changes during refrigerated storage may contribute to a better understanding of the processes responsible for quality losses and subsequent spoilage. Several reports are available on microbial population changes in iced shrimp. Campbell and Williams (1952) reported that species of *Micrococcus*, *Achromobacter*, *Pseudomonas* and *Flavobacterium* were predominant in fresh Gulf shrimp. During ice storage for 16 days, *Micrococcus* and *Flavobacterium* species decreased and *Achromobacter* increased steadily. Carroll et al. (1968) reported a similar pattern of population changes in white, brown and pink shrimp. According to Vanderzant et al. (1970), coryneforms, *Pseudomonas*, *Moraxella* and *Micrococcus* species predominated in Gulf shrimp. Refrigerated storage usually caused an increase in *Pseudomonas* species.

Endogenous enzymic activity also causes quality deterioration in shrimp (Cobb and Vanderzant, 1971; Cobb et al., 1974). Total volatile nitrogen (TVN) is produced at a low but constant rate in shrimp until bacterial levels exceed 10^6 per g. TVN levels then increase rapidly at a rate which is dependent upon the species of bacteria growing on the shrimp. At the same time, TVN along with free amino acids (AA-N) is leached from the shrimp. Melanosis or blackening of shrimp is another deleterious effect of endogenous enzymic activity (Bailey et al., 1960). These reports, however, do not present information on the microbial and chemical changes in deep columns of ice and shrimp as practiced on shrimp boats. Under these conditions there is considerable washing of shrimp by the melting ice. The present study reports on microbial and chemical changes in shrimp stored mixed with commercial ice in glass columns (121.9 cm deep) for 12–15 days to mimic the effect of melting ice on shrimp. Microbial population and chemical changes of the "drip" (melted ice collected at the bottom of the column) are also presented.

EXPERIMENTAL

Shrimp

Fresh shrimp (*Penaeus setiferus* and *P. aztecus*) were obtained directly from fishing boats, packed in ice and shipped to the laboratory. Experiments were started 9 hr or less after the shrimp had been caught. Samples were designated A through F in order of receipt. Collection of all samples, except sample E, was supervised by trained personnel.

Glass column

A 17.8 × 121.9 cm insulated glass column, supported by an angle iron framework, was packed with shrimp tails and commercial ice (1 part shrimp to approximately 2 parts ice) and stored in a cold room (1–3°C). The bottom of the glass column was fitted with a rubber gasket and inserted into a 18.7 cm Buchner funnel equipped with a loose plate for easy cleaning. Before each experiment the Buchner funnel and column were carefully washed with a hot detergent solution and sterilized by rinsing with 95% ethanol, then allowed to air-dry. A layer of ice (about 10 cm thick) was added to prevent contact of shrimp with the Buchner funnel, then the shrimp and ice were added. The column was topped off by an additional 10 cm layer of ice and covered with sterile aluminum foil. Shrimp were kept about 2 cm from the column walls by mechanical manipulation with a sterile brass rod. Sterile distilled water was allowed to percolate through the center of the column at approximately 50 ml/hr (calculated to simulate total melt of ice in 18 days). As ice melted near the glass walls it was replaced from the top of the column. Drip was collected at the bottom of the Buchner funnel and measured. Columns were allowed to run until spoilage odors became evident (12–15 days). At the end of the experiment, shrimp tails were removed from the top, bottom and intermediate areas of the column for analyses. Six different batches of shrimp (6 columns) were evaluated.

Organoleptic evaluation

Shrimp were judged on the basis of odor as acceptable or unacceptable by a 5-member trained panel. Shrimp receiving both acceptable and unacceptable ratings were classified as borderline.

Microbiological procedures

For Aerobic Plate Counts (APC), SWYE medium (Kaneko and Colwell, 1973) and Trypticase Soy Agar (TSA, BBL) were used. SWYE medium consisted of 0.3% yeast extract, 1% proteose peptone, 2.4% NaCl, 0.7% $MgSO_4 \cdot 7H_2O$, 0.07% KCl, 0.53% $MgCl_2 \cdot 6H_2O$, 2.0% agar, distilled water 1 liter, pH 7.2–7.4. Appropriate dilutions of sample in 0.1 ml quantities were placed on the surface of prepoured agar plates which had been dried overnight at 35°C. The sample dilutions were spread evenly over the agar surface with a sterile bent glass rod. Duplicate plates were incubated at 25°C for 3 days. To determine microbial types, approximately 30–40 colonies were picked at random from countable plates. Diagnostic procedures and schemes for identification were presented in a previous report (Vanderzant and Nickelson, 1969).

Shrimp samples were prepared for plating by blending for 2 min 50g of shrimp and 450 ml sterile salts solution in a Waring Blendor. The salts solution consisted of 2.4% NaCl, 0.7% $MgSO_4 \cdot 7H_2O$, 0.07% KCl, 0.53% $MgCl_2 \cdot 6H_2O$, pH 7.2, in distilled water. The sterile salts solution was also used as diluent to prepare serial dilutions of samples. Plating media and salts solution were heat sterilized at 121°C for 15 min.

Chemical analyses

Shrimp tails and drip were analyzed for total volatile nitrogen

(TVN) and free amino nitrogen (AA-N) by the method of Cobb et al. (1973). At least 20 shrimp tails were used for each analysis. The tendency to form black pigment (melanin) in the drip was determined by exposing 200 ml drip in a 250-ml Erlenmyer to bright light (two 3400 Kelvin photoflood bulbs at 40 cm distance) until color development ceased (approximately 40 min) and reading the absorbancy in a Coleman 295 Spectrophotometer at 425 nm. The degree of melanosis in shrimp was expressed by estimating the amount of blackened area (%) of each of 100 shrimp. Bisulfite was measured by the free sulfurous acid procedure (AOAC, 1970).

RESULTS

Bacteriological analyses of ice

Aerobic plate counts of ice ranged from < 1 to 7,800 per ml (Table 1). Counts of 4 of the 6 samples were slightly higher on TSA agar (mean count on TSA 1,400/g, on SWYE 760/g). The counts of the other 2 ice samples on TSA and SWYE were identical. *Micrococcus* species were isolated from 4 of 5 samples; *Moraxella-Acinetobacter* and *Flavobacterium* species from 3 samples; *Pseudomonas, Staphylococcus, Lactobacillus, Corynebacterium* and *Microbacterium* species from 2 samples; and *Enterobacteriaceae, Achromobacter* and *Arthrobacter* species from 1 sample.

Organoleptic evaluation of shrimp

Samples A, B, C, E and F were judged as unacceptable when the experiment was terminated. Sample D received both acceptable and unacceptable ratings. For this reason sample D was chosen to illustrate changes in microbial flora and chemical content of drip during the storage period.

Bacteriological analyses of shrimp

Initial counts of shrimp on SWYE agar ranged from 3.8×10^4 to 3.2×10^6 and on TSA from 6.9×10^4 to 3.7×10^6 per g. Counts of identical samples on the two media were similar, the mean count on SWYE and TSA was 8.1×10^5 per g. Storage on ice for 12–15 days increased counts on SWYE from 0.5–4.6 logs, on TSA from 0.5–4.3 logs. The smallest increases were in the samples with highest initial counts stored for 12 days, the largest increases in the samples with the lowest initial counts stored for 15 days. The mean increase in count was 2.4 logs on SWYE and 2.2 logs on TSA. The changes in levels and types of microorganisms of sample D (*P. setiferus*) are presented in Table 1.

With few exceptions, the major bacterial types (comprising 10% or more of total isolates) from SWYE and TSA plates of identical samples were similar. The initial microbial flora of the 6 shrimp samples consisted primarily of *Vibrio, Pseudomonas* and *Moraxella-Acinetobacter* species. In 4 of the 6 samples *Vibrio* species predominated, *Pseudomonas* and *Moraxella-Acinetobacter* species were dominant in 1 sample each Other genera present (<10% of isolates) in some samples included *Alcaligenes, Achromobacter, Flavobacterium, Staphylococcus, Corynebacterium, Microbacterium* and *Bacillus.* Following 12–15 days of refrigerated storage, *Vibrio* species had disappeared completely and *Pseudomonas* species predominated followed by *Moraxella-Acinetobacter* species. Other bacterial types (<10% of isolates) present in some ice-stored shrimp samples were *Flavobacterium, Staphylococcus, Aeromonas, Lactobacillus* and *Corynebacterium.*

Bacteriological analyses of drip

Initial bacterial counts of the drip ranged from 1.2×10^2 to 6.4×10^4 per ml. After 9–13 days of storage, counts of the drip ranged from 6.2×10^5 to 2.6×10^8 per ml. In most samples the increase in bacterial count of the drip over a 11–13 day period was 3–4 logs (mean increase in count was 3.4 logs).

Changes in microbial types of the drip during refrigerated storage are presented in Table 2. Only those types that comprised at least 10% of the initial microbial population of the drip are presented. Initially *Moraxella-Acinetobacter, Pseudo-*

Table 1—Changes in bacterial counts and types in shrimp and melting ice (drip) during iced-storage period

Sample[a] D	Stored (days)	APC/g	Percentage distribution[b]										
			Ps	Vi	Flav	M-A	Micr	Staph	Lact	Cor	Micrb	Bac	Y
Shrimp	0	9.9×10^4 [c]		100									
		6.9×10^4 [d]	31.8	67.2		0.5		0.5					
Ice	0	<1											
		3 X 10					66.7	33.3					
Drip	3	1.4×10^2	7.1		7.1	21.4	28.7	35.7					
		2.7×10^2	22.2	3.7	3.7	11.1	14.8			14.8			29.7
Drip	6	1.1×10^2				54.5	18.2		9.2	18.1			
		1.9×10^2	5.3		10.5	15.8	10.5			10.5	15.8		31.6
Drip	8	1.8×10^2	88.9			11.1							
		3.7×10^2	51.3		2.5	10.3			5.2	10.2	7.7	5.1	7.7
Drip	10	2.5×10^3	87.8		8.4	3.4					0.4		
		4.2×10^3	78.6						11.9	9.5			
Drip	13	6.2×10^5	100										
		9.7×10^5	95.8						2.1	2.1			
Shrimp (top)	14	8.1×10^5	100										
		1.1×10^6	92.5							7.5			
Shrimp (bottom)	14	3.4×10^6	100										
		4.9×10^6	98.0						2.0				

[a] Sample D was borderline acceptability, other shrimp samples were unacceptable when the experiments were terminated.
[b] Ps = Pseudomonas, Vi = Vibrio, Flav = Flavobacterium, M-A = Moraxella-Acinetobacter, Micr = Micrococcus, Staph = Staphylococcus, Lact = Lactobacillus, Cor = Corynebacterium, Micrb = Microbacterium, Bac = Bacillus, Y = yeast.
[c] Aerobic Plate Count on SWYE
[d] Aerobic Plate Count on TSA (0.5% NaCl)

Table 2–Changes in microbial types of the melted ice collected at the beginning and end of the refrigerated storage of 6 samples of shrimp

Drip of shrimp sample	Microbial types[a]								
	Ps (5)[b]	Alc (1)	Vi (2)	Flav (4)	M-A (6)	Micr (3)	Staph (1)	Cor (2)	Y (1)
A			0[c]	0	>	0			
B	=			0	<				
C	=	0	0	0	>	0			
D	>				0	0	0	<	0
E	>				=			=	
F	>				=				

[a] Ps = Pseudomonas, Alc = Alcaligenes, Vi = Vibro, Flav = Flavobacterium, M-A = Moraxella-Acinetobacter, Micr = Micrococcus, Staph = Staphylococcus, Cor = Corynebacterium, Y = yeast

[b] Pseudomonas sp. initially present in drip of 5 of 6 shrimp samples, constituting at least 10% of isolates

[c] During the refrigerated storage period (11–13 days) the following changes occurred in microbial types: no marked changes (=); a decrease (<); an increase (>); failed to detect (0).

Table 3–Grouping of 214 Moraxella-Acinetobacter isolates of shrimp, drip and ice in phenons 3 and 4 of nonmotile coccoid rods of Thornley (1968)

Sample	Percentage of strains belonging to phenon[a]				
	3	4-I	4-II	4-III	Mor. lwoffi 37 str.
Fresh shrimp	33.3	28.6	23.8	9.5	4.8
Stored shrimp	41.7	37.5			20.8
Ice		77.8			22.2
Drip	33.2	50.6	<1	<1	15.0

[a] Some of the major characteristics to separate phenons 3, 4-I, 4-II, 4-III, and Mor. lwoffi-37 str. and their reactions are: Oxidative acid production from glucose, galactose, arabinose, xylose, and lactose (+----); oxidase (++--+); NO_3 reduction (++--+); Simmon's citrate (----+); gelatin liquefaction (-----); Penicillin (2.5I.U./ml) sensitivity (+++-+); growth at 0°C (+++++); growth at 37°C (-----); H_2S from cysteine (+-+++).

monas, Flavobacterium, Micrococcus, Vibrio, Corynebacterium, Staphylococcus, Alcaligenes and yeast were present in the drip samples. At the end of the ice storage period, *Alcaligenes, Vibrio, Flavobacterium, Micrococcus, Staphylococcus* species and yeast could not be detected in the drip samples. *Pseudomonas* and *Moraxella-Acinetobacter* usually maintained their presence or in some samples increased in concentration during refrigerated storage. When all microbial types (including those present at <10% of total isolates) present in the drip initially and after 9–13 days refrigerated storage are compared, the disappearance of *Vibro, Flavobacterium* and *Micrococcus* species stands out.

Gram-negative coccoid rods. An examination of the microbial flora of shrimp and drip often showed the presence of aerobic nonmotile, gram-negative cocci, coccoid or short rods frequently irregular in form. In this group both oxidase-positive and oxidase-negative species were present. In Table 1 these cultures are included under *Moraxella-Acinetobacter* species. The characteristics of 214 of these isolates were compared with the properties of phenons 2, 3 and 4 (*Acinetobacter* sp.) described in a study on nonmotile cocci, coccoid or short rods by Thornley (1968). The data in Table 3 show that the isolates from shrimp and drip belonged primarily to phenons 3, 4-I and 4-*Mor. lwoffi* (37 str.). Strains belonging to phenons 4-II and 4-III initially present on shrimp disappeared after ice storage for 12–15 days. A comparison of the strains in phenons 2, 3 and 4 with the description of *Moraxella* and *Acinetobacter* in the 8th ed. of Bergey's Manual (Buchanan and Gibbons, 1974) indicates that they possibly could have been classified as follows: phenon 3 *Moraxella*-like taxa, phenon 4-I *Moraxella*, phenons 4-II and 4-III *Acinetobacter* and phenon 4-*Mor. lwoffi* (37 str.)-*Moraxella*.

Chemical analyses of shrimp

TVN and AA-N values for shrimp are listed in Table 4. Only four samples are listed because in samples A and B AA-N values were uncertain due to black pigment formation in the drip. In sample C initial TVN values were low and AA-N values high, giving a low TVN-AA-N ratio. Initial TVN content of shrimp samples D, E and F was high, ranging from 26.1–38.2

Table 4–Total volatile nitrogen (TVN) and free amino nitrogen (AA-N) contents and balance of shrimp and drip, free amino acid loss rate constant (k) and TVN-AA-N ratio of shrimp

Sample	Days on ice	Shrimp Avg wt (g)	Avg drip ml/day	TVN (mg N/100g)			
				Shrimp			Balance
				Initial	Final	Loss in drip	(Final + loss in drip) – Initial
C	12	2.87	1343	13.5	18.5	12.4	17.4
D	14	5.80	1294	26.1	14.6	18.2	6.7
E	15	15.90	869	38.2	59.0	35.1	55.9
F	14	11.46	549	30.3	73.6	16.7	60.1

Sample	AA-N				k[a]	Shrimp TVN-AA-N Ratio mg N/mmol	
	Shrimp Initial mmol/100g	Shrimp Final mmol/100g	Loss in drip mmol/100g	Balance (Final + loss in drip) – Initial mmol/100g	1 / liter $(g)^{1/3}$	Initial	Final
C	26.0	9.2	10.3	−6.5	0.236(0.198)[b]	0.52	2.01
D	28.6	10.2	15.4	−3.0	0.192	0.91	1.43
E	17.0	12.5	8.3	3.8	0.0611	2.24	4.72
F	29.4	17.0	9.0	−3.4	0.192	1.02	4.35

[a] $k = \frac{\text{Log } \frac{n_0}{n}}{V(w)^{1/3}}$, where n_0 is initial AA-N concentration, n is final AA-N concentration, V is amount of drip expressed in liters (l), $(w)^{1/3}$ is cube root of shrimp tail weight.

[b] When calculated on the basis of square root of weight (see Discussion).

mg N/100g. AA-N values were also high in samples D and F, resulting in low ratios for TVN-AA-N. In sample E the AA-N level was relatively low, giving a high initial TVN-AA-N ratio of 2.24 mg N/mmol which is indicative of spoilage (Cobb and Vanderzant, 1975). Sample E had a low initial APC (6.8 X 10^4/g) and contained 0.233% SO_2 (from sodium bisulfite). Other samples did not contain measurable SO_2.

During the period of ice storage, TVN levels increased in samples C, E and F but decreased in sample D. However, considering the TVN loss in the drip there was a net increase in total TVN (TVN-balance) ranging from 6.7—60.1 mg N/100g. AA-N levels in the shrimp decreased in all of the experiments. Total AA-N levels (AA-N balance) decreased in experiments C, D and F, and increased by 3.8 mmol/100g in sample E. Small differences both in TVN and in AA-N existed in shrimp taken from different parts of the column but no particular trend was apparent from the top to the bottom of the column.

The AA-N data for samples D and F fit the equation

$$kV(w)^{1/3} = \log\frac{n_o}{n}$$

where V is the volumn of drip in liters, $(w)^{1/3}$ is the cube root of shrimp tail weight, n_o is the initial concentration of AA-N and n is the final concentration of AA-N. The AA-N data for sample C fit the equation

$$kV(w)^{1/2} = \log\frac{n_o}{n}$$

where $(w)^{1/2}$ is the square root of the weight. The data for sample E did not fit the equations, probably because of proteolysis of the shrimp protein or bisulfite treatment.

Chemical analyses of drip

The amount of TVN and AA-N appearing in the drip from sample D (expressed per 100g of shrimp) during the period of the experiment is presented in Figure 1. The amount of TVN and AA-N in the drip was largely a function of the amount of drip, with decreases in TVN and AA-N as storage progressed. The TVN-AA-N ratio of the drip did not increase significantly until bacterial levels reached 10^7/ml of drip (Fig. 2). The increase in the TVN-AA-N ratio was caused by increased TVN levels of the drip. As was true with shrimp, the TVN-AA-N ratio of the drip from sample E was initially high and remained high throughout the experiment.

Melanosis was evident in the shrimp in only two experiments. In sample A, 7—15% (approximately 13% average) of the shrimp tail surface area was blackened, in sample B, 10—35% (approximately 24% average). Some of these shrimp placed in the refrigerator without ice storage had almost 100% of their surface area blackened. Melanin precursor was removed rapidly in the drip, with approximately 60% of the total amount lost in the first 3 days of the experiment (Fig. 3).

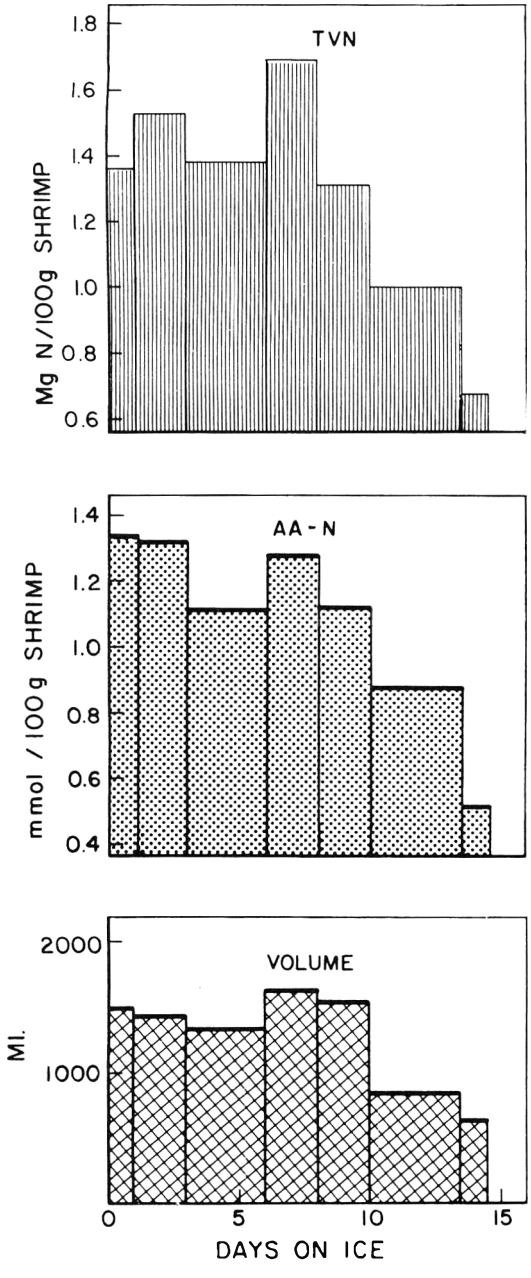

Fig. 1—Total volatile nitrogen and AA-N content and volume of shrimp drip during 14 days of ice storage.

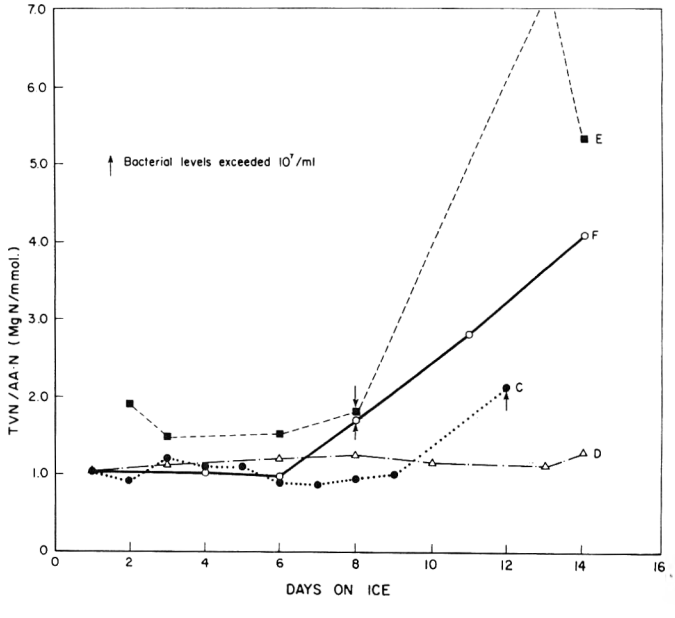

Fig. 2—TVN-AA-N ratio of the drip during 14 days of ice storage.

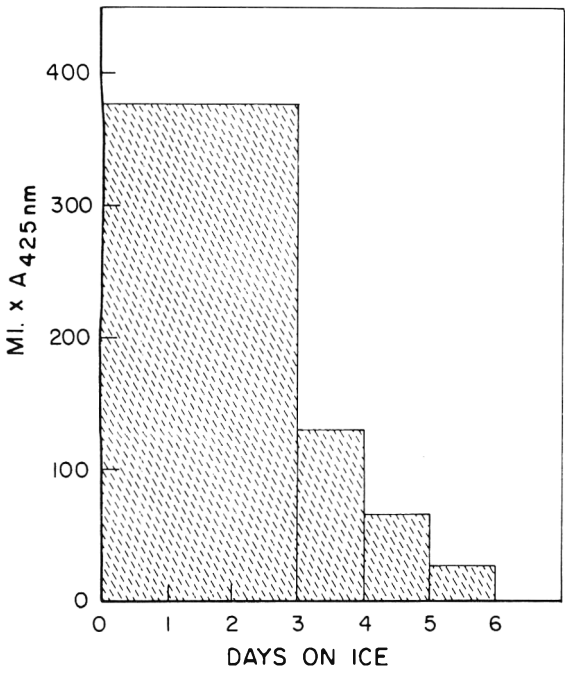

Fig. 3–Melanin precursor in shrimp drip.

Melanosis in the shrimp and development of color in the drip were negligible in the other experiments.

DISCUSSION

IN THIS STUDY *Vibrio, Pseudomonas* and *Moraxella-Acinetobacter* species predominated in fresh Gulf shrimp. In other reports, *Micrococcus, Achromobacter, Pseudomonas* and *Flavobacterium* (Campbell and Williams, 1952); coryneforms, *Pseudomonas, Moraxella* and *Micrococcus* (Vanderzant et al., 1970); or *Acinetobacter-Moraxella, Flavobacterium* and *Pseudomonas* species (Harrison and Lee, 1968) were dominant in fresh shrimp. Some of these differences in microbial flora of shrimp can probably be attributed to differences in shrimp species, marine environment, handling practices on board, and time and temperature during ice storage. The lower percentage of *Achromobacter* and increased significance of *Moraxella* and *Acinetobacter* species in recent reports reflects a change in taxonomic status (Davidson et al., 1973; Harrison and Lee, 1968; Lee and Harrison, 1968; Thornley, 1968; Vanderzant and Nickelson, 1969; Vanderzant et al., 1970). Some of the gram-negative, biochemically inert coccoid rods formerly assigned to *Achromobacter* are now classified as *Moraxella* or *Acinetobacter* species. In addition to the above mentioned factors, differences in microbial flora reported for fresh shrimp may involve differences in composition of plating media and conditions of plate incubation. In the present study in which TSA (with 0.5% NaCl) and SWYE with a salts solution (NaCl, $MgSO_4$, KCl, $MgCl_2$) were used as plating media, *Vibrio* species were dominant in fresh Gulf shrimp. In an earlier study (Vanderzant et al., 1970) in which Standard Methods Agar (without NaCl) was used coryneforms were dominant in addition to *Pseudomonas, Moraxella* and *Micrococcus.* In this respect, some species of the natural microbial flora of shellfish exhibit a partial or complete salt dependence as demonstrated by the growth stimulating effect of NaCl added to standard media (Colwell and Liston, 1960). Furthermore, one seldom knows the relative contribution of marine and terrestrial species to the total microflora of a seafood sample. Hence, differences in recovery conditions (media, time and temperature of plate incubation) may be responsible for differences in level and microbial types reported for seafood samples.

The increased significance of *Pseudomonas* and *Moraxella-Acinetobacter* species in ice-stored shrimp is in agreement with previous reports (Campbell and Williams, 1952; Carroll et al., 1968; Vanderzant et al., 1970). The disappearance of *Vibrio* species during storage on ice may be associated with a reduction of the salinity of the shrimp because of the washing effect of the melting ice. The disappearance of *Micrococcus* and *Flavobacterium* species in ice-stored shrimp observed by Campbell and Williams (1952) may be attributed to this phenomenon (Carroll et al., 1968). The increases in total viable population of the shrimp samples during iced-storage are similar to those reported previously (Campbell and Williams, 1952; Carroll et al., 1968; Vanderzant et al., 1970).

The steady increase in total viable population of the drip during refrigerated storage of the shrimp samples reflects the removal by the melting ice of microorganisms developing on the shrimp in the column. The varied microflora of the drip during the first few days of storage probably represents removal of bacteria already present on the shrimp. The dominance of *Pseudomonas* and *Moraxella-Acinetobacter* species in the drip at the end of the storage period is not surprising since these species grew extensively on the shrimp during refrigerated storage. This is shown by their dominance in the microbial flora of stored shrimp. The loss of *Vibrio, Flavobacterium* and *Micrococcus* species in the drip collected toward and at the end of the storage period probably reflects their inability to develop on shrimp after washing of the surface by water of the melting ice. Carroll et al. (1968) attributed the reduction of *Micrococcus* and *Flavobacterium* species in iced shrimp to a reduction of the salinity of the shrimp. On the other hand, it is possible that changes in nutritional properties of the surface of the shrimp or interactive microbial activities are involved in changes of the microbial flora of shrimp and/or melting ice. In addition, some organisms (*V. parahaemolyticus* for example) are very sensitive to refrigerated storage.

Nonmotile, gram-negative coccoid rods now classified as *Moraxella* or *Acinetobacter* species are frequently isolated from refrigerated meat, fish and poultry (Davidson et al., 1973; Harrison and Lee, 1968; Lee and Harrison, 1968; Thornley, 1968). The 214 isolates in this study were similar to organisms included in phenons 3, 4-I and 4-*Mor. lwoffi* (37 str.) of Thornley (1968). A group of 110 nonmotile, gram-negative coccoid aerobic rods isolated from meats (Davidson et al., 1973) belonged primarily to phenons 4-I, 4-II and *Mor. lwoffi* (37 str.). In view of the recent recommendations in Bergey's Manual (Buchanan and Gibbons, 1974) those gram-negative coccoid rods from fresh shrimp include both *Moraxella* and *Acinetobacter* species. In iced shrimp and in the melting ice *Moraxella* species dominated.

This study also illustrates both the beneficial and deleterious aspects of ice storage on the chemical composition of shrimp. High TVN levels in shrimp due to endogenous enzymic or bacterial activities (Cobb and Vanderzant, 1974) were lessened by the loss in the drip. The physical removal of melanin pigment diminished the level of melanosis in the shrimp. However, the loss of amino nitrogen represented a serious loss of flavor agents as amino acids are related to the fresh sweet flavor of shrimp (Hashimoto, 1965; Nair and Bose, 1965).

With small shrimp tails (<7.5g) in ice, the amino acid glycine and presumably AA-N leached from shrimp at a logarithmic rate which was a function of the time on ice and the square root of the weight of the shrimp tail (Cobb et al., 1974). The amount of leaching (drip) which the shrimp would be exposed to is a function of time. Thus the volume of drip substituted into the original equations developed by Cobb et al. (1974) should give a constant value if glycine and AA-N

leaching were similar. However, the present data and unpublished data on the amino acid content of brown and white shrimp indicate that when the shrimp tail is about 5g or greater, the rate of leaching becomes a function of the cube root of tail weight. Since variation in the rate of AA-N loss from shrimp is opposite to that expected if simple diffusion or surface area were the determining factors, the reason for this phenomenon has not been established.

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IMPROVED ACCEPTANCE AND SHELF LIFE OF FROZEN MINCED FISH WITH SHRIMP

ABSTRACT

Earlier studies indicated that the incorporation of shrimp (*Pandalus jordani*) in a fish-shrimp portion increased the shelf life of machine-separated fish muscle. This investigation was initiated to determine if shrimp could reduce the undesirable changes known to occur during frozen storage in one particular species of rockfish (*Sebastes melanops*). After mixing different ratios of shrimp with minced fish muscle, the portions were frozen at −34°C for 2 hr, packed loosely in polyethylene bags and held at −18°C until analyzed. The all-fish portion was found to be unacceptable after 11 wk of frozen storage. Increasing the amount of shrimp in the portion markedly improved the acceptability and shelf-life stability of the minced fish. This was directly related to the decreased formation of malonaldehyde and peroxides. The beneficial effects of incorporating shrimp with minced fish was attributed to substance(s) extractable in ethanol that exhibited antioxidant properties.

INTRODUCTION

A RECENT STUDY (Babbitt et al., 1974) indicated that the incorporation of shrimp (*Pandalus jordani*) in a fish-shrimp portion greatly enhanced the acceptability of mechanically deboned minced fish muscle. Furthermore, the results suggested that the incorporation of shrimp extended the shelf life of the frozen fish muscle.

One particular species of black rockfish (*Sebastes melanops*) is characterized by an extremely short shelf life during frozen storage.

If shrimp could reduce the undesirable changes that occur during frozen storage of fish, the utilization of certain species, such as black rockfish, could be greatly increased. This investigation was initiated to determine if shrimp could reduce the undesirable changes known to occur during frozen storage in black rockfish.

EXPERIMENTAL

ROCKFISH (*Sebastes melanops*) and shrimp pieces (*Pandalus jordani*) were obtained from local commercial sources. Minced flesh was prepared by grinding fillets removed from the rockfish in the late stages of rigor through a 3/16-in. plate. The minced fish and freshly processed shrimp pieces were mixed in varying ratios in a mechanical mixer (48 rpm) until thoroughly blended. Half-ounce portions were formed using a Hollymatic 200 Patty machine. The portions were held in a −34°C blast freezer for 2 hr and then 1-lb quantities were sealed in 2 mil polyethylene bags. All the samples were sealed in a larger 4 mil polyethylene bag and held at −18°C until analyzed. A mixture of antioxidants was prepared by mixing 0.75g butylated hydroxy anisole (BHA), 0.75g butylated hydroxy toluene (BHT), 13.5g propylene glycol and 10.0g Tween 20 (Eastman Kodak). One gram of this mixture was diluted to 50 ml with distilled water and the antioxidants were incorporated into the 25% shrimp-75% fish portion by spraying a known volume of the mixture while blending the fish and shrimp to achieve a level of 0.01% BHA-BHT/lipid (100 ppm BHA-BHT) in the portion.

Just prior to sampling, three-fourths of a 1-lb sample was ground in a meat chopper to ensure homogeneity of the sample. Moisture, ash, protein (total N × 6.25) and fat were determined, according to AOAC procedures (AOAC, 1965). Peroxide values of the chloroform extracted lipid (Dyer and Morton, 1956) were also determined by AOAC methods (AOAC, 1965). Malonaldehyde was determined by a 2-thiobarbituric acid procedure (Yu and Sinnhuber, 1957) with appropriate modifications (Yu and Sinnhuber, 1962). The simplified method of Miller (1971) was used to evaluate the antioxidant properties of an ethanol (4:1) extract of the shrimp pieces. The ability of the antioxidant to prevent the oxidative destruction of the carotene solution (50 μg/ml) was expressed as the decrease in absorbance (470 nm) × 100 during a 60 min reaction period.

For desirability (acceptance) evaluations, the frozen portions were wrapped in aluminum foil and baked in a preheated oven at 233°C for 15 min. The same group of five panelists evaluated the desirability of the portions throughout the study using a 9-point hedonic scale ranging from 9, "extremely desirable," to 1, "extremely undesirable."

RESULTS & DISCUSSION

SINCE OFF-FLAVOR, texture and desirability have been found to vary significantly between mechanically deboned

Table 1—Proximate composition of fish-shrimp portions

Treatment[a]	Percent[b]			
	Moisture	Ash	Lipid	Protein
4F	78.28	1.96	2.23	18.50
3F1SA	78.51	1.90	1.93	18.03
3F1S	78.39	1.92	1.91	18.70
2F2S	78.83	1.91	1.63	18.31
1F3S	78.73	1.87	1.44	18.52
4S	78.73	1.77	1.28	19.22

[a] Treatments: 4F (100% fish); 3F1SA (75% fish, 25% shrimp + antioxidant); 3F1S (75% fish, 25% shrimp); 2F2S (50% fish, 50% shrimp); 1F3S (25% fish, 75% shrimp); 4S (100% shrimp)
[b] Mean of duplicate samples

Table 2—Effect of an ethanol extract of shrimp on the rate of carotene decolorization[a]

Treatment	Decrease in carotene absorbance[b] (ΔA_{470nm} X 100 for 60 min)
Ethanol control	50.7
BHT (8 mg/L)	12.8
BHT (4 mg/L)	28.0
Shrimp ethanol extract (1:4)	14.8

[a] Carotene conc (50 μg/ml)
[b] Mean of duplicate samples

minced fish muscle and intact fish portions (Crawford et al., 1972), minced (ground) fillets were used in this study.

The substitution of shrimp pieces in the portion altered the lipid content from 2.23% in the 100% fish (4F) portion to 1.28% in the 100% shrimp (4S) portion (Table 1). Substitution of shrimp also slightly increased the moisture and protein levels in the portion. The substitution of shrimp in the portion improved the desirability and also improved the frozen shelf life of the portion even when only 25% shrimp was incorporated (Fig. 1).

The antioxidants, BHA and BHT, used in this study have proved beneficial in other fishery systems (Yu et al., 1973; Sweet, 1973). Although the formation of peroxides and malonaldehyde were lowered by the antioxidant treatment (Fig. 2 and 3), the benefits of the antioxidants could not be detected by the taste panelists.

The incorporation of shrimp pieces with fish in the portion markedly reduced the formation of peroxides and malonaldehyde particularly as the concentration of shrimp was increased (Fig. 2 and 3). The increased desirability of the portions with increasing levels of shrimp was partially attributed to the improved texture given to the minced fish by the shrimp pieces. Also, the flavor and decreased formation of "rancid" off-flavors in the portions during frozen storage may also be attributed to the shrimp.

From Figures 2 and 3, one could conclude that the decreases in malonaldehyde and peroxide levels were proportional to the level of shrimp substituted in the portion. However, an ethanol extract of the shrimp pieces exhibited strong antioxidants properties (Table 2). These antioxidant properties may be due to the presence of natural carotenoids, tocopherols, phenolic compounds (Bailey et al., 1960) and/or the synergistic properties of TMAO (Ishikawa and Yuki, 1974).

The combined advantages of improved texture, flavor and reduction in off-flavors formed during frozen storage of the fish-shrimp portion offer a potential for increasing the utilization of certain species of fish. Interestingly, the prolonged shelf life of the portion may be due to certain antioxidants naturally present in shrimp.

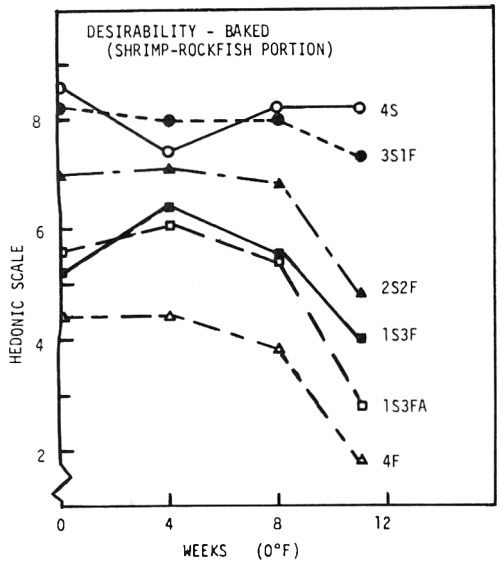

Fig. 1–Desirability of fish-shrimp portions stored at –18° C. Treatments: 4F (100% fish); 3F1SA (75% fish, 25% shrimp + antioxidant); 3F1S (75% fish, 25% shrimp); 2F2S (50% fish, 50% shrimp); 1F3S (25% fish, 75% shrimp); 4S (100% shrimp).

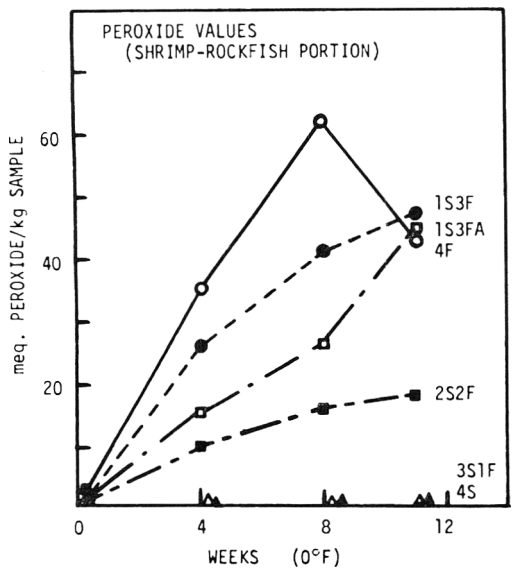

Fig. 2–Peroxide determination of fish-shrimp portions stored at –18° C. Treatments: 4F (100% fish); 3F1SA (75% fish, 25% shrimp + antioxidant); 3F1S (75% fish, 25% shrimp); 2F2S (50% fish, 50% shrimp); 1F3S (25% fish, 75% shrimp); 4S (100% shrimp). Mean of duplicate analyses for two samples.

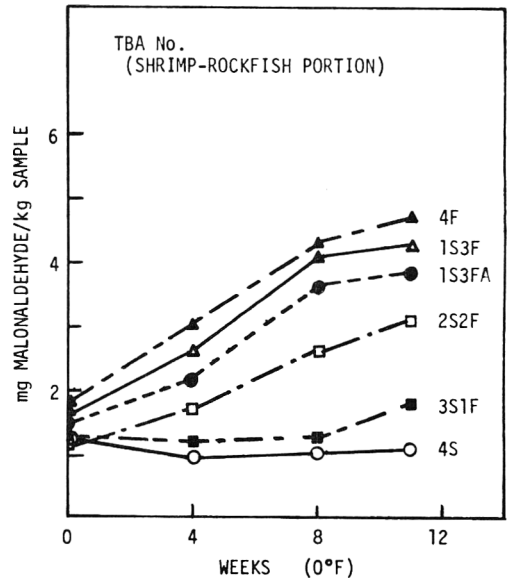

Fig. 3–TBA determination of fish-shrimp portions stored at –18° C. Treatments: 4F (100% fish); 3F1SA (75% fish, 25% shrimp + antioxidant); 3F1S (75% fish, 25% shrimp); 2F2S (50% fish, 50% shrimp); 1F3S (25% fish, 75% shrimp); 4S (100% shrimp). Mean of duplicate analyses for two samples.

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CRYSTALLIZATION KINETICS OF ALPHA LACTOSE

ABSTRACT

Rates of lactose crystallization from solutions of constant alpha lactose concentration were determined at temperatures of 15–50°C. Commercially produced crystalline lactose was used as the seed material. Surface integration rather than diffusion of alpha lactose to the crystal surface was the rate controlling step under the experimental conditions. Correlations suitable for exploratory design of commercial crystallizers are given.

INTRODUCTION

RATES of precipitation of alpha lactose from solutions at various conditions have been determined by a number of workers. Troy and Sharp (1930) showed that precipitation from seeded solutions at 25°C proceeded more rapidly at both low and high pH and concluded that this was due to the effect of pH on mutarotation rate. Whittier and Gould (1931) compared concentration changes of seeded solutions of lactose and other sugars in the temperature range 0–30°C. Haase and Nickerson (1966) determined the changes in concentration of unseeded pure solutions and condensed whey at 0–25°C. Twieg and Nickerson (1968) investigated the effect of temperature and pH on the precipitation rate from seeded solutions and also the effect of seed mass relative to solution mass on both the precipitation rate and the proportions of alpha and beta lactose in solution at 25°C. Under the conditions employed in the latter experiments they found that the ratio of alpha to beta lactose remained considerably lower than the equilibrium value until crystallization was nearing completion. It can be expected from this work that the rate of mutarotation of beta to alpha lactose would significantly effect crystallization rates in commercial practice where high ratios of surface area to solution mass are employed.

Growth rates of various faces of single lactose crystals at 30°C and two relative supersaturation levels, 55% and 120%, were measured by Van Krevald and Michaels (1965). The low rates of crystallization of alpha lactose, compared with sucrose for example, have been shown to be due largely to a retarding effect of beta lactose present (Michaels and Van Krevald, 1966; Van Krevald, 1969).

Crystallization rates per unit crystal surface area have been reported by Jelen and Coulter (1973) based on the increase in mass of large individually perfected crystals. These authors determined specific crystallization rates at four levels of supersaturation in the temperature range 30–70°C.

Most published data on lactose crystallization kinetics is expressed in variables which are inappropriate for crystallizer design. In many cases the experimental conditions employed were too specific to allow the data to be used for general design purposes, or variables such as alpha lactose concentration and the extent of nucleation were not measured. There is a need for data on crystal growth rates, expressed as a mass rate per unit surface area, over a range of temperature and alpha lactose supersaturation. The objective of this work was to obtain such data for the growth of commercially produced crystals in a slurry.

EXPERIMENTAL

Experimental procedure

Crystallization rates were determined from the change in mass of seed crystals under conditions of essentially constant lactose concentration and hence constant alpha lactose driving force. The crystals were contained in conical mesh baskets, 5 cm long and of 2 cm maximum diameter, from which nuclei formed were washed by the mother liquor. In this manner the growth rate of new crystals was excluded from the calculated crystallization rates.

B.P. grade lactose ("Wyndale" brand, produced by The Lactose Company of New Zealand Ltd.) was used to prepare solutions and as seed crystals. Solutions were prepared at 70°C using distilled water, filtered, reheated to 70°C, held at this temperature for at least 10 minutes to ensure complete dissolution, then cooled to the desired crystallization temperature in a thermostat bath. Cooling took about 10 min. Crystallization was carried out at the natural pH of the solution, approximately pH 4. The seeds, initially 124–147 μm sieve aperture diameter, were pretreated by rotating the seed basket for 5 min in slightly supersaturated lactose solution to remove fines and surface impurities; growth under these conditions was negligible. The basket was then drained and immediately immersed in the crystallizer vessel where it was rotated for a period of time dependent on concentration and temperature.

Thorough mixing was achieved with the basket inclined from the vertical and rotated with a gyratory motion; crystal breakage was found to be insignificant. After crystallization the baskets were dried at 50–55°C to constant weight, usually overnight. The loss of water of crystallization at these conditions was confirmed to be negligible. For all weighings the baskets were kept in air-tight containers.

Average alpha and total lactose concentrations were determined for the crystallization period, the changes being very slight as only about 1g of crystals in 3 kg of solution was used in each experiment and a reasonably close approach to mutarotation equilibrium was attained despite the rapid cooling of solution from 70°C. Samples for analysis were withdrawn from the crystallizer through a sintered glass filter and passed to a density meter and polarimeter. A thermostat bath was used to maintain a constant temperature (25°C or 35°C) in the analysis circuit. The density meter, manufactured by Anton Paar K.G., utilizes the effect of sample mass and hence density on the resonant frequency of a glass tube containing the sample. By calibration with lactose solutions of known concentration, sample concentrations were obtained directly from the digital counter of the instrument. After applying temperature corrections the estimated absolute error in total lactose determinations was ± 0.1 wt%; repeatability under similar conditions was considerably better. The polarimeter was a Zeiss instrument with the scale reading to ±0.01°. Overall accuracy of alpha lactose determinations was estimated to be ± 0.2 wt% with repeatability slightly better.

Calculation procedure

The mass of an individual crystal is given by

$$M = \rho f_v D^3 \quad (1)$$

where the constant f_v, the volume shape factor, is defined by

$$f_v = \frac{V}{D^3} \quad (2)$$

and ρ, D and V are the density of alpha lactose monohydrate, a representative crystal dimension (taken here as the sieve aperture diameter) and crystal volume respectively, in consistent units.

The rate of increase in diameter with time due to crystallization is given by equation (3):

$$\frac{dD}{d\tau} = \frac{dM}{d\tau} / \frac{dM}{dD} \tag{3}$$

Now

$$\frac{dM}{d\tau} \equiv \left(\frac{dM}{d\tau} \cdot \frac{1}{S}\right) f_s D^2 \tag{4}$$

where S is the crystal surface area and the constant f_s, the surface shape factor, is defined by

$$f_s = \frac{S}{D^2} \tag{5}$$

By differentiating equation (1),

$$\frac{dM}{dD} = 3\rho f_v D^2 \tag{6}$$

Hence, combining equations (3), (4) and (6),

$$\frac{dD}{d\tau} = \left(\frac{dM}{d\tau} \cdot \frac{1}{S}\right) \frac{f_s}{3\rho f_v}$$

$$\text{i.e.} \quad \frac{dD}{d\tau} = \left(\frac{dM}{d\tau} \cdot \frac{1}{S}\right) \frac{F}{3\rho} \tag{7}$$

where F is the surface:volume shape factor.

With the assumption that the specific crystallization rate and shape factor do not change with time, which is reasonable for this type of experiment, equation (7) may be integrated to give

$$\left(\frac{dM}{d\tau} \cdot \frac{1}{S}\right) = \frac{3\rho}{F\tau} (D_f - D_o) \tag{8}$$

where D_o and D_f are the initial and final crystal sieve aperture diameters respectively, and τ is the duration of crystallization.

Specific crystallization rates (in g $h^{-1} m^{-2}$) were calculated using equation (8) with the appropriate factor for conversion of units and converted to an anhydrous lactose basis.

The value taken for D_o of the seed crystals was the mean of the apertures (136 μm) of the two screens used to prepare the material. The final diameter was calculated from the initial diameter and the ratio of the final to the initial mass of crystals. This is valid as the number of crystals in a basket during the crystallization period remained substantially constant. The number of crystals, N, (approximately 0.7 million/g solid) is given by

$$N = \frac{W_o}{\rho f_v D_o^3} = \frac{W_f}{\rho f_v D_f^3} \tag{9}$$

where W_o and W_f are the initial and final crystal mass respectively.

From equation (9),

$$D_f = D_o \left(\frac{W_f}{W_o}\right)^{1/3} \tag{10}$$

The initial crystal mass was taken as 98% of the mass of dry crystals weighed into the baskets, the loss of fines during pretreatment having been found in separate experiments to be quite constant and averaging only 2% of the charge. It was also found in these experiments that no further loss of fines occurred when pretreatment was extended beyond 5 min. In determining the final crystal mass, W_f in equation (10), the mass of lactose precipitated on drying of solution adhering to the crystals after removal of the basket from the bulk solution was allowed for in each experiment. This was calculated from the mass of water evaporated and the solution concentration.

Reverting to equation (8), values used for ρ and F were 1.525g cm^{-3} and 10 respectively. The value for F was calculated from the length: width:thickness ratio of 1:0.622:0.219 reported by Whittier (1944). Microscopic examination of seed crystals showed them to be geometrically similar to this and to be generally undamaged.

The fraction of lactose present as alpha lactose in the crystallizing solution, and hence the alpha lactose concentration, was calculated by the standard method using values of Sharp and Doob (1941) for the specific rotations of alpha and beta lactose at 25°C, 89.5° and 35.0° respectively. Total lactose concentrations (C_v) in g anhydrous lactose/100 ml solution required in this method were obtained from the measured concentrations (C, wt%) using equation (11), determined experimentally at 25°C:

$$C_v = 2.965 + 0.7670C + 0.008638C^2 \tag{11}$$

For crystallizer temperatures of 35°C and above, analyses were carried out at 35°C to minimize nucleation. The above data for 25°C were used but a small correction (+0.6%), based on comparisons of known solutions at 25°C and 35°C, was applied to the measured angles of rotation.

For calculation of alpha lactose supersaturation total lactose solubility data of Hudson (1908) were used, together with values for the equilibrium constant of the mutarotation reaction,

$$\text{alpha lactose} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{beta lactose}$$

calculated from thermodynamic data used by Kendrew and Moelwyn-Hughes (1940). Solubilities and equilibrium constants are shown in Table 1.

RESULTS

IN ALL, 72 experiments were carried out at temperatures of 15–50°C. The duration of crystallization ranged from 10–272 min and the increase in seed mass (initially 1g approximately) from 28 to 187%. Solution concentrations ranged from 22.9 wt% to 47.8 wt% and absolute alpha lactose supersaturations from 1.9 wt% to 6.4 wt%. Specific crystallization rates were in the range 1–90 g $h^{-1} m^{-2}$, the highest rate being at 50°C. Maximum rates achievable were limited by solution cloudiness and decrease in concentration due to nucleation. Experiments in which alpha lactose and total lactose measurements varied by more than 0.3 wt% and 0.1 wt% respectively were rejected.

Table 1—Lactose solubilities and mutarotation equilibrium constant

Temp (°C)	Lactose solubility (wt%)		Equilibrium constant
	Total	Alpha	
15	14.42	5.58	1.583
25	17.78	6.71	1.650
35	22.02	8.11	1.716
40	24.53	8.93	1.748
50	30.47	10.84	1.810

Table 2—Crystallization order and rate constant

Temp °C	Order, n	Rate constant,[a] k_c g $h^{-1} m^{-2}$ $(wt\%)^{-n}$
15	3.0	0.107
25	2.9	0.196
35	3.1	0.156
40	3.4	0.106
50	4.1	0.073

[a] For alpha lactose supersaturations in wt%

Specific crystallization rates for each experiment are shown in log-log plots, Figures 1–3. At each temperature the plots are linear showing that the relationship usually employed for calculation of overall crystallization rates, equation (12), is appropriate:

$$\frac{dW}{d\tau} \cdot \frac{1}{A} = k_c(C_\alpha - C_{\alpha s})^n \qquad (12)$$

where W (g) is the mass of crystals (anhydrous), A(m^2) is the crystal surface area, C_α and $C_{\alpha s}$ (wt%) the concentration and solubility of alpha lactose respectively; k_c in g h^{-1} m^{-2} (wt%)$^{-n}$ and n are the overall crystallization rate constant and order respectively. Experimental values for the latter are shown in Table 2. With the order of crystallization in the range 3–4, considerable variation in experimental values for order and rate constant could be expected. However, there does appear to be an increase in order with temperature.

Figure 4 shows the effect of relative alpha lactose supersaturation $[(C_\alpha - C_{\alpha s})/C_{\alpha s}]$ on specific crystallization rate. For a rate of only 5 g h^{-1} m^{-2} relative supersaturations ranging from 0.25 (at 50°C) to 0.65 (at 15°C) are required, corresponding to *absolute* supersaturations of 2.7 and 3.6 wt% alpha lactose, respectively. The high level of supersaturation necessary before crystallization rates become significant and the rapid increase in rates thereafter are clearly demonstrated.

The above data were determined with pure solutions in which alpha and beta lactose were present in approximately equilibrium proportions. During commercial crystallization the mutarotation reaction may be far from equilibrium and, quite apart from assumptions required on impurity effects, this data cannot be applied directly. Data of Van Krevald (1969) show that in solutions containing alpha lactose and water in a given ratio the presence of a small fraction of beta lactose causes a large reduction in crystallization rate. As beta lactose concentration is increased, the rate of crystallization decreases, reaching a constant value when the ratio of beta to alpha lactose is greater than one. In most practical cases the ratio of beta to

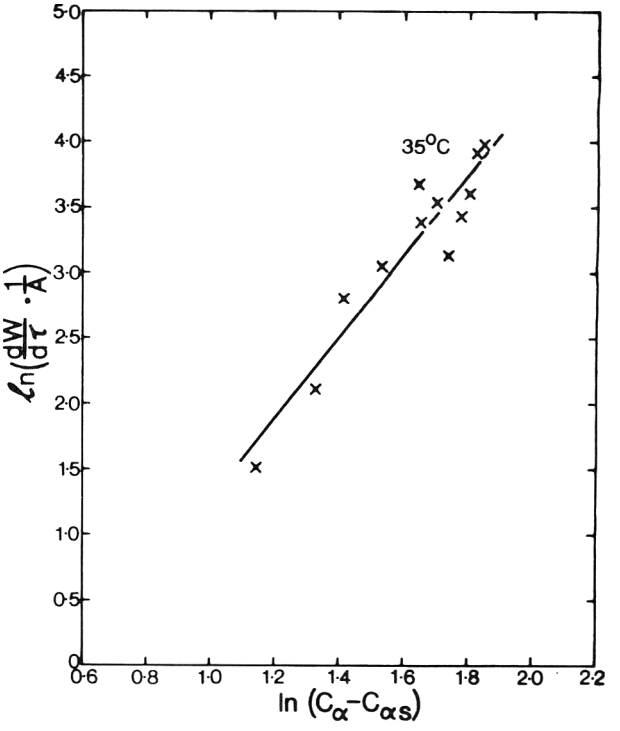

Fig. 2—Logarithmic plot of specific crystallization rates—35° C.

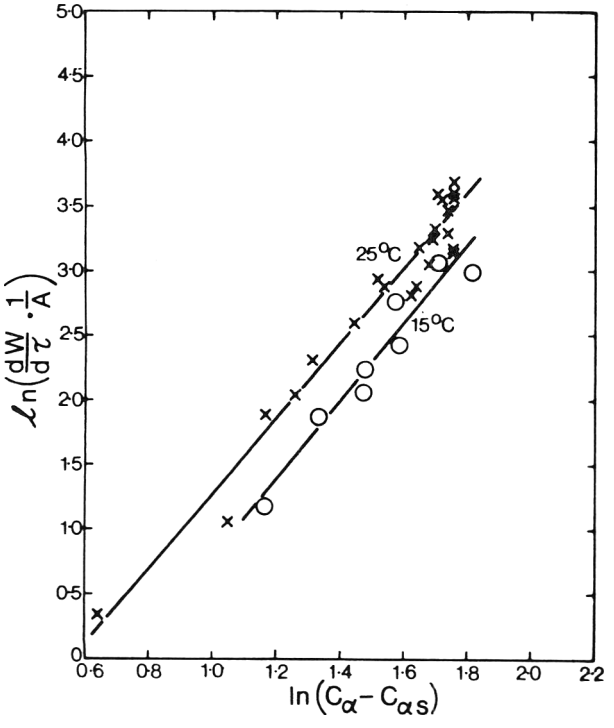

Fig. 1—Logarithmic plot of specific crystallization rates—15° C, 25° C.

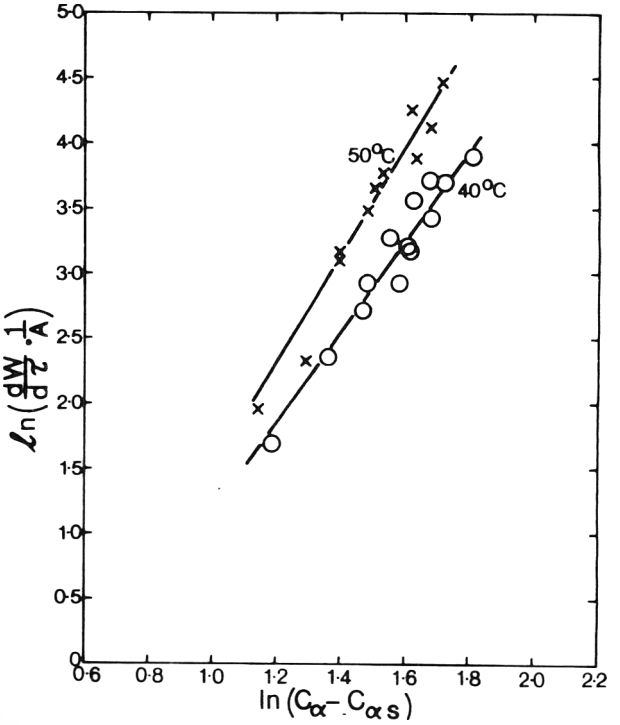

Fig. 3—Logarithmic plot of specific crystallization rates—40° C, 50° C.

alpha lactose would be considerably greater than one and it is realistic to regard beta lactose as having a constant, although very significant, effect on crystallization rate. The relevant driving force for the overall crystallization process is therefore the alpha lactose supersaturation calculated on a beta-free basis. Concentrations in all experiments were recalculated accordingly and correlated with specific crystallization rates. This resulted in a slight (0.2–0.3) decrease in order over the experimental temperature range. Order and rate constant were found to be satisfactorily expressed by the following empirical equations, for temperatures (t°C) of 15–50°C.

$$n' = 2.5 - 0.018t + 0.88 \times 10^{-3} t^2 \quad (13)$$

$$\ln k'_c = -2.3 + 0.05t - 1.70 \times 10^{-3} t^2 \quad (14)$$

where k'_c in g h^{-1} m^{-2} $(wt\%)^{-n'}$ and n' are the overall crystallization rate constant and order respectively, for alpha lactose supersaturation expressed in wt% on a beta-free basis.

Deviations between experimental rates and rates calculated using orders and rate constants from these equations are small, except at 15°C and high supersaturations. For example, at this temperature the deviation reaches 7.5% with a supersaturation corresponding to 20 g h^{-1} m^{-2} specific crystallization rate.

DISCUSSION

CRYSTAL GROWTH is often regarded as a process consisting of a diffusion step followed by a surface integration step in which the solute molecules arrange themselves into the crystal lattice. These two steps are represented by equations (15) and (16).

$$\left(\frac{dW}{d\tau} \cdot \frac{1}{A}\right) = k_d (C_\alpha - C_{\alpha i}) \quad \text{(diffusion)} \quad (15)$$

$$\left(\frac{dW}{d\tau} \cdot \frac{1}{A}\right) = k_i (C_{\alpha i} - C_{\alpha s})^j \quad \text{(surface integration)} \quad (16)$$

where k_d is the diffusion mass transfer coefficient, k_i the rate constant for surface integration, $C_{\alpha i}$ the concentration of alpha lactose at the crystal/solution interface and j the order of the integration step.

The mass transfer coefficients for diffusion under conditions of dissolution and crystallization can be expected to be of the same order of magnitude. To confirm that diffusion did not have a significant rate-limiting effect on crystallization under the conditions of agitation in the crystallization experiments, the rate of dissolution of lactose was measured, in a solution slightly undersaturated with alpha lactose at 25°C. Dissolution was too rapid for accurate data to be obtained but it was concluded that the rate was at least 165 g h^{-1} m^{-2} (wt% alpha lactose undersaturation)$^{-1}$. In comparison the specific crystallization rate at 25°C with 1.0 wt% alpha lactose supersaturation was negligible. Although at higher driving forces the difference would be reduced, it could be expected that dissolution would remain far more rapid than crystallization for driving forces of equal magnitude. On the assumption that the mass transfer coefficient for diffusion in the dissolution experiment was of similar magnitude to those in the crystallization experiments it is concluded that the diffusion step did not significantly influence the overall crystallization rate.

That diffusion was not rate-limiting is confirmed by the high order of crystallization. High orders (with respect to total lactose concentration) were also found by Jelen and Coulter (1973). Conversion of their concentrations to wt% alpha lactose supersaturations, assuming their solutions were at mutarotation equilibrium, gives an overall order of 2.8 at 30°C. Specific crystallization rates of these authors at 30°C are also in satisfactory agreement with interpolated values from this work although their rates at 50°C are considerably higher.

The above representation of the crystallization process is simplified. It does not specifically take into account such steps as surface diffusion, desolvation of lactose molecules and counter-diffusion of water. Neither does it account for increased growth rates in the presence of nuclei, observed by Van Krevald and Michaels (1965), or for the inhibiting effect of the beta lactose present (Van Krevald, 1969).

Calculated solubilities of alpha lactose depend on values taken for the mutarotation equilibrium constant. In this work the latter were calculated from thermodynamic data used by Kendrew and Moelwyn-Hughes (1940) and are in close agreement with values determined experimentally by these authors at seven temperatures in the range 20–50°C. The values used increase from 1.58 at 15°C to 1.81 at 50°C. There are other data which indicate a decrease in equilibrium constant with temperature. Data of Gillis published in 1920 is commonly quoted, for example by Nickerson (1956). These are 1.78 at 0°C (also confirmed by Nickerson), 1.71 at 25°C and 1.63 at 50°C. Reasons for this disagreement are not apparent, but sufficient data are given in the present paper for the recalculation of alpha lactose supersaturations on a different basis if this becomes desirable.

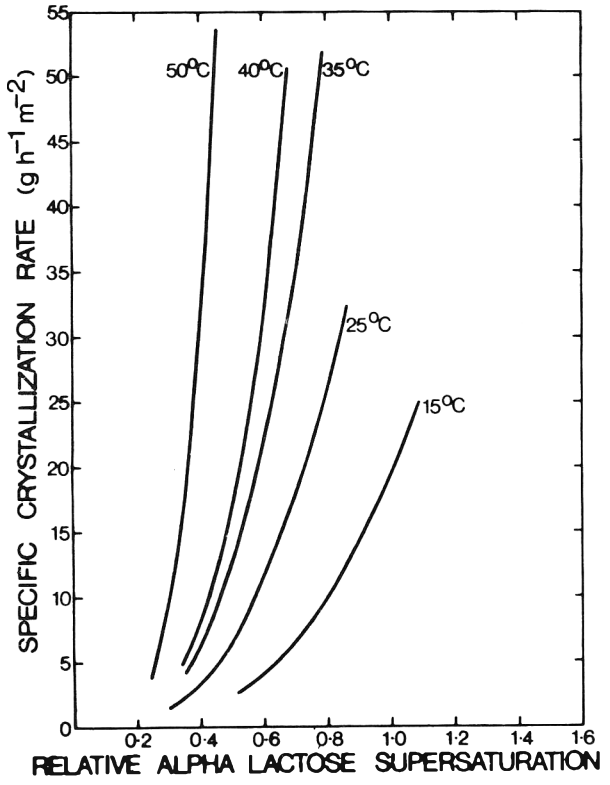

Fig. 4–Effect of relative saturation and temperature on specific crystallization rate.

CONCLUSIONS

FOR A GIVEN alpha lactose supersaturation an increase of temperature in the range 15–50°C increases crystallization rate. Rates at any temperature are low (less than 2 g h^{-1} m^{-2})

at absolute alpha lactose supersaturations of less than 2.0 wt%.

The overall order of crystallization with respect to alpha lactose supersaturation was found to vary from about 3 to 4 in the temperature range 15–50°C and appears to increase with temperature. Under the experimental conditions surface integration was the rate controlling step. Diffusion of alpha lactose to the crystal surface is rapid compared to surface integration, provided that agitation is sufficient to keep crystals in suspension.

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LACTOSE CRYSTALLIZATION: INVESTIGATION OF SOME PROCESS ALTERNATIVES

ABSTRACT

A number of alternative methods of lactose crystallization, including the commonly used batch tank method, were explored with a view to identifying potential process improvements and to provide a better understanding of the significance of mutarotation under conditions relevant to commercial practice. Results of simulations with various operating conditions are given. A combined process consisting of continuous crystallization at moderate temperature followed by low temperature batch crystallization appears to be the best method for obtaining high yields of lactose from whey ultrafiltrate.

INTRODUCTION

THE WORLDWIDE consolidation of dairy factories and greater awareness of environmental aspects of cheese and casein whey disposal have increased the incentives to develop new methods of utilizing whey and to improve well-established processes such as the production of lactose. Currently the most common method of producing lactose involves evaporation of whey to approximately 60 wt% solids content followed by cooling in large tanks. Crystallization of alpha lactose is slow and influenced, amongst other factors, by the simultaneous mutarotation of beta to alpha lactose. One objective of the present work was to determine the significance of mutarotation, the rate of which depends directly on temperature and indirectly on crystallization rate and hence crystal surface area. The main objective however was to model different crystallization methods with a view to identifying process improvements. The needs and the scope for this have both increased due to the expected availability of by-product whey ultrafiltrate from protein concentrate production. Removal of proteins results in a feed more suitable for lactose crystallization and less attractive for other products such as dried whey powder. Use of ultrafiltrate also increases incentives for high lactose recovery as markets for spent mother liquor containing little protein are very limited.

Process alternatives

Twieg and Nickerson (1968) demonstrated that in a crystallizing solution the ratio of alpha to beta lactose can be considerably lower than the equilibrium value. Unpublished work of the present authors confirmed that significant reductions in the ratio of alpha to beta lactose occur during crystallization even when crystal surface area is relatively low.

From these observations it is apparent that maintaining high alpha lactose supersaturation is the key factor in achieving reasonable rates of crystallization and hence in decreasing lactose production costs. Several options for accomplishing this can be suggested. For example, evaporative crystallization, continuous makeup of concentrated feed to the crystallizer and provision of a reactor in which conditions are more favorable for mutarotation. The following batch, semicontinuous and continuous alternatives were selected for this study:

Decreasing-temperature batch crystallization. This model represents batch tank crystallization of highly concentrated lactose solutions by cooling, and approximates the present commercial process. Initial seeding is assumed, although this is not general commercial practice. As relevant data on nucleation rates are not available, it is assumed that formation of new crystals can be controlled and is kept such as to maintain constant specific crystal surface area (i.e., the area per unit crystal mass).

Semi-continuous crystallization. Mother liquor from the crystallizer (represented as a stirred tank in Fig. 1) is circulated through an evaporator and is cooled, together with fresh feed, to the desired constant crystallizer temperature. Fresh feed rate and evaporation rate are adjusted to maintain constant solution mass and alpha lactose concentration (on a beta lactose-free basis). Thus the specific crystallization rate, i.e., the rate per unit crystal surface area, is held constant at a value set by considerations such as nucleation rate or required product purity. Constant surface area is assumed, product being withdrawn continuously. Feeding of the crystallizer would continue until the mother liquor impurity concentration reached an unacceptable level. An optional baffled tank reactor to increase alpha lactose content is included in the mother liquor circuit.

Continuous crystallization. The crystallizer in this arrangement is fed continuously with concentrate from an evaporator. Feed rate, mother liquor withdrawal rate, crystal surface area and temperature are all held constant.

Constant low-temperature batch crystallization. This batch operation is intended as a second stage of crystallization with low to medium initial lactose concentration. Specific crystal surface area is assumed constant.

With the exception of the continuous process model which was amenable to hand calculation, the models of these process variations were programmed for solution by the simulation program DARE III-B, developed at the University of Arizona (Trevor and Wait, 1972).

PROCESS MODELLING

ALL CONCENTRATIONS are expressed as weight fractions on a salt-free basis. Total lactose solubilities are given by equation (1), for temperature (t°C) in the range 0–90°C, based on data of Hudson (1908).

$$C_s = \begin{matrix} 0.1058 + 0.221 \times 10^{-2} t + 0.184 \\ \times 10^{-4} t^2 + 0.34 \times 10^{-6} t^3 \end{matrix} \quad (1)$$

Alpha lactose solubilities have been calculated by equation (2), and converted to a beta-free basis as in equation (3):

$$C_{\alpha s} = \frac{C_s}{K + 1} \quad (2)$$

$$C_{\alpha\alpha s} = \frac{C_{\alpha s}}{1 - C_s + C_{\alpha s}} \quad (3)$$

where K is the equilibrium constant for the mutarotation reaction,

$$\text{alpha lactose} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{beta lactose},$$

and is given by equation (4) derived from thermodynamic data used by Kendrew and Moelwyn-Hughes (1940). These authors also give a relationship for k_1 from which equation (5) for k_2 in pure solutions has been derived:

$$\log K = 0.7382 - \frac{155}{T} \quad (4)$$

$$\log k_2 = 41.728 - 10.318 \log T - \frac{4945}{T} \quad (5)$$

In accord with the findings of Haase and Nickerson (1966) for a synthetic whey solution, values of k_2 from equation (5) have been doubled. This increase in the mutarotation rate constants, attributed to a catalytic effect of salts present in natural whey, is expected to occur in both whey and ultrafiltrate. It has also been assumed that the mutarotation equilibrium is not significantly affected by the presence of salts.

Empirical relations for the order of crystallization, n', and rate constant, k'_c, reported by Thurlby (1976) have been used, as given in equations (6) and (7) respectively.

$$n' = 2.5 - 0.018t + 0.88 \times 10^{-3} t^2 \quad (6)$$

$$\ln k'_c = -2.3 + 0.05t - 1.70 \times 10^{-3} t^2 \quad (7)$$

where k'_c and n' apply to alpha lactose supersaturation in wt% on a beta-free basis.

Batch models

Total lactose concentration at any time, was calculated by material balance. Thus,

$$C = \frac{C_o G_o - (W - W_o)}{G_o - W_h} \quad (8)$$

where C_o is the initial concentration, G_o the initial solution mass and W_o the mass of crystals present initially (as anhydrous lactose). W is the mass of crystals present (as anhydrous lactose) and G, the denominator of equation (8), is the solution mass, at any time. The mass of hydrated lactose (W_h) which has precipitated is calculated from W and W_o.

Crystallization rate, which by integration gives W, is given by equation (9):

$$\frac{dW}{d\tau} = \frac{k'_c A}{1000} [100(C_{\alpha\alpha} - C_{\alpha\alpha s})]^{n'} \quad (9)$$

where $C_{\alpha\alpha}$, the alpha lactose concentration on a beta-free basis, is calculated similarly to $C_{\alpha\alpha s}$ in equation (3). Crystal surface area has been calculated assuming a constant specific surface area (s) by equation (10):

$$A = \frac{sW}{\mu} \quad (10)$$

where $\mu = 0.95$, the molecular weight ratio of anhydrous and hydrated alpha lactose. This arbitrary assumption implies a certain degree of nucleation and/or attrition. The objective in commercial crystallizers is to produce just sufficient nuclei early in the crystallization period to result in product crystals of suitable size for recovery. However as representation of this would be equally arbitrary due to the lack of suitable data on the extent and rate of nucleation, the assumption of constant specific surface area has been chosen.

Alpha lactose concentration was found by integration of equation (11) which gives the net rate of change in alpha lactose concentration due to mutarotation and crystallization.

$$\frac{dC_\alpha}{d\tau} = k_2 [C - (1 + K)C_\alpha] - \frac{dW}{d\tau} \cdot \frac{1}{G} \quad (11)$$

Equations representing cooling policies were included in the model of the decreasing-temperature batch process. New values for the temperature dependent properties were calculated at each iteration.

Semi-continuous model

Total lactose concentration in the crystallizer was calculated as for the batch processes above with allowance for lactose entering in the feed and for the increase in concentration of liquor recycled via the evaporator. Alpha lactose concentration on a beta-free basis was determined using equation (9) for a chosen specific crystallization rate. In the absence of the reactor it has been assumed that the ratio of alpha to beta lactose in the recycle does not change although the residence time and increased temperature in the evaporator would have some effect.

Continuous model

Crystallization rate in this steady-state process can be calculated from the overall lactose material balance around the crystallizer,

$$\frac{dW}{d\tau} = \frac{F(C_f - C)}{1 - C/\mu} \quad (12)$$

where F and C_f are the feed rate and concentration respectively. For a chosen specific crystallization rate, alpha lactose concentration on a beta-free basis can be calculated using equation (9) and from this the alpha concentration on a total solution basis. The net rate of change in alpha lactose concentration, as given by equation (13), is zero for this case:

$$\frac{dC_\alpha}{d\tau} = k_2 [C - (1 + K) C_\alpha] - \frac{dW}{d\tau} \cdot \frac{1}{G} + C_{\alpha f} \frac{F}{G} = 0 \quad (13)$$

where $C_{\alpha f}$ is the alpha lactose concentration in the feed. By rearrangement of equation (13) the required mass of crystallizer solution can be found directly. Thus,

$$G = \left(\frac{dW}{d\tau} - C_{\alpha f} F\right)/k_2 [C - (1 + K) C_\alpha] \quad (14)$$

SIMULATION RESULTS

Decreasing-temperature batch crystallization

As an example of this process consider crystallization of a solution initially containing 0.55 g/g lactose including 0.186 g/g alpha lactose, slightly less than the mutarotation equilibrium value. The effects of cooling from 68°C to 25°C at three constant rates and maintaining temperature at 25°C thereafter have been investigated. In each case the initial seed area has been taken to be 0.5 m^2/kg solution, equivalent to about 1g of 140 μm crystals per kg solution.

As would be expected, increasing the cooling rate greatly increases the rate of crystallization as shown by the slopes of the concentration curves in Figure 2. Specific crystallization rate decreased very rapidly (see Fig. 3) for the first 2 hr, reflecting the high order of crystallization. Despite the low specific rates after a few hours, absolute crystallization rates remained significant due to the large surface area available by then. This is apparent from the slopes of the upper curves in Figure 3, for instance at four hours. Figure 3 also shows the change in alpha lactose supersaturation expressed in absolute units and as a percentage of the potential supersaturation, i.e., as a percentage of the supersaturation which would exist if the solution was in mutarotation equilibrium at the prevailing temperature. The absolute supersaturation changed slowly at first, mainly due to the low initial surface area and hence low absolute crystallization rate. This factor, together with relatively high initial temperatures and hence high mutarotation rate constants, also resulted in a low initial rate of decrease of the crystallization driving force expressed as a percentage of the potential supersaturation. The latter reached a minimum value for each cooling rate shortly after the final temperature of 25°C was reached. At the minimum, crystallization and mutarotation were in balance. Thereafter the relative driving force increased as the ratio of alpha to beta lactose increased towards the equilibrium value at 25°C. The retarding effect of mutarotation on the progress of crystallization was not as great as could be expected from these departures from equilibrium. This is demonstrated in Figure 2 by a concentration curve (shown dashed) determined for mutarotation rate constant values approaching infinity and a cooling rate of 5.0°C h^{-1}.

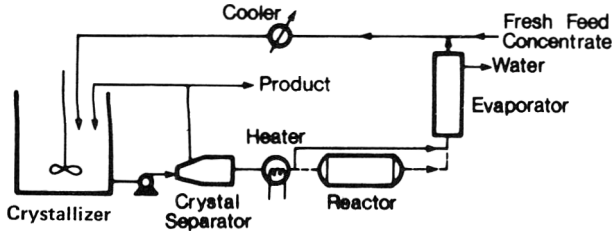

Fig. 1–Simplified flowsheet for semi-continuous process.

Initial seed area was increased fivefold to 2.5 m^2/kg solution. The effect of this (not shown in the Figures) was slight. There was a proportionate increase in absolute crystallization rate initially but this was short-lived as alpha lactose concentration and hence specific crystallization rate decreased more rapidly with the higher initial area. Thus the rate of generation of new crystal surface area (which soon far exceeds the initial area) became almost independent of the initial area.

Semi-continuous crystallization

The main variable of interest in simulations of this process is the mass of solution required relative to crystallization rate, which is an indicator of crystallizer size requirements. Its relationship to specific crystallization rate and steady-state lactose concentration is shown in Figure 4 for a crystallization temperature of 25°C and crystal surface area of 14 m^2/kg solution, which is equivalent to 300g of 140 μm crystals per kg solution. In this example the required relative solution mass increased rapidly as lactose concentration was reduced below 0.30 g/g. This process could only be useful as a first stage of crystallization or where the value of by-product liquor is relatively high. To test the effect of increasing the rate of mutarotation using an external reactor, the favorable assumption was made that the ratio of alpha to beta lactose in the circulating liquor could be increased to the equilibrium value at 25°C. On this basis the required relative solution mass was approximately halved when the crystallizer liquor was circulated through the reactor six times per hour.

Continuous crystallization

The required residence time of solution in the crystallizer is shown in Figure 5 for a range of operating conditions. The rapid increase as concentration was decreased is apparent. The optimum temperature for a given concentration depends on the balance between decreasing driving force due to increasing solubility and both increasing mutarotation rates and improved crystallization kinetics, with increasing temperature. Optimum temperature increased with concentration; specific crystallization rate changed from 2.6 to 9.6 g h^{-1} m^{-2} as concentration was changed from 0.26 to 0.34 g/g.

Constant low-temperature batch crystallization

The progress of batch crystallization is best shown by the decrease in solution concentration with time. Examples are given in Figure 6 for solutions of 0.24 and 0.32 g/g initial concentration (0.088 and 0.113 g/g alpha lactose respectively) at 25°C. For an initial seed area of 4 m^2/kg solution both

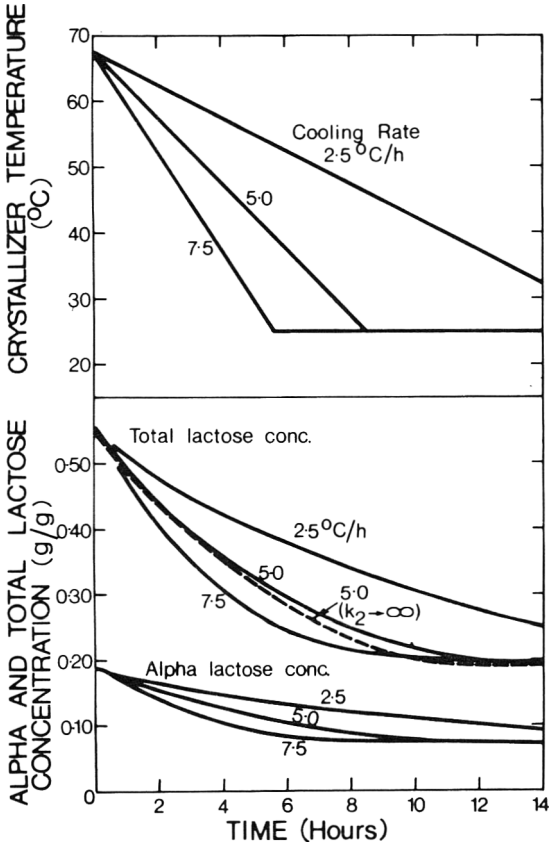

Fig. 2–Decreasing-temperature batch process: effect of cooling rate on rate of crystallization. Initial crystal surface area 0.5 m^2/kg solution.

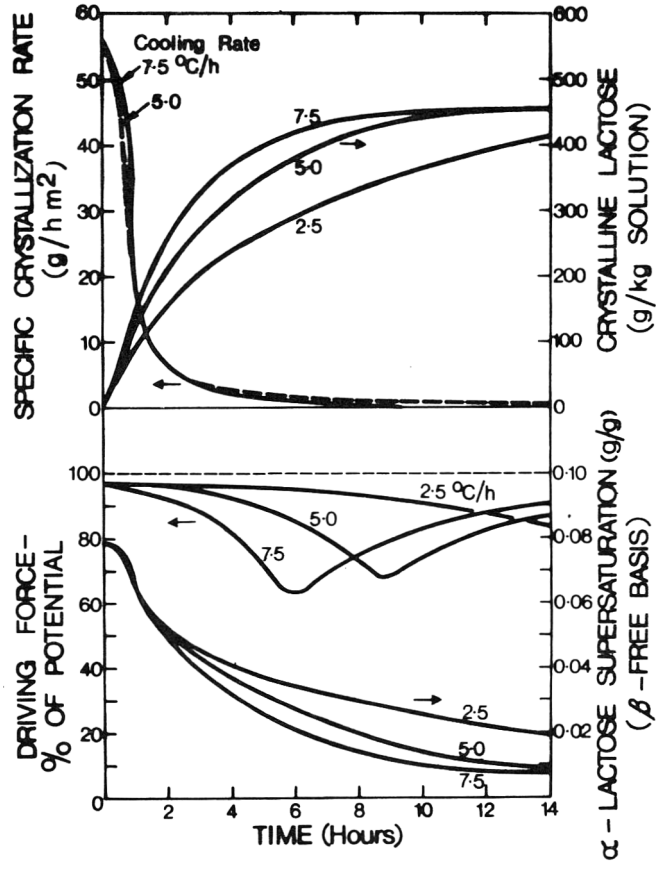

Fig. 3–Decreasing-temperature batch process: change in specific crystallization rate, quantity of crystalline lactose and driving force. Initial crystal surface area 0.5 m^2/kg solution.

solutions reached the same concentration in less than 7 hours; initial specific crystallization rates and generation of new surface area were much greater for the 0.32 g/g solution.

In commercial practice there is a limit on the final crystal slurry density and comparison of the crystallization of solutions with different initial concentrations to the same final crystal surface area is of interest. Concentration curves are shown in Figure 6 for solutions of initial concentrations 0.32 g/g and 0.24 g/g, seeded at levels of 7 and 18 m^2/kg solution respectively. These initial seed areas were chosen such that in both solutions crystal surface area reached 24 m^2/kg solution when the concentration reached 0.20 g/g. As could be expected, concentrations always remained lower for the solution of lower initial concentration.

The manner in which alpha lactose driving force as a percentage of potential driving force varied with time is shown in Figure 7. For the lower initial surface area and lower initial concentration the crystallization rate was always less than the mutarotation rate and the ratio of alpha to beta lactose increased steadily towards the equilibrium value. For all other cases this ratio (and thus the percentage driving force) decreased rapidly initially, reaching minimum values at less than 1.5 hr from commencement.

DISCUSSION

CRYSTALLIZATION KINETICS data for pure solutions have been used in these simulations. Rates may be different in whey or ultrafiltrate depending on the promoting or inhibiting effects of proteins and some salts naturally present. Estimation of rates at relatively low supersaturations and at temperatures greater than 50°C has involved extrapolation of experimental data. Also some basic assumptions concerning nucleation have been made as previously mentioned. However, as the main objective of this work was a comparison of process alternatives, rather than crystallizer design, these assumptions are not inappropriate.

From the simulation results and simplicity of the process, decreasing-temperature batch crystallization appears preferable

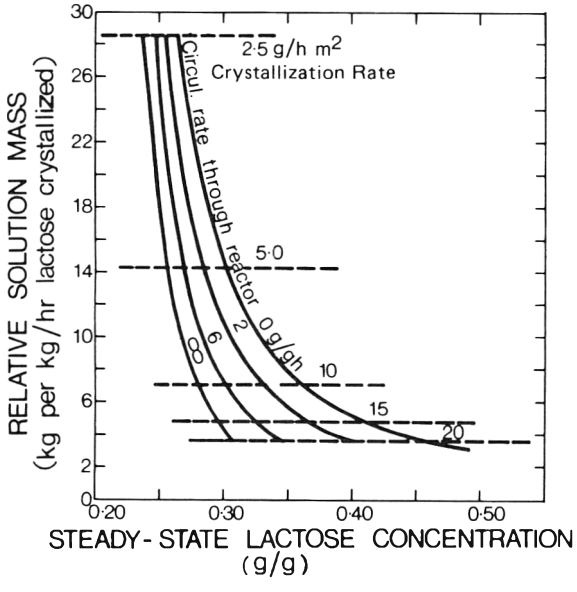

Fig. 4–Semi-continuous process: effect of concentration, specific crystallization rate and circulation rate through reactor on mass of crystallizing solution. Constant crystal surface area of 14 m^2/kg solution. Crystallizer and reactor temperature 25° C. Feed concentration 0.55 g/g (0.186 g/g alpha lactose).

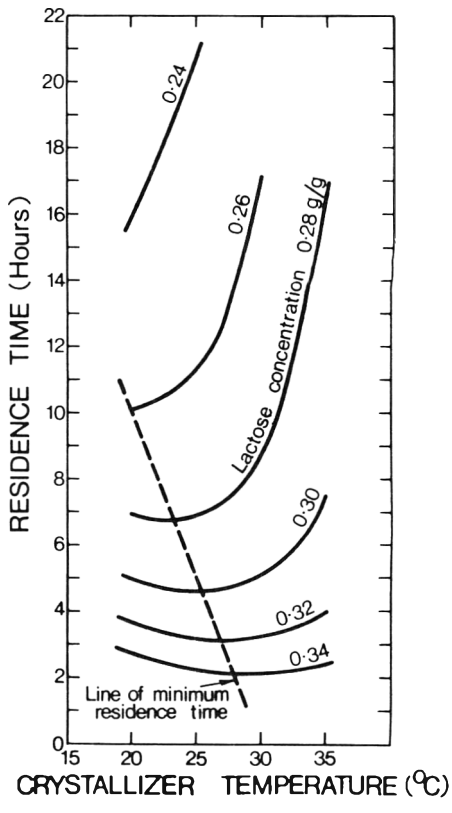

Fig. 5–Continuous process: effect of temperature and concentration on residence time. Feed concentration 0.55 g/g (alpha lactose 0.186 g/g). Crystal surface area 15 m^2/kg solution.

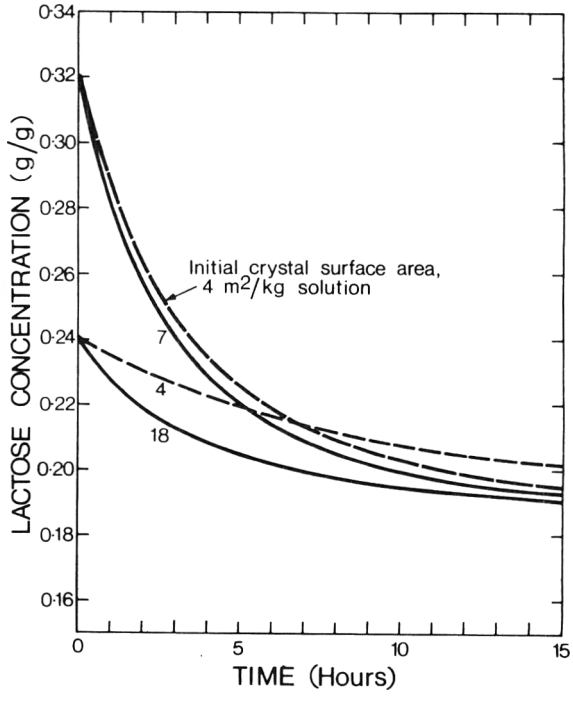

Fig. 6–Low-temperature batch process: effect of initial lactose concentration and initial crystal surface area on rate of crystallization. Crystallizer temperature 25° C.

to the alternatives studied when fairly high yields are desired. However, in practice, extremely long residence times (in the order of a few days to weeks) are necessary for increasing the size of crystals to give satisfactory recoveries and allow adequate washing in a centrifuge. On the basis of 14 days total residence time, storage is required for approximately 750 kg of solution (0.55 g/g initial lactose content) per kg/h average lactose crystallization rate. Satisfactory control of nucleation to avoid high residence times is not practical in this process. Yield is also limited by the degree of concentration practicable in one pass through an evaporator and by the crystal content of the final slurry. On the other hand, alternatives such as the semi-continuous and continuous processes investigated do offer reasonable control of the number of new crystals formed as only moderate supersaturations need be employed. Also nuclei which are formed can be removed by recycling of liquor to the evaporator, preferably through a heater, at little additional cost. By good control of crystal size it may be possible to employ a less expensive type of centrifuge or even screens.

The semi-continuous process is not favored because of its variable evaporation rate requirement. Although this could be overcome, the operational difficulty involved in intermittent filling and emptying of the two or more crystallizers still remains a serious disadvantage.

These features do not occur with continuous crystallization. However, continuous crystallization also requires moderate solution concentrations and on its own would only be suitable in the unusual situation where the value of the spent mother liquor is relatively high. Where the latter is low or where costs must be incurred in disposal of spent liquor, overall yield could be increased by use of a second stage (low-temperature) batch crystallizer. Seed crystals used can be a recycled product of the required size range or be grown in the second stage crystallizer. Liquor to the second stage could be heated to increase the alpha to beta ratio and to destroy nuclei and thus improve control of second stage crystal size. Three second stage vessels would be needed: one filling and cooling, the second crystallizing at the minimum practical constant temperature and the third feeding a centrifuge. With such an arrangement total solution holdup would be in the order of 50–60 kg per kg/h lactose crystallized. A reactor to increase mutarotation rate is not justified on either stage, although some holdup of the heated first stage recycle stream may be useful for the dissolution of nuclei.

A continuous first stage and batch second stage scheme is flexible with regard to product grade and recovery. For example the first stage can be operated to produce a high purity product, mother liquor being re-concentrated batchwise before second stage crystallization. In this way overall yield can be increased, at the expense of a lower purity second stage product. This may be recrystallized if warranted. The greater flexibility and lower solution holdup for a given yield are significant advantages over the conventional process. However, for small scale production particularly, these are offset to some extent by the need for better control and more operator attention.

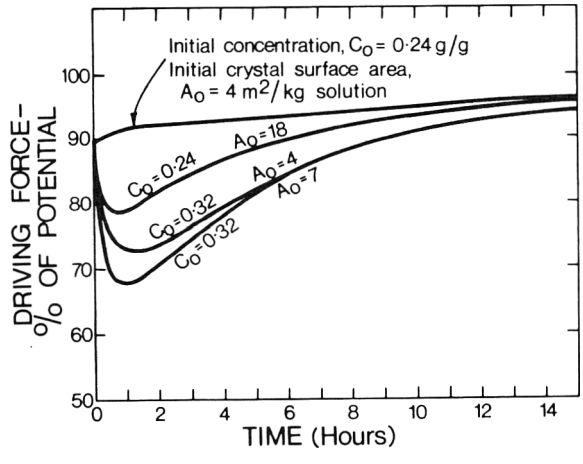

Fig. 7—Low-temperature batch process: change in actual crystallization driving force as % of potential. Crystallizer temperature 25° C.

CONCLUSIONS

IN THE CONVENTIONAL batchwise process for crystallization of lactose from whey, departures from mutarotation equilibrium can be significant if relatively high cooling rates are employed. However, this is of little practical significance as the residence time is dictated by crystal size requirements for satisfactory recovery and washing, rather than crystallization rate. Solution residence time can be reduced considerably with better control of nucleation or destruction of nuclei. Both are possible with continuous crystallization at moderate supersaturation and with recycle of mother liquor to the evaporator. Where high yields are required as would usually be the case for lactose production from whey ultrafiltrate this continuous crystallization stage must be followed by low temperature batch crystallization. Such a scheme is likely to be cheaper than the simpler batch process in general use and offers greater flexibility in lactose product purity and yield.

LIST OF SYMBOLS

A	Crystal surface area (m^2)
C	Total lactose concentration (g/g)
C_f	Total lactose concentration in feed (g/g)
C_o	Initial lactose concentration (g/g)
C_s	Total lactose solubility (g/g)
C_α	α-Lactose concentration (g/g)
$C_{\alpha f}$	α-Lactose concentration in feed (g/g)
$C_{\alpha s}$	α-Lactose solubility (g/g)
$C_{\alpha\alpha}$	α-Lactose concentration on β-free basis (g/g)
$C_{\alpha\alpha s}$	α-Lactose solubility on β-free basis (g/g)
F	Feedrate (kg h^{-1})
G	Mass of solution (kg)
G_o	Initial mass of solution (kg)
k_1	Rate constant for α to β lactose conversion (h^{-1})
k_2	Rate constant for β to α lactose conversion (h^{-1})
K	Equilibrium constant for mutarotation
k'_c	Crystallization rate constant [g h^{-1} m^{-2} $(wt\%)^{-n'}$]
n'	Crystallization order [defined by equation (9)]
s	Specific surface area of crystals (m^2 kg^{-1})
t	Temperature (° C)
T	Absolute temperature (° K)
W	Mass of crystals (anhydrous) (kg)
W_h	Mass of hydrated lactose precipitated (kg)
W_o	Initial mass of crystals (anhydrous) (kg)
μ	Molecular weight ratio of anhydrous and hydrated α-lactose
τ	Time (h)

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COWPEA POWDERS DRIED WITH METHIONINE: PREPARATION, STORAGE STABILITY, ORGANOLEPTIC PROPERTIES, NUTRITIONAL QUALITY

ABSTRACT

Cowpea (*Vigna sinensis*) was soaked, dehulled, ground, supplemented with various levels of DL-methionine, drum dried and the powders evaluated fresh and after storage. Chemical determinations indicated that the protein, amino acid composition, available lysine, methionine retention and thiamine content of the cowpea powders, compared to raw cowpeas, were not appreciably affected by the processing and storage conditions, including 24 wk at temperatures up to 37°C. Fresh and stored powders supplemented with up to 0.6% methionine were evaluated in a traditional moin-moin recipe by a taste panel of members from several African countries and well rated in terms of color, flavor, consistency and overall acceptability. Nutritional studies indicated that drum drying eliminated trypsin inhibitors and that methionine supplementation produced a concentration dependent effect on protein quality as gauged by weight gains of rats and PER values, which increased from 1.64 without methionine supplementation to 2.65 at the 0.6% addition level. At the 0.6% level, however, fatty infiltration of rat livers occurred. This was also evident but much less pronounced at a level of 0.4% added methionine. Cowpea powders stored 24 wk showed reductions in PER values but even at 37°C protein quality, as measured by PER, was largely retained in methionine-supplemented powders.

INTRODUCTION

DESPITE the high protein content of legumes, their maximum contribution to nutrition has not been fully exploited in many parts of the world because of problems of antinutritional factors such as trypsin inhibitors, their low levels of sulfur amino acids, particularly methionine, and in many instances their inconvenience of preparation into local dishes (Aykroyd and Doughty, 1964; Anonymous, 1973a). Apart from groundnut, which is cultivated primarily for oil and export, cowpea (*Vigna sinensis*) is the most important food legume in many parts of Africa where the cost of protein from cowpea can be one-tenth that from milk or meat.

Cowpeas are consumed in various ways including cooking with maize, as a supplement to other carbohydrate staples, and in the preparation of native dishes following overnight soaking, manual removal of seed coats and mashing. Moin-moin (steamed mash) and akara (fried mash) are relished dishes in West Africa (Aykroyd and Doughty, 1964). Because of the frying involved, the nutritive value of the akara is inferior to moin-moin which has been recommended as a suitable bean preparation for young children (Welbourn, 1956; Morley, 1958). Although cowpea yields have increased in West Africa in recent years (Taylor, 1964; Ebong, 1968), one of the consequences of rapid urbanization is that cowpea and other crops are not being fully utilized because of inconvenience of preparation into local dishes. Converting cowpea into a highly nutritious convenient form for use in moin-moin, weaning foods, or as a supplement to bread formulas and other preparations would therefore appear to have numerous advantages.

For reasons of economy and simplified operation the double drum dryer has been favored for the preparation of quality legume powders (Morris, 1961; Rolland et al., 1966; Bakker-Arkema et al., 1967; Kon et al., 1974). Several powders obtained from chickpeas, lentils, kidney beans and other legumes have been prepared without seed coat removal and with pregelatinization of the beans prior to drum drying. In the case of cowpea, seed coats must be removed prior to powder preparation, otherwise they leave black specks which are not acceptable for many uses, and gelatinization prior to drying does not appear necessary or desirable for several applications of the powder.

It is well known that the first limiting amino acid in most legumes is methionine and that the protein quality of legume foods can be substantially improved by the addition of small amounts of DL-methionine (Sherwood et al., 1954; Parthasarathy et al., 1964; Graham, 1971; Jansen, 1973; Zucker, 1973; Bookwalter et al., 1975). However, there is a narrow range between the effective and toxic levels of methionine and this has prompted the FAO to revise downward the recommended level of this amino acid (Anonymous, 1973b; Anonymous, 1974). Another problem with methionine supplementation has to do with objectionable flavors that can be produced by this amino acid (Balance, 1961; Beigler, 1969; Dunlap et al., 1974).

Since very little has been reported on the processing of cowpea, the present study was undertaken to prepare high quality convenience cowpea powders and to determine the effects of methionine supplementation, processing and storage on their functional, organoleptic and nutritional properties.

MATERIALS & METHODS

Cowpea preparation and drying

Cowpeas (*Vigna sinensis*), commonly called black-eyed peas or "bean" in West Africa, were soaked in water in the ratio of 1:4 at 23°C for 18 hr. Seed coats were separated by hand rubbing and removed by flotation. Dehulled cowpeas were rinsed and ground in a mill with a worm screw through a plate with 6 mm openings. Water was added as needed to facilitate passage through the plate. Puree was further diluted to a solids content of 25% to facilitate handling on the drum dryer. Dilution water contained BHT and Myverol 1806 emulsifier (Eastman Kodak) at levels to yield 10 ppm and 0.1%, respectively, based on bean solids. DL-methionine also was dissolved in the dilution water at levels to add 0.2, 0.4 and 0.6%, corresponding to 0.8, 1.6 and 2.4 g/16g N to the cowpea solids. To uniformly disperse these additives, batches of puree were blended with a Hobart mixer (Model A-200) fitted with a wire whip for 5 min at medium speed.

The cowpea puree was dehydrated on a Blaw-Knox laboratory atmospheric double drum dryer with two 6 in. diam × 7 5/8 in. length drums. Drum speeds were varied between 2–4 rpm with a clearance between the drums of approximately 0.016 in. The drums were heated internally by steam at a pressure of 45 psi. The puree was applied in splashes at the top of the drums to prevent accumulation and excessive gelatinization. Samples of dried cowpea sheet from the drums were crumbled into flakes and comminuted into powder through a No. 18 Standard Sieve (Tyler equivalent, 16 mesh), stored in air-tight jars within incubators at 23, 30 and 37°C and evaluated at 8-wk intervals up to 24 wk.

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Chemical and biological properties

Moisture and protein (N × 6.25) contents of purees and powders were determined by the methods of the AOAC (1970).

For amino acid analyses, samples containing 80 mg protein were hydrolyzed with 5 ml 6N HCl under vacuum at 110°C for 22 hr. Hydrolyzates were filtered, evaporated to dryness and made to volume with pH 2.2 sodium citrate buffer. Amino acids were determined by ion-exchange column chromatography using a Model 120C Beckman amino acid analyzer. Tryptophan was determined separately by the method of Horn and Jones (1945) and methionine and cystine by the method of Herrick et al. (1972). Available lysine was estimated by the method of Finley and Friedman (1973). The nitrogen solubility index of cowpea powders in water was determined by the method of the AOCS (1970).

Trypsin inhibitor activity of raw cowpeas and cowpea powders was determined by the method of Sambeth et al. (1967) using benzoyl-dl-arginine-p-nitroanilide as the substrate for trypsin. Thiamine was determined by the thiochrome method described by the AOVC (1966).

The biological values of fresh and stored cowpea powders supplemented with different levels of methionine were determined by growth experiments using 21-day old male weanling rats of the Sprague-Dawley strain. Rats were randomly assigned to each experimental diet after feeding them for 3 days on a commercial stock diet. The rats were kept in individual wire bottomed cages and given food and water ad libitum. Experimental diets contained 10% protein supplied by the different cowpea powders or by casein, 10% Cerelose, 6% mineral mix (Jones and Foster, 1942), 2% vitamin fortification mix (General Biochemicals, Chagrin Falls, Ohio), 5% corn oil, 4% crude fiber and the balance as cornstarch. Individual feed consumption and body weights were recorded at 2-day intervals during experiments which lasted 21 days. For the last 10 days fecal output was collected and oven dried to calculate apparent dry matter digestibility.

At the end of the test period rats were euthanized and the liver and pancreas of each rat were removed and weighed fresh. These organs were then fixed in Bouin's solution for 36 hr and in 10% phosphate buffered formalin, pH 7.4, for histopathology. Portions of liver and pancreas were embedded in paraffin, sectioned at 6μ, stained in hematoxylin and eosin and examined microscopically.

Organoleptic and physical properties

Powder bulk densities were determined by measuring the loose volume occupied in a graduated cylinder.

Colors of stored powders were determined objectively with the Hunterlab Color and Color Difference Meter (Hunterlab Associates) and changes expressed as total color differences (ΔE values).

Cowpea powders were made into moin-moin by mixing 50g with 190 ml water, 12g unmodified tapioca starch (Stein-Hall) to firm texture, 3g cooking oil (Wesson) and 0.5g salt. This recipe steamed in aluminum foil for 20 min gave acceptable texture compared to moin-moin conventionally prepared from overnight-soaked, dehulled, ground cowpeas. Controls were prepared replacing the cowpea powder plus starch with an equivalent weight of solids entirely from soaked-ground cowpeas in the above recipe and steaming for 35 min. Consistencies after steaming were measured at 30°C with a Brookfield Helipath Stand Viscometer, Model HB, fitted with a TB cross-bar spindle turning at 20 rpm.

Quality characteristics including color, consistency, flavor and overall acceptability of moin-moin products were assessed by taste panel preference tests (Amerine et al., 1965). The panel consisted of 13 members of the African community at Cornell University who were familiar with the traditional cowpea product. Preference scores, based on a 9-point hedonic scale, were analyzed statistically.

RESULTS & DISCUSSION

Chemical and physical properties of cowpea powders

Typical operational data for the preparation of cowpea powders are shown in Table 1. Independent of the level of methionine supplementation, these data were much the same for each batch of powder produced. The weight of 94.5% solids powder obtained from 10 lb of raw cowpea was 5.5 lb. The total solids loss, including that of the hulls, hardshells, embryos and broken cotyledons, plus the drying loss, represented about 43% of the solids of the raw cowpeas. The yield of cowpea powder solids based on puree solids approached 90% recovery. Moisture content of powders increased gradually from 5.5 to 6.5% as drum speeds were changed from 2 to 4 rpm. A speed beyond 4 rpm gave poor quality powder due to excessive moisture. The relatively high bulk density of sieved powder indicates that it can be packaged economically.

Soaking and drum drying were without major effects upon protein and thiamine contents of cowpea, but drying sharply decreased trypsin inhibitor activity (Table 2), consistent with the fact that trypsin inhibitors and other antinutritional factors in legumes generally are destroyed by heat (Liener, 1962; Kakade et al., 1973). The slight decrease in nitrogen solubility index from drying indicates that some denaturation of the protein occurred, but this should be of minor consequence in applications where high solubility is not crucial. Stability of thiamine was further studied over a 24-wk storage period at temperatures of 23, 30 and 37°C with powders of 5.5 and 6.5% moisture containing 0.2 and 0.6% levels of methionine supplementation. In no case did thiamine decrease below a level of 0.74 mg/100g of cowpea solids, representing a retention of at least 90% in all cases.

The amino acid compositions of the raw cowpea puree and the freshly dried and stored powders of 5.5% moisture are seen in Table 3. Appreciable changes in compositions due to drum drying did not occur and storage for the 24-wk period at 30 and 37°C was essentially without further effects. Virtually identical results were obtained for the 24-wk period at a storage temperature of 23°C and for similar powders of 6.5% moisture stored 24 wk at each of these temperatures. Methionine in the raw cowpea puree was detected at a level of 1.2 g/16g N which is in the range commonly reported for legumes. Supplemental levels of methionine were retained through drying and storage to the extent that 85–93% of added methionine was analytically recoverable after 24 wk from 5.5 and 6.5% moisture powders at all temperatures of storage. This

Table 1—Operational data for the preparation of cowpea powders

Wt of raw cowpea grain (91.5% solids)	10 lb
Wt of the cowpeas soaked 18 hr	20 lb
Wt of the dehulled cowpeas	16 lb
Wt of bean puree diluted to 25% solids	24 lb
Drum speed	2–4 rpm
Steam pressure	45 psi
Clearance between the drums	0.016 in.
Wt of cowpea powder (94.5% solids)	5.5 lb
Bulk density (through 16 mesh, loose fill)	0.65 g/cc

Table 2—Effects of soaking and drum drying on trypsin inhibitor activity and other properties of cowpeas[a]

Treatment	Protein %	Trypsin inhibitor Units/g bean	Trypsin inhibitor Units/g protein	NSI[b]	Thiamine mg/100g
None (raw cowpeas)	23.9	23.6	98.7	22.0	0.92
Soaked 18 hr and dehulled	23.9	24.0	100.4	21.8	0.97
Drum dried cowpea (6.5% moist)	22.5	4.2	18.7	–	0.82
Drum dried cowpea (5.5% moist)	22.5	4.0	17.8	17.0	0.79

[a] All values are on a total solids basis.
[b] Nitrogen solubility index of protein determined at pH 6.9.

Table 3—Amino acid compositions of cowpea puree and dried and stored powders of 5.5% moisture

Amino acids (g/16g N)	Cowpea puree	Initial powders			Powders stored 24 wk at 30° C			37° C		
		Methionine supplementation (% of solids)								
	0.0	0.2	0.4	0.6	0.2	0.4	0.6	0.2	0.4	0.6
Lysine	6.7	7.6	7.6	7.5	7.5	7.5	7.5	6.6	6.9	6.7
Histidine	2.6	3.3	3.4	3.5	3.6	3.0	3.2	3.0	3.2	3.1
Arginine	7.8	7.3	7.1	7.3	6.5	6.3	6.5	6.5	6.2	6.4
Aspartic acid	12.7	11.2	12.8	11.7	12.7	12.2	11.7	11.7	12.0	12.2
Threonine	4.5	4.2	4.1	4.2	3.4	3.5	3.5	3.6	3.4	3.4
Serine	5.3	6.9	7.0	6.8	5.3	5.4	5.8	5.9	5.9	5.5
Glutamic acid	19.2	20.7	19.3	19.2	20.0	18.6	19.0	18.7	18.6	19.0
Proline	6.0	4.9	4.9	4.9	4.3	4.5	4.4	4.5	4.6	4.8
Glycine	4.3	4.6	5.0	4.9	4.6	5.0	4.8	4.9	5.0	4.8
Alanine	4.4	5.1	5.2	5.1	5.2	5.1	5.3	5.1	5.5	5.4
Valine	5.1	6.5	6.7	6.6	2.6	6.6	6.5	6.6	6.4	6.4
Methionine	1.2	1.9	2.7	3.6	1.7	2.6	3.2	1.7	2.4	3.2
Isoleucine	4.8	3.7	3.4	3.5	3.6	3.3	3.5	3.6	3.4	3.5
Leucine	7.7	6.5	6.3	6.5	7.0	7.3	7.2	7.6	8.0	7.2
Tyrosine	3.0	3.2	3.4	3.5	3.2	3.3	3.4	3.3	3.2	3.3
Phenylalanine	5.3	6.0	5.8	6.0	5.9	5.7	5.6	5.6	5.7	5.9
Tryptophan	1.7	1.0	1.1	1.1	1.0	1.1	1.1	1.1	1.1	1.1
Half cystine	1.8	1.0	1.2	1.0	1.1	1.0	1.0	1.0	1.0	1.1

stability of added methionine agrees with the fact that methionine is heat stable and shows that it is feasible to incorporate the methionine prior to drying, which in many instances can be simpler and less costly than post-dehydration dry blending.

Although lysine levels in Table 3 remained high through drying and storage as measured on acid hydrolyzates, the possibility of changes in lysine availability, as may occur when the epsilon amino group of lysine enters into Maillard reactions, also was investigated. Available lysine contents of freshly dried and stored powders are given in Table 4. Available lysine content of freshly dried powders was of the order of 97% of total lysine and this value decreased only very slightly to 94% at 37°C in 24 wk. A very similar order of stability in terms of available lysine was obtained with powders of 6.5% moisture.

Color changes were monitored as another measure of storage stability and ΔE values between freshly dried powders and corresponding samples stored 24 wk are presented in Table 5. Regardless of storage temperature between 23 and 37°C, level of added methionine, and the small difference in moisture content between powders, ΔE values in no case exceeded 6.0 and such color changes as occurred were not of a magnitude that could be readily detected visually. This observation is consistent with the data on lysine availability which indicated that the stored powders did not undergo substantial Maillard-type changes.

Organoleptic properties of cowpea powders

The consistency of steamed moin-moin made from soaked-ground cowpeas (native cowpea control) was more firm than from cowpea powders cooked without starch (100 vs 55 Brookfield Units). The tapioca starch in the recipe increased firmness slightly to 65 Brookfield Units which was quite acceptable to taste panel members.

The taste panel scores for moin-moin made from native cowpea and freshly dried powders are given in Table 6, and from stored powders are given in Table 7. All data represent the average scores from 13 tasters. Preference scores were analyzed by Dunnett's multiple comparison technique. The data indicate that with respect to color, consistency and overall acceptability, such differences as occurred between recipes made with freshly dried powders and native cowpea did not significantly ($P > 0.05$) affect preference. However, with respect to flavor, 0.6% and zero-methionine powders were less preferred ($P < 0.05$) than those with intermediate levels of methionine.

Table 4—Available lysine content[a] of freshly dried and stored cowpea powders of 5.5% moisture

Lysine (g/16g N)	Initial powders			Powders stored 24 wk at 30° C			37° C		
	Methionine supplementation (% of solids)								
	0.2	0.4	0.6	0.2	0.4	0.6	0.2	0.4	0.6
Total	7.6	7.6	7.5	7.5	7.5	7.5	6.6	6.9	6.7
Bound	0.2	0.2	0.2	0.3	0.3	0.3	0.3	0.4	0.4
Available	7.4	7.4	7.3	7.2	7.2	7.2	6.3	6.5	6.3
% Available	97.4	97.4	97.3	96.0	96.0	96.0	95.5	94.2	94.0

[a] Determined by the method of Finley and Friedman (1973)

Table 5–Color changes (ΔE) of stored cowpea powders[a]

Powder moisture	Powders stored at 23°C, Methionine supplementation (% of solids) 0.2	0.4	0.6	30°C 0.2	0.4	0.6	37°C 0.2	0.4	0.6
				Stored 8 wk					
5.5%	4.0	4.6	5.0	4.7	4.9	5.1	4.3	4.8	5.3
6.5%	5.2	5.5	5.6	5.1	5.3	5.3	5.8	5.7	5.8
				Stored 24 wk					
5.5%	4.3	4.9	5.1	4.9	5.0	5.2	5.0	4.9	5.6
6.5%	5.3	5.7	5.8	5.5	5.8	5.7	6.0	6.0	5.9

[a] Corresponding powders freshly dried were used as standards.

Table 6–Taste panel scores for moin-moin prepared from native cowpea and freshly dried cowpea powders of 5.5% moisture[a]

Attribute	Native cowpea	Cowpea powders, Methionine supplementation (% of solids) 0	0.2	0.4	0.6
Color	5.0	5.6	5.5	5.4	5.7
Consistency	6.1	5.8	5.9	5.8	5.9
Flavor	6.0	4.4	5.5	5.6	4.8
Overall acceptability	6.1	5.4	5.9	5.9	5.8

[a] Scores are based on the scale 1 = dislike extremely, 9 = like extremely.

Table 7–Taste panel scores for moin-moin prepared from stored cowpea powders of 5.5% moisture[a]

Temp (°C)	8 wk, Methionine supplementation (% of solids) 0.2	0.4	0.6	16 wk 0.2	0.4	0.6	24 wk 0.2	0.4	0.6
				Color					
23	6.4	6.7	6.9	6.9	6.7	6.4	6.4	6.4	5.8
30	6.7	6.8	6.6	6.6	6.4	6.4	6.4	5.6	5.7
37	6.6	6.4	6.4	6.3	6.6	6.6	6.9	6.9	6.1
				Consistency					
23	5.7	5.7	5.7	6.0	5.9	6.0	5.8	5.8	5.9
30	5.7	6.0	5.7	5.8	5.9	6.0	5.9	5.9	5.9
37	5.7	5.8	5.8	5.8	5.9	5.8	5.4	5.8	5.9
				Flavor					
23	4.9	5.1	5.6	6.3	6.4	6.9	6.3	6.1	6.3
30	5.7	4.9	5.0	6.6	6.1	6.4	5.6	5.3	5.6
37	4.3	5.0	5.3	4.6	6.0	6.4	4.3	6.2	6.0
				Overall acceptability					
23	5.1	5.4	6.2	6.3	6.2	6.6	6.5	6.0	6.0
30	6.0	5.6	5.5	5.8	6.7	6.6	6.1	5.8	5.9
37	5.1	5.1	5.7	6.2	6.6	5.0	5.9	6.3	6.0

[a] Scores are based on the scale 1 = dislike extremely, 9 = like extremely.

Tasters' scores for methionine-supplemented powders stored up to 24 wk show that storage temperatures between 23 and 37°C did not appreciably affect color, consistency, flavor and overall acceptability. Factorial analysis indicates that color of the recipe was significantly ($P < 0.05$) influenced by time and temperature and the interaction between time and temperature of storage was significant ($P < 0.05$). Flavor of the recipe was significantly ($P < 0.05$) affected by storage time, and the interaction between time and temperature on flavor also was statistically significant ($P < 0.05$). These effects, however, were very small and may be due as much to panel inconsistency between testing periods as to changes in the powders. There was no evidence for methionine-related off-flavor development with time even at 37°C. Overall acceptability of the recipe was not substantially affected by the time and temperature of powder storage nor the level of methionine added up to 0.6%. Thus, the organoleptic tests indicate that cowpea powder of 5.5% moisture, supplemented with up to 0.6% DL-methionine, would be acceptable in a typical African moin-moin dish even after storage at a temperature of 37°C for at least 24 wk.

Biological properties of cowpea powders

Data on weight gains, protein efficiency ratios (PER), dry matter digestibility, and liver and pancreas weights of rats fed various cowpea diets are given in Table 8. PER values have been corrected to that of casein (2.50). Weight gains of rats on diets containing methionine-supplemented *fresh cowpea* powders were significantly greater ($P < 0.05$) than those on either the raw cowpea puree diet or the diet containing cowpea powder without added methionine. Further, weight gains, food consumption, and efficiency of food utilization all improved with increased level of methionine supplementation. Dry matter digestibility of methionine-supplemented powders also was greater than that of raw cowpea puree or cowpea powder without added methionine. PER values of raw cowpea puree and unsupplemented powder diets were significantly poorer ($P < 0.05$) than those with methionine-supplemented powders, and PER values increased with level of added methionine up to 0.6%. Both 0.4 and 0.6% levels gave PER values comparable to that of casein.

Liver and pancreas weights also increased with added methionine level and exceeded the weights of these organs on the casein diet. Microscopic examination of the livers revealed that their weight increase was paralleled by fat deposition, which was minimal on the casein and unsupplemented cowpea powder diets, moderate on the raw cowpea puree diet, and mild to moderate going from 0.2 to 0.4% methionine. At the 0.6% level, hepatic cells near portal veins were distended by large fat globules, indicating that cowpea powder supplemented with 0.6% (and possibly 0.4%) methionine may produce adverse effects (Benevanga, 1974, and others) or stimulate food intake, resulting in excessive calories in relation to balanced protein, which can increase fat synthesis (Harper et al., 1970; Shannon et al., 1972). In the case of pancreas, cellular abnormalities generally associated with trypsin inhibitors of uncooked legumes (Melmed et al., 1973) occurred on the raw cowpea puree diet. All other diets produced histologically normal pancreas, which together with low values for trypsin inhibitor activity (Table 2) confirms that trypsin inhibitor was effectively eliminated by drum drying.

Protein efficiency ratios were further determined on cowpea powders stored 24 wk using a 3-way factorial design involving 3 temperatures, 3 levels of methionine and 2 levels of moisture. Analysis of variance on the data summarized in Table 9 shows that PER values of stored powders were significantly ($P < 0.01$) improved as methionine supplementation level increased from 0.2 to 0.6%, as was the case with fresh powders. A statistically significant ($P < 0.01$) interaction occurred between storage temperature and level of methionine

Table 8–Biological response of rats fed various cowpea diets[a,b]

Experimental Diet	Wt gain (g)	Food consumed (g)	PER	Dry matter digestibility (%)	Liver wt (g)	Pancreas wt (g)	Wt gain /day (g)	Wt gain /g food (g)
Casein	63.2	259.1	2.50	83.0	4.80	0.40	3.01	0.24
Cowpea puree, raw	26.3	202.0	1.34	74.0	4.11	0.25	1.25	0.13
Drum-dried cowpea	29.3	182.8	1.64	70.1	3.86	0.36	1.40	0.16
Drum-dried cowpea + 0.2% methionine	51.0	228.1	2.29	81.0	5.00	0.45	2.43	0.22
Drum-dried cowpea + 0.4% methionine	53.3	224.0	2.44	80.3	5.71	0.47	2.54	0.24
Drum-dried cowpea + 0.6% methionine	64.8	251.0	2.65	80.5	6.47	0.54	3.09	0.26

[a] All values represent averages from 7 rats per diet.
[b] Duration of experiment 21 days

Table 9–Protein efficiency ratios of stored and fresh cowpea powders[a]

	Powders stored 24 wk at								
	23°C			30°C			37°C		
	Methionine supplementation (% of solids)								
Powders	0.2	0.4	0.6	0.2	0.4	0.6	0.2	0.4	0.6
5.5% moisture	1.37	2.39	2.31	1.60	2.32	2.50	1.85	2.16	2.50
6.5% moisture	1.24	2.18	2.02	2.00	2.32	2.50	1.81	1.80	2.20

	Fresh vs stored powders Methionine supplementation (% of solids)		
	0.2	0.4	0.6
Freshly dried[b]	2.29	2 44	2.65
Stored 24 wk[c]	1.64	2 19	2.34

[a] All PER values for stored powders represent averages from 6 rats per d et corrected to casein standard of 2.50.
[b] Values from Table 8
[c] Overall means of PER values for both powder moistures at all temperatures

supplementation; however, the improvement in PER with increased methionine was present at all storage temperatures. No other main effects or interactions were significant. Comparing the overall means of PER values for all stored powders of a given methionine level (independent of temperature or moisture) with PER values of freshly dried powders at similar levels of methionine it is apparent that decreases occurred during storage. However, powders containing 0.4 and 0.6% levels of added methionine retained PER values of 2.19 and 2.34, indicating that major changes in protein quality of these products did not occur in the 24 wk period.

From the above studies it may be concluded that cowpea powders of improved protein quality can be produced by methionine supplementation of soaked raw cowpeas prior to drum drying. Such powders are convenient to use, perform well in traditional African dishes such as moin-moin and have good storage stability for at least 24 wk at temperatures up to 37°C. This stability encompasses organoleptic properties as well as substantial retention of thiamine, added methionine, available lysine and protein quality as measured by PER. At a level of 0.6% methionine supplementation, however, cowpea powder produced fatty infiltration of livers. More work is needed to affirm limits of methionine free of adverse effect in such powders.

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PROTEIN CONTENT AND AMINO ACID COMPOSITION IN GROATS AND HULLS OF DEVELOPING OATS (Avena sativa)

ABSTRACT

Protein content and amino acid composition were determined in groats and hulls of three oat cultivars harvested at four stages of development. Protein content was slightly higher in mature than in immature groats. In the hulls, protein content decreased during development to about one-third the content of immature hulls. The large decrease in protein content of the hulls was accompanied by little change in amino acid composition. In the groats, however, there were consistent and large decreases in concentrations of lysine, threonine and aspartic acid and an increase in glutamic acid. The results suggest that in addition to deposition of storage protein in the groat, amino acids are translocated from the hulls to the groats.

INTRODUCTION

CHANGES in protein content and amino acid composition of developing oats have been the subject of few investigations (Pomeranz, 1973). According to Wiggans and Frey (1958), the relative proportion of prolamines does not increase in developing oats. Sedova and Pleshkov (1968) reported only small changes in the content of essential amino acids, including lysine, during the development of oat grains. Brown et al. (1970) showed that differences in amino acid content of 10-day, 15-day, and mature oats were small and inconsistent. Relative lysine content declined only slightly with increasing development. Brown et al. (1970) inferred that since the amount of lysine-poor prolamine does not increase during development, the lysine content of the total oat proteins would be expected to remain relatively constant, and that the pattern in developing oats differs from patterns in developing wheat, barley and corn. The lysine content of corn endosperm protein dropped from 4.7% at 17 days after pollination to 3.4% at 21 days, and 1.7% at development (unpublished data of C.M. Wilson, cited by Brown et al., 1970). During development of wheat (Pomeranz et al., 1966) and barley (Pomeranz and Robbins, 1972), concentrations of lysine, aspartic acid, threonine, serine, glycine, alanine, valine and isoleucine decreased, and concentrations of glutamic acid, proline, cystine, tyrosine and phenylalanine increased.

Oat groat protein content and amino acid composition have been determined in 289 samples of oat groats covering a wide range of genetic material (Robbins et al., 1971), but there is little information on the protein content and amino acid composition in oat hulls (Pomeranz et al., 1973). We know of no study on changes which occur in the proteins of hulls of developing oats.

MATERIALS & METHODS

Materials

Three cultivars of oats, *Avena sativa* L., were grown in three replicates of 4-row nursery plots 305 cm long, 30.5 cm apart, at the University of Wisconsin Charmany Experimental Farm. Because of wet soil, seeding date was May 1, 1971, several days later than average. Seeds were hand-spaced at 15 cm intervals (9.3 kg/ha) or funnel-seeded at a rate of 72 kg/ha (spaced at 15 cm). Soil was fertilized with 336 kg/ha of 10-10-10 fertilizer broadcasted before soil preparation for planting, and a top dressing of 112 kg/ha of nitrogen in the form of ammonium nitrate, applied just before plant heading to one of the duplicate plots seeded at 72 kg/ha. The top dressing was worked into the soil with a hoe. The three cultivars were Dal and Goodland (X1656-1, CI 9202) with high contents of groat protein and Froker with intermediate protein content. Spikelet samples were collected one week after anthesis and weekly through the fourth week.

Methods

Groats and hulls were separated manually. Oats harvested at earliest stages of development were difficult to separate completely.

Moisture and protein were determined according to Methods of Analysis of the American Society of Brewing Chemists (1958). Crude protein is reported as Kjeldahl–N × 6.25, %, on a moisture-free basis.

Seventeen amino acids and ammonia were determined on a Beckman 121 automatic amino acid analyzer (Pomeranz and Robbins, 1972) and are expressed in grams of amino acid per 100 grams amino acids recovered. Recoveries in groats ranged from 67.1–97.9% (mean 86.9%) and in hulls from 40.5–83.9% (mean 65.3%). Recoveries were generally lowest in samples harvested at earliest stages of development. The low recoveries in hulls and in groats from early stages of development were attributed to the relatively high concentrations of non-protein nitrogenous compounds in these samples.

RESULTS & DISCUSSION

TO SIMPLIFY PRESENTATION of data, values of lysine, aspartic acid, threonine, glutamic acid and methionine only, from the 72 kg/ha seeding rate treatment without added nitrogen at heading, are recorded in Tables 1 and 2. This treatment was found representative of the three studies treatments. Lysine, threonine and methionine were selected as representatives of the nutritionally limiting amino acids in proteins of most cereal grains including oats. Glutamic acid is the major amino acid of cereal proteins and the main component of storage proteins (prolamines and glutelins). Aspartic acid is a key intermediate in the biosynthesis of lysine in bacteria, algae and higher plants (Vogel, 1965).

In the groats (Table 1), protein content generally was lower during early stages of development and was highest in mature samples. A similar trend was found in developing barley (Pomeranz and Robbins, 1972).

Protein content increased more rapidly during late than during early stages of development; changes in concentrations of amino acids were rapid shortly after anthesis and much slower thereafter.

Concentration of lysine in the proteins of groats decreased during development by about one-third; the decrease was similar to that observed in other cereal grains. Decreases were relatively smaller in aspartic acid and threonine than in lysine.

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The average concentration of glutamic acid was about 35% higher in mature than in immature oat groats. Absolute amounts of glutamic acid were greater in mature than in immature groats because of the large increase in groat weight during development and because glutamic acid is the major amino acid of groat proteins at all stages of maturity.

Average (for the three cultivars) concentrations of histidine (about 2.3%), leucine (about 7.6%), and tyrosine (about 3.3%) in groat proteins changed little during maturation. Average concentrations of the following amino acids increased: arginine (6.0 to 6.9%), cystine (1.4 to 1.9%) and phenylalanine (4.8 to 5.4%); concentration of ammonia increased from 2.4 to 2.8% and reflects to a large degree the increase in the concentration of glutamic acid. Concentrations of the following amino acids decreased: serine (4.7 to 4.3%), proline (6.5 to 5.4%), glycine (5.1 to 4.8%), alanine (6.2 to 4.7%), valine (6.0 to 5.7%) and isoleucine (4.3 to 4.0%). Concentrations of methionine changed inconsistently and were around 2.5%.

At final harvest, kernel weight was near 32 mg/kernel of which 72–75% was groat. Protein content of groat was 21.5, 22.9 and 19.3 for Goodland, Dal and Froker, respectively.

Protein content in hulls decreased consistently during development of oats (Table 2). Generally, protein content in the hull at maturity was less than one-third of that 1 wk after anthesis. This decrease and the fact that hull weight increases little during development, indicate that some of the proteins are translocated from the hull into the groat.

Most amino acid concentrations in the hull remained fairly constant during development (Table 2). Average (for the three cultivars) concentrations of histidine, arginine, serine, alanine, methionine, isoleucine, tyrosine and phenylalanine in hull proteins changed little during maturation. The largest increases

Table 2—Changes in protein content[a] and concentration of certain amino acids[b] in proteins of hulls from three oat cultivars grown in Madison, Wis. in 1971 and harvested at four stages of development

Variety and parameter	Weeks after anthesis 1	2	3	4
Goodland				
Protein	8.0	7.3	4.3	3.0
Lysine	5.9	6.7	6.9	6.7
Aspartic acid	15.1	16.6	14.5	11.3
Threonine	4.7	5.1	5.2	5.1
Glutamic acid	12.6	13.3	14.1	15.3
Methionine	2.1	1.1	2.6	1.8
Dal				
Protein	9.4	5.1	3.2	2.5
Lysine	6.2	6.9	6.9	5.9
Aspartic acid	13.5	12.5	12.1	12.0
Threonine	5.0	5.3	5.4	5.3
Glutamic acid	11.9	13.4	13.4	15.6
Methionine	1.7	2.6	1.8	0.8
Froker				
Protein	7.1	5.3	2.6	2.1
Lysine	6.1	7.2	7.6	6.4
Aspartic acid	13.7	12.5	11.7	11.6
Threonine	4.8	5.1	5.3	5.2
Glutamic acid	12.5	14.1	13.6	15.8
Methionine	1.5	2.5	2.1	0.9

[a] N X 6.25, %, dry matter basis
[b] Grams amino acid per 100g amino acids recovered

Table 1—Changes in protein content[a] and concentrations of certain amino acids[b] in proteins of groats from three oat cultivars grown in Madison, Wis. in 1971 and harvested at four stages of development

Variety and parameter	Weeks after anthesis 1	2	3	4
Goodland				
Protein	19.5	19.3	20.5	21.5
Lysine	6.3	4.9	4.3	4.2
Aspartic acid	11.0	8.5	8.2	8.3
Threonine	4.3	3.6	3.2	3.1
Glutamic acid	15.7	20.3	21.9	23.0
Methionine	2.4	2.3	2.9	2.6
Dal				
Protein	17.2	18.6	19.9	22.9
Lysine	6.2	4.9	4.4	4.0
Aspartic acid	8.8	8.1	8.1	8.2
Threonine	4.3	3.5	3.4	3.1
Glutamic acid	17.5	20.3	22.1	24.1
Methionine	2.4	3.2	2.9	1.9
Froker				
Protein	17.1	16.1	16.8	19.3
Lysine	5.8	5.2	4.5	4.4
Aspartic acid	9.7	8.5	8.4	8.6
Threonine	4.1	3.6	3.3	3.3
Glutamic acid	18.0	20.0	22.4	21.9
Methionine	2.4	3.2	2.5	2.2

[a] N X 6.25, %, dry matter basis
[b] Grams amino acid per 100g amino acids recovered

Table 3—Means, standard deviations (S.D.) and coefficients of variability (C.V.) in protein content[a] and amino acid composition[b] of groats and hulls from three oat cultivars grown in Madison, Wis. in 1971 and harvested at four stages of development

Parameter	Groats Mean	S.D.	C.V.	Hulls Mean	S.D.	C.V.
Protein	19.4	2.20	11.4	5.1	2.18	42.8
Lysine	4.9	0.80	16.3	6.6	0.53	8.0
Histidine	2.2	0.12	5.2	2.0	0.23	11.5
Ammonia	2.6	0.21	7.8	2.8	0.55	19.3
Arginine	6.5	0.37	5.7	4.8	0.47	9.7
Aspartic acid	8.7	0.70	8.0	13.2	1.68	12.7
Threonine	3.5	0.40	11.3	5.1	0.24	4.7
Serine	4.3	0.30	6.8	4.9	0.34	6.9
Glutamic acid	20.7	2.41	11.7	13.9	1.27	9.1
Proline	5.8	0.62	10.7	3.9	2.66	67.8
Half-cystine	1.7	0.27	15.8	0.2	0.20	115.1
Glycine	4.9	0.17	3.4	5.9	0.55	9.3
Alanine	5.3	0.61	11.5	7.5	0.49	6.6
Valine	5.8	0.17	3.0	7.2	0.77	10.7
Methionine	2.6	0.44	16.7	1.8	0.50	27.6
Isoleucine	4.2	0.16	3.7	4.7	0.28	5.9
Leucine	7.6	0.09	1.1	8.4	0.52	6.2
Tyrosine	3.3	0.13	3.9	2.3	0.24	10.5
Phenylalanine	5.1	0.26	5.0	4.6	0.24	5.1
SLTM[c]	11.1	1.37	12.4	13.6	0.78	5.8

[a] N X 6.25, %, dry matter basis
[b] Grams amino acid per 100g amino acids recovered
[c] Sum of lysine, threonine and methionine

Table 4 - Protein content and concentration of essential amino acids[a] in proteins of selected food legumes, cereals, oat groats and oat hulls

Protein and amino acid	Whole egg[b]	Soybean[b]	Peas[b]	Beans[b]	Wheat[b]	Rice[b]	Corn[b]	Oat groats I[c]	Oat groats M[d]	Oat hulls I	Oat hulls M
Protein (%)	12.8	34.9	23.8	21.4	12.3	7.5	10.0	18.1	21.9	7.9	2.9
Lysine	6.4	6.9	7.3	7.4	2.8	4.0	2.9	6.0	4.1	6.4	6.2
Methionine	3.1	1.5	1.2	1.0	1.5	1.8	1.9	2.6	2.3	1.8	1.5
Threonine	5.0	4.3	3.9	4.3	2.9	3.9	4.0	4.1	3.1	4.9	5.3
Isoleucine	6.6	5.9	5.6	5.7	4.3	4.7	4.6	4.3	4.0	4.5	4.8
Leucine	8.8	8.4	8.3	8.6	6.7	8.6	13.0	7.6	7.7	7.8	8.7
Tyrosine	4.3	3.5	4.0	3.9	3.7	4.6	6.1	3.3	3.3	2.3	2.3
Phenylalanine	5.8	5.4	5.0	5.5	4.9	5.0	4.5	4.8	5.4	4.5	4.6
Valine	7.4	5.7	5.6	6.1	4.6	7.0	5.1	6.0	5.7	6.3	8.1

[a] % of total protein
[b] The data for egg, legumes, wheat, rice, and corn are from U.S. Dept. Agr., Home Econ. Res. Rept. No. 4, 1966.
[c] Immature
[d] Mature

were in glycine (5.2 to 6.6%), valine (6.3 to 8.1%) and leucine (7.6 to 8.7%). Those increases were accompanied by very large decreases in concentrations of proline (7.1 to 1.7%) and cystine (0.4 to 0.1%).

Means and variability (grouped together for the three cultivars at four stages of development) in protein content and amino acid composition of hulls and groats are compared in Table 3. Standard deviations and coefficients of variability given in this table represent the sum total of the assay procedure and inherent variability. Average coefficient of variability attributable to the assay procedure in groats is 5.5% (Pomeranz and Robbins, 1972). The coefficient is somewhat larger in sulfur-containing amino acids, but is considerably smaller in other amino acids, such as leucine and phenylalanine. In hulls, the assay variability is greater due to the low protein content and reduced amino acid recovery. Despite the large coefficient of variability in protein of hulls, concentrations of lysine, aspartic acid, threonine and glutamic acid varied little (Table 3). The average coefficients of variability in lysine and threonine in proteins of groats were over twice as high as average coefficients of variability of those amino acids in hulls. Apparently, the proteins deposited in the groat have higher values of lysine and threonine shortly after anthesis, and lower values toward maturity.

The large decrease in proline in the oat hull proteins was accompanied by a small decrease of proline in the oat groat proteins. During development of wheat (Pomeranz et al., 1966) and barley (Pomeranz and Robbins, 1972) concentrations of proline increased. Concentrations of proline in groats from mature oats is only about 5%; concentrations of proline in proteins of barley are 10.7% and of wheat, 10.0%. The increase in concentration of glutamic acid in oat groat proteins was much smaller (from 17.1–23.0%) than in proteins of wheat (from about 27.9–33.8%). The relatively small increase in glutamic acid and decrease in proline during development of oats, along with a fairly uniform and high protein content, are some of the important factors which contribute to the excellent amino acid balance of groat proteins in mature oats.

The excellent amino acid balance of oat proteins is shown in Table 4. This table lists the average (for the 3 varieties, 3 replications and 3 treatments) values for essential amino acids (except trytophan) in groats and hulls from 1 and 4 wk after anthesis. The values are compared with those of essential amino acids in a reference whole egg, soybeans, legumes and the main cereal grains (wheat, rice, and corn). The protein content of groats from mature oats is comparable to that of peas and beans and substantially higher than that of the main cereal grains. The oat groat proteins are much more balanced, in terms of essential amino acid contents, than the wheat and corn proteins. Oat maturation decreased concentration of lysine in the proteins of the groat but not in the hulls; the concentrations of the other essential amino acids in the groats changed little. The nutritional superiority of oat groat proteins, over the wheat or corn proteins, can be attributed to the relatively low concentration in oats of the nutritionally poorly balanced prolamines (Mosse, 1966). The avenin content of oat proteins is about 12%, compared to 45% gliadin in wheat and 50% zein in corn (gliadin and zein are both prolamines). This low concentration of prolamine in oats is accompanied by a high concentration of the nutritionally more balanced globulins in oat proteins (about 78%); wheat and corn contain, respectively, only 10 and 2% globulins (Whitehouse, 1973).

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ILLINOIS PROCESS FOR PREPARATION OF SOYMILK

ABSTRACT

A beverage consisting of water, whole soybeans (including hulls), sugar and flavor has been developed. Preparation includes soaking and then blanching the whole soybeans in 0.5% sodium bicarbonate, grinding with water in a hammermill, heating the slurry to 200°F, homogenizing, neutralizing, dilution, addition of sugar and flavor, pasteurizing and re-homogenizing. Enzyme inactivation by blanching prior to grinding of soaked beans was found to completely prevent formation of painty (oxidized) flavor and result in a bland flavored product. Trypsin inhibitors were also inactivated by blanching. A sufficient degree of tenderization of soybean tissue during the soak and blanch treatments was necessary to obtain good mouth feel and colloidal stability. Homogenizing conditions such as temperature and pressure were also important; when the soybeans had been blanched to a LEE-Kramer Tenderometer reading of 300 lb or below and homogenization was done at 200°F and 3500 psi, the resulting beverage showed zero separation after 2 months refrigerated storage. Dilution to below 1% protein had no effect on colloidal stability. Coulter Counter measurements of the beverage indicated that 81% of the particles fell between 3.4–7.3 microns which is larger than the defined colloidal particle range. Recoveries of protein and total solids based on the raw soybean were 99% and 90%, respectively.

INTRODUCTION

Soybeans are high in protein which contains a well balanced amino acid pattern (Smith and Circle, 1972). The soybean has served as an important source of protein in the diets of Oriental people for many centuries. Despite these nutritional and prior use credits, beverage preparation from whole soybeans has been quite limited in other areas, principally because of the undesirable beany or painty off-flavor and odor. Prior attempts to overcome this problem have, at best, been only partially successful. The undesirable flavor and odor are principally caused by an enzyme system, lipoxygenase (Wilkens et al., 1967). The reaction takes place quickly whenever the beans are ground and exposed to moisture at temperatures below instant enzyme inactivation (Nelson et al., 1971). Bland soybean milk with high protein recovery is of particular interest because it could be an economical protein source for infants, children and adults. It can also be used for all individuals who are intolerant to lactose in cow's milk (PAG, 1972).

Soybean "milks" are conventionally made in the Orient by soaking, grinding in water, filtering to remove sediment and then heating the extract (Piper and Morse, 1923). Although this process is simple, the resulting beverage has a distinct painty (linseed oil) off-flavor and odor. Numerous modifications of this traditional Oriental process have been reported to partially improve the off-flavor and odor defects. Some of these modifications of cold water extracts are: hot water extraction (Wilkens et al., 1967), acid grinding (Kon et al., 1970; Al-Kishtaini, 1971), and alkali soaking (Badenhop et al., 1970). These modifications gave some flavor improvement but generally resulted in lower protein recovery than that obtained with the traditional Oriental process.

Other attempts were made to improve flavor or protein recovery or both. These methods (Hand et al., 1964; Mustakas et al., 1972; Lo, 1971; Miles, 1966) for soybean beverage preparation use dehulled soybeans, are often partially desludged, and some contain added stabilizers for colloidal stability.

The process reported here starts with hydration of the whole soybeans and inactivation of the lipoxygenase enzyme to completely prevent formation of any painty flavor. Thereafter, the soybeans are ground to a slurry and further processed to the finished beverage without filtration or other loss of protein. The effect of the processing steps used on trypsin inhibitor and lipoxygenase inactivation and on colloidal stability was studied. Samples were evaluated by appropriate subjective and objective methods.

MATERIALS & METHODS

Soybeans

The soybean beverage was prepared with Wayne variety soybeans procured from commercial sources. The soybeans were dry cleaned to remove husks, stems, cracked beans and other foreign material with a sample grader (Key Equipment Co., Milton-Freewater, OR). The clean beans contained 11.4% moisture. Trypsin inhibitor assay was performed in duplicate on ground, freeze-dried soybeans following various soaking-blanching treatments using a modification (Hetrick, 1970) of the Kunitz (1947) procedure. In this method, the sample is digested with trypsin, formalin is added, and the carboxyl groups are titrated with sodium hydroxide.

Beverage preparation

The soybean beverages were manufactured according to the method of Nelson et al. (1975). The procedure is as outlined in Figure 1.

Soybean beverage made from dehulled beans. For batches made with dehulled beans, the procedure was as follows: The blanched and drained beans were dehulled with a Buhr mill (Bauer Bros. Co., Springfield, OH). The spacing between plates in the Buhr mill was adjusted to tear and separate the hulls from the cotyledons with minimum damage to the cotyledons. The hulls were separated from the cotyledons by water flotation. The cotyledons were ground with the hammermill and the process continued as described in Figure 1.

Organoleptic evaluation

The organoleptic evaluations were made by five staff members who were quite experienced in judging throat and mouth feel characteristics. The samples were organoleptically evaluated for mouth feel by discussion of product characteristics to arrive at a consensus opinion. Product characteristics were evaluated as follows:

Throat and mouth feel. All samples were shaken just prior to tasting. Particular emphasis was placed on the effect of product coating the oral cavity which produced a drying or catching in the mouth and throat. This objectionable mouth or throat feeling was considered as highly undesirable by the experienced tasters. The consensus rating used the following descriptions: a very smooth product which was similar in mouth feel to high quality cow's milk was described as excellent; this was followed by very good, good, acceptable, not acceptable and poor ratings in decreasing order of desirability.

Apparent colloidal stability. Samples for evaluation had remained undisturbed for at least 24 hr. Judges were instructed to determine whether a visible line of demarcation existed between settled and remaining portions of the beverage. Apparent colloidal stability was indicated by separation at the top of a five inch total height of soy beverage. Separation was measured in inches from the top of the beverage as indicated by a visible demarcation line between two layers.

Objective evaluations

Protein separation. Beverages, after standing undisturbed at 34°F for

a period of time, were sampled using a pipet. Top sample was taken at a point about one-half inch below the surface of the sample, while bottom sample was taken at a point about one-half inch above the bottom. Approximately 15 ml portions were withdrawn at the specified points. The mixed sample (5-in. ht) was removed from the bottle after the contents were thoroughly shaken. The micro-Kjeldahl method (AOAC, 1970) was used to determine total nitrogen which was multiplied by 6.25 to obtain protein.

Viscosity measurement. A falling ball viscometer (Model G, National Instrument Co., Inc. of Baltimore, MD) was used to measure viscosity of the soybean beverage at 20°C after dilution to various protein concentrations.

Analysis of free and bound oil. Free oil in the beverage was determined on a freeze-dried sample using a Soxhlet extraction apparatus with hexane. Total oil was determined on the liquid sample using a modified Babcock test (Al-Kishtaini, 1971). The difference between total and free oil is defined as bound oil.

Tenderness measurement of blanched beans. Tenderness of the blanched beans was measured with the L.E.E. Kramer Shear Press (L.E.E., Inc., Washington, DC). 100g (wet wt) of beans were placed in the test cell (No. C274) and shear force was determined using a 14.5 sec stroke and a 3000 lb test ring. The results are presented as tenderometer values and are expressed as maximum shear force developed.

Particle size distribution. Particle size distribution in the finished soybean beverage was determined with a Coulter Counter according to Wilson (1973).

RESULTS & DISCUSSION

OVER 100 SMALL BATCHES of soybean beverage have been prepared according to the procedure outlined in Figure 1. Many organoleptic evaluations of the product have been made using the laboratory panel. According to our records, at least 1,000 visitors from industry, government and other countries have sampled the beverage. Invariably, the rating and comments regarding flavor and off-flavor have been very good. The product is rated as very bland and free from painty off-flavors. Thus, in the study reported here, the organoleptic factors of flavor and off-flavor are not included.

Trypsin inhibitor destruction

In the Figure 1 process, a 30-min blanch in 0.5% $NaHCO_3$ was used for the whole beans. After blanching, the beans were ground into a slurry which was never held for any length of time at temperatures above 200°F. Therefore, destruction of trypsin inhibitor after various combinations of soaking and blanching was investigated. The results, Table 1, indicated that destruction of trypsin inhibitor is more easily accomplished by blanching of soaked (hydrated) as compared to nonsoaked (dry) beans. Also, trypsin inhibitor can be more readily destroyed when $NaHCO_3$ is added to the blanch water regardless of soaking treatment (Albrecht et al., 1966). The beans soaked overnight in either 0.5% $NaHCO_3$ or water and then given a 5-min boiling blanch in either 0.5% $NaHCO_3$ or water were negative for trypsin inhibitor. Thus, a 30-min blanch in 0.5% $NaHCO_3$ following an overnight soak, as described in Figure 1, would result in a beverage completely free from trypsin inhibitor.

Lipoxygenase enzyme inactivation

When raw tissue of the soybean cotyledons is damaged or broken, the lipoxygenase enzyme as well as the lipid substrate are liberated. As long as the tissue is dry (about 13% moisture) the enzyme does not catalyze the instant oxidation of the substrate. However, if water is added, a reaction takes place rapidly and produces a highly objectionable beany or oxidized odor and flavor which are acceptable only to Oriental groups who have developed a tolerance to it (Nelson et al., 1971).

Problems associated with inactivation or control of this enzyme system were recognized as the development of the process (Fig. 1) was undertaken. It was found that sound, whole, raw beans could be soaked for 8—12 hr until their weight was doubled prior to blanching without developing the beany, painty off-odors and flavor. However, it was necessary to handle the soaked beans gently during draining and handling. The soaked beans were blanched by dropping directly into boiling water. After this prescribed blanch (Fig. 1), the beans were bland in taste and completely free from all off-flavor and odor.

Alternatively, whole, dry, raw beans could be dropped into boiling water for hydration and blanching in one operation. This blanched product was equally desirable but longer blanch times were required, as shown below.

Quality of beverage

Effect of soaking, blanching and homogenization condi-

Fig. 1—Preparation of beverage from whole soybeans

1.	Soybeans	Dry whole soybeans (Wayne variety)
2.	Soak	Soak overnight in tap water (84 ppm $CaCO_3$ hardness) solution of 0.5% $NaHCO_3$ (1:3::bean:solution)
3.	Drain	Drain
4.	Blanch	Blanch in fresh tap water solution of 0.5% $NaHCO_3$ for 30 minutes (1:3::original dry beans:solution)
5.	Drain	Drain and tap water rinse
6.	Add water	Grind through hammermill (Fitzpatrick, Model D) with sufficient tap water to make 12% bean solids,
7.	Grind	first through 0.25 inch opening screen then through 0.028 inch opening screen
8.	Heat	Heat slurry to 200° F in a steam jacketed kettle
9.	Homogenize	Homogenize at 3500 psi (first stage pressure) 500 psi (second stage pressure with a Gaulin Model 15 M8TA)
10.	Add water	Mix slurry with tap water to adjust protein content to desired level
11.	Neutralize	Neutralize with 6 *N* HCl to pH 6.8 to 7.2
12.	Formulate	Add Sugar, Salt and Flavoring 5.0% Sucrose 0.2% NaCl 0.02% Vanillin 0.007% starter distillate
13.	Heat	Heat to 180° F
14.	Homogenize	Homogenize as above
15.	Bottle	Bottle
16	Cool	Cool and Store at 34° F

Table 1—Soybean Trypsin Inhibitor (STI) assay of soaked and unsoaked blanched beans

Soak treatment	Blanch soln	Blanch time (min)	mg STI per g soybeans	% STI destroyed
Overnight 0.5% $NaHCO_3$	0.5% $NaHCO_3$	5	0.0	100
		10	0.0	100
Overnight water	water	5	0.0	100
		10	0.0	100
Overnight water	0.5% $NaHCO_3$	5	0.0	100
		10	0.0	100
None	water	10	6.0	79
		20	0.0	100
None	0.5% $NaHCO_3$	10	4.4	85
		20	0.0	100
0.5% $NaHCO_3$ for 4 hr	0.5% $NaHCO_3$	5	0.6	98
		10	0.0	100
None	None	None	28.2	0.0

Table 2—Tenderometer value after blanching of unsoaked or soaked soybeans and quality of beverage after homogenizing under various conditions

Blanch treatment	Tenderometer value, lb per 100g	Homogenization conditions Two passes Temp °F	Pressure psi	Quality of beverage
Unsoaked soybeans				
30 min, water	310	180	3500	poor mouthfeel, separated on standing
30 min, 0.5% $NaHCO_3$	185	180	3500	borderline on mouthfeel and stability
30 min, 0.5% $NaHCO_3$	185	210	4500	good mouthfeel and stability
Soybeans soaked 8 hr in 0.5% $NaHCO_3$				
5 min, 0.5% $NaHCO_3$	250	180	3500	borderline mouthfeel and stability
5 min, 0.5% $NaHCO_3$	250	210	8000(1st) 3500(2nd)	highly acceptable mouthfeel and excellent colloidal stability
20 min, 0.5% $NaHCO_3$	165	180	3500	Highly acceptable mouthfeel and excellent colloidal stability

tions. As previously discussed, the need to inactivate lipoxygenase and trypsin inhibitor was apparent early in the development work. However, the degree of blanching required to achieve an acceptable tenderness of the tissue for effective milling and homogenization to prepare beverage of good quality, required considerable investigation. The results are summarized in Table 2.

Starting with unsoaked soybeans and simultaneously hydrating and blanching for 30 min in hot tap water resulted in a tenderometer reading of 310. Subsequent homogenization at 180°F and 3500 psi pressure (2 passes) resulted in a final product of poor mouth feel and colloidal stability. Direct hydration and blanching in 0.5% $NaHCO_3$ solution resulted in a blanched bean tenderometer reading of 185. When the homogenizing pressure was 3500 psi, the finished product was borderline as regards mouth feel and colloidal stability. However, increasing homogenization pressure from 3500 to 4500 psi resulted in much improved mouth feel and colloidal stability which was rated as very good. Thus, the data (Table 2) demonstrated the favorable effects of 0.5% $NaHCO_3$ in the blanch solution as well as increased homogenization pressure when starting with unsoaked beans.

Table 2 also presents data on the effect of blanching time in 0.5% $NaHCO_3$ solution and homogenization temperatures and pressures after the beans had been soaked overnight in 0.5% $NaHCO_3$ solution. A 5-min blanch resulted in a tenderometer reading of 250 and when the bean slurry was heated to 180°F and homogenized at 3500 psi (2 passes), the final product was borderline in colloidal stability and mouth feel. However, using the same blanch conditions with a 210°F slurry temperature and 8000 psi on first homogenization and 3500 psi on second homogenization, resulted in a beverage of very good colloidal stability and mouth feel. A 20-min blanch in $NaHCO_3$ solution of the beans soaked in $NaHCO_3$ solution resulted in a tenderometer reading of 165. This slurry, at a temperature of 180°F, was homogenized at 3500 psi (2 passes) and the resulting product was rated as very good to excellent in colloidal stability and mouth feel.

This study showed the advantage of a presoak of whole beans before blanching, as well as the effect of 0.5% $NaHCO_3$ on improving tenderization of soybeans. Also, it is apparent that a substantial increase in homogenization pressure can counteract the effects of the firmer texture of beans resulting from a shorter blanch. Thus, the quality of beverage, especially colloidal stability and mouth feel, as shown in Table 2, indicated that the tenderometer value of blanched beans was not solely responsible for the acceptability of the final product. Beans with high tenderometer value can be made into colloidally stable – good mouth feel product by raising either homogenization pressure or temperature. Therefore, the interaction between number of passes through the homogenizer, homogenizing pressure and slurry temperature at time of homogenization were investigated.

The results (not shown) indicated that a single homogenization at 200°F can yield a satisfactory product from the standpoint of colloidal stability and mouth feel provided the homogenization pressure is increased to at least 6000 psi. Table 3 shows the effect of temperature at time of homogenization. None of these samples showed separation. Double homogenization, both cold, gave an acceptable mouth feel and colloidal stability. A combination of hot homogenization and then cold homogenization or hot-hot homogenization resulted in a beverage of better colloidal stability and mouth feel than cold homogenization followed by hot. Table 4 shows the interaction between first and second homogenization pressures on beverage quality. Otherwise, the preparation was as in Figure 1. The results indicated that with either 1500 or 2500 psi first homogenization pressure, increasing pressure of second homogenization improved colloidal stability and mouth feel. When 1500 psi initial pressure was used, a second pressure of 5000 psi was required to give very good quality product, while at 2500 psi initial pressure, a good product was obtained with 4000 psi second pressure. Thus, with double homogenization, an important consideration seems to be a summation of the two pressures.

Mouth feel determination data, as presented in Tables 3 and 4, cannot be considered as absolute. However, the trends shown in the data are clear and the effects of various combinations of pretreatments, number of passes through the homogenizer and the homogenizer pressures are evident. Certain-

Table 3—Effect of temperature at time of homogenization

Max temp before first homogenization at 3500 psi, °F	Max temp before second homogenization at 3500 psi, °F	Mouth feel
60	60	Good
60	180	Very good
200	60	Excellent
200	180	Excellent

ly, very high homogenization pressures are desirable to obtain very good mouth feel in the finished product. However, a commercial process based on exceedingly high homogenization pressure presents obvious practical drawbacks. Thus, the use of 3500 psi was finally selected (Fig. 1) because it gave good results and was reasonable from a processing standpoint.

Colloidal stability. Instability of a beverage colloidal system can be a severe problem, particularly in case of soy-based beverages. Therefore, it was important to evaluate the stability of the present beverage after standing quiescent at 34°F for various times. Organoleptic evaluation was done by (1) tasting the product and judging for mouth feel, and (2) visual observation of the bottle for evidence of settling. Objective evaluation was by determining protein (1) at the top and (2) bottom of the quiescent bottle, and (3) after thoroughly mixing the bottle contents to obtain a composite reading; uniformity of protein among the three samples was taken to mean lack of settling and good stability. Soymilks were prepared using either whole (with hull) or dehulled soybeans; dilution of soybean solids was also a variable.

The data in Table 5 showed that standing times at 34°F for as long as 7 days had no effect on the organoleptic properties of mouth feel and visual separation. In case of the objective results (not shown), the protein contents at top and bottom of the bottles were the same as in the mixed sample. Thus, a good colloidal stability was obtained both with and without the presence of hull, which contains much fiber. Also, the stability was not related to soybean protein concentration. Viscosity of the beverage may be affected by both of these processing variables. To study this, samples were prepared with and without hulls at several solids contents and the viscosity determined. The data in Table 6 show that, indeed, presence of hull and higher solids increased the viscosity. Therefore, it was inferred that stability of the beverage is not related to its viscosity.

Subsequent observations have shown the pasteurized beverage, with or without hulls, to remain stable at 34°F with no separation for about 2 months; microbial spoilage with acid formation and gelation takes place before separation (Nelson et al., 1975). The question arose as to whether particle size was responsible for this amazing stability. Coulter Counter measurements, Table 7, showed that about 10% of the particles were over 10 microns and 80% of the particles were 3.4–7.3 microns in diameter. Even more interesting was the absence of particles 2.7 microns and smaller. This size is well above that normally considered the upper limit for colloidal particles and falls in the range of *un*homogenized milk. Thus, this system is not a true colloidal system and particle size distribution is not solely responsible for the good stability of this beverage.

The Wayne variety soybean used in this experiment contained approximately 40% protein and showed 20% oil by a Soxhlet extraction with hexane; however, freeze-dehydrated beverage containing 3.1% protein on a liquid basis showed no oil recovered by the same method. When the dry beverage was digested with sulfuric acid according to the modified Babcock test, 1.7% oil was recovered; this is about the same ratio of fat to protein as in the soybeans. Thus, it appears that the oil in this soybean beverage is bound by complex chemical forma-

Table 4—Interaction between first and second homogenization pressures

Homogenization pressure		Colloidal stability	
First (psi)	Second (psi)	in. of separation	Mouth feel
1500	0	1/2	Poor
1500	500	1/2	Not acceptable
1500	1000	1/4	Not acceptable
1500	2000	1/8	Not acceptable
1500	3500	1/8	Not acceptable
1500	5000	0	Very good
1500	6000	0	Excellent
2500	0	1/2	Not acceptable
2500	500	1/2	Not acceptable
2500	1000	1/2	Not acceptable
2500	2000	1/4	Not acceptable
2500	4000	0	Good
2500	5000	0	Excellent

Table 5—Evaluation of beverages prepared with and without hulls and allowed to stand undisturbed at 34° F for various times

Sample	Time of standing	Throat and mouth feel	Separation measurement in. of separation
With hulls	4 hr	Good	0.3
Dehulled	4 hr	Very good	0
With hulls	16 hr	Very good	0
Dehulled	16 hr	Very good	0
With hulls	5 days	Very good	0
With hulls	7 days	Good	0

Table 6—Viscosity of soy beverage samples prepared with and without hulls and to cover a range of protein contents

Protein conc (%)	Viscosity CPS at 25°C
With hulls	
2.8	10.8
2.0	7.0
1.5	3.7
1.0	3.1
Dehulled	
4.6	14.0
2.8	6.3
1.5	3.0

Table 7—Particle size distribution by Coulter Counter of whole (not dehulled) soybean beverage

Diameter in microns	Count[a]	% of Total
2.7	0	0
3.4	577	10.6
4.6	2,141	39.3
5.8	962	17.6
7.3	764	14.0
9.2	430	7.9
10.5	301	5.5
11.6	179	3.2
13.5	56	1.0
>13.5	42	0.8
Total	5,452	99.9

[a] 50 mμ of sample after a beverage of 3.5% protein content was diluted 4000 times.

tion. Therefore, it is presently believed that tenderization of the soybeans in combination with homogenization of the slurry results in the formation of hydrophilic protein-lipid complexes which are responsible for the beverage stability.

Protein and solids recovery

Two lb of Wayne variety soybeans were soaked 10 hr at ambient temperature in 0.5% $NaHCO_3$ in 3:1 solution:bean ratio. The final soak solution (3.6 lb) contained 0.94% dry matter and 0.17% protein, wet basis. After blanching the soaked soybeans in fresh 0.5% $NaHCO_3$ for 30 min, the solution weighed 2.6 lb and contained 5.27% dry matter and 1.09% protein, wet basis. The blanched soybeans, as calculated by difference, contained 1.57 lb dry matter and 0.696 lb protein. On this basis, 95% of the protein and 89% of the total solids in the raw beans were recovered in the blanched beans. Thereafter, there are no further losses of protein or other solids due to processing.

This 5% loss of protein was investigated to determine what proportion was actually protein since a certain amount of nonprotein nitrogen should be expected to leach out of the soybean. The Kjeldahl determination included, of course, inorganic N and organic nonprotein N, as well as protein, but the Biuret test is specific for the peptide bond. Therefore, the soak and blanch solutions were analyzed for protein with Biuret; the results showed 0.034% and 0.16%, respectively. Calculations showed that the total weight of Biuret protein in the two solutions was less than 1% of the crude protein (Kjeldahl N x 6.25) in the soybeans. This shows that the protein loss found above was essentially nonprotein constituents. From this it was concluded that the final beverage contains essentially 100% of the protein present in the soybean.

However, the loss of total bean solids was calculated to be 11%. Thus, this process did remove bean constituents other than protein, such as oligosaccharides. Substantial extraction of oligosaccharides during hydration and cooking of the soybeans has been demonstrated (Ku, 1972).

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EFFECT OF SODIUM ALKALIS AND SALTS ON pH AND FLAVOR OF SOYMILK

ABSTRACT

Sodium hydroxide, sodium carbonate and sodium bicarbonate were added to soymilk made by the "boiling-water grind" process. NaOH caused a rapid increase in pH; Na_2CO_3 a moderate increase and $NaHCO_3$ a slight increase in pH. After sterilization in bottles the pH of the soymilks containing alkali decreased, with the decrease least with the $NaHCO_3$ treatment and greatest with the NaOH treatment. The pH of the untreated soymilk did not change during sterilization. An experienced taste panel showed greater acceptability for soymilk adjusted to pH 7.0–7.5 with NaOH but noted a soapy flavor and gave lower scores for milks with pH greater than 7.5. Soymilks adjusted to pH 7.0–7.5 with Na_2CO_3 or $NaHCO_3$ were disliked by the panel. We theorized that the improved acceptability with NaOH could be due to the increase in sodium ion concentration rather than the change in pH. Soymilks prepared with Na_2CO_3, $NaHCO_3$, $NaNO_3$, Na_2SO_4, Na acetate and Na citrate additions at the same levels as that amount of NaOH required to raise the pH to 7.2 were given approximately the same scores by the panel as the NaOH-treated sample even when the pH was not in the range of 7.0–7.5. This evidence supports the theory that the sodium ion concentration is the effective mechanism in improving the flavor of soymilk rather than the change in pH.

INTRODUCTION

SOYMILK is the aqueous extract of dry, mature soybeans. It is made by grinding the beans with water and filtering off the insoluble residue. The product is a milky-looking liquid that usually contains about 80% of the protein and about 60% of the fat of the whole bean. The basic process has been known in the Orient for about two milleniums.

Since soymilk is relatively easy to prepare, and has a protein content and quality that approximates that of cow's milk, it has been the subject of considerable interest to those organizations that wish to make a low-cost protein beverage available to those areas of the world where protein malnutrition is a problem. Unfortunately soymilk has a strong, characteristic "beany" flavor that makes it unacceptable to non-Oriental populations. The poor acceptability of soymilk has thwarted efforts to introduce this nutritionally valuable beverage to low-income populations.

Wilkens et al. (1967) found that the off-flavors of soymilk were not present in the dry soybean but were formed during the processing and that grinding the beans in boiling water prevented the formation of the strong beany flavors. They attributed this result to the rapid heat-inactivation of the lipoxidase in the soybean precluding its attacking the unsaturated fatty acid chains in the soybean oil to form a number of lower molecular weight compounds that have objectionable flavor impact. Many of the volatiles found in soymilk prepared by the conventional grinding at ambient temperatures have been isolated and identified (Mattick and Hand, 1969; Wilkens and Lin, 1970).

Soymilk made correctly by the boiling-water grind method is free of the strong beany flavors; it has a faint, pleasant cereal-like flavor. Since the objectionable flavor can be eliminated it seems that soymilk should be reconsidered as a low-cost protein beverage for developing countries. Steinkraus et al. (1968) and Bourne (1970) extended the new process to a pilot plant operation in the Philippines and studied factors that would affect the widespread acceptance of soymilk in a tropical country.

There is a common belief that the use of a mild alkali such as sodium bicarbonate improves the eating quality of cooked common dry beans, although it is not always clear whether the improvement is in the flavor, the texture, or both (e.g., Buckeye Cookery, 1883; Snyder, 1936). The use of alkali seems to be partly effective in improving the organoleptic quality of conventional cold-grind soymilk because there are frequent references to the use of sodium bicarbonate (baking soda) in recipe books that describe how to make soymilk (e.g., Monahan and Pope, 1915).

Badenhop and Hackler (1970) studied the effects of soaking soybeans in sodium hydroxide solutions on various aspects of soymilk quality using the boiling-water grind technique to prepare the soymilk. They found that as the pH of the soymilk increased from 6.55 (water soak) to 9.18 (soak in 0.097N NaOH) the protein efficiency ratio (PER) decreased from 2.41 to 1.70; niacin increased from 3.96 mg/100g at pH 6.55 to 4.98 at pH 8.04 and declined to 3.69 at pH 9.18. They also noted that a taste panel composed of Americans preferred the flavor of soymilk of pH 7.37 (soybeans soaked in 0.048N NaOH) over samples with higher or lower pH.

Steinkraus et al. (1968) studied some effects of alkali on soymilks in the Philippines using a Filipino taste panel. They found with soymilk prepared from Taichung variety of soybeans the panel was unanimous in preferring milk made from beans that had been soaked in 0.1% NaOH to those soaked in water. However, with the Hsieh-Hsieh variety the panel was evenly divided in preference between soymilks made from water-soaked and 0.1% NaOH-soaked beans. They further noted that the taste panel was unanimous in preferring soymilk containing 0.15% $NaHCO_3$ to those containing no sodium bicarbonate. In a subsequent study, Puertollano et al. (1970) examined the effect of changes in formulation of soymilk on its acceptability by Filipino school children. They reported that soaking soybeans in 0.1% NaOH prior to the boiling water grind process gave a soymilk with a slight, but not significantly higher acceptability over the soymilk that had been prepared from beans soaked in water only. They also noted that adult Filipinos noted a "soapy" flavor in soymilk made from beans soaked in 0.1% NaOH and concluded that the questions of alkali additions and flavor of soymilks required further study.

Khaleque et al. (1970) soaked soybeans in solutions of Na_2CO_3, $NaHCO_3$, NaOH, Na_2SO_3, Na_2HPO_4, Na_3PO_4, NaCl and a mixture of $Na_2S_2O_5$ plus NH_4OH in concentrations of 0.2–0.8M and then made soymilk from the soaked beans. They concluded that soymilk made from beans that had

been soaked in 0.4M Na_2CO_3 had the lowest level of beany flavor. These authors questioned the lipoxidase theory of beany flavor formation because all their soymilks had some beany flavor. However, it seems that they did not achieve a temperature sufficiently high enough to inactivate the lipoxidase. They soaked 100g dry soybeans in solution, removed the skins, and then ground the beans in a blender with 250 ml of boiling water. After grinding, the volume was made to 700 ml. Since the soaked weight of the beans would have been about 200g, the mixture of beans at ambient temperature and 250 ml of boiling water would have a temperature considerably below the minimum of 80°C required to prevent beany flavor formation. A strong beany flavor would have developed in the grinding operation under these conditions, and subsequent heating and dilution would not remove it completely. This is confirmed by their results; they found that all samples had the beany flavor.

Koski and Smith (1972) describe a process for making soymilk in which the soy ingredient was defatted flakes. The flakes are extracted in a sodium hydroxide solution with a pH of about 12, then the solution is brought back to a pH of 7–8 by adding an acid such as citric acid. The use of alkali in this case is presumably to assist in solubilizing the protein rather than to produce a bland flavor.

This experiment reports on studies on the effect of alkali additions to the bland-flavored soymilk made by the boiling-water grind process, using an experienced taste panel of eight young adult Filipinos to evaluate the products.

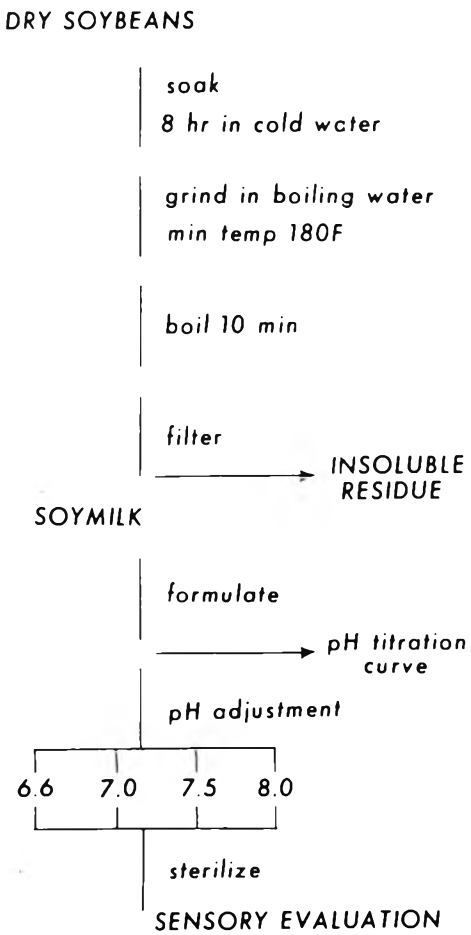

Fig. 1–Schematic representation of experimental procedure.

EXPERIMENTAL

Preparation of soymilk

Soybeans of the Biyeloxi 256 variety, obtained from the Philippine Bureau of Plant Industry were soaked for approximately 8 hr in plain water at ambient temperature, then drained, washed in fresh water and drained again. A Rietz Disintegrater fitted with a stainless steel screen with 0.032 in. perforations was preheated by passing a quantity of boiling water through it. Small quantities of soaked beans and boiling water were metered into the grinder at frequent intervals using almost all of the required water. A food-grade silicone antifoam spray was used to control foaming. Great care was taken to maintain the temperature of the bean-water slurry above 80°C at all times during the grinding operation because previous experience had shown that if the temperature falls below 80° C for even a short period of time some beany flavor will be present. The slurry was then boiled with constant stirring in a steam-jacketed kettle for 10 min, after which the total weight was adjusted to give a bean:water ratio of 1:10 by adding that small amount of water necessary to give the correct final weight (1 kg of dry beans yields 11 kg of slurry). The resulting slurry was filtered through a plate-and-frame filter press. The insoluble residue was discarded and the soymilk was formulated by adding 7% sugar and 20 ppm of a commercial essence of a vanilla. This was the basic product that was used in all subsequent tests.

pH titration curve

300 ml samples of soymilk were titrated with 1.25N solutions of NaOH, $NaHCO_3$ and Na_2CO_3, using a glass electrode pH meter. The sample was stirred continuously be means of a magnetic stirrer during the titration.

Effect of alkali and heat

A row of 600 ml beakers was set up with 300 ml of soymilk in each beaker. A calculated amount of alkali (based on the pH titration curve) was added to each beaker to raise the pH by approximately 0.2 units over the preceding beaker. Sodium hydroxide and sodium carbonate were added in the form of 1.25N solutions. Sodium bicarbonate was added as a dry powder because the volume of solution needed to achieve the desired pH level would have caused excessive dilution of the soymilk. The pH of each sample was measured after the alkali addition. Approximately 210 ml of each sample were transferred to a 7-fl oz bottle, sealed with a crown seal, and sterilized in steam in a retort using a process of 12 min at 121.1°C (250° F). The pH of the sterilized products was measured the following day.

Flavor study

Based on the results of the work mentioned above, calculated amounts of the three alkalis were added to soymilk to give pH values of 7.0, 7.5 and 8.0 after sterilization in the bottle. The actual pH was measured just before presentation to the panel. The samples were stored for approximately 2 wk at ambient temperature before submission to the panel. Figure 1 is a schematic representation of the preparation method.

The panelists were mostly young adult Filipinos with approximately equal numbers of each sex. The members of the panel had had considerable previous experience with tasting soymilks. A 9-point hedonic scale was used; a score of 1 = dislike extremely, a score of 5 = neither like nor dislike, and a score of 9 = like extremely.

When the results with the three sodium alkalis suggested that pH was not the cause of flavor improvement, we studied the effect of adding sodium salts (nitrate, sulfate, acetate and citrate) at levels that would give sodium ion concentrations of 1.50, 2.62 and 3.75 milliequivalents in the soymilk. Sodium chloride was not used because it was found that the salty flavor of the chloride ion would mask any effects of the sodium ion.

RESULTS & DISCUSSION

FIGURE 2 shows the pH titration curves for the three alkalis. As would be expected from the relative strengths of the alkalis, NaOH gave the most rapid increase in pH, followed by Na_2CO_3 and $NaHCO_3$ in that order. The addition of only 3.5 ml of the NaOH solution raised the pH of the soymilk to 10. Five ml of the Na_2CO_3 solution were required to raise the pH to about 9.0 after which further additions gave only a slight increase in pH. The highest pH obtainable with Na_2CO_3 addition was about 9.5. The $NaHCO_3$ raised the pH very slowly; about 30 ml of solution were required to raise the pH to 8.0

and further additions had negligible effects on the pH. A mixture of 100 ml of 1.25N $NaHCO_3$ and 300 ml of soymilk showed a pH of 8.2.

Figures 3, 4 and 5 show the effect of the combination of alkali addition and a heat process of 12 min in live steam at 121.1°C (250°F). The pH of the alkali-treated soymilks fell as a result of the heat treatment but there was no change in pH as a result of the heat treatment of the alkali-free soymilks. For NaOH the pH decrease became larger as the amount of added NaOH increased up to a maximum of about 2.5 ml of NaOH solution when the pH difference was 1.0 pH unit. The pH difference remained constant at 1.0 unit with additions greater than 2.5 ml of NaOH solution. A similar effect was found with the addition of Na_2CO_3; the pH after sterilization was lower than before sterilization, and the increase became greater as the amount of added Na_2CO_3 increased to about 4.5 ml of the solution when the pH difference was 0.9 unit. With further additions of Na_2CO_3, the pH difference remained at 0.9 pH unit. The $NaHCO_3$-treated soymilks showed a small drop in pH after sterilization. The addition of 5 ml of $NaHCO_3$ solution gave a pH difference of 0.16 unit, and the difference remained at this level with all greater additions. It is likely that the reactivity of the alkali on some constituent of the soymilk is responsible for this type of behavior. For example, at pH 7.5 before heat sterilization the following decreases were observed after sterilization: for NaOH, a fall of 0.45 to give a pH of 7.05; for Na_2CO_3, a fall of 0.40 to give a final pH of 7.10; and for $NaHCO_3$, a fall of 0.15 to give a final pH of 7.35. Some representative pH difference figures are shown in Table 1.

The results of the acceptability score vs pH after sterilization are shown in Figure 6. For NaOH-treated milks there was an increase in the acceptability score at pH 7.0 and 7.5 over the untreated soymilk but this was followed by a marked decrease in acceptability at pH 8.0. For Na_2CO_3-treated soymilks the acceptability decreased with every increase in pH; there was no initial increase in acceptability similar to that shown by the NaOH. The $NaHCO_3$-treated soymilk showed no initial increase in acceptability; the acceptability decreased and at a much faster rate than for the Na_2CO_3 treatments. The panelists noted that flavors described as "bitter," "soapy" and "sticky after-taste" were present in the soymilks at the higher pH values. Some curdling of the soymilk was observed in the sample that had its pH raised to 7.5 by the addition of $NaHCO_3$.

The data in Figure 6 could lead to the conclusion that Na_2CO_3 and $NaHCO_3$ are completely unsuited for flavor improvement of soymilk. It will now be shown that such a conclusion is both superficial and incorrect.

If the acceptability of soymilk were a function of pH, then the flavor score should be the same regardless of which alkali was used to adjust the pH. The evidence in Figure 6 leads to the conclusion that the flavor acceptability of soymilk is not

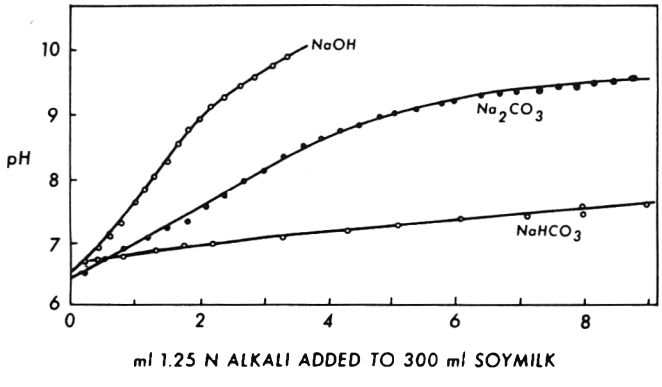

Fig. 2—pH titration curves for three alkalis and soymilk.

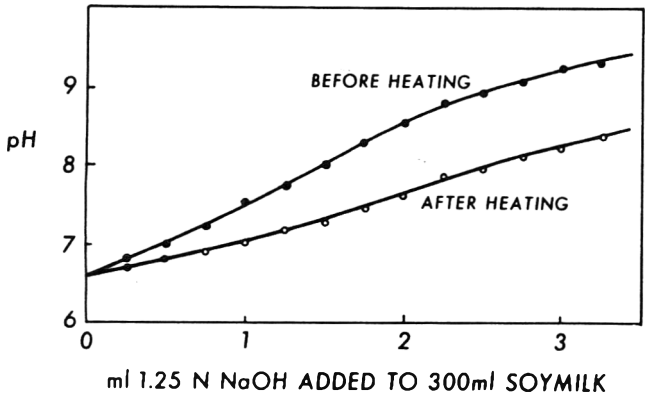

Fig. 3—Effect of heat processing on pH values of NaOH-treated soymilk.

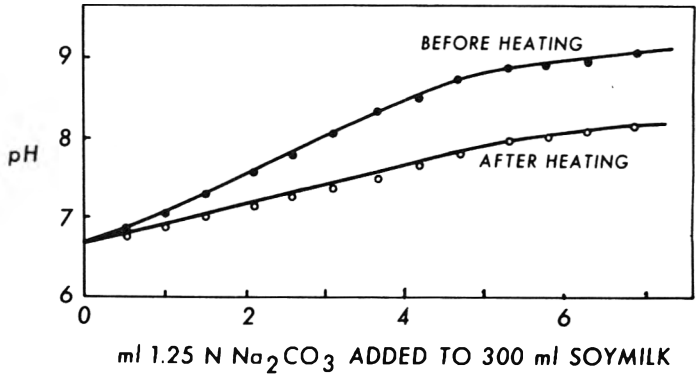

Fig. 4—Effect of heat processing on pH values of Na_2CO_3-treated soymilk.

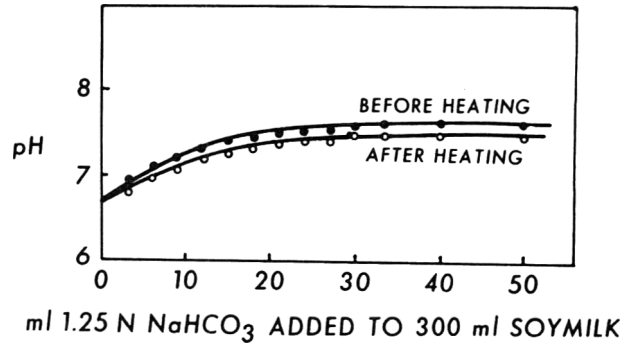

Fig. 5—Effect of heat processing on pH values of $NaHCO_3$-treated soymilk.

related directly to the pH since at each pH there are widely different acceptability scores, depending on which particular alkali was used to adjust the pH.

A question then arises as to the factor responsible for the observed changes in flavor of soymilk as the result of alkali additions. One significant factor is the amount of each alkali required to achieve a given pH. It requires 1.0 ml of NaOH, 1.8 ml of Na_2CO_3, and 7 ml of $NaHCO_3$ solutions (1.25N) to raise the pH of 300 ml of soymilk to pH 7.0 after sterilization. We hypothesized that the sodium ion concentration could be the key factor in these flavor studies. The added sodium ion concentrations from the NaOH-treated soymilks of pH 7.0, 7.5 and 8.0 are respectively 1.50, 2.62 and 3.65 milliequivalents; therefore, we made a new series of alkali-treated soymilks in which 1.50, 2.62 and 3.65 milliequivalents of sodium ion were added to soymilks, with the sodium being provided respectively by NaOH, Na_2CO_3 and $NaHCO_3$. An additional series of experiments was run in which the same levels of sodium ion were added to the soymilk with the sodium supplied by four salts: $NaNO_3$, Na_2SO_4, Na acetate and Na citrate.

The results for the addition of three alkalis at equal sodium ion concentrations are shown in Figure 7. The striking feature here is the similarity in acceptability scores for all alkalis. In each case there is an increase in acceptability at the 1.50 and 2.62 milliequivalent sodium ion levels, followed by a decrease at the 3.65 milliequivalent level. This evidence supports the hypothesis that it is the sodium ion concentration that affects the flavor of soymilk rather than the pH.

Figure 8 shows the effect on acceptability of the same three levels of sodium ion concentration in soymilk with the sodium supplied by four sodium salts. The sodium citrate and sodium sulfate gave curves that are similar to those obtained with the alkalis. The nitrate and acetate salts appeared to have no effect on the acceptability scores at the levels tested.

An analysis of variance of the pooled data shown in Figures 7 and 8 shows that the differences in acceptability between sodium ion concentrations is highly significant (1% level on the F-test), while the differences between sodium compounds is not significant.

This evidence is interpreted as an indication that the sodium ion is the principal causative factor in the effects of sodium alkalis or sodium salts on the flavor of the bland-tasting soymilks that are made by correctly using the boiling-water grind method of preparation. Although not conclusive, it seems probable that the anion associated with the sodium ion may have a secondary effect. It should be noted that in the case of the alkalis the flavor improvement decreased in a reverse sequence to the alkali strength. The invarying acceptability of the nitrate and acetate with change in concentration is unexplained. The higher score for the sodium citrate, although not significant in this experiment, suggests that the citrate ion may exert an improvement in the flavor in addition to the improvement given by the sodium ion. Additional

Table 1–Effect of heat and alkali on pH of soymilk

pH before heating	pH after heating		
	NaOH	Na_2CO_3	$NaHCO_3$
6.60	6.60	6.60	6.60
7.00	6.80	6.82	6.84
7.50	7.05	7.10	7.35
8.00	7.25	7.35	–
8.50	7.60	7.60	–
9.00	8.00	8.10	–
9.50	8.50	–	–

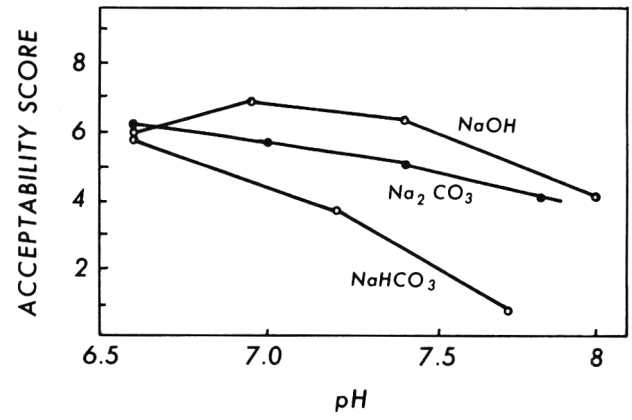

Fig. 6–Acceptability scores and pH values of soymilks treated with NaOH, Na_2CO_3 and $NaHCO_3$.

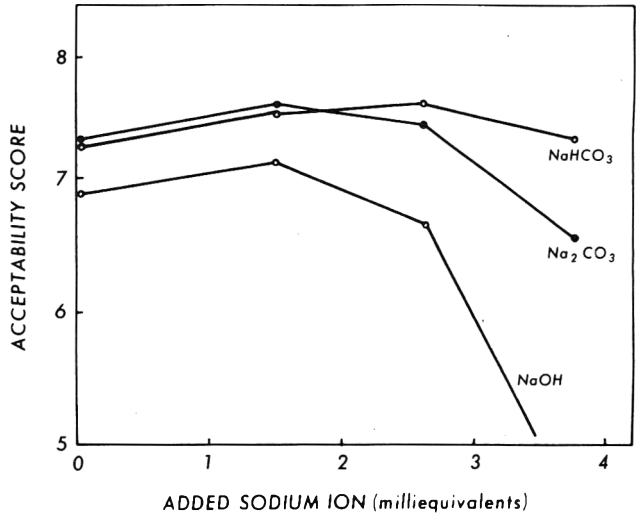

Fig. 7–Acceptability scores and added sodium ion concentration of soymilks treated with NaOH, Na_2CO_3 and $NaHCO_3$ (the final point for the NaOH curve lies outside the graph).

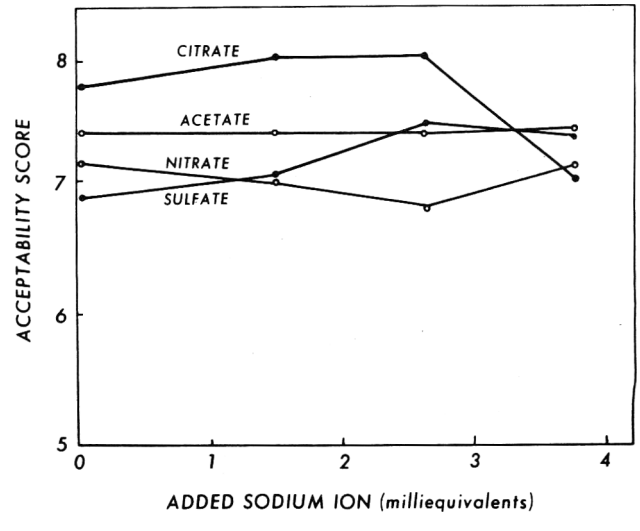

Fig. 8–Acceptability scores and added sodium ion concentration of soymilks treated with $NaNO_3$, Na_2SO_4, Na citrate and Na acetate.

studies are needed to determine the cause of the anomalous behavior of the nitrate and acetate ions, and whether the effect of the citrate ion is additive or synergistic to the sodium ion.

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DOUGH CONDITIONERS FOR 12% SOY-FORTIFIED BREAD MIXES

ABSTRACT

Dough conditioners in waxy solid and fluid forms were prepared by (1) reactions of starch or lactose with glycerol or propylene glycol; (2) alkoxylation of polyol glycosides with ethylene oxide or its mixture with propylene oxide; and (3) transesterification with methyl esters of oleic, stearic or palmitic acid or triglyceride mixtures. Dry mixes of these experimental dough conditioners were prepared by chilling and grinding the waxy conditioners to powder form and blending with defatted soy flour. Grinding of the waxy products was facilitated by incorporation of up to 10% fully hydrogenated soybean oil flakes (HSF). Fluid conditioners were added directly and blended with soy flour. When tested at the 0.5% level in 12% soy-fortified wheat bread, functional properties were the same whether the experimental dough conditioners were added to the bread formula either by dissolving in water or as a dry mix with the soy flour and wheat flour. Baking results were not affected by the incorporation of up to 10% HSF with the waxy products. The 12% soy-fortified bread containing waxy or fluid dough conditioner had substantially improved loaf volume, grain and crumb color when compared to control bread with no conditioner. FDA approval of these new dough conditioners for food use has not yet been obtained.

INTRODUCTION

WHEAT FLOURS can be fortified with soybean flours to improve their nutritive value (Pomeranz, 1966). Defatted soy flour contains about 52% protein and 3.6% lysine, an essential amino acid. Wheat flour intended for breadmaking contains about 12% protein and 0.38% lysine. When wheat flour is fortified with 12% soy flour, the levels of protein and lysine are about 16.8% and 0.76%, respectively (Tsen, 1974).

Fortification of bread wheat flour with soy flour can cause undesirable effects on dough properties and bread quality (Tsen et al., 1971a). Most noticeable in finished bread products are: darker crumb color, more open and coarser grain and reduced loaf volume. Several fatty acid derivatives reportedly improve the baking characteristics of bread made with soy-fortified wheat flour. The addition to wheat flour of natural glycolipids (Pomeranz et al., 1969a, b) from wheat or quaking grass or of sucrose esters permitted fortifying with up to 16% soy flour without a substantial loss in the bread's physical properties. The addition of sodium stearoyl-2-lactylate (SSL) to wheat flour fortified with as much as 12% soy flour also maintained high-quality bread characteristics (Tsen and Hoover, 1971b). The 12% level of soy fortification of wheat flour containing SSL was found by previous workers (Tsen et al., 1971a) to be the maximum level to maintain acceptable physical quality characteristics. This work resulted in the development of a 12% soy-fortified bread flour which is used in export programs (USDA, 1972).

Bean et al. (1973) tested mono- and di-fatty esters of polyethoxylated and polypropoxylated propylene glycol and of glycerol glycosides prepared from starch as improvers for wheat flour fortified with 6% soy flour. The fatty esters were added to the bread formula by dissolving them in dough water. The products tested showed promise for improving loaf volume and grain characteristics of soy-fortified bread. All the dough conditioners were in fluid or waxy solid forms which were not conveniently added to baking formulas.

We undertook to prepare fatty esters of polyethoxylated and polypropoxylated propylene glycol and of glycerol glycosides derived from starch and lactose, investigate methodology for incorporating these experimental dough conditioners into a dry mix with wheat and soy flour, and to evaluate their dough-conditioning action in bread containing wheat flour fortified with 12% soy flour.

MATERIALS & METHODS

Experimental dough conditioners

Glycosides of glycerol and propylene glycol were prepared from starch (Otey et al., 1968) and from lactose (Mehltretter and Wilham, 1973). From starch, the reaction products were mixtures of polyol glucosides and higher oligomeric polyol glycosides. From lactose, the reaction products were polyol glucosides and polyol galactosides. The mixtures were alkoxylated with ethylene oxide or with mixtures of ethylene oxide and propylene oxide and transesterified with methyl esters of oleic, stearic and palmitic acids and by triglyceride mixtures (Crisco®, Proctor and Gamble Co.). The conceptual structure of one of the possible products from a random mixture is illustrated in Figure 1. This stearic acid ester of polyethoxylated glycerol α-D-glucoside was prepared by the sequential reactions of starch with glycerol, ethylene oxide and methyl stearate. The nine preparations (A to I) for this study are shown in Table 1. The variables in these compositions are: starch or lactose starting materials, glycerol or propylene glycol, levels of ethylene oxide or its mixtures with propylene oxide, methyl esters of various fatty acids and triglycerides. All final products were either waxy solid or fluid forms. These experimental dough conditioners do not have FDA approval for food use.

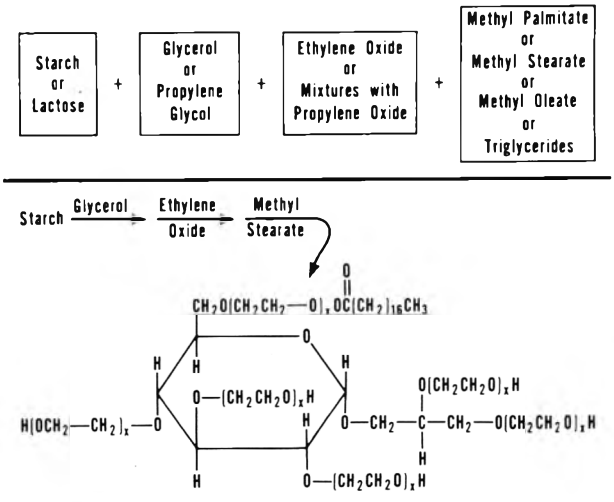

Fig. 1—Possible combinations of reactants that can produce experimental dough conditioners. Conceptual structure of stearic acid ester of polyethoxylated glycerol α-D-glucoside prepared by the sequential reactions of starch with glycerol, ethylene oxide and methyl stearate.

Dry mixes

The physical state of the experimental dough conditioners (Table 1) required methodology to facilitate their incorporation in fully formulated dry mixtures of wheat and soy flours. In export programs (USDA, 1972), a dry prepared mix is required. The waxy materials were heated to 65°C and cast into 1/4 in. × 1/8 in. ribbons which after hardening were cut to 1/4 in. × 1/4 in. × 1/8 in. pieces. These pieces were chilled to −12°C and ground (-20 U.S. Standard mesh) in a similarly chilled impeller mill. The chilled powder was immediately blended with defatted soy flour. One dough conditioner hardened by the addition of 5 and 10% fully hydrogenated soybean oil flakes was reduced to powdered form by this procedure. The fluid dough conditioners were heated to 65°C and added directly to defatted soy flour in small droplets accompanied by mixing in a Hobart mixer. This step was followed by screening (-14 U.S. Standard mesh) and remixing. The defatted soy flour-dough conditioner blends were then in a form suitable for preparing a dry mixture of wheat flour, soy flour and dough conditioner.

Baking formula and procedure

The experimental dough conditioners were evaluated in bread baking tests at the 12% level of soy fortification. Comparisons were made between dough conditioners (A to H), added to the bread formula either in dry mix form with the soy flour and wheat flour or with the dough water. Baking tests were made according to the procedure specified for soy-fortified bread flour used in export programs (USDA, 1972). The bread formula is as follows:

	%	g
Wheat flour	88	200
Soy flour, defatted, lightly toasted	12	
Dough conditioner[a]	0.5[b]	1
Yeast, compressed	2.5[b]	5
Salt	2.0[b]	4
Sugar	4.0[b]	8
Water (containing 10 ppm KBr)	68.0[b]	136

[a] Premixed with soy flour or water.
[b] Percent of wheat-soy flour.

The bread wheat flour contained 11.3% protein, 0.71% ash and 12.7% moisture. The dough scaling weight was 150g; this amount yielded a pup loaf weighing about 125g after baking. Each batch produced two pup loaves. Each of the experimental dough conditioners and controls containing no fat were tested by this procedure. The experimental bread was evaluated for grain score and crumb color according to standard laboratory practice (Bookwalter et al., 1971). Bread volume was measured by standard AACC (1962) procedures.

Chemical analyses were made following standard procedures (AACC, 1962). Analysis of variance (Snedecor and Cochran, 1968) and Duncan's (1955) multiple-range test were used to examine results. Statistical significance is at the 5% probability-of-error level wherever the term "significant" appears in this paper.

RESULTS & DISCUSSION

Dry mix preparation

Experimental fluid and waxy solid dough conditioners A through H (Table 1) were satisfactorily blended into dry mix form by the methods described. Waxy sample I was too soft to grind as evidenced by sticking in the impeller mill and plugging the screen. The waxy samples required more processing steps and controlled conditions than did the fluid samples. Waxy sample B produced a drier, less sticky powder after grinding when 5 or 10% fully hydrogenated soybean oil flakes were incorporated. Waxy samples C and G produced the driest powders after grinding. These results indicate that preparation of dough conditioners in dry mix form is facilitated by increased hardness of the waxy samples or by the use of fluid samples. Increased hardness may be achieved by varying the types and amounts of components during preparation or by the addition of fully hydrogenated soybean oil flakes. Using available equipment, a commercial scale process for incorporating the experimental fluid dough conditioners in a soy-wheat mix could be readily achieved. Additional process development would be required for scaling up the waxy type.

Bread baking tests

Baking results of 12% soy-fortified bread flour with and without experimental dough conditioner types A through H are compiled in Table 2. These results demonstrate the effec-

Table 1—Composition of experimental dough conditioners

Dough conditioner type	Moles/mole glycoside EO[a]	PO[a]	Glycoside	Physical state
A	8.0	3.8	GG[b] Monooleate	L[c]
B	5.0	0	GG Monostearate	W
C	5.0	0	PGG Monostearate	W
D	5.0	0	GG Fatty ester, mixed[d]	L
E	8.2	0	GG Fatty ester, mixed	L
F	5.0	0	GL Monopalmitate	W
G	2.5	0	GL Monopalmitate	W
H	4.0	0	PGL Monopalmitate	W
I	6.6	0	PGL Monopalmitate	W

[a] EO = ethylene oxide; PO = propylene oxide.
[b] GG = glycerol glycoside from starch; PGG = propyleneglycol glycoside from starch; GL = glycerol glycoside from lactose; PGL = propyleneglycol glycoside from lactose.
[c] L = liquid; W = waxy solid (at ambient temperatures).
[d] Mixed = mixed esters (transesterified with Crisco®, Proctor and Gamble Co.).

Table 2—Effect of experimental dough conditioners on the baking quality of 12% soy-fortified bread

Dough conditioners and their premixes with soy flour		Grain score (15 max)	Crumb color[a]	Loaf vol[b] (cc)	Vol increase (% over nonfat control)	Baking quality ranking[c]
A[d]	(SF)[e]	12	95	603 lmn[f]	26	2
A	(W)[g]	12	95	630 klmn	31	
B	(SF)	12	95	630 klmn	31	2
B	(W)	12	95	635 jkl	32	
B + 5% HF[h]	(SF)	12	95	665 jk	38	
B + 10% HF	(SF)	12	95	632 klm	32	
C	(SF)	12	95	642 jkl	34	1
C	(W)	12	95	637 jkl	33	
D	(SF)	12	95	585 n	22	3
D	(W)	11.5	95	587 mn	22	
E	(SF)	12.5	95	632 klm	32	2
E	(W)	12.5	95	637 jkl	33	
F	(SF)	12	95	625 klmn	30	2
F	(W)	12	95	627 klmn	30	
G	(SF)	12.5	95.5	600 lmn	25	2
G	(W)	12	95	600 lmn	25	
H	(SF)	12.5	95	665 jk	38	1
H	(W)	12.5	95.5	680 j	42	
None		10	92	475 p	—	4
None		10	92	487 p	—	
None		10	92	480 p	—	

[a] 100 = white; 95 = creamy
[b] Average of two
[c] 1 = best
[d] Experimental dough conditioner type A through H (Table 1)
[e] (SF) = dough conditioners premixed with soy flour, then preblended with dry ingredients.
[f] Coded means (j to p) with no letter in common differ significantly (5% level)
[g] (W) = dough conditioners dissolved in dough water.
[h] HF = fully hardened soybean oil flakes.

tiveness of the experimental dough conditioners in improving the baking characteristics of 12% soy-fortified bread. Paired comparisons are shown for experimental dough conditioners premixed with soy flour (SF) and samples added with the dough water (W). There were substantial differences in baking quality between bread made with and without 0.5% dough conditioner. Grain scores were about two points lower, color values were three shades darker and loaf volume was significantly lower in bread made with no dough conditioner. Volume increase of the bread made with dough conditioners ranged from 20–42% over the nonfat control. The least significant difference for comparing two mean loaf volume values in Table 2 is 42.6 cc. There was no significant difference in baking quality between experimental conditioners added as a dry mix with (SF) or with (W). These results are indicative of no change in baking potential when the waxy or fluid dough conditioners are converted to a dry mix form.

The baking quality of waxy sample B was not changed when as much as 10% fully hardened soybean oil flakes were incorporated. This result suggests that hard fat may be added to a waxy sample to improve milling properties without affecting baking quality. In an overall baking quality ranking (Table 2), experimental dough conditioners C and H rated best while sample D rated lowest. This result may be related to the use of propylene glycol in preparation of samples C and H and glycerol in sample D or to the presence of monoglycerides and mixed esters in sample D.

This work demonstrates that glycosides prepared from starch or lactose are very effective dough conditioners in 12% soy-fortified bread. High functionality is indicated when added to a bread formula with either the dough water or as a premix with the soy and wheat flours.

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CONSUMER PREFERENCES FOR VEGETABLES GROWN UNDER "COMMERCIAL" AND "ORGANIC" CONDITIONS

ABSTRACT

The present study was designed to provide experimental data which would bear on undocumented claims as to the superior flavor of so called "organic" or "natural" foods. Four vegetables (lettuce, green beans, broccoli greens, and carrots) were grown under three conditions: depleted soil (no additional fertilizer or pesticides); commercially fertilized (plus pesticides), and organically fertilized (manure, no pesticides). Fifty consumer subjects evaluated the four vegetables individually in separate sessions, using a 1 to 9 hedonic rating scale. A fifth evaluation of beans, broccoli and carrots was conducted on vegetables grown under just the commercial and organic conditions, but with products correctly and incorrectly labelled commercial or organic. Analysis of variance indicated no significant differences for lettuce or green beans due to growing conditions. There was a significant preference for the commercial and depleted samples of carrots over the organic sample in the separate sessions, but not in the label study. Organic broccoli was preferred to depleted and commercial samples in the separate sessions study, and to commercial in the label study. In the latter study there was a significant difference for type of vegetable (beans and carrots preferred to broccoli) and for label (organic preferred to commercial). The data indicated that the consumer would not be getting a more acceptable product if organically grown vegetables were purchased.

INTRODUCTION

DURING the last 5 yr consumers have been increasingly interested in the quality of so-called "natural" or "organic" foods. Many individuals and groups which espouse the "health food" movement have claimed superior properties for organically grown vegetables. An IFT expert panel, in a status report on organic foods, reported that there were no known benefits to organic foods in the area of nutrients or cost (IFT Panel, 1974). Several other scientists who have examined claims concerning the benefits of organic foods have concluded that the value of organically grown foods is doubtful (Leiser, 1973; Seelig, 1972; von Elbe, 1972). One area of undocumented properties is superior flavor (White, 1972). The consumer, without any evidence, may then purchase such products on the basis of these claims. We believed a study in which growing conditions, harvesting and postharvest treatment were carefully controlled would yield valid evidence for comparing the acceptance properties of vegetables grown under commercial and organic conditions.

Four vegetables–lettuce, green beans, broccoli and carrots–representing a wide range of types, were chosen for study. One advantage of the vegetables selected was that two could be prepared in cooked form (beans and broccoli), and two could be served raw (lettuce and carrots). In addition to growing conditions, the "label" effect of the word organic was evaluated, as it could be hypothesized that for many people just the information that a product was organically grown could serve as a preference bias.

MATERIALS & METHODS

Growing conditions

The vegetables were grown on a Yolo fine sandy loam soil which had not received any fertilizer, nor had the crops grown received any pesticides, for more than 2 yr previous to the test. Three treatments were used: (1) no fertilizer (depleted); (2) 20 tons manure per acre (organic); and (3) 200 lb of N plus 150 lb of P_2O_5 per acre, derived from ammonium sulfate and treble superphosphate, respectively (commercial). In treatment 3, the crops were sprayed with Lannate for worm control, but crops of the other treatments did not receive any pesticide.

The manure and fertilizers were applied broadcast and harrowed into the soil on July 16, 1974. The soil was then immediately furrow irrigated. It was bedded up, and the crops were planted in two rows on 42-in. lettuce beds on July 26. The crops were irrigated, thinned and grown in accordance with the usual commercial practice in the area. The plots were 50 ft long and 42 ft wide, and each treatment was replicated three times. There were sufficient beds of each crop that border effects between crops were minimized.

The cultivars grown were: lettuce, 'Great Lakes'; snap beans, 'Tendergreen'; carrots, 'Imperator'; and broccoli, 'Raab'. The latter cultivar has small, immature flowers, and the leaves are used as well as the flower parts (only the leaves were used for acceptance evaluation).

The crops were harvested as nearly as possible at prime market condition. This was a judgement made on the basis of size, color and solidity. Because growth was greatly accelerated by N from the ammonium sulfate or the manure, it was sometimes necessary to harvest crops of the various treatments on different dates. Only the lettuce was harvested on the same date, with 20 of the best heads per plot being used for evaluation.

Although the purpose of this study was to evaluate consumer preferences, not yield, it would be remiss not to mention that the yields with the depleted and organic growing conditions were much lower (about 25%) than those with the commercial condition, for all vegetables.

Storage of vegetables

Lettuce. The whole heads of lettuce were stored in refrigerators at 6°C for 6 days before being evaluated.

Green beans and broccoli. The beans were trimmed and cut into 1-1/4 in. lengths; the broccoli was trimmed and the leaves were cut into several pieces. The products were water blanched in 1-lb lots (water temp 98–100°C) with a blanch time of approximately 3 min, or until no enzymic activity was indicated by standard peroxidase test. The material was frozen in liquid Freon at atmospheric pressure. The beans were submerged for about 45 sec or until a center temperature of −18°C. The broccoli was submerged 2–3 min. The products were packaged in 1-lb lots in polyethylene bags, and were wire tied. Storage was at −18°C.

Carrots. The carrots were stored in a 6°C refrigerator for 6 days before being evaluated.

Preparation of product for serving

Lettuce. The lettuce samples were prepared by removing wrapper leaves, washing the remaining leaves, and cutting into 1-in. squares. These were refrigerated at 6°C until needed.

Green beans. The frozen content of a bag was rinsed in cold water, cut into 1-in. pieces, and cooked in 1 cup of boiling water to which 1/4 teaspoon of salt had been added, for 8–10 min. The beans were then drained and stored in foil-lined styrofoam containers. Two batches were prepared during the testing session in order to minimize holding time.

Broccoli. The broccoli was prepared in the same way as the green beans, except that 1/2 cup of boiling water was used, and the leaves were first cut into 1-in. squares. Four batches were prepared during the testing session in order to minimize holding time.

Carrots. The carrots were washed and cut into slices 3/8 thick and 3/4 to 1-1/4 in. in diameter. The ends were not used. The slices were kept refrigerated at 6°C until served.

Table 1–Summary of analysis of variance for four vegetables

	Source of variation	df	Mean square				F				Test source
			Lettuce	Beans	Broccoli	Carrots	Lettuce	Beans	Broccoli	Carrots	
A	Sex	1	2.613	72.030	11.213	3.853	0.818	8.890**	0.955	0.599	D
B	Treatment	2	0.863	3.203	37.510	15.160	0.520	1.065	16.315***	9.205***	DB
C	Plots	1	0.213	2.430	24.653	3.000	0.116	1.470	9.931***	1.908	DC
D	Subjects	48	3.196	8.102	11.738	6.435	–	–	–	–	–
AB	Sex X Treatment	2	2.823	3.070	0.963	3.453	1.702	1.021	0.419	2.097	DB
AC	Sex X Plot	1	0.053	12.703	0.853	0.853	0.029	7.505**	0.344	0.543	DC
DB	Subject X Treatment	96	1.659	3.008	2.299	1.647	–	–	–	–	–
DC	Subject X Plot	48	1.842	1.653	2.482	1.572	–	–	–	–	–
BC	Treatment X Plot	2	0.163	3.270	11.603	2.720	0.091	1.700	4.388*	2.621	DBC
ABC	Sex X Treatment X Plot	2	0.443	1.043	0.643	0.493	0.247	0.542	0.243	0.348	DBC
DBC	Subject X Treatment X Plot	96	1.793	1.924	2.644	1.419	–	–	–	–	–
Total		299									

* 5% level of significance
** 1% level of significance
*** 0.1% level of significance

Subjects

Fifty student volunteers, 25 male and 25 female, ranging in age from 18–24, participated in the acceptability evaluations. The subjects were each paid $5. dollars as an incentive for completing all five tests.

Test design

Five evaluations were conducted, one for each of the four vegetables (single-vegetable), and a fifth in which the "storable" products (carrots, beans, broccoli) were evaluated under two instruction conditions (multiple-vegetable). They were: (1) the vegetabels correctly identified as to growing condition (commercial or organic) and (2) the vegetables incorrectly identified as to growing condition, thus making four testing situations for each vegetable. To minimize the number of samples to be tested at one session, only two plot replications were utilized for the single-vegetable tests and one for the multiple-vegetable test. In addition to allowing for the evaluation of a "label" effect, the multiple-vegetable study provided a replication of single-vegetable results.

Presentation conditions

Three lettuce and carrot samples were served in 2-oz souffle cups. The beans and broccoli were also served in 2-oz cups which were placed in styrofoam containers and covered with aluminum weighing dishes. Three lettuce, carrot, and beans pieces and 6 tablespoons of broccoli were placed in the individual containers.

The samples were coded with a 3 digit code and presented to subjects one at a time in a random order. For the single-vegetable studies this resulted in six samples per person, and for the multiple-vegetable study, 12 samples per person. Approximately 1 min was the time between samples. Rinse water was available for use on an ad libitum basis.

Temperature of the carrots and lettuce was approximately 10°C, and that of the green beans and broccoli 28°C at time of serving.

The testing took place in an air-conditioned room (22 ± 1°C) in individual testing booths, illuminated with incandescent light.

The rating instrument used was the 9-point hedonic scale (Peryam and Pilgrim, 1957).

The subjects were told that they were to evaluate vegetables that had been grown under different conditions. They were not told what the products would be in the succeeding evaluations, only that there were five tests. In the multiple-vegetable study the subjects were told they would evaluate vegetables grown under commercial and organic conditions. The serving containers were labelled either "commercially fertilized" or "organically fertilized," in addition to having a 3 digit code.

Testing took place in the afternoons in 10 sessions, one every 20 min, with 5 subjects per session. The vegetables were tested on successive weekly intervals in the following order: lettuce, green beans, broccoli, carrots. The final multiple-vegetable study took place two weeks after the carrot test.

RESULTS & DISCUSSION

THE RATINGS were converted to numbers, 1–9, with 1 designating the dislike end of the scale and 9 the like end.

Analysis of variance for single-vegetables

A mixed model analyses of variance in which sex was nested within subjects was conducted for each vegetable evaluation (Winer, 1971).

Lettuce. There were no significant differences for any of the effects (Table 1). The overall mean of 6.3 indicated that the lettuce was generally well liked, even though it was served without salt or dressing. Thus for this vegetable, growing cultural practices had no measurable influence on acceptability.

Green beans. The summary of the analysis of variance is shown in Table 1. There were two significant effects for beans, sex as a main effect and a sex-by-plot interaction. The means for these effects are shown in Table 2. The Duncan test (Duncan, 1955) was used for determining significant differences among means. Examination of the interaction means reveals that there was no order change, but that the difference was greater between males and females for plot 2 than it was for plot 1. This difference between sexes is probably best explained as a learned attitude. Again, there was no evidence for an acceptability difference related to growing conditions.

Broccoli. The summary of the analysis of variance is shown in Table 1. There were three significant effects, treatments, plots and treatment-by-plots (means shown in Table 3). The

Table 2–Significant bean effect means[a]

Sex (N = 150)		Sex X Plot (N = 75)		
Male	Female		Plot 1	Plot 2
5.6	6.6	Male	5.9	5.3
		Female	6.4	6.7

[a] All values not underlined or bracketed together are significantly different at the 0.05 level.

treatment effect gives evidence of a preference for the depleted and organic samples over the commercial sample. However, examination of the treatment-by-plot interaction indicates that this is true only for plot 2. The subjects' comments contained a higher level of bitter comments for the commercial sample than for the other two samples. Interpretation of this difference is difficult as it occurred in only one plot, and since results obtained from the depleted condition do not differ from those obtained in the organic condition. Also, the ratings on this vegetable were very low, probably reflecting the unfamiliarity of the subjects with broccoli greens, and the resemblance of the product to spinach.

Carrots. The summary of the analysis of variance is shown in Table 1. Treatment was the only significant effect with the organic sample rated lower than the commercial or depleted sample (means shown in Table 4). Some indication as to the cause of this difference can be found in the subjects' comments. The organic sample had fewer mentions of good color and more negative mentions about toughness.

Analysis of variance for multiple-vegetables

Table 5 gives the summary of the analysis of variance for this evaluation. There were three significant main effects—vegetable, treatment, and instructions—and two significant interaction effects, vegetable-by-treatment and vegetable-by-treatment-by-instructions. The means for these effects are shown in Tables 6, 7 and 8. The vegetable difference was expected, based on the results of the single-vegetable evaluations in which broccoli was highly disliked as compared to the beans and carrots. The instruction mean difference indicates, that, overall, products labelled organic rate higher than products labelled commercial, although a difference of only 0.2 scale points is not of practical significance. The treatment effect indicates that organic vegetables rate higher than commercial; however, examination of the vegetable-by-treatment interaction means reveals that this difference is due entirely to broccoli. This confirms the results from the single-vegetable evaluation of broccoli. The three way interaction means (Table 8) are more difficult to interpret, but it appears that the effect of labelling a vegetable organic was more pronounced in beans and carrots, and that in broccoli it was less consistent.

Looking at the results for all the evaluations, the major finding was the lack of difference in acceptance due to growing conditions. There is some indication that, for the broccoli greens, there was a slight but significant preference for the organic product over the commercial samples; but this difference is difficult to interpret, as all products were disliked.

Table 5—Summary of analysis of variance for multiple vegetable study

	Source of variation	df	MS	F	Test source
A	Sex	1	0.167	0.020	E
B	Vegetable	2	601.222	149.475***	EB
C	Treatment	1	10.667	6.640*	EC
D	Instructions	1	10.140	6.204*	ED
E	Subjects	48	8.347	–	–
AB	Sex X Vegetable	2	9.712	2.415	EB
AC	Sex X Treatment	1	0.060	0.037	EC
AD	Sex X Instructions	1	0.240	0.147	ED
BC	Vegetable X Treatment	2	9.312	6.031**	EBC
BD	Vegetable X Instructions	2	0.455	0.350	EBD
BE	Subject X Vegetable	96	4.022	–	–
CD	Treatment X Instructions	1	0.007	0.006	ECD
CE	Subject X Treatment	48	1.606	–	–
ED	Subject X Instructions	48	1.634	–	–
ABC	Sex X Vegetable X Treatment	2	0.915	0.593	EBC
ABD	Sex X Vegetable X Instructions	2	1.445	1.111	EBD
BCD	Vegetable X Treatment X Instructions	2	6.552	4.680*	EBCD
BCE	Vegetable X Treatment X Subject	96	1.544	–	–
ACD	Sex X Treatment X Instructions	1	0.427	0.404	ECD
EBD	Subject X Vegetable X Instructions	96	1.301	–	–
ECD	Subject X Treatment X Instructions	48	1.057	–	–
ABCD	Sex X Vegetable X Treatment X Instruction	2	0.922	0.658	EBCD
EBCD	Subject X Vegetable X Treatment X Instruction	96	1.400	–	–
	TOTAL	599			

* 5% level of significance
** 1% level of significance
*** .1% level of significance

Table 3—Significant broccoli effect means[a]

Treatment (N = 100)		Plots (N = 150)		Treatment X Plot (N = 50)		
Commercial	3.7	1	2		Plot 1	Plot 2
Depleted	4.4	4.6	4.0	Commercial	4.3	3.0
Organic	4.9			Depleted	4.5	4.3
				Organic	5.0	4.8

[a] All values not underlined or bracketed together are significantly different at the 0.05 level.

Table 4—Significant carrot effect means[a]

Treatment (N = 100)		
Organic	Commercial	Depleted
6.5	7.0	7.2

[a] All values not underlined or bracketed together are significantly different at the 0.05 level.

Table 6—Significant three vegetable effect means[a]

Vegetable (N = 200)			Instruction (N = 300)		Treatment (N = 300)	
Broccoli	Beans	Carrots	Commercial	Organic	Commercial	Organic
3.6	6.4	6.8	5.6	5.8	5.5	5.8

[a] All values not underlined or bracketed together are significantly different at the 0.05 level.

Table 7—Vegetable X Treatment means (N = 100)[a]

	Commercial	Organic
Broccoli	3.3	4.0
Beans	6.4	6.5
Carrots	6.9	6.9

[a] All values not underlined or bracketed together are significantly different at the 0.05 level.

Table 8—Vegetable X Treatment X Instruction means (N = 50)[a]

	Commercial treatment		Organic treatment	
	Comm	Org	Comm	Org
Broccoli	3.4	3.1	3.7	4.3
Beans	6.1	6.7	6.5	6.5
Carrots	6.7	7.1	6.7	6.9

[a] All differences between means greater than 0.5 are significant at 0.05 level.

Since the difference showed up in both the single-vegetable study and the multiple-vegetable evaluation, it appears to be real for this study. Additional work is needed to clarify this result. The carrot difference found in the single-vegetable evaluation disappeared in the multiple study. This could be the result of a change in age of the samples, or the first result could have been due to variability in preparation factors.

The fact that labeling vegetables organic causes a small but statistically significant increase in ratings is not surprising for this particular subject population. One would expect that a difference of this type would vary with cultural background, and geographic location.

Although generalizing to all vegetables and consumers would not be warranted, the data from this study show that the consumer would not be receiving a product of higher acceptability when purchasing an organically grown product.

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USE OF EXPANDED COLOR SCALES TO PREDICT CHEMICAL AND VISUAL CHANGES IN SOLUTIONS

ABSTRACTS

Cranberry juice cocktail was chosen as a typical red fruit juice. Samples were heated at 65°C to simulate pigment degradation on storage. The heated samples were ranked for color visually and objectively using a Hunter Color Meter, a Gardner Color Meter and a G.E. recording spectrophotometer. Most functions of color obtained from the instrument correlated highly with visual color except dominant wavelength, hue, and Hunter and Gardner b. Most instrumental values also correlated well with anthocyanin concentration except the three above. The expanded L, a*, b* scales, which were developed in previous work to eliminate the inversion of tristimulus scales with pigment concentration with dark beverages, also gave very high correlations with visual evaluations and with anthocyanin concentrations.

INTRODUCTION

ADVANCES IN TECHNOLOGY and instrumentation in the field of colorimetry have provided many excellent contributions in the area of foods. However, there are several areas of importance in Food Colorimetry which, as of yet, have not been explored. One such area involves the need for colorimetric techniques to predict both visual and chemical changes occuring in food, using either raw or reduced data which would eliminate time consuming chemical analyses combined with visual evaluations.

Nearly all colorimetric work involving foods, and especially cranberry products, is intimately associated with the type and quantity of pigment present in the food. Cranberries owe their color to four major anthocyanin pigments: cyanidin galactoside, peonidin galactoside, cyanidin arabinoside and peonidin arabinoside (Zapsalis and Francis, 1965). Two other anthocyanins, cyanidin and peonidin monoglucoside are present at less than 1% of the total red pigment (Fuleki and Francis, 1967). Six yellow flavanoid pigments are also present and many contribute to the color (Puski and Francis, 1967).

A great amount of work has been aimed at developing relatively simple chemical methods to measure the anthocyanin content of berries. An early method was based on an optical density measurement of an alcohol-hydrochloric acid extract at 535 nm. At that time, the identities of the pigments were unknown, thus a secondary standard had to be used to calibrate the absorption readings. A standard which was often used for red solutions was Congo Red but it had the disadvantage that various commercial samples of Congo Red produced slightly different calibration curves. In order to avoid this problem, a relationship between mg of Congo Red and optical density units was used (Sondheimer and Kertesz, 1948). Zapsalis and Francis (1965) identified the pigments and determined their absorption coefficients, thus allowing the abandonment of the Congo Red method. A method developed by Fuleki and Francis (1968a) reported actual mg of anthocyanin pigment per 100g of product. This method was extended by Fuleki to individual pigments in berries, sauce or juice (Fuleki and Francis, 1968b, c, d).

A great deal of success in objective color measurement to simulate and predict visual assessment of foods has been achieved. Francis and Clydesdale (1970) have reviewed this work for cranberries and cranberry products which are typical anthocyanin containing foods and are greatly dependent on color appeal. Investigations aimed at using color for predicting chemical changes have been carried out, albeit to a lesser extent. Little (1969) proposed a method which relates a chromaticity shift and CIE Y to chemical changes in canned tuna. Using Y with a chromaticity changes has the advantage of indicating the "available" pigment. If not, it may be an indication of improper processing, such as browning, scorching, stackburn or oxidative degradation. A small change in chromaticity with a low Y value would be indicative of the dark color resulting from something other than the normal pigment. Staples and Francis (1968) conducted a feasibility study for the continuous color measurement of cranberry juice cocktail. It was found that a function of pigment content expressed as O.D. (515–420) nm correlated very well with visual color and it was concluded that such a system could work in a continuous in-line process. Joslyn and Little (1967) investigated the relationship of objective color measurements to changes in chemical composition of rosé wines. It was found that a decrease in dominant wavelength indicated pigment loss and an increase in purity indicated browning. Also, color changes as evaluated visually paralleled the colorimetrically determined differences in the psychophysical quantities with generally good agreement. However, in one set of circumstances, a change in chromaticity was not visually perceived.

Eagerman et al. (1973a, b) demonstrated that currently used color scales do not relate to pigment concentration in dark colored juices. The scales in use are "reflectance scales." It was found that with dark colored juices, an area of confusion occurs in which an increase in pigment concentration results in a decrease in the color quality parameters when certain color scales are used. New scales were developed with the aid of a computer to account for this effect. This area of confusion was explained by an increase in the pigment concentration causing a decrease in the luminosity of the sample. The decrease in light causes the photocell of the colorimeter to be less sensitive. This problem was encountered when data in the CIE system were converted to the Lab system. This work developed a color scale to account for the inversion problem as far as color value versus pigment concentration was concerned, but did not include a correlation with visual evaluation. The present work examined this important factor.

EXPERIMENTAL

Preparation of samples

Cranberry juice cocktail obtained from a local retail outlet was mixed into 2000 ml bulk lots in round bottomed flasks and heated at 65°C in a controlled temperature water bath for 48 hr. Initial samples were taken prior to heating and immersed in an ice bath prior to analysis. When the cocktail reached a constant temperature of 65°C another sample was taken and then samples were withdrawn at various intervals for each of the three experiments. In Expt. I and II, samples were withdrawn every hour for 8 hr. In Expt. II, samples were also withdrawn at 12 and 24 hr. In Expt. III, samples were withdrawn every

hour for 12 hr and then at 24, 36 and 48 hr. These treatments created samples which simulated the range of color values which might be obtained upon room storage of cranberry cocktail. After sampling, the aliquots were immediately immersed in an ice bath to cool to approximately room temperature (24°C) and held until further analysis.

Pigment analysis

Total anthocyanins in the cranberry juice cocktail samples were analyzed by the method of Fuleki and Francis (1968b) which minimizes interference due to brownish colored degradation products. The method is based on the measurement of the absorbance at 510 nm on samples diluted with pH 1.0 and pH 4.5 buffers, KCl-HCl and sodium acetate-acetic acid, respectively. The pigment content is calculated in absolute quantities with extinction coefficients established for the cranberry anthocyanins dissolved in the buffers. Each sample of cocktail representing a time interval was analyzed. Samples were stored at room temperature. Optical density readings were performed on a Hitachi Perkin-Elmer Model 139 UV-VIS Spectrophotometer using 10 mm cells.

Subjective evaluation of color

Visual judgments for each set of samples were performed with 10 panel members. All panel members were instructed to rank the samples in order of redness acceptability of the cranberry juice cocktail samples. Visual evaluations were not done in Expt. I since this experiment served as a preliminary test in order to establish pigment degradation with time. 25 ml samples in 50 ml beakers were presented to the panel for examination. Controlled illumination of the samples during visual examination was accomplished with the use of a MacBeth Examolite (MacBeth Daylighting Corp., Newburg, N.Y.) with daylight illumination. All samples were ranked in duplicate and the results averaged. These average results were then rank averaged to produce an average for each sample representing the entire panel.

Objective evaluation of color

Instrumental color data were obtained with three different instruments: a Hunterlab D25 Color Difference Meter (Hunter Associates Laboratory, Inc., Fairfax, Va.), a Gardner XL-10 Color Difference Meter (Gardner Laboratories Inc., Bethesda, Md.) and a General Electric Recording Spectrophotometer (Diano Corp., Foxboro, Ma.) with a tristimulus integrator (Davidson and Hemmendinger, Inc., Easton, Pa.).

The standards used with each instrument were as follows:

Hunterlab D 25: white tile with tristimulus values
L = +92.5, a = −0.4, b = +2.3

Gardner XL-10: distilled water blank with
L = +100, a = 0.0, b = 0.0

GERS: pressed barium sulfate standard

Duplicate measurements were made on each set of samples on the same day as the visual examinations. All samples were removed from the ice bath and allowed to come to room temperature prior to evaluation. The average values of the duplicates were used in the analysis of the data. The cranberry cocktail samples were measured in a 3 mm cell on the GERS and Hunterlab D25 and in a 10 mm cell on the Gardner XL-10 in order to evaluate cell thickness differences.

Analysis of data

Instrument readings and reduced data were correlated with both pigment concentration and visual ranking. Data from the CIE system were converted to the L, a*, b* system using the computer program developed by Eagerman et al. (1973a, b). Data obtained from this program were then correlated with pigment concentration and visual ranking.

RESULTS & DISCUSSION

FIGURE 1 shows the spectral curves of selected cranberry juice cocktail samples from Expt. III. It can be seen that as heating continues, more energy is transmitted through the 500–550 nm region indicating the presence of less anthocyanin pigment. Correlations of optical density versus optimum wavelength for anthocyanins and browning compounds and for optical density versus visual rank were all quite high ($r > 0.96$). However, the relationship of the increase in browning and decrease in anthocyanin concentration is not always one to one relationship. Thus, these high correlations may not occur in every case.

In order to present a background as to how well the raw and reduced color functions correlated with visual rank and anthocyanin concentration, Tables 1 and 2 are provided. Table 1 indicates the correlation coefficients for visual ranking versus

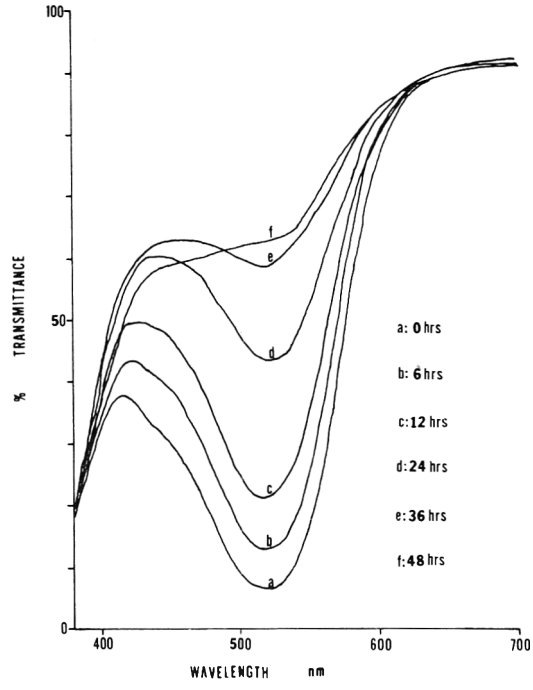

Fig. 1—Spectral curves of cranberry juice cocktail heated at 65° C for the times indicated in Expt. III.

Table 1—Correlations of visual rankings versus instrumental tristimulus data for cranberry juice cocktail

Correlation of visual ranking with:		Correlation coefficient Expt. II (8 samples)	Expt. III (10 samples)
G.E.	X	0.999	0.957
	Y	0.993	0.944
	Z	0.988	0.964
$(X^2 + Y^2)^{1/2}$		0.991	0.948
$(X^2 + Z^2)^{1/2}$		0.994	0.955
$(Y^2 + Z^2)^{1/2}$		0.999	0.962
purity		−0.987	−0.935
dominant wavelength		0.631	0.632
x		−0.991	−0.980
y		0.941	0.808
Gardner	L	0.971	0.913
	a	−0.946	−0.882
	b	−0.016	−0.780
hue		−0.776	−0.542
chroma		−0.973	−0.901
Hunter	L	0.995	0.950
	a	−0.985	−0.937
	b	0.692	−0.654
hue		−0.978	−0.534
chroma		−0.995	−0.945

the various instrumental functions for each of the two separate experiments. The sample size is reduced in these experiments because several samples were within the just "noticeable color difference" in the color solid and the panel could not distinguish between them. This reduced the sample size to 8 and 10 for experiments II and III, respectively. A visual evaluation was not performed for Expt. I because it was found that the sampling conditions gave less than 50% degradation of the pigment. Thus, any visual evaluation would be valid for only the upper limits of the pigment concentration. This might have biased the results. In Expts. II and III, approximately 80% and 90% of the pigment degraded, respectively.

From this table, it may be seen that most functions gave high correlations with the exception of dominant wavelength, hue and *b*. In a study of rosé wines, Joslyn and Little (1967) noted that visual color changes correlated well with instrumental values. Dominant wavelength was included here, but the design of the experiment did not allow for a statistical evaluation of the results as only two samples of each wine were rated subjectively. Over a wider range of pigment degradation it was shown in the present study that dominant wavelength does not provide an accurate description of predicted visual rank. However, results may depend on the type of product being examined.

Table 2 shows the correlations of anthocyanin concentration versus instrumental functions for the three experiments. It is seen here that all functions except dominant wavelength, hue and *b* provide high correlations. Dominant wavelength and hue are analagous. The hue of a partially degraded system is due to a combination of effects brought about by the intact pigments and the degradation products (browning compounds). These two factors are not perfectly correlated, thus hue is not a good indicator of either. The reason that *b* is a poor index might be explained in the conversion equation:

$$b = 7.0\,(Y - 0.847Z)/Y^{1/2}$$

Anthocyanins absorb maximally near 535 nm and would affect the Y function. The browning compounds absorb at 400 nm and would affect the Z function, but to a greater extent. Thus, these two values change at different rates and give a low correlation to *b*. The cell thickness does not seem to markedly affect the results as seen by a comparison of the Gardner data to the Hunter data which were done with different cell thicknesses. Correlations that were high for functions from one instrument were also high for the other instrument. The same was true for the functions giving low correlations.

Eagerman et al. (1973a, b) proposed an expanded color scale (L, a*, b*) due to the fact that some dark colored beverages showed an "inversion area" in the color quality parameters (*a* and *b*) when luminosity decreased due to an increase in pigment concentration. New scales were developed to compensate for this effect to eliminate this inversion area. These scales worked well when correlations of color parameter with pig-

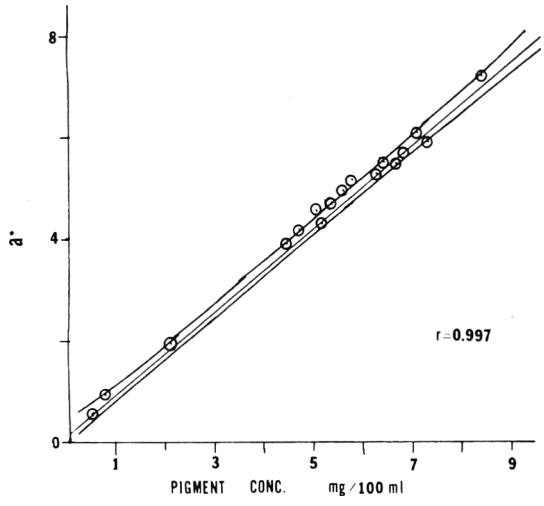

Fig. 2—Line of best fit and 95% confidence limits obtained by regression analysis for total anthocyanin concentration versus a in Expt. III.*

Table 2—Correlations of anthocyanin concentration versus instrumental tristimulus data for cranberry juice cocktail

Correlation of anthocyanin conc with:		Correlation coefficient I[a]	II	III
G.E.	X	−0.990	−0.960	−0.993
	Y	−0.991	−0.974	−0.981
	Z	−0.994	−0.939	−0.993
$(X^2 + Y^2)^{1/2}$		−0.991	−0.967	−0.986
$(X^2 + Z^2)^{1/2}$		−0.994	−0.949	−0.989
$(Y^2 + Z^2)^{1/2}$		−0.993	−0.965	−0.992
purity		0.991	0.966	0.966
dominant wavelength		−0.947	−0.678	−0.642
	x	0.994	0.959	0.992
	y	−0.974	−0.949	−0.839
Gardner	L	−0.985	−0.966	−0.961
	a	0.983	0.958	0.915
	b	0.982	0.059	0.928
hue		−0.941	0.863	0.272
chroma		0.985	0.973	0.933
Hunter	L	−0.992	−0.972	−0.985
	a	0.992	0.975	0.973
	b	0.885	−0.736	0.658
hue		−0.490	0.976	0.235
chroma		0.990	0.973	0.978

[a] I, II and III refer to three separate experiments having 10, 12 and 16 samples, respectively.

Table 3—Correlation coefficients for anthocyanin concentration versus a* and visual mean rank versus a* for Expt. II and III

Correlation with a*	Correlation coefficient Expt. II	Expt. III
Anthocyanin conc	0.970	0.997
Visual mean rank	−0.994	−0.961

Table 4—Color differences and hue ranges for Expts. I, II and III using Gardner and Hunter data

	Gardner			Hunter		
Expt.	ΔE	a/b	arctan a/b	ΔE	a/b	arctan a/b
I	16.7	0.75	3.6	40.2	5.63	4.3
II	47.3	2.37	27.7	30.6	6.30	43.3
III	59.3	2.29	13.5	50.4	5.55	52.1

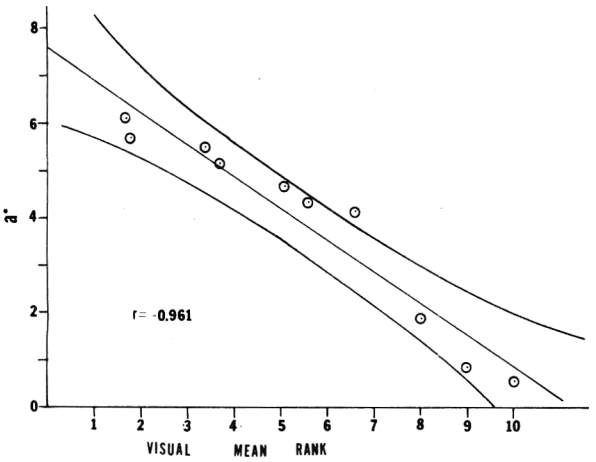

Fig. 3–Line of best fit and 95% confidence limits obtained by regression analysis for visual mean rank versus a in Expt. III.*

ment concentration were performed. However, these scales were not tested against visual estimations of color. Data from the present study were used to determine whether these new scales do, in fact, reflect visual perception.

Data from the cranberry cocktail experiments in the CIE system were put through the SEARCH program in order to find the conversion formula which gave the highest correlation of converted data against anthocyanin concentration. After obtaining the converted L, a*, b* data, correlations of converted a* values versus visual mean rank for the corresponding experiment were calculated. Table 3 shows the correlations of a* versus anthocyanin concentration and versus visual rank for the two sets of data. Graphs of the data with statistical information are provided for a* versus pigment concentration (Fig. 2) and a* versus visual rank (Fig. 3) in Expt. III. It can be seen that high r values were obtained for both types of correlation.

The conclusion is that the scales generated do indeed indicate what the eye is seeing. The equation generated was:

$$a^* = 170\,(1.02X - Y)/Y^{1.60} + 200$$

This equation would probably have to be adjusted for different types of foods depending upon such factors as physical state, type of pigment, scattering, etc. Table 4 shows the ΔE values and hue ranges for the three experiments using the Gardner and Hunter data. High correlations using conventional color functions could be due to the relatively large differences between the samples. It can be seen that the range of hue for the samples is relatively small, which could be a factor in the hue and dominant wavelength functions having low correlations.

As well, it should be pointed out that the reason for the high correlation using conventional techniques was that the samples utilized were not dark enough to carry the measurements into the "area of confusion" as described by Eagerman et al. (1973a, b).

The methodology described in this paper is suitable to produce more functional color scales for many foods. Such scales might add a more versatile dimension to current colorimetric practices.

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PRODUCTION OF A BETACYANINE CONCENTRATE BY FERMENTATION OF RED BEET JUICE WITH Candida utilis

ABSTRACT

Recent limitations on the use of artificial red food colorants has prompted extensive research into finding suitable natural red pigments as color additives. Investigations have shown that water-based concentrates or powders of red beet root (*Beta vulgaris*) are applicable colorants in some food systems. However, presently available commercial beet preparations have relatively low pigment concentration; therefore, large quantities are required to obtain the desired color strength. Fermentation of ultrafiltered red beet juice by *Candida utilis* under partial anaerobic conditions was shown to substantially reduce solids, resulting in a five- to sevenfold increase in betacyanine content on a dry basis. A 6–8% betacyanine content was achieved in the final dry product. The fermentable carbohydrate, nitrates and 80% of the Kjeldahl nitrogen were assimilated by *Candida utilis* after 6.5 hr of fermentation by maintaining the pH at 5.0 with NaOH and HCl solutions. After drying, the product lacked the characteristic beet flavor and aroma and was readily water soluble. This dry betacyanine concentrate may have application as a red food colorant.

INTRODUCTION

INTEREST in natural food colorants has increased markedly in the past few years, particularly because of intensified consumer awareness and questions about the safety of some artificial dyes. Dehydrated red beets or beet juice concentrates are permitted as colorants under the 1960 Color Additive Amendment to the Food Drug and Cosmetic Act of 1938, and have been shown to be applicable as colorants in some food systems (von Elbe et al., 1974; Pasch et al., 1975; von Elbe, 1975). However, commercially available beet concentrates contain only 0.2–1.0% (w/w) betacyanine (Anon., 1973). This low pigment concentration requires adding large amounts to foods to obtain the desired tinctorial strength. In addition, since the concentrates are unrefined, they contribute undesirable organoleptic properties. Therefore, a more concentrated and bland betacyanine preparation would be advantageous.

Product cost and environmental impact should be considered in the production of a betacyanine concentrate since only 1% (average betacyanine content) has coloring value (von Elbe et al., 1972). Since 80% of the solids of beet juice consist of fermentable carbohydrate and nitrogenous compounds, a yeast fermentation would seem to be a reasonable approach to decrease total solids and proportionately increase betacyanine concentration on a solids basis. *Candida utilis* was chosen as the fermentation organism for its utilization of a wide variety of carbon and nitrogen substrates (Van Uden and Buckley, 1971). In addition, the resultant yeast cells could be utilized as a human food supplement or could be added to the beet pulp for use as animal feed. The purpose of this study was to obtain a betacyanine concentrate of high pigment content by fermentation.

EXPERIMENTAL

Laboratory fermentation (1 liter)

Fresh red beets (*Beta vulgaris*) were obtained from a commercial source. The beets were washed, diced (3/8 in.), ground up (Fitzpatrick Model D Mill, 3/4 in. diam perforated screen), pressed, and centrifuged to yield a clarified juice. Sufficient ascorbic acid was added prior to milling to yield approximately 100 ppm ascorbic acid in the juice.

The juice was ultrafiltered at room temperature under 8 psi nitrogen with a hollow fiber unit (c/HFU-10 Mini-Plant Ultrafilter, cellulose acetate, 10,000 cm^2 nominal area, 30,000 nominal molecular weight cut-off, Dow Corning Corp., Midland, Mich.). Sufficient antifoam (no. 72, aqueous nonionic silicon emulsion, General Electric, Waterford, N.Y.) was added to prevent foaming during fermentation.

The inoculum for fermentation was prepared by transferring the cells from 1-day-old Plate Count Agar slants of *Candida utilis* (Strain 3, Bacteriology Dept., University of Wisconsin) to a sterile broth (50g sucrose/1, 2g yeast extract/1, 5g NaH_2PO_4/1, 2g $(NH_4)_2SO_4$/1, pH 5.4) and incubating the broth at room temperature on a platform shaker for 16 hr prior to the start of the fermentation. The total fermentation inoculum was determined by measuring the absorbance per milliliter of the broth containing the yeast at 650 nm (1 cm cell, H_2O ref) using a 1% NaH_2PO_4 solution as diluent. The number of milliliters of broth to be used as inoculum was calculated as yeast cells equivalent to 10,000 absorbance units. This starter culture was then centrifuged to yield an inoculum of spun cells.

The fermentation apparatus consisted basically of a 2.5 liter jar containing an air inlet system, pH electrodes, dissolved oxygen monitoring probe (Johnson et al., 1964), access for sampling, and connections to introduce acid or base solutions to maintain pH. During the fermentation the following conditions were maintained: pH 5.0 ± 0.3 with either 7% NaOH or 5% HCl solutions, stirrer speed 1000 rpm, air flow 1750 cc/min, and temperature 30 ± 2°C. The temperature and relative dissolved oxygen concentration were continuously monitored. At the end of the fermentation time the juice was centrifuged and the volume measured. The yeast cell concentration was determined by centrifuging and washing the cells in 1% NaH_2PO_4 solution twice, making up to original volume, diluting, and measuring the absorbance at 650 nm as before.

Pilot plant fermentation

Fresh whole beets were exposed to saturated steam at atmospheric pressure for approximately 5 min and the outer surface was removed in an abrasion peeler. The peeled beets were diced, ground up in a Fitzpatrick mill (Model D), and the pulp was expressed in an experimental screw-type continuous press (8 in. diam). The pulp was then wetted and pressed again. Sufficient ascorbic acid was added prior to milling to yield approximately 100 ppm ascorbic acid in the combined juice. The juice was clarified in a juice finisher, pasteurized in a plate heat exchanger (95°C, 20 sec), and centrifuged (Type AS-16 P, 4 in. diam, 15,000 rpm, Sharples Corp. Centrifugation Engineers, Philadelphia, Pa.) to remove the majority of microflora. Ultrafiltration was accomplished using pilot plant equipment employing four ultrafiltration modules (30,000 nominal atomic weight cut-off, Osmotik Module 701810-103-213, Fluid Systems Div., Universal Oil, San Diego, Calif.). Because of limitations in equipment capacity, this process of preparing 285 gal for the pilot plant fermentation required 10 days. During this processing period, the juice was stored in polyethylene bags in 10 gal milk cans at 5°C prior to ultrafiltration and under CO_2 atmosphere at 0°C in a bulk milk cooler after ultrafiltration.

The inoculum for the fermentation was cultured on the broth described for the laboratory fermentations in the following successive increments: three slant cultures, three 1-liter shaker flasks, three 15-liter stirred aerated fermenters, one 500-gal fermenter (experimental 750-gal baffled stirred fermenter, Biochemistry Dept., University of Wisconsin). The centrifugation of the broth (PX 213, DeLaval Separator Co., Poughkeepsie, N.Y.) yielded 45 gal of yeast cell suspension (approximately 20% yeast cells).

This suspension served as inoculum for the 285 gal of beet juice. During the fermentation, the temperature was maintained at 30°C, the stirrer speed was 100 rpm, and the pH was maintained at 5.0 ± 0.2 with 30% NaOH and 15% H_2SO_4 solutions. The percent yeast cells was determined by centrifugation of 10 ml samples of fermentation broth in a graduated centrifuge cup for 3 min (Gyro Test Unit, DeLaval Separator Co., Chicago, Ill.). The air flow was set originally at 4 ft^3/min and increased gradually to maintain a low dissolved oxygen concentration (less than 20% saturation). After 3.5 hr the air flow was fixed at the maximum of 45 ft^3/min. Sufficient Antifoam B (Dow Corning Corp., Midland, Mich.) was added to prevent excessive foaming. After fermentation, the yeast cells were removed by centrifugation and the supernatant medium was concentrated to 5.5% dissolved solids in a falling film evaporator and subsequently spray dried (experimental design, Babcock Hall, University of Wisconsin).

Chemical analysis

Total solids and ash. 20-g juice samples were weighed into 40 ml ceramic crucibles and the total solids and ash content were determined sequentially according to AOAC (1970) procedures for vegetable products.

Crude protein. Percent nitrogen of 1 ml juice samples was determined according to micro-Kjeldahl AOAC (1970) procedures. Crude protein was estimated by multiplying the percent nitrogen by the factor of 6.25.

Reducing sugar and sucrose. Alcohol was added to a known weight of juice to yield an 80% ethanol solution. Each solution was filtered to remove alcohol insoluble solids, heated to evaporate ethanol, treated with saturated lead acetate solution, and filtered according to AOAC (1970) procedure to yield a protein-free solution. This solution was analyzed separately for reducing sugars and sucrose. Reducing sugar content was determined by employing the technique of Somogyi (1945) and Nelson (1944) using glucose as a standard. Sucrose was estimated after inversion (4% HCl solution, 70°C, 7.5 min) of the protein-free solution and expressed as the difference between the reducing sugar content before and after inversion.

Betacyanine. Betacyanine was determined by electrophoresis of 10 µl samples and subsequent densitometry (von Elbe et al., 1972).

Nitrate. The method of Bremmer and Keeney (1965) was utilized to determine ammonia, nitrate and nitrite content of 5 ml samples. The results were expressed as nitrate.

Total phosphorus. The procedures of AOAC (1970) for samples larger than 10 ppm and of Fiske and Subbarow (1925) for samples under 10 ppm were utilized. These analyses were conducted by WARF Institute, Inc., Madison, Wisc.

RESULTS & DISCUSSION

A PRELIMINARY SURVEY of the effects on the fermentation of pH control, enzymatic hydrolysis and ultrafiltration prior to and during fermentation was conducted to determine the greatest reduction of beet juice solids with the least degradation of betacyanine. In addition, the effect of the initial betacyanine concentration in beets on the final betacyanine content on solid basis was determined. From these experiments (Adams, 1974), it was concluded that the use of:

(a) NH_3 to maintain the desired pH value decreased the utilization of indigenous nitrogenous material during fermentation;

(b) a pectinase treatment prior to fermentation did not reduce the total solids after fermentation;

(c) proteolytic enzymes did not noticeably increase the utilization of crude protein;

(d) enzymatic hydrolysis treatments under anaerobic conditions prior to fermentation produced substantial betacyanine losses; and

(e) beets with high betacyanine content (optimum canning beets) resulted in a greater betacyanine percent increase on a solids basis than large-sized, low-betacyanine content beets.

Table 1 presents a comparison of beet juice constituents prior to and after fermentation of the juice with and without ultrafiltration (UF). The beet juice for fermentation A (without UF) was heated to 75°C to inactivate oxidative enzymes while the juice in fermentation B was not heated. The UF process produced a slight dilution (fermentation B) because of retained water in the UF unit. The fermentation was terminated at the point at which the relative dissolved oxygen increased to near saturation value (Fig. 1).

In fermentation A there was an 82% reduction in total soluble solids (0.0714 to 0.0127 g/g sample). This reduction in juice solids was attributed to the yeast utilization of sucrose and 79% of the crude protein. The ash content increased from 0.0056 to 0.0061 g/g sample (7.9% and 45% of T.S., Table 1). This increase was the result of the addition of NaOH and HCl solutions to maintain pH. The betacyanine content decreased from 0.00072 to 0.00065 g/g sample, or approximately 10% of the pigment was lost during fermentation. On a total solids basis, however, there was a fivefold increase in betacyanine concentration.

In fermentation B the process of ultrafiltration resulted in a reduction of total solids from 0.0788 to 0.0701 g/g sample. The reduction, in part, can be explained by the retained water in the UF unit during ultrafiltration (max 2%). Since the crude protein was not appreciably reduced (0.0076 to 0.0073 g/g sample), the reduction in total solids was attributed to the removal of excluded macromolecules of pectinaceous material or colloidal solids not removed by centrifugation. Ultrafiltration had the additional beneficial effect of reducing foaming during fermentation.

The analysis of fermentation B paralleled those of fermentation A. The higher betacyanine concentration (6.8% vs 5.1%) and lower unaccountable fraction (25% vs 32%) on a solids basis of fermentation B were attributed to ultrafiltration.

Table 1—Beet juice analyses prior to and after partial anaerobic fermentation by Candida utilis[a]

	Total solids (TS) (mg/g sample)	Ash (% of TS)	Crude protein (% of TS)	Sucrose (% of TS)	Glucose (% of TS)	Betacyanine (% of TS)	Unaccountable fraction (% of TS)
Fermentation A							
Initial juice	71.4 ± 0.1[b]	7.9 ± 0	12 ± 0	69 ± 4	1.6 ± 0.1	1.0 ± 0.1	8 ± 4
After fermentation	12.7 ± 0.1[c]	45 ± 0	14 ± 1	0	3.7 ± 0.2	5.1 ± 0.1	32 ± 1
Fermentation B							
Initial juice	78.8 ± 0.2	7.8 ± 0.1	9.6 ± 0.1	77[d] ± 2	—	0.94 ± 0.06	5 ± 3
After ultrafiltration	70.1 ± 0.4	7.9 ± 0.3	10.4 ± 0.2	79 ± 4	1.7 ± 0.1	1.1 ± 0.1	0
After fermentation	12.1 ± 0.1[c]	53 ± 2	12 ± 0	0	4.1 ± 0.1	6.8 ± 0.6	25 ± 3

[a] Conditions: 1 liter, 30°C, pH 5.0
[b] Maximum estimated error based on minimum of triplicate determinations or use of internal standards (sugars)
[c] Corrected for volume change during fermentation
[d] Total reducing substances after inversion expressed as sucrose

After freeze drying, the powder from fermentation B dissolved readily in water while the powder from fermentation A aggregated, increasing the dissolving time. Both powders lacked the characteristic beet flavor and aroma.

To further optimize the fermentation of beet juice, it was desirable to follow the consumption pattern of some beet juice constituents. In another 1-liter fermentation (C), with juice preparation similar to that of fermentation B, 10 ml samples were collected throughout the fermentation and analyzed for reducing sugars after inversion expressed as sucrose, % Kjeldahl nitrogen, betacyanine, yeast cell count, and relative dissolved oxygen. The results of these analyses were plotted against time in Figure 1.

Fermentation C was considered completed after 11 hours as shown by the increase in relative dissolved oxygen. The betacyanine content stayed relatively constant throughout the fermentation. Total reducing substances after inversion expressed as sucrose and Kjeldahl nitrogen were essentially utilized after approximately 6.5 hr. In addition, at this time the yeast multiplication rate decreased. The sudden rise and fall in relative dissolved oxygen suggested a change in metabolic activity. The resumption of oxygen consumption suggested the utilization of fermentation by-products not determined in analyses, or the metabolic consumption of intercellular substrates. Nitrate content is known to be relatively high (0.4–1.8% of total solids, Peck et al., 1974) and therefore may account for this metabolic activity. Because of the limited sample size collected in fermentation C, a similar fermentation was conducted and 10 ml samples were collected throughout the fermentation for nitrate analysis. These analyses indicated that all nitrates had been utilized prior to the 6.5 hr time. Therefore, the increased metabolic activity could not be attributed to nitrate utilization.

To determine if there was a phosphorus deficiency in the fermentation of beet juice, small quantities of NaH_2PO_4 were added at various points of a 100-liter fermentation to observe changes in dissolved oxygen concentration. In the latter portion of the fermentation, this addition resulted in a sudden decrease in dissolved oxygen concentration, suggesting that phosphate might be a limiting growth factor.

Pilot plant fermentation

The pilot plant fermentation, approximately 285 gal, was undertaken to show that large scale fermentations are possible to obtain a product of high betacyanine content compared to presently available beet products; to allow for sequential samples large enough to conduct all previous analyses on the same sample; and to provide enough betacyanine concentrate to allow further studies on pigment stability and purification techniques. Table 2 shows the results of the analyses of total solids, ash, % yeast cells, Kjeldahl nitrogen, nitrate and total phosphorus. The data show that after 6.5 hr of fermentation, 96% of the final reduction in total solids had taken place, the yeast cells content had reached a maximum level, and the Kjeldahl nitrogen, nitrate and total phosphorus had been utilized. The rapid depletion of total phosphorus might indicate that phosphorus was a limited growth factor although a phosphorus mass balance was not determined. Thus, the metabolic activity previously observed in the latter portion of fermentation (Fig. 1) may be attributed to the utilization of intercellular substrates.

In Figure 2, the % betacyanine content expressed on a dry weight basis and nonash content on a wet basis are plotted against fermentation time. As in earlier fermentations, the loss

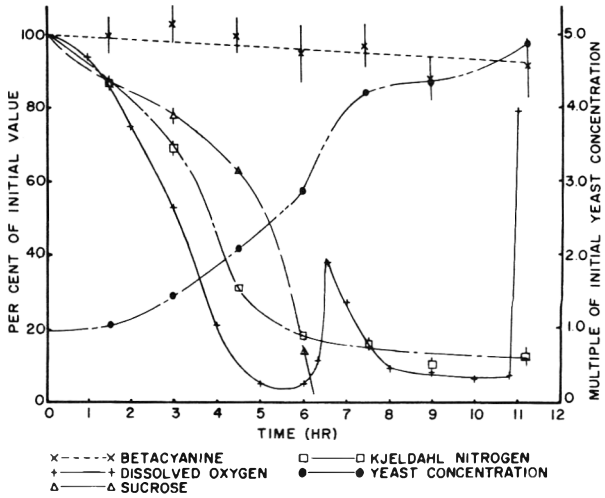

Fig. 1—Constituent analyses during beet juice fermentation by Candida utilis (30° C, pH 5.0).

Table 2—Beet juice analyses during partial-anaerobic fermentation by Candida utilis[a]

Fermentation time (hr)	Total solids (mg/g sample) ± 0.2[b]	Ash (mg/g sample) ± 0.2	Yeast cells (volume %) ± 0.2	Kjeldahl nitrogen (mg/g sample) ± 0.02	Nitrate (mg/g sample)	Total phosphorus (μg/g sample)
0	78.8	5.1	2.0	1.60	0.52 ± 0.05	22.1 ± 1.1
1.5	75.8	4.8	2.4	1.46	0.61 ± 0.04	16.6 ± 0.8
3.0	69.3	5.0	5.4	1.08	0.70 ± 0.08	3.2 ± 0.4
4.5	58.2	4.9	8.7	0.49	0.87 ± 0.02	0.7 ± 0.1
5.0	46.8	4.9	9.8	0.35	0.21 ± 0.01	–
6.0	24.6	5.3	13.0	0.28	0.01 ± 0	0.3 ± 0.0
6.5	19.2	5.7	15.0	0.27	c	–
7.5	17.9	5.7	15.0	0.27	c	0.3 ± 0.0
8.5	17.8	6.5	15.0	0.24	c	–
9.5	17.3	6.6	15.0	0.19	c	–
11.0	17.0	6.6	15.0	0.18	c	0.4 ± 0.1

[a] Conditions: 285 gal beet juice, 30° C, pH 5.0.
[b] Maximum estimated error based on triplicate determinations
[c] Below limits of methods of analysis

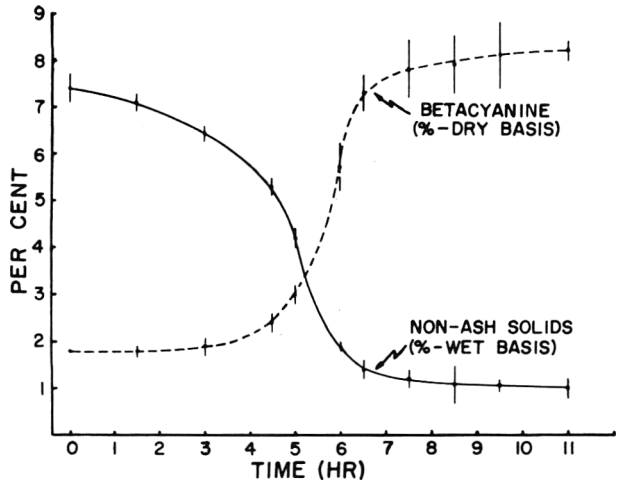

Fig. 2—Percent betacyanine and nonash solids as a function of fermentation time.

in betacyanine content was minimal and a maximum content of 8.2% betacyanine on a dry weight basis was achieved. Since after 6.5 hr of fermentation 96% of the total solid reduction had occurred, maximum betacyanine content was essentially obtained at this point. Therefore, to optimize betacyanine concentration in an industrial process, a 6.5 hr fermentation could be chosen for economic consideration. To shorten the fermentation time, further optimization could possibly be achieved by the addition of phosphate salts, or other growth factors not determined in these experiments.

CONCLUSION

THE RESULTS of these experiments have shown that fermentation of beet juice to remove solids is a possible method to increase betacyanine content in beet concentrates. Fermentation of ultrafiltered red beet juice by *Candida utilis* under partial anaerobic conditions was shown to substantially reduce solids, resulting in a five- to sevenfold increase in betacyanine content on a dry basis and achieving up to an 8.2% betacyanine content on a dry basis. The fermentable carbohydrates, nitrates, and 80% of the Kjeldahl nitrogen were assimilated after approximately 6.5 hr of fermentation. The fermentation medium was maintained at 30°C and at a pH of 5.0 with NaOH and HCl solutions. In addition, total phosphorus was determined and the preliminary data suggest phosphorus is a limiting growth factor. After drying, the product lacked the characteristic beet flavor and aroma, and was readily water soluble. This dry betacyanine concentrate may have application as a red food colorant.

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ENZYME USE TO REDUCE VISCOSITY AND INCREASE RECOVERY OF SOLUBLE SOLIDS FROM CITRUS PULP-WASHING OPERATIONS

ABSTRACT

Soluble solids (SS) recovered by countercurrent washing of citrus juice pulp are concentrated by evaporation and sold primarily for use in beverage bases and other food uses. On a pilot plant scale, treatment of the finisher pulp with pectolytic enzymes resulted in increasing the yield of recoverable SS from 65–80 kg SS/ton pulp (oranges) and from 45–50 kg SS/ton (grapefruit). High viscosity of pulp-wash liquids and other citrus processing liquids was found to be a deterrent during concentration by evaporation. Enzyme-treated liquids were concentrated in a medium-temperature evaporator to over 70° Brix and relative viscosities in the range of 1700–2200 centipoises; however, due to gel formation, nontreated liquids had to be pumped out of the evaporator at 25–35° Brix with a relative viscosity of over 20,000 centipoises. Careful use of enzyme to increase SS yields or lower viscosity during pulp-washing did not adversely affect the °Brix, acid, pH or clouding ability of the concentrated pulp-wash.

INTRODUCTION

CITRUS FRUIT is processed into several product streams at the time of juice extraction for the unit operations of juice manufacture, peel oil recovery and manufacture of dried cattle feed. The extracted juice immediately goes to finishers where part of the pulp is removed. This finisher pulp consists largely of the ruptured juice vesicles or "sacs" and may be manufactured into products used by the beverage and food industries (Kesterson and Braddock, 1973).

Because there are still juice and sugar-containing soluble solids (SS) remaining in the finisher pulp, further processing is necessary to recover this material. Recovery of SS from the finisher pulp is accomplished by multiple stage countercurrent washing with water, wherein, the water soluble materials are leached from the pulp. This process is known in the citrus industry as "pulp-washing" and recovered solution as "pulp-wash." The pulp-wash is concentrated in an evaporator similar to raw single-strength juice. Pulp-wash concentrate is an integral part of the citrus by-products industry and is sold worldwide for use as cloud, flavor or beverage base purposes.

Engineering data concerning processing technology of pulp-washing have been published by Belk (1964) and by McKinnis et al. (1964). These authors describe their respective countercurrent washing processes in details, discussing such factors as pulp flow, liquid flow, finisher variables, evaporation and cleanup. Other data concerning pulp washing and recovery of SS from pulp have been recently published by Braddock and Kesterson (1974).

Because of considerable variation in the types and maturities of the citrus fruit being processed, such factors as pulp softness (or firmness) and pectin content can make system control to obtain maximum yield of low viscosity liquids very difficult. For example, tight finisher settings during extraction of pulp might greatly increase the amount of pectin in the wash liquid. This liquid, during concentration in an evaporator could be expected to be difficult to handle because of high viscosity resulting from concentration of the pectin. There are now on the market reasonably priced, pectolytic enzymes which are compatible with citrus pulp and wash liquids. These enzymes may be used effectively to increase SS yields and eliminate viscosity problems during pulp washing, but data are scarce relating to actual uses in the citrus industry. This paper reports the use of pectolytic enzymes to increase the recovery of SS from citrus juice finisher pulp and describes their use to lower viscosity of pulp-wash liquids.

EXPERIMENTAL

Pulp recovery and quality analysis

Two major types of commercial juice extractors and finishers are currently used in the Florida citrus industry. Both systems were used during this experiment. Finisher pulp was recovered from oranges extracted with either of two types of commercial juice extractors (Model 700, Automatic Machinery Corp., Winter Haven, Fla.; Model 391, FMC Corp., Lakeland, Fla.) and finishers (AMC Model 3600; FMC Model 35). Finisher screen sizes were 0.5 mm. Experiments were also performed using finisher pulp recovered during two different seasons from grapefruit processed by the FMC system. In the discussion, pulps A and B were obtained from 204 kg fruit samples and differed by the type of commercial extractor which was used to process the fruit.

Various quality analyses referred to are common in the citrus industry. SS were determined as °Brix by refractometer, titratable acidity as % citric acid and cloud by measuring % light transmittance at 650 nm of liquids. Work was performed in the Agricultural Research and Education Center pilot plant and laboratory facilities where control of fruit variety and accurate extractor and finisher variables could be maintained.

Enzyme treatment for SS recovery

Laboratory scale washing experiments were performed using 2:1 water to finisher pulp ratios; wherein, 400g water and 200g pulp were mixed and allowed to stand for varying lengths of time, with or without appropriate enzymes. Consecutive washings were performed by screening the pulp-water mixtures on a #30 U.S. Standard sieve (0.59 mm mesh) allowing a 2 min drain time followed by rewashing. The liquid was recovered quantitatively, and the SS were determined by refractometer and put on a kg solids/ton of pulp basis. Similar washing experiments were performed on a pilot plant scale using 13.6 kg samples of pulp and 27.2 kg of water, mixed in 95 liter containers and finished to recover SS and liquid.

Enzyme treatment for viscosity reduction

Relative viscosities of various citrus pulp-wash concentrates and liquids were determined using a Brookfield Model LVF viscometer and the appropriate rpm and spindle. Concentrate viscosities were determined generally with the #3 spindle at 12 rpm, 25°C and 250 ml of sample in a 300 ml Berzelius beaker. In cases of extremely high viscosities, slower rpm and smaller spindles were used. For dilute pulp-washes and samples with low viscosities, sample size was 250 ml and the viscosity was measured using the Brookfield UL adaptor at 25°C.

Enzyme treatments of various 3–10° Brix pulp-wash liquids were used to reduce viscosity in order that the liquid could be concentrated to a higher °Brix by evaporation. Enzyme was added to 757–1514 liter samples of the dilute liquid to be concentrated. Enzyme concentration necessary for viscosity reduction was generally in the 50–100 ppm ranges and was based on the total SS contained in the tank. Holding times after enzyme addition were from 15–30 min before pumping the

sample to the evaporator at a rate of about 11.4–18.9 liter/min. Viscosities of evaporator pump-out at various stages of concentration were determined as described above.

The evaporator used was an experimental type (Vincent Processes, Inc., Tampa, Fla.) designed for an evaporative capacity of about 363 kg H_2O evaporation/hr with triple effect economy at product temperatures no higher than 62.8°C. Concentrated evaporator pump-out was obtained under predetermined conditions and ranged from 45° Brix up to 73° Brix depending on the experiment. All dilute pulp-washes were heat stabilized at 87.8°C for 0.5 sec and cooled immediately to 62.8°C in a heat exchanger (Junior Paraflow, A.P.V. Corp., Buffalo, N.Y.) for pumping to the evaporator. This step was necessary to inactivate natural and added enzymes in the pulp-wash.

Enzymes

Enzymes used were commercial pectolytic preparations and were obtained from the following sources: Irgazyme 100 (Ciba-Geigy), Klerzyme Liquid 200 (Wallerstein) and Pectinex R-Super Concentrate (Novo). Enzyme manufacturers' specifications as to optimum pH, temperature, etc. were generally found to be valid. Concentration of enzyme needed for a given experiment (e.g., SS recovery or viscosity reduction) differed according to manufacturer and had to be established, depending on the enzyme used.

RESULTS & DISCUSSION

RECOVERY of additional SS from citrus juice pulp is complicated by such variables as the type of juice extractor, finisher pressures, fruit maturity and variety of fruit being processed. The main types of fruit processed for juice and pulp-wash include oranges, grapefruit and mandarin-types (tangerines, tangelos, etc.). Each type of fruit has certain chemical and physical properties which contribute to different processing characteristics. For example, viscosity due to soluble pectin may cause handling problems during pulp-washing of less mature grapefruit; while, SS recovery from pulp-washing of mature tangerines may be affected by pulp softness causing problems during washing.

Pulp yield and SS recovery

The amount of SS which can be washed from finisher pulp depends largely on the quantity of pulp available from juice extraction of the various fruits. Generally, the highest yields of pulp were recovered from Duncan and Marsh grapefruit (59.6–129.5 g/kg fruit), followed by Pineapple (56.1–87.8 g/kg fruit), Hamlin (48.8–78.0 g/kg fruit) and Valencia (43.9–56.1 g/kg fruit) oranges.

Experiments were done to determine whether or not holding time before washing affected the quantity of SS which could be washed from the pulp. Examination of data in Table 1 will show that where washing and enzyme addition occurred immediately after pulp recovery, the SS yield increased by 15–18% for either pulps A or B. However, when the pulp was held in a 95-liter pot at 26.6°C for 1 hr before washing and enzyme treatment, a 17% SS increase occurred after enzyme treatment of pulp A, with no SS increase from pulp B.

In Table 1, the difference between the enzyme-related SS increase from pulps A and B was found to be reproducible and thought to be related to physical differences in the juice extraction processes, resulting in greater natural enzyme activity in pulp B. Since Rouse et al. (1962, 1964, 1965) have reported that orange and grapefruit juice sacs contain considerably more pectinesterase activity than membrane and seed components, there may be more pectinesterase activity in pulp B than pulp A. This reasoning is logical because pulp B was almost 100% juice sacs, while pulp A also contained some seeds and membrane components.

Pulp-wash properties

Some common citrus industry quality control tests were made on dilute pulp-wash liquids recovered in our pilot plant. Examination of Table 2 will show the effect of enzyme treatment on certain properties of pulp-wash liquids. These data are averages from six experiments with Valencia oranges and three experiments with Marsh grapefruit pulp and are representative of commercial pulp-wash liquids. Enzyme treatment time of the pulp before washing was 1 hr. This treatment resulted in significant improvement of SS yield and wash liquid recovery. In most instances, the SS yield increase was less for grapefruit pulp-washing; however, higher enzyme concentrations or longer holding times may be used to further increase the yield. It should be mentioned that citrus pulp will readily ferment, resulting in microbial spoilage, so holding times greater than 1 hr may not be commercially practical. Because of increased recovery of wash water and SS, an additional benefit of enzyme treatment of the pulp may be use of less water for pulp-washing. This could result in considerable savings in handling and waste treatment.

From Table 2, one can see that enzyme use lowers the pH and Brix/acid ratio of both orange and grapefruit pulp-washes. The acidity increase in both samples was slight and may be a result of enzymatic pectin breakdown to galacturonic acid residues. Generally, lowering the ratio of a citrus juice by 1 unit would not significantly affect the quality, except where the ratio would fall below the minimum for a particular grade standard.

One of the main uses for citrus pulp-wash is to impart cloud to certain fruit drinks and beverages. Enzyme use to increase SS yield from pulp-washing lowers the clouding ability of the liquid recovered (Table 2). However, if the liquid is properly heat-stabilized (87.8°C) during processing, cloud loss is usually not sufficient to be of concern and has been discussed for orange juice by Baker and Bruemmer (1971).

Table 1–Comparison of soluble solids yield from Valencia pulp obtained by commercial extraction processes

	Solids recovered (kg/ton pulp)			
	I[a]		II	
	No enz	Enz	No enz	Enz
Pulp A	59.88	70.86	56.39	65.87
Pulp B	67.37	77.35	74.85	75.35

[a] Washing and enzyme treatment occurred immediately (I) and 1 hr (II) after juice extraction and pulp recovery.

Table 2–Properties of pulp-wash liquids from Valencia orange and Marsh grapefruit juice pulps treated with enzymes[a]

	Orange		Grapefruit	
	No enz	Enz	No enz	Enz
Soluble solids (kg/ton pulp)	66.9	80.8	46.4	50.9
Wash liquid recovery (%)	77	83	76	86
Brix	3.0	3.2	2.8	2.7
Acid (%)	0.14	0.16	0.23	0.24
Ratio (°Brix/Acid)	21.4	20.0	12.2	11.3
Serum viscosity (cps)	1.4	1.1	3.2	1.1
Cloud (% T at 650 nm)	5	10	10	17
pH	4.59	4.50	3.55	3.52

[a] Enzyme concentrations to effect the same SS increase were Irgazyme (50 ppm), Klerzyme and Pectinex (500 ppm). Enzyme ppm was based on the pulp weight.

Table 3—Effect of enzyme treatment on the viscosity of various concentrated citrus liquids

Product	°Brix	Relative viscosity (centipoises) No enz	Enz (50 ppm)
Orange	17	1,200	90
pulp wash	36	20,000	108
	57	Gel	190
	71	–	1,710
Lemon peel	1.1	14	2[a]
leach water	15	Gel	16
	40	–	160
	55	–	1,200
Grapefruit	10	87	4
pulp wash	30	12,000	150
	50	Gel	1,170
	65	–	2,200

[a] 100 ppm enzyme was used in the lemon leach water.

Viscosity reduction

Citrus pulp-washes are concentrated in large commercial evaporators to 50–55° Brix. Because the washing operation leaches soluble pectic substances from the pulp, high viscosity of the liquid sometimes causes difficulties and prevents concentration to high °Brix. Pectolytic enzymes can be used to reduce the viscosity of liquids after recovery from the pulp.

In contrast to enzyme use to increase SS during pulp-washing, much less total amounts of enzyme are needed for viscosity reduction. For example, based on the total SS (from Brix tables) in 11,400 liters of a 3° Brix liquid, 37.5g of enzyme (Irgazyme) was sufficient to lower the viscosity enough that the liquid could be concentrated to 60° Brix. Prior to enzyme addition, concentration attempts of this particular liquid failed, and the liquid had to be pumped out of the evaporator at about 20° Brix because of high viscosity.

The effect of enzyme treatment of pulp-wash liquids on concentration in our pilot evaporator is shown in Table 3. Concentration to high Brix was easily done for enzyme-treated orange and grapefruit pulp-washes; whereas, gel formed at much lower Brix without enzyme treatment. Lemon peel leach water is a difficult-to-handle waste obtained during manufacture of dried pectin pomace. Concentration of this liquid to 55° Brix (or higher) was facilitated by use of pectolytic enzymes.

Following laboratory and pilot plant usage of pectolytic enzymes to lower viscosity, some trials were made at a commercial citrus processing plant (Alcoma Packing Co., Inc., Lake Wales, Fla.) where pulp-washing is performed. The pulp was recovered from large quantities of fruit and washed through a commercial pulp wash system (AMC, Winter Haven, Fla.). Data listed in Table 4 show the results of these commercial scale tests using different concentrations of two enzyme products. Enzyme solutions were added to the pulp-wash liquids in an evaporator feed tank and held 15–30 min before pumping to the evaporator.

The most significant results shown in Table 4 of adding enzyme to pulp-wash liquids were the viscosity reductions of the final pump-out concentrate from the pulp-wash evaporator. The sample to which no enzyme was added had a much higher viscosity than any of the enzyme-treated samples. Enzyme concentration also affected viscosity, noticeably in the case of Klerzyme, and to a lesser extent by Irgazyme. When the concentrated pulp-wash was diluted to 10° Brix, quality analyses showed a loss of cloud, which was not considered significant, due to enzyme treatment. Also, the lower viscosities noticed in the concentrates were reflected in the serum viscosities of the 10° Brix reconstituted products.

CONCLUSIONS

ADAPTATION of pectolytic enzyme treatment to increase recovery of SS during citrus pulp-washing is feasible and could be made common industry practice. However, certain modifications in the conveying system to increase pulp handling times might be necessary in existing commercial pulp-washing

Table 4—Process variables and quality data obtained when enzymes were added to pulp-wash liquids during commercial operations[a]

Cultivar[b]	P	P/J	P	P/V	P	P
Enzyme brand name	None	Klerz	Klerz	Klerz	Irgaz	Irgaz
Enzyme (ppm of tot. solids)	0	693	1,324	1,382	58	75
Pulp wash conc						
°Brix	48.4	53.4	52.4	53.2	50.6	50.8
Acid (mg/100g)	2.7	2.9	2.9	2.9	2.5	2.5
Viscosity (cps)	9,400	4,260	1,100	1,000	1,000	940
Conc diluted to 10°B						
Serum viscosity (CS)	11.4	4.0	2.8	2.5	3.1	3.0
Cloud (% T at 650 nm)	6.1	7.5	8.5	9.0	9.4	9.5
Sinking pulp (%)	2.0	2.0	2.0	3.0	3.0	3.0
Amount of fruit (ton)	332	377	328	353	377	209
Wash liq. recovered (liter)	45,333	43,217	30,352	28,141	37,210	32,755
°Brix of liquid	4.5	4.7	5.3	4.5	4.5	4.5
Evaporator feed (liter/min)	97.7	101.8	108.3	104.1	103.3	93.5

[a] These data were obtained through cooperation with a commercial processing plant (Alcoma Packing Co., Inc., Lake Wales, Fla.) and the technical assistance of R. Waters and H. Jones (AMC Corp., Winter Haven, Fla.).

[b] Cultivar names are as follows: P = pineapple orange; P/V = 9:1 blend of pineapple to Valencia oranges; P/J = 5:4 blend of pineapple to Jaffa oranges.

operations to allow time for the enzyme to react with the substrate. Enzymes should be added directly to the pulp based on the weight of pulp being treated. Additional conclusions regarding enzyme concentration, reaction times and SS yield were published by Braddock and Kesterson (1974). Since the quantity of SS recovered from pulp-washing is directly proportional to the quantity of pulp obtained from the fruit, juice extraction processes which obtain the greatest amount of pulp will result in the greatest economic return to the processor.

Use of enzymes to lower viscosity of pulp-wash liquids to facilitate concentration to higher °Brix is inexpensive and simple. Enzyme concentration is calculated on the basis of total SS in the liquid. The appropriate quantity of enzyme needs only to be metered into a surge tank or stream going to the evaporator. A short holding time (ca. 15 min) is necessary before feeding the evaporator. When enzyme is added to the pulp-wash liquid after the washing operation, the viscosity is lowered because of pectin degradation in the liquid. At lower liquid viscosities, pulp can be finished tighter, resulting in recovery of more liquid and increasing the yield of SS. Lower viscosities also will allow the evaporators to operate more efficiently at lower temperatures, thus saving on fuel cost.

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PEELING OF CHINESE WATERCHESTNUTS

ABSTRACT

The extensive hand labor required in peeling Chinese waterchestnut corms has dicouraged their production in this country. After cutting the apical and basal ends from the corms, the peel was removed by softening in hot alkali, followed by wet brushing. The lignaceous pigmentation which stains the corms during peeling was decolorized by treatment with hydrogen peroxide. A nomograph to optimize peel softening in terms of alkali concentration, time and temperature is presented. This nomograph shows solutions to the equation 6.76 = [0.038 (°F) – (6.51/% NaOH) – (9.81/min of treatment)].

INTRODUCTION

THE CHINESE WATERCHESTNUT, *Eleocharis dulcis* (Fig. 1), was introduced into this country in 1934. In paddy culture, reported yields vary from 8–23 tons per acre (Hodge and Bissett, 1955; Twigg et al., 1957). Despite this attractive yield, the horticulture of waterchestnuts has not been pursued because of difficulties in peeling. In 1973, 18 million pounds of hand-peeled waterchestnut corms were imported from Formosa (Tariff Schedules, 1973). The 1974 production of waterchestnuts in Formosa decreased because of government encouragement of rice production.

A prototype mechanical peeling system was developed at the USDA Western Utilization Research Laboratory, ARS (Shepherd et al., 1958). This system consists of two parts. The first produces a central cylinder from the corms by removing the apical and basal ends with rotating circular knives. The second abrades the peel from this cylinder. The chief disadvantage of this system is its low rate of operation. Two workers at best could peel only 40 corms a minute. In the years since its development, a machine of this type has seen no commercial application.

Preliminary histochemical investigation of the corms revealed a multicellular epidermal layer. This heavily lignified layer is morphologically discrete from the adjacent cortical cells (Fig. 2). The lignified nature of the peel prompted us to pursue peeling schemes based on pulp and paper technology. Preliminary work showed that the wood pulping agents, sodium sulfide and sodium sulfite, did promote peel softening; however, data analysis indicated that the same effect could be achieved with higher concentrations of sodium hydroxide. In addition, the bleaching of paper pulp suggested this approach for decolorizing alkali peeled corms.

The peel removal technique developed from this approach consists of five steps. First, the apical and basal ends of the corm are mechanically removed. Second, the peel is softened in hot alkali. Third, the softened peel is removed by wet brushing. Fourth, the peeled corms are bleached to decolorize lignaceous pigmentation. Finally, the corms are rinsed to remove excess alkali and bleach.

MATERIALS & METHODS

WATERCHESTNUT CORMS were obtained from the Plant Introduction Station, USDA, ARS, Savannah, Ga., and from Formosa, courtesy of RJR Foods, Inc. All chemicals were technical grade or better.

Ends were removed by three methods. In hand trimming, all basal

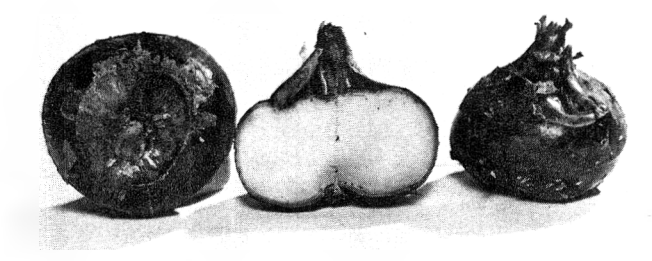

Fig. 1—Chinese waterchestnuts. Shown are the basal scar from rhizome attachment (left), a vertical section through the corm with the recessed basal scar at the bottom and apical shoot at the top (middle), and an intact corm inclined to accentuate apical shoot tissue (right).

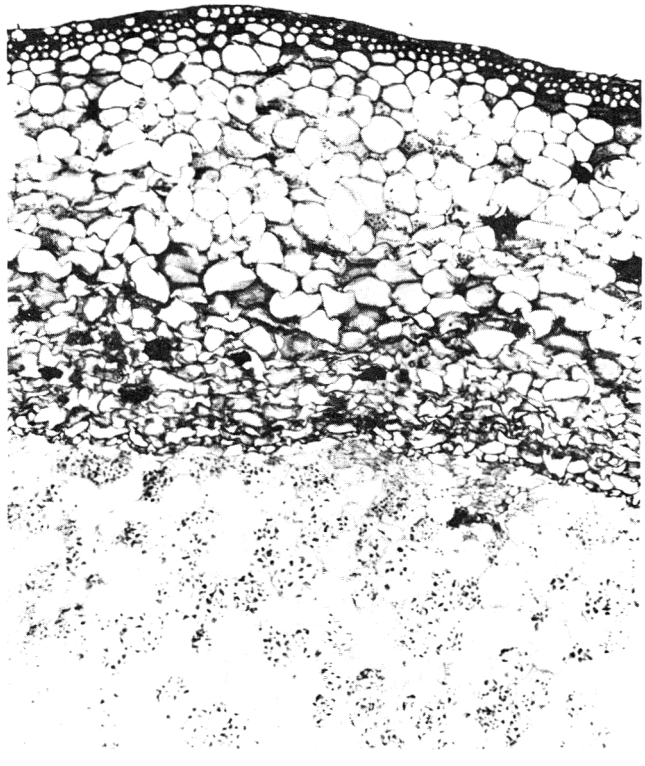

Fig. 2—This light micrograph shows the sharp line of demarcation between the waterchestnut peel and the adjacent cortical cells. The darkly stained walls of the 0.75 mm thick peel are heavily lignified.

scar and apical shoot tissue was removed. The end trimming device, developed as part of the mechanical peeler of Shepherd et al. (1958) was used at a knife separation of 0.5 inches. A waterchestnut end cutting machine purchased from A.C.E., Haddock, Ga., was also used. This machine was set to remove all the basal scar tissue and produce a first apical cut 0.6 inches above the basal cut. If the first apical cut did not expose the white cortical tissue, a photosensitive mechanism activated a second cut at 0.5 inches.

The peel was alkali softened in a steam-heated tank, thermostatically controlled to within 2°F. The corms were submerged during this treatment.

After peel softening, the corms were wet brushed by tumbling in a right cylindrical drum (20.5 in. × 22 in. diam) lined with Monsanto CH-4 landscape material (Fig. 3).

Peel removal on individual corms was scored as follows: 0 = peel intact, 1 = ⩽ 50% peel removed, 2 = > 50% peel removed, 3 = 100% peel removed. Lots of 30 corms each were used to determine average peel removal score. The consumption of hydrogen peroxide during the bleaching step was determined by iodometric titration.

RESULTS

A SERIES OF TESTS were run to determine the effect of sodium hydroxide concentration, temperature and processing time on peel removal by brushing (Table 1). Treatments of 15 min or longer in hot alkali caused hyalinization of the outer cortex of the corms, regardless of temperature or sodium hydroxide concentration over the ranges tested. This hyaline layer is not removed by subsequent brushing.

Brushing seemed to remove the softened peel better than water abrasion. Although a 2-min brushing period was used in all tests for peel softening by sodium hydroxide, 1 min of brushing was equally effective. Brushing for 5 min or longer caused abrasive damage on the peeled corms.

The percent of the original material recovered at this step was 50% for hand-trimmed corms, 35% for corms cut 0.5 in. thick and 50% for corms cut on the A.C.E. machine. The majority of the product loss was caused by end removal. Hand peeling corms resulted in a recovery of 50%.

Table 1—The effect of NaOH concentration, temperature and processing time on waterchestnut peel removal after 2 min brushing

% NaOH	Temp (°F)	Alkali treatment time (min)	Removal score[a]
5	210	10	1.67 ± 0.09
5	210	15	2.27 ± 0.11
11	198	10	2.50 ± 0.09
11	200	15	2.73 ± 0.08
11	189	30	2.77 ± 0.08
12	210	10	2.67 ± 0.09
12	210	5	1.67 ± 0.09
12	210	15	2.93 ± 0.05
16	190	15	2.53 ± 0.09
16	200	10	2.17 ± 0.11
16	210	5	2.13 ± 0.06
16	210	10	2.80 ± 0.07
16	165	10	1.13 ± 0.06
16	175	10	1.43 ± 0.09
16	184	10	1.57 ± 0.09
16	195	10	1.77 ± 0.12
16	200	10	2.33 ± 0.09
16	205	10	2.43 ± 0.09

[a] 1 = ⩽ 50% peel removed; 2 = > 75% peel removed; 3 = 100% peel removed.

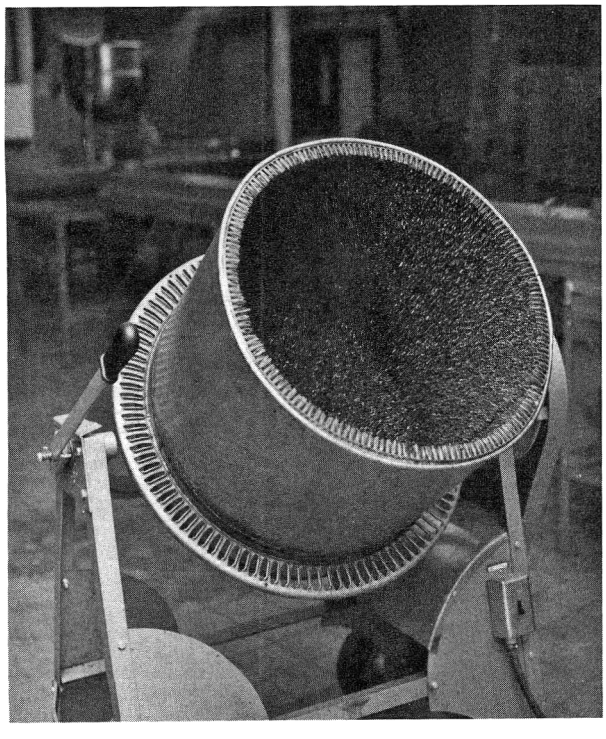

Fig. 3—The brush was constructed from a portable concrete mixer. The conical drum was replaced with a right cylindrical drum, the sides and bottom of which were covered with CH-4 landscape material. During brushing, approximately 3 gal of water were added to flush the removed peel from the bristles. This apoaratus can accommodate 30 pounds of corms.

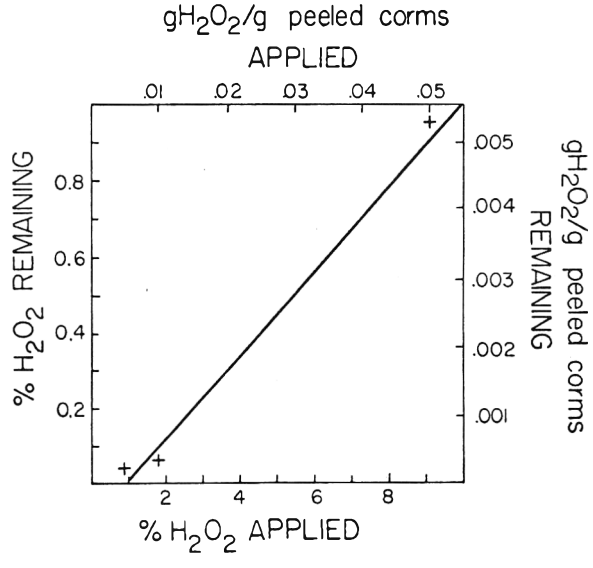

Fig. 4—Hydrogen peroxide remaining after 16 hr treatment at room temperature of peeled waterchestnut corms. The x-axes show rate of application in terms of % (W:V) H_2O_2 covering the corms and quantity of H_2O_2 applied as a weight fraction of the corms treated. The y-axes show the quantity of H_2O_2 in the same terms after treatment.

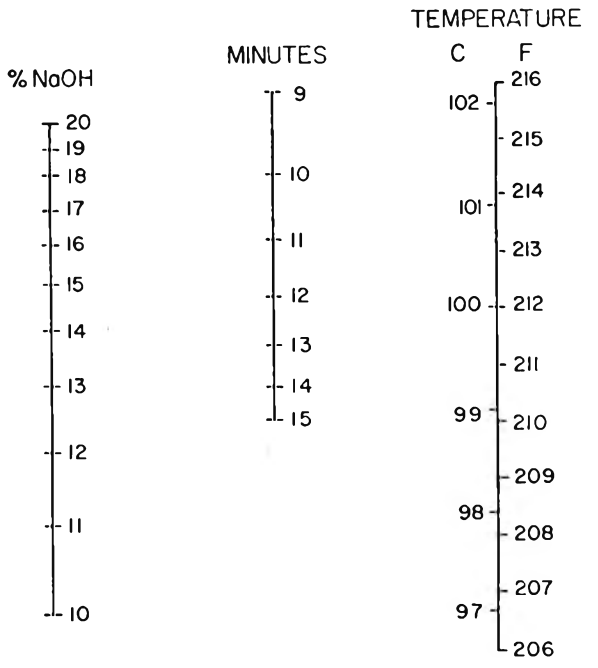

Fig. 5—Nomograph of NaOH concentration (W:V) and treatment time and temperature for optimum peel softening of waterchestnuts. A straightedge intersection all three columns estimates conditions for nonagitated treatment of submerged corms. Treatment times longer than 15 min gelatinize the starch in the outer part of the corm. This nomograph is based on the following regression equation solved for an average peel removal score of 3: Peel removal score = [0.038 (° F) − (6.51/% NaOH) − (9.81/min) − 3.76].

Corms peeled in alkali are orange to rust in color. This lignaceous pigmentation was decolorized by bleaching. Hydrogen peroxide proved to be much more satisfactory than hypochlorite. In addition, hydrogen peroxide is approved for bleaching of food.

The rate of decolorization by H_2O_2 was limited by its diffusion into the corms. The rate of diffusion was approximately 2 mm per hr at room temperature. However, once in contact with the colored area the bleaching is very rapid. Satisfactory H_2O_2 bleaching was achieved by overnight treatment in 0.5–3% H_2O_2. The consumption of H_2O_2 during 16 hr of treatment at room temperature is indicated in Figure 4. Peroxide levels of 5% or higher erode the surface of peeled corms resulting in product loss.

DISCUSSION

STATISTICAL ANALYSIS of the effect of sodium hydroxide concentration, temperature and time on peel removal by brushing resulted in the following equation:

$$\text{Peel removal score} = 0.038\ (°\text{F}) - \frac{6.51}{\%\ \text{NaOH}} - \frac{9.81}{\text{min}} - 3.76.$$

The standard error of the estimate for this equation is 0.2. This equation solved for a peel removal score of 3, 100% of the peel removed by brushing, provided for the construction of the nomograph shown in Figure 5. The experimental tests outlined in Table 1 were used to establish the mathematical relationships between treatment conditions and peel removal. These relationships have been used to extrapolate to concentrations of sodium hydroxide and treatment temperatures higher than those used in the original tests. However, the nomograph has been tested at its upper limits of these two conditions, as well as several others, and has been found to serve as a convenient guide for peel softening prior to brushing.

The hyaline layer formed on the outer surface of the waterchestnut during treatment times longer than 15 min appears to be due to the thermal gelatinization of the cellular starch granules. This processing defect can be entirely eliminated by limiting the duration of treatment in hot alkali.

The decolorization of the alkali-peeled waterchestnuts by overnight treatment in H_2O_2 does not seem to produce any off flavors. This has been corroborated by a commercial user of processed waterchestnuts. The surface erosion of the peeled corms at concentrations of H_2O_2, the rate of oxygen release is probably low enough for the liberated oxygen to remain solubilized.

After bleaching the residual alkali can be eliminated by neutralization. Any remaining H_2O_2 would be rapidly decomposed during heat treatment. This would happen during canning or cooking of the product.

At the present, we are investigating the effect of H_2O_2 concentration, treatment time, and water wash rate and time on the whitening of alkali-peeled Chinese waterchestnuts.

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A SIMULATED FRUIT GEL SUITABLE FOR FREEZE DEHYDRATION

ABSTRACT

Acceptance of new food resources may be achieved by simulation of traditional foods. Sodium alginate gels may be used to produce a desirable fruit-like texture useful as a base for fabricated nutrient-controlled foods. A two-step gelation process has been used to produce fruit-like textured products. These products can be flavored as desired and utilized without further processing to simulate fresh fruit pieces, or they may be either frozen and thawed, or freeze dried and rehydrated without loss of desirable texture. The freeze-dried materials rehydrate rapidly and when incorporated into food products such as yogurt, the rehydrated material compares favorably with rehydrated freeze-dried fruits or currently available commercial fruit yogurts.

INTRODUCTION

ACCEPTANCE of new food resources may be achieved by simulation of traditional foods through food analogs. In the field of food analog fabrication, most attention has been focused on protein-based food products (Inglett, 1975). Less work has been done on simulated fruits or vegetables, and less still regarding the processing stability and properties of simulated fruits and vegetables. Studies on the molecular basis of structural properties of fabricated foods have also focused on proteins (Stanley et al., 1972; Cumming et al., 1972; Buttkus, 1974).

Szczesniak (1968) showed that nonuniform cellular structures which simulate fruits and vegetables may be prepared by dialyzing certain alkaline earth metal salts, such as the acid salts of calcium or magnesium, at a uniform rate into an aqueous solution of water soluble alginate salt of suitable viscosity and concentration. Rapp and Ziemba (1972) employed corn syrup, invert sugars, carrageenan, color and flavor to produce structured, colored and flavored bits as ready-to-use replacements for fruits and confections in frozen and baked goods. A method of preparing a fruit product having a nonuniform texture simulating that of soft fruits has been developed by Wood et al. (1974). In this process drops of fruit pulp or puree incorporating dissolved calcium or aluminum ions is brought into contact with an alginate or pectate solution to form the "skin" of the fruit. Recently also a series of papers has been published by Russian workers who have used polysaccharides and/or proteins to produce textured systems. Alginate gels as well as gelatin-polysaccharide gel systems were used in these studies, which resulted in formation of anisotropic gels simulating various food products (Tolstogusov, 1974). The delicate texture of fruits or simulated fruit systems usually cannot sustain freeze drying treatment, becoming either spongy or rubbery after rehydration. The development of a method for producing a food matrix system which simulates fruit texture with good sensory quality and processing stability is reported here. In addition, some properties of the food matrix system are also noted.

EXPERIMENTAL

Gel formation and preparation

Development of formulation. The materials used for these studies were sodium alginate (Kelco Gel LV, KGLV 2475-52, Kelco Co.), calcium lactate (N.F. Powder, Mallinckrodt Chemical Works), citrus pectin (Sigma Chemical Co.), gelatin (Knox unflavored gelatin), Avicel and sucrose.

Initial tests with controlled interaction of sodium alginate (water soluble alginate salt) and calcium lactate (source of calcium ions) produced calcium alginate gels which had a crisp cucumber-like texture. However, after a freeze-thaw cycle, a product with undesirable rubbery and spongy texture was obtained. Another defect of the simple calcium alginate system was the poor breakdown properties of the gel toward the end of mastication. While the product had the cucumber-like crisp texture on the first bite, upon further chewing it became progressively drier rather than maintaining juiciness like natural cucumber. It was also somewhat unpleasant to swallow. To improve the sensory quality of the calcium alginate gel upon chewing, compounds of high water holding capacity were incorporated. These included dextran, starch, sucrose and pectin, tried either singly or in combinations.

The addition of pectin and sucrose to the alginate solution prior to the crosslinking process resulted in a gel with improved chewing quality. Pectin can absorb large quantities of water which is probably the main reason for its effectiveness.

Gelatin was added to the sample mixture when the two-step gelation procedure was developed. The gelatin allows the system to be thermally gelled prior to the chemical crosslinking with calcium ions.

In order to minimize textural damage due to mechanical forces exerted by the expanding ice crystals during freezing, the following procedures were adopted.

Avicel, a water insoluble microcrystalline cellulose was incorporated to create nucleation sites thus increasing their number and decreasing the size of ice crystals. The crosslinked matrix was also partially dehydrated prior to freezing either by air drying or an osmosis treatment against a 50% sucrose solution. The water content was reduced by 20–30% prior to freezing. With the addition of these procedures the freeze-thawed matrices were no longer cracked into pieces, nor were mushy, but instead, had a texture almost equal to the fresh matrices.

When this system was tested for retention of desirable texture following freeze drying and rehydration, a variable degree of success was achieved. For some samples good texture retention was found, while in others, the texture was significantly degraded. Comparative organoleptic evaluations showed that changes in texture noted in rehydrated samples were due mainly to changes which occurred during freeze drying or rehydration. If the matrix had collapsed during freeze drying a poor quality product was invariably obtained. Collapse also affected the appearance of the freeze dried matrices, and increased the rehydration time significantly.

A number of possible causes for this collapse phenomenon could be identified:

(1) High sucrose concentration due to sucrose incorporated initially in the matrix system;

(2) Collapse of one or more of the matrix polymeric components;

(3) Reduced mass transfer due to surface sucrose sorbed by the matrix during osmosis; and

(4) Partial melting of the frozen matrix during freeze drying.

A series of experiments showed that all these factors except collapse of the macromolecular matrix components had some influence. Most critical was an apparent melting during the early stages of freeze drying. Melting could be reduced or prevented by chilling the frozen samples in liquid nitrogen prior to insertion in the freeze dryer, and/or by precooling the freeze dryer plates for the initial drying period. The rate of freezing also played an important role, with rapid freezing giving somewhat increased collapse of the structure when compared to identically treated samples which had been slowly frozen.

The calcium alginate gel which could be successfully frozen and

thawed without loss of texture could also be successfully freeze dried and rehydrated without changing composition, if the freeze drying process was conducted so as to prevent matrix collapse. In general, this involved slow freezing and insuring that the sample was well chilled during the initial stage of freeze drying.

Matrix formation procedures

Initial studies were conducted using a mold and membrane system to contain the alginate solution during a one-step gelation procedure. This led to a number of difficulties especially because of a long gelation time, and a two-step gelation procedure has been developed. The crosslinking reaction resulting from controlled diffusive contact of the sodium alginate mixture with calcium ions from the calcium lactate solution is now preceded by a thermal gelation step. System preparation involves chilling the gelatin-containing alginate mixture at refrigeration temperatures to obtain a soft gelatin gel. The minimum concentration of gelatin which is required to preshape the alginate mixture is 1.5% (w/w). It appears that at this concentration gelatin does not interfere with the subsequent crosslinking of the alginate. The soft gelatin gel is sliced and then placed directly into calcium lactate solution at room temperature for sufficient time to completely crosslink the alginate. The time required for matrix formation using this two-step gelling procedure depends on the size of the soft gelatin slices, which can be varied easily according to needs.

Textural quality of the fresh matrix as determined by a three person panel was satisfactory, being equal to the better quality samples obtained earlier with the nonthermally pre-gelled process.

Advantages of the two-step gelation procedure over the one-step crosslinking are:

(1) Time required for matrix formation is greatly reduced.

(2) Simplified preparation, as no molds and nylon membrane needed.

(3) Increased flexibility, since the size and shape of the final matrix can be easily varied according to needs instead of being limited by the size and shape of the molds used.

In addition, the two-step gelation procedure simplifies scale up of the production of the matrices.

RESULTS & DISCUSSION

Rate of matrix formation

The formation of the matrix from sodium alginate and calcium lactate occurs through crosslinking of calcium ions with carboxylic groups of the alginate molecules. In order to get a successful gel, calcium ions have to diffuse slowly into the alginate solution. The rate at which this matrix forms in the thermally gelled system was studied by observing the time dependence of the thickness of the cross-linked region. This region can easily be differentiated visually since the thermal gel has a soft consistency which becomes firm after the formation of crosslinks. When the shortest dimension of a three dimensional matrix piece has been fully crosslinked, the crosslinking step is regarded as complete.

The time required for complete crosslinking of a given size of matrix is related to the length of the shortest dimension by

$$t = k\,d^2 \qquad (1)$$

where t is time in minutes, d is one-half of the shortest dimension in centimeters, and k is a constant, which is a function of sample composition, calcium ion concentration and temperature, and is equal to the reciprocal of the diffusion coefficient (d) using the unidirectional Fickian model with the usual simplifying assumptions (Treybal, 1955).

Alginate solutions containing pectin (2%), gelatin (1.5%), Avicel (0.25%) and sucrose (20%) were tested in a calcium lactate bath of 4.5% at alginate concentrations of 1.0–3.0%. An alginate concentration of 1.0% produced a matrix which was not firm enough for measurements to be made.

With alginate concentrations from 1.5–3.0%, there was no observable difference in rate of crosslink formation which was expressible as

$$t = 820\,d^2 \qquad (2)$$

This value corresponds to a value of D of 1.2×10^{-3} cm^2/min (2×10^{-5} cm^2/sec), which is in the range of expected values for diffusion of electrolytes in water and food gels (Karel, 1975).

The influence of calcium ion concentration on rate of matrix formation is shown in Figure 1 for an alginate solution of 2.5% alginate, 2.0% pectin, 1.5% gelatin, 0.25% Avicel and 20% sucrose. These rates can be expressed by the following equations:

$t = 1{,}520\,d^2$	when	$[Ca^{++}] = 1.5\%$
$t = 1{,}120\,d^2$		$[Ca^{++}] = 3.0\%$
$t = 820\,d^2$		$[Ca^{++}] = 4.5\%$

Alteration of the sucrose concentration also influences the rate of matrix formation (Fig. 2) when the concentration reaches levels above 20%. The retardation of crosslink formation due to sucrose can be expressed by:

$t = 768\,d^2$	when	sucrose = 0%
$t = 821\,d^2$		sucrose = 20%
$t = 1{,}150\,d^2$		sucrose = 30%

The influence of calcium ion concentration gradient and sucrose concentration on the rate of matrix formation suggests that the rate of matrix formation is controlled by the rate of diffusion of calcium ions, which is independent of alginate concentrations in the ranges used.

Properties of the food matrix

Structure. The matrix microstructure was investigated using optical and scanning electron microscope techniques.

Grains of the freeze-dried fabricated food matrix were examined using the optical microscope. When immersed in oil and examined at 600 × magnification, the grains appeared

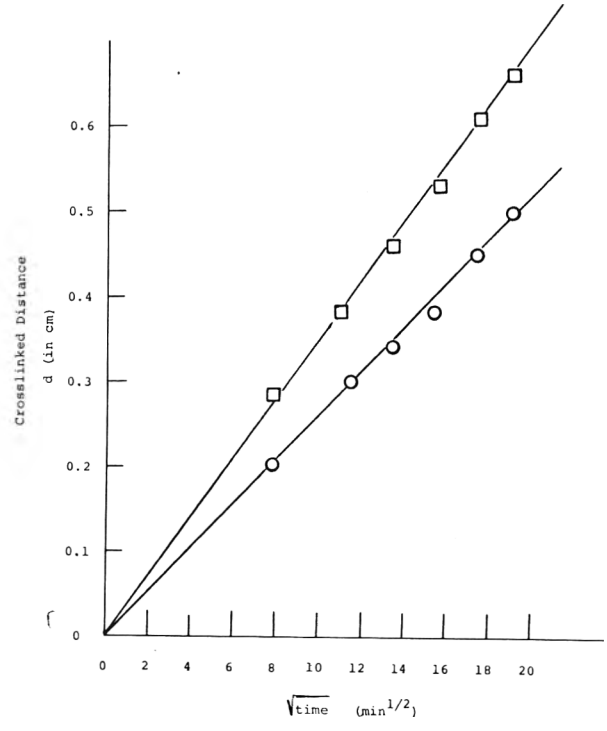

Fig. 1—Effect of calcium ion concentration on the rate of matrix formation: ○ Calcium lactate 1.5% (w/w); □ Calcium lactate 4.5% (w/w).

homogeneous. However when crossed polarizers were used, anisotropic regions were clearly distinguished from the remainder of the grain. When water was added, swelling of the grain was observed, and the Avicel microcrystals which will not dissolve or swell in water, were seen to be dislocated by the swelling of the food matrix. The strongly anisotropic regions remained following the rehydration. It can be assumed therefore that these regions are Avicel, since any sucrose crystals would disappear after rehydration.

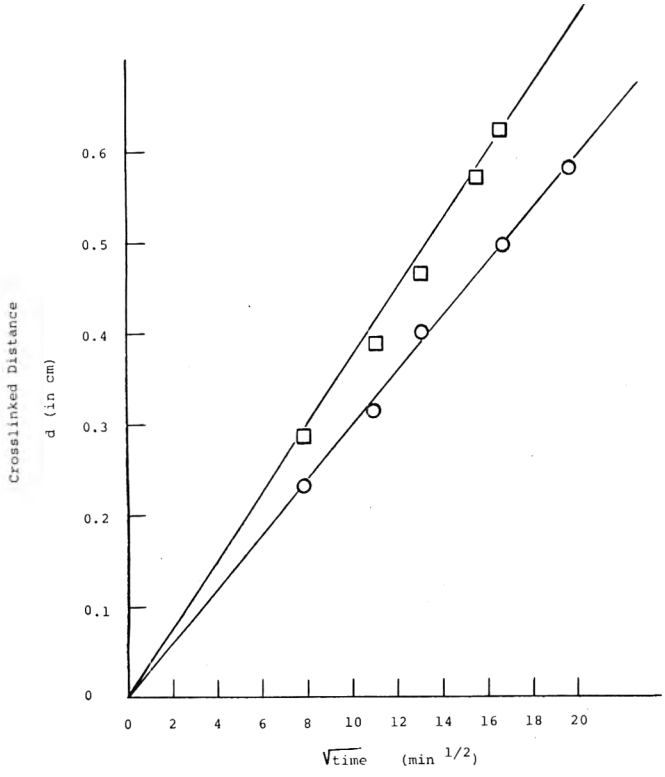

Fig. 2–Effect of sucrose concentration on the rate of matrix formation: ○ Sucrose 30% (w/w); □ Sucrose 0%.

A weak anisotropy of the food matrix which decreases upon rehydration and returns after re-evaporation of water was also observed. This appears to be due to a certain degree of polymeric alignment in the dried crosslinked matrix.

Scanning electron micrographs of the surface structure of food matrices which were freeze dried with and without matrix collapse are shown in Figure 3 and 4, respectively. In each sample, many surface pores were observed. However, it can be seen that there is a sizable difference in the size of the pores between collapsed and noncollapsed samples, with the average diameter of noncollapsed samples about tenfold larger than the average diameter of the holes of the collapsed samples. The difference results in an approximately 100-fold difference in the pore cross section area. The two conditions differ therefore very greatly in vapor or liquid flow resistance, and have different structural arrangements of the matrix units which in turn can be expected to alter the overall strength. These factors are probably the reason why the collapsed samples are so tough and hard to rehydrate.

Rehydration. Rehydration behavior of freeze-dried food matrices was studied by measuring weight gain after fixed times of rehydration. To measure sucrose loss during rehydration the dry matrix weight before and after rehydration (i.e., re-freeze dried) was determined.

Percent (%) rehydration compares the weight of water per unit weight of solids after rehydration to the weight of water per unit weight of solids of the product after the osmotic treatment. This can be expressed as

$$\% \text{ Rehydration} = \frac{\dfrac{w_3 - w_4}{w_4}}{\dfrac{w_1 - w_2}{w_2}} \times 100$$

where all weights are for a given sample after the indicated treatment: w_1 = weight after osmotic treatment; w_2 = weight of osmotically treated sample after freeze drying; w_3 = weight after rehydration; and w_4 = weight of rehydrated sample after re-freeze drying. It was noted that samples having different pretreatments showed differing rehydration behavior. During rehydration fast and slowly frozen osmotically treated samples

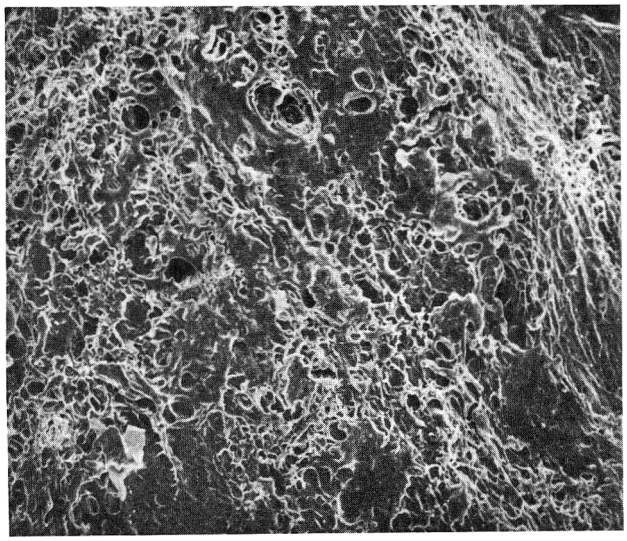

Fig. 3–Surface structure of freeze-dried food matrix as viewed by scanning electron microscope (Collapsed sample–100X).

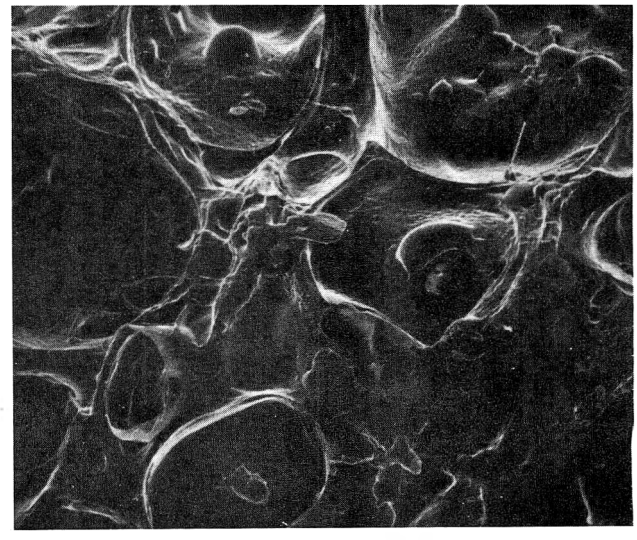

Fig. 4–Surface structure of the freeze-dried food matrix as viewed by scanning electron microscope (Uncollapsed sample–100X).

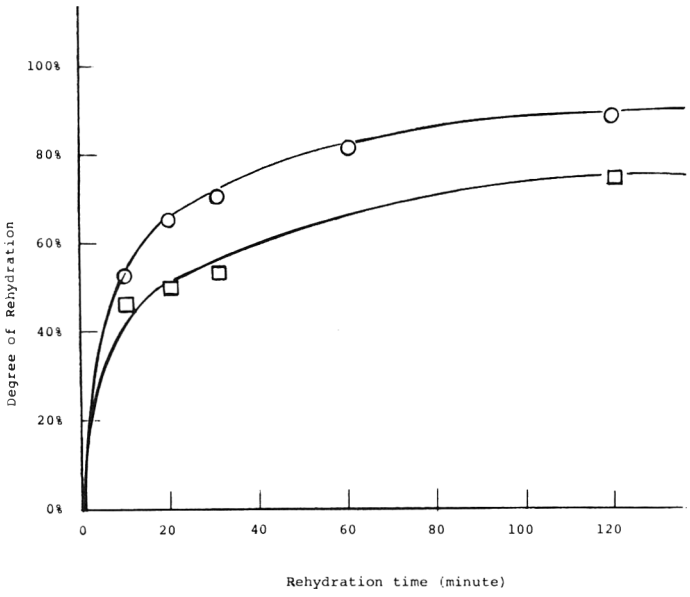

Fig. 5–Rehydration of freeze-dried food matrix (with osmosis treatment): ○ Slow freezing; □ Fast freezing.

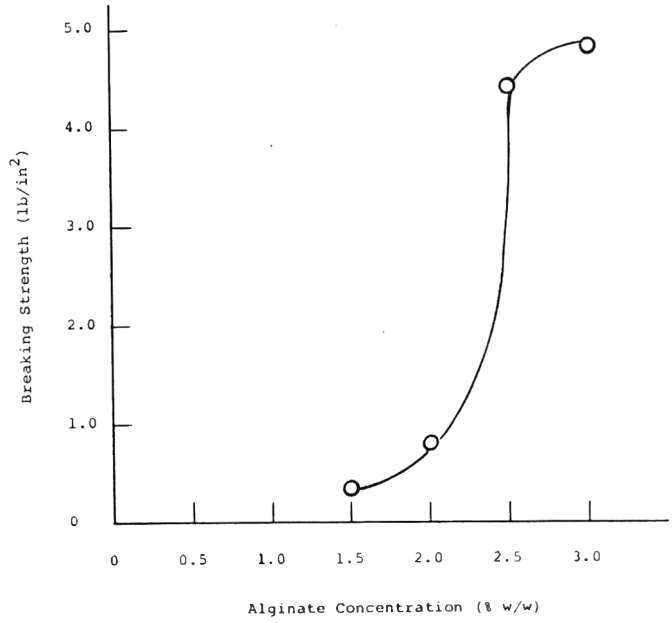

Fig. 6–Effect of alginate concentration on the breaking strengths of the fabricated food matrix.

showed similar loss of sucrose (41 and 45% of the initial sucrose, respectively) while the nonosmotically treated samples showed some differences (54% loss of sucrose for slowly frozen vs 33% loss for fast frozen). In Figure 5 it is seen that a slow freezing prior to freeze drying resulted in samples with a higher rehydration rate than samples rapidly frozen. It was also noted that samples without osmosis treatment rehydrated faster than samples with osmosis treatment. For example after 30 min rehydration osmotically treated samples showed a rehydration of 53% while samples without osmotic treatment had 71%.

Rheology. Changes of rheological properties of the food matrix system brought about by different processing procedures have been evaluated using the Instron Universal Testing Machine. At present any relationship or organoleptic evaluations of texture with rheological properties of the fabricated food matrix is limited in scope and the reported evaluation serve primarily to indicate the influence of process and composition variations on mechanical properties of the matrix. The changes of matrix breaking strength at different alginate concentrations are shown in Figure 6. It is seen that at alginate concentrations lower than 2.0%, the crosslinking is not extensive enough to give a sufficiently strong three-dimensional network, resulting in soft, weak matrices which will not sustain further processing.

Figure 7 shows changes of elastic moduli, breaking strengths and fractural energy densities of samples at various stages of the preparation and use process.

It can be seen that the organoleptically desirable final products, fresh matrix (A), freeze-thawed matrix (without osmotic treatment) (B) and rehydrated freeze-dried matrix (with osmotic treatment and no collapse) (E) have similar values for elastic moduli, breaking strengths and fractural energy densities. Freeze-dried and rehydrated samples which have not been osmotically treated have high elastic moduli, breaking strengths and fractural energy densities, probably due to rearrangement of the macromolecular components of the food matrix which occurred during freeze drying which causes increased stiffness and toughness of the rehydrated samples. Osmotically treated samples freeze dried without shelf refrigeration during the first period of freeze drying, differed in physical properties from the same sample freeze dried with shelf refrigeration. Partial melting and collapse probably gives a more dense, rigid structure, resulting in the much higher elastic modulus, breaking strength and fractural energy density. Samples prepared with 30% sucrose in the initial alginate mixture show much smaller variation in rheological properties than

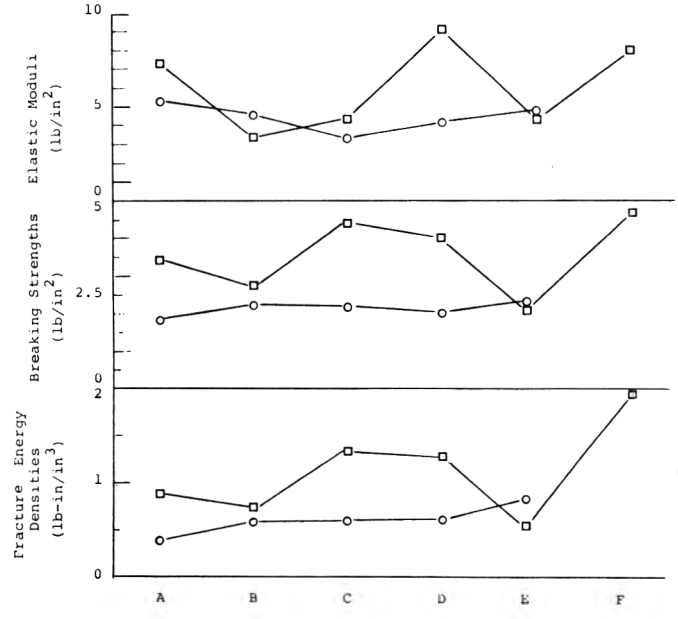

Fig. 7–Effect of processing on the rheological properties of fabricated food matrices: (A) Fresh samples; (B) Frozen-thawed samples; (C) Frozen-thawed samples (with osmosis treatment); (D) Freeze-dried and rehydrated samples; (E) Freeze-dried and rehydrated samples (with osmosis treatment); (F) Freeze-dried and rehydrated sample (with osmosis treatment, partially collapsed): ○ 30% Sucrose; □ 0% Sucrose.

Table 1—Organoleptic scores for products containing food matrices

Product	Number of panelists	Organoleptic score: Taste $\bar{x}$[b]	Taste σ[c]	Texture $\bar{x}$	Texture σ	Ranking[a]: Rank	Ranking $\bar{x}$	Ranking σ
Peach Yogurt								
Yogurt with food matrices[d]		4.36	1.21	3.70	1.57	2	−0.08	0.60
Yogurt with freeze-dried fruit[e]	11	3.82	1.17	4.10	1.37	3	−0.31	0.69
Commercial yogurt[f]		4.64	1.03	4.20	1.23	1	+0.39	0.70
Strawberry Jello								
Jello with food matrices		4.17	1.03	3.83	0.72	2	−0.14	0.71
Jello with freeze-dried fruit	12	4.50	1.00	3.83	1.34	1	+0.28	0.66
Jello with frozen fruit		4.00	1.21	4.17	1.03	2	−0.14	0.71
Pineapple Yogurt								
Yogurt with food matrices		3.42	1.16	4.25	1.14	3	−0.28	0.55
Yogurt with freeze-dried fruit	12	4.42	1.56	4.67	0.98	1	+0.43	0.77
Commercial yogurt		3.92	1.44	4.30	1.37	2	−0.14	0.61
Banana Yogurt								
Yogurt with food matrices		3.46	1.05	3.31	0.95	2	−0.33	0.55
Yogurt with freeze-dried fruit	13	3.62	1.04	3.69	0.85	2	−0.33	0.55
Yogurt with fresh fruit		5.00	0.91	4.62	1.26	1	+0.65	0.51
Pineapple Yogurt								
Yogurt with food matrices		3.92	1.00	3.33	1.23	2	−0.21	0.38
Yogurt with canned fruit	12	5.08	0.67	5.00	0.74	1	+0.71	0.33
Commercial yogurt		2.83	1.11	3.58	1.08	3	−0.50	0.67
Pineapple Yogurt (Evaluated as dry snack)								
Yogurt with food matrices		4.08	1.38	4.00	1.40	1	+0.23	0.80
Yogurt with freeze-dried fruit	12	3.58	1.31	3.83	1.48	2	−0.07	0.74
Commercial yogurt		3.50	1.08	3.83	1.27	3	−0.14	0.57

[a] Values given for ranks are: first (0.81), second (0) and third (−0.81).
[b] $\bar{x}$ = mean.
[c] σ = standard deviation.
[d] Yogurt with food matrices: Plain yogurt with sugar added containing rehydrated food matrices of desired flavor and color.
[e] Yogurt with freeze-dried fruit: Plain yogurt with sugar added containing rehydrated freeze-dried fruit.
[f] Commercial yogurt: Commercial fruit yogurt of desired variety.

those without sucrose included. Sucrose seems to play an important role in stabilizing the texture of the matrix during processing.

Applications of food matrices

Organoleptic evaluations have been conducted on products containing the food matrices as a substitute for fruit products. Comparisons were made with products containing freeze-dried fruits prepared in our laboratory, and to equivalent commercially available products. Organoleptic quality was evaluated using a difference analysis test having a six-point hedonic scale running from excellent (6) to very poor (1) and a ranking preference test with the most preferred first. The results presented in Table 1 are encouraging. Only fresh banana and canned pineapple were rated better than the fabricated food matrices which had been freeze dried and rehydrated.

A number of advantages which result when using the food matrices as fruit substitutes are:

(a) Controllable size and shape which are more uniform than freeze-dried fruits.

(b) No discoloration problems, such as due to enzymatic browning.

(c) When the product is consumed as a dry snack the food matrix is less chewy or sticky to teeth and more crunchy.

The results presented here demonstrate the feasibility of preparation of satisfactory fruit-simulating gels as components of freeze-dried products. The economic value of this approach will depend on future needs for engineered foods with capability for controlling texture and nutrient content.

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EFFECT OF A PROTECTED LIPID SUPPLEMENT ON FLAVOR PROPERTIES OF BEEF

ABSTRACT

Beef with subcutaneous fat containing about 20% linoleic acid was produced by supplementing rations of 9-month old Angus steers with a formaldehyde-treated sunflower seed-casein preparation for 2 months. A laboratory taste panel compared the flavor of this beef with that from steers fed a conventional diet. In addition the cooked-meat flavor volatiles recovered by vacuum distillation were examined by gas chromatography. High-linoleic beef was preferred less and was significantly different from conventional beef in possessing a characteristic 'oily' odor and flavor, probably attributable to elevated levels of *trans, trans*-deca-2,4-dienal found in lipid portions of the cooked meat. However, it did not possess the objectionable sweet odor sometimes found in high-linoleic lamb produced using the same supplement.

INTRODUCTION

THE PROPORTION of linoleic acid to total fatty acids in the subcutaneous lipids of ruminant animals can be increased more than tenfold over normal concentrations by including in their diet a polyunsaturated oil or oilseed, protected from ruminal hydrogenation by a formaldehyde-treated protein coating (Scott et al., 1970, 1971). Sheep meats containing about 20% of linoleic acid (high-linoleic meat) in subcutaneous fat were obtained by supplementing alfalfa-grain rations with a formaldehyde-treated sunflower seed-casein preparation and the cooked flavor examined (Ford et al., 1975). The odor and flavor of this meat was markedly different from that of normal lamb or mutton in possessing 'sweet' and 'oily' characteristics. These characteristics were attributed by Park et al. (1974, 1975) to the presence of abnormal quantities of *cis*-γ-dodec-6-enolactone and *trans,trans*-deca-2,4-dienal, respectively, in the fatty portions from the cooked meat.

This report describes a laboratory evaluation of the flavor properties of meat from 11-month old beef animals fed a diet similar to that fed previously to sheep (Ford et al., 1975).

EXPERIMENTAL

Animals and feed

Eight Angus steers approximately 9-month old were divided into two groups of four which were housed in large, covered pens and allocated to one of two dietary treatments for a period of 67 days.

One group, of mean liveweight 161 kg, was given a ration of equal weights of chopped alfalfa hay and oats. Initially, the steers were allocated 4.5 kg feed/head/day. This was increased gradually to 9 kg/head/day over a period of 12 days and this level maintained until slaughter.

The other group, of mean liveweight 179 kg, was given a ration of chopped alfalfa hay, oats and formaldehyde-treated sunflower seed-casein mixed in the proportions 50:20:30 (Scott et al., 1972). This diet was fed at 4.5 kg/head/day initially, raised gradually to 9 kg/head/day over 12 days and maintained at this level until slaughter. These diets were nearly equal in energy and calorific value.

The steers were slaughtered at a local abattoir and the carcasses halved and chilled to about 1°C, while suspended from the obturator foramen to minimize between-carcass differences in tenderness (Bouton et al., 1973). The carcasses were held at 1°C for 24 hr before quartering and boning out. One rump portion (principally gluteus medius muscle) from each animal was stored in a polyethylene bag at 0–2°C for up to 7 days prior to taste panel evaluation, while the alternate rump was stored at −30°C (3–4 wk) and then thawed at 5–6°C for 18–24 hr before use.

Samples of subcutaneous fat from the rump and perinephric fat were removed and the lipid extracted and its fatty acid composition determined by gas chromatography of the methyl esters (Ford et al., 1975).

Taste panel evaluations

Evaluations, employing a selected 20-member laboratory taste panel (Ford et al., 1975), were carried out on both the chilled and frozen rump portions with subcutaneous fat attached. Each rump was halved and one portion prepared as boiled, ground meat served hot (Park et al., 1972) and the other as oven-grilled steaks. The latter, 18–20 mm thick complete with subcutaneous fat were cooked in an air-circulating oven at 230°C for 20 min, sliced into 50 × 10 mm portions each containing some subcutaneous fat and served hot. Samples were presented in pairs, one from each dietary treatment which were matched by ranking weights while the order of tasting within pairs and order of presentation of pairs were randomized. Panel members scored the flavor properties previously employed (Park et al., 1972) i.e., intensity of meat odor, 'different' odor, meat flavor, 'different' flavor and flavor acceptability. An intensity rating scale 0 = zero to 8 = very strong was used for the first four of these properties and a hedonic scale of 0 = very poor to 8 = very good for rating of flavor acceptability. Panel members entered their scores on computer cards (Gipps and Casimir, 1973) and the data analyzed for differences arising from the dietary treatments, by analysis of variance. Taste panel members were also asked to provide descriptive comments to assist in characterizing the nature of any unusual odor or flavor perceived in the samples. They were provided with a list of descriptive terms to assist in describing such odor or flavor qualities (Ford et al., 1975).

Examination of volatile flavor components

The volatile components were recovered from the steam-volatile and lipid fractions of the boiled, ground beef and these examined by gas chromatography, using the methods described for examination of sheep meat volatiles (Park et al., 1975). Gas chromatographic examinations were carried out using the Carbowax 20M-coated SCOT and OV101

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Table 1—Mean percent composition of individual fatty acids in subcutaneous and perinephric lipids of beef from high linoleic and control dietary treatments

Treatment	Lipid	Constituent fatty acids[a] 14:0	16:0	16:1	18:0	18:1	18:2
High linoleic	subcutaneous	3.8 ±0.3*	21.2 ±1.0	3.9 ±0.5	15.8 ±1.7	34.2 ±2.7	19.1 ±1.4
	perinephric	1.7 ±0.2	19.7 ±0.7	1.1 ±0.2	27.4 ±0.7	28.1 ±0.8	21.4 ±1.3
Control	subcutaneous	2.6 ±0.4	28.9 ±1.0	4.2 ±0.2	18.6 ±1.6	43.6 ±1.9	1.4 ±0.2
	perinephric	2.1 ±0.4	27.8 ±0.3	2.6 ±0.6	34.9 ±2.6	31.3 ±1.7	0.9 ±0.3

* Standard error

[a] Fatty acids below 14:0 or greater than 18:3 were not measured here and 18:3 concentrations were all less than 1% of total fatty acids.

packed columns under the temperature programming conditions described for the relevant distillation fractions, while the columns were fitted for olfactory examination of eluting components. Identification of components was by comparison of retention and odor characteristics with those in similar fractions previously examined by GC-MS techniques (Murray and Park, 1975).

RESULTS & DISCUSSION

THE MEAN concentrations of individual fatty acids in the subcutaneous (rump) and perinephric lipids of the beef animals from the two dietary treatments, expressed as a percentage of the total acids, are given in Table 1. Fatty acids below 14:0 and greater than 18:3 were not recorded under the conditions of analysis. The subcutaneous lipid linoleic acid levels of the group consuming the protected sunflower seed preparation as the principal dietary lipid source exceeded those for the control group by a factor of more than 10 while the perinephric lipid linoleic levels were increased more than twenty-fold. The mean liveweights at slaughter of the animals in the high-linoleic and control groups were, respectively, 245 and 219 kg and the corresponding carcass weights 128 and 109 kg. The average daily liveweight gains of the two groups were 985 and 866 g/day, respectively. It has been assumed that these small weight differences between the treatment groups would have no consequence to flavor evaluations.

Taste panel evaluations

The mean taste panel scores for the beef from high-linoleic and control dietary treatments are summarized in Table 2. The responses to the chilled and frozen meat are pooled since no significant effects of storage appeared in the analyses. The panel found a significant difference in aroma and flavor properties between the high-linoleic and control beef samples. These results are very similar to those reported for high-linoleic and control lamb and yearling mutton (Ford et al., 1975). The agreement of the responses to the two cooking methods also parallels the experience with sheep meat and lends support to the use of boiled beef as a source of the volatile flavor components contributing to the flavor differences between the two beef diet treatment groups. The influence of methods of cooking on taste panel responses was not measured here.

The odor and flavor descriptions given to the high linoleic beef are somewhat different to those given for sheep meat. The principal odor and flavor description given to high-linoleic beef was 'oily' and this was applied equally to boiled meat and to grilled steaks. However the term 'sweet' was applied consistently only by one panellist to this beef, whereas in high-linoleic sheep meat, 'sweet' was applied as frequently as 'oily.'

The significantly lower acceptability scores given for high-linoleic beef demonstrate that this panel preferred the conventional beef flavor. However, the views of a trained and experienced laboratory panel such as employed here in assessing differences by paired comparison tasting may not represent the views of any group of consumers (Ford et al., 1975).

Examination of volatile flavor components

The relative yields of the more significant volatiles found in the steam-volatile and cold finger fractions of the lipid portions from the boiled meat are shown in Table 3. The qualitative composition of the distillation fractions obtained from the boiled ground beef was similar to that of the corresponding fractions from lamb fed similar diets (Park et al., 1975). However, the cold finger fractions from distillation of the high-linoleic beef fat were different from the corresponding fractions from high-linoleic lamb fat in one very significant respect. The concentrations of *cis*-γ-dodec-6-enolactone in the fractions from beef fat were less than 0.1 ppm compared with 1.0 ppm or greater in high linoleic lamb fat. It is considered unlikely that the lactone would make any significant individual contribution to the aroma or flavor properties of the beef fat at the concentrations found here (Park et al., 1975). The total amount of all lactones found in the high-linoleic beef fat was about 0.25 ppm and below the odor or flavor threshold of any one. It is thus considered unlikely that any additive effect

Table 2—Mean taste panel ratings for intensity of aroma and flavor and scaling of acceptability of flavor of high-linoleic and control beef

Cooking method	Dietary treatment	Flavor property: Meat[a] aroma	Meat[a] flavor	Different[a] aroma	Different[a] flavor	Acceptability[b] of flavor
Grilled steaks (frozen or chilled storage)	high-linoleic	3.12**	3.50**	2.53***	2.55***	3.75***
	control	3.66	4.13	1.03	0.68	5.35
Boiled, ground (frozen or (chilled storage)	high-linoleic	3.33**	3.20***	1.96**	2.84***	3.27***
	control	3.73	3.97	0.69	0.92	4.79

[a] Intensity rating from 0 (zero), through 4 (moderate), to 8 (very strong intensity)

[b] Hedonic scale from 0 (very poor), through 4 (moderate), to 8 (very good acceptability)

** Significant dietary treatment effect, $P < 0.01$

*** Significant dietary treatment effect, $P < 0.001$

Table 3—Relative yields of selected volatiles from both high-linoleic and conventional boiled beef

Volatile compound	Source[a]	Relative yield from distillate fraction[b]	
		Conventional beef	High-linoleic beef
Pentanal	Aqueous	35	13
Hexanal	Aqueous	10	30
2-heptenal	Aqueous	1	4
2-octenal	Aqueous	1	,6
Pentanol	Aqeuous	20	15
Deca-2,4-dienal	Fat	2	35
γ-dodecalactone	Fat	5	3
Cis-γ-dodec-6-enolactone	Fat	2	6

[a] Either aqueous, steam-volatile fraction or cold finger distillate from high vacuum degassing of the fat.
[b] Yield as a percentage of total volatiles recovered from the distillate fraction concerned.

of the lactones would make a significant contribution to the meat flavor. This is in agreement with the lack of use of the term 'sweet' in the odor and flavor descriptions given by most panellists to the high-linoleic beef.

The relative quantities of *trans, trans*-deca-2,4-dienal found in the high-linoleic beef fat distillate fractions (1–3 ppm) were similar to those recovered from high-linoleic lamb, probably accounting for the 'oily' descriptions given to aroma and flavor of the meat. The relative amounts of individual lower carbonyls and like components in the steam-volatile fractions from high-linoleic beef were also similar to those from high-linoleic lamb, indicating a likely origin from oxidation of linoleic acid. The distillate fractions from control beef had lower quantities of the unsaturated lactone (< 0.1 ppm in fat) and carbonyl compounds than those from high-linoleic beef and generally similar to those of corresponding control or conventionally-fed lamb fractions.

The boiled high-linoleic beef examined here has been found to contain elevated levels of several carbonyl compounds, notably *trans, trans*-deca-2,4-dienal and slightly elevated levels of *cis*-γ-dodec-6-enolactone. The occurrence of lower quantities of the unsaturated lactone in bovine than in ovine meat from animals consuming qualitatively the same feed remains unexplained, but has obvious consequences in the aroma and flavor properties of the meat.

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FURTHER EVALUATION OF CONVENTIONAL AND HOT-BONED BOVINE LONGISSIMUS DORSI MUSCLE EXCISED AT VARIOUS CONDITIONING PERIODS

ABSTRACT

The objective of this study was to evaluate the tenderness of bovine longissimus dorsi muscles conditioned in the intact half at 16°C and excised at 6, 8 or 10 hr postmortem (hot boning) as compared to the same muscles chilled at 2°C and excised at 48 hr postmortem (cold boning). Fifteen choice and good grade heifers were utilized in this study. When each postmortem holding period was evaluated, statistically nonsignificant differences ($P > 0.10$) were observed between hot- and cold-boning means for shear force, myofibrillar protein extractability and sarcomere length. A statistically significant difference ($P < 0.10$) between hot- and cold-boning fiber diameter means was observed at the 6-hr holding period. Fiber kinkiness means for the hot-and cold-boning treatments were also statistically different at the 6- ($P < 0.01$) and 8-hr ($P < 0.10$) holding periods. These data indicate that conditioning intact halves at 16°C and excising bovine longissimus dorsi muscles at 8 hr postmortem can yield a product of acceptable tenderness. The authors recommend the 8-hr holding period as a precautionary measure realizing that the 6-hr holding period did not give greatly different results.

INTRODUCTION

DUE TO CURRENT INTEREST in processing beef carcasses soon after slaughter (Schmidt and Gilbert, 1970; Kastner et al., 1973; Schmidt and Keman, 1974; Kastner and Russell, 1975; Henrickson, 1975), the effects of selected postmortem holding periods, prior to fabrication, should be further evaluated.

Several workers (Lowe and Stewart, 1946; Ramsbottom and Strandine, 1949; Locker, 1960; Goll et al., 1964; Herring et al., 1965a; Herring et al., 1967; Gillis and Henrickson, 1969a; Cagle and Henrickson, 1970; McCrae et al., 1971; Henrickson et al., 1974) have shown that muscles excised soon after death and allowed to freely contract are less tender than those muscles restrained during the development of rigor or excised post-rigor. As the degree of muscular contraction increases, tenderness as measured by the Warner-Bratzler shear apparatus decreases and fiber diameter increases (Herring et al., 1965a, b; Gillis and Henrickson, 1969a, b). In addition to increased fiber diameters, increases in shear force appear to be related to increased muscle fiber kinkiness (Gillis and Henrickson, 1969a; Henrickson et al., 1974). Herring et al. (1965b) and Hostetler et al. (1972) have shown that muscle restraint during the onset of rigor mortis affects sarcomere length. Sarcomere length, in turn, was negatively correlated to tenderness as measured by shear force. Sarcomere length was used by Smith et al. (1969) to evaluate cold shortening in avian muscle held at selected temperatures, and they found shortening to be minimal at 12–18°C and maximum at 20°C. Marsh (1972) also described this same condition and the resulting toughness due to sarcomere shortening. Minimum shortening in fresh ox muscle was observed by Locker and Hagyard (1963) when the ambient temperature, during the time course of rigor mortis, was 14–19°C. Cassens and Newbold (1967) showed that the delay phase of rigor mortis increased as the temperature was decreased from 37°C to 15°C, but this phase decreased as the temperature was decreased from 15°C to 1°C. Sink et al. (1965) noted that when the delay phase of rigor mortis was of short duration, shortening at the onset of rigor mortis was quite severe, but when the delay phase of rigor was of long duration, the sarcomere shortening was somewhat less.

The effects of postmortem aging are accelerated as temperatures are increased above 2°C. Henderson et al. (1970) found Z-line degradation to occur more rapidly upon storage at 25°C or 37°C versus storage at 2°C or 16°C. Buckley et al. (1974) demonstrated that myofibrillar protein solubility increased upon storage of aseptically prepared porcine muscle samples. Decreases in extractable protein have been correlated to the onset of rigor mortis (Khan and van den Berg, 1964; Disney et al., 1967; van Eerd, 1972) as well as to increase in shear force values (Hegarty et al., 1963; Buck et al., 1970; Ma and Addis, 1973). Goll et al. (1964), however, found no relationship between extractable protein and tenderness.

This study was conducted to evaluate the differences in shear force, fiber diameter, fiber kinkiness, sarcomere length and myofibrillar protein extractability for bovine longissimus dorsi muscles excised at 6, 8, 10 or 48 hr postmortem.

EXPERIMENTAL

Hot and cold boning

Fifteen choice and good grade heifers, ranging in weight from 367–501 kg, were utilized in this study. Five animals were assigned to each of three postmortem holding periods (6, 8 or 10 hr). Within 2 hr postmortem, one-half of each carcass was randomly assigned to either hot or cold boning. Upon exsanguination, the time was recorded and all postmortem holding periods were initiated at this zero time.

After the specified postmortem holding period (6, 8 or 10 hr) at 16°C, the longissimus dorsi muscle was excised, placed in a Cryovac bag (B620), and held unrestrained at 2°C until 48 hr postmortem (hot boning). At 48 hr postmortem, the same intact muscle was removed from the cold-boned halves which were chilled at 2°C.

Sampling

At 48 hr postmortem, samples for the various parameters under investigation were cut from corresponding locations on the hot- and cold-boned test muscles. The maximum length of each longissimus dorsi muscle (taken from the fifth rib to anterior tip of ilium) was measured and divided by two; thus, the location of the midline of each muscle was determined. Steaks for each determination were taken in respect to this midline location. On the anterior side of the midline, steaks for shear force (5.0 cm thick) and myofibrillar protein extractability (2.5

cm thick) were taken 5.0 and 15.0 cm, respectively, from the midline. On the posterior side of the midline a 2.5 cm thick steak for fiber diameter, fiber kinkiness and sarcomere length determinations was taken adjacent to the midline, whereas a steak for shear force determinations (5.0 cm thick) was removed at a distance of 2.5 cm from the midline. Samples for shear force and extractable myofibrillar protein determinations were packaged, frozen and held at −40°C until evaluated.

Histological determinations

After removing the 2.5 cm steak from the location described above, two 1.2 cm cores were removed from each steak, derived from hot- and cold-boning treatments and placed in 10% buffered formalin (Humanson, 1967). The samples were held at 2°C prior to evaluation. Samples for fiber diameter and fiber kinkiness were cut from each "fixed" core and placed in a Waring Blendor (blades reversed). Upon disruption of the sample (3 min at low speed), the muscle fibers were harvested and 50 fibers were evaluated for each parameter using 430× magnification on a light microscope equipped with an ocular micrometer. Each fiber was measured at its widest point and the total of the 50 fibers was averaged to obtain a representative fiber diameter. Utilizing the fiber kinkiness standards presented by Cagle (1967), a subjective score of 1–7 was assigned to each of 50 fibers depending upon the state of kinkiness. The total for 50 fibers was divided by 350 (the maximum score that 50 fibers could receive) and multiplied by 100 to obtain a representative percent kinkiness score (Gillis and Henrickson, 1969a; Cagle and Henrickson, 1970). Sarcomere lengths were measured by initially dislodging the myofibrils for 4 min at low speed in a Waring Blendor. Suspended myofibrils were placed on a glass slide, covered with a cover slip and evaluated at 1250× utilizing a Zeiss phase contrast microscope equipped with an ocular micrometer. Sarcomere length was determined as an average of 25 myofibrils (Gillis and Henrickson, 1969a).

Shear force determinations

Steaks (5.0 cm thick) for Warner-Bratzler shear force determination were taken from storage at −40°C and thawed at 2°C for 48 hr. The thawed steaks were then oven roasted at 163°C to an internal temperature of 72°C and chilled at 2°C for 24 hr. Three 2.5 cm cores were then removed from each steak and sheared three times for shear force determinations (Kastner and Henrickson, 1969).

Myofibrillar protein extractability

Prior to analysis of myofibrillar protein extractability, all samples were transferred to frozen storage at −25°C. Samples of the longissimus dorsi, from both the hot- and cold-boned muscles of the same carcass, were removed from frozen storage and allowed to partially thaw at 3°C for 24 hr. Upon removal, sample steaks were allowed to complete thawing at 3°C for an additional 24 hr. Excessive fat and connective tissue were removed and the remaining muscle ground through 4.5 mm pores in a precooled Kitchen Aid grinder. The ground sample (ca. 20g) was transferred to a Sorvall Omnimixer (17150) canister (50 ml) and chopped to a homogeneous sample at moderate speed (4), and the speed and chopping time were consistent throughout the study. Duplicate 4-g samples for extraction and three 0.2-g samples for total nitrogen determination were weighed. The temperature was maintained at 5°C throughout the sampling and extraction procedures.

Sarcoplasmic proteins were extracted first using 0.3M potassium phosphate buffer, pH 7.4 and the myofibrillar protein fraction was then extracted with 1.1M KCl, 0.1M potassium phosphate buffer, pH 7.4 (Helander, 1957). Each fraction was extracted twice in a buffer: sample ratio of 10:1 (v/w). The extraction was initiated by blending the sample and buffer in a Sorvall Omnimixer (17150) canister (50 ml) for 2 min at speed (3), again, with this speed being consistent throughout the study. The canister shaft was fitted with a teflon baffle plate to prevent foaming and the same canister assembly was used for each sample. The extraction was then continued by mechanical stirring of the solution for 45 min.

Following each extraction, the sample was centrifuged at 5°C for 15 min at 25,000 × G to obtain the sarcoplasmic fraction and 35,000 × G to obtain the myofibrillar fraction. The myofibrillar supernatants were decanted carefully through glass wool to remove insoluble components, the volume recorded and the protein content determined by the biuret method (Gornall et al., 1949). Extractable myofibrillar protein content was expressed as a percentage of the total protein as determined by the micro-Kjeldahl method.

Statistical analysis

The sources of variation in the analysis for a postmortem holding period were treatments, animals and animal × treatment so that means for shear force, percent kinkiness, fiber diameter, sarcomere length and myofibrillar protein extractability had five observations (animals).

RESULTS & DISCUSSION

Shear force

When each postmortem holding period was evaluated, the differences between the corresponding hot- and cold-boned longissimus dorsi means (Table 1) were statistically nonsignificant ($P > 0.10$). Considering the test muscle, these data indicate that comparable tenderness exists between the two treatments regardless of the postmortem holding period for the hot-boned halves. Even though no statistical differences were observed at each postmortem holding period, the hot-boning treatment yielded smaller mean shear forces values than the corresponding cold-boned samples (Table 1).

Fiber diameter

Hot- and cold-boning fiber diameter means were statistically different ($P < 0.10$) in the 6-hr holding period; however, the same comparisons were not statistically significant ($P > 0.10$) in the 8- and 10-hr holding periods (Table 1). Considering the extent to which fiber diameter and Warner-Bratzler shear force are related and the level of statistical significance observed at the 6-hr holding period, these results agree with the lack of statistical differences between shear force means for the hot- and cold-boning treatments.

Fiber kinkiness

Fiber kinkiness means for hot versus cold boning were sta-

Table 1—Parameter means for hot- and cold-boned longissimus dorsi muscles by postmortem holding periods

Holding periods (hr)	Shear force (kg)			Fiber diameter (μ)			Fiber kinkiness (%)			Extractable myofibrillar protein (%)			Sarcomere length (μ)		
	Hot		Cold[b]	Hot		Cold[b]	Hot		Cold[b]	Hot		Cold[b]	Hot		Cold[b]
6[a]	9.0		9.4	70.5[c]		72.8	66.7[d]		38.7	24.0		23.0	1.56		1.68
$S\bar{x}$		0.37			0.72			3.75			0.72			0.04	
8[a]	9.8		11.5	78.2		73.3	65.9[c]		46.9	22.2		21.9	1.57		1.59
$S\bar{x}$		0.84			1.75			5.09			0.62			0.04	
10[a]	7.7		8.8	71.4		73.1	64.9		57.7	24.4		23.9	1.57		1.61
$S\bar{x}$		0.30			3.49			3.60			0.61			0.02	

[a] Postmortem holding periods 6, 8 or 10 hr for hot-boned halves.
[b] Cold-boned halves held 48 hr postmortem for all holding periods.
[c] ($P < 0.10$).
[d] ($P < 0.01$); Significant difference between hot and cold boning.

tistically different at the 6- ($P < 0.01$) and 8-hr ($P < 0.10$) holding periods. A statistically nonsignificant ($P > 0.10$) difference was observed between hot- and cold-boning fiber kinkiness means in the 10-hr holding period (Table 1). Hot-boning means were consistently larger than their cold-boned counterparts. Even though a statistical difference was observed between hot- and cold-boning means in the 8-hr holding period a probability level of 0.10 is not overly convincing. Statistical analyses of the other parameters at the 8-hr holding period do not support this difference (Table 1). In addition, the statistical difference observed in the 6-hr holding period is only weakly supported by the statistical difference ($P < 0.10$) observed for fiber diameter for hot versus cold boning.

Extractable myofibrillar protein

Interpretation of data on the extractability of salt-soluble proteins requires careful scrutiny of extraction conditions. Salts, pH, ionic strength and degree of comminution greatly affect extractability of these proteins (Helander, 1957). Baliga et al. (1969), in their studies of fish muscle, measured a decrease in protein extractability during 5 days storage on ice. Further storage resulted in increased solubility. Minimum salt-soluble protein extractability coincided with the highest level of actomyosin formation and the maximum rigidity of rigor.

Statistically nonsignificant ($P > 0.10$) differences were observed between hot- and cold-boning means at each postmortem holding period (Table 1). Considering the suggested relationship between extractable myofibrillar proteins and shear force (Hegarty et al., 1963; Buck et al., 1970; Ma and Addis, 1973), these statistically nonsignificant results agree with the shear force data (Table 1). In addition, the hot-boning means for extractable myofibrillar proteins were consistently larger than corresponding cold-boning means at each postmortem holding period (Table 1).

Sarcomere length

Hot- and cold-boning means were not statistically different ($P > 0.10$) at each postmortem holding period (Table 1). In general, these data agree with the other nonsignificant differences observed between hot- and cold-boning means considering the other test parameters even though the hot-boning means were consistently smaller than the corresponding cold-boned means.

Hot-boned longissimus dorsi muscle treated as outlined in this study can yield a product that is comparable to its cold-boned counterpart with respect to shear force, fiber diameter, fiber kinkiness, myofibrillar protein extractability and sarcomere length. Therefore, to the extent that these parameters are related to tenderness, bovine longissimus dorsi muscle held at 16°C and excised at 8 hr postmortem can yield a product of acceptable tenderness. These results agree with previous work by Kastner et al. (1973) and Kastner and Russell (1975). The authors recommend the 8-hr holding period as a precautionary measure realizing that the 6-hr holding period did not give greatly different results. Any tenderness differences experienced at the 6-hr holding period might be alleviated by subsequent aging prior to evaluation. Reduced postmortem holding periods for carcasses to be hot boned may be utilized (Schmidt and Gilbert, 1970 and Schmidt and Keman, 1974) if subsequent conditioning is employed. The determination of the optimum postmortem holding period appears to be dependent on economic and processing requirements. Both of these areas should be investigated in more detail if this process of hot boning is to be seriously considered by the meat processing industry.

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EFFECT OF MUSCLE TYPE ON RESIDUAL NITRITE IN CURED MEAT

ABSTRACT

When a cured meat model system was made from white muscle it contained less residual nitrite than if it were made from a red muscle; this effect is due to the lower pH of white muscle. If the pH of red and white muscle is similar then the slightly lower residual nitrite in products made from red muscle probably is due to the greater content of myoglobin in red muscle.

INTRODUCTION

CONSIDERABLE ATTENTION has been focused recently on the use of nitrite in foods. The reason for this is twofold. First, a potential exists for reaction of nitrite with amines or amides to form N-nitroso compounds which are carcinogenic. Second, the residual nitrite content of cured meats represents one source of nitrite consumed by humans. Since such residual nitrite is a known measurable source, it deserves some scrutiny in terms of not only significance but also in regard to procedures for influencing the level of residual nitrite in the finished product.

Some of the nitrite added to meat for curing disappears—that means it cannot be identified analytically as residual nitrite nor can it be accounted for via known reaction pathways. A review of work pertinent to the fate of nitrite has been made by Cassens et al. (1974), and a complete account of aspects regarding the use of nitrite in meat products is found in the proceedings of the Symposium edited by Krol and Tinbergen (1974).

Muscles are composed of muscle fibers; muscle fibers consist of at least two distinctly different types known generally as red and white (Cooper et al., 1969). The gross properties of a muscle result from the ratio of the two types of fibers present. Visual color, for example, is a reflection of predominant fiber type within a muscle, and usually varies consistently among different muscles. This is readily apparent on the cross-sectional surface of a ham which shows several muscles of different degrees of color. Red muscle fibers have a higher myoglobin content, more lipid and higher activity of oxidative enzymes than do white fibers. White muscle fibers contain more glycogen than red muscle fibers; therefore, postmortem glycolysis results in a lower pH in white muscle (predominance of white fibers) than in red muscle.

The objective of the work reported herein was to determine the effect of muscle type on residual nitrite in a model cured meat system. The rationale was to develop information about the possibility of regulating residual nitrite in cured meat by utilizing the natural characteristics of the fresh meat selected for further processing.

MATERIALS & METHODS

LONGISSIMUS WAS used as a white muscle, Trapezius as a red muscle, and white and red portions were taken from the Semitendinosus (Beecher et al., 1965). All samples were excised from the carcass 1 day postmortem, frozen in liquid nitrogen and pulverized with a blendor. After pH was measured, 5 ml of solution containing sodium nitrite was added to 10g of muscle sample to make final 156 ppm nitrite and mixed well with a plastic rod. In other aliquots of some samples, pH was adjusted; lactic acid was used to lower pH and sodium carbonate was added to raise pH. Thus the pH values of the original red portions were adjusted to those of the white portions and vice versa.

Since the above systems were dilute, other experiments were conducted in which water was added to the sample at a rate of 3% by weight of the meat. The water contained only sodium nitrite or it was made up as a curing agent. Thus samples contained only 156 ppm sodium nitrite, or 156 ppm sodium nitrite plus 0.4% disodium phosphate, and 0.47% ascorbate (3/4 oz ascorbate to 100 lb meat).

Triplicate samples were prepared in all cases. After mixing, samples were stored at 3°C for 15 hr and then cooked at 65°C in a water bath for 2 hr. Residual nitrite contents were determined by the AOAC (1970) method after either 2 or 3 days storage.

Mitochondria were isolated (Greaser et al., 1969) from 30g each of Trapezius, Longissimus and the white and red portions of the Semitendinosus from a freshly slaughtered pig. After isolation, mitochondria were suspended in 6 ml of 0.25M sucrose with a Potter-Elvehjem homogenizer. To 8 ml of pH 5.4 buffer solution were added 2 ml of each mitochondrial suspension from the white and red portions of the Semitendinosus. To 8 ml of pH 6.6 buffer solution were added 2 ml of each suspension from the Longissimus and Trapezius. The combined 10 ml solution contained 0.2 mM myoglobin, 2 mM nitrite (138 ppm) and 0.2 mM NADH. The buffer solutions were made as follows: 22.2 ml of 0.1M citric acid and 27.8 ml of 0.2M Na_2HPO_4 were mixed and diluted to 100 ml to make pH 5.4 buffer solution. 13.6 ml of 0.1M citric acid and 36.4 ml of 0.2M Na_2HPO_4 were diluted to 100 ml to make pH 6.6 buffer solution.

All the solutions were kept at 0–3°C until they were used and triplicate samples were prepared for each suspension. The samples were stored under a nitrogen atmosphere in a 3°C cooler for 24 hr and then residual nitrite levels were determined.

RESULTS & DISCUSSION

THE RESULTS for Semitendinosus muscle are shown in Table 1. All of the samples were stored at 3°C for 2 days after cooking. pH of the white portion of the muscle was about 0.3 units lower than for the red portion. The red portion contained about 6 ppm more residual nitrite than did the white. We believe that this difference results from the higher pH of the red portion of the muscle, since nitrite depletes more rapidly at lower pH (Nordin, 1969; Olsman and Krol, 1972).

The muscles used in this work were characterized earlier by Beecher et al. (1965) in terms of fiber type composition and myoglobin. When red fibers are expressed as a percentage of total fibers, the red portion of Semitendinosus had 47%, the white portion of the Semitendinosus had 20%, Longissimus had 25% and Trapezius had 48%. Myoglobin as mg/g was 12.2 for the red portion of Semitendinosus, 3.5 for the white portion of Semitendinosus, 3.0 for Longissimus and 11.5 for Trapezius.

Table 1—Residual nitrite content of white and red portions of the Semitendinosus muscle[a]

	White portion		Red portion	
	pH	Nitrite (ppm)	pH	Nitrite (ppm)
Sample 1	5.63	117	5.99	126
Sample 1[b]	5.99	131	5.63	111
Sample 2	5.30	91	5.50	100
Sample 3	5.32	90	5.58	88
Sample 4	5.48	95	5.72	100

[a] All results are a mean from three different preparations
[b] pH adjusted as explained in text

When the pH of the white portion was adjusted to that of the original red portion (pH 5.99) then the adjusted white portion had about 5 ppm higher residual nitrite than the original red portion. When the pH of the red portion was adjusted to that of the original white portion (pH 5.63) then the original white portion had a higher residual nitrite than the adjusted red portion. This supports the concept that a lower pH results in a lower residual nitrite and suggests that if the pH difference is removed, then the redder muscle (more myoglobin) will have a lower residual nitrite.

Table 2 shows the results from the same type of experiment except that samples were taken from different muscles instead of from different regions within the same muscle. pH in the Longissimus, which was taken as a typical white muscle, was lower than in the Trapezius which was used as a typical red muscle. The residual nitrite content in the red muscle was higher than in the white muscle. When pH adjustments were made, a result similar to that in Table 1 was obtained. The difference in residual nitrite was greater when two different muscles were compared (Table 2) than when two different portions of the same muscle were compared (Table 1).

Since the above systems were dilute compared to cured meat, other samples from red and white portions of the Semitendinosus were prepared with 3% added water or with 3% added water containing disodium phosphate and ascorbate. All samples contained 156 ppm nitrite. Residual nitrite was determined after 3 days of storage and the results are shown in Table 3; residual nitrite is much lower than in the diluted system. This was an expected difference since the systems used were more concentrated than the previous ones. The pH effect again was present; red muscle was about 0.35 units higher in pH and had approximately 31 ppm more residual nitrite than did white muscle (Samples 1 and 2). Olsman and Krol (1972) reported a difference of 30–40 ppm when residual nitrite was measured on homogenized meat samples differing in pH by 0.3 units. In the presence of phosphate (Samples 3[c] and 4[c] in Table 3) the pH of red and white portions was the same and the red portion had a lower residual nitrite. This result again emphasizes the influence of myoglobin and possibly other compounds when pH is similar.

Table 2—Residual nitrite content of white (Longissimus) and red (Trapezius) muscles[a]

	Longissimus		Trapezius	
	pH	Nitrite (ppm)	pH	Nitrite (ppm)
Sample 1	5.39	100	5.80	116
Sample 1[b]	5.80	126	5.39	93
Sample 2	5.31	85	5.67	106
Sample 3	5.36	87	6.00	108
Sample 4	5.38	85	5.69	100

[a] All results are a mean from three different preparations
[b] pH adjusted as explained in text

Table 3—Residual nitrite content of white and red portions of the Semitendinosus muscle[a,b]

	White portion		Red portion	
	pH	Nitrite (ppm)	pH	Nitrite (ppm)
Sample 1	5.8	45	6.1	71
Sample 2	5.35	56	5.75	93
Sample 3	6.1	81	6.1	54
Sample 3[c]	6.4	68	6.4	47
Sample 4[c]	6.4	83	6.4	73

[a] All results are a mean from three different preparations
[b] Conducted in a system more similar to cured meat than for the experiments reported in Tables 1 and 2
[c] Contains phosphate and ascorbic acid as explained in text

It has been reported that mitochondria are involved in transferring nitric oxide from nitrite and thus decreasing nitrite content (Walters et al., 1967). We examined the effect of mitochondria since mitochondrial content in red muscle is several times higher than in white ones. Nitrite was incubated in the presence of mitochondria isolated from various muscles. No difference was found for residual nitrite at pH 6.6 for the white portion (137 ppm) and red portion (137 ppm) of Semitendinosus or at pH 5.4 for the Longissimus (136 ppm) or Trapezius (137 ppm) muscles. Thus, mitochondria had little effect, in terms of nitrite loss, in that case.

CONCLUSIONS

LEVELS of residual nitrite were different when white and red muscles were used in a model cured meat system, and residual nitrite was different even when white and red portions from the same muscle were used. This fact should receive due consideration during selection of raw material for experimentation and sampling for analysis. White muscles had 19 ppm lower residual nitrite (a difference of 6 ppm for red and white portions of the same muscle) content in diluted systems. However, the white portion contained about 31 ppm less nitrite than the red portion in more concentrated systems. The lower residual nitrite was due mainly to lower pH in the white muscles. The mechanism of action of pH, in this circumstance, is not known. It is unlikely that the pH range considered here exerts much effect on the dissociation curve of nitrous acid. We favor the idea, therefore, that the pH difference between red and white muscle alters the reaction of nitric oxide with some component of meat or makes some component of meat more available for reaction. When pH was adjusted to the same value, red muscles had lower nitrite contents than white muscle. The lower nitrite in the red muscle, when pH is similar, is probably due to a higher amount of myoglobin. Mitochondria had little effect on loss of nitrite under the conditions of our experiment.

The possibility exists, therefore, that a processor may exert some control on residual nitrite content of cured meat by selecting the type of muscle used and thereby utilizing natural inherent properties of the meat. White muscle with a low pH produces a lower residual nitrite content than does red muscle with a high pH. The use of phosphate may, of course, alter the situation by its effect on pH.

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RELATIONSHIP BETWEEN CONTRACTILITY AND SOME BIOCHEMICAL PROPERTIES OF MYOFIBRILS PREPARED FROM NORMAL AND PSE PORCINE MUSCLE

ABSTRACT

Contractility and ATPase activity of myofibrils from normal and PSE porcine muscles were studied. Studies were also made on the extractability of proteins from myofibrils isolated from normal and PSE muscles. Normal muscles contracted instantaneously after the addition of Mg^{2+}-ATP, while PSE muscles contracted in part or did not contract. ATPase activity of myofibrils isolated from muscle having higher pH value was higher than that of myofibrils isolated from muscle having lower pH value. When using either Hasselbach-Schneider or KI solutions to extract myofibrillar proteins, the proportion of protein extracted was significantly higher for normal muscles when compared to those from PSE muscles.

INTRODUCTION

THE PSE CONDITION in porcine muscle is characterized by extremely rapid biochemical changes after slaughter such as a rapid pH fall and an increase in rate of ATP hydrolysis (Wismer-Pedersen, 1959; Briskey, 1964; Briskey et al., 1966; Sybesma, 1972). It has been considered that myofibrillar proteins in PSE muscle might be denatured by the combination of high temperature and low pH in postmortem muscle, based on the findings that rabbit myofibrils and glycerinated muscle fibers lost their ATPase activity in a considerable extent by treating these materials with a medium of high temperature and low pH (Penny, 1967; Yasui et al., 1973). This consideration was supported in part by the findings on the extractability of myofibrillar proteins from porcine muscle (Sayre and Briskey, 1963; Briskey and Sayre, 1964; Penny, 1969) and ATPase activity of porcine myofibrils (Galloway and Goll, 1967; Penny, 1969; Greaser et al., 1969).

In the present study, we investigated ATPase activity of myofibrils and the extractability of myofibrillar proteins from normal and PSE muscles in relation to the contractility of myofibrils, and discussed the interrelationship between the contractility of myofibrils and the properties of contractile proteins of normal and PSE muscles.

MATERIALS & METHODS

Materials

The materials used in this study were mainly obtained from the crossbred of Landrace and Hampshire. Selection of normal and PSE muscles was conducted on longissimus dorsi muscle of the porcine carcasses stored for 3–4 hr at room temperature and then stored in a cold room for 20–24 hr after slaughter by measuring pH (the pH of PSE muscles was below 5.44) and visual subjective evaluation. In evaluating pork quality, scores were given for color and texture of meat. For scoring, a scale of 4 was used: 4, denoting red color and fine texture; 3, denoting slightly pale color and good texture; 2, denoting pale color and poor texture; 1, denoting pale color and extremely poor texture. Scores 2 and 1 were classified as PSE.

Contractility of myofibrils

Contractility test was conducted on approximately 50 carcasses. The longissimus dorsi muscle was excised from porcine carcasses and was minced with a meat chopper. Approximately 0.2g of the minced muscle was homogenized in 20 ml of 0.1M KCl, 0.039M borate buffer solution (pH 7.1) with a Waring Blendor for 30 sec. A drop of the muscle suspension was placed on a slide glass and then a cover glass placed on it. Thereafter, a drop of Mg^{2+}-ATP solution (1 mM $MgCl_2$, 1 mM ATP, 10 mM Tris-maleate, pH 7.0) was instilled at one edge of a cover glass. The appearance of myofibrils in the suspension before and after the addition of the Mg^{2+}-ATP solution was observed at about 20°C with a phase-contrast microscope (Olympus microscope FHT, Olympus Ltd.). Photographs were taken with an Olympus model PM-10-M camera, attached to the microscope. The number of contracted and uncontracted myofibrils in the suspension after the addition of the Mg^{2+}-ATP solution were counted. Contractility of myofibrils in the muscle suspension was expressed as percentage of the contracted myofibrils to the total myofibrils counted. In each case, more than 200 myofibrils were counted.

Preparation of myofibrils

Myofibrils were prepared from porcine longissimus dorsi muscles classified as normal and PSE by the method of Briskey and Fukazawa (1971).

ATPase activity

ATPase activity of myofibrils was measured at 25°C in the following reaction mixtures: (a), 0.1M KCl, 0.02M Tris-maleate (pH 7.0), 1 mM $MgCl_2$ and 1 mM ATP; (b), 0.1M KCl, 0.02M Tris-maleate (pH 7.0), 0.1 mM ethyleneglycol-*bis*-(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), 1 mM $MgCl_2$ and 1 mM ATP; (c), 0.1M KCl, 0.02M Tris-maleate (pH 7.0), 0.1 mM EGTA, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM ATP; (d), 0.1M KCl, 0.02M Tris-maleate (pH 7.0), 4 mM $CaCl_2$ and 1 mM ATP; and (e), 0.5M KCl, 0.02M Tris-maleate (pH 7.0), 4 mM $CaCl_2$ and 1 mM ATP. After the reaction was stopped by the addition of trichloroacetic acid (final concentration 5%), the liberated phosphorus was determined by the method of Fiske and Subbarow (1925).

Extractability of myofibrillar proteins from myofibrils

Myofibrillar proteins were extracted from myofibrils with the following solutions: (1), Guba-Straub solution (0.3M KCl, 0.15M K-phosphate, pH 6.5); (2), Hasselbach-Schneider solution (0.6M KCl, 0.01M sodium pyrophosphate, 0.1M K-phosphate, 1 mM $MgCl_2$, pH 6.4); and (3), KI solution (0.6M KI, 6 mM sodium thiosulfate, 2 mM dithiothreitol (DTT), 0.5 mM ATP, 20 mM Tris-HCl, pH 7.5). Myofibril pastes (10g) containing approximately 10% protein were suspended in 5 vol of each solution, and then stirred gently for 10 min, followed by centrifugation (1500g, 15 min). Protein concentration of the supernatant was measured by the biuret method (Gornall et al., 1949) standardized against crystalline bovine serum albumin. Extractability of myofibrillar proteins was expressed as percentage to the total proteins in the suspension.

SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis (5% gel) of the extracts from myofibrils with various solvents were used according to the method of Weber and Osborn (1969) with a slight modification (Ito and Ando, 1975). The extracts were dialyzed against 0.01M sodium phosphate buffer (pH 7.0) containing 0.1% SDS and 0.1% β-mercaptoethanol, and then boiled in 1% SDS, 1% β-mercaptoethanol, 0.1M sodium phosphate (pH 7.0) and 25% glycerol for 3 min, and adding a

small volume of 0.05% Bromophenol Blue. Electrophoresis was run at 8 mA per tube for 3 hr. Gels were stained with 0.25% Coomassie Brilliant Blue in 45.4% methanol and 9.2% acetic acid for 2 hr. The gels were destained in a solution containing 7.5% acetic acid and 5% methanol with several changes. Densitometry of the gels at a wavelength of 610 nm was performed with a Gilford spectrophotometer 2400S using the absorbance range of 0–3. The amount of each band was obtained by weighing the tracing of each band.

pH determination

Minced muscle (5g) was homogenized in 10 ml of distilled water with a Waring Blendor for 3 min. The pH of the muscle homogenate was determined with a Hitachi-Horiba pH meter F-7 at 20°C.

RESULTS

MYOFIBRILS from porcine muscles showed various contractile patterns after the addition of the Mg^{2+}-ATP solution depending on pH of the muscles. The contractility of myofibrils was plotted against pH of the muscles, from which myofibrils were prepared (Fig. 1). The myofibrils from normal muscles whose pH was 5.45 or above exhibited 100% contractility, while those from PSE muscles with intermediate pH values (5.30–5.44), increased in proportion to pH of the muscles, although a few exceptions were found as such that myofibrils from PSE muscles whose pH was around 5.35 showed 100% contractility. This result is consistent with the findings on superprecipitation of myosin B from porcine muscles which denoted that superprecipitating activity of myosin B prepared from normal muscles having higher pH values was higher than that of myosin B prepared from PSE muscles having lower pH values (Park et al., 1975). As to the speed of contraction, myofibrils from normal muscles contracted within a second after the addition of Mg^{2+}-ATP solution, while the speed of contraction of myofibrils from PSE muscles with intermediate pH values was much slower as such that about 10 sec are required for contraction, even in the myofibrils in which the contractility was 100%. The appearance of myofibrils after the addition of the Mg^{2+}-ATP solution was divided typically into three types according to pH of the muscles, from which myofibrils were prepared (Fig. 2). All of myofibrils from normal muscles showed a supercontraction band exhibiting a pattern of alternating light and dark bands (Fig. 2a), while no contraction band was found in the myofibrils from PSE muscles with lower pH values (Fig. 2c). On the other hand, most of the myofibrils from PSE muscles with intermediate pH values showed a dark band in the middle of the Z-band, indicating that thin filament overlaps and hence myofibrils have contracted slightly by the addition of the Mg^{2+}-ATP solution. In addition, contracted myofibrils and uncontracted myofibrils were often found at the same time in the same myofibrils preparation (Fig. 2b). From the findings on the contractility of myofibrils, it appears that ATPase activity of myofibrils may be related to pH of the muscles, since ATPase activity of myofibrils can readily be related to muscle contraction (Marsh, 1952).

ATPase activities of myofibrils at various conditions were shown in Figures 3 and 4. The lines in these figures were drawn according to the equation obtained from the plots by the calculation of the least squares method. As shown in Figure 3, Mg^{2+}-modified ATPase activity at low ionic strength also depended upon pH of the muscle; Mg^{2+}-modified ATPase activity of myofibrils prepared from muscle having higher pH value was higher than that of myofibrils prepared from muscle having lower pH value. This result is consistent with those of Galloway and Goll (1967) and Penny (1969). However, in the presence of Mg^{2+}-ion only, with EGTA present to chelate out all traces of Ca ions, the ATPase activity of myofibrils increased only a little as pH of the muscle increased, so that the activity of myofibrils in this medium was much less than that of myofibrils in the presence of traces of Ca ions. Consequently, the higher the pH the larger the differences between these two types of the activities. Mg^{2+}-modified ATPase activity of myofibrils in the presence of 4×10^{-4}M Ca ions showed a similar tendency to that of Mg^{2+}-modified ATPase activity without adding Ca ions, but showed a little higher values. This result suggests that the Ca ions concentration in myofibril preparations was just below the threshold level of Ca ions enough to inhibit the troponin function. In the presence of Ca ions alone, myofibrils split ATP at a much higher rate than that of Mg^{2+}-modified ATPase activity. In this case too, the ATPase activity of myofibrils increased proportionally as pH of the muscles from which myofibrils were prepared increased. At high ionic strength, the ATPase activity in the presence of Ca ions (4 mM) also depended upon pH of the muscles; the ATPase activity of myofibrils prepared from muscle having higher pH value was higher than that of myofibrils prepared from muscle having lower pH value. This result indicates that the activity of myosin decreased as pH of the muscle decreased, so that myosin in muscle cells might be denatured concomitant with the pH fall. However, Quass and Briskey (1968) reported that ATPase activity of myosin isolated from PSE muscle at death was greater than that of normal. In view of this, it seems that myosin in muscle cells might be denatured during early stage of postmortem aging, since pH of the muscle declined due to glycolysis during aging.

The extractability of proteins from myofibrils with KI, Hasselbach-Schneider and Guba-Straub solutions were plotted against pH of the muscles and the regression lines are shown in Figure 5. As in the case of ATPase activities, the extractability of myofibrillar proteins with Hasselbach-Schneider solution also depended on pH of the muscles; the higher the pH of the muscle from which myofibrils was prepared the more the extractability. So, the extractability of myofibrillar proteins with this solution from PSE muscles were much less than that of normal muscles. Similar results were also obtained in the case of KI solution, but showed somewhat higher values than that of normal muscles. These results suggest that myofibrillar proteins of PSE muscles were subjected to denaturation and the extent of the denaturation depended on pH of the muscles,

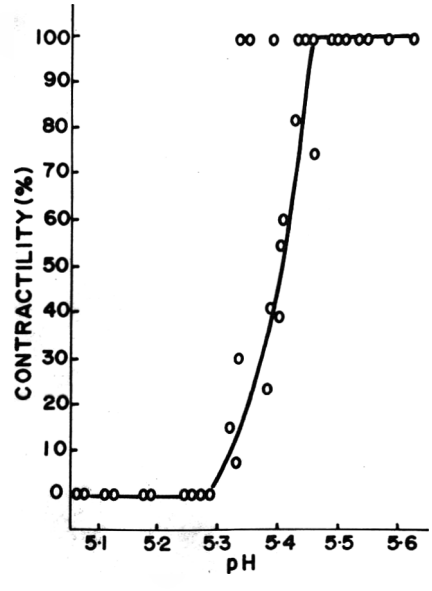

Fig. 1–Contractility of porcine muscles. [The contractility was expressed as percentage of contracted myofibrils in muscle to the total myofibrils after addition of Mg^{2+}-ATP solution (1 mM $MgCl_2$, 1 mM ATP, 0.01M Tris-maleate, pH 7.0), and plotted against pH of the muscles.]

i.e., the lower the pH the more the denaturation. Therefore, these results reconfirm the findings on the ATPase activities of myofibrils shown in Figures 3 and 4. In addition, these results also coincide with the results of Sayre and Briskey (1963) and Briskey and Sayre (1964). However, the extractability of proteins with Guba-Straub solution was much less than that of KI and Hasselbach-Schneider solutions, and no significant differences were observed in the extractability of myofibrillar proteins between normal and PSE muscles.

In order to elucidate whether or not which myofibrillar

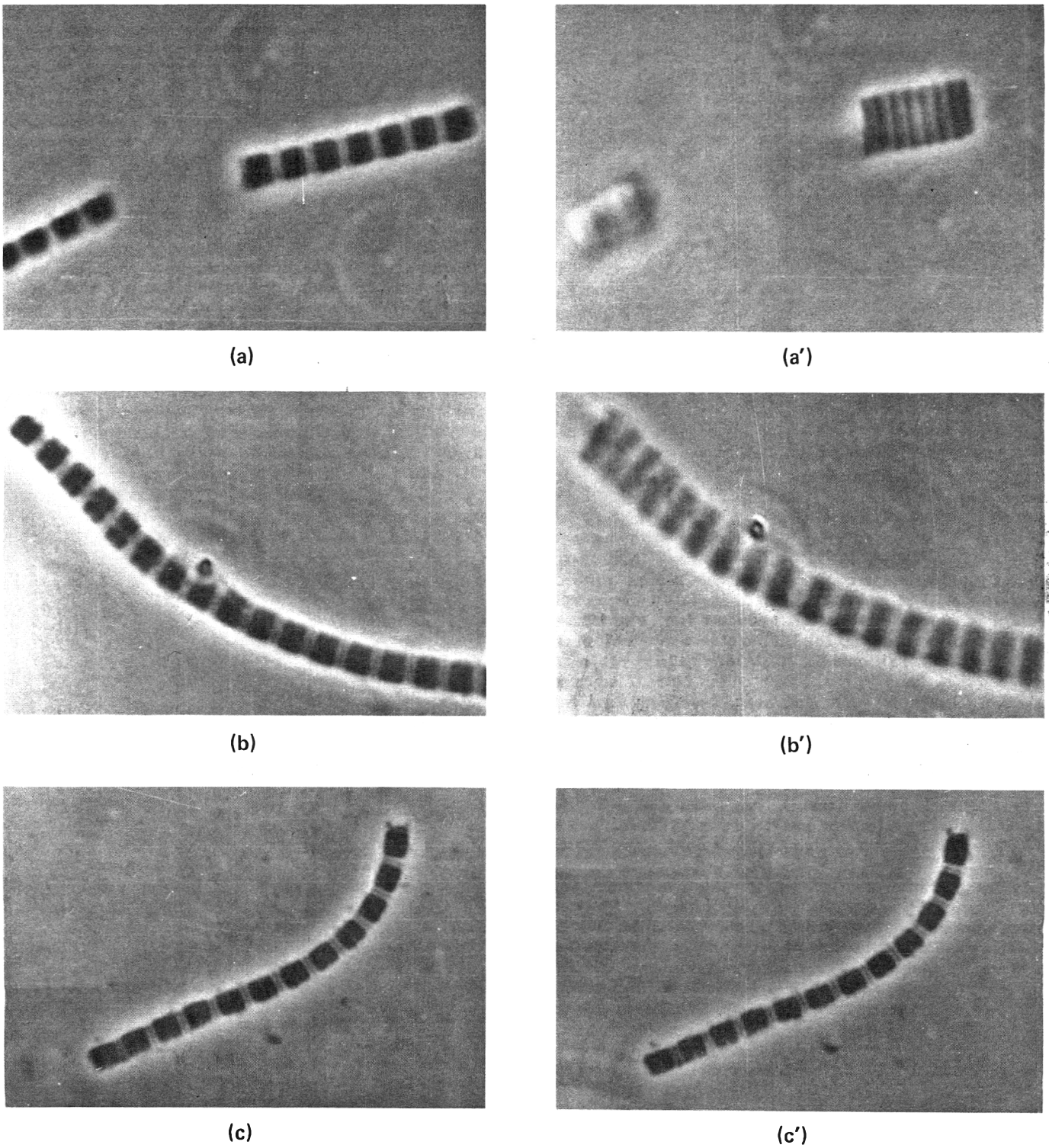

Fig. 2–Phase contrast micrographs of the myofibrils before (a, b, c) and after (a', b' c') the addition of the Mg^{2+}-ATP solution. [a, a', normal myofibril (pH 5.53); b, b', PSE myofibril (pH 5.40); c, c', PSE myofibril (pH 5.08); Magnification: X2,512.]

protein had been denatured in PSE muscles, a comparative study was made on SDS polyacrylamide gel electrophoresis of the extracts obtained from myofibrils of normal and PSE muscles. In comparing the densitograms of the gels, remarkable differences were found in the amounts of myosin and actin in the extracts with Hasselbach-Schneider solution between normal and PSE muscle. A typical result is shown in Figure 6. As shown in Figure 6, the myosin to actin ratio (M/A ratio) of the extract from normal muscle (pH 5.7) was much higher than that of PSE (pH 5.37), i.e., the M/A ratio of the extract from normal muscle was 11.3, whereas that of PSE was 5.6. In addition, the amount of band 3 component, whose molecular weight was about 150,000, in the extract from PSE muscle was more than that of normal (Fig. 6). Similar results were also obtained in the extracts with KI solution, but the values of the data obtained were larger and somewhat more variable than those of Hasselbach-Schneider solution. However, in the case of Guba-Straub solution, no significant difference was found in the electrophorograms of the extracts between normal and PSE muscle. The amounts of myosin and actin in the extracts can be calculated by multiplying the total amount of extractable proteins and the percentage of the bands of myosin heavy chain and actin in the extracts obtained from densitograms (In calculation of the amount of myosin, only the percentage of the myosin heavy chain was used for convenience.). Figure 7 shows the extractabilities of myosin and actin from myofibrils with Hasselbach-Schneider solution. The data based on densitometry of SDS polyacrylamide gels can easily be in error unless electrophoresis is done under a very complex set of control. So, the conditions in electrophoresis may involve the accuracy of the data in Figure 7. The extractability of myosin decreased proportionally as pH of the muscle from which myofibrils were prepared decreased, whereas that of actin was almost the same value at any pH of the muscle. Therefore, the decrease in the extractability of myofibrillar proteins from myofibrils with Hasselbach-Schneider solution might be due mainly to the denaturation of myosin. Similar results were also obtained in the case of KI solution. On the other hand, in the case of Guba-Straub solution, the extractabilities of myosin and actin were always constant in spite of the variance of pH of the muscles.

DISCUSSION

Mg^{2+}-ATP causes the actin-myosin interaction which gives rise to muscle contraction, wherever the contractile proteins in the myofibrils retain their function. Myofibrils from normal muscles whose pH is higher than 5.45 contract instantaneously by the addition of the Mg^{2+}-ATP solution, suggesting that contractile proteins in normal muscles fully retain their function, even for

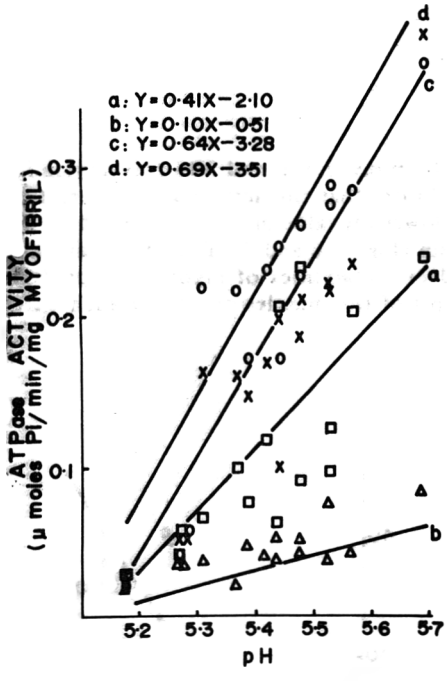

Fig. 3—ATPase activities of myofibrils from porcine muscles at low ionic strength. ATPase activity of myofibrils (0.5 mg/ml) was measured at 25°C in the following media; (a) 0.1M KCl, 0.02M Tris-maleate (pH 7.0), 1 mM $MgCl_2$ and 1 mM ATP, □; (b) 0.1M KCl, 0.02M Tris-maleate (pH 7.0), 0.1 mM EGTA, 1 mM $MgCl_2$ and 1 mM ATP, △; (c) 0.1M KCl, 0.02M Tris-maleate (pH 7.0), 0.1 mM EGTA, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM ATP, ×; (d) 0.1M KCl, 0.02M Tris-maleate (pH 7.0), 4 mM $CaCl_2$ and 1 mM ATP, ○. The ATPase activity of myofibrils was plotted against pH of the muscles.

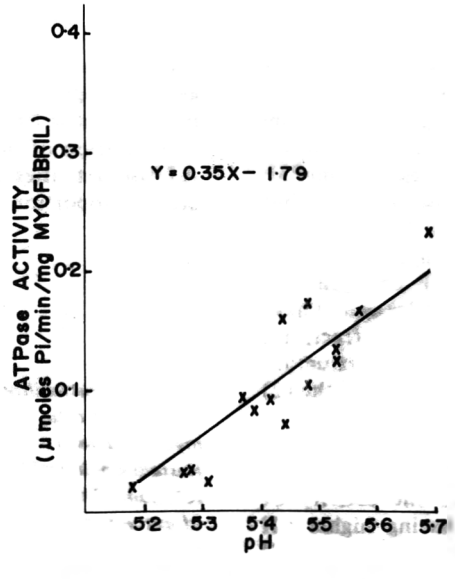

Fig. 4—ATPase activity of myofibrils at high ionic strength. [ATPase activity of myofibrils (0.5 mg/ml) was measured at 25°C in 0.5M KCl, 0.02M Tris-maleate (pH 7.0), 4 mM $CaCl_2$ and 1 mM ATP. The ATPase activity was plotted against pH of the muscles.]

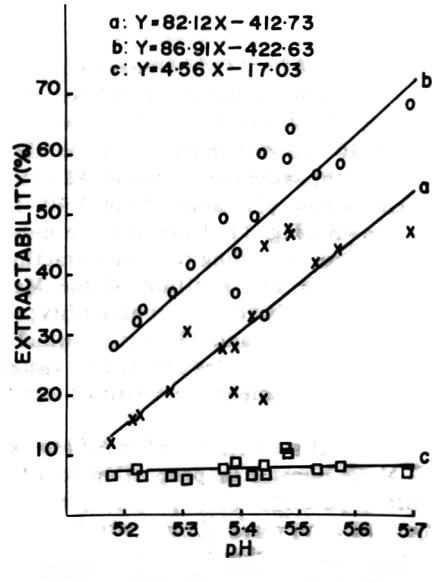

Fig. 5—Extractabilities of proteins from myofibrils with various solutions. The extractabilities of myofibrillar proteins were expressed as percentage of the extractable proteins to the total. [(a) With Hasselbach-Schneider solution, ×; (b) with KI solution, ○; (c) with Guba-Straub solution, □.]

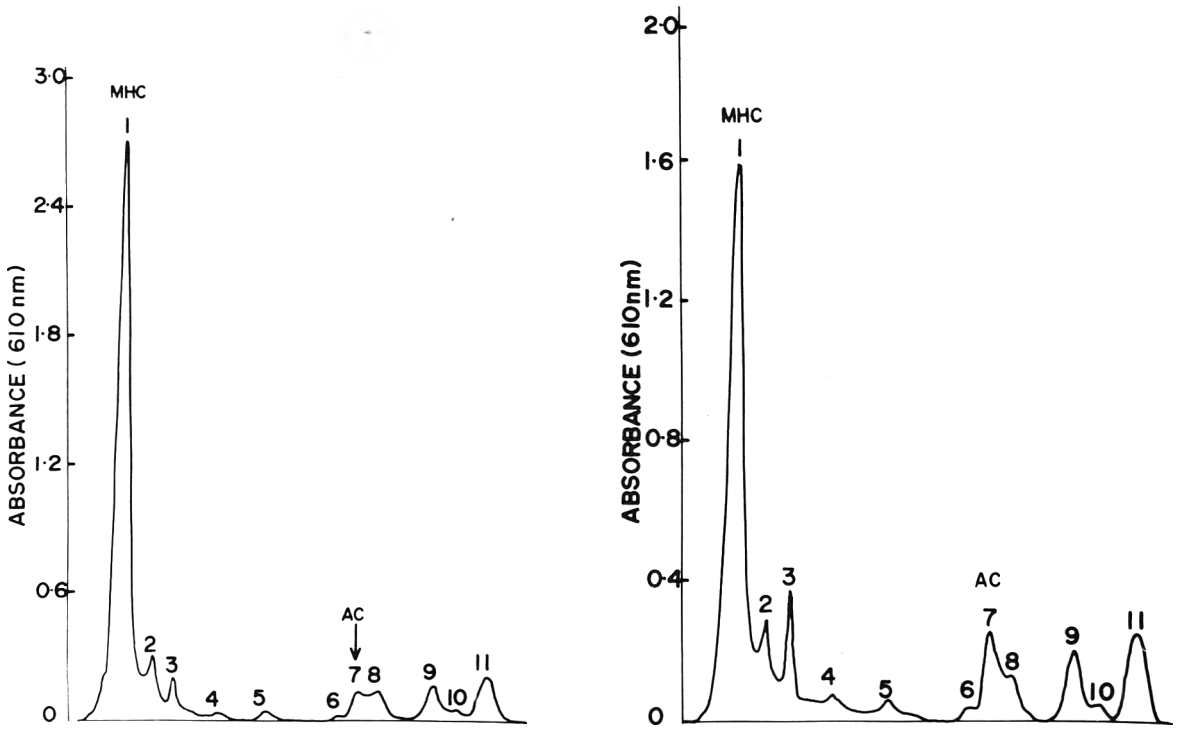

Fig. 6–Densitograms of the SDS polyacrylamide gel electrophoresis of the extracts from myofibrils with Hasselbach-Schneider solution. [Left: Extract from normal myofibril (pH 5.57); Right: extract from PSE myofibril (pH 5.37).] Each band was numbered from left to right (from larger molecular weight to smaller). MHC, myosin heavy chain; AC, Actin.

muscles stored for 24 hr or longer. On the other hand, myofibrils from PSE muscles whose pH is lower than 5.30 are not able to contract. Myofibrils from PSE muscles whose pH ranges from 5.30–5.40 contract in part, or some fibers contract and others do not in the same muscle tissue. All of the myofibrils from PSE muscles whose pH ranges from 5.40–5.44 also contract, but very slowly (Fig. 1). Thus, porcine muscles are classified into three groups according to the contractility of myofibrils in the muscles after the addition of the Mg^{2+}-ATP solution: (1) muscles which have high contractility; (2) muscles which have low contractility; and (3) muscles which have no contractility. Therefore, PSE muscles selected by visual subjective evaluation and pH measurement are classified into two groups of the (2) and (3).

Mg^{2+}-ATPase activity of myofibrils in the presence of 4×10^{-4} M Ca ions is in proportion to pH of the muscles from which myofibrils were prepared, and hence the activity of myofibrils prepared from normal muscle having higher pH value is much higher than that of PSE muscle having lower pH value, while in the absence of Ca ions (in the presence of 10^{-4} M EGTA) the Mg^{2+}-ATPase activity is almost the same at any pH of the muscle, indicating that actomyosin ATPase activity decreased in proportion to pH of the muscle, while residual ATPase activity is almost the same at any pH of the muscle (Fig. 3). This suggests that the decrease in Mg^{2+}-ATPase activity of myofibrils might be brought about by the inactivation of actin-myosin system. These results are consistent in part with the findings on superprecipitation of myosin B from PSE muscles which denoted that both myosin and actin in myosin B preparation from PSE muscles might be denatured and the denatured actin remaining in the supernatant after ultracentrifugal separation of myosin B might take part in lowering superprecipitating activity of myosin B (Park et al., 1975). In addition, the results on the extractability of myofibrillar proteins from myofibrils and SDS polyacrylamide gel electrophoresis indicates that the actin-myosin interaction in PSE muscle having lower pH value might be inhibited by the denaturation of myosin (Fig. 5, 6 and 7). Thus, the changes in ATPase activities and extractabilities of myofibrillar proteins are in proportion to pH of the muscles and hence each curve

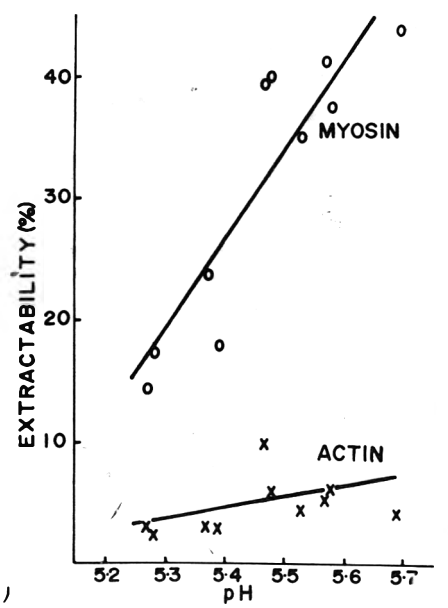

Fig. 7–Extractability of myosin and actin from myofibrils with Hasselbach-Schneider solution. (The amounts of extracted myosin and actin were calculated by multiplying the total amounts of extractable protein and the percentage of the bands of myosin heavy chain and actin in the densitograms. The extractability of myosin and actin was expressed as percentage of these proteins to the total in the myofibrils, and plotted against pH of the muscle.)

representing the ATPase activities (Mg^{2+}-ATPase activity in the presence of Ca^{2+}-ions, Ca^{2+}-ATPase activity) vs pH of the muscles show gentle slopes (Fig. 3 and 4). However, the curve representing the contractility of myofibrils vs pH of the muscles was so steep in the narrow pH range of 5.30–5.45 (Fig. 1), that a distinct difference is found between the ATPase vs pH curve and the contractility vs pH curve. This difference implies that the loss of contractility of PSE muscle having lower pH value cannot readily be explained only by the decrease of ATPase activity. Therefore, it may be speculated that some changes might occur within myofibrils in PSE muscle as such that muscle contraction might be inhibited by an unknown factor other than the inactivation of ATPase activity.

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CYCLIC AMP IN LONGISSIMUS MUSCLE FROM CONTROL AND STRESS SUSCEPTIBLE PIGS

ABSTRACT

As part of a continuing study to define the etiology of porcine stress syndrome and pale, soft, exudative pork, cyclic AMP (c-AMP) levels in longissimus muscle from stress susceptible (SS) and control (C) pigs were compared. At 3 min postmortem, levels of c-AMP were significantly higher for SS than for C pigs; thereafter differences were not significant. Variations in initial levels of glycogen affected lactate, pH and color of the postmortem muscles but did not affect c-AMP levels. Initial levels of glycogen were higher for SS pigs killed in the morning than for those killed in the afternoon. Results of this study indicate that further study, using greater numbers of pigs, is warranted to establish whether the 3 min postmortem level of c-AMP can be used as an objective measure of stress susceptibility.

INTRODUCTION

THE ETIOLOGY of pale, soft and exudative (PSE) pork appears to be associated with an abnormal rate of glycogenolysis (Briskey, 1964). An approach to defining the etiology is to determine the rate-determining step associated with glycogenolysis. Kastenschmidt and coworkers (1968) partially defined the mechanism of glycolysis in the "fast-" and "slow-glycolyzing" porcine muscles but the rate-determining step was not identified. A major difficulty associated with undertaking such a systematic approach for defining the mechanism is the complexity of the glycogenolytic process. Extracellular variables such as hormone levels and intracellular effects such as aerobic and anaerobic enzymic activities are involved. Selection of an optimal area for study, thus, becomes extremely important.

Discovery of the mediating effect of c-AMP on epinephrine activity (Sutherland et al., 1965; Sutherland and Robinson, 1966) was especially pertinent to the selection of an area of study for characterizing the etiology of porcine stress syndrome (PSS) and PSE. By defining the relationships between epinephrine and c-AMP concentrations to rate of glycogenolysis in PSS pigs, it might be possible to determine whether the rate-determining step which controls the abnormal glycogenolytic rate occurs extra or intracellularly in the skeletal muscle. Positive correlation between rate of glycogenolysis and level of c-AMP would suggest that the rate-determining step was extracellular or immediately intracellular, the site of major control of c-AMP synthesis. If, however, rate is not related to c-AMP concentration, the rate-determining step must occur after c-AMP synthesis, possibly involving the Ca^{++} sequestering and phosphorylase activation mechanisms.

Concentrations of c-AMP in muscles from control (C) and *stress* susceptible (SS) pigs and their relationships to rate of glycogenolysis is the topic of this report.

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EXPERIMENTAL

Animal handling

Pigs which included barrows and gilts and each weighing about 90 kg were obtained from the Iowa State University Swine Breeding Farm. A Poland China sire and York-Landrace cross dams were used to produce the litters for this study. Selection for stress susceptibility was based on positive reaction to halothane anesthesia (Sybesma and Eikelboom, 1969; Berman et al., 1970). Six pigs, three C and three SS, were delivered to the abbatoir during the afternoon and fasted until slaughtered the next day. During the holding period at the abbatoir, the C and SS pigs were allowed to mingle freely with each other, without crowding. A SS pig was isolated from the others, shackled and exsanguinated at about 8:00 a.m. (I), followed shortly by a C pig. A second set was slaughtered at about 10:30 a.m. (II) and a third set at about 2:00 p.m. (III). The SS pigs were slaughtered first in each set. Two days later the process was repeated with three C and three SS pigs.

Muscle sampling

Longissimus muscles were excised at 3, 30, 60, 120 and 180 min postmortem and immediately frozen in liquid nitrogen. Samples were pulverized with a Sorvall Omnimixer and the resulting powder was stored at $-50°$ F until ready for use.

Extraction and assay

Without modification, muscles were extracted and metabolites assayed by published methods (Ono and Woods, 1974). Muscle pH was determined by blending 2g of the muscle powder in 20 ml of 0.1M iodoacetate, pH 7.5, and reading the resulting pH with a combination electrode.

Percent reflectance was measured by the method of Marple and coworkers (1969). Subjective color evaluation was classified as pale, normal or dark.

Statistical analysis

Analysis of variance was used to test for significance of difference between overall means while the Duncan's new Multiple Range Test was used for mean separation analyses.

RESULTS & DISCUSSION

CONCENTRATIONS of c-AMP in longissimus muscle obtained from C and SS pigs are shown in Figure 1. Means between C and SS samples differed significantly only at 3 min postmortem. Since sampling of muscle at less than 3 min postmortem was not possible, it is difficult to conclude definitely that the 3 min c-AMP levels for C and SS samples were elevated from the basal levels, although Posner et al. (1965) found basal levels of c-AMP ranging from 600–700 picomoles/g muscle for rat and frog skeletal muscles. Thus, if there is elevation in c-AMP level resulting from the stress of slaughter, it may be highly transient and 3 min postmortem may not reflect the magnitude of the effective difference between C and SS samples. Exton and coworkers (1971) demonstrated that response of c-AMP to epinephrine can occur within 30 sec. Topel (1972) measured plasma epinephrine levels in C and SS strain of Danish Landrace pigs and found that levels increased significantly within 30 sec after electrical stimulation with levels for SS higher than for C. Thus differences in circu-

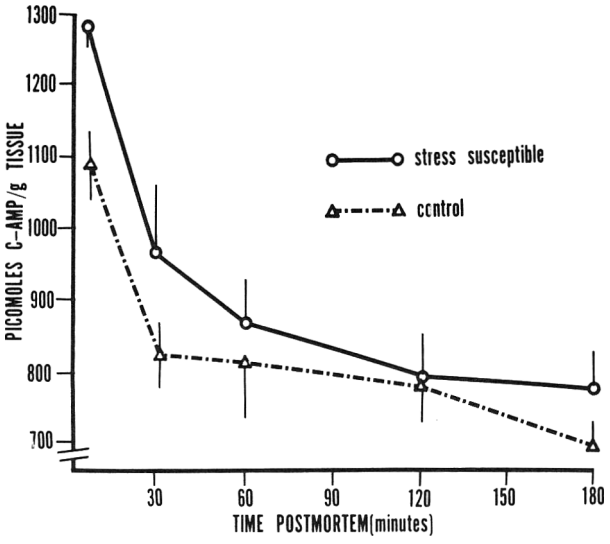

Fig. 1–Cyclic AMP concentrations in porcine longissimus muscle. Vertical bar represents standard error of the mean.

lating levels of epinephrine may be reflected in different c-AMP levels at 3 min. The implication of ATP concentration in regulating c-AMP levels must be considered also. Although details of ATP concentrations will be reported as part of an experiment involving adenyl cyclase, comparison of mean ATP concentrations indicate that SS had significantly (P = 0.05) lower ATP levels than C muscles up to 60 min postmortem. Kastenschmidt and coworkers (1968), similarly, found lower levels of ATP in "fast-" than in "slow-glycolyzing" muscles of pigs. Since muscles from SS pigs had higher levels of c-AMP and lower levels of ATP than from C pigs, it appears that concentration of ATP was not the limiting factor in controlling c-AMP levels during this period. Biopsy sampling techniques are being investigated to define, more precisely, the time-course of c-AMP elevation. It is also possible that total c-AMP concentration does not represent the effective level and once a minimum level is reached, the rate-determining step of glycogenolysis occurs after c-AMP synthesis. Studies by Aberle and Merkel (1968a, b) and by Pearson and coworkers (1973) indicate that stress applied during slaughter produces enough epinephrine to cause a saturated system with respect to potential for activating phosphorylase b. The glycogen (Table 1) and c-AMP (Fig. 1) values partially support this view. The values indicate that rates of glycogenolysis are fairly constant for C and SS muscles, despite rapid disappearance of c-AMP between 3–30 min. This might imply that there is an excess amount of c-AMP than what is needed for phosphorylase activation. For reasons which are not obvious, glycogen levels were significantly (P = 0.05) higher for SS than C pigs until 60 min postmortem. Exton (1975) proposed a novel concept which runs counter to the accepted mechanism of c-AMP mediated glycogenolysis. His data indicate that, in rat liver, stimulation of glycogenolysis and gluconeogenesis by epinephrine is mediated principally by α-adrenergic receptors and does not involve an increase in c-AMP. The pertinence of this concept to our study needs to be established.

Another variable which needs consideration in relating rate of glycogenolysis to PSS and PSE muscle is the variation in nutritional state of the pig at slaughter. Due to this variation, some of the results from our experiment point out the difficulties associated with relating levels of c-AMP, glycogen, lactate and pH.

The time of slaughter affected initial glycogen levels (Table 2). Pigs sacrificed later in the day had lower glycogen levels than those sacrificed in the morning, although c-AMP levels did not differ with time of slaughter. As expected, SS pigs accumulated lactate at faster rates than C pigs. Except at 3 min postmortem, when difference was not significant, SS pigs had significantly (P = 0.05) higher levels of lactate than C pigs. The time of slaughter associated with nutrient intake affected lactate levels for SS pigs which was a logical reflection of the initial glycogen level. At 30 min postmortem, SS pigs killed at I and II had significantly higher levels of lactate than C pigs (Table 3).

The pH values generally reflected the results of lactate concentrations. Within 30 min postmortem, SS pigs had significantly lower pH than C pigs (Table 4). Initial levels of glycogen significantly influenced pH values at 30 min within the SS group. Values at I, II and III (Table 5) for this group differed significantly (P = 0.05) from each other.

Table 1–Glycogen concentration versus time postmortem and stress susceptibility

Min postmortem	μmoles glucosyl units per g tissue[a]	
	Control	Stress susceptible
3	15.62b	32.77a
30	17.80b	30.47a
60	11.52b	27.52a
120	10.13bc	17.61b
180	5.40c	10.40bc

[a] All means with different letters are significantly different (P = 0.05) from each other. n = 6 each for control and stress susceptible.

Table 2–Glycogen concentration at 3 min postmortem versus time of slaughter and stress susceptibility

Time of slaughter	μmoles glucosyl units per g tissue[a]	
	Control	Stress susceptible
I (8:00 a.m.)	17.70bc	54.75a
II (10:30 a.m.)	16.85bc	28.05b
III (2:00 p.m.)	11.10c	15.50bc

[a] All means with different letters are significantly different (P = 0.05) from each other. n = 2 each for control and stress susceptible at each time.

Table 3–Lactate concentration at 30 min postmortem versus time of slaughter and stress susceptibility

Time of slaughter	μmoles lactate per g tissue[a]	
	Control	Stress susceptible
I (8:00 a.m.)	40.4c	75.5a
II (10:30 a.m.)	39.9c	65.2b
III (2:00 p.m.)	48.6c	48.1c

[a] All means with different letters are significantly different (P = 0.05) from each other. n = 2 each for control and stress susceptible at each time.

Table 4—pH versus time postmortem and stress susceptibility

Min postmortem	pH[a] Control	pH[a] Stress susceptible
3	6.40a	6.28ab
30	6.36a	6.04c
60	6.12bc	5.77d
120	5.82d	5.53e
180	5.62e	5.44e

[a] All means with different letters are significantly different (P = 0.05) from each other. n = 6 each for control and stress susceptible.

Table 5—pH at 30 min postmortem versus time of slaughter and stress susceptibility

Time of slaughter	pH[a] Control	pH[a] Stress susceptible
I (8:00 a.m.)	6.41a	5.89c
II (10:30 a.m.)	6.38a	5.95b
III (2:00 p.m.)	6.30a	6.28a

[a] All means with different letters are significantly different (P = 0.05) from each other. n = 2 each for control and stress susceptible at each time.

Percent reflectance and subjectively evaluated color of the longissimus muscle were also affected by initial glycogen level (Table 6) for the SS group. Despite the limited number of hogs tested, a trend is evident; pigs killed about 2.5 hr after initiation of agitation in the morning tended to produce normal muscles. The significance of this to industrial application should be evaluated.

The results illustrate the difficulties associated with relating c-AMP levels to rate of glycogenolysis and, also, the importance of initial glycogen levels in the relation between concentrations of glycolytic metabolites and PSS and PSE muscle. Thus, future studies of relating c-AMP concentrations to glycolytic metabolites should be conducted under conditions which minimize variations in initial glycogen levels. The effects of high initial concentration of substrate on rate of metabolite accumulation also should be determined. Perhaps substrate becomes rate-determining only after a stress stimulus that activates the adenyl cyclase system. Since initial levels of glycogen were the same for all C pigs (Table 2), substrate effects could not be tested.

Table 6—Percent reflectance and color versus time of slaughter and stress susceptibility

Time of slaughter	% Reflectance[a] – Color[b] Control	% Reflectance[a] – Color[b] Stress susceptible
I (8:00 a.m.)	21.1-normal	29.1-pale
II (10:30 a.m.)	21.0-normal	24.0-normal
III (2:00 p.m.)	21.0-normal	18.8-normal, dark

[a] Scale calibration: >27-pale; 20—24-normal; <18-dark.
[b] Evaluated subjectively. n = 2 each for control and stress susceptible at each time.

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MICROBIOLOGY OF PALE, DARK AND NORMAL PORK

ABSTRACT

Pale, soft, exudative (PSE), dark cutting, and normal colored pork were compared to determine differences in susceptibility to microbial growth. Chops were packaged and stored under simulated retail conditions. Color, marbling, pH, numbers of total aerobic mesophiles, psychrotrophs, proteolytic and lipolytic bacteria and fluorescent *Pseudomonas* and incidence of *Salmonella* and coagulase positive *Staphylococcus* were recorded for pork chops as storage time progressed. Data were statistically analyzed. Differences in pH between PSE and normal chops, after aging, were significant, and dark chops had significantly higher pH values than normal chops. Development of microorganisms during storage was influenced by pH. Most rapid growth of organisms occurred on dark meat and slowest bacterial growth on PSE pork, although exudative conditions of the latter provided the highest expressible juice. pH of the meat also seemed to have a selective action on the microflora. Marbling of the meat did not influence growth of lipolytic bacteria. Relevance of various stages of processing as sources of contamination for the meat is discussed.

INTRODUCTION

COLOR CONTRIBUTES to the subjective evaluation of palatability and it is a very important quality characteristic of meat in obtaining a favorable judgement from the consumer at the retail level.

Extreme differences in color may result from abnormal glycolysis and stress on meat animals (Briskey, 1964; Topel et al., 1968; Ashmore et al., 1972). Examples of these extremes are pale, soft, exudative (PSE) musculature and dark cutting meat. The latter condition is considered undesirable for marketing fresh beef because the consumer may associate the dark color with that of meat from old animals or relate it to the appearance of beef held under adverse conditions. A preliminary report on a survey of consumer acceptance (Topel et al., 1973) indicates that color also has a great influence in determining preferences of the consumer for pork.

Attributes such as firmness, high water-holding capacity, tenderness and juiciness make dark pork meat very acceptable for processing. At the other extreme, pork with low pH has decreased water-holding capacity, a soft texture and a poor salt retention and is not desirable for emulsion type products (Wisner-Pedersen, 1962) or for ham manufacture (Briskey, 1964).

Predictions of the bacteriological condition of meat with these abnormal conditions based on certain phenomena associated with abnormal glycolysis, and pH have been published (Jay, 1966). However, differences in microbial spoilage of dark or pale muscles have not been described.

The present investigation was conducted to elucidate differences in susceptibility to microbial spoilage of dark, pale and normal pork.

EXPERIMENTAL

Samples for comparison between normal and pale pork

28 "wholesale" loins from pigs raised at the University Farm were used for comparisons of PSE and normal pork. One-half of these loins were classified as pale with scores of 1 and 2 for color and firmness according to the Standards for Pork Color prepared by Rust and Topel (1969). The remaining loins were classified as normal pork with scores between 3 and 4. On the day after slaughter, the loins were removed from the carcasses and stored at 2°C for 9 days. On the tenth day after slaughter, 1-in. chops were cut from each loin, placed in plastic trays and wrapped with 195 MSAD-80 cellophane (E.I. DuPont de Nemours and Co., Inc., Wilmington, Del.). This material is highly permeable to oxygen. The packages were stored at 5°C in an open display case under soft white fluorescent lights to simulate retail conditions. Samples were analyzed after cutting and after 1, 2 and 3 days of storage.

Samples for comparisons between normal and dark pork

22 loins purchased from a commercial meat packing plant were used for these comparisons. The loins were transported to the laboratory 1 day after slaughter. At the laboratory they were stored at 2°C for 9 days, cut into chops and wrapped and stored under the same conditions described above.

Table 1—Bacteriological determinations

Type of organisms	Growth media	Technique	Incubation
Quantitative analyses			
Psychrotrophs	TSA agar	Pour plates	5°C for 15 days
Mesophiles	TSA agar	Pour plates	30°C for 3 days
Fluorescent *Pseudomonas*	King's agar	Surface plates	15°C for 6 days
Lipolytic organisms	NBS agar	Surface plates	15°C for 14 days
Proteolytic organisms	S&G agar	Surface plates	15°C for 4 days
Qualitative analyses			
Type of organisms	**Growth**	**Technique**	**Incubation**
Coagulase positive *Staphylococcus*	Procedure of Wilson et al. (1959)	Procedure of Herman and Morelli (1960)	Tube coagulase
Salmonella	Procedure for meats (Ga ton et al., 1968)	BGS agar (Galton et al., 1968)	TSI and agglutination (Galton et al., 1968)

Analytical procedures

The following observations were recorded for the samples: expressible juice, percent reflectance, color score, marbling score, pH, numbers of psychrotrophs, mesophiles, proteolytic, lipolytic and fluorescent bacteria, incidence of *Salmonella* and coagulase positive *Staphylococcus*.

Color and marbling were subjectively evaluated by comparing the samples with the standards for pork color mentioned above. Expressible juice, pH and percent light reflectance were obtained from each loin immediately after the loins were cut into chops. Methods reported by Matsushima and Topel (1969) were used. These values were used to classify loins into three quality groups and provided pertinent information regarding samples.

A Beckman Zeromatic pH meter (Beckman Instruments, Inc., Fullerton, Calif.) was used for measuring pH by placing the electrodes on the surface of the meat for pH values obtained after 1, 2 and 3 days of storage in the retail display case.

The bacteriological procedures used are summarized in Table 1. Abbreviations in the table are: TSA for trypticase soy agar (Baltimore Biological Laboratories Division of Bio Quest, Cockeysville, Md.); King's agar for medium B of King et al. (1954); NBS agar for a modification of nile blue sulfate agar of Goldman and Rayman (1952) as described by Rey (1968), using lard as the lipid material; S and G for the gelatin-agar medium of Smith and Goodner (1958); BGS agar for brilliant green agar (BBL) with sulfadiazine added and TSI for triple sugar iron agar (BBL).

A swab technique was used for sampling. Sterile aluminum templates were employed to delineate 10 cm^2 of the surface of the chops for enumeration of bacteria per cm^2. After this first swab, two other swabs were taken, one on each side of the chops for detection of *Salmonella* and coagulase positive *Staphylococcus* respectively.

Statistics

Bacterial counts were evaluated for significance after logarithmic transformation by means of analysis of variance, Duncan's multiple range tests and correlation coefficients tested under the null hypothesis Ho; R = 0. Chi-square tests for independence were used to determine significance of incidence of coagulase positive *Staphylococcus*.

RESULTS & DISCUSSION

THE ANALYSES OF VARIANCE of bacterial counts are presented in Table 2. For the comparison of PSE and normal pork, the animals were slaughtered at the Meat Laboratory and the slaughtering period was considered as a source of variation (Table 2a). For the comparison of dark and normal pork, there was no control of slaughtering conditions since the animals were slaughtered in a commercial operation. However, cutting and preparation of the chops were done at the Meat Laboratory and cutting period during the course of this study was segregated as a source of variation in this comparison (Table 2b). Essentially, both sources (slaughtering or cutting period) were considered to reflect variability due to handling.

The two analyses of variance for bacterial counts show significant variability for all bacterial counts due to handling during slaughtering or cutting and preparation of the retail cuts. Another significant source of variation for bacterial counts was the length of storage under simulated retail conditions which shows that the bacterial densities increased significantly during refrigerated storage for all types of pork; such increases were to be expected.

The analyses of variance in Table 2 indicated that the variability of the overall mean of bacterial numbers that could be attributed to meat type (pale, normal or dark) was not significant. Figure 1 (b through f) compares growth of different types of bacteria on normal and pale pork. The level of initial contamination was practically the same for both kinds of meat. One might predict that the watery condition on the surface of the PSE pork would favor the development of bac-

Table 2—Analyses of variance of bacterial counts

A. Comparison between pale and normal pork

Source of variation	DF	Mean squares				
		Psychrotrophs	Mesophiles	Proteolytic	Lipolytic	Fluorescent
A = Slaughtering period	3	32.42**	34.80**	40.57**	48.31**	31.25**
B = normal vs pale pork	1	6.52	9.23	,8.10	7.12	9.88
A × B (error a)	3	1.08	1.12	1.11	1.03	1.06
C = days in storage	3	45.01**	37.97**	49.36**	38.87**	40.23**
B × C	3	1.08	0.89	0.59	1.15	1.24
A × C	9	1.47*	4.09**	6.72**	8.34**	5.89**
Residual (error b)	89	0.61	0.41	0.56	0.58	0.55
Total	111					

B. Comparison between dark and normal pork

Source of variation	DF	Mean squares				
		Psychrotrophs	Mesophiles	Proteolytic	Lipolytic	Fluorescent
A = cutting period	10	38.03**	21.22**	30.96**	36.23**	34.23**
B = normal vs dark pork	1	9.26	6.17	13.59A[a]	3.20	27.52**
A × B (error a)	10	2.66	1.87	,3.18	2.03	2.40
C = days in storage	3	70.95**	57.51**	77.91**	57.96**	68.94**
A × C	30	2.04**	2.01**	2.31**	1.87**	2.28*
B × C	3	0.49	0.79	0.76	0.97	0.75
Residual (error b)	118	0.64	0.55	0.64	0.63	0.78
Total	175					

*P ≤ 0.05
**P ≤ 0.01
A = approaching 5% level

terial spoilage due to the availability of water. However, a longer lag phase and a slower rate of growth was observed for all types of bacteria on pale than on normal chops. This resulted in much lower bacterial densities on pale than on normal pork by the third day of storage. Differences in numbers of bacteria due to differences in pH of the meat are discussed later.

Figure 2 (a through e) compares bacterial growth on normal and dark pork. Again the initial contamination was practically the same for either normal or dark chops. However, the longest lag phase and the slowest rate of growth was associated with the normal colored chops. The differences in lag phase and rate of bacterial multiplication on pale, normal and dark pork resulted in remarkable differences in bacterial numbers among the three types of meat by the second and third days of storage. PSE pork showed the lowest bacterial densities, normal meat was intermediate and dark meat exhibited the highest numbers of all types of bacteria assayed. These differences were tested for significance by multiple range tests. The differences were significant at the 5% level of probability (Table 3).

The analyses of variance (Table 4) also showed that the difference in pH between pale and normal pork was highly significant, as was that between normal and dark pork. Average pH values of the three types of meat are plotted in Figure 1a. The relative levels of pH for the meats were consistent with known values for muscle showing these color differences: highest pH for the dark, intermediate for the normal, and lowest for the pale. The pH of the dark and normal pork increased during storage. As mentioned earlier, the packaging material used (195-MSAD 80) has good permeability to oxygen. Under aerobic conditions, development of certain psychrotrophic species is selectively favored in refrigerated meats (Pierson et al., 1970). This type of growth is generally accompanied by an increase in pH (Adamcic and Clark, 1970) because end products from the metabolism of proteinaceous materials (Masurovsky et al., 1963).

Our results show that the differences in bacterial counts observed between the three types of pork chops are concomitant with differences in pH (Fig. 1 and 2). Correlation coeffi-

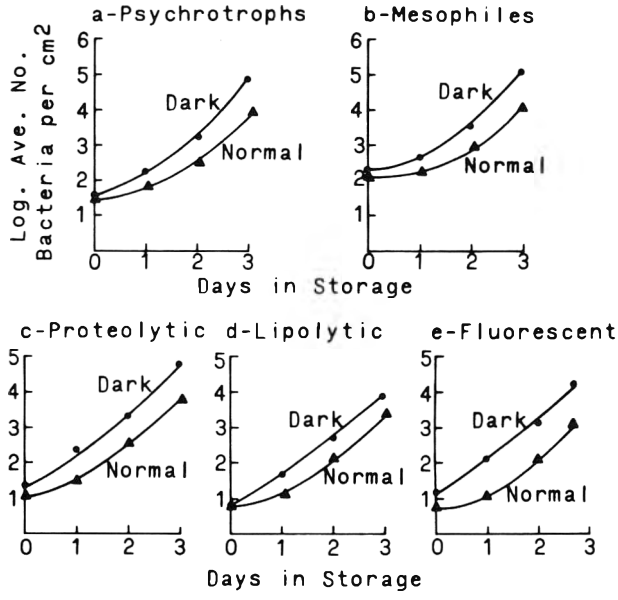

Fig. 2—Numbers of bacteria on normal and dark pork during storage at 5° C.

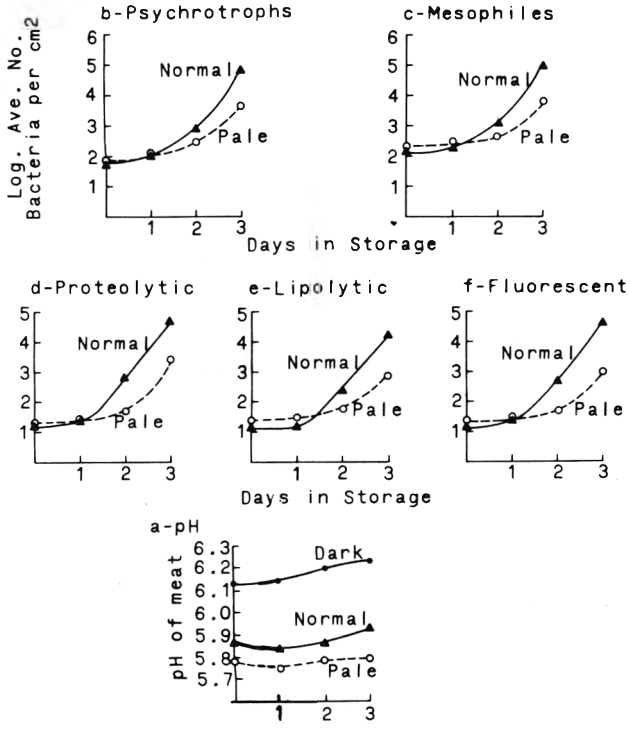

Fig. 1—(a) pH changes for pale, normal, and dark pork during retail storage conditions; (b—f) Numbers of bacteria on normal and pale pork during storage at 5° C.

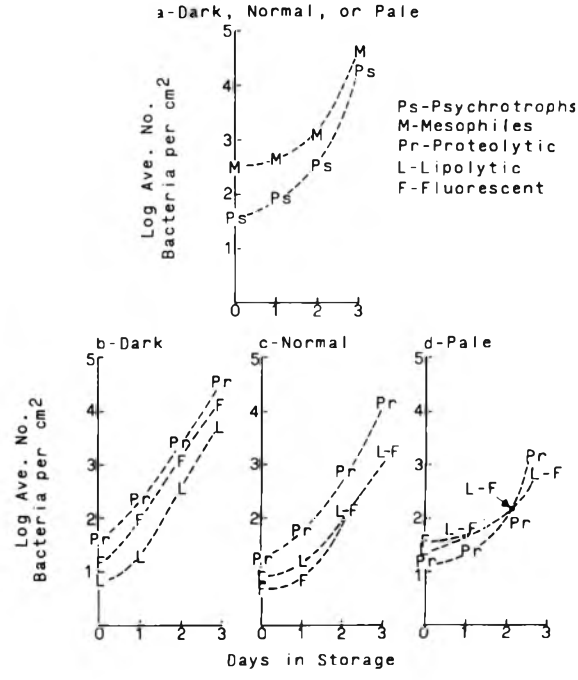

Fig. 3—Growth of various types of bacteria on dark, normal and pale pork: (A) mesophiles and psychrophiles on the three types of meat; proteolytic, lipolytic, and fluorescent bacteria on (b) dark, (c) normal, and (d) pale pork.

Table 3—Multiple range tests for differences in bacterial numbers and in pH between types of pork

Observations	Comparison	Differences between means			
		Day 0	Day 1	Day 2	Day 3
Psychrotrophs	Dark minus normal	0.17	0.44	0.57	0.66*
	Normal minus pale	0.06	0.12	0.41	1.03*
Mesophiles	Dark minus normal	0.17	0.33	0.25	0.76*
	Normal minus pale	0.04	0.36	0.20	1.05*
Proteolytic	Dark minus normal	0.24	0.86*	0.77*	0.65*
	Normal minus pale	0.09	0.02	1.26*	1.12*
Lipolytic	Dark minus normal	0.12	0.28	0.59	0.34
	Normal minus pale	0.15	0.36	0.65*	1.21*
Fluorescent *Pseudomonas*	Dark minus normal	0.43	0.97*	0.76*	1.00*
	Normal minus pale	0.06	0.11	0.84*	1.39*
pH	Dark minus normal	0.270*	0.300*	0.340*	0.320*
	Normal minus pale	0.080	0.090	0.070	0.100

* $P \leq 0.05$

cients calculated for all types of bacterial counts with pH were positive and significant ($P < 0.0001$). These results suggest that the effect of pH is to produce conditions whereby normal meat is more susceptible than the abnormal PSE pork to bacterial spoilage of the type that is likely to predominate under retail conditions and the dark cutting meat with significantly higher pH is even more prone to this type of spoilage than the normal colored meat. However, it should be mentioned that normal pork came from the two sources previously described for comparisons with pale and dark pork. This may have affected results observed. Further investigation of the role of pH might involve detailed analysis within color groups to determine effects on bacterial growth. Such studies would be helpful in elucidating the significant correlations between pH and bacterial counts in this work.

A comparison of the densities of the various types of bacteria recovered from the different types of pork is presented in Figure 3. Mesophilic and psychrotrophic counts from the three kinds of meats were averaged and are shown in Figure 3a. Averages were adopted for this comparison since the relative densities for these two types of bacterial counts were the same for PSE, normal or dark pork.

Initial contamination with mesophilic bacteria was always higher than with psychrotrophs. Since the loins had been aged under refrigeration for more than 1 wk before preparation of the chops, it may have been expected that these storage conditions would have favored the predominance of psychrotrophs on the chops by the time of preparation (Day 0). However, since mesophiles were predominant on Day 0 it may be inferred that the greatest proportion of the initial contamination of chops was derived from equipment, utensils and handling during cutting and preparation of the chops.

Figure 3b, c and d shows the relative densities of bacteria with proteolytic and lipolytic activity or ability to fluoresce. This comparison shows that organisms having these three biochemical characteristics of spoilage were quite restricted on the pale pork as compared to normal or dark pork. The somewhat higher pH observed for normal pork compared with pale pork allowed for an increase in rate of multiplication of proteolytic species and to a lesser degree also for an increase in rate of growth of lipolytic and fluorescent bacteria. At the higher pH recorded for the dark meat a further increase in rate of growth was evident for all three types of organisms. One of the most remarkable differences in growth rate of bacteria on the different meat types was that observed for fluorescent *Pseudomonas* which were greatly restricted on the PSE pork (most acidic) but seemed to thrive on the dark colored pork (highest pH). Differences in pH among meats had a selective action on the type of microflora that developed on the samples.

The influence of marbling on the densities of lipolytic organisms was tested and it was found that the degree of marbling of the meat was not significantly related to lipolytic bacteria ($P > R = 0.39$).

Potential pathogens

Salmonellae were not recovered from any samples, indica-

Table 4—Analysis of variance of reflectance, pH and juice expressed

Source of variation	df	Mean squares		
		Reflectance	pH	Juice extracted
Pale vs Normal	1	880.32**	1.15**	11.09**
Residual	26	3.84	0.04	0.16
Total	27			
Normal vs Dark	1	64.80**	0.59**	0.91**
Residual	18	2.51	0.03	0.06
Total	19			

**Significant at 1% level

ting that conditions for slaughter and subsequent handling of the meat were excellent in controlling this type of contamination.

Coagulase positive *Staphylococcus* were recovered from 12 chops of the 112 analyzed from the animals slaughtered at the Meat Laboratory. These were the samples used for comparison between PSE and normal pork. Seven of the 12 chops yielding *Staphylococcus* were PSE pork and the other 5 were normal, showing no significant trend or difference between the two types of meat. However, 12 positive samples came from the same slaughtering and cutting period, indicating close dependence of staphylococcal contamination of specific handling procedures.

Coagulase positive *Staphylococcus* were recovered frequently from chops used for comparisons between dark and normal pork. The meat came from a commercial plant where the animals were slaughtered and cut into wholesale cuts before transportation to the laboratory. Chi-square tests showed that frequency distribution of these organisms was independent of the type of meat ($P \leqslant 0.29$) or days in storage under simulated retail conditions ($P \leqslant 0.72$). However, there was a significant relationship between the period of cutting and preparation of the chops and the incidence of coagulase positive *Staphylococcus* ($P \leqslant 0.0001$). Figure 4a shows the frequency of recovery of these organisms at each cutting time for dark and normal pork. There are no outstanding differences between types of pork but the great variability between cutting periods illustrates the influence of the procedures used for cutting and preparation of the retail cuts on contamination of the meat with coagulase positive *Staphylococcus*.

The analysis of variance had shown that cutting period was a highly significant source of variation. Average numbers of mesophilic microorganisms on chops from different cutting periods are presented in Figure 4b. Although not presented here, graphs for numbers of psychrotrophs, lipolytic, proteolytic and fluorescent bacteria showed the same trends for each cutting period as that for the mesophiles, which indicates the possibility of a common source of contamination. However, comparison of Figure 4a and b reveals that the direct source of contamination with coagulase positive *Staphylococcus* during cutting and preparation was different than for the other organisms. Therefore, meat prepared and handled under conditions that yield retail cuts having low numbers of saprophytic microorganisms does not necessarily imply freedom from contamination by potential pathogens such as coagulase positive *Staphylococcus*. This observation concurs with that of Hans et al. (1973) for beef steaks. These authors found no correlation between total numbers of bacteria and numbers of potential pathogens such as salmonellae and staphylococci.

Statistical analysis of reflectance, pH and expressible juice is given in Table 4. Significant differences in muscle color (expressed as percent reflectance) existed between the three groups of pork loins. The pale (high percent reflectance) loins had lower pH and higher expressible juice values than the normal colored loins. The normal colored loins also had significantly lower muscle pH and higher expressible juice than the dark colored loins. These data indicate that major differences exist in quality for the three groups of loins used in this study.

From these results, the following summary statements may be made: (1) the average pH of normal pork after aging was significantly higher than the pH of PSE pork, while the average pH of dark pork was significantly higher than that of normal colored meat; (2) the differences in pH between the three types of meat influenced the proportions of microorganisms of various types that developed on the samples; (3) length of the lag phase and rate of multiplication of saprophytic microflora that developed under the simulated retail conditions were related to the pH of the meat; (4) from this relation, it may be expected that abnormal, dark colored pork would be more susceptible than normal meat, and normal pork more susceptible than PSE pork to the type of bacterial spoilage that generally predominates under conditions of storage similar to those used in a retail store display case; (5) additional work on pH within color groups may be useful in explaining the role of pH on bacterial numbers on the different types of pork; and (6) the frequency of isolation of coagulase positive *Staphylococ-*

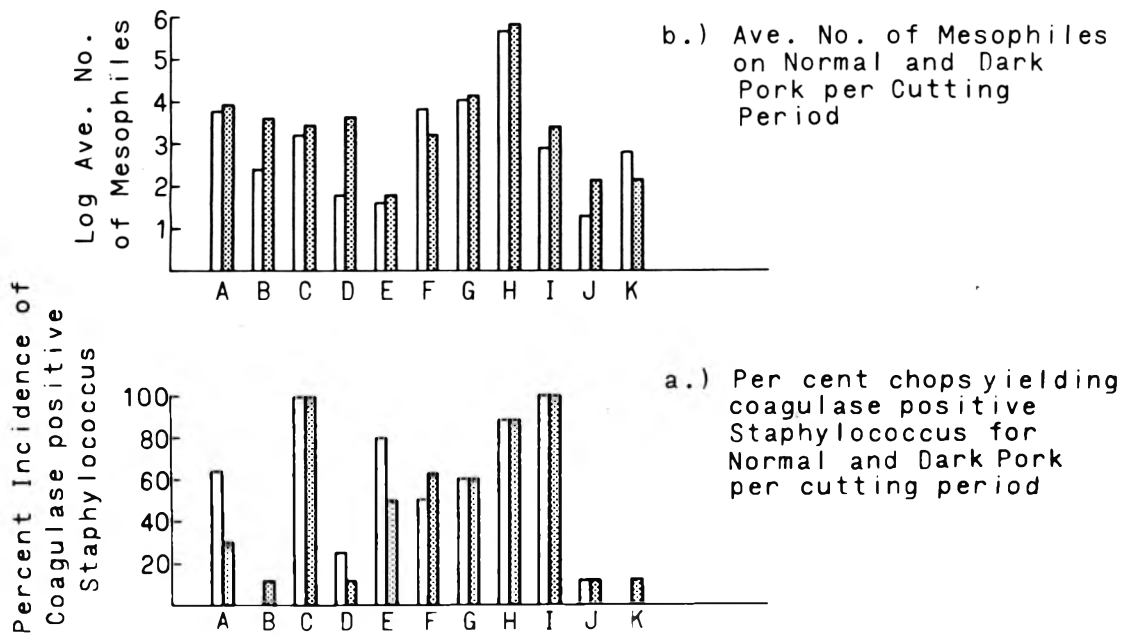

Fig. 4–(a) Number of mesophiles on normal and dark pork at each cutting period; (b) Percent of normal and dark pork chops yielding coagulase positive staphylococci at each cutting period. [Normal = white bar; Dark = shaded bar].

cus did not bear a direct relationship to the type of meat but depended on the conditions of handling during cutting and preparation of the retail cuts.

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RELATIONSHIP OF Vibrio parahaemolyticus IN OYSTERS, WATER AND SEDIMENT, AND BACTERIOLOGICAL AND ENVIRONMENTAL INDICES

ABSTRACT

Of 153 samples of oysters, water and sediment tested 94 (61.4%) were positive for *V. parahaemolyticus*. In 87% of the positive oyster samples the level of *V. parahaemolyticus* was < 100 per g. Standard Plate Counts of oysters ranged from 5.0×10^3 to $> 3.0 \times 10^7$ (mean 2.5×10^6) per g, Aerobic Plate Counts from 3.0×10^3 to $> 3.0 \times 10^7$ (mean 1.8×10^6) per g. No seasonal distribution of *V. parahaemolyticus* was apparent. No statistically significant relationship existed between *V. parahaemolyticus* count of oysters and bacteriological (Standard Plate Count, Aerobic Plate Count, coliform, fecal coliform and *E. coli* counts) and environmental parameters (water temperature, salinity, pH, dissolved O_2, sampling depth, tide, wind and rainfall).

INTRODUCTION

Vibrio parahaemolyticus is a potential pathogen associated with marine species and their environments. It is found primarily in water, sediment and marine species in coastal waters and estuaries. Since 1951, isolations of *V. parahaemolyticus* have been reported in Japan where it is a major cause of gastroenteritis associated with the consumption of seafoods, particularly during summer months (Sakazaki, 1969). In recent years this organism has been isolated from shellfish and marine environments in the United States and other countries. Although *V. parahaemolyticus* was the probable cause of several unconfirmed outbreaks of foodborne illness in the United States in 1969 (USDHEW, 1969), the first confirmed outbreaks occurred in 1971 which involved consumption of crab (Molenda et al., 1972; USDHEW, 1971). Since 1971, *V. parahaemolyticus* has been responsible for eight confirmed and five unconfirmed outbreaks of foodborne illness in this country. More than 1200 persons were involved in these outbreaks. Of the 13 outbreaks, nine were associated with the ingestion of crabs, two with oysters, and one each with shrimp and lobster.

Foodborne illness caused by *V. parahaemolyticus* in Japan usually is associated with consumption of raw seafoods. In the United States most seafoods are subjected to some heat treatment before consumption. Under these conditions outbreaks were caused by gross mishandling of the food. The oyster is the only mollusc that is generally eaten raw. Isolations of *V. parahaemolyticus* from oysters have been reported from widely different marine environments in the United States (Baross and Liston, 1970; Bartley and Slanetz, 1971; Landry et al., 1970; Thomson and Trenholm, 1971; Vanderzant and Nickelson, 1972). A seasonal incidence of this organism with a peak during the summer months is reported for oysters and/or water from Puget Sound (Baross and Liston, 1970), Great Bay and Little Bay areas of New Hampshire (Bartley and Slanetz, 1971), and Chesapeake Bay (Kaneko and Colwell, 1973). To evaluate the potential of oysters in foodborne illness, information is needed concerning the distribution of *V. parahaemolyticus* in oysters from sub-tropical waters such as the Northern Gulf of Mexico. Little is known about the effect of environmental factors on the presence of *V. parahaemolyticus* in coastal waters and in marine species. This report provides information on the concentration of *V. parahaemolyticus* in oysters, water and sediment from sub-tropical waters of the Northern Gulf of Mexico, and possible relationships between *V. parahaemolyticus* and bacteriological and environmental indices.

MATERIALS & METHODS

ALL SAMPLES were taken from areas in the Galveston Bay system (Fig. 1) by personnel of the Texas State Dept. of Health at LaMarque or of the Marine Lab., Texas A&M University at Galveston, Texas. Oysters were collected in sterile plastic bags and transported to the laboratory in an insulated container at 5–10°C. Samples usually were examined within 6–12 hr after collection. Preparation of oysters for microbiological examination was as described in APHA (1970).

The shucked oysters were blended for 2 min in a sterile Waring Blendor. A 50-g portion was then blended for 1.5 min with 450 ml of sterile 0.1% trypticase peptone (BBL) with 3% NaCl. Bacterial counts were determined with the spread plate method by placing 0.1 ml of appropriate dilutions on trypticase soy agar (TSA, BBL) plates with 3% NaCl (incubation at 25°C for 2 days-Aerobic Plate Count), and on Plate Count Agar (PCA, BBL) plates (incubation at 35°C for 2 days-Standard Plate Count). Water and sediment samples were transported and plated in the same manner as the oysters.

Isolation and enumeration of *V. parahaemolyticus* was accomplished with the 3-tube MPN technique by placing appropriate quantities of oyster-trypticase-peptone homogenate (equivalent to 1 to 10^{-4} g of oyster) in glucose salt teepol broth (GSTB). After 24 hr at 35°C, the tubes were streaked with a wire loop onto thiosulfate citrate bile salts sucrose agar (TCBS, BBL). After incubation for 24 hr at 35°C, blue-green colonies from TCBS medium were picked as suspect *V. parahaemolyticus*. With some samples both GSTB and trypticase soy broth (TSB, BBL) with a total of 7% NaCl were used as enrichment media. Tests used for confirmation of isolates are described in a previous report (Vanderzant et al., 1973), and in USDHEW (1972) and included Gram reaction, morphology, cytochrome oxidase, catalase, starch hydrolysis, triple sugar iron agar, lysine decarboxylase, ornithine decarboxylase, NH_3 from arginine, growth in 1% trypticase broth with 0, 3, 7 and 10% NaCl, indole production, methyl red, Voges-Proskauer, motility, nitrate reduction, citrate utilization, urease, gelatine liquefaction, sensitivity to pteridine 0/129, acid from sucrose, lactose, mannitol, arabinose and cellobiose. Coliform, fecal coliform and *Escherichia coli* counts were determined using the 3-tube MPN method as recommended in USDHEW (1972). Salinity, temperature and dissolved O_2 of the waters from which samples were taken were measured with a YSI Temperature, Salinity and Conductivity Meter (Yellowstone Instrument Co., Yellow Springs, Ohio). Wind direction and velocity and rainfall data were obtained from the U.S. Weather Service. The pH of the water was measured with a portable pH meter (W.A. Taylor Co., Baltimore, Md.).

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RESULTS & DISCUSSION

THE NUMBER of oyster, water and sediment samples with confirmed *V. parahaemolyticus* is presented in Table 1. Data on the concentration of *V. parahaemolyticus* in these samples are presented in Figure 2. 32 (59.3%) of the 54 oyster samples, 24 (49%) of the 49 water samples, and 38 (76%) of the 50 sediment samples contained *V. parahaemolyticus.* Of a total of 591 suspect colonies picked from TCBS plates as *V. parahaemolyticus,* 285 (48.2%) were confirmed. The concentration of *V. parahaemolyticus* ranged from 0–1100 per g in oyster samples, from 0–9.3 in water samples, and from 0–2400 per g in sediment samples. In 87% of the positive oyster samples, the level of *V. parahaemolyticus* was < 100 per g, 73% of the positive oyster samples contained < 10 per g. Of the positive sediment samples, 94% contained < 100 *V. parahaemolyticus* per g.

Table 1—Number of samples of oysters, sediment and water with confirmed V. parahaemolyticus as determined after enrichment in Glucose Salt Teepol Broth (GSTB) and plating on TCBS agar

Sample	No. of samples	Samples positive for V. parahaemolyticus No.	(%)
Oysters	54	32	(59.3)
Sediment	50	38	(76.0)
Water	49	24	(49.0)
TOTALS	153	94	(61.4)

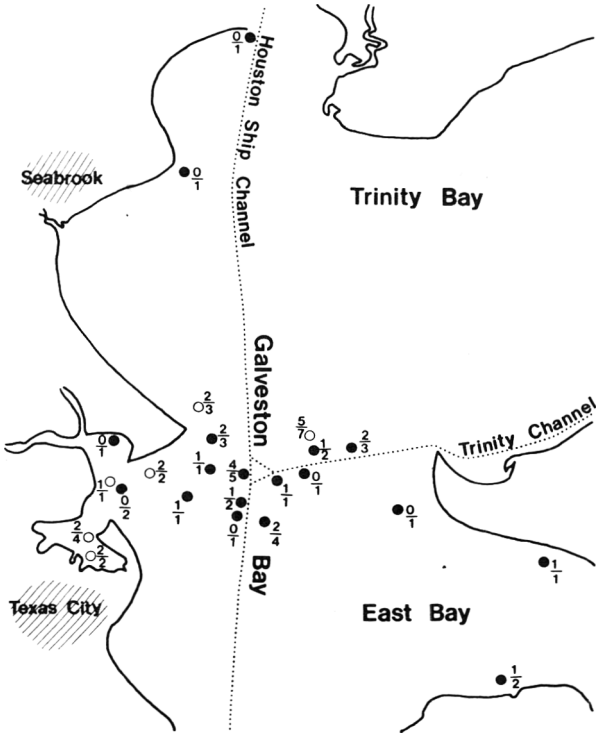

Fig. 1—Areas in the Galveston Bay system from which samples were collected (numerator = number of oyster samples containing V. parahaemolyticus; denominator = number of oyster samples collected at this site during this study; ○ areas from which one or more oyster samples contained > 10 V. parahaemolyticus per g).

With 18 oyster samples, *V. parahaemolyticus* counts were determined with enrichment in GSTB and TSB and subsequent plating on TCBS agar. In eight of the 18 samples (44.4%), the MPN/GSTB per g was larger than the MPN/TSB per g, in seven samples (38.9%) the two were approximately equal, and in 3 samples (16.7%) the GSTB/MPN per g was less than the TSB/MPN per g. The mean GSTB/MPN was 10.6, that of TSB/MPN 1.9 per g. GSTB was used as the enrichment medium with all other samples. The concentration of *V. parahaemolyticus* in oysters, water and sediment samples in this study was somehwat lower than those reported by Baross and Liston (1968, 1970) and Johnson et al. (1971). This discrepancy probably resulted from a difference in classification of *V. parahaemolyticus.* Although the concentration of *V. parahaemolyticus* in the samples was similar, the percentage of positive oyster, water and sediment samples was smaller in this study (1973–74) than reported in a preliminary study (Vanderzant et al., 1973). Differences in enrichment media (GSTB versus TSB with 7% NaCl) probably were not involved because the present study shows that enrichment in GSTB usually resulted in higher levels of *V. parahaemolyticus* than with TSB (7% NaCl). Of the environmental parameters, differences were noted in the salinity of the waters. In 1972, salinities ranged from 7–26 ppt and in 1974 from 1–16.9 ppt. However, statistically the relationship between *V. parahaemolyticus* count and salinity of the water was not significant.

Within the limits of this study, no relationship could be detected between classification of shellfish harvesting areas (open or closed) and presence of *V. parahaemolyticus* in oysters. Of 16 samples from 10 closed areas, 50% contained *V. parahaemolyticus.* However, about 60% of the samples from open (approved) areas also contained *V. parahaemolyticus.* Oysters with relatively high initial *V. parahaemolyticus* levels (>10/g) were among samples from both approved and closed areas. In most cases, samples taken from these areas at other times (without change in classification of harvesting area) had lower levels of *V. parahaemolyticus* or *V. parahaemolyticus* was absent. In one case, however, three of the seven samples taken at one station (Redfish Isle) in May, Sept. and Nov. had *V. parahaemolyticus* levels exceeding 10/g.

The Standard Plate Count is used as an index of bacterial quality of shucked oysters at the wholesale level. High counts indicate improper handling or inadequate refrigeration during harvesting, processing and shipping. To be acceptable, the Standard Plate Count must not exceed 5.0×10^5 per g. The Standard Plate Count (PCA, plate incubation 2 days at 35°C) of 54 oyster samples, 49 water samples and 50 sediment samples is presented in Figure 3. The Standard Plate Counts of oyster, water and sediment samples ranged as follows: oysters, from 5.0×10^3 to $> 3.0 \times 10^7$ per g (mean 2.5×10^6 per g); water, from $< 3.0 \times 10^3$ to 3.3×10^6 per g (mean 1.1×10^5 per g); sediment, from 3.0×10^3 to $> 3.0 \times 10^7$ per g (mean 2.6×10^6 per g). Based on the legal standard for oysters which are to be sold commercially, 11% of the samples would have been unacceptable shortly after harvesting.

The Aerobic Plate Count (TSA + 3% NaCl, plate incubation 2 days at 25°C) of 54 oyster samples, 49 water samples, and 50 sediment samples is presented in Figure 4. The ranges of the Aerobic Plate Counts were: oysters, from 3.0×10^3 to $> 3.0 \times 10^7$ per g (mean 1.8×10^6 per g); water, from $< 3.0 \times 10^3$ to 4.0×10^6 per g (mean 8.9×10^4 per g); sediment from $< 3.0 \times 10^3$ to $> 3.0 \times 10^7$ per g (mean 1.1×10^6 per g).

A comparison of the Standard Plate Count versus the Aerobic Plate Count of oysters is presented in Figure 5. In 17 of the 54 samples (31.5%) the Aerobic Plate Count was approximately equal (difference ≤ 0.25 log) to the Standard

Plate Count. In 11 samples (20.4%) the Aerobic Plate Count was smaller, and in 26 samples (48.2%) the Aerobic Plate Count was greater than the Standard Plate Count. For the samples with higher Aerobic Plate Counts, the difference between the Aerobic Plate Count and the Standard Plate Count was $<$ 1 log in 15 samples, and 1–2 logs in 11 samples. For sediment samples the Aerobic Plate Count and Standard Plate Count compared as follows (Fig. 6): in 14 of the 50 samples (28%) the Standard Plate Count was approximately equal to the Aerobic Plate Count, in 10 samples (20%) the Standard Plate Count was less than the Aerobic Plate Count, and for 26 samples (52%) larger than the Aerobic Plate Count. With 30 (61.2%) of the 49 water samples, the Standard Plate Count was approximately equal to the Aerobic Plate Count. In four (8.2%) the Standard Plate Count was smaller than the Aerobic Plate Count and in 15 (30.6%) samples the Standard Plate Count was larger than the Aerobic Plate Count. The differences in effect of plating medium and plate incubation on the bacterial counts of oysters as compared with waters and sediments may have been caused by differences in composition of the microbial flora. Higher bacterial counts of oysters on TSA may have resulted from improved recovery of sublethally injured cells. Straka and Stokes (1959) reported higher recovery of cold-injured cells on TSA than on simpler media. In addition, microbiological recovery conditons for total counts must reflect the characteristics of the food and the environment in which the microorganisms ordinarily proliferate. For some psychrotrophic bacterial species in seafoods, a temperature of 32–35°C may constitute the upper limit for growth on laboratory media. Furthermore, many microbial species of marine origin exhibit a partial or complete salt dependence as demonstrated by the growth stimulating effect of NaCl added to standard media (Colwell and Liston, 1960).

One of the principal objectives of enumeration procedures is that the count reflects as nearly as possible the microbial load of the food. The results of this study indicate that bacterial counts of oyster frequently would be greater with plate incubation at 25°C and salt added to the plating medium. Which of the bacterial counts, at 35°C on PCA without salt or at 25°C on TSA with salt, provides more useful information is a controversial point and to a certain degree depends upon the level and type of marine and terrestrial species that make up the total population at the time of analysis. High bacterial counts at 35°C in seafoods usually indicate unsanitary handling. With plate incubation at 25°C high bacterial counts

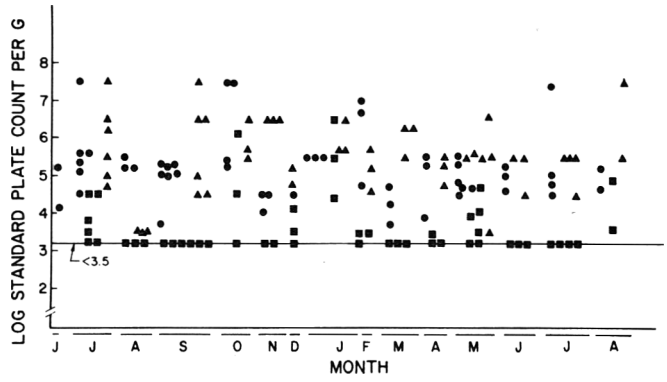

Fig. 3—Standard plate count (35° C) of oysters (●), water (■) and sediment (▲).

reflect increases in count of psychrotrophic and some mesophilic species initially present on the freshly harvested seafood or acquired by contact with contaminated equipment or surfaces in the plant. These species are frequently responsible for quality deterioration during refrigerated storage. For some of these species a temperature of 35°C may constitute the upper limit of growth on laboratory media. Bacterial counts of oysters $> 10^7$ per g were encountered in July, Oct. and Feb., only one of which is a summer month.

Coliform counts (MPN per g) of oyster, water and sediment samples are presented in Figure 7. Coliform counts in oyster samples ranged from 0–2400 (mean 81.3) per g. 49 (90.7%) of the 54 samples were positive for coliform bacteria. Coliform counts in water samples ranged from 0–2.8 (mean 0.37) per g. 12 (24.5%) of the 49 samples were positive for coliform bacteria. Coliform counts in sediment samples ranged from 0–2400 (mean 62.9) per g. 28 (56%) of the 50 samples were positive for coliform bacteria.

Fecal coliform counts (MPN per g) of oyster, water and sediment samples are presented in Figure 8. Fecal coliform counts in oyster samples ranged from 0–430 (mean 28.2) per g. 40 (74.1%) of the 54 samples were positive for fecal coliforms. High fecal coliform counts generally were associated

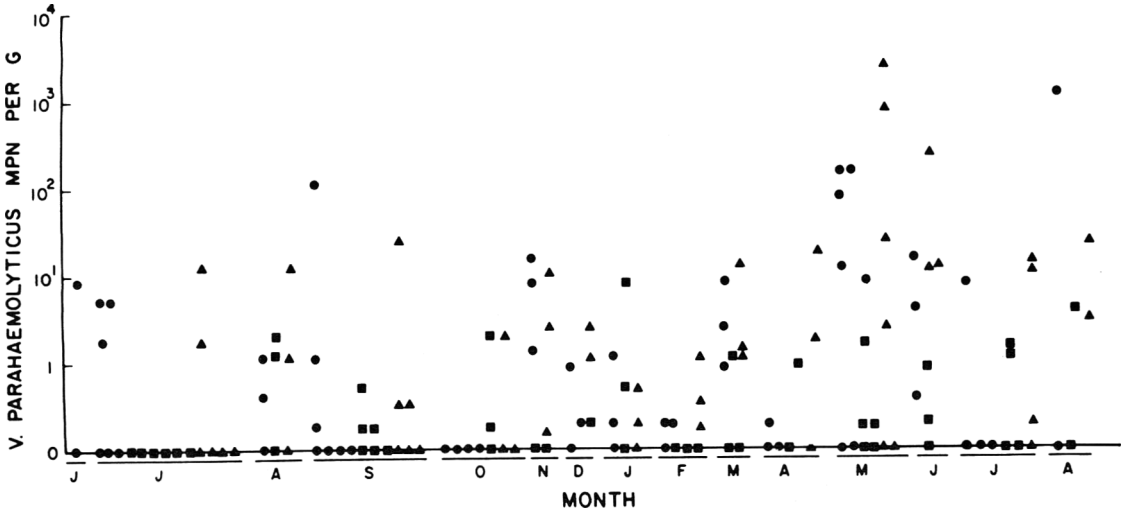

Fig. 2—Vibrio parahaemolyticus in oysters (●), water (■) and sediment (▲).

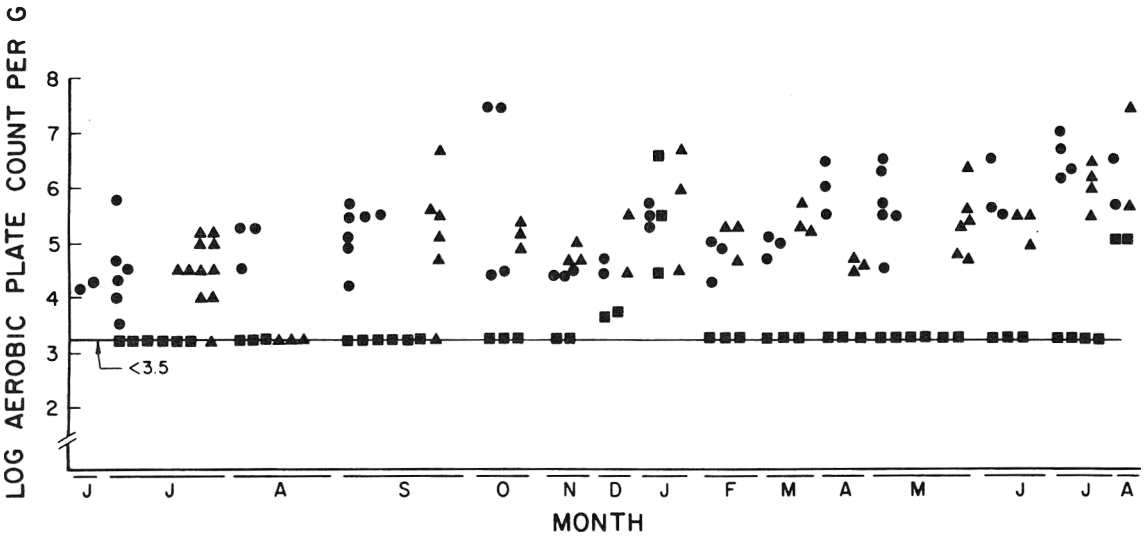

Fig. 4—Aerobic plate count (25° C) of oysters (●), water (■) and sediment (▲).

with high coliform counts, as well as with high Standard Plate Counts. High coliform counts were often indicative of high fecal coliform counts, whereas high Standard Plate Counts were not. The fecal coliform counts in water samples ranged from 0–2.3 (mean 0.17) per g. Only 8 (16.3%) of the 49 samples were positive for fecal coliforms. There was little relation between coliform and/or Standard Plate Counts and fecal coliform counts. The fecal coliform counts in sediment samples ranged from 0–2400 (mean 57.8) per g. 19 (38%) of the 50 samples were positive for fecal coliforms. No relationship could be detected between fecal coliform count and Standard Plate Count.

E. coli counts (MPN per g) of oyster, water and sediment samples are presented in Figure 9. *E. coli* counts of oyster samples ranged from 0–230 (mean 8.2) per g. 30 (55.6%) of the 54 samples were positive for *E. coli*. Samples with high *E. coli* counts invariably had high coliform and fecal coliform counts. Little relationship was observed between *E. coli* count and Standard Plate Count. *E. coli* counts of water samples ranged from 0–2.1 (mean 0.13) per g. Seven (14.3%) of the 49 samples were positive for *E. coli*. Standard Plate Counts could not be related to *E. coli* counts. The *E. coli* count in sediment ranged from 0-2400 (mean 57.2) per g. 17 (34%) of the 50 samples were positive for *E. coli*. Standard Plate Counts could not be related to *E. coli* levels. No seasonal distribution of coliform, fecal coliform or *E. coli* counts was apparent.

Information in the literature is scarce on the relationship in seafoods of coliform, fecal coliform and *E. coli* counts and

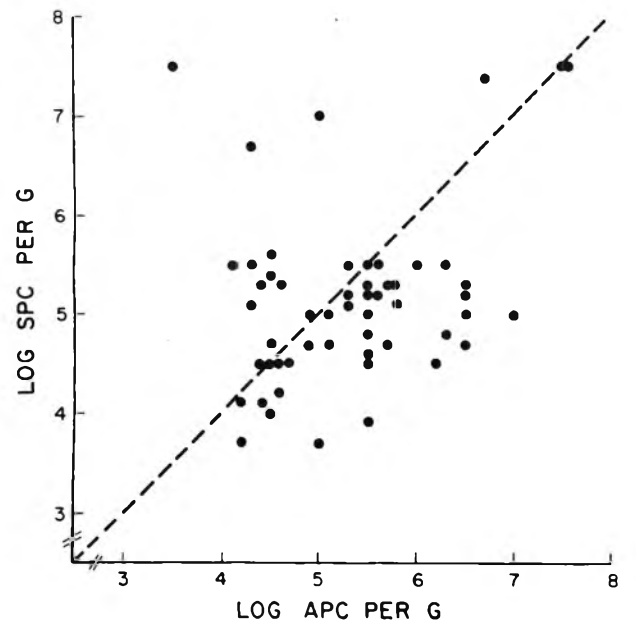

Fig. 5—Comparison of Standard plate count (35° C) and aerobic plate count (25° C) of oysters.

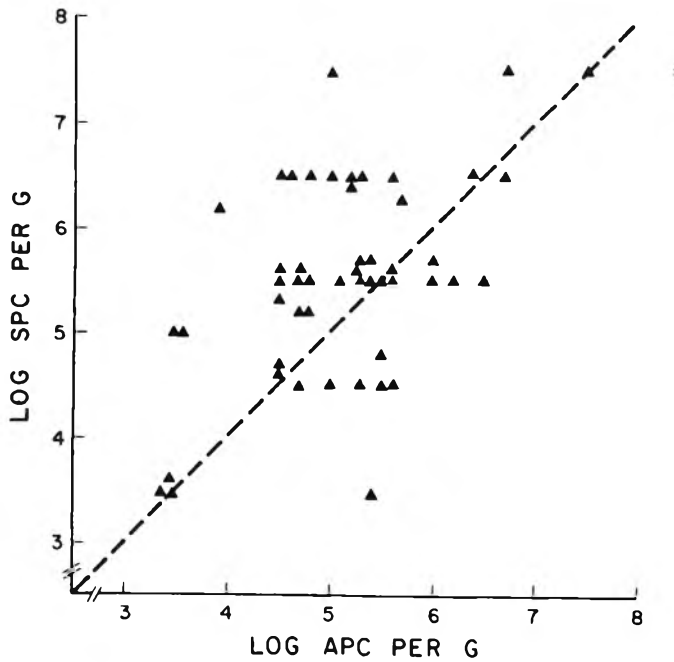

Fig. 6—Comparison of Standard plate count (35° C) and aerobic plate count (25° C) of sediment.

counts of *V. parahaemolyticus*. It has been suggested (Baross and Liston, 1970) that some correlation may exist between pollution indices and *V. parahaemolyticus* counts. Kaneko and Colwell (1973), however, reported that no relationship existed between *V. parahaemolyticus* counts and *E. coli* counts of water from Chesapeake Bay. The results of the present study are in agreement with this report. No statistically significant relationship could be established between coliform, fecal coliform, and *E. coli* counts and *V. parahaemolyticus* counts of oysters, water or sediment samples.

Salinities of waters from which samples were taken ranged from 1.0 ppt in Feb. to 16.9 ppt in Aug. (Fig. 10). A comparison of *V. parahaemolyticus* count of oysters and water salinity showed that samples with *V. parahaemolyticus* counts of 1 per g or greater were from waters with salinities ranging from 6–16 ppt. When counts were equal to or greater than 10 per g, salinities ranged from 10–13 ppt.

Water temperatures for the 15 month sampling period (Fig. 11) ranged from a low of 11.6 in Feb. to a high of 31.1 in Aug. Reports by Baross and Liston (1968, 1970), Bartley and

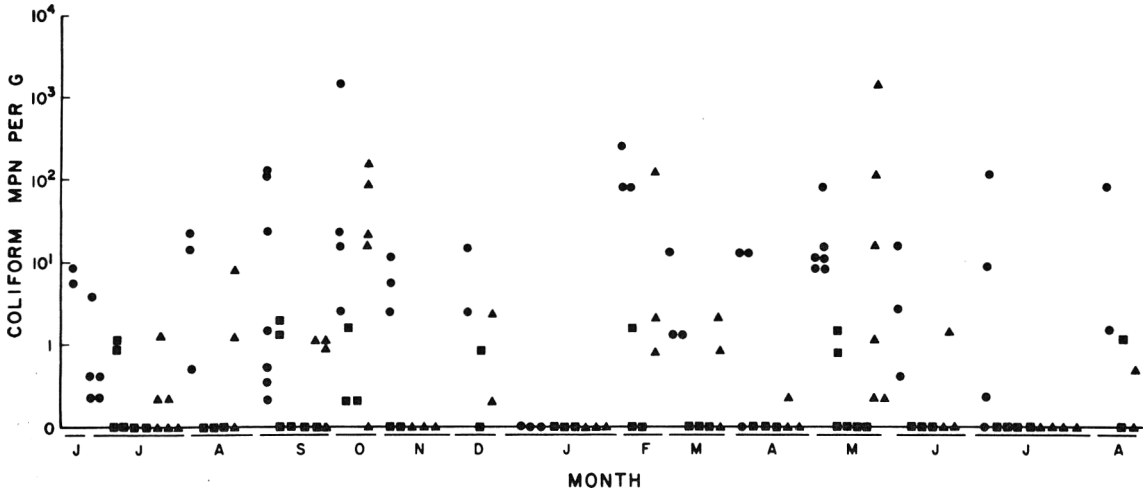

Fig. 7—Coliform count of oysters (●), water (■) and sediment (▲).

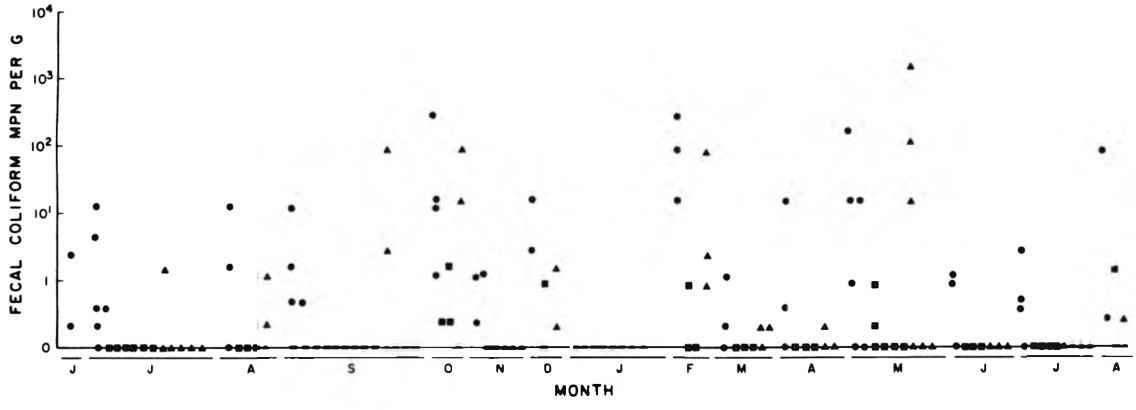

Fig. 8—Fecal coliform count of oysters (●), water (■) and sediment (▲).

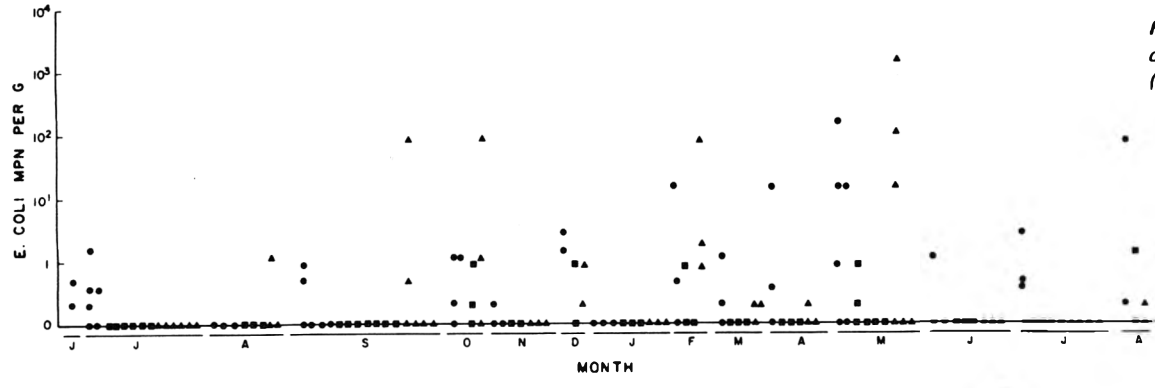

Fig. 9—Escherichia coli count of oysters (●), water (■), and sediment (▲).

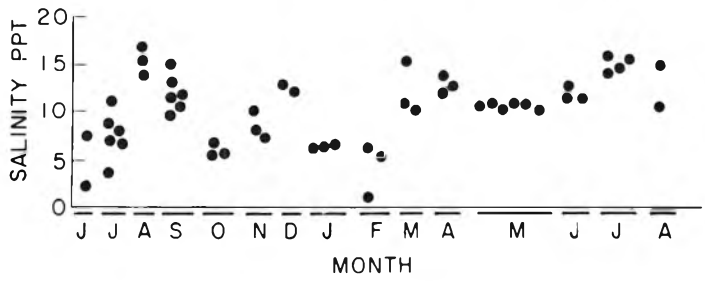

Fig. 10–Salinity of waters from which samples were taken.

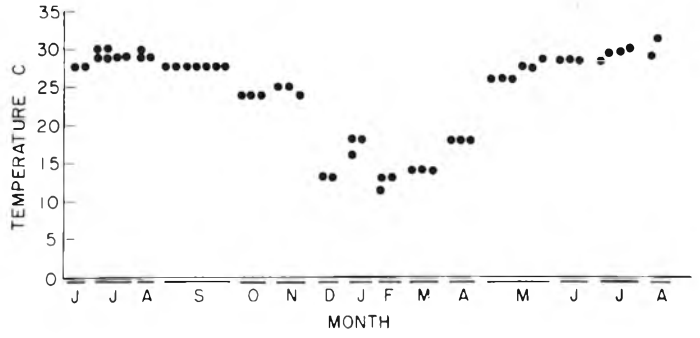

Fig. 11–Temperature of waters from which samples were taken.

Slanetz (1971), Kaneko and Colwell (1973), and Miyamoto et al. (1962) indicated a seasonal distribution of *V. parahaemolyticus*. Numbers were greater in the warm summer months and lower in early spring and fall. The results of this study do not show a definite seasonal effect on the concentration of *V. parahaemolyticus* in oysters. It is likely that the relatively high water temperature in the Northern Gulf of Mexico during the period Nov. to May fails to cause drastic reduction in *V. parahaemolyticus*. For example, Kaneko and Colwell (1973) and Liston and Baross (1973), reported lower counts of *V. parahaemolyticus* in marine environments when the water temperature dropped below 15°C. In the present study, water temperatures of the sampling areas dropped below 15°C (but never below 10°C) in Dec., Feb. and March.

A comparison of *V. parahaemolyticus* count of oysters and water temperature showed that samples with *V. parahaemolyticus* counts of 1 per g or greater were from waters with temperatures ranging from 14–30°C. Only when water temperatures were equal to or above 24°C, did *V. parahaemolyticus* counts exceed 10 per g. However, even at temperatures above 24°C the majority of the samples contained less than 10 organisms per g. No seasonal distribution of *V. parahaemolyticus* was apparent with respect to temperature or salinity. Although the occurrence of plankton blooms in various harvesting areas over the entire experimental period was not recorded, 36 zooplankton and 4 phytoplankton samples were collected in Galveston Bay during Aug. and Sept., 1974. No relationship existed between the proportion of types of zooplankton or diatoms and *V. parahaemolyticus*. The details of this study will be presented in a separate paper.

Data on other environmental parameters were as follows: the pH levels of waters from which samples were taken ranged 8.0–8.8. Lowest pH values were recorded in Oct., Jan. and Feb.; highest pH values were observed in Sept. Dissolved oxygen levels (ppm) ranged from 7.0 in Oct. to 11.5 in July. Sample depth varied from 3–10 ft, with most samples being harvested at the 3-ft level. Changes in tide were recorded as each sample was taken. The number of samples collected during incoming and outgoing tide was approximately equal. Wind velocity ranged from 3 mph in Sept. to 25 mph in Nov. Wind direction varied greatly around most possible compass points; however, no North winds were experienced during sample collections. Rainfall for 24 hr periods ranged from 0–3 in; during 7-day periods from 0–4 in. Heaviest rainfall occurred in Sept.

Statistical analyses (correlation analyses) were made comparing *V. parahaemolyticus* counts (MPN per g) to the following parameters: Standard Plate Count, Aerobic Plate Count, Coliform, Fecal Coliform, *E. coli* counts, water temperature, salinity, pH, dissolved O_2, sample depth, rainfall, tide and wind velocity. No significant correlation existed between *V. parahaemolyticus* count and the various bacteriological and environmental parameters.

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EFFECT OF PROCESSING, DISTRIBUTION AND STORAGE ON Vibro Parahaemolyticus AND BACTERIAL COUNTS OF OYSTERS (Crassostrea virginica)

ABSTRACT

Levels of *V. parahaemolyticus* did not increase greatly in most samples of shellstock held for 3 days at 25°C while total bacterial counts frequently increased 1–2 logs. The data from nine market surveys indicated that *V. parahaemolyticus* counts of oysters usually decreased during processing and subsequent storage. On the other hand, Standard Plate Counts increased in the majority of samples after storage at refrigeration temperatures. *V. parahaemolyticus* counts of composite samples (12 oysters) usually were lower than the mean count of the individual oysters.

INTRODUCTION

TO EVALUATE the potential of *V. parahaemolyticus* to cause foodborne illness through the consumption of oysters, information is needed about the presence of this organism in freshly harvested oysters and the effects of handling, storing and processing on the concentration of *V. parahaemolyticus*. In a previous paper (Thompson et al., 1976) we reported that *V. parahaemolyticus* is present in 59.3% of freshly harvested oysters from the Northern Gulf of Mexico usually at levels ranging from 1–100 per gram. No statistically significant relationship existed between *V. parahaemolyticus* counts of oysters and other bacteriological indices such as total bacterial count, coliform, fecal coliform and *E. coli* counts. In addition, *V. parahaemolyticus* counts were not related to environmental parameters such as water temperature, salinity, dissolved O_2, depth of harvesting, rainfall, wind direction and velocity, and tide. Although many reports indicate that *V. parahaemolyticus* is often present in oysters, little is known about the effect on the viable population when shellstock (oysters in the shell) or shucked oysters are held either inadequately refrigerated or not refrigerated at all. In addition, little is known about the effect of commercial processing and marketing procedures on the concentration of *V. parahaemolyticus*. Johnson et al. (1973) reported that growth of *V. parahaemolyticus* occurred in oyster shellstock held for 2–3 days at 35°C. In a preliminary report, Vanderzant et al. (1973) indicated that *V. parahaemolyticus* usually decreased when oysters were processed properly and were adequately refrigerated.

This report provides information on *V. parahaemolyticus* levels and total bacterial counts when oysters are stored at ambient temperatures (25°C). The effect of processing and marketing on these bacteriological indices is also presented. In addition, data are presented on the level of *V. parahaemolyticus* in individual oysters as compared to a composite sample.

MATERIALS & METHODS

METHODS OF COLLECTION, transportation and bacteriological analysis of the oyster samples are presented in a previous paper (Thompson et al., 1976). Standard Plate Counts (SPC) were made on Plate Count Agar (PCA, BBL) with plate incubation at 35°C for 2 days. For Aerobic Plate Counts (APC), Trypticase Soy Agar (BBL, 3% NaCl) was used and the plates were incubated for 2 days at 25°C.

Each of the 21 samples of shellstock for the storage trials consisted of 48 oysters collected at a confined sampling site. Samples were taken from different sites in Galveston Bay from March to August, 1974. The oyster shellstock was held in clean plastic containers at 25°C. At each sampling interval (0, 1, 2, 3 days), 12 oysters were taken at random for bacteriological analyses. Oysters for the storage experiments with shucked oysters were collected in the same manner. They were shucked in the laboratory under aseptic conditions and stored at 25°C in closed sterile glass jars. Commercial oyster samples for the market surveys were obtained from two processing plants in the Texas Gulf Coast area. In each of the plant-processing trials, a lot of oyster shellstock was tagged and sampled (12 oysters). These oysters were subsequently shucked in the laboratory. Plant samples (1 pt) of the tagged lot then were collected after shucking, washing, and from 1-gal tagged containers immediately after icing and after 7 days at 3°C. Environmental parameters were determined as described previously (Thompson et al., 1976).

RESULTS

HARVESTING DATA and changes in *V. parahaemolyticus* counts of shellstock stored for 3 days at 25°C are presented in Table 1. Of the fresh shellstock 20 samples (95%) contained < 1000 *V. parahaemolyticus* per g; 18 (86%) < 100 organisms per g; and 15 (71%) < 10 organisms per g. Seven of the eight samples of shellstock initially negative for *V. parahaemolyticus* became positive (range 1.1 – > 11,000 per g) after storage at 25°C for 3 days. After 1 day at 25°C, *V. parahaemolyticus* increased in 16 (76%) samples. Over a 3-day period, *V. parahaemolyticus* increased in 13 samples (62%), decreased in seven samples and remained the same in one sample. Except for three samples in which the count had increased to > 11,000 per g, increases in counts after 3 days at 25°C were relatively small. A certain pattern of changes in *V. parahaemolyticus* was detected in about 50% of the samples. In seven samples some increases in count occurred after 1 day at 25°C followed by large increases after 2 days and significant decreases after 3 days. In five samples, large increases in *V. parahaemolyticus* count occurred after 1 day at 25°C with decreases on the second and/or third day of storage. Storage of shucked oysters (Table 2) for 36 hr at 25°C increased *V. parahaemolyticus* counts of three out of five samples. None of the counts after 36 hr exceeded 11,000 per g.

Standard Plate Counts (PCA, plates incubated for 2 days at 35°C) and Aerobic Plate Counts (TSA-3% NaCl, plates incubated for 2 days at 25°C) of shellstock and shucked oysters initially and after storage at 25°C are presented in Figures 1 and 2. Initial Standard Plate Counts of shellstock ranged from 5.5×10^3 to 2.5×10^7 per g, with only one sample exceeding 5.0×10^5 per g, the legal limit set for oysters which are to be

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Table 1—Harvesting data and changes in V. parahaemolyticus counts of 21 samples of shellstock before and after holding for up to 3 days at 25°C

V. parahaemolyticus (MPN/g) after holding at 25°C for				Environmental characteristics		
0 day	1 day	2 days	3 days	Water temp (°C)	Salinity (ppt)	Harvest date (month)
0	2.3	19	0	31.1	15	Aug
0	9.3	110	1.1	28.3	12.5	June
0	21	11,000	2.7	17.7	12.2	April
0	39	24,000	93	27.8	10.8	May
0	30	150	440	30	15.2	July
0	0	530	530	28.3	14	July
0	0	11,000	530	29.4	14.5	July
0	93	160	>11,000	28.9	10.1	May
0.3	9	5,300	24	17.7	12.9	April
0.6	290	0	2.3	17.7	12.2	April
0.9	0	0	0	14	11	March
4.3	0	0	0	14	15.1	March
6	1,600	93	>11,000	28.3	11.5	June
9.1	30	11,000	420	29.4	16	July
9.3	0	0	0	14	10.9	March
11	160	>11,000	0	27.8	10.9	May
15	530	23	>11,000	28.3	11.5	June
93	11,000	4,600	2,400	26.1	10.2	May
240	11,000	73	36	26.1	10.1	May
240	4,600	4,600	210	26.1	10.8	May
1,100	11,000	11,000	290	28.9	10.5	Aug

Table 2—Changes in V. parahaemolyticus counts of five samples of shucked oysters before and after holding for up to 36 hr at 25°C

V. parahaemolyticus (MPN/g) after holding at 25°C for				Environmental characteristics		
0 hr	12 hr	24 hr	36 hr	Water temp (°C)	Salinity (ppt)	Harvest date (month)
0	150	93	0	28.9	10.1	May
0	420	0	0	31.1	15	Aug
0	0	200	0.36	30	15.2	July
6	230	2,400	11,000	28.3	11.5	June
9.1	0	94	1,600	29.4	16	July

sold commercially. After storage of shellstock at 25°C for 3 days, the Standard Plate Count of four of 21 samples increased by at least 2 logs, in 10 samples by 1–2 logs and in four samples < 1 log. In two samples little or no increase in count was detected. After storage for 36 hr at 25°C the Standard Plate Count of four out of five samples of shucked oysters increased 2–3 logs. Storage of shellstock for 3 days at 25°C (Fig. 2) increased Aerobic Plate Counts of 16 of 21 samples. In 11 samples the increase in count was at least 1 log. A similar increase in Aerobic Plate Count was observed for three of the shucked oyster samples. The Aerobic Plate Count of fresh shellstock was usually higher than the Standard Plate Count. In 12 samples the difference in count was at least 1 log. After storage for 3 days at 25°C, the Aerobic Plate Count of shellstock usually exceeded the Standard Plate Count. In six samples, the difference in count exceeded 1 log. After 36 hr at 25°C, the Aerobic Plate Counts of shucked oysters were equal to the Standard Plate Counts.

V. parahaemolyticus count, Standard Plate Count, and Aerobic Plate Count were determined on nine samples of oysters during various stages of processing and marketing. Changes in *V. parahaemolyticus* are presented in Table 3. Four of the fresh shellstock samples contained *V. parahaemolyticus* at low levels (MPN 0.6–16 per g). Processing (shucking, washing and packaging) of these samples caused the following changes: in two samples the count decreased to 0 per g, in one it remained approximately the same, and in one it increased to 290 per g. When shucked oysters were held for 7 days at 3°C, six of the nine samples did not contain *V. parahaemolyticus*. In the other three samples, *V. parahaemolyticus* was present in low numbers (MPN 0.36–0.62 per g). The effect of processing on the total bacterial counts (SPC, APC) was not consistent. Both minor increases or decreases in count occurred. Of the nine retail samples, five had a Standard Plate Count $> 5.0 \times 10^5$ per g, which is the legal standard for oysters to be sold commercially. After storage of shucked oysters for 7 days at 3°C, eight samples showed an increase in Standard Plate Count. In four samples the count increased < 1 log, in four 1–2 logs, and in one sample the count decreased. In six of the nine refrigerated oyster samples, the Aerobic Plate Count also increased. A comparison of the Standard Plate Count and Aerobic Plate Count of 45 sub-samples of oysters (nine samples, sampled 5x, Table 4) during processing and refrigerated storage showed that the Aerobic Plate Count exceeded the Standard Plate Count in 78% of the samples. In most cases, the difference in count was < 2 logs.

V. parahaemolyticus count, Standard Plate Count and Aerobic Plate Count were determined on six individual oysters and on a composite of 12 oysters from the same sample collected at a confined sampling site in Galveston Bay. Twenty-

Table 3—V. parahaemolyticus count of nine samples of oysters in various stages of processing and marketing

Sample	Changes in V. parahaemolyticus (MPN/g) during processing of sample								
	1	2	3	4	5	6	7	8	9
In shell before shucking[a]	0	0.62	0	0	0	0	9	15	16
After shucking	0	200	0.36	30	19	190	0	44	44
After shucking and washing	0	190	3.6	91	0	0	0	0	15
At retail level									
0 days at 3°C	0	290	0	0	3	>11,000	0	0	15
7 days at 3°C	0	0.6	0	0	0	0.36	0	0	0.62

[a] Subsequently shucked in laboratory under aseptic conditions

five trials were made over a 1-yr period involving 150 individual oysters and 25 composite samples. The data for one sample are given in Table 4 and the *V. parahaemolyticus* counts for all samples in Table 5. In 21 of 25 trials, the *V. parahaemolyticus* count of the composite sample was lower than the mean count of the individual oysters. In nine of the 25 composite samples, *V. parahaemolyticus* could not be detected. However, in five of these samples, four or more of the six individual oysters contained *V. parahaemolyticus*. When the counts of individual oysters are low, the discrepancy in presence of *V. parahaemolyticus* in individual oysters and absence in the composite sample can be explained. For example, in four samples when *V. parahaemolyticus* was not detected in the composite sample, the ranges of *V. parahaemolyticus* count for the individuals were low: 0–0.36 (mean 0.06), 0–0.72 (mean 0.18), 0–2.3 (mean 0.6) and 0–3 (mean 0.7) per g. In five other samples, however, *V. parahaemolyticus* could not be detected in the composite samples when the counts of the individual oysters ranged from 0–29, 0.94–23, 0–350, 0–360 and 0–430 per g. The means (MPN per g) for these samples were respectively: 13.5, 15.2, 92.2, 60.2 and 115.1. An examination of each of the six oysters in a sample revealed that in 17 samples (68%), five or six of the individuals were positive for *V. parahaemolyticus*. In the other samples, one to four of the individuals contained *V. parahaemolyticus*.

A comparison of the mean Standard Plate Count of six individual oysters and the Standard Plate Count of the composite sample (Fig. 3) showed a difference in count exceeding 1 log in only four of the 25 samples. When a similar comparison was made with the Aerobic Plate Count (Fig. 4), the count of the composite sample and the mean count of the individual oysters were within 1 log in 23 of the 25 samples. An examination of the range of the Standard Plate Count within each of 21 samples of six individual oysters showed that the difference in count was < 1 log in 15 (60%) of the samples. Difference in count range of 1–2 logs, and > 2 logs were found in five samples each, representing 40% of the samples.

A comparison of the Standard Plate Count and Aerobic Plate Count of each of 150 individual oysters and 25 composite samples showed that in most cases (87%) the Aerobic Plate Count exceeded the Standard Plate Count. The difference in count usually was < 2 logs.

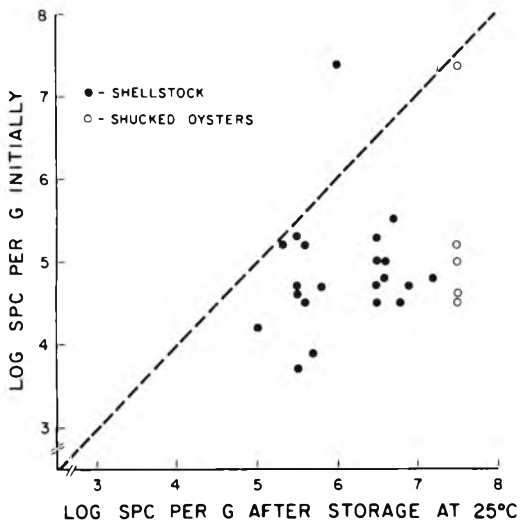

Fig. 1—Standard Plate Counts (35°C) of shellstock and shucked oysters before and after storage at 25°C (Shellstock 3 days, shucked oysters 36 hr)

DISCUSSION

INFORMATION on the effect of ambient temperatures on the level of *V. parahaemolyticus* in oysters is needed to determine the impact of storage conditions as a possible public health hazard. A recent study (Presnell, 1970) showed that oysters harvested in the Gulf Coast area may be subjected to temperatures of about 27°C on board and during storage prior to shucking. For this reason, shellstock and shucked oysters were stored at 25°C. In most cases, storage of shellstock or shucked

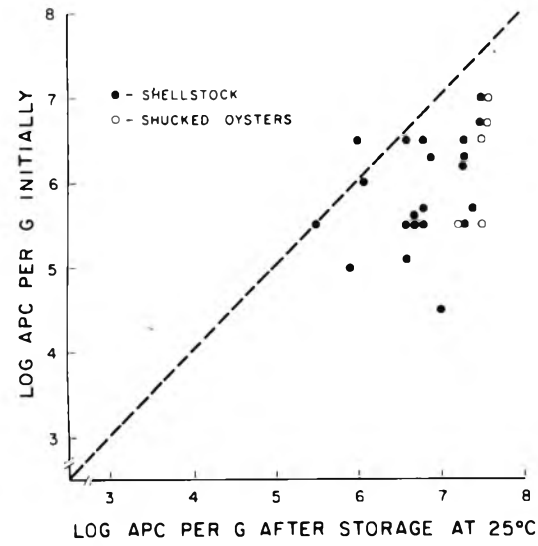

Fig. 2—Aerobic Plate Counts (25°C) of shellstock and shucked oysters before and after storage at 25°C (shellstock 3 days, shucked oysters 36 hr)

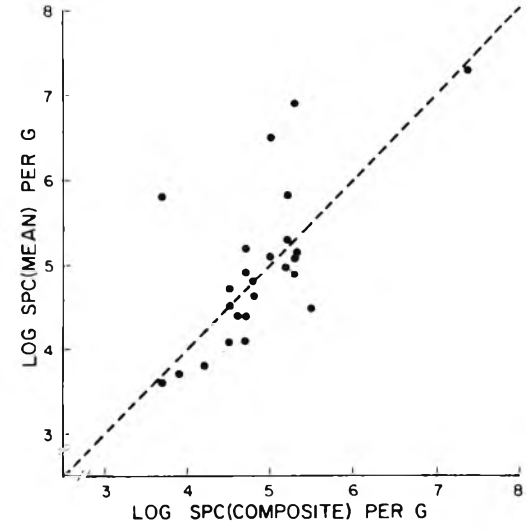

Fig. 3—Comparison of mean Standard Plate Count (35°C) of six individual oysters and Standard Plate Count (35°C) of a composite of 12 oysters from the same sample (25 samples).

oysters at 25°C did not cause large increases in *V. parahaemolyticus* counts. It is possible that other organisms inhibited the development of *V. parahaemolyticus*. Goatcher and Westhoff (1975) reported that *V. parahaemolyticus* levels decreased significantly at 25°C in the presence of *Pseudomonas* species. Although these tests were carried out in artifical media they may explain the changes in *V. parahaemolyticus* count in oysters stored at 25°C. On the other hand, Johnson et al. (1973) reported growth of *V. parahaemolyticus* in shellstock held at 35°C for 2–3 days. Interactive phenomena at this temperature may differ from those occurring at 25°C. Only one sample had a Standard Plate Count of $> 5.0 \times 10^5$ per g at time of harvesting, the legal limit set for oysters to be sold commercially. These data agree with those of a previous study (Presnell, 1970) on bacteriological quality of oysters at time of harvesting. However, after storage at 25°C for 3 days, the Standard Plate Counts of 14 samples increased 1–2 logs. In fresh and stored shellstock the Aerobic Plate Count usually exceeded the Standard Plate Count. The lower plate incubation temperature likely was more favorable for the psychrotrophic bacteria common among the marine bacteria from these samples. In addition, added NaCl may have satisfied the need of the slightly halophilic bacteria among the marine species. High bacterial counts of shucked oysters after storage were most likely due to the absence of the protective shell. Statistical analysis showed that no significant relationship existed between *V. parahaemolyticus* count, Standard Plate Count, or Aerobic Plate Count and temperature or salinity of the waters from which the samples were collected (Thompson et al., 1976). This was probably due to the less drastic changes in these parameters in the Gulf of Mexico, as compared with more northern waters such as Chesapeake Bay or Puget Sound.

Data on the levels of *V. parahaemolyticus* in oysters usually are based on samples of 10–12 oysters each. The *V. parahaemolyticus* count of a composite sample usually was lower than the mean count of the individual oysters. In nine composite samples, *V. parahaemolyticus* could not be detected, yet one to six of the individual oysters were positive for *V. parahaemolyticus*. This is not suprising when counts of the individual oysters were low. However, in more than half of the samples where this occurred, maximum *V. parahaemolyticus* counts of the individual oysters in the samples ranged from 23–430 per g. This variation in *V. parahaemolyticus* count of individual oysters in a sample may have been caused by differences in their feeding pattern and status of their health. For

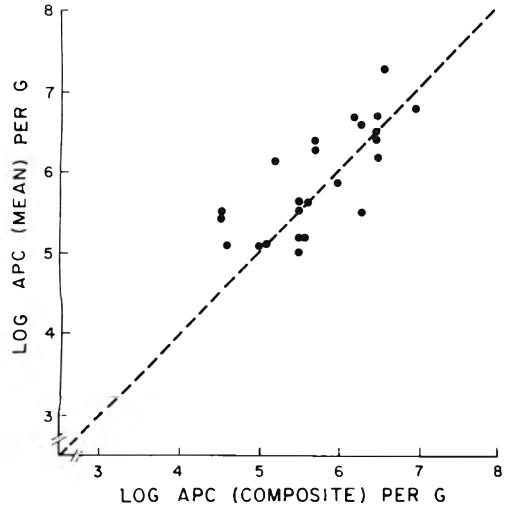

Fig. 4—Comparison of mean Aerobic Plate Count (25°C) of six individual oysters and Aerobic Plate Count (25°C) of a composite of 12 oysters from the same sample (25 samples).

Table 4—V. parahaemolyticus count, Standard Plate Count, and Aerobic Plate Count of 6 individual oysters and of a composite of 12 oysters from the same sample, collected in September 1973 at a water temperature of 27.7°C and a salinity of 15 ppt.

Sample[a]		V. parahaemolyticus MPN/g—GSTB/TCBS[b]	SPC[c]/g	APC[d]/g
Oyster	1	29	1.8×10^5	2.0×10^5
	2	0	3.5×10^4	1.1×10^5
	3	15	3.4×10^4	4.3×10^4
	4	7.3	3.0×10^5	8.0×10^4
	5	9.4	3.1×10^4	1.0×10^5
	6	20	5.0×10^4	2.9×10^5
Mean		13.5	1.1×10^5	1.4×10^5
Composite		0	2.0×10^5	2.9×10^5

[a] 18 oysters were obtained from one confined sampling site; 12 were used for the composite sample and six for individual samples.
[b] 3-tube MPN enrichment in GSTB. Tubes showing growth were streaked on TCBS agar. Suspect colonies from TCBS were confirmed by biochemical tests.
[c] Standard Plate Count—Count on Plate Count Agar with plate incubation at 35°C for 2 days
[d] Aerobic Plate Count—Count on Trypticase Soy Agar (3% NaCl) with plate incubation at 25°C for 2 days

Table 5—V. parahaemolyticus count of six individual oysters (mean and range) and of a composite of 12 oysters from the same sample

Sample	V. parahaemolyticus (MPN/g) Composite	Mean	Range	Positive/ Total
1	0	0.06	0 – 0.36	1/6
2	0	0.18	0 – 0.72	2/6
3	0	0.6	0 – 2.3	3/6
4	0	0.7	0 – 3	4/6
5	0	13.5	0 – 29	5/6
6	0	15.2	0.94 – 23	6/6
7	0	60.2	0 – 360	2/6
8	0	92.2	0 – 350	5/6
9	0	115.1	0 – 430	5/6
10	0.3	15.6	0.3 – 39	6/6
11	0.36	10	0 – 30	2/6
12	0.6	89.7	0.3 – 230	6/6
13	0.94	6	0 – 24	5/6
14	4.3	39.8	2 – 110	6/6
15	6	49.1	2.8 – 93	6/6
16	9.1	629.5	0 –2400	5/6
17	9.3	0.22	0 – 0.62	3/6
18	11	153	0 – 910	3/6
19	15	81.6	0.3 – 430	6/6
20	30	42.7	0 – 93	5/6
21	93	1014	21 –4600	6/6
22	130	42.9	0 – 92	5/6
23	240	118.7	23 – 240	6/6
24	240	368	150 –1100	6/6
25	1100	306.3	11 –1100	6/6

example, oysters do not have a consistent feeding schedule. While some oysters are filtering phytoplankton and organic detritus, others are not in the feeding process. Plankton is an excellent source of *V. parahaemolyticus* (Kaneko and Colwell, 1973). Presumably, because of the high rate of depuration of an oyster, it would be possible to select a sample of oysters, none of which would be positive for *V. parahaemolyticus*. At the same time, some oysters may be sick or moribund, thereby lowering their natural defenses to bacterial invasion. This type of oyster would have a less effective immune system, and be more susceptible to an invasion by *V. parahaemolyticus*. After storage of shucked oysters for 7 days at 3°C, *V. parahaemolyticus* was found in three of nine samples at a level ranging from 0.36 to 0.62 per g. These data agree with those of others (Covert and Woodburn, 1972; Johnson and Liston, 1973; Matches et al., 1971), who reported decreases in numbers of *V. parahaemolyticus* in seafoods stored at refrigeration temperatures. Survival of the organism in oyster shellstock for 3 wk at 4°C is reported by Johnson et al. (1973).

Food poisoning resulting from consumption of the wholesale and retail oysters of the present market surveys would appear highly unlikely because of the relatively low numbers of *V. parahaemolyticus*. However, the possibility of this occurring cannot be entirely dismissed since infectious concentrations of *V. parahaemolyticus* may in some cases (Table 3, > 1,000 per g) develop if these shellfish are mishandled and held at elevated temperatures for prolonged periods of time. Moreover, raw oysters containing low numbers of *V. parahaemolyticus* could contaminate other foods which may not receive further thermal processing.

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EFFECT OF SODIUM ACETATE ON GROWTH AND AFLATOXIN PRODUCTION BY Aspergillus parasiticus NRRL 2999

ABSTRACT

The effect of sodium acetate and propionate on growth and aflatoxin production by *A. parasiticus* NRRL 2999 was studied in AMY medium (modified AM medium + 2% yeast extract) to determine the possible use of this compound as a means of controlling aflatoxin production. At pH 4.5, concentrations of sodium acetate ≥ 1.0g/100 ml completely inhibited growth and prevented aflatoxin production, while levels of 0.6 and 0.8g/100 ml partially inhibited growth and decreased aflatoxin production by 70% and 99%, respectively. Examination of the effect of initial pH on the inhibitory action of sodium acetate indicated that the extent of inhibition was a function of the initial pH. The effect of propionic acid on growth and aflatoxin production was also examined, and this compound was found to be more inhibitory than acetate. The role of sodium acetate and propionic acid as a means of controlling aflatoxin production appears to be promising.

INTRODUCTION

IN THE PAST 15 yr aflatoxins have been extensively studied, particularly in regard to their production, detoxification and incidence in foods and feeds. However, little research has been directed towards characterizing agents that could be used to control the growth and aflatoxin production by *A. flavus* and *A. parasiticus*. It has long been known that various fatty acids have significant antimicrobial properties, and acetic acid, propionic acid, and several of their salts and derivatives have been successfully employed for their antimicrobial activity (Chichester and Tanner, 1968). Kirby et al. (1937) demonstrated that at pH 3.5, 0.2% acetic acid was an effective inhibitor of a wide range of molds involved with the spoilage of bread. Hoffman et al. (1939) studied the inhibitory properties of fatty acids against molds, and reported that at a given pH, the antimicrobial activity of fatty acids increased with increasing chain length. They demonstrated that propionic acid was significantly more inhibitory to mold growth than acetic acid.

These earlier studies indicated that these acids might offer a means of controlling aflatoxin production. The objective of the present study was to determine and characterize the effect of sodium acetate on an aflatoxin-producing strain of *A. parasiticus* and determine if acetic acid and propionic acid have potential as a means of controlling aflatoxin.

EXPERIMENTAL

Microorganisms

A. parasiticus NRRL 2999 was used throughout the study. Other strains employed during the course of the investigation were *A. parasiticus* ATCC 15517 and *A. flavus* PC42, PC65, PC101, PC120, PC125 and M-pl(99). The *A. flavus* strains designated PC were originally isolated from pecans and strain M-pl(99) was isolated from a country-cured ham. All cultures were maintained on YES agar slant (Davis et al., 1966).

Medium

The basal medium of Adye and Mateles (1964) with the modifications of Hsieh and Mateles (1971) was further modified by increasing the glucose concentration from 50g/liter to 60g/liter and supplementing with yeast extract at a level of 20g/liter. This semisynthetic medium (designated AMY medium) was used throughout the study. Adjustments of pH and supplementation with sodium acetate, propionic acid, sodium chloride, or monobasic potassium phosphate were performed prior to sterilization. Acetic acid supplements were added to the medium after sterilization. The pH of the medium was adjusted using 12N HCl and 1N NaOH. Unless otherwise specified, the initial pH of the medium was 4.5.

Culture techniques

The microorganism was cultured using either flask or tube cultures. Flask cultures were prepared by transferring 25-ml aliquots of medium containing the appropriate levels of the desired supplement to 125 ml Erlenmeyer flasks. The flasks were then sealed with foam plugs and sterilized by autoclaving for 15 min at 15 psi. In the preliminary examination of sodium acetate, the medium was also sterilized by micropore filtration (Gelman filter GN-6, 0.45 μm). The pH of the medium was checked before and after sterilization to insure that there was no change. Flask cultures were inoculated using 1.0 ml of a suspension of 10^6 spores/ml, and were incubated without agitation at 28°C for 7 days. In the determination of the effect of acetate on growing cultures of *A. parasiticus*, acetic acid was used instead of sodium acetate to avoid contamination and dilution effects. At 0, 3, or 5 days, 1.0 ml of an acetic acid stock solution was added to the appropriate cultures, which were then returned to the incubator to complete the 7-day incubation period. An initial pH of 4.0 was used in this study in order to produce a large acetate effect. Similarly, in the determination of the effect of phosphate on aflatoxin production, an initial pH of 5.5 was used in order to limit osmotic effects. Growth in the flask cultures was measured by the mycelial dry weights.

Tube cultures were employed in a similar manner, according to the method of Halls (personal communication). Culture tubes (18 × 150 mm) received 4.0 ml of medium supplemented with the appropriate amounts of sodium acetate. The tubes were sealed with plastic caps and autoclaved for 15 min at 15 psi. The tubes were inoculated with 0.1 ml of a suspension of 10^6 spores/ml, and incubated without agitation for 15 days at 28°C. Growth was estimated by visual observation. The tube culture technique was only used for the screening of *A. parasiticus* and *A. flavus* strains for their growth response to sodium acetate.

Aflatoxin analysis

Cultures were anlayzed for aflatoxin production according to the method of Eppley (1968). Chloroform (25 ml) was added to the culture, which was then agitated on a rotary shaker (Model 6130, Eberbach Corp., Ann Arbor, Mich.) for 10 min. The liquid was then transferred to 125 ml separatory funnels and agitated vigorously. The chloroform layer was drawn off into a 500 ml round bottom flask. The extraction procedure was repeated twice with the three extracts being combined in the round bottom flask. The extracts were concentrated to a volume of 10 ml using a flash evaporator. Aflatoxin assays were by thin-layer chromatography using Absorbosil-1 plates developed with the chloroform-acetone (9:1). The aflatoxins were quantitated using a fluorodensitometer (Model 52C, Photovolt Corp., New York, N.Y.), and the sum of the B_1, B_2, G_1 and G_2 fractions was reported as the amount of aflatoxin present.

RESULTS & DISCUSSION

PRELIMINARY EXAMINATION of the effect of sodium acetate on growth and aflatoxin production in heat and filter sterilized AMY medium (pH 4.5) indicated that concentrations ≥ 1.0g/100 ml completely inhibited growth and aflatoxin production. No differences between autoclaved and filter steri-

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lized media were found, and autoclaving was thereafter employed as the method of sterilization.

Sodium acetate levels ⩽ 1.0g/100 ml were examined to determine the minimum concentration needed to inhibit *A. parasiticus* (Fig. 1). Concentrations ⩾ 0.6g/100 ml inhibited growth and aflatoxin production. These findings are in agreement with those of Hoffman et al. (1939) whose results predicted that acetic acid in media at pH 4.5 would be inhibitory to mold growth at a level equivalent to 0.5g NaAc/100 ml. The agreement between these results clearly points out the interchangeability, on a molar basis, of sodium acetate and acetic acid (Chichester and Tanner, 1968). The decrease in aflatoxin production at this pH appeared to be primarily due to the decrease in growth.

Kirby et al. (1937) reported that acetic acid was inhibitory to mold growth in an acidic environment. Hoffman et al. (1939) demonstrated that acetic acid inhibited mold growth at initial pH levels of 5.0, with the amount of acetic acid needed to produce a no-growth response being a function of the initial pH. In the present study (Fig. 2a-c), sodium acetate was strongly inhibitory at acidic pH's. Concentrations of 1.0g/100 ml and 2.0g/100 ml completely inhibited growth in all samples having initial levels of 4.5 and 5.0, respectively. At initial pH levels of 5.0 and 5.5, growth was completely inhibited in some of the samples containing 1.0g/100 ml and 2.0g/100 ml, respectively. At higher pH levels, acetate appeared to slightly inhibit growth while more definitely decreasing the amount of aflatoxin. Examination of aflatoxin production per unit growth indicated that the decrease in aflatoxin production at the higher pH levels was not primarily due to inhibition of growth; instead the acetate seems to have affected aflatoxin production more directly.

Reddy et al. (1971) examined the effect of initial pH on the production of aflatoxin. They reported that maximal production of aflatoxin in SL medium occurred at an initial pH of

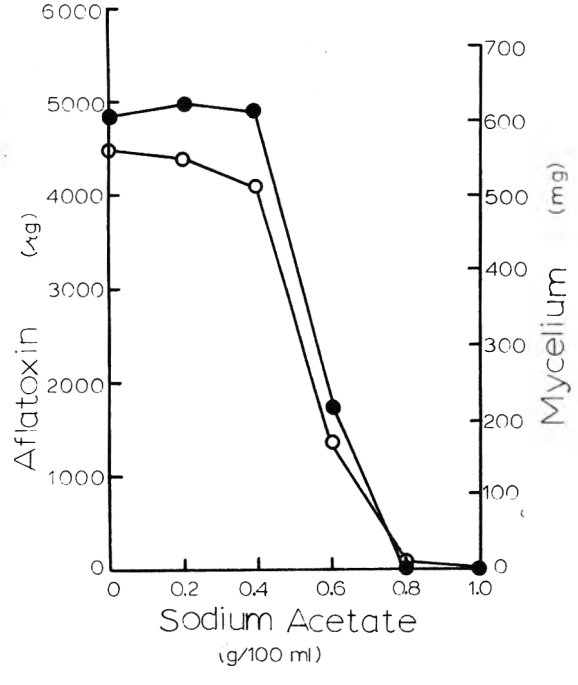

Fig. 1—Effect of sodium acetate concentration on growth (•) and aflatoxin production (○) per 25 ml medium by A. parasiticus NRRL 2999. Cultures were incubated in AMY (pH 4.5) at 28° C for 7 days, and each point represents the average of six cultures.

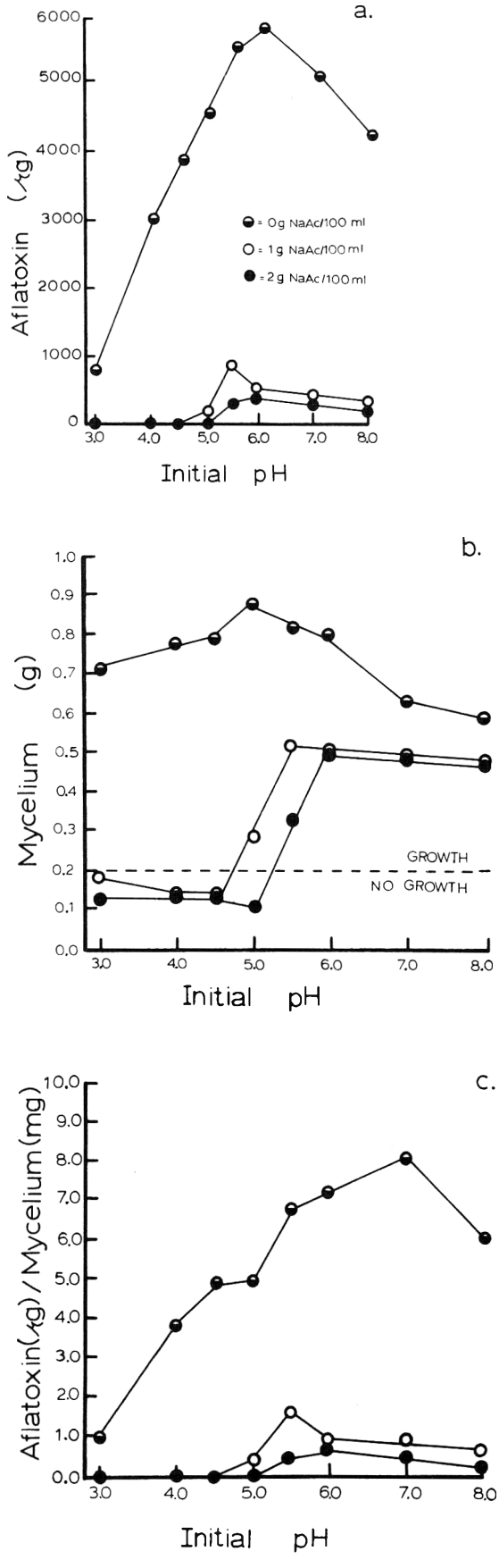

Fig. 2—Effect of sodium acetate concentration and initial pH on (a) aflatoxin production, (b) growth, and (c) aflatoxin production per unit growth per 25 ml medium by A. parasiticus NRRL 2999. Cultures were incubated at AMY at 28° C for 7 days, and each point represents the average of six cultures.

4.5, and that maximal production per unit growth occurred at an initial pH of 5.5. However, results in the present study indicate that the optimum initial pH for aflatoxin production may be dependent on the medium used for cultivation. Using AMY medium, maximal growth, aflatoxin production, and aflatoxin production per unit growth occurred at initial pH levels of pH 5.0, 6.0 and 7.0, respectively. These differences may reflect the presence and absence of yeast extract in AMY and SL media, respectively.

Addition of acetic acid to cultures of *A. parasiticus* at various times during the mold's growth cycle was examined to determine the effectiveness of acetate against growing cultures (Table 1). Acetic acid was most effective when added prior to inoculation, but did demonstrate an inhibitory action against cultures that were partially grown (3-day samples). The addition of acetic acid to cultures that had attained abundant growth (dense mycelium covering entire surface of medium) did not inhibit aflatoxin production, and may have stimulated it as indicated by the cultures receiving 1.0g/100 ml after 5 days. These results indicate that acetic acid has a potential for preventing aflatoxin production, but would have little effect in material already heavily spoiled by *A. parasiticus*.

The effect of sodium chloride supplementation (2.0g/100 ml) of AMY medium was examined to determine if the additional sodium content of the medium due to added sodium acetate was partially responsible for the inhibitory action of sodium acetate (Fig. 3). The addition of sodium chloride did not appear to have any inhibitory effect against aflatoxin production by *A. parasiticus* and may have actually stimulated production of the toxin. This lack of inhibition by NaCl is in agreement with results cited by Kulik and Hanlin (1968) who reported that 15% NaCl was needed to significantly inhibit the growth of *A. parasiticus*.

Halls and Ayres (1975) reported that in GMS medium (pH 4.8), 0.05M sodium acetate inhibited the production of sterigmatocystin by *A. versicolor* while 0.05M sodium acetate plus 0.05M Na_2HPO_4 stimulated the production of the mycotoxin. The presence and absence of KH_2PO_4 in AMY medium (pH 4.5) was examined to determine if the buffer had any effect on acetate-induced inhibition of *A. parasiticus* (Table 2). Cultures containing 1.0g NaAc/100 ml, with or without KH_2PO_4, did not grow or produce aflatoxin; while similar cultures without sodium acetate grew abundantly and produced equivalent amounts of aflatoxin. Apparently phosphate does not play a role in acetate inhibition of *A. parasiticus*.

Reddy et al. (1971) reported that increasing the concentration of KH_2PO_4 in SL medium from 0.075g/100 ml to 1.0g/100 ml produced an approximate 94% reduction in aflatoxin accumulation, and they concluded that phosphate is inhibitory to aflatoxin production. The effect of the KH_2PO_4 content of AMY medium (pH 5.5) on aflatoxin production was examined (Fig. 4) in the present study. Concentrations of 0.00, 0.01, 0.10 and 1.00g/100 ml produced no differences in aflatoxin accumulation. Concentrations of 5.00 and 10.00g/100 ml produced relatively small decreases and in-

Table 1—Effect of the addition of acetic acid at various time intervals on the growth and aflatoxin production by A. parasiticus NRRL 2999. The cultures were incubated in AMY medium (pH 4.0) at 28°C for a total of 7 days

Acetic acid (g/100 ml)	Days of incubation prior to addition of acetic acid	% of control	
		Mycelium[a]	Aflatoxin[a]
0.0	–	100.00	100.00
1.0	0	0.00	0.08
1.0	3	30.00	22.00
1.0	5	110.00	129.70
2.0	0	0.00	0.13
2.0	3	8.00	18.00
2.0	5	90.00	105.00

[a] Each value is the average of three cultures.

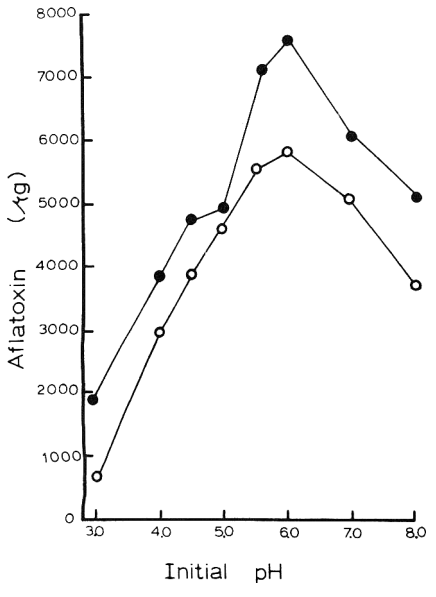

Fig. 3—Production of aflatoxin per 25 ml medium by A. parasiticus NRRL 2999 incubated at 28° C for 7 days in AMY (pH 4.5) containing 0.0g/100 ml (○) and 2.0g/100 ml (●) NaCl. Each point represents the average of six cultures.

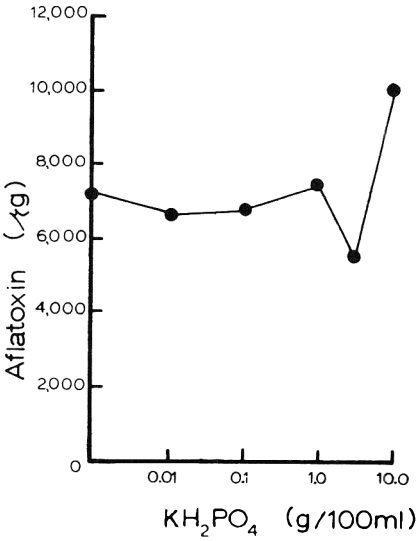

Fig. 4—Effect of KH_2PO_4 concentration on aflatoxin production per 25 ml medium by A. parasiticus NRRL 2999 incubated in AMY (pH 5.5) at 28° C for 7 days. Each point represents the average of six cultures.

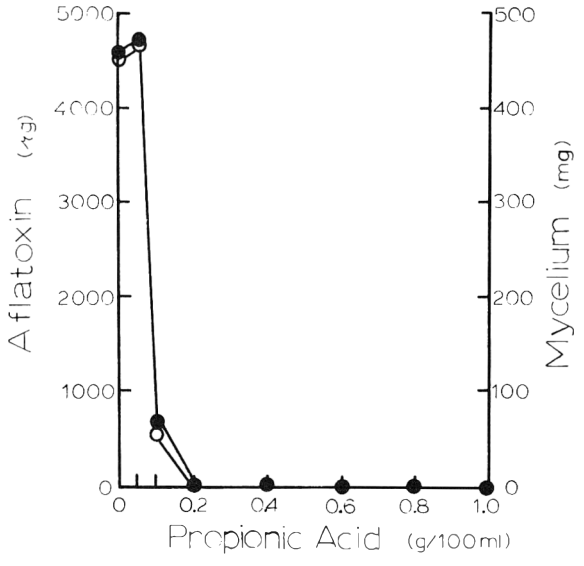

Fig. 5—Effect of propionic acid concentration on growth (•) and aflatoxin production (○) per 25 ml medium by A. parasiticus NRRL 2999 incubated in AMY (pH 4.5) at 28° C for 7 days. Each point represents the average of three cultures.

Table 2—Effect of 1.0g KH_2PO_4/100 ml on sodium acetate (1.0g/100 ml) inhibition of A. parasiticus NRRL 2999 incubated in AMY medium (pH 4.5) at 28° C for 7 days

Sample	Mycelium[a] (g)	Aflatoxin[a] (μg)/25 ml
− phosphate − acetate	0.661	3529.2
+ phosphate − acetate	0.783	3419.7
− phosphate + acetate	0.172[b]	Trace[c]
+ phosphate + acetate	0.134[b]	Trace[c]

[a] Each value is the average of three cultures.
[b] No visible growth
[c] Amount of aflatoxin was approximately 1 μg per 25 ml of medium.

creases in aflatoxin, respectively. The present study could not demonstrate any substantial phosphate-induced inhibition of aflatoxin production. A possible explanation of this difference may lie in the composition of the SL medium employed by Reddy et al. (1971). One of the mineral constituents of SL medium was calcium chloride ($CaCl_2 \cdot 2H_2O$). Preliminary studies in our laboratory (Halls, personal communication) have indicated that medium buffered with a combination of $CaCO_3$ and KH_2PO_4 strongly inhibited aflatoxin production although neither compound when used alone was inhibitory. Apparently the presence of both calcium and phosphate ions in the medium has some inhibitory effect against aflatoxin production by *A. parasiticus*. It has been well established that the absorption of calcium, phosphate, iron, magnesium, manganese and zinc in mammalian systems is highly interdependent (Guthrie, 1971), and a similar relationship in *A. parasiticus* may be a factor in the phosphate inhibition of aflatoxin production reported by Reddy et al. (1971).

To insure that the responses were not strain specific, six strains of *A. flavus* and two strains of *A. parasiticus* were screened in tube cultures to determine the effect of sodium acetate on their growth in AMY medium (Table 3). Both aflatoxin positive and negative strains were included among the *A. flavus*. All strains of *A. flavus* and *A. parasiticus* tested were susceptible to the inhibitory action of acetate.

As previously mentioned, Hoffman et al. (1939) demonstrated that propionic acid was significantly more inhibitory to mold growth than acetic acid. The present study examined the effectiveness of propionic acid against the growth and aflatoxin production by *A. parasiticus* (Fig. 5). Propionic acid concentrations of 0.1g/100 ml were partially inhibitory, while levels of 0.2g/100 ml completely inhibited mold growth and aflatoxin production. Results using these concentrations are in general agreement with those of Hoffman et al. (1939) who reported that 0.3g/100 ml inhibited mold growth. Propionic acid was more inhibitory than acetic acid against *A. parasiticus*, and seems to offer even greater potential as a means of controlling aflatoxin.

A. flavus and *A. parasiticus* are classified as spoilage molds, and their production of alfatoxin in commodities is usually related to improper harvesting and storage techniques (Diener and Davis, 1969). Kirby et al. (1937) demonstrated that "painting" the surface of bread with acetic acid prior to storage dramatically prevented surface spoilage by molds. The results of the present study indicate that a similar technique might be successfully employed as a means of preventing aflatoxin production in stored commodities. Surface application of acetic acid, propionic acid, or other suitable fatty acids by

Table 3—Effect of sodium acetate on growth of A. flavus and A. parasiticus strains incubated in AMY tube cultures for 15 days at 28° C. Each response represents observations of three cultures[a]

		pH 4.0			pH 5.0			pH 6.0		
Sodium acetate (g/100 ml)		0.0	1.0	2.0	0.0	1.0	2.0	0.0	1.0	2.0
A. flavus:	PC 65	++	–	–	++	+[b]	–	++	++	++
	PC 120	++	–	–	++	+[b]	–	++	++	++
	M-pl(99)	++	–	–	++	+[b]	–	++	++	++
	PC 101	++	–	–	++	+[b]	–	++	++	++
	PC 42	++	–	–	++	++	–	++	++	++
	PC 125	++	–	–	++	+[b]	–	++	++	++
A. parasiticus:	NRRL 2999	++	–	–	++	++	–	++	++	++
	ATCC 15517	++	–	–	++	++	–	++	++	++

[a] Code: – = no visible growth; + = limited growth; ++ = abundant growth.
[b] Some samples had heavy surface growth while other samples had only a small amount of submerged growth.

spraying, dipping or flotation prior to storage may be a potential means of preventing the growth and aflatoxin production by *A. flavus* and *A. parasiticus* in the product. Studies are now being conducted to evaluate the potential of this technique.

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INFLUENCE OF PROTEIN SUPPLEMENTS ON GROWTH OF *Staphylococcus aureus* AND PRODUCTION OF ENTEROTOXINS

ABSTRACT

Influences of soy protein (promine D; SP), fish protein concentrate (FPC) and Brewer's Yeast or Torula Yeast on aerobic growth of *S. aureus* (Z88) and production of enterotoxins A and D were evaluated in liquid media containing equivalent amounts (1.8%) of protein from each source. *S. aureus* grew faster (29 min generation time) and reached a final population of $2-7 \times 10^9$/ml in yeast media as compared to soy or fish protein concentrate; 50–56 min generation time and a final population of 2×10^8/ml in SP or FPC. Production of enterotoxins by *S. aureus* was enhanced in yeast media. Enterotoxin A was detectable in unconcentrated yeast preparations after 6 hr and both toxins (A and D) after 12 hr incubation. Enterotoxin A was detected only in FPC concentrated 50-fold and after 24 hr incubation. Neither enterotoxin A nor D was detected in SP concentrated 100-fold after 24- or 48-hr incubation. Addition of Brewer's Yeast (2% w/v) to whole milk enhanced production of enterotoxins A and D; there was at least a 100-fold higher amount of enterotoxin in milk + yeast as compared to simply milk.

INTRODUCTION

SUPPLEMENTATION of foods with less expensive sources of protein and the increased usage of various isolated protein fractions in fabricated foods are receiving considerable attention primarily from the standpoint of economics involved in food production and preservation. Soy protein or isolated soy protein concentrates, fish protein concentrates, single cell proteins or dried whole yeast, cotton seed or sunflower seed protein isolates are some of the items considered and/or used in human foods. Though the nutritional and functional properties of some of these proteins have been evaluated, their effects on microbial growth have not been adequately assessed. Stimulative effects of certain soy protein preparations have been noted (Schroder and Busta, 1973) on growth of *Clostridium perfringens*. While thermal processing of foods may eliminate most vegetative cells or spores of bacteria of public health concern, growth of some bacteria before processing, especially *Staphylococcus aureus* could lead to accumulation of thermostable enterotoxins which may withstand these treatments and cause food poisoning. Therefore, it is essential to evaluate the effects of the protein supplements in supporting growth of *S. aureus* and production of enterotoxins. There is very limited information available on the effects of protein supplements on growth of *S. aureus* or production of enterotoxins A or D, although enterotoxin B production was reported enhanced by fish protein concentrate (Fung, 1972).

The purpose of this study was to evaluate the effects of isolated soy protein concentrate, fish protein concentrate, and yeast protein on growth of *S. aureus* and production of the most commonly encountered enterotoxins (A and D) in food poisoning outbreaks.

EXPERIMENTAL

Conditions of growth of *S. aureus* (Z88)

Staphylococcus aureus (Z88) which produces enterotoxins A and D, was grown aerobically in a shaker water bath (model G-76, New Brunswick Scientific Co., Inc., New Brunswick, N.J.) at 150 rpm for 18–25 hr at 37°C. A predetermined amount of this culture grown in the respective test medium was inoculated into the appropriate test medium to yield an initial *S. aureus* population of $1 \times 10^4 - 1 \times 10^5$/ml. The experimental flasks (500 ml Erlenmeyer flask containing 300 ml of test medium) were incubated in the shaker water bath at 37°C. *S. aureus* population was determined at various intervals of incubation by plating on Trypticase Soy agar (Difco) and incubating plates at 37°C for 48 hr.

Composition of the test medium

Equivalent amount of protein from each source 1.8%, dipotassium phosphate 0.25%, NaCl 0.5%, and dextrose 0.25%, pH 7.0.

Source of protein

Promine D (soy protein), Central Soy Co., Chicago; fish protein concentrate (whole hake fish–isopropyl alcohol extract), U.S. National Marine and Fisheries Service, College Park, Md.

Yeast: Instant Brewer's Yeast, Formula 450, Plus Products, Los Angeles, Calif. Torula Dried Yeast NFXIII, St. Regis, Rhinelander, Wisc.

Enterotoxin analyses

Supernatant from culture growth (spent medium) obtained by centrifuging for 15 min at 14,000 rpm in a Sorvall RC-2B centrifuge, was used to detect the presence of enterotoxin A or D by the microslide in microdiffusion procedure of Casman and Bennett (1965). In the case of milk or milk + yeast, casein was removed by adjusting pH to 4.6 with 1N HCl and centrifuging as stated above and the supernatant was used for enterotoxin analyses. To demonstrate the small amounts of enterotoxins, the supernatant was concentrated by dialyzing against 40% polyethylene glycol at 4°C and lyophilizing. The lyophilized material was rehydrated in 0.25–0.40 ml saline. The rehydrated preparation and/or its further dilutions in saline was used for enterotoxin assay.

Determination of generation time

The generation time was determined by the use of the formula $g = (0.3 \times t)/(\log a - \log b)$; where g is generation time in minutes and t is the elapsed time in minutes (during exponential growth between 4 and 8 hr of incubation), a is *S. aureus* population after 8 hr; and b is population after 4 hr of incubation.

RESULTS & DISCUSSION

GROWTH RESPONSE of *Staphylococcus aureus* (Z88) in each of the three substrates (soy, fish and yeast), is shown in Figure 1. These data are average values from two separate trials. As can be seen from Figure 1, *S. aureus* grew faster and reached a higher final population (about 30-fold) of 7×10^9/ml in yeast as compared to 2×10^8/ml in soy or fish protein concentrate (SP or FPC). *S. aureus* always reached a final population of 3×10^9/ml in yeast regardless of the initial inoculum level of 10^2, 10^4, or 10^5/ml (results not shown here). As shown in Table 1, *S. aureus* showed slowest growth in FPC (56 min generation time) as compared to SP (50 min) or yeast (29 min). The faster rate of growth and attainment of a higher final *S. aureus* population in yeast may have been due to differences in these substrates relative to the availability of more readily utilizable nutrients such as free amino acids, peptides and vitamins or cofactors. Trichloroacetic acid (TCA) supernatant (a final concentration of 5% TCA) from Brewer's yeast showed a twofold higher nitrogen content per ml (Kjel-

Table 1—Effects of protein source on growth of S. aureus (Z-88) and production of enterotoxins

Protein source	Generation time (min)	Max S. aureus population reached (24–48 hr)	Enterotoxin A or D in the supernatant from spent medium after 24 hr incubation[a]		
			Concentrated (× original)	Unconcentrated (original)	Diluted (× original)
Brewer's yeast	29	7.1×10^9	1, +	+	5, +
Fish protein concentrate	56	2.0×10^8	50, +	ND	
Soy protein	50	2.0×10^8	100 (ND)	ND	
Whole milk	31	2.0×10^9	25, +	ND	
Whole milk plus brewer's yeast (2% w/v)	27	7.0×10^9	1, +	+	5, +

[a] + detected; ND not detected

dahl analysis) than similar supernatant from FPC or SP. Yeast preparations used in this study contained high levels of various vitamins (thiamine, niacin, biotin, riboflavin, etc., from manufacturer's label of contents) as compared to the absence of these in soy or fish. As can be seen from Table 1, production of detectable amounts of enterotoxins A or D was influenced by the substrate. In SP, despite the attainment of a similar population of 2×10^8/ml as in FPC, neither toxin was present in detectable level even in SP concentrated 100-fold. In FPC, only enterotoxin A was produced in detectable amounts after 12 or 24 hr incubation; enterotoxin was detected in FPC concentrated 50 and not 25-fold. On the other hand, in both yeast preparations enterotoxins A and D were detected in the unconcentrated medium after 12 hr incubation. In fact, enterotoxin A was first detected in both yeast preparations after 6–7 hr incubation in the unconcentrated as well as the spent medium diluted fivefold with attainment of about 5×10^7 to 1×10^8 *S. aureus*/ml. When *S. aureus* reached similar populations after 12 or 24 hr in fish or soy, very little or no detectable amounts of enterotoxins respectively, were produced. These data indicate that yeast enhanced production of both enterotoxins. SP supported growth of *S. aureus* and not production of enterotoxins. It is not known whether the lack of vitamins such as niacin and thiamine which are present in yeast and/or some amino acids essential for enterotoxin production (Bergdoll, 1970) might have been responsible for the absence of detectable amounts of enterotoxins in SP. This aspect is currently under investigation.

The results with FPC were in contradiction to those reported (Fung, 1972) with another source of FPC (Viobin) in which there was stimulation of enterotoxin B production. The FPC employed in this study was an isopropyl alcohol extract and reported to have contained about 115 ppm isopropyl alcohol among the 2.4% residual volatiles present. It is possible

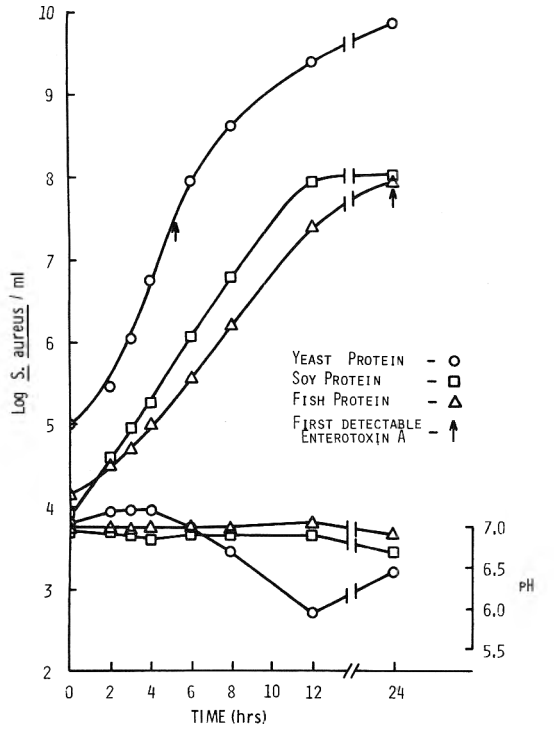

Fig. 1—Growth of S. aureus (Z88) in fish, soy and yeast proteins under aerobic conditions at 37° C.

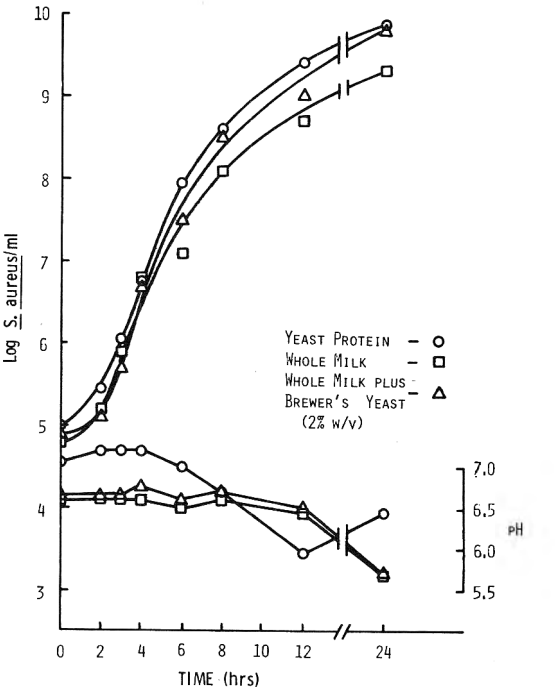

Fig. 2—Growth of S. aureus (Z88) in yeast, milk and milk plus yeast under aerobic conditions at 37° C.

that either the presence of some volatile components or the absence of vitamins or cofactors used in Fung's study, may have contributed to the poor production of enterotoxins in the FPC. Vitamins (thiamine and niacin) that affect the oxidative metabolism of pyruvate significantly influence production of enterotoxin B (Morse and Baldwin, 1971). However, such inhibitory effects (due to lack of vitamins) have not been reported for enterotoxins A or D. In our preliminary study (based on a single trial and results not shown here), when vitamins (thiamine, riboflavin, niacin, and biotin) present in yeast were added to the FPC at comparable levels, no significant difference was noted in terms of the generation time, maximum *S. aureus* population reached or production of detectable amounts of enterotoxins A or D.

The effect of addition of Brewer's yeast to whole milk was evaluated relative to growth of *S. aureus* and production of enterotoxins. The Brewer's yeast is marketed for use with milk, fruit juices, soups, meat loaf, etc. Results are in Figure 2. While there would not appear to be much difference in growth of *S. aureus* in milk, milk + yeast or yeast there is a substantial increase in the production of both enterotoxins in milk + yeast as compared to simply milk. Enterotoxin A or D was detected in the unconcentrated as well as a fivefold diluted supernatant from milk + yeast after 12 or 24 hr incubation. On the other hand, detectable amounts of enterotoxins were not present in the unconcentrated supernatant from milk incubated 24 hr. Enterotoxins A and D were, however, detected in the supernatant from milk (12 or 24) which was concentrated 25 and not 10-fold. Thus, addition of Brewer's yeast to milk enhanced production of enterotoxins by at least 100-fold. This aspect of enhancement of staphylococcal enterotoxin production by yeast should be borne in mind while formulating and preparing foods so that precautions could be instituted by the processor in limiting hazards from staphylococcal enterotoxins.

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THERMAL INJURY AND RECOVERY OF Saccharomyces cerevisiae

ABSTRACT

This investigation was undertaken to determine the effect of mild heat treatments on the survival, injury and recovery of *Saccharomyces cerevisiae*. Cells of *S. cerevisiae* strain Y-25 were grown in yeast extract-malt extract, harvested by centrifugation, washed 2X, and suspended in 0.1M phosphate buffer at pH 7.0. Cell suspensions were heated in a water bath at various temperatures and survivors were determined by plating on Plate Count Agar (PCA) and Potato Dextrose Agar (PDA) with or without acidification. The heated suspensions were stored at room temperature (20–22°C) and sampled at various periods to monitor the recovery of injured cells. Using the system described above, heat-injured cells of *S. cerevisiae* were not recovered when plated on PCA or PDA immediately after heating. Heat-injured cells were recovered on the plating media after storage in buffer at room temperature, but 5–7 days were required for maximum recovery. Initially, the rate of recovery on PCA was greater than on PDA; this was not attributable to the differences in pH of the media, but apparently was due to nutritional factor(s). Resting cells of *S. cerevisiae* did not undergo a comparable recovery when heated and stored in either water or buffer.

INTRODUCTION

THIS INVESTIGATION was undertaken to study the thermal injury and recovery of *S. cerevisiae*. While thermal destruction of microbial cells has been the subject of numerous investigations, only during the last decade has there been intensive research on the thermal injury and recovery of microorganisms. The major portion of this work has utilized systems for studying the thermal injury and recovery of *Staphylococcus aureus* and other bacteria.

There have been few studies reported on thermal injury and recovery of yeasts. Nelson (1972) investigated the effect of pH on the apparent survival of sublethally stressed yeasts, while Tsuchido et al. (1972) utilized the classical two-media system to demonstrate thermal injury and recovery of *Candida utilis*. In the latter study cells were subjected to a sublethal heat treatment (45°C for 15 min) prior to plating on a complete medium with or without 7% NaCl. The recovery of cells in water or buffer during storage appeared to be dependent upon resynthesis of RNA and protein. The present report concerns preliminary investigation of the thermal destruction and survival of *S. cerevisiae*. Significantly, immediately after thermal stress the injured cell populations which we studied were unable to grow on a complete medium, i.e., a "stress" medium was not utilized for differentiation of injured and noninjured cells.

MATERIALS & METHODS

Saccharomyces cerevisiae Y-25 was maintained on Yeast extract-malt extract (YM) agar slants at 25°C. For preparation of inocula, growth from a 24-hr YM slant was used to inoculate 250 ml of YM broth in a 500-ml Erlenmeyer flask. The flask was incubated at 25°C on a gyrotary shaker (New Brunswick Scientific Co.) operating at 100 rpm. For actively growing cells, 25 ml of the YM-broth culture was transferred to 250 ml of YM broth after ca. 12 hr, and the new culture was incubated for an additional 4-hr period. For resting cells the YM broth cultures were incubated for ca. 72 hr. Cells were harvested by centrifugation at 4,000 × G, washed 2× with distilled H_2O and suspended in 0.1 M sodium phosphate buffer, pH 7.0.

For the heating trials, 1 ml of cell suspension was added to a flask containing 99 ml of preheated buffer in a water bath adjusted to the appropriate temperature. Samples were removed at various intervals and immediately mixed with 9 volumes of distilled water or buffer equilibrated at room temperature (20–22°C).

For the recovery experiments, the diluted samples were stored at room temperature for 7–9 days and samples were withdrawn at various intervals during storage. Appropriate dilutions of the samples were plated in duplicate or triplicate on Plate Count Agar (PCA) and Potato Dextrose Agar (PDA). Some of the samples were also plated on PCA and PDA after acidification with 1 part 10% tartaric acid per 100 parts media (APCA and APDA at pH 5.5 and 3.5, respectively).

To serve as controls, unheated samples were diluted 1:10 with distilled H_2O and stored at room temperature.

RESULTS & DISCUSSION

FIGURE 1 represents survivor curves obtained when vegetative cells of *S. cerevisiae* were heated at 52°C. The number of apparent survivors is greatly influenced by the plating media and the use of a 5-day storage period prior to plating. This "delayed plating" system was used in an attempt to enumerate injured cells; Patrick and Haynes (1964) had found a 5-day recovery period yielded maximum recovery of *S. cerevisiae* cells which had been subjected to X-irradiation. The differing numbers obtained when heated cells were plated on PCA and

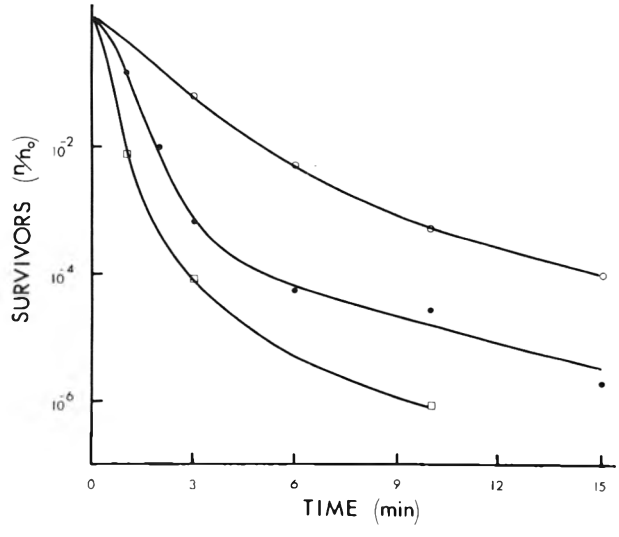

Fig. 1—Survivor curve for Saccharomyces cerevisiae cells heated at 52°C. Apparent fraction of cells surviving as determined by plating immediately on PDA (□——□) and PCA (●——●), or by plating on PCA after storage for 5 days in phosphate buffer (○——○). (n_0 = ca. 10^8 cells/ml).

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PDA was initially considered to be due to difference in pH (7.0 and 5.6, respectively). Nelson (1972) described the influence of pH on yeasts subjected to sublethal heat treatments. Also, the effect of the pH of media on enumeration of yeasts has been reported by others (Mace and Koburger, 1967; Koburger, 1971; Jarvis, 1973). However, in studies using acidified and nonacidified PCA and PDA we found the differences in survivors were not significantly affected by the pH, but presumably were affected by nutritional differences between the PCA and PDA.

In order to further study the recovery process, *S. cerevisiae* cells were heated at 52°C for 3 min followed by enumeration on PCA and PDA after various storage periods (Fig. 2). When enumerated immediately after heating, ca. six-fold higher counts were obtained on PCA as compared to counts on PDA; however, at 2 days and thereafter, there was no substantial difference in the counts obtained using both media.

The recovery process was slow requiring 5–7 days; when compared to day 0, the counts of survivors after 7–9 days represented an increase of approximately 3 log cycles. The counts of control samples containing nonheated cells initially remained constant and then decreased slightly during the latter part of storage. Due to the long period required for recovery, one might postulate that the increases in cell numbers were due to utilization of material from dead cells and subsequent multiplication rather than recovery. However, counts of additional controls containing nonheated cells along with cells destroyed by heating at 52°C for 30 min exhibited a pattern similar to the other controls. Thus, the increases in counts after storage of the heated samples were due to recovery of injured cells rather than cannabalism and multiplication of cells. Work is currently underway to determine the specific type(s) of injury incurred under our experimental conditions.

Resting cells were much more resistant to thermal destruction than actively growing cells (Fig. 3). This phenomenon has been described by others (Rosenberg and Wood, 1957; Tsuchido et al., 1972). Unlike the results reported for actively growing cells, after resting cells were heated, the numbers of apparent survivors did not increase during storage (unpublished data). This difference in ability to repair the thermal injury during storage may be linked to the utilization of endogenous reserves present in the actively growing cells. In any event the ability to repair the drastic thermal injury sustained in these experiments, or the nature of the injury itself, was highly dependent upon the physiological state of the cells. In contrast to our results, Tsuchido et al. (1972) were able to show recovery of injured and uninjured populations of resting cells using milder conditions for thermal injury (45°C) and differentiation of injured and uninjured cell populations employing a stress medium.

The results of this investigation show thermal stress can affect the apparent numbers of viable yeast cells. Therefore the ability of analytical methods to detect sublethally stressed cells is of great importance to the food industry, particularly for food processors utilizing mild heat treatments.

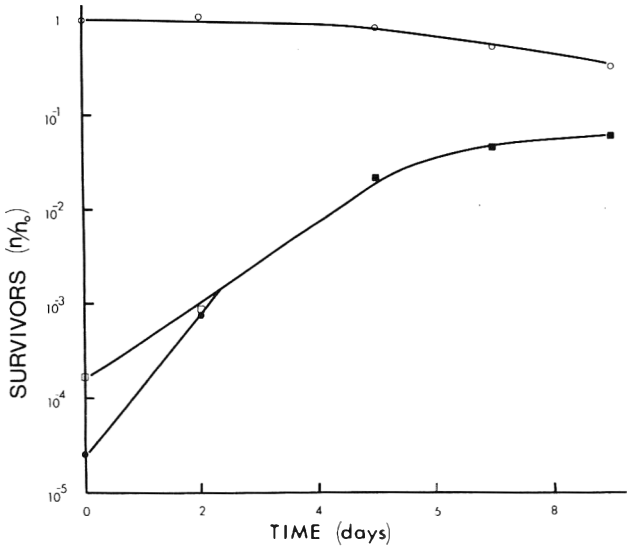

Fig. 2—Effect of storage in phosphate buffer on the apparent number of survivors of Saccharomyces cerevisiae cells heated for 3 min at 52° C and enumerated on PDA (●——●) or PCA (□——□); nonheated control on PCA (○——○). (n_o = ca. 10^8 cells/ml).

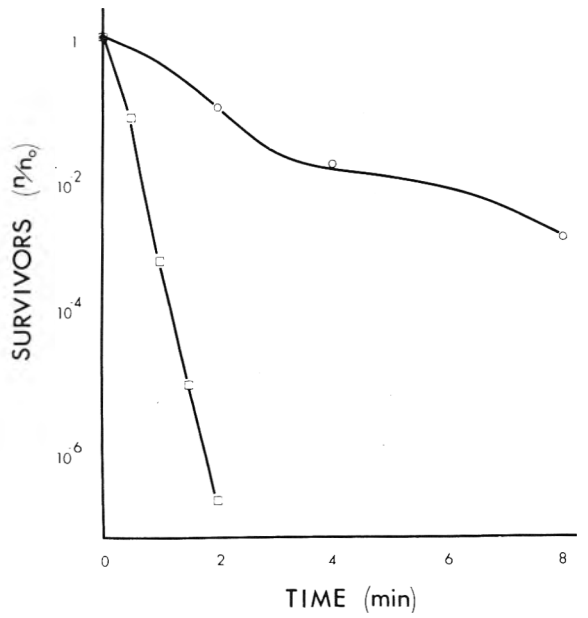

Fig. 3—Survivor curve for Saccharomyces cerevisiae cells heated at 56° C. Apparent fraction of cells surviving as determined by plating immediately on PCA; actively growing cells (□——□); resting cells (○——○). (n_o = ca. 10^8 cells/ml).

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ACTIVITY AND STABILITY OF α- AND β-AMYLASE AT TEMPERATURES FROM 4° TO −23°C

ABSTRACT

α- and β-Amylase activity were determined at 4°, −13°, −18° and −23°C in model systems and in a sweet potato puree system for different periods of time. In the model systems, commercially purified swine pancreatic α-amylase and sweet potato β-amylase at 0, 0.5, 1.0 and 1.5 μg/0.5 ml concentrations were used to react with 0.5 ml of a 2% soluble potato starch substrate for 112 days. In the sweet potato puree system, samples prepared from cured and noncured roots were frozen and stored for 56 days. As an index of amylase activity, accumulated maltose was determined after different periods of storage. Also, the stability of the enzymes as affected by low temperatures was determined in the sweet potato puree. In the model systems, α- and β-amylase showed constant activity at 4°, −13°, −18° and −23°C for different periods of storage, up to 113 days. Amylase activity did increase with enzyme concentration. Both enzymes were active at −23°C. In the noncured sweet potato puree system, α- and β-amylase were active at 4°C for up to 28 days, but were essentially inactive at −13°, −18° and −23°C. In the cured sweet potato puree system, α- and β-amylase were stable for up to 28 days at 4°C. Enzyme stability in samples from noncured roots was not affected for up to 14 days of storage at 4°C, but decreased considerably thereafter. α- and β-Amylase stability in all sweet potato puree samples stored for 56 days at −13°, −18° and −23°C was not affected.

INTRODUCTION

LITERATURE REPORTS indicate that temperatures in the range from −6.7° to −25°C and ice formation do not inactivate but retard enzyme activity (Cobet et al., 1961; Hartzler and Guerrant, 1952; Kiermeier, 1952; Lund and Halvorson, 1957; Maier and Tappel, 1954; Pallavicini et al., 1970; Tappel, 1966). Makoto and Motohiro (1955) working with rice koji, and Veselov et al. (1970) working with a starch paste model system reported that α-amylase activity decreased as the temperature decreased.

Appreciable amounts of α-amylase (Ikemiya and Deobald, 1966) and β-amylase (Giri, 1934) were found in sweet potatoes. Culpepper and Magoon (1926) and Gore (1923) demonstrated that the principal sugars resulting from starch hydrolysis in sweet potato roots are maltose and dextrin. Deobald et al. (1969) showed that maltose formation in sweet potato puree was associated equally with the degree of heat treatment above starch gelatinization temperatures and with the amount of amylolytic activity in the purees. Hasselbring and Hawkins (1915) found that at 15.5° and 5°C the rates of conversion of starch into sugars are slow and the equilibrium is shifted to permit a greater concentration of sugar to accumulate.

There is little published information on the activity and stability of α- and β-amylase at low temperatures. The present study was undertaken to determine (a) the activity of purified α- and β-amylase in a soluble potato starch system, and (b) the activity and stability of naturally occurring α- and β-amylase in sweet potato puree. The temperatures included ranged from 4° to −23°C over storage periods of 0−112 days.

EXPERIMENTAL

Enzymes

Commercially prepared, purified and standardized, swine pancreatic α-amylase (Worthington Biochemical Corp., Freehold, N.J.) was used. Swine pancreatic α-amylase was availabe as a suspension in 0.5 saturated sodium chloride in 0.003M calcium chloride. Sweet potato β-amylase (Worthington Biochemical Corp., Freehold, N.J.) was suspended in 0.6 saturated ammonium sulfate at pH 3.0.

Suspensions containing 2941 μg/10 ml of swine pancreatic α-amylase, and 3333 μg/10 ml of sweet potato β-amylase were prepared by diluting each of the original enzyme suspensions with triple distilled, deionized water. The suspensions were then transferred into polyethylene bottles and stored at 4°C. Subsequent aliquots were drawn from these stock suspensions to prepare solutions of the required enzyme concentrations.

Sweet potato roots

Two lots of Centennial variety sweet potatoes (*Ipomoea batatas*)

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Table 1—Effect of holding time at 4°C on accumulated maltose at different concentrations of purified swine pancreatic α-amylase and purified sweet potato β-amylase with 2% soluble starch substrate

Holding time (min)	Accumulated maltose (μmoles maltose)[a] α-Amylase conc (μg/ml)			Accumulated maltose (μmoles maltose)[a] β-Amylase conc (μg/ml)		
	0.5	1.0	1.5	0.5	1.0	1.5
0	0.0820 a	0.1003 a	0.1590 a	0.0967 a	0.1487 a	0.1557 a
15	0.2110 b	0.3551 b	0.5470 b	0.2230 b	0.4553 b	0.6683 b
30	0.3766 c	0.6021 c	0.9728 c	0.4102 c	0.8404 c	1.1019 c
45	0.5326 d	1.0257 d	1.4492 d	0.5565 d	1.2459 d	1.8066 d
60	0.7546 e	1.3326 e	1.9191 e	0.7395 e	1.5889 e	2.5506 e
75	0.8825 f	1.7395 f	2.4773 f	0.9792 f	1.9373 f	2.9593 f

[a] Data are mean of three replicates. Means within columns followed by a common letter are not significantly different at 5% level as determined by Duncan's Multiple Range Test.

from the Eastern Shore area of Virginia were used. They were freshly dug and treated approximately 72 hr after harvest. The first lot of roots was placed at 26.7°C and 80% relative humidity for 10 days to "cure." Then the lot was put in storage at 12.8°C and 80% relative humidity for 7 months before they were made into purees. The second lot of roots was made into purees immediately.

Sweet potato purees were prepared by washing and preheating the roots in a 54.5°C water bath for 30 min. The preheated roots were then submerged in a 12% caustic soda solution heated to 100°C for 5 min, washed to remove the lye, trimmed and then ground through a disintegrator fitted with a 1/32-in. screen.

Substrate

Soluble potato starch (Fisher certified A.C.S. grade) at 2% concentration was used as substrate. It was dissolved in the following appropriate buffer solutions when used as substrate for different enzymes: (1) 0.02M phosphate buffer, pH 6.9, for purified swine pancreatic α-amylase; (2) 0.02M phosphate buffer, pH 6.0, for crude sweet potato α-amylase; and (3) 0.016M acetate buffer, pH 4.8, for commercially purified and crude sweet potato β-amylase.

Sample preparation for storage tests

In the model systems, 0.5 ml of 2% soluble potato starch in appropriate buffer and at desired pH was pipetted into a polyethylene bottle and treated with 0.5 ml of either swine pancreatic α-amylase or sweet potato β-amylase at 0, 0.5, 1.0 and 1.5 μg concentrations. The treated samples were quickly frozen by immersing the samples immediately in a mixture of dry ice and ethanol at −60°C ± 5°C for 10 sec.

In the sweet potato puree system, approximately 8–10 oz of sweet potato puree samples were tightly packed in polyethylene bags and quickly frozen in a similar manner for 20 min.

After all the treated samples appeared solid, they were put into storage at various temperatures.

Enzyme assays

α- and β-Amylase activity in the model systems was determined by using the procedures described by Bernfeld (1951, 1955) and modified by Worthington Biochemical Corp. (Anonymous, 1972). The basic procedures were the determinations of the increase in maltose in the soluble potato starch solution using 3,5-dinitrosalicylic acid reagent at pH 6.9 and 4.8 for α- and β-amylase, respectively. Data on α- and β-amylase activity in the model systems were expressed as μmoles of maltose.

For the extraction of α- and β-amylase from the sweet potato puree, 5g of a puree sample were vigorously hand shaken for 1 min with 50 ml of distilled, deionized water. The suspension was then filtered through a Whatman No. 1 filter paper. The filtrate was diluted to a suitable con-

Table 2—Comparative effect of enzyme concentration at 4°C on activity of purified swine pancreatic α-amylase measured as accumulated maltose after equal holding times using a 2% soluble starch substrate

Enzyme conc	Accumulated maltose (μmoles maltose)[a]					
	Holding time (min)					
	0	15	30	45	60	75
1.0 μg/ml	0.1003	0.3551	0.6021	1.0257	1.3326	1.7395
1.5 μg/ml	0.1590	0.5470	0.9728	1.4492	1.9191	2.4773
Mean Difference[b]	0.0587*[c]	0.1919**	0.3707**	0.4235**	0.5865**	0.7378**
0.5 μg/ml	0.0820	0.2110	0.3766	0.5326	0.7546	0.8825
1.0 μg/ml	0.1003	0.3551	0.6021	1.0257	1.3326	1.7395
Mean difference[b]	0.0183	0.1441**	0.2255**	0.4931**	0.5780**	0.8570**
0.5 μg/ml	0.0820	0.2110	0.3766	0.5326	0.7546	0.8825
1.5 μg/ml	0.1590	0.5470	0.9728	1.4492	1.9191	2.4773
Mean difference[b]	0.0770**	0.3360**	0.5962**	0.9166**	1.1645**	1.5948**

[a] Data are mean of three replicates.
[b] Tabulated L.S.D. values of enzyme concentrations X storage time required for significance at 5% and 1% levels were 0.0523 and 0.0702, respectively.
[c] Asterisks indicate that means are statistically different at 5% (*) and 1% (**) level.

Table 3—Comparative effect of enzyme concentration at 4°C on activity of purified sweet potato β-amylase measured as accumulated maltose after equal holding times, using a 2% soluble starch substrate

Enzyme conc	Accumulated maltose (μmoles maltose)[a]					
	Holding time (min)					
	0	15	30	45	60	75
1.0 μg/ml	0.1487	0.4553	0.8404	1.2459	1.5889	1.9373
1.5 μg/ml	0.1557	0.6683	1.1019	1.8066	2.5506	2.9593
Mean difference[b]	0.0070	0.2130**[c]	0.2615**	0.5607**	0.9617**	1.0220**
0.5 μg/ml	0.0967	0.2230	0.4102	0.5565	0.7395	0.9792
1.0 μg/ml	0.1487	0.4553	0.8404	1.2459	1.5889	1.9373
Mean difference[b]	0.0522*	0.2323**	0.4302**	0.6894**	0.8494**	0.9581**
0.5 μg/ml	0.0967	0.2230	0.4102	0.5565	0.7395	0.9792
1.5 μg/ml	0.1557	0.6683	1.1019	1.8066	2.5506	2.9593
Mean difference[b]	0.0592**	0.4453**	0.6917**	1.2501**	1.8111**	1.9801**

[a] Data are mean of three replicates.
[b] Tabulated L.S.D. values of enzyme concentrations X storage time required for significance at 5% and 1% levels were 0.0438 and 0.0588, respectively.
[c] Asterisks indicate that means are statistically different at 5% (*) or 1% (**) level.

Table 4—Effect of storage time at −13°C on accumulated maltose at different concentrations of purified swine pancreatic α-amylase and purified sweet potato β-amylase with 2% soluble starch substrate

Storage time (days)	Accumulated maltose (μmoles maltose)[a] α-Amylase conc (μg/ml)			Accumulated maltose (μmoles maltose)[a] β-Amylase conc (μg/ml)		
	0.5	1.0	1.5	0.5	1.0	1.5
0	0.1377 a	0.1487 a	0.1563 a	0.0893 a	0.1413 a	0.1590 a
1	0.4539 b	0.7702 b	1.1489 b	0.6028 b	1.0799 b	1.4352 b
2	0.7894 c	1.4915 c	2.2515 c	0.8307 c	1.4244 c	1.9128 c
3	1.0489 d	1.8338 d	2.8487 d	1.0222 d	1.7161 d	2.3085 d

[a] Data are mean of three replicates. Means within columns followed by a common letter are not significantly different at 5% level as determined by Duncan's Multiple Range Test.

centration. Maltose was determined as an index of α- and β-amylase activity in sweet potato puree, and calculated by using the formula:

$$\text{Naturally occurring maltose} = \frac{\mu\text{mole maltose} \times \text{dilution factor}}{\text{g sample}}$$

α- and β-Amylase in sweet potato puree were measured by using the same procedures described earlier in the model systems. However, phosphate buffer, pH 6.0, as described by Ikemiya and Deobald (1966) was used to determine α-amylase activity. The formula used to calculate crude α- and β-amylase activity at 25°C was:

$$\text{Units of activity} = \frac{\frac{\mu\text{mole M} - \text{NOM}}{\text{min}} \times \text{DF}}{\text{g sample}}$$

where: M = Maltose; NOM = Naturally Occurring Maltose; and DF = Dilution Factor.

Storage temperature and storage time

Four storage temperatures, namely 4°, −13°, −18° and −23°C were employed throughout the experiment.

Storage times of the model sysems varied among the different storage temperatures studied. Treated samples held at 4°C were kept at that temperature for only 0, 15, 30, 45, 60 and 75 min before being analyzed for enzyme activity. Samples stored at −13°C were kept for 0, 1, 2 and 3 days while those stored at −18° and −23°C were kept for 0, 28, 56, 84 and 112 days.

Samples for the sweet potato puree system were stored at the same temperatures as in the model systems. α- and β-Amylase and maltose were extracted after 0, 14, 28, 42 and 56 days storage at those temperatures.

The zero storage time samples were those which were treated with enzymes. They were immediately frozen by the rapid freezing technique, and enzyme activity was immediately determined. A representative amount of sweet potato puree was frozen by the same method used for the model systems. α- and β-Amylase and maltose were immediately extracted. The zero storage time results were used as controls to compare with those samples which had been stored.

Enzyme inactivation after storage

Of primary importance when samples were removed from storage was immediate inactivation of the enzymes. This was to insure that subsequent activity measurement reflected activity during storage and not after samples were removed from storage.

In the model systems, when the samples were removed from storage, their enzyme activities were terminated by immediately adding 3,5-dinitrosalicylic acid solution, and then the rest of the procedures followed.

The stability of the α- and β-amylase in the sweet potato puree sytem was the main objective for a part of this study; therefore, the enzyme was not inactivated after the samples were removed from storage. They were promptly prepared for water extractions of α- and β-amylase and maltose.

Analysis of research data

Triplicate samples were used throughout the experiment. Research

Table 5—Comparative effect of enzyme concentration at −13°C on activity of purified swine pancreatic α-amylase measured as accumulated maltose after equal storage times, using a 2% soluble starch substrate

Enzyme conc	Accumulated maltose (μmoles maltose)[a] Storage time (days)			
	0	1	2	3
1.0 μg/ml	0.1487	0.7702	1.4915	1.8338
1.5 μg/ml	0.1563	1.1489	2.2515	2.8487
Mean difference[b]	0.0076	0.3787**[c]	0.7600**	1.0149**
0.5 μg/ml	0.1377	0.4539	0.7894	1.0489
1.0 μg/ml	0.1487	0.7702	1.4915	1.8338
Mean difference[b]	0.0110	0.3163**	0.7021**	0.7849**
0.5 μg/ml	0.1377	0.4539	0.7894	1.0489
1.5 μg/ml	0.1563	1.1489	2.2515	2.8487
Mean difference[b]	0.0186	0.6950**	1.4621	0.7998**

[a] Data are mean of three replicates.
[b] Tabulated L.S.D. values of enzyme concentrations X storage time required for significance at 5% and 1% levels were 0.0237 and 0.0322, respectively.
[c] Asterisks indicate that means are statistically different at 5% (*) or 1% (**) level.

Table 6—Comparative effect of enzyme concentration at −13°C on activity of purified sweet potato β-amylase measured as accumulated maltose after equal storage times, using a 2% soluble starch substrate

Enzyme conc	Accumulated maltose (μmoles maltose)[a] Storage time (days)			
	0	1	2	3
1.0 μg/ml	0.1413	1.0799	1.4244	1.7161
1.5 μg/ml	0.1590	1.4352	1.9128	2.3085
Mean difference[b]	0.0177	0.3553**[c]	0.4884**	0.5924**
0.5 μg/ml	0.0893	0.6028	0.8307	1.0222
1.0 μg/ml	0.1413	1.0799	1.4244	1.7161
Mean difference[b]	0.0520*	0.4771**	0.5937**	0.6939**
0.5 μg/ml	0.0893	0.6028	0.8307	1.0222
1.5 μg/ml	0.1590	1.4352	1.9128	2.3085
Mean difference[b]	0.0697**	0.8324**	1.0821**	1.2863**

[a] Data are mean of three replicates.
[b] Tabulated L.S.D. values of enzyme concentrations X storage time required for significance at 5% and 1% levels were 0.0476 and 0.0647, respectively.
[c] Asterisks indicate that means are statistically different at 5% (*) or 1% (**) level.

Table 7—Effect of storage time at −18° C on accumulated maltose at different concentrations of purified swine pancreatic α-amylase and purified sweet potato β-amylase with 2% soluble starch substrate

Storage time (days)	Accumulated maltose (μmoles maltose)[a] α-Amylase conc (μg/ml)			Accumulated maltose (μmoles maltose)[a] β-Amylase conc (μg/ml)		
	0.5	1.0	1.5	0.5	1.0	1.5
0	0.0893 a	0.1487 a	0.1563 a	0.0893 a	0.1413 a	0.1590 a
28	0.2173 b	0.3579 b	0.5799 b	0.3477 b	0.5463 b	0.5995 b
56	0.4877 c	0.6659 c	0.9333 c	0.4312 c	0.6229 c	0.7643 c
84	0.5518 d	0.9456 d	1.1560 d	0.5551 d	0.7976 d	1.0132 d
112	0.6556 e	1.1582 e	1.3816 e	0.7005 e	0.9633 e	1.1887 e

[a] Data are mean of three replicates. Means within columns followed by a common letter are not significantly different at 5% level as determined by Duncan's Multiple Range Test.

data were analyzed statistically. Statistical analysis was conducted by using a combined analysis of the Randomized Complete Block design, analysis of variance, Least Significant Difference Test, and Duncan's Multiple Range Test.

RESULTS & DISCUSSION

α- and β-Amylase activity in model systems at 4°, −13° and −18°C

Tables 1 through 9 present data on the effects of holding or storage time, storage temperature, and enzyme concentration on α- and β-amylase activity as measured by accumulated maltose. Analyses of the data show that at 4°, −13° and −18°C the accumulated maltose at all concentrations of α- and of β-amylase studied generally increased with storage time (Tables 1, 4, and 7).

Data in the tables also show that the increase in accumulated maltose at all time intervals was statistically significant as determined by Duncan's Multiple Range Test.

α- and β-Amylase at different levels of concentration and storage times reacted differently. In general, as the enzyme concentration increased, there was a statistically significant increase in the rate of product formation as determined by the Least Significant Difference Test (Tables 2, 3, 5, 6, 8, and 9). Enzyme concentration was probably the limiting factor affecting the rate of maltose accumulation. Thus the activity was lower in the lower concentration treatments at the end of all storage periods.

α- and β-Amylase activity in the model system at −23°C

The accumulated maltose showed a trend to increase with storage time (Table 10). However, the increase in activity at 28-day intervals, as shown by maltose accumulation, was not statistically significant at all levels studied with the exception of 0 5 μg concentration, where α-amylase activity at the end of 112 days of storage was significantly higher than that at 0, 28 and 56 days storage periods. A similar trend for β-amylase, though not statistically significant, was observed at 28-day intervals up to 56 days of storage. The increase in activity for

Table 8—Comparative effect of enzyme concentration at −18° C on activity of purified swine pancreatic α-amylase measured as accumulated maltose after equal storage times, using a 2% soluble starch substrate

Enzyme conc	Accumulated maltose (μmoles maltose)[a] Storage time (days)				
	0	28	56	84	112
1.0 μg/ml	0.1487	0.3579	0.6659	0.9456	1.1582
1.5 μg/ml	0.1563	0.5799	0.9333	1.1560	1.3816
Mean difference[b]	0.0076	0.2220**[c]	0.2674**	0.2104**	0.2234**
0.5 μg/ml	0.0893	0.2173	0.4877	0.5518	0.6556
1.0 μg/ml	0.1487	0.3579	0.6659	0.9456	1.1582
Mean difference[b]	0.0594*	0.1406**	0.1782**	0.3938**	0.5026**
0.5 μg/ml	0.0893	0.2173	0.4877	0.5518	0.6556
1.5 μg/ml	0.1563	0.5799	0.9333	1.1560	1.3816
Mean difference[b]	0.0670**	0.3626**	0.4456**	0.6042**	0.7260**

[a] Data are mean of three replicates.
[b] Tabulated L.S.D. values of enzyme concentrations X storage time required for significance at 5% and 1% levels were 0.0472 and 0.0635, respectively.
[c] Asterisks indicate that means are statistically different at 5% (*) or 1% (**) level.

Table 9—Comparative effect of enzyme concentration at −18° C on activity of purified sweet potato β-amylase measured as accumulated maltose after equal storage times, using a 2% soluble starch substrate.

Enzyme conc	Accumulated maltose (μmoles maltose)[a] Storage time (days)				
	0	28	56	84	112
1.0 μg/ml	0.1413	0.5463	0.6229	0.7976	0.9633
1.5 μg/ml	0.1590	0.5995	0.7643	1.0132	1.1887
Mean difference[b]	0.0177	0.0532*[c]	0.1414**	0.2156**	0.2254**
0.5 μg/ml	0.0893	0.3477	0.4312	0.5551	0.7005
1.0 μg/ml	0.1413	0.5463	0.6229	0.7976	0.9633
Mean difference[b]	0.0520*	0.1986**	0.1917**	0.2425**	0.2628**
0.5 μg/ml	0.0893	0.3477	0.4312	0.5551	0.7005
1.5 μg/ml	0.1590	0.5995	0.7643	1.0132	1.1887
Mean difference[b]	0.0697**	0.2518**	0.3331**	0.4581**	0.4882**

[a] Data are mean of three replicates.
[b] Tabulated L.S.D. values of enzyme concentrations X storage time required for significance at 5% and 1% levels were 0.0500 and 0.0674, respectively.
[c] Asterisks indicate that means are statistically different at 5% (*) or 1% (**) level.

Table 10—Effect of storage time at −23°C on accumulated maltose at different concentrations of purified swine pancreatic α-amylase and purified sweet potato β-amylase with 2% soluble starch substrate

Storage time (days)	Accumulated maltose (μmoles maltose)[a] α-Amylase conc (μg/ml)			Accumulated maltose (μmoles maltose)[a] β-Amylase conc (μg/ml)		
	0.5	1.0	1.5	0.5	1.0	1.5
0	0.0893 a	0.1487 a	0.1563 a	0.0893 a	0.1413 a	0.1590 a
28	0.0893 a	0.1520 a	0.1523 a	0.0820 a	0.1303 a	0.1760 a
56	0.0967 a	0.1447 a	0.1660 a	0.0893 a	0.1663 a	0.2080 a
84	0.1377 ab	0.1770 a	0.1950 a	0.1917 b	0.2880 b	0.3364 b
112	0.1660 b	0.2113 a	0.2173 a	0.1737 b	0.3173 b	0.3499 b

[a] Data are mean of three replicates. Means within columns followed by a common letter are not significantly different at 5% level as determined by Duncan's Multiple Range Test.

β-amylase after 84 and 112 days of storage was statistically different from that at 0, 28 and 56 days of storage. There was no statistical difference in β-amylase activity between 84 and 112 days of storage.

Maltose accumulation increased as enzyme concentration increased. The amount of accumulated maltose was still lower in the lower enzyme concentration treatments. α- and β-Amylase in the model systems were still active at temperatures as low as −23°C. However, rates of maltose accumulation were significantly lower as storage temperatures decreased for both swine pancreatic α-amylase and sweet potato β-amylase in the model systems (Tables 11 and 12).

α- and β-Amylase activity in sweet potato puree samples at 4°, −13°, −18° and −23°C

Table 13 indicates that the cured and noncured sweet potatoes of the Centennial variety which were pureed and stored at 4°C for a period of 14, 28, 42 and 56 days showed an increase in the amount of accumulated maltose over that of the zero storage time sample. The increase reached its peak at 28 days of storage and decreased thereafter. The decrease in accumulated maltose could be due to growth of microorganisms. Microbiological growth could also cause a change in the chemical composition of the sweet potato puree. The pureed samples stored at −13°, −18° and −23°C for 14, 28, 42 and 56 days, showed no statistically significant difference in amount of accumulated maltose during the 56 days of storage.

In cured and noncured sweet potatoes the amount of accumulated maltose at −13°, −18° and −23°C was significantly different from that observed at 4°C (Tables 14 and 15). Apparently, α- and β-amylase remained essentially inactive at storage temperatures of −13°, −18° and −23°C.

Table 11—Comparative effect of storage temperature on activity of different concentrations of purified swine pancreatic α-amylase measured as accumulated maltose after equal storage times, using a 2% soluble starch substrate

Enzyme conc	Accumulated maltose (μmoles maltose)[a] Storage time (days)				
	0	28	56	84	112
0.5 μg/ml					
at −18°C	0.0893	0.2173	0.4877	0.5518	0.6556
at −23°C	0.0893	0.0893	0.0967	0.1377	0.1660
Mean difference[b]	0.0	0.1280**[c]	0.3910**	0.4141**	0.4896**
1.0 μg/ml					
at −18°C	0.1487	0.3579	0.6659	0.9456	1.1582
at −23°C	0.1487	0.1520	0.1447	0.1770	0.2113
Mean difference[b]	0.0	0.2059**	0.5212**	0.7686**	0.9469**
1.5 μg/ml					
at −18°C	0.1563	0.5799	0.9333	1.1560	1.3816
at −23°C	0.1563	0.1523	0.1660	1.1950	0.2173
Mean difference[b]	0.0	0.4276**	0.7673**	0.9610**	1.1643**

[a] Data are mean of three replicates.
[b] Tabulated L.S.D. values of storage temperatures X storage time required for significance at 5% and 1% levels were 0.0541 and 0.0721, respectively.
[c] Asterisks indicate that means are statistically different at 5% (*) or 1% (**) level.

Table 12—Comparative effect of storage temperature on activity of different concentrations of purified sweet potato β-amylase measured as accumulated maltose after equal storage times, using a 2% soluble starch substrate

Enzyme conc	Accumulated maltose (μmoles maltose)[a] Storage time (days)				
	0	28	56	84	112
0.5 μg/ml					
at −18°C	0.0893	0.3477	0.4312	0.5551	0.7005
at −23°C	0.0893	0.0820	0.0893	0.1917	0.1737
Mean difference[b]	0.0	0.2657**[c]	0.3419**	0.3634**	0.5268**
1.0 μg/ml					
at −18°C	0.1413	0.5463	0.6229	0.7976	0.9633
at −23°C	0.1413	0.1303	0.1663	0.2880	0.3173
Mean difference[b]	0.0	0.4160**	0.4566**	0.5096**	0.6460**
1.5 μg/ml					
at −18°C	0.1590	0.5995	0.7643	1.0132	1.1887
at −23°C	0.1590	0.1760	0.2080	0.3364	0.3499
Mean difference[b]	0.0	0.4235**	0.5563**	0.6768**	0.8388**

[a] Data are mean of three replicates.
[b] Tabulated L.S.D. values of storage temperatures X storage time required for significance at 5% and 1% levels were 0.0541 and 0.0721, respectively.
[c] Asterisks indicate that means are statistically different at 5% (*) or 1% (**) level.

Table 13—Effect of storage time at different temperatures on accumulated maltose in cured and noncured sweet potatoes of the Centennial variety

Storage time (days)	Cured sweet potatoes Accumulated maltose (μmoles maltose/g puree)[a] Storage temperature				Noncured sweet potatoes Accumulated maltose (μmoles maltose/g puree)[a] Storage temperature			
	4°C	−13°C	−18°C	−23°C	4°C	−13°C	−18°C	−23°C
0	238.30 a	238.30 a	238.30 a	238.30 ab	131.25 a	131.25 a	131.25 a	131.25 a
14	289.70 b	229.80 b	221.65 b	229.80 a	170.00 b	131.63 a	133.10 ab	132.39 ac
28	311.70 c	249.63 c	249.63 c	246.80 b	206.29 c	137.21 b	132.39 ac	135.32 bc
42	302.77 d	242.55 ac	231.33 a	235.47 a	122.91 d	137.56 b	136.08 bc	133.10 ab
56	252.47 e	243.97 ac	235.47 a	229.80 a	80.78 e	136.43 b	136.45 b	136.81 b

[a] Data are mean of three replicates. Means within columns followed by a common letter are not significantly different at 5% level as determined by Duncan's Multiple Range Test.

Table 14—Comparative effect of storage temperature on accumulated maltose in cured sweet potatoes of the Centennial variety after equal storage times

Storage temperature	Accumulated maltose (μmoles maltose/g puree)[a] Storage time (days) 0	14	28	42	56
4°C	238.30	289.70	311.70	302.77	252.47
−13°C	238.30	229.80	249.63	242.55	243.97
Mean difference[b]	0.0	59.90**[c]	62.07**	60.22**	8.50*
4°C	238.30	289.70	311.70	302.77	252.47
−18°C	238.30	221.65	249.63	231.33	235.47
Mean difference[b]	0.0	68.05**	62.07**	71.44**	17.00**
4°C	238.30	289.70	311.70	302.77	252.47
−23°C	238.30	229.80	246.80	235.47	229.80
Mean difference[b]	0.0	59.90**	64.90**	67.30**	22.67**

[a] Data are mean of three replicates.
[b] Tabulated L.S.D. values of storage temperatures X storage time required for significance at 5% and 1% levels were 8.5054 and 11.3798, respectively.
[c] Asterisks indicate that means are statistically different at 5% (*) or 1% (**) level.

Table 15—Comparative effect of storage temperature on accumulated maltose in non-cured sweet potatoes of the Centennial variety after equal storage times

Storage temperature	Cumulative maltose (μmoles maltose/g puree)[a] Storage time (days) 0	14	28	42	56
4°C	131.25	170.00	206.29	122.91	80.78
−13°C	131.25	131.63	137.21	137.56	136.43
Mean difference[b]	0.0	38.37**[c]	69.08**	14.65**	55.65**
4°C	131.25	170.00	206.29	122.91	80.78
−18°C	131.25	133.10	132.39	136.08	136.45
Mean difference[b]	0.0	36.90**	73.90**	13.17**	55.67**
4°C	131.25	170.00	206.29	122.91	80.78
−23°C	131.25	132.39	135.32	133.10	136.81
Mean difference[b]	0.0	37.61**	70.97**	10.19**	56.03**

[a] Data are mean of three replicates.
[b] Tabulated L.S.D. values of storage temperatures X storage time required for significance at 5% and 1% levels were 3.7160 and 4.9720, respectively.
[c] Asterisks indicate that means are statistically different at 5% (*) or 1% (**) level.

Table 16—Effect of storage time at different temperatures on activity of α-amylase and β-amylase in cured sweet potatoes of the Centennial variety

Storage time (days)	α-Amylase — Units of activity (μmoles maltose/min/g puree at 25°C)[a] Storage temperature				β-Amylase — Units of activity (μmoles maltose/min/g puree at 25°C)[a] Storage temperature			
	4°C	−13°C	−18°C	−23°C	4°C	−13°C	−18°C	−23°C
0	550.27 a	550.27 a	550.27 b	550.27 b	569.81 a	569.81 a	569.81 a	569.81 a
14	561.04 a	555.84 a	608.71 a	593.38 a	534.14 d	570.68 a	573.47 a	569.18 a
28	559.30 a	578.50 a	575.02 d	594.32 a	555.49 ad	576.37 a	576.20 a	581.88 a
42	40.11 b	574.94 a	597.29 ad	616.38 a	28.25 b	576.13 a	555.43 a	575.48 a
56	15.77 c	566.91 a	545.14 b	527.58 b	21.74 b	567.69 a	553.58 a	525.68 b

[a] Data are mean of three replicates. Means within columns followed by a common letter are not significantly different at 5% level as determined by Duncan's Multiple Range Test.

Table 17—Effect of storage time at different temperatures on activity of α-amylase and β-amylase in noncured sweet potatoes of the Centennial variety

Storage time (days)	α-Amylase — Units of activity (μmoles maltose/min/g puree at 25°C)[a]				β-Amylase — Units of activity (μmoles maltose/min/g puree at 25°C)[a]			
	Storage temperature				Storage temperature			
	4°C	−13°C	−18°C	−23°C	4°C	−13°C	−18°C	−23°C
0	411.66 a	411.66 a	411.66 a	411.66 a	399.13 a	399.13 a	399.13 a	399.13 a
14	409.04 a	375.99 b	362.97 b	381.61 b	359.41 b	365.89 b	339.08 b	352.37 bc
28	167.76 d	390.22 ab	395.67 a	395.96 ab	146.29 c	380.58 ab	378.48 ac	379.95 ac
42	22.74 b	410.81 a	405.01 a	389.15 ab	11.72 d	384.26 ab	376.55 c	370.07 c
56	9.57 b	408.27 a	408.26 a	406.88 ab	9.55 d	385.10 ab	381.30 ac	382.53 ac

[a] Data are mean of three replicates. Means within columns followed by a common letter are not significantly different at 5% level as determined by Duncan's Multiple Range Test.

Effect of storage conditions on the stability of α- and β-amylase in sweet potato puree

α- and β-Amylase were extracted from cured and noncured sweet potato puree samples. At 4°C, the enzymes were still active after storing the noncured puree samples for 14 days and the cured samples for 28 days (Tables 16 and 17). Aging effects and microbiological growth in the puree samples could have contributed to the decrease in α- and β-amylase stability after prolonged storage at 4°C. Visual observation indicated that spoilage of the samples from noncured sweet potatoes occurred more readily than in samples from cured roots. Higher microbiological loads of the samples from noncured roots may have been the reason. Both α- and β-amylase activities decreased approximately ninefold after 42 and 56 days of storage and were statistically significant from the activities observed after 0, 14 and 28 days of storage. In samples from noncured roots the activities of both enzymes decreased considerably after 28, 42 and 56 days, and were statistically different from the activities observed in samples stored for 0 and 14 days.

α- and β-Amylase were still stable in samples of purees prepared from cured and noncured sweet potatoes after storage at −13°, −18° and −23°C for 14, 28, 42 and 56 days.

The activity of α- and β-amylase from samples of cured and noncured roots stored at 4°, −13°, −18° and −23°C after 14, 28, 42 and 56 days was compared. It was found that the activity of both enzymes extracted after at least 28 days of storage at 4°C was significantly lower than that of samples stored at −13°, −18° and −23°C. There was no statistically significant difference in the stability of the enzymes from samples stored at −13°, −18° and −23°C.

Comparison of α- and β-amylase activity in the model systems and in the sweet potato puree system

As storage temperatures were lowered, it was anticipated that enzyme activity would decrease. Such was the case in the model systems (Tables 1, 4, 7 and 10). In the sweet potato puree system, a decrease in enzyme activity was also observed when storage temperature was lowered from 4° to −13°C (Table 13). However, no enzyme activity was detected at −13°C and lower storage temperatures.

Natural α- and β-amylase in the sweet potato puree acted differently from the purified enzymes in the model systems. α and β-Amylase present in the sweet potato system were more sensitive to low temperature treatments and showed no activity. In the model systems a measurable amount of activity was still observed at −23°C.

In accordance with the Q_{10} theory, reaction rates are lower when temperatures decrease. Results of this study indicate that the enzymes in the model systems appear to follow this theory more closely than the enzymes in the sweet potato puree system. The higher chemical complexity of the sweet potato puree system may be a factor contributing to the different behavior of the two systems. It is known that some enzymes exist in the total system that regulate other enzymes. Consequently, results obtained are not to be unexpected. This could be explained also by having the product of enzyme hydrolysis either being active in a "feed-back" inhibition or by having the product affect amylase or another enzyme directly or indirectly.

If starchy foods in general behave like sweet potatoes, at −13°C amylase activity would be essentially negligible in those foods.

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CARBONYL-PROPYLENE GLYCOL INTERACTIONS IN FLAVOR SYSTEMS

ABSTRACT

Variable organoleptic qualities and GC composition of a commercial cherry flavor were traced by GC-MS to four variable components, benzaldehyde, tolualdehyde and their corresponding propylene glycol acetals. The formation of flavorless acetals was shown to consume 40–70% of the most important cherry flavor components between manufacture and use. The formation of acetals in propylene glycol flavor solvents was investigated with a variety of flavor-important aldehydes. Acetals are quite stable under neutral and alkaline conditions and are only hydrolyzed by dilute mineral acids or moderate heat treatment. Utilization of flavors containing an unsuspected and unknown amount of acetal in food applications where hydrolysis does not take place can result in variable product flavor quality.

INTRODUCTION

A CONSISTENT and reproducible flavor system is an important consideration in the formulation of a food product. This reproducibility can only be achieved if the flavoring material used is of consistent quality and composition. In most commercial artificial and natural flavors, a complex blend of chemical substances are compounded to give a desired flavor effect. These concentrated mixtures usually are composed of components having a variety of chemical functional groups in an appropriate solvent and interactions between them are possible. In many instances flavor interactions are desirable, i.e., aging of wine (Stevens et al., 1969), brandy etc. but if these interactions are not desirable or unknown, serious alteration of a desired flavor composition can occur.

It is the purpose of this communication to discuss one such known interaction between flavor components, acetal formation, and describe methods to determine its presence and effect on finished product.

EXPERIMENTAL

GAS CHROMATOGRAPHIC analysis was performed on a Hewlett-Packard 7620A equipped with flame ionization detectors and 3370B electronic integrator. All runs were made on either of two columns:

(1) 1/8 in. × 10 ft, 10% (w/w) Carbowax 20M on Gas Chrom Q programmed from 85°C to 220°C at 4°/min.
(2) 1/8 in. × 10 ft., 10% SF-96 on Gas Chrom Q programmed from 135°C to 220°C at 4°/min.

Flow rates were 30 cc/min of nitrogen, 35 cc/min of hydrogen and 250 cc/min of air.

Mass spectrometry was performed on a Hewlett-Packard 5930A quadrupole mass spectrometer coupled to a 5700 series gas chromatograph via a silicone membrane interface. All spectra were obtained at an electron energy of 70 eV and ion source temperature of 220°C. GC conditions and columns were identical to those above.

Sample preparation

1,2-propandiol (BP. 186–187°C) was purchased from Matheson, Coleman and Bell. All the aldehydes used were the purest grade commercially available. The original commercial cherry flavor samples were diluted to 5% v/v with ethanol for gas chromatographic analysis.

Development of acetals was studied in a system that contains 2% of the appropriate aldehyde in a 9:1 propylene glycol:H_2O solvent with and without the presence of 0.05% W/V citric acid. Solutions were made in 10 cc volumetric flasks and stored at ambient temperature in the absence of light for 7 days. GC and mass spectrometry was performed on the solutions directly.

RESULTS & DISCUSSION

DURING THE DEVELOPMENT of a product containing a commercial liquid artificial cherry flavor, variable organoleptic properties were found between two lots of flavor. These lots differed in age with one being fresh (2 wk old) and the other 3 months old. They had been stored in brown bottles at ambient

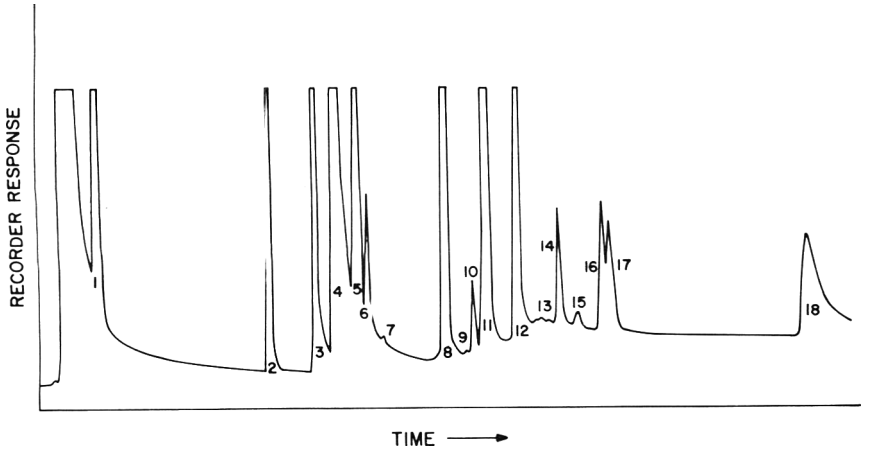

Fig. 1—Gas chromatographic analysis of commercial cherry flavor on C-20M.

temperature during this period. The differences were primarily in organoleptic strength with some difference in character notes. The fresher sample was stronger in flavor when tasted in a 6% sucrose-milk medium and also in the finished product. Figure 1 shows a gas chromatogram of the cherry flavor on a C-20M column. The total integrated areas of peaks 3, 5, 8 and 11 were quantitatively variable and all other peaks were quantitatively constant between the two samples. It was also found that in the fresh sample, peaks 3 and 5 were 35% larger and peaks 8 and 11 were correspondingly smaller than those in the 3 month old sample. In addition, the sums of peaks 3 + 8 and 5 + 11 were constant between the two samples.

GC-MS analysis confirmed peaks 3 and 5 as benzaldehyde and tolualdehyde and gave the spectra in Figure 2 for peaks 8 and 11 respectively. From the fragmentation pattern and molecular weight, these were identified as the propylene glycol acetals of benzaldehyde and tolualdehyde and verified with authentic acetals. Four other commercial cherry flavors in propylene glycol were examined for acetal content and were found to contain some acetal.

These results can readily explain the variable organoleptic properties of the two original lots of cherry flavor. Since these acetals are essentially odorless and flavorless (Arctander, 1969), there is considerably more flavor intensity to be expected from the fresh sample. An inquiry to the flavor manufacturer revealed neither of the acetals had been added to the flavor composition and therefore, had been formed between manufacture and use.

In order to examine the scope of the problem, 2% solutions of various flavor-important aldehydes were allowed to stand in 9:1 propylene glycol:H_2O for 7 days and were examined by GC-MS for acetal content.

Table 1 summarizes the relative ratios of acetal to aldehyde found in the solutions in 7 days. In all cases acetals did form and in the presence of 0.05% citric acid formed to a greater degree. The data shown are only relative in that it is the ratio of integrator counts present under each peak. The presence of propylene glycol acetal in the new peak formed was verified by mass spectrometry in each case.

For comparison purposes, the ratio of acetal to benzaldehyde for the 3 month old commercial cherry flavor was 2.34 and for the fresher sample was 1.28. These flavors did not contain citric acid. Differences in original aldehyde concentration would change the rate of acetal formation and the position of equilibrium. Even a ketone, 2-heptanone, formed a propylene glycol ketal under acid catalysis conditions.

Table 2 details some of the analytical parameters obtained from the GC-MS analysis of the acetal forming solutions. Under the GC conditions employed, the retention time of each corresponding acetal is greater than that of the aldehyde. The relative retention time of each acetal to the corresponding aldehyde is summarized in Table 2.

Table 1—Ratio of amounts of acetal formed in 7 days

Aldehyde	PG:H_2O Acetal / Aldehyde	PG:H_2O:Citric Acetal / Aldehyde
iso-valeraldehyde	0.20	1.05
n-octanal	0.04	0.89
neral	0.09	0.23
geranial	0.10	0.19
vanillin	0.02	0.13
benzaldehyde	0.18	1.27
tolualdehyde	0.07	—
cuminic aldehyde	0.02	0.09
p-t-butyl benzaldehyde	0.03	0.09
cinnamaldehyde	0.22	0.31
2-heptanone	0.00	0.12

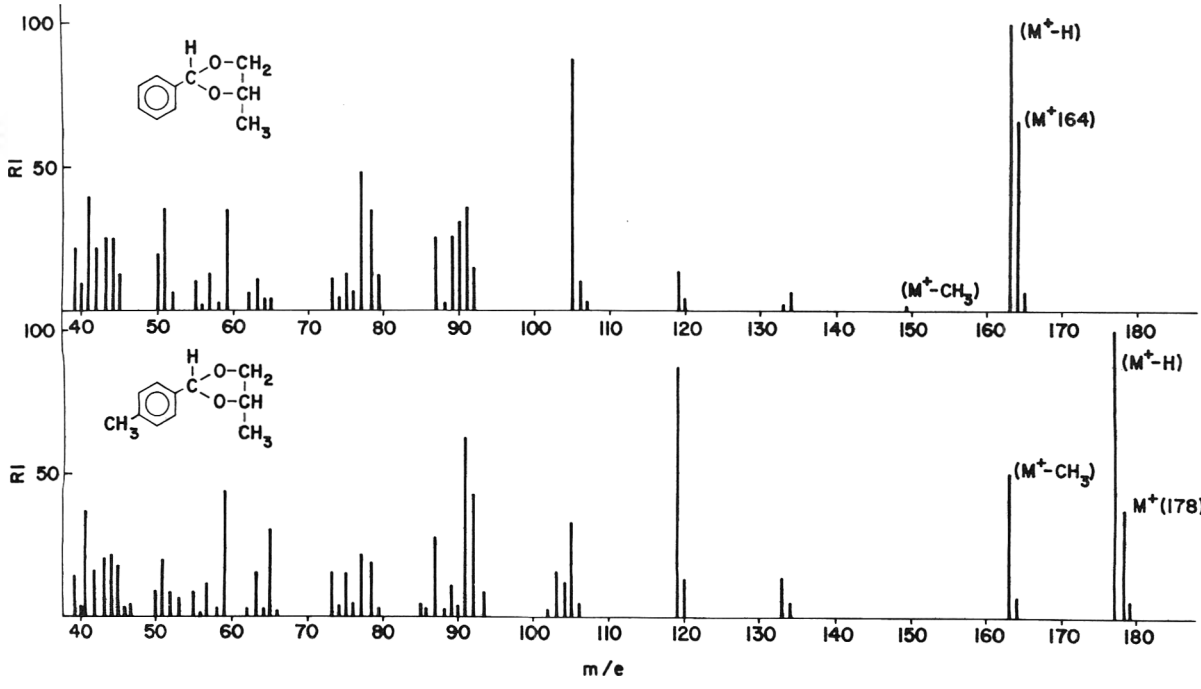

Fig. 2—Mass Spectra of Figure 1 peak 8 (upper) and peak 11 (lower).

Table 2—GC-MS characteristics of propylene glycol acetals

PG Acetal	RRT Acetal/Aldehyde SF-96	C-20M	Characteristic MS peaks[c]
benzaldehyde	2.94	1.45	Fig. 2 (upper)
tolualdehyde	–	1.39	Fig. 2 (lower)
cuminic aldehyde	1.91	–	205[a](100)[b], 147(85), 163(70), 105(54), 206(25)
p-t-butyl benzaldehyde	1.76	–	219(100), 119(68), 161(67), 163(56), 220(35), 91(30)
cinnamic	–	1.27	104(100), 190(85), 115(41), 131(38), 107(17), 77(12), 78(10), 189(9)
valeraldehyde	–	2.40	87(100), 31(90), 41(87), 45(84), 59(68), 43(65), 43(65), 143(9)
iso-valeraldehyde	–	2.69	87(100), 31(82), 41(73), 59(66), 43(32), 143(5)
octanal	–	1.62	87(100), 41(47), 31(40), 59(38), 43(13), 185(7)
neral	1.64	–	41(100), 87(84), 69(59), 55(47), 141(38), 59(32), 209(4)
geranial	1.80	–	41(100), 87(87), 69(57), 55(48), 141(38), 59(32), 209(4)
2-heptanone	–	1.43	113(100), 101(53), 41(18), 157(16), 45(15), 99(12)

[a] m/e
[b] Relative abundance
[c] Parent ion underlined

The mass spectra of the acetals obtained from these experiments are also summarized in Table 2. The acetals formed from aromatic aldehydes are characterized by strong molecular ions (M^+) and a base peak at M^+-1 Which is typical for 1,3-dioxolan compounds (Porter and Baldas, 1971). In the series of p-alkyl-substituted benzaldehyde acetals, the p-substituent is lost to give the stable 2-phenyl, 4-methyl, 1,3-dioxolan ion (m/e 163) in all cases examined where the phenyl ring is in the 2-position on the dioxolan ring. The acetal spectra obtained from the alkyl aldehydes are typical of that expected for alkyl-substituted 1,3-dioxolans. Weak or nonexistent parent ions are observed and weak M^+-1 ions are seen.

The formation of acetals from aldehydes and propylene glycol is an equilibrium phenomena and is catalyzed by acids but not bases (Roberts and Caserio, 1964). Therefore, in flavoring compositions, acetal formation can be retarded or stopped by the use of neutral or slightly basic conditions. A patent on the use of $NaHCO_3$ to stabilize propylene glycol solutions of carbonyl-based flavorings has been issued (Levinson et al., 1968).

Our results indicate that small amounts of citric acid hasten acetal formation in flavor systems but in product usage, acidic conditions and heat would favor hydrolysis and regenerate full flavor effect. This can be readily demonstrated by adding 0.5% cherry flavor containing acetal to a soft drink type solution containing 11% sucrose and 0.8% citric acid. The solution was stirred, extracted with ethyl ether and gas chromatography of the ether extract showed no traces of acetal. However, in products at neutral pH, lacking heat processing or containing both water and oil phases, total hydrolysis may not occur and variable batch-to-batch flavoring in product can occur. This was the case in the product where the initial observation was made. The flavoring was added to a water-vegetable oil mixture and presumably the acetal was more soluble in the oil environment and did not hydrolyze under these conditions.

The presence of an unknown and unsuspected amount of acetal in flavoring compositions can lead to serious nonuniformity in the flavor of finished products. Of special concern are products produced under conditions which do not allow hydrolysis of the acetal back to the free aldehyde. The production of a continually uniform flavor in food products is especially dependent on the uniformity of the flavoring components. Flavor component-solvent interactions should be evaluated as to their effect on the finished product flavor and quality control techniques should be sensitive enough to detect these interactions.

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PRODUCTION OF FLAVOR VOLATILES IN ENZYME AND SUBSTRATE ENRICHED PEANUT HOMOGENATES

ABSTRACT

The role of enzymes in the production of flavor volatiles from raw peanuts was investigated through the use of enzyme- and substrate-enriched peanut homogenates. Enzymatic control of the production of the flavor volatiles was shown by the generation of the volatiles upon addition of peanut acetone powders to an enzyme-inactivated sample. Lipoxygenase was shown to be primarily responsible for production of the flavor volatiles in model systems of purified peanut lipoxygenase and linoleic acid which produced gas chromatographic profiles almost identical to those of peanut homogenates. Typical lipoxygenase intermediates were demonstrated in extracted lipid material from raw peanut homogenates by UV absorption peaks at 234 nm and 275 nm, whereas heat-inactivated samples showed only trace absorption peaks. The optimum pH for production of the flavor volatiles was between 6.5 and 7.0. Pentane production was inhibited by propyl gallate, hydroquinone and ascorbic acid. Exogenous aliphatic alcohols added to peanut homogenates before blending were converted to their corresponding aldehydes, indicating that an alcohol-to-aldehyde conversion reaction exists in peanuts.

INTRODUCTION

MECHANISMS by which flavor components arise have interested flavor chemists for many years. Recently, attention has been focused upon enzymic reactions. Raw peanuts have a simple volatile profile, in which hexanal is the prime flavor contributor (Pattee et al., 1969), and offer a unique opportunity to study the enzymatic production of a complete flavor volatile profile. Suggestive evidence that the total profile might be enzymatically produced was provided by the correlation between volatile flavor components and levels of enzyme activity across maturation stages of the peanut seed (Pattee et al., 1970). Evidence in that study also suggested that pentane is a major product of the lipoxygenase reaction. Pattee et al. (1974) showed pentane to be a product of the peanut lipoxygenase-linoleic reaction. A lipoxygenase-mediated pentane-producing system from soybeans also has been characterized by Johns et al. (1973).

The role of lipoxygenase in the production of carbonyl compounds has been studied by Grosch and Schwencke (1969) who reported that pentanal, hexanal, hept-2-enal, oct-2-enal, nona-2,4-dienal and deca-2,4-dienal were produced by a pH 7 soybean lipoxygenase reacting with linoleic acid. Kazeniac and Hall (1970) reported that in tomatoes, hexanal and hexenals, important flavor contributing compounds, were enzymatically formed and Jadhav et al. (1972) found hexanal was produced when ^{14}C-labeled linoleic and linolenic acid were supplied as substrate. Carbonyl compounds produced by cucumber homogenates are also enzymatically formed and a requirement for molecular oxygen was demonstrated by Fleming et al. (1968). Grosch and Schwarz (1971) showed linoleic and linolenic acids to be precursors of carbonyl compounds responsible for cucumber flavor and attributed formation of these compounds to a lipoxygenase reaction. Grosch (1967) showed that a wide range of carbonyl compounds were produced when pea lipids were reacted with a crude pea enzyme isolate. Siddiqi and Tappel (1956) implicated lipoxygenase in the production of off-flavor components in underblanched peas.

The conversion of alcohols to aldehydes can be very important to the overall flavor balance in many food products. Meigh et al. (1966) showed that tomato tissues enzymatically produce acetaldehyde, propanal and acetone from the corresponding alcohol homologs. Similar results have been reported by MacLeod and MacLeod (1968) on cabbage volatiles and by Ralls et al. (1965) on green-pea volatiles.

This paper reports on the production of flavor volatiles by a lipoxygenase-mediated reaction in raw peanut homogenates and the conversion of exogenous alcohols to their corresponding aldehyde homologs.

EXPERIMENTAL

Preparation of acetone powders

Cured peanuts (variety NC-2) were obtained from the Peanut Belt Research Station, N.C. Dept. of Agriculture, Lewiston, N.C. and stored at 10°C and 70% RH until used. Acetone powders were prepared by extracting a 100g peanut sample three times with 500 ml of cold acetone (−20°C) per extraction in a Waring Blendor. The powders were collected on filter paper in a Buchner funnel and washed successively with 200 ml of cold acetone, 100 ml of cold ether, and 500 ml of ether at room temperature. Acetone powders were dried, placed in stoppered glass vessels, and stored at −20°C.

Sample preparation and gas liquid chromatography analysis

Control samples were prepared by boiling 110g of peanuts for 5 min, decanting the water, and air drying the sample on paper towels for 10 min. In some experiments, 5g of acetone powder were added to the control sample before blending. Control or raw samples (110g) were blended with 300 ml of distilled water for 1 min in a Waring Blendor and immediately placed in a 500-ml Erlenmeyer flask fitted with a septum-rubber stopper combination to facilitate vapor phase sampling. Samples used for determining the effect of pH on pentane and hexanal production were prepared by adjusting the pH of the distilled water with appropriate amounts of solid citric acid, concentrated HCl, or a 50% solution of NaOH before blending. The pH of the homogenate was measured after blending and recorded. In the alcohol conversion studies, each alcohol was added, 0.1 ml per 300 ml distilled water, before blending. Various concentrations of inhibitors and antioxidants were used in the inhibition study. For volatile development, samples were held at room temperature before being placed in a 65°C water bath for 5 min. Vapor phase sampling and analysis were by published procedures (Singleton et al., 1975). At least three replications were performed on all experiments. Within a given experiment the variation was about 5%.

Lipid extraction for UV spectral analysis

The methods of Galliard (1970) were used to extract lipids from control and raw peanuts. For the control, 110g of boiled peanuts were blended in 300 ml distilled water and immediately placed in refluxing MeOH-H_2O for 5 min; raw samples were stirred for 15 min after blending and then placed in refluxing MeOH-H_2O. The lipid fraction was dissolved in absolute EtOH (0.1g/100 ml) and scanned from 370 nm to 190 nm with a Coleman model 124 double beam spectrophotometer.

Enzyme purification and gel chromatography

Peanut lipoxygenase was extracted from acetone powders and purified as described (Pattee et al., 1974).

RESULTS & DISCUSSION

THE EXTENT of enzyme involvement in the production of a raw peanut flavor volatile profile was shown through a series of experiments (Table 1). The control sample formed only minute amounts of volatile compounds, but the addition of 5g of acetone powder increased the amount of volatiles. When water extracts of acetone powders, rather than the powder, were added to the control, volatile production increased, especially pentane and hexanal. When the water extract was heated at 100°C for 5 min and added to the control sample, the volatile profile was comparable to that of the control alone. The addition of linoleic acid to raw samples before blending increased production of pentane and hexanal, but not as much as might have been expected, probably because of inadequate dispersion of the fatty substrate in an aqueous medium. Kazeniac and Hall (1970) reported enhancement of hexanal when linoleic acid was added to tomato homogenates. The increase in production of pentane and hexanal by addition of linoleic acid to raw peanut homogenates suggested that lipoxygenase is involved. To elucidate the extent of lipoxygenase involvement a purified lipoxygenase preparation isolated from raw peanuts was added to a model system containing linoleic acid. The volatiles from the model system (Fig. 1A) were almost identical in peak position to those from the raw homogenate (Fig. 1B) indicating that peanut lipoxygenase is primarily responsible for the production of the flavor volatiles in raw peanuts.

Lipoxygenase reacts with unsaturated fatty acids with the 1,4-pentadiene linkage to produce hydroperoxides and subsequently carbonyl compounds, conjugated diene fatty acid derivatives, and dimeric fatty acids of the reaction products (Garssen et al., 1972). Absorbance at 234 nm has been used to measure hydroperoxide content in lipids, and also as a measure of lipoxygenase activity. Conjugated diene compounds absorb between 275 nm and 285 nm. UV spectra of the lipid material isolated from raw and control aqueous peanut homogenates (Fig. 2) differed distinctly. The absorption spectra of lipid material showed absorption peaks at 234 nm and 275 nm for the raw sample (Fig. 2A), but not for the control (Fig. 2B). Thus volatile profile analysis and UV spectral analysis of the lipid material from aqueous peanut homogenates (control and raw) suggested that the production of lipid hydroperoxides in aqueous peanut homogenates preludes the formation of the volatile profile and further implicates the enzyme lipoxygenase. Similar findings, with potato tubers, have been reported by Galliard (1970, 1972).

Antioxidants, chelates and enzyme inhibitors are used widely in the food processing industry. To further elucidate the enzymatic production of volatiles in aqueous peanut homogenates, selected chemical compounds of the above types, some of which are lipoxygenase inhibitors, were tested for their effects on volatile production. Results of inhibitor effects on production of volatiles are shown in Table 2. Reduction in the amount of pentane produced was used as a measure of inhibition since it has been shown to be lipoxygenase mediated (Johns et al., 1973). Propyl gallate, an effective lipoxygenase inhibitor in model systems (Siddiqi and Tappel, 1956), inhibited pentane production 58%. However, inhibitors are more effective in model than in natural systems possibly because of a protective effect of protein in food homogenates. Hydroquinone, an antioxidant, reduced pentane production 27%. Ethylenediaminetetraacetic acid (EDTA), a chelate, had no effect on pentane production, but increased the carbonyls. These results are similar to those reported by Jadhav et al. (1972) who found EDTA to be an ineffective inhibitor of lipoxygenase activity. Nordihydroguaiaretic acid (NDGA) did not inhibit pentane production. However, NDGA is relatively insoluble in aqueous systems and concentration may have been a factor. NDGA (0.4 mM) was reported by Khan (1961) to inhibit all types of oxidation in model systems. Ascorbic acid, widely used in the food industry as an antioxidant and chelate, is a competitive inhibitor of wheat lipoxygenase (Walsh et al., 1970). Pentane production was reduced 82% by 56.8 mM ascorbic acid, but lower concentrations had no effect. Hexanal, also lipoxygenase mediated (Pattee et al., 1974), was not inhibited by ascorbic acid, but appears to have been stimulated by concentrations up to 56.8 mM. Hexanal is produced almost instantaneously when peanuts are macerated (Singleton et al., 1975) and therefore would be difficult to inhibit, whereas pentane production requires more time to reach its maximum.

The pH levels of homogenates have been adjusted to produce bland soymilk (Kon et al., 1970), consistency in tomato products (Becker et al., 1972) and for extraction of high qual-

Table 1—Effects of acetone powder, aqueous acetone powder extracts and linoleic acid on volatile production in peanut homogenates[a]

	Acetaldehyde	Pentane	Pentanal	Hexanal
	Peak areas in integrator units X 10^{-3}			
Raw	13	168	16	115
Contro	1	2	7	22
Contro + 5g of acetone powder	23	131	12	35
Contro + water extract from 5g acetone powder	15	306	7	65
Contro + heat inactivated water extract from 5g acetone powder	9	15	4	24
Raw + 0.1 ml of linoleic acid	14	192	15	212

[a] Each value is the mean of three replications.

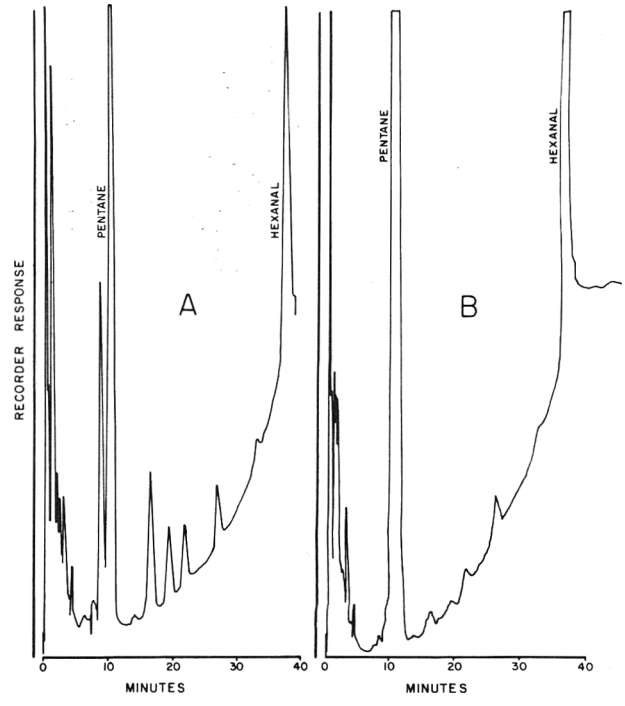

Fig. 1—Typical chromatogram of volatiles from a peanut lipoxygenase-linoleic acid model system (A) and a raw peanut homogenate (B).

ity protein and oil from aqueous peanut slurries (Rhee et al., 1972). The effect of pH on the production of pentane and hexanal in peanut homogenates is shown in Figure 3. Essentially no volatiles were produced below pH 3.5. Pentane production ceased between pH 8 and 9, while hexanal was still produced at pH 10. These results show that volatiles in peanut homogenates were produced over a pH range of 3.5 thru 10. The consistency of the homogenate also thickened with increasing pH over this range. Control of volatile production and consistency could be very important in the development of new peanut products.

Schormuller and Grosch (1965) reported that an aldehyde-alcohol relationship exists in tomato volatiles; further Meigh et al. (1966) showed that acetaldehyde, propionaldehyde and acetone are enzymatically produced from the corresponding alcohol. It was of interest in this study to determine whether an aldehyde-alcohol relationship existed in peanut homogenates. Data in Table 3 show that a homologous series of aliphatic alcohols were converted partially to the corresponding aldehydes. For each alcohol added to the peanut homogenate the concentration of the corresponding aldehyde increased in the volatile profile. Propanal and butanal were also produced from their alcohol homologs even though these aldehydes are not normally present in peanut volatiles. Hexanal production decreased when alcohols other than hexanol were added, possibly from partial inhibition of lipoxygenase by aliphatic alcohols (Mitsuda et al., 1967). These data suggest that an alcohol-aldehyde relationship may exist in the production of peanut volatiles.

Results of this study and/or previous work (Singleton et al., 1975) show that the flavor volatiles of raw peanuts are enzymatically produced and that molecular oxygen is required for the reaction. Inhibition of pentane production by certain inhibitors, requirement for molecular oxygen, and the fact that a lipoxygenase-linoleic acid model system produces a volatile profile almost identical to that found in raw peanut homogenates indicate that lipoxygenase mediates volatile production in raw peanuts. These results show that controlled enzyme reactions, certain antioxidants, and substrate specificity could effectively alter flavor volatiles of peanuts and thus might be useful in product development.

Table 2—Effects of antioxidants and chelates on production of volatiles by peanut homogenates[2]

Antioxidant chelate	Conc (mM)	Acetaldehyde	Pentanal	Hexanal	Pentane	Pentane Production (% of Control)
		Peak areas in integrator units X 10^{-3}				
None		40	23	258	210	100
Hydroquinone	1.5	17	20	274	153	73
Propyl gallate	1.5	34	16	273	88	42
NDGA	0.1	—	—	294	241	115
EDTA	1.5	64	31	391	227	108
Ascorbic acid	2.4	68	20	340	237	113
	18.9	32	36	367	229	109
	56.8	21	57	405	38	18
	94.7	3	28	256	5	2

[a] Each value is the mean of three replications.

Table 3—Conversion of exogenous alcohols to aldehydes by peanut homogenates[a]

Added alcohol (0.1 ml/300 ml vol)	Acetaldehyde	Propionaldehyde	Butanal	Pentane	Pentanal	Hexanal
	Peak areas in integrator units X 10^{-3}					
None	12	—	—	205	16	194
Ethanol	81	—	—	204	12	151
n-Propanol	7	241	—	190	10	168
n-Butanol	7	—	240	223	43	116
n-Pentanol	12	—	—	195	139	73
n-Hexanol	20	—	—	220	22	283

[a] Each value is the mean of three replications.

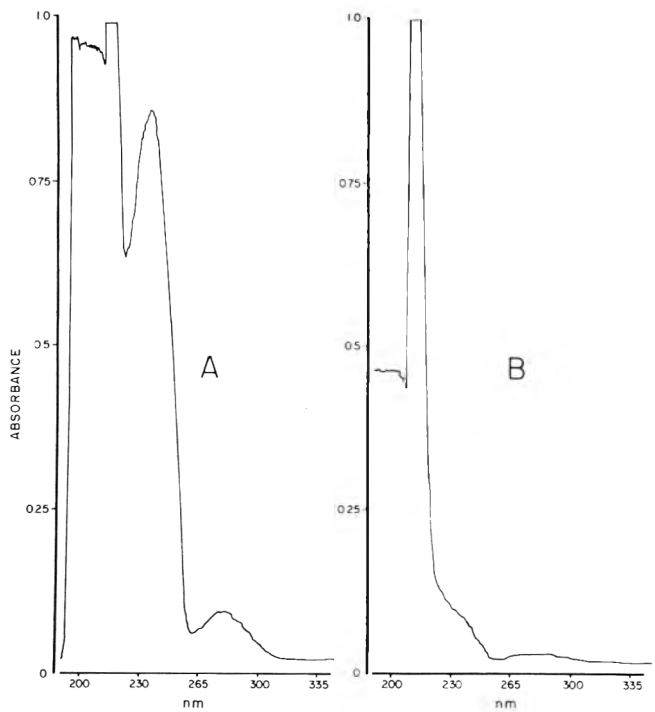

Fig. 2—UV spectra of lipids extracted from aqueous peanut homogenates (A) raw sample; (B) control sample.

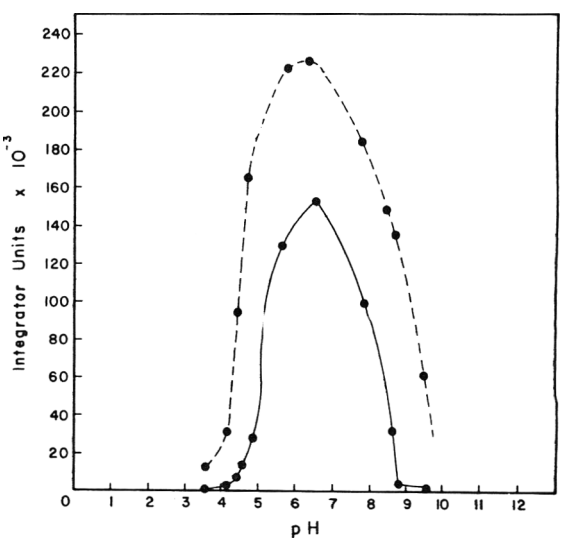

Fig. 3—Effect of pH on the production of pentane (●——●) and hexanal (●——●) in aqueous peanut homogenates.

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EFFECTIVE HEAT CAPACITIES FOR THE FREEZING AND THAWING OF FOOD

ABSTRACT

Based on a modified form of the freezing point depression equation we have derived a set of rigorous, broadly applicable equations for effective heat capacity during the freezing and thawing of foods and biological materials. The equations have been integrated with respect to temperature, thereby providing a set of useful equations for enthalpy during freezing and thawing. The validity and utility of the equations are demonstrated using data from the literature. Methods for adjusting the equations to account for changes in water content and fat content are presented. The enthalpy equations are useful for calculating heat-transfer loads during freezing and thawing, and the heat capacity equations can be advantageously used in differential equations for calculating freezing and thawing heat-transfer rates.

THE CALCULATION of cooling and heating loads and rates are central problems in designing food freezing and thawing systems. To facilitate such calculations we have developed a generalized equation for effective heat capacity during freezing and thawing. It provides much of the same information as the enthalpy – moisture content – temperature diagrams developed by Riedel (1956; 1957a, b), but it is more compact and convenient, and is directly usable in a broader range of problem situations.

Foods start to freeze at lower temperatures than pure water. Their freezable water content changes to ice over a range of temperatures rather than at a single temperature. Since the ice forms over a range of temperatures, the associated latent heat effect per degree can be added to the normal heat capacity, yielding an effective heat capacity C_e which is a function of temperature. Values of C_e vs temperature have been calorimetrically determined for meat and fish by Jason and Long (1955), Fleming (1969) and by Rolfe (1968) using the calorimetric data of Riedel (1956; 1957a, b). Heldman (1974a, b) has used the freezing point depression equation to calculate ice content and enthalpy changes for small temperature changes during freezing, and from these values he calculated values for C_e. We utilize the same equation in a somewhat different fashion in this paper.

Freezing point depression

Foods, during freezing, obey the freezing point depression equation. The differential and integral forms of this equation are:

$$\frac{\partial \ln a_w}{\partial T} = \frac{1}{a_w}\frac{\partial a_w}{\partial T} = \frac{18\Delta H_T}{RT^2} \quad (1)$$

$$\ln a_w = \frac{18\Delta\bar{H}(T - T_o)}{RT_oT} \quad (2)$$

where a_w is the water activity, T_o the freezing point of pure water, T the freezing point of the solution (T and T_o are absolute temperatures), R the gas law constant (1.987 BTU/lb mole °R or 1.987 cal/g mole °K), ΔH_T the heat of fusion per unit weight at T and $\Delta\bar{H}$ the average heat of fusion over the range between T_o and T.

Since $\Delta H = H_w - H_I$, where H_w and H_I are the specific enthalpy of water and ice respectively; $\partial\Delta H/\partial T$ the temperature coefficient of latent heat = $\partial H_w/\partial T - \partial H_I/\partial T = C_w - C_I = \Delta C_P$, where ΔC_P is the difference between C_w the heat capacity of water and C_I the heat capacity of ice. By integrating the temperature coefficient of latent heat between T_o and T it can be shown that $\Delta H_T = \Delta H_o - \Delta Cp\,(T_o - T)$ and that $\Delta\bar{H} = H_o - 0.5\,\Delta Cp\,(T_o - T)$, where ΔH_o is the heat of fusion at T_o.

At low solute concentrations foods can be treated as ideal solutions and a_w can be set equal to x_w the mole fraction of available water in solution. The following equation relates x_w and hence a_w to n_w the unfrozen water content and n_s the nonaqueous solids content per unit weight of food.

$$a_w \approx x_w = \frac{n_w - bn_s}{n_w + (E - b)n_s} \quad (3)$$

In Eq. (3) b may be regarded as the amount of water which is bound per unit weight of solids and is unavailable for freezing at any temperature. $E = 18/M_s$, where M_s is the effective molecular weight of the solids. For solutes whose component molecular weights M_i and weight fractions n_i are known: $En_s = 18\sum_i n_i/M_i$ or $\sum_i n_iE_i$. This summation should not include insoluble components. Heldman (1974b) has used an expression which is similar to Eq. (3) in the freezing point depression equation to successfully correlate freezing point data and ice contents for a wide variety of foods. For these foods he effectively treated M_s, and hence E, as an empirical constant which is determined from the freezing point itself. In essence, b might also be regarded as an empirical constant which simultaneously accounts for the amount of bound water and corrects for deviations from ideality. Eq. (2) and (3) can be used to correlate the freezing behavior of fruit juices and juice concentrates (Heldman, 1974b), and published freezing point data for sugar solutions (Hoynak and Bollenback, 1966), and coffee extract (Barnett, 1973). We have found that established techniques for accounting for nonideality, e.g., the symmetric van Laer equation also successfully correlate the freezing point behavior of these solutions but these techniques are inconvenient to use when calculating C_e.

Evaluation of freezing point parameters

If either E or b is known for a food the remaining value can

be determined from the initial freezing point T at a known water content and Eq. (3) in revised form

$$E = (N - b)(1 - a_w)/a_w \tag{4}$$

where N is the weight of water per unit weight of dry solids and a_w is determined by using the initial freezing point of T in Eq. (2). Approximate values for b have been determined by Duckworth (1971) using differential thermal analysis. These can be used when b is not known and can't be conveniently determined. For meat and fish muscle his b values range from 0.24–0.27 and for vegetables from 0.18–0.25.

If freezing points are available at two water contents, as can be arranged by partially drying or concentrating a portion of the food prior to freezing, Eq. (4) can be applied twice and solve simultaneously using the two freezing temperatures T_1 and T_2 and the corresponding water contents N_1 and N_2 to obtain b

$$b = \frac{N_1 a_{w2} - N_2 a_{w1} + a_{w1} a_{w2} (N_2 - N_1)}{a_{w2} - a_{w1}} \tag{5}$$

Here a_{w1} and a_{w2} are obtained from Eq. (2) at T_1 and T_2 respectively.

We have used freezing point data for glucose and sucrose (Hoynak and Bollenback, 1966) to test Eq. (4) and (5). The calculated values of b and E vary somewhat, depending on the pair of points used in calculating them. Regardless of this variation the E and b values obtained from temperature pairs separated by roughly 4°C or more provide good freezing point correlations over a wide range of temperatures and concentrations. For glucose, b is about 0.15–0.2 and for sucrose about 0.3.

Heat capacity derivation

Assuming heat of solution effects are negligible, the enthalpies of food components are additive. In such a case, if the enthalpy per unit mass during freezing is:

$$H = H_s n_s + H_w n_w + H_I n_I \tag{6}$$

where H_I, H_w and H_s are the enthalpies per unit mass of ice; water and solids respectively and n_I is the weight fraction of ice. Differentiating with respect to T at constant pressure we obtain:

$$\frac{\partial H}{\partial T} = C_e = C_s n_s + C_w n_w + C_I n_I + H_w \frac{\partial n_w}{\partial T} + H_I \frac{\partial n_I}{\partial T} \tag{7}$$

where C_s is the heat capacity of the solids. Since $dn_I = -dn_w$, $\Delta H_T = H_w - H_I$, and $n_I = n_{wo} - n_w$, where n_{wo} is the weight fraction of water prior to freezing, we obtain:

$$C_e = C_s n_s + n_{wo} C_I + n_w \Delta C_P + \frac{\partial n_w}{\partial T} \Delta H_T \tag{8}$$

Further if C_m is the heat capacity of the food prior to the onset of freezing $C_s n_s = C_m - n_{wo} C_w$. Substituting for $C_s n_s$ in Eq. (8).

$$C_e = C_m - n_{wo} \Delta C_P + n_w \Delta C_P + \frac{\partial n_w}{\partial T} \Delta H_T \tag{9}$$

Both n_w and $\partial n_w/\partial T$ can be related to a_w, and through a_w, in turn, can be related to T. Eq. (3) rearranged yields

$$n_w = \frac{E n_s (a_w)}{1 - a_w} + b n_s \tag{10}$$

Further using Eq. (1) in revised form

$$\frac{\partial n_w}{\partial T} = \frac{\partial a_w}{\partial T} \Big/ \frac{\partial a_w}{\partial n_w} = \frac{18 \Delta H_T}{RT^2} a_w \Big/ \frac{\partial a_w}{\partial n_w} \tag{11}$$

and $\partial a_w/\partial n_w$ from Eq. (3).

$$\frac{\partial a_w}{\partial n_w} = \frac{E n_s}{[n_w + (E - b) n_s]^2} = \left[\frac{E n_s}{n_w + (E - b) n_s}\right]^2 \frac{1}{E n_s} \tag{12}$$

Using Eq. (3) it can be shown that

$$(1 - a_w) = \frac{E n_s}{n_w + (E - b) n_s} \tag{13}$$

Substituting Eq. (13) in (12), and (12) in (11) yields

$$\frac{\partial n_w}{\partial T} = \frac{E n_s a_w}{(1 - a_w)^2} \frac{18 \Delta H_T}{RT^2} \tag{14}$$

Substituting Eq. (10) and (14) in Eq. (9) there is obtained

$$C_e = C_m + (b n_s - n_{wo}) \Delta C_P + \frac{E n_s a_w}{(1 - a_w)} \left[\Delta C_P + \frac{18 (\Delta H_T)^2}{(1 - a_w) RT^2}\right] \tag{15}$$

Lescano (1973) differentiated H with respect to T and obtained a complicated expression for C_e which is in many respects similar to Eq. (15). No details of the derivation were provided. Lescano's term corresponding to a_w is more complex than that used in Eq. (15) and though the minor terms involving a_w appear to be numerically correct, the most important term, that corresponding to $a_w/(1 - a_w)^2$ is numerically $1/n_s$ times too large. Because of the lack of details it is impossible to determine the cause of this discrepancy.

By using Eq. (2) numerical values of a_w can be obtained as a function of T, and by substituting these values in Eq. (15), values of C_e vs T can be obtained. Alternative forms which relate C_e to T more directly can also be obtained by noting that $\ln(a_w) \approx a_w - 1$ and that therefore from Eq. (2)

$$(1 - a_w) \approx \frac{18 \Delta \bar{H}}{RTT_o} (T_o - T) \tag{16}$$

Using this relationship to substitute for $(1 - a_w)$ and, in rearranged form, for a_w and the further simplifications that $\Delta H_T/RT^2 \approx \Delta \bar{H}/RTT_o \approx \Delta H_o/RT_o^2$ (which are fortuitously true for the freezing of water) Eq. (15) yields

$$C_e = C_m + (b n_s - n_{wo}) \Delta C_P + E n_s \left[\frac{RT_o^2}{18 (T_o - T)^2} - \frac{\Delta H_o}{(T_o - T)}\right] \tag{17}$$

If the approximation $\ln a_w \approx (a_w - 1)$ had been used in Eq. (1) and hence the relationship

$$\frac{\partial n_w}{\partial T} = \frac{\partial (a_w - 1)}{\partial T} \Big/ \frac{\partial (a_w - 1)}{\partial n_w} = \frac{18\Delta H_T}{RT^2} \Big/ \frac{\partial (a_w - 1)}{\partial n_w} \quad (18)$$

had been instead of Eq. (11), the following slightly different equation for C_e would have been obtained

$$C_e = C_m + (bn_s - n_{wo})\Delta C_P + En_s \left[\frac{RT_o^2}{18(T_o - T)^2} - \Delta C_P \right] \quad (19)$$

Since Eq. (17) and (19) involve approximations which are not used in Eq. (15), the three equations were numerically evaluated and compared. Eq. (19) provided better agreement with the more rigorous Eq. (15) than did Eq. (17). A slight empirical adjustment of Eq. (19)

$$C_e = C_m + (bn_s - n_{wo})\Delta C_P + En_s \left[\frac{RT_o^2}{18(T_o - T)^2} - 0.8\Delta C_P \right] \quad (20)$$

provides even better agreement with Eq. (15)—within 0.03% over the temperature range from −1°C to −60°C. Numerically, $RT_o^2/18 = 26721$ when T is in °R and 8,239 when T is in °K. In both cases $\Delta C_P \approx 0.5$. Once these substitutions have been made, the °R expression can be used with Fahrenheit temperatures, and the °K expression with Centigrade temperatures. For most purposes the term $0.8\ En_s\Delta C_P$ in Eq. (20) can be dropped because contribution to C_e is negligibly small compared to the contribution of the other terms in the equation.

Comparison with published data

Values of C_e calculated using Eq. (20) were compared to the C_e data of Fleming (1969), and data derived from Riedel (1956, 1957a). These values are compared in Tables 1 and 2.

Duckworth's rough average b value for meat and fish, 0.25, was used for b in calculating C_e. Since En_s was not known for the materials tested by Fleming and Riedel it was evaluated from the experimental C_e and C_m values by using Eq. (20) in the following revised form:

$$En_s = \frac{C_e - C_m - (bn_s - n_{wo})\Delta C_P}{[RT_o^2/18(T_o - T)^2 - 0.8\Delta C_P]} \quad (21)$$

The values of En_s calculated using the first three or four usable C_e values below the freezing point were averaged to provide the En_s value used to calculate C_e at all T values for

Table 1—Calculated and experimental C_e (Derived from Riedel's data)

Lean beef		Cod muscle	
N_{wo}	= 0.74	n_{wo}	= 0.82
n_s	= 0.26	n_s	= 0.18
C_m	= 0.83	C_m	= 0.90
E	= 0.0266	E	= 0.0347

Temp °C	Calc C_e	Exp C_e	Temp °C	Calc C_e	Exp C_e
−1.5	25.82	28.0	−1.5	23.37	24.5
−2.5	9.61	9.0	−2.5	8.74	9.0
−3.5	5.14	5.0	−3.5	4.39	4.5
−4.5	3.29	3.0	−5	2.57	2.5
−5.5	2.37	2.0	−7	1.56	1.8
−6.5	1.84	1.5	−9	1.14	1.3
−7.5	1.50	2.0	−13	0.81	0.8
−8.5	1.28	1.0	−18	0.67	0.6
−9.5	1.12	1.0	−25	0.59	0.5
−11	0.96	1.0	−35	0.55	0.45

Table 2—Calculated and experimental C_e (Fleming's data)

	Lamb kidneys		Calf veal		Lamb loin (lean)		Lamb loin (mod. fat)		Lamb loin (fat)	
n_{wo}	0.798		0.775		0.649		0.525		0.444	
n_s	0.202		0.225		0.351		0.475		0.556	
n_f	0.029		0.044		0.117		0.284		0.394	
$n_s - n_f$	0.173		0.181		0.234		0.191		0.162	
C_m	0.90		0.86		0.81		0.70		0.84	
E	0.0433		0.0165		0.0176		0.00903		0.00485	
E_i	0.0505		0.0205		0.0263		0.0231		0.0167	
Temp °F	Calc	Exp	Calc	Exp	Calc	Exp	Calc	Exp	Calc	Exp
30	58.92	50.0	24.77	20.00	20.5*	23.0	14.70*	14.0	9.00*	8.0
28	15.12	12.0	6.69	7.0	10.80	11.0	7.84	8.5	5.10	4.8
26	7.01	7.2	3.25	3.2	5.08	5.0	3.73	3.3	2.6	2.6
24	4.17	4.8	2.04	2.0	3.03	3.1	2.30	2.2	1.72	1.8
20	2.14	2.1	1.19	1.3	1.65	1.6	1.27	1.3	1.10	1.1
10	1.00	1.1	0.70	0.82	0.85	0.86	0.70	0.72	0.76	0.65
0	0.74	0.76	0.59	0.61	0.67	0.59	0.52	0.51	0.67	0.43

* Calculated average C_e values for the 28—30° F range.

the material. Because of the rapidity with which C_e changes near the initial freezing point it is difficult to measure there. Thus for that region, the starred points in Table 2, average C_e values for the 28–30°F range were computed and used for comparison with the experimental C_e data. Average C_e values between T_1 and T_2, as can be shown by appropriate derivation, are obtained by using $(T_o - T_1)(T_o - T_2)$ for the $(T_o - T)^2$ term in Eq. (20). Fleming's C_e data below 0°F appear to be erroneously low and were not utilized.

The average absolute percentage difference between the calculated and experimental C_e results is 9.1%. The maximum average percentage difference for any product is 13%.

Despite moderate differences between certain individual pairs of calculated and experimental C_e in Tables 1 and 2, the overall agreement is so marked that we can regard Eq. (20) as experimentally confirmed.

It may be argued that the procedure for determining En_s automatically insures good agreement between the experimental and the predicted C_e values. This argument is valid only if Eq. (20) is correct–otherwise, no matter how En_s was determined, the agreement would be poor. The correctness of Eq. (20) is demonstrated by the ability to obtain good agreement through a proper choice of En_s

Fat content

E was determined by dividing En_s by n_s. It varies markedly. Part of this variation appears to be due to the variation in fat content. Values of the fractional fat content n_f, if known, are listed in Tables 1 and 2. Since fat is not soluble in water it should not appear in the right hand side of the formulation $En_s = \Sigma n_i E_i$. We can lump the various nonfat terms in this summation yielding $En_s = E_i(n_s - n_f)$, or $E_i = En_s/(n_s - n_f)$. Wherever n_f is known, values of E_i have been calculated and tabulated. It can be seen that E_i varies much less than E, particularly for a given type of meat. If En_s is known for a given type of meat of known fat content, the relative constancy of E_i suggests that En_s can be roughly estimated for the same type of meat at a different fat content by the formula

$$(En_s)_2 = (En_s)_1 (n_s - n_f)_2/(n_s - n_f)_1 \qquad (22)$$

where the subscripts 1 and 2 refer to the different fat contents.

Enthalpies

Eq. (20) multiplied by dT can be integrated between a chosen reference temperature T_R and temperatures in the freezing range to provide specific enthalpy values at these temperatures, i.e.,

$$H = (T - T_R)\left\{C_m + (bn_s - n_{wo})\Delta C_P + En_s\left[\frac{RT_o^2}{18(T_o - T_R)(T_o - T)} - 0.8\Delta C_P\right]\right\} \qquad (23)$$

The combined term in curved brackets is the mean heat capacity between T and T_R. Eq. (23) can be solved for En_s if experimental enthalpy data are available at T. Because enthalpy data are more reliable than C_e data it is preferable to calculate En_s and E from enthalpy values using Eq. (23) rather than from corresponding values of C_e.

When E is calculated for lean beef using Eq. (23) and Riedel's enthalpy data for $n_{wo} = 0.74$ and T = –2°C, a value of 0.0247 is obtained, which differs from the E value of 0.0266 obtained from C_e data by 7%. When E = 0.0247 is substituted in Eq. (23) and enthalpies at temperatures other than –2°C are calculated based on T_R = –40°C, good agreement with Riedel's enthalpy data is obtained. For example, the calculated enthalpy at –12°C is 15.9 cal/g vs an experimental value of 15.5 cal/g.

Water content

Since E should be constant for a given type of meat one can use Eq. (23) even when the water content changes. If the subscripts 1 and 2 refer to the original water content and the changed water content respectively, then at the changed water content $C_{m2} = [C_{m1}(1 - n_{wo2}) + C_w(n_{wo2} - n_{wo1})]/(1 - n_{wo1})$ should be substituted for C_m, $(1 - n_{wo2})$ for n_s, and n_{wo2} for n_{wo}. When this is done for lean beef at a 50% water content and the enthalpy is calculated at –5.6°C, for example, the calculated enthalpy is 32.1 cal/g which is close to Riedel's value of 30.0. Thus it can be seen that through the use of C_m, C_e and Eq. (23), one can obtain all of the same type of data that is available in a highly detailed enthalpy-water content-temperature diagram.

Alternative expressions

A pair of convenient expressions for C_e and H based on n_{wo} and T_1, the initial freezing point, rather than E and n_s can be obtained. Using Eq. (10) in rearranged form, one solves for En_s in terms of $\ln a_w$.

$$En_s = (n_w - bn_s)\left(\frac{1 - a_w}{a_w}\right) \approx -(n_w - bn_s)\ln a_w \qquad (24)$$

The $\ln a_w$ approximation in Eq. (24) is valid near the initial freezing point where a_w is close to 1.0. Using Eq. (2), one substitutes for $\ln a_w$ in Eq. (24) at the initial freezing point, where $n_w = n_{wo}$:

$$En_s = (n_{wo} - bn_s)\,18\frac{\Delta\bar{H}(T_o - T_1)}{RT_oT_1} \approx \frac{18(n_{wo} - bn_s)\Delta H_o}{RT_o^2}(T_o - T_1) \qquad (25)$$

Substituting this expression for En_s in Eq. (20), dropping negligibly small term $0.8\,En_s\Delta C_P$, and further substituting $(1 - n_{wo})$ for n_s there is obtained:

$$C_e = C_m + [n_{wo}(1 + b) - b]\left\{\frac{\Delta H_o(T_o - T_1)}{(T_o - T)^2} - \Delta C_P\right\} \qquad (26)$$

It can be seen that only four parameters C_m, n_{wo}, b and T_1 are required to correlate C_e. The group $[n_{wo}(1 + b) - b]$ is the initial free water content. Since the peak value of C_e occurs at the initial freezing point T_1 it can be seen that the peak rise is proportional to the initial free water content and inversely proportional to the initial freezing point depression $(T_o - T_1)$.

For convenience the various constants in Eq. (25) can be lumped together yielding the following simple equation

$$C_e = A + B/(T_o - T)^2 \qquad (27)$$

where $A = C_m - [n_{wo}(1 + b) - b]\Delta C_P$ and $B = [n_{wo}(1 + b)$

$- b]\Delta H_o(T_o - T_i)$. This two-parameter equation is particularly convenient for use in empirical data fitting and when carrying out unsteady state heat transfer calculations.

By integrating Eq. (26) between T_R and T the following expression for H is obtained

$$H = (T - T_R)\left\{C_m + [n_{wo}(1 + b) - b]\left[\frac{\Delta H_o}{(T_o - T_R)}\left(\frac{T_o - T_i}{T_o - T}\right) - \Delta C_P\right]\right\} \quad (28)$$

Its simplified lumped parameter counterpart is

$$H = (T - T_R)\left[A + \frac{B}{(T_o - T_R)(T_o - T)}\right] \quad (29)$$

Based on empirically formulated and fitted relationships between unfrozen water content and temperature and solution heat capacity and temperature, Heldman (1966) and Heldman and Hedrick (1970) developed and used an expression for H as a function of T for the freezing of ice cream. The equation requires six empirically fitted constants and powers of T up to the 13th power, is quite complex and not functionally similar to Eq. (28) and (29). Eq. (26), (27), (28) and (29) are convenient to use at fixed water and fat contents. Eq. (20) and (23) on the other hand can be readily adjusted to take into account variations in fat and water content.

CONCLUSIONS

BECAUSE of the generality of their derivation, Eq. (20), (26), (27), (28) and (29) should be applicable not only to the freezing of foods but also to the freezing of most biological systems and many nonbiological systems. The only theoretical restrictions appear to be that a_w should be reasonably well represented by Eq. (3), that any deviations from phase equilibrium should be negligible, and that any heat of solution or heat effects caused by the formation of solid phases other than ice should be negligible.

These equations provide useful bases for correlating effective heat capacities and ethalpies during the freezing and thawing of food and biological material; and calculating heat transfer loads for such freezing and thawing. Eq. (26) and its simplified counterpart Eq. (27) provide convenient expressions for C_e for use when analytically and numerically solving differential equations describing heat transfer during freezing and thawing.

Since the equations are based on the existence of phase equilibrium, they provide reference standards for detecting and measuring the magnitude of nonequilibrium effects, such as sugar glass formation, and the occurrence of unanticipated phase changes. They therefore should prove useful in analyzing data obtained through differential thermal analysis tests.

The derived equations can be readily adjusted to compensate for changes in water content and fat content. The parameters required for the equations can be readily determined by means of simple moisture content, freezing point and calorimetric measurements.

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EFFECT OF CALIBRATION PROCEDURES ON ACCURACY AND PRECISION OF AUTOMATED KJELDAHL NITROGEN ANALYSIS IN SOME FORMULATED FOODS

ABSTRACT

The Technicon AutoAnalyzer total nitrogen system was studied to determine some of the limitations and special modifications of the method that pertain to the analysis of formulated foods with one or more high protein ingredients. Assorted instant breakfasts and meat analogs were used as test products. Specifically, the effects of composition of calibration standards, heat of continuous digestion, and nitrogen concentration on the agreement between AutoAnalyzer and manual macro-Kjeldahl were studied. Resistance of particular protein types to continuous digestion did not constitute a major limiting factor in the precision and reproducibility of the automated method, so that fixed temperatures could be used for diverse proteins. Standard deviations from 0.21–0.47% protein were obtained with the AutoAnalyzer for 5–8 replicates of several different food products compared to about 0.22–0.37% for manual Kjeldahls. Of more importance than qualitative composition of standards was the maintenance of a narrow range of concentration (100 ppm N) for sample solutions and calibration with dual standards corresponding to the lower and upper limits of the concentration range. Better accuracy and reproducibility were achieved with calibration standards prepared from dispersible or soluble ingredients that formed stable, homogeneous acid mixtures. Although the method is not recommended as the best means of obtaining accurate nutritional composition data for formulated foods, the automated method may be used for less demanding applications, including rough proximate analyses.

INTRODUCTION

RAPID, ACCURATE METHODS of determining crude protein in complex fortified and formulated foods are needed to meet the demands of research, quality control and labelling practices. To date the continuous analysis of total nitrogen using the Technicon AutoAnalyzer has been successfully applied to raw food commodities with various modifications, including meat products (McNeal et al., 1970; Gantenbein, 1973; Schmidhofer et al., 1973), fertilizers and feeds (Gehrke et al., 1971), grains and grain flours (Uhl and Lancaster, 1971; Deschreider and Maes, 1968), and miscellaneous canned produce and processed foods (Lento and Daugherty, 1971). The success in applying the method to these products has been reportedly dependent on composition of calibration standards (Uhl and Lancaster, 1971; Brisson, 1965; Cox and Harmon, 1966; Gantenbein, 1973) or on digestion conditions (Davidson et al., 1970; Kramme et al., 1973; Lento and Daugherty, 1971; McNeal et al., 1970).

The current study was undertaken to determine some of the limitations and special modifications of the AutoAnalyzer method that relate to the analysis of formulated foods with one or more high protein constituents. Specifically, the effects of composition of calibration standards, heat of continuous digestion, and nitrogen concentration on the agreement between AutoAnalyzer and macro-Kjeldahl total nitrogen values were studied.

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EXPERIMENTAL

Apparatus and reagents for AutoAnalyzer

AutoAnalyzer components. Sampler II for delivering aliquots of sample solutions to the system, continuous digestor with rotating glass helix for digestion of successive sample aliquots, proportioning pumps for delivering reagents to the system through precision bore flexible tubing; heating bath/delay coil for incubating the ammonia containing color complex prior to its colorimetric measurement, and single channel colorimeter connected to a single pen recorder for obtaining absorbance or concentration directly. Components were assembled following standard manifold no. 116-D092-01 (Technicon, 1972). 8.5 ml (60 × 15 mm) Nalgene centrifuge tubes served as sample cups. The sample pick-up assembly at the output end of the digestor was modified (Fig. 1) to allow brisker flow and less erratic sampling.

Sulfuric acid for sample preparation. To 550 ml distilled water were added 450 ml concentrated sulfuric acid (AR grade, 1.84 sp gr with a nitrogen assay of 0.001% or less).

Digestor mix. To 10 ml 70% perchloric acid (ACS certified) and 70 ml distilled water together with 3.5g selenous acid (AR grade) were added 900 ml AR grade sulfuric acid. After cooling, the mixture was diluted carefully to 1 liter.

Alkaline phenol. To 50 ml 5N NaOH were added 27.6 ml phenol (90%, ACS certified) with mixing and cooling. The mixture was diluted to 200 ml with distilled water, to which was added 0.1 ml Brij-35 surfactant (Atlas Chemical Co.). This quantity of mixture was sufficient for roughly 150 samples, but was discarded after 2 days to prevent color build-up in the reagent.

Sodium hypochlorite. Commercial grade bleach with at least 5% available chloride.

Water and wash fluid. About 0.1 ml Brij-35 was added to ca 2 liters of water.

Sample and standard preparation

Solid sample and standard materials were weighed to the nearest 0.001g. Dried infant cereals and textured vegetable protein samples were ground prior to weighing in a WIGL-BUG apparatus (Crescent Dental Mnfg. Co.). Meat analog samples were comminuted in a Waring Blendor. For short term storage (1 wk or less) the sample powders and flours were stored in stoppered vials to limit water vapor pick-up.

100 ml of 45% sulfuric acid were added slowly to flasks containing the samples with stirring and mild heating to char and solubilize the samples. Thus, excessive heating and severe clumping and charring that would result from direct addition of concentrated acid to water slurries was avoided. After the sample and standard solutions cleared and attained a stable color, they were stoppered and cooled. Sample solution aliquots for analysis were transferred to the Nalgene sample cups mounted on the Sampler II unit.

Operating procedures for AutoAnalyzer

The start-up and shut-down procedures were carried out according to the manufacturers recommended method (Technicon, 1972). When a new protein type (wheat, milk, etc.) or new product type (instant breakfast, meat analog, etc.) was introduced into the system, the heat applied to the two stages of the continuous digestor system was adjusted to give maximum nitrogen recovery (absorbance) by varying stage 1 and 2 helix amperage settings independently. Sample flow through the system was interrupted for 15 min after each change in temperature settings in order to stabilize the helix temperature before the sample was re-introduced. A 20 per hour sampling rate and a 6:1 sample-to-wash ratio were selected for normal operation.

Calibration with standards

Calibration was performed with the system at stable operating conditions with all reagents flowing through their respective lines giving a steady baseline trace. The sample line was immersed in the standard solution for 4 min or long enough to generate a concentration plateau for approximately 1 min.

One standard solid material or one mixture of solid materials at two different nitrogen concentrations in 45% acid was used to set the minimum and maximum concentration limits on the recorder chart using a single calibration setting on the colorimeter. For standard solution pairs where the two std cal. (colorimeter calibration dial) settings differed by more than 0.05 unit, linearity of settings with absorbance over the concentration range was assumed, and a std cal. setting representing the average of the two was selected, corresponding to the mid-range concentration.

Determination

The nitrogen concentration reading in ppm was converted to percent crude protein, when appropriate, by means of an equation of the general form,

$$\frac{(\text{wt of sample} + \text{wt of added soln}) \times \text{ppm N} \times \text{factor}}{\text{wt of sample}}$$

where the factor is generally 6.25×10^{-4} or 6.38×10^{-4}. The weight of added solution was computed from the specific gravity and volume of solution delivered. Computing protein strictly on a weight basis in this manner eliminated the necessity for making up sample-acid mixtures to a fixed volume and correcting for dilution errors based on the measured moisture content of the samples.

Kjeldahl nitrogen determination

Kjeldahl nitrogen contents were determined in duplicate for product sample materials and in triplicate for standard solid materials using AOAC (1970) method 2.051.

Soy and milk test mixtures and standards

Two series of sample solutions of one carefully assayed soy isolate (SPI) in 45% sulfuric acid were prepared to give weight concentrations of nitrogen in solution ranging from 100–300 ppm for series i and from 200–300 ppm for series ii. A similar procedure was followed for preparing two series of solutions of nonfat dry milk (NFDM), except that a 100–200 ppm nitrogen series was prepared in lieu of series ii. These various dilution series served as test solutions for assessing the suitability of calibration standard solutions.

Six different standard solutions were prepared for calibration purposes using the SPI and/or NFDM materials. The relative proportions of dry material as percent of the total weight of dry material were as follows:

Standard	A	B	C	D	E	F
SPI	100	80	60	40	20	0
NFDM	0	20	40	60	80	100

The pairs of calibration standard solutions, A–F, used for analyzing the soy protein test series i contained solids at the levels of 100 and 300 ppm nitrogen to set the upper and lower concentration limits, whereas standards A–F used for soy protein series ii contained 200 and 300 ppm. Standards A–F were prepared at levels of 100 and 300 ppm as standards for the dry milk test series which ranged from 100–300 ppm and at levels of 100 and 200 ppm nitrogen as standards for the dry milk series which ranged from 100–200 ppm.

Instant breakfast test mixtures and standards

An assortment of 12 distinct instant breakfast products were assayed by AOAC Kjeldahl and used to prepare 12 test sample solutions in the range of 200–300 ppm nitrogen. The solutions were analyzed as a group 6 times using each of the standard solutions, A–F, in turn. Standard solution pairs contained 200 and 300 ppm nitrogen.

Mean analog test mixtures and standards

An assortment of 8 meat analog products was similarly analyzed against 6 standards using 3 different concentration levels for each product: 230, 260 and 290 ppm nitrogen. The 6 standard solutions, G–L, were formulated according to the following proportions of dry material as percent of the total weight of dry material:

Standard	G	H	I	J	K	L
Textured vegetable protein	100	50	50	0	0	0
Egg white solids	0	50	0	100	50	0
Whole wheat flour	0	0	50	0	50	100

Each standard solution was prepared in pairs to give 200 and 300 ppm nitrogen.

RESULTS & DISCUSSION

TABLE 1 shows readings at various digestor current settings for soy isolate and dry milk. Other materials such as egg solids and wheat flour showed peak absorbances in the same general range as these soy and milk products: 4.0–4.2 for stage 1 and

Table 1—Effect of digestor current settings on AutoAnalyzer readings for ppm nitrogen in two protein products

Current, amp		ppm N	
Stage 1	Stage 2	SPI	NFDM
3.8	4.0	320	–
3.9	4.0	322	155
4.0	4.0	322	155
4.0	4.5	323	155
4.2	4.0	325	153
4.2	4.5	325	153
4.2	5.0	324	152
4.2	5.5	318	150

Table 2—Average standard deviations and coefficients of variability for five or more replicates of AutoAnalyzer and Kjeldahl determinations for various products

	AutoAnalyzer			Kjeldahl		
Product	Replicates[a]	Std dev % protein	COF[b]	Replicates	Std dev % protein	COF
Dry milk	8	0.38	0.9%	8	0.25	0.7%
TVP	5	0.47	1.0%	5	0.31	0.6%
Meat analog	8	0.30	0.7%	8	0.30	0.7%
Instant breakfast	5	0.21	1.2%	5	0.22	1.3%
Egg solids	5	0.29	0.4%	5	0.34	0.4%
Wheat flour	6	0.26	1.9%	6	0.37	2.6%

[a] Simple replicate aliquots from the same sample solution
[b] Coefficient of variability

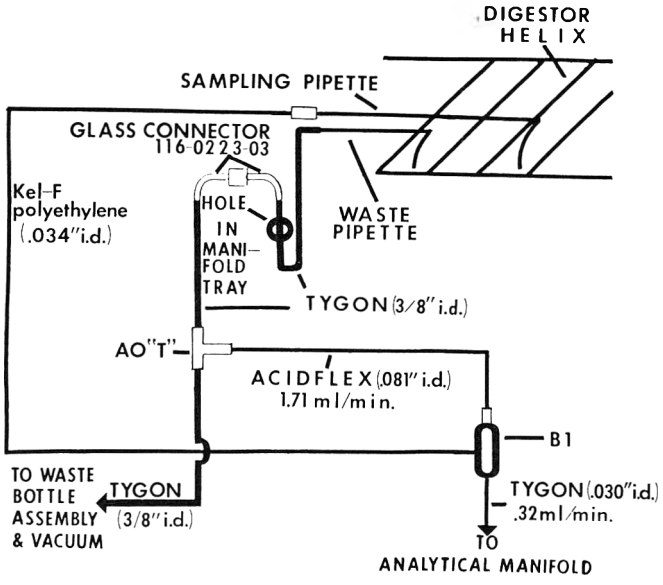

Fig. 1—Assembly for sample pick-up at output end of continuous digestor.

4.0–5.0 for stage 2. Thus, these products do not appear to differ significantly with respect to the effect of temperature on nitrogen losses, and recovery maxima tend to occur in the same temperature range, concurring with similar observations by Marten and Catanzaro (1966). For most products, amperage settings of 4.2 and 4.0–4.5 for stages 1 and 2, respectively, were deemed appropriate.

Examples of scatter among simple replicate determinations under fixed operating conditions are shown in Table 2 in the form of standard deviations and coefficients of variability. The standard deviations shown range from 0.26–0.47% protein. Statistical comparison of sample variances does not show any significant variability from one product to another, indicating that scatter among replicates is probably not strictly linked to the digestion procedure. The standard deviations obtained for the manual Kjeldahl data for these products ranged from 0.22–0.37%.

Products which exhibit recovery maxima occurring at the same temperature may actually show different absolute percent nitrogen recoveries at this temperature. These differences, whether arising from different digestibilities of particular proteins, selenium catalyst effects (Willits et al., 1949), interferences, or the like would not be detectable by this procedure. Such difference could be better assessed by the extent to which one material can serve as a calibration standard for another, since particular calibration settings for particular concentration readings operationally assume a fixed recovery among standards and samples.

Since ammonium sulfate as a standard almost invariably gave poor agreement between AutoAnalyzer and Kjeldahl values for food products in this laboratory, it was decided to investigate the parameters of standard selection, first, to gain an understanding of the analytical effects involved and, secondly, to optimize conditions for analysis of two types of high-protein formulated foods.

AutoAnalyzer nitrogen values are plotted against Kjeldahl nitrogen values in Figures 2 and 3 for soy protein concentration series i and ii, respectively (100 parts per million = 0.1 part per thousand). Each line represents the best regression line through data obtained using a different standard from among standards A–F, which varied from 100% SPI to 100% NFDM. The cluster of lines forms a tighter configuration about the theoretical unit slope line in the case of the 200–300 ppm plot. A similar phenomenon was observed for the two dry milk series, indicating that use of the narrower concentration range (200–300 ppm vs 100–300 ppm) improves overall agreement between the two methods. Calibration spans a narrower range, and potential error in the std cal. setting is not as great. Also, the potential distortion problem encountered in proceeding from large to small peaks on the recorder strip chart is eliminated when concentrations are constrained to fall within the narrower range. Despite the wide latitude in concentrations permitted by the method (Technicon, 1972), where good accuracy is desired and approximate compositions are known it seems advisable to prepare sample and standard solutions to fall within a 100 ppm nitrogen range.

Table 3 presents data for comparison of the two-parameter regression lines plotted in Figure 3 to Y = x (45° line) by the statistical method of Youden (1962). Comparison of the plotted two-parameter regression lines with one-parameter lines obtained either by setting the slope equal to unity or the intercept equal to zero is also presented. None of the lines associated with particular standards permits acceptance of Y = x, i.e., equivalence of the two methods as measured by the quantity percent reduction in SS dev, the sum of squared deviations of experimental y values from the regression line: $\Sigma(Y - y_i)^2$. Table 3 shows that percent reduction values were comparably high for the dry milk series.

Moreover, if similarity of standards and unknowns were a critical requirement for optimizing agreement between the two methods for these data, we would expect the differences in summed deviations for Y = x and the regression line, Y = ax + b, to decline from standards A through F for the dry milk series and to increase from A through F for the soy protein and dry milk series. The quantity percent reduction in SS dev, however, shows no such progression for either the soy or milk series, although of course, if such progressions were apparent, the smallest percent reduction values would have to fall below

Table 3—Comparison of equations for describing fit of AutoAnalyzer data to Kjeldahl data for soy and milk concentration series[a]

Standard	A	B	C	D	E	F
% SPI	100	80	60	40	20	0
% NFDM	0	20	40	60	80	100
Soy isolate series 200–300 ppm N						
Percent reductions in SS devs:						
Eq II vs I	90.5	92.8	97.1	98.5	98.4	95.6
Eq II vs III	70.1	57.2	88.2	89.8	94.0	83.9
Eq II vs IV	43.8	30.7	79.6	77.7	89.8	73.8
Dry milk series 100–200 ppm N						
Percent reductions in SS devs:						
Eq II vs I	92.7	84.6	91.0	91.3	95.0	88.3
Eq II vs III	82.3	81.5	85.3	85.9	86.7	79.4
Eq II vs IV	67.7	76.4	77.8	78.7	73.8	68.2

[a] Critical percent = 77.1 (95% confidence level)—lower level for demonstrating significant statistical difference between equation types I and II, III and II and IV and II as models for data (df = 4)

Equations: I Y = x III Y = x + b
II Y = ax + b IV Y = ax

the critical level to fully substantiate this effect. Comparison of the two-parameter regression equations with one-parameter equations (Y = ax and Y = x + b) for both the soy and dry milk samples indicated that, for most every standard solution, the one-parameter equations should be rejected in favor of the two-parameter equation, i.e., neither a unit slope, nor zero intercept are adequate for explaining the disposition of data points.

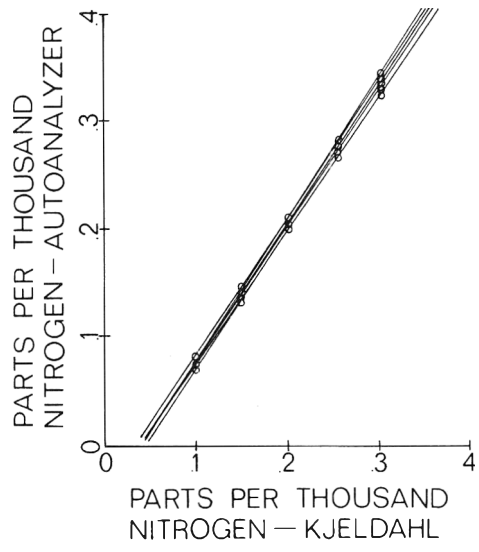

Fig. 2—The best Y = ax + b lines through 6 plots of AutoAnalyzer vs Kjeldahl nitrogen for series i solutions of a soy isolate using 6 different soy/milk standard solution sets, A—F. Solution concentration range: 100—300 ppm nitrogen (0.1—0.3 parts per thousand). Circles arranged vertically indicate the 6 AutoAnalyzer values for each Kjeldahl value.

The 6 plots of AutoAnalyzer percent crude protein vs Kjeldahl protein for 12 distinct instant breakfast-type products using the 6 different standards, A—F, are shown in Figure 4. Comparison of the two-parameter equations for these lines with the best fit one-parameter lines (Table 4) shows that, for the data associated with standards E (80 NFDM/20 SPI) and F (100 NFDM), the percent reductions in SS dev in comparing Y = ax + b to Y = x fall far short of the critical level needed to reject Y = x as a suitable equation to describe the agreement between AutoAnalyzer and Kjeldahl. Also, the one-parameter equations, statistically, are just as valid as the two-parameter equations for all of the data sets (lines). Such one-step, one-factor correction models, if generally valid, might represent an acceptable deviation from the ideal condition, Y = x, provided *a* does not differ greatly from 1 or provided *b* does not differ greatly from 0.

While there is no steady trend in the change of values for percent reduction in SS dev for II vs I as standard composition is varied, there is nevertheless a distinct decline in these values for standards containing a high proportion of dry milk (D, E and F). Since dry milk is the major protein constituent in these products, the presence of a standard specific effect could not be discounted without examining additional data.

To this end, analysis of the series was repeated once using the same samples and a fresh standard set with a composition identical to E, and twice using the same samples and a fresh standard set F. Figure 5 is a comparison plot showing the regression lines obtained for repeat runs with each standard solution set. The variability of the data in terms of relative shifts and biases in these lines is nearly as great as that observed among all standards, A—F, with a common standard deviation (AOV) of 0.52%. In addition, percent reduction in SS dev values for the two-parameter lines associated with runs E_2, F_2 and F_3 (Table 4) are of the same magnitude as those for the earlier runs with standards A—D, exceeding the critical level for rejection of Y = x. The results suggest that the cause

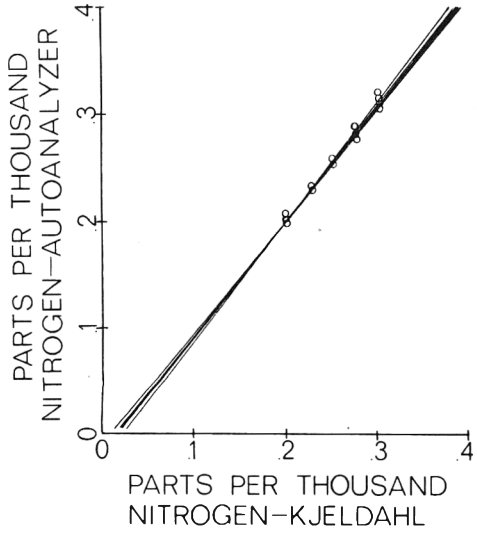

Fig. 3—The best Y = ax + b lines through 6 plots of AutoAnalyzer vs Kjeldahl nitrogen for series ii solutions of a soy isolate using 6 different soy/milk standards, A—F. Solution concentration range: 200—300 ppm nitrogen (0.2—0.3 parts per thousand). Circles indicate the 6 AutoAnalyzer values for each Kjeldahl value.

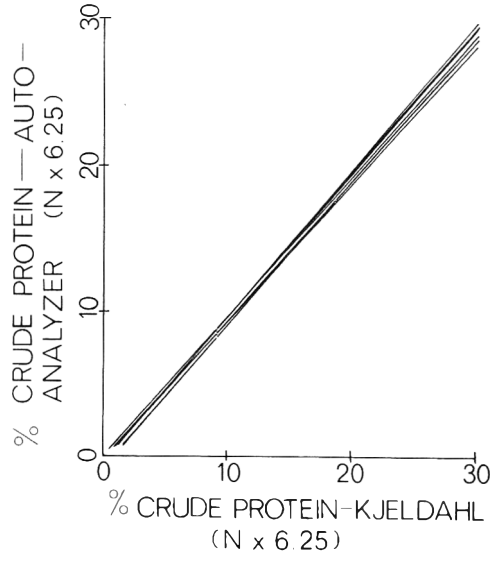

Fig. 4—The best Y = ax + b lines through 6 plots of AutoAnalyzer vs Kjeldahl crude protein for 12 instant breakfast powders obtained using 6 standards, A—F. Solution concentration range: 200—300 ppm nitrogen. Crude protein = N X 6.25.

of the variability probably does not lie in the standard composition and that a fundamental problem of reproducibility may exist, connected with factors other than calibration procedures.

To confirm the absence of a standard composition effect and to amass additional data on application of the two methods to formulated products, a series of eight "meat analog"-type products was analyzed at three concentrations. Table 5 shows the comparison of equations for best lines through the AutoAnalyzer nitrogen vs Kjeldahl nitrogen plots obtained using calibration standard solutions G–L. Lines corresponding to standards H (50% textured vegetable protein/50% egg white solids) and J (100% EWS) represent the closest approximations to Y = x, and, the unit slope line, representing equivalence of the two methods, can be accepted for these sets of data.

Again, little evidence was found to indicate that compositional factors governed the success or failure of the individual standards. For instance, in the case of meatball and frankfurter meatless analog samples, the standard solutions producing best agreement were those corresponding to the major protein constituents. On the other hand, standard H (50% TVP/50% EWS) failed to outperform standard J (100% EWS) in the case of the chicken analog even though its chief ingredients were textured vegetable protein and egg white solids.

It also appears that the wheat flour standards do not provide any more accurate calibration for wheat containing products than for other products. The dark color background and nonhomogeneous physical character of the wheat flour suspensions probably accounted for the variations obtained with these standards. It was assumed that the homogeneity, solubility and purity of the standards were more important than the type of protein. To test this assumption the wheat flour standard was replaced with readily dispersible, purified wheat gluten preparations, designated L' (1) and L' (2). The results in Table 5 show that the one parameter equations are just as valid as the two parameter equations for the gluten standards, indicating better agreement between methods.

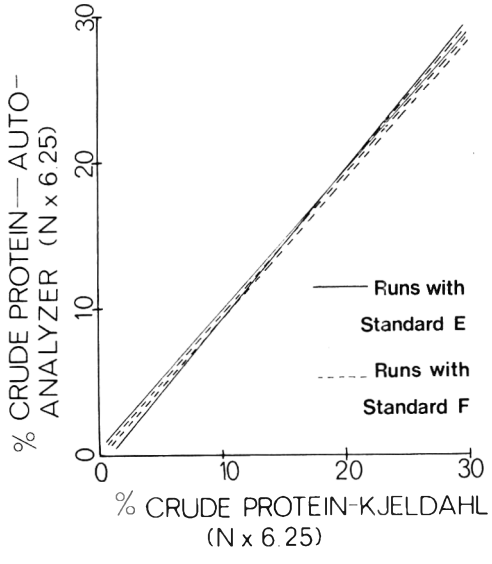

Fig. 5—The best Y = ax + b lines through 3 plots of AutoAnalyzer vs Kjeldahl crude protein for 12 instant breakfast powders obtained from one repeat run with standard solution set E and 2 repeat runs with standard solution set F. Solution concentration range: 200–300 ppm nitrogen.

Using the optimum digestion conditions and standards along with a sampling rate of 20 per hour with a 6:1 sample: wash ratio, AutoAnalyzer and Kjeldahl data for instant breakfasts, meat analogs, soy isolate and flour, dry milk, and some dried cereal grains were pooled to give 61 comparisons. The evaluation of differences is shown in Table 6 for the complete data. The large net difference for the instant breakfast powders compared to the other products is attributable to an inherent problem of reproducibility of analysis with instant breakfasts and probably not to simple mechanical calibration errors. A barely significant difference was observed for the combined instant breakfast-meat analog data, while no significant difference was found between the two methods when the other data were included, i.e., the mean difference = 0.

Table 4—Comparison of equations for describing fit of AutoAnalyzer data to Kjeldahl data for assorted instant breakfast powders[a]

Initial determinations with standards A–F						
Standard	**A**	**B**	**C**	**D**	**E**	**F**
Percent reductions in SS devs:						
II vs I	83.2	83.8	92.5	53.6	8.5	2.1
II vs III	3.5	11.3	26.5	1.0	4.3	0.4
II vs IV	0.3	1.1	2.5	0.2	5.3	0.2

Repeat determinations with standards E & F					
Standard	**E(1)**	**E(2)**	**F(1)**	**F(2)**	**F(3)**
Percent reductions in SS devs:					
II vs I	8.5	45.3	2.1	79.3	59.2
II vs III	4.3	31.4	0.4	1.2	6.6
II vs IV	5.3	4.7	0.2	1.4	1.3

[a] Critical percent = 33.2; 95% level df = 11
Equations: I Y = x III Y = x + b
II Y = ax + b IV Y = ax

Table 5—Comparison of equations for describing fit of AutoAnalyzer to Kjeldahl data for assorted meat analogs

Initial determinations with standards G–L for 8 products at ca 230, 260, 290 ppm N[a]						
Standard	**G**	**H**	**I**	**J**	**K**	**L**
Percent reductions in SS devs:						
II vs I	88.0	11.2	72.9	15.3	59.1	89.6
II vs III	1.5	7.2	0	0.6	0.7	2.7
II vs IV	26.3	9.1	9.3	1.9	1.9	1.3

Repeat determinations with gluten standard, L', for 8 products[b]			
Standard	**L'(1)**	**L'(2)**	**L'(1) & L'(2) combined**
Percent reductions in SS devs:			
II vs I	0.2	0	0.03
II vs III	0.01	0	0
II vs IV	0.01	0	0

[a] Critical percent = 15.7; 95% level df = 24
[b] Critical percent = 50.0; 95% level df = 7
Equations: I Y = x III Y = x + b
II Y = ax + b IV Y = ax

Table 6—Overall comparison of automated and manual Kjeldahl crude protein analysis for formulated foods (optimum conditions)

Sample material	No. samples	Net difference
Meat analog products	16	−1.1%
Instant breakfast powders	24	−6.3%
Soy derivatives	7	−3.8%
Milk and egg protein derivatives	6	+1.7%
Wheat protein products	5	−1.0%
Oat cereal	1	0%
Barley cereal	1	+0.2%
Rice cereal	1	−0.1%
	61	

Meat analogs + Instant breakfasts		Overall	
Total mean error	= 0.15	total mean error	= 0.02
std dev difference	= 0.444	std dev difference	= 0.552
std error difference	= 0.069	std error difference	= 0.071
t	= 2.181	t	= 0.232

Thus it is possible to "optimize" conditions to obtain near equivalence of the two methods (zero mean difference) for these formulated foods, but it is essential that such conditions be adhered to, since a certain amount of variability is inherent in the continuous flow method under any conditions. In fact, results obtained in a preliminary study on 60 samples showed greater variability, presumably due to inadequate preparation of samples or improper technique.

CONCLUSIONS

VARIATION of the heat input amperages for the continuous digestor unit for the Technicon AutoAnalyzer total nitrogen method revealed that maximum nitrogen recovery can be achieved for several protein-containing products using nearly identical settings of 4.0–4.2 amp for stage 1 and 4.0–4.5 amp for stage 2. Thus, recovery maxima tend to occur in the same temperature range for the products tested. The estimates of scatter (standard deviations) among replicates for dry milk, textured vegetable protein, meat analogs, instant breakfasts, egg solids and wheat flour did not differ significantly, suggesting that the resistance of particular proteins to continuous digestion is not a major limiting factor in the precision and reproducibility of the method.

The accuracy of automated Kjeldahl nitrogen analysis of soy and dry milk samples did not depend on the qualitative composition of the standard solutions used in any consistent fashion. Of more importance was the maintenance of a narrow range of concentration (100 ppm nitrogen) for sample solutions and calibration with a pair of standard solutions corresponding to the lower and upper limits of the concentration range. In most cases, the nitrogen source or "matrix" seems less critical than the homogeneity and stability of the standard acid solution.

The data indicate that one can obtain results with the automated method that are comparable to the manual method results. To achieve such comparable results with the automated method, the analyst must pay especial attention to the preparation of the samples and the calibration. Because of the difficulty in controlling all of the critical variables this method is not recommended as the best method for obtaining accurate results on formulated foods. However, the method can be very useful if speed is of primary importance and a 2–4% error in reproducibility can be tolerated.

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A PROCESS FOR THE PURIFICATION OF LUTEIN-FATTY ACID ESTERS FROM MARIGOLD PETALS

ABSTRACT

A process for the purification of lutein-fatty acid esters from marigold petals based on isopropanol precipitation is described. This procedure removed over 65% of the lipids from marigold extracts. The precipitated fraction contained 51.3% lutein esters, and a second precipitation from isopropanol-petroleum ether (80 + 20) gave a product over 65% pure. The precipitated lutein esters were soluble in vegetable oil to the extent of 20% (w/w) at 60°C and were miscible with vegetable oil above 80°C. Lutein esters produced yellow to orange color in foods (Model system), and hue range can be extended to red by the addition of synthetic FDA-approved carotenoids.

INTRODUCTION

THE AVAILABILITY of synthetic carotenoids has decreased the use of natural carotenoids as food colorants, although they are still being used as specific colorants. Annatto, for example, is used for coloring dairy products. The low concentration and complexity of carotenoids in natural materials makes them unsuited to economical extraction, and availability of pure carotenoids at a reasonable price is a prerequisite for their widespread use as commercial colorants.

Marigold (*Tagetes erecta*) is commercially cultivated in Central America and the dried marigold flower petals are used as a pigment source for poultry. Marigold can be cultivated easily, and marigold petals are the most concentrated common source of xanthophylls (Scott et al., 1968). Lutein, which accounts for over 60% of the xanthophylls of marigold flowers, occurs, acylated with palmitic and myristic acids, and the high solubility of lutein-fatty acid esters in vegetable oil compared to the FDA approved synthetic carotenoids is an important factor in the commercial application of xanthophyll esters for coloring foods (Philip and Berry, 1975).

Carotenoids from natural sources can be extracted with an organic solvent, and the limiting factor in their commercial production is the lack of economical purification methods. The conventional purification procedures involve saponification and column chromatography. These methods, though suitable for laboratory scale purification, are tedious and costly.

This paper describes a procedure for the purification of lutein esters from marigold flowers and evaluation of lutein esters as food colorants.

MATERIALS & METHODS

Extraction

Commercial dried marigold petals (1 kg) were packed in a column (1200 × 6.5 cm) and extracted with petroleum ether (3 liters, 30–60° C) at room temperature. The extract was evaporated to dryness under vacuum at 50° C (yield 65g).

Purification

The efficiency of alcohols (C_2 to C_4) in precipitating lutein esters from marigold petals extracts was tested as follows: A weighed amount of extract (1.0g) was dissolved in 25 ml hot alcohol and cooled to 15°C. The precipitate was filtered and dried in a desiccator. The dried precipitate was analyzed for lutein ester content.

A larger scale precipitation was carried out as follows: Crude extract from marigold petals (40g) was dissolved in hot isopropanol (1 liter) and the solution cooled to 15°C. The precipitated lutein esters were filtered through a sintered glass funnel and the filtrate concentrated to one-fourth its volume. The lutein esters were again precipitated by cooling to 15°C and filtered. The filtrate was evaporated to dryness under vacuum at 50°C (mother liquor, yield 27g). The combined precipitate was dried in a vacuum oven at 30°C (lutein esters, yield 13g).

Carotenoid analysis

Dried marigold petals (5.0g) were blended with acetone in a Waring Blendor and filtered. The residue was extracted exhaustively with acetone. The combined acetone extracts (250 ml) were evaporated to dryness under vacuum at 50°C. The residue was dissolved in carbon disulfide and absorbance measured after appropriate dilution: The precipitate and mother liquor were analyzed by measuring the absorbance of a weighed quantity in carbon disulfide.

Lutein esters were purified according to the method of Philip et al. (1971). The purified lutein esters (a mixture of dipalmitate and dimyristate) gave an $E^{1\%}_{cm}$ value of 1070 in carbon disulfide at 475 nm. The carotenoids were calculated based on this value.

The concentration of individual lutein esters was calculated as follows: A chloroform solution of pigments was applied as a thin strip on silica gel G thin-layer plates and developed with petroleum ether + acetone (95 + 5). The major separated bands were quantitatively scraped off, eluted and the absorbance measured in carbon disulfide at the wavelength of maximum absorption. The percentage of individual esters was calculated based on relative absorbance.

Fatty acid analysis

The fatty acid analyses were carried out according to the method of Alam et al. (1968).

Solubility studies

A weighed quantity of lutein esters was slowly added with stirring to commercial hydrogenated vegetable oil (m.p. 43–48°C) contained in a beaker maintained at different temperatures on a water bath. The addition of lutein esters was continued until saturation.

Color measurement

The suitability of lutein esters as food colorants was evaluated as follows: a 5% stock solution (w/w) of lutein esters was prepared in hydrogenated vegetable oil containing distilled mono- and diglycerides (1 + 1). The stock solution was diluted with acetone to obtain 1.0% lutein ester solutions. Appropriate amounts of diluted lutein ester solutions were added to microcrystalline cellulose (Avicel PH-101) to give concentrations ranging from 0.003–0.570% lutein ester. The cellulose was coated with lutein ester uniformly by slow evaporation under vacuum. The dry cellulose containing lutein esters was poured onto a petri dish (100 × 15 mm) and color measured. Cellulose was used as a colorant base, because a similar preparation using citrus juice sacs was found to be a suitable colorant base and/or clouding agent for liquid, semi-liquid and solid foods (Philip, 1975).

Instrumental analysis

Visible spectrum: Perkin-Elmer 202 UV-Visible Spectrophotometer.

Gas chromatography: Wilkens Instrument and Research Inc., Model 600C with FID.

Color measurement: Hunter D25 Color and Color Difference Meter with 2 in. viewing area.

RESULTS & DISCUSSION

The dried marigold petals contained 1.6% carotenoids and lutein esters accounted for 89.6% of the total carotenoids. The

carotenoid composition of marigold petals is given in Table 1. Lutein which occurs acylated with palmitic and myristic acids in marigold petals is readily extracted with hydrocarbon solvents. Petroleum ether (30–60°C) in the ratio 1:3 extracted 89.6% of the total carotenoids.

A petroleum ether extract of marigold petals is mostly lipids, and lutein esters, being epiphasic, readily precipitate from an alcoholic solution. The efficiency of different alcohols in precipitating lutein esters is tabulated in Table 2. Isopropanol gave the best yields and was used in large scale precipitation.

The properties of isopropanol-precipitated lutein esters and mother liquor are given in Table 3. Mother liquor contained 12.0% lutein esters and can be used as a pigment source for poultry. The isopropanol precipitate contained 51.3% lutein esters (a mixture of lutein dipalmitate and lutein dimyristate in the ratio 5:3). The fatty acid composition of lutein esters indicates that saturated triglycerides precipitate along with lutein esters. Lutein esters are miscible with vegetable oil above 80°C and thus offer a convenient way for commercial application. Lutein ester content of the precipitate can be increased to 65% or more by reprecipitation from isopropanol containing petroleum ether (80 + 20).

Microcrystalline cellulose coated with lutein esters gave visual colors ranging from bright yellow to bright orange depending on concentration. As the lutein ester concentration increases to 0.2%, the Hunter b value (yellowness) increases to a maximum (50.0) and above this concentration, the observed color shifts to orange (positive Hunter a values). Addition of β-apo 8′-carotenal and canthaxanthin to lutein esters extended the hue range to red. However, addition of these carotenoids are limited by their solubility in lipids.

Table 1—Composition and properties of marigold petal carotenoids

Carotenoid	%	Rf-value[a]	Visible absorption in CS_2, nm	
Lutein dipalmitate	52.1	0.46	474	505
Lutein dimyristate	31.2	0.45	474	505
Lutein monoesters	6.3	0.10	475	506
Unknowns (by difference)	10.4	–	–	–

[a] Acetone + pet. ether, 5 + 95

Table 2—Properties of lutein esters precipitated by different alcohols

Solvent	Extract: alcohol	Yield (%)	Lutein ester (%)
n-Propanol (b.p. 97.1°C)	1:25	28	43.8
Isopropanol (b.p. 82.4°C)	1:25	32	51.3
n-Butanol (b.p. 117.5°C)	1:25	24	43.8
tert-Butanol (b.p. 82.5°C)	1:25	20	34.8
Ethanol (b.p. 78.5°C)	1:35	26	41.4

Table 3—Properties of isopropanol-precipitated lipids and mother liquor

Property	Precipitated lipids	Mother liquor
Yield (g/kg)	21	45
Lutein ester content, %	51.3	12.0
Absorption maxima (CS_2), nm	473, 504	472, 502
Melting range, °C	45–53	15–20
Sol in hydrogenated fat at 60°C (w/w), %	20	miscible
Fatty acid composition, %		
C10:0	–	1.5
C12:0	2.4	3.5
C14:0	17.1	15.9
C16:0	30.4	31.8
C18:0	15.3	16.4
C18:1	5.6	5.1
C18:2	12.7	11.8
C18:3	4.7	3.4
C20:0	–	1.4
Unknown	11.8	9.2

CONCLUSION

LUTEIN ESTERS can be purified by isopropanol precipitation of marigold extracts, and hue ranging from yellow to red is possible by mixing lutein esters with other carotenoids.

Carotenoid extracts from marigold petals are commercially produced as a pigment source for poultry, and isopropanol precipitation of lutein esters involves three additional unit operations (precipitation, filtration and evaporation). These operations can be added to existing industry without large capital investment. The mother liquor obtained from the precipitation process can still be used as a pigment source for poultry.

Lutein is reported to be present in spinach (Purcell and Walter, 1968), pineapples (Morgan, 1966), prunes (Curl, 1963), tomatoes (Mallia et al., 1964), bell peppers (Curl, 1964a), muskmelons (Curl, 1966), peaches (Curl, 1959), apricots (Curl, 1960a), persimmons (Curl, 1960b), citrus fruits (Curl and Bailey, 1956; 1957; Yokoyama and White, 1967) and several low carotenoid fruits (Curl, 1964b), and is considered to be nontoxic for human consumption.

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AN AUTOMATED RAPID TEST FOR Escherichia coli IN MILK

ABSTRACT

A method was developed to rapidly enumerate *Escherichia coli* in milk, using a Technicon AutoAnalyzer II system. The automated system was used to assay for the presence of glutamate decarboxylase (E.C. 4.1.1.15), which is found only in *E. coli* and a few other bacterial species that are highly unlikely to be found in milk. Coliforms other than *E. coli* were shown not to have this enzyme. This system was capable of detecting as few as 50,000 *E. coli* organisms/ml. The most probable number (MPN) technique was combined with the automated system to quantitate *E. coli* in milk. With pure cultures it was possible to determine the positive and negative MPN tubes in 8 hr, but a 10-hr incubation was required for the analysis of milk samples. Since the enzyme is specific for *E. coli,* the nonselective medium trypticase soy broth and the mildly selective medium lauryl tryptose broth were used in the MPN tubes. It was shown that these media allowed the growth of heat-injured organisms and the detection of organisms that could not be detected using violet red bile agar. The three advantages of this test over the current violet red bile plating procedure are (1) it is more rapid, (2) it is specific for *E. coli* and (3) it enumerates process-injured organisms.

INTRODUCTION

COLIFORMS have long been used as indicator organisms for a variety of conditions in a variety of menstrua. Three improvements in the coliform test procedure are now being actively pursued: (1) the reduction in the time for the test (Andrews et al., 1975; Francis et al., 1974); (2) modifications that will allow the test to detect process stressed and injured organisms (Hartman et al., 1975; Mossel and Ratto, 1970; Speck et al., 1975; Warseck et al., 1973); and (3) to detect fecal coliforms (nominally *Escherichia coli*) and not just coliforms (Andrews et al., 1975; Rose et al., 1975). The procedure we are presenting and investigating in this paper accomplishes all three objectives and in addition is partially automated.

Following the original work done by Gale (1940) on glutamate decarboxylase (EC 4.1.1.15) in *E. coli*, Trinel and Leclerc (1972) devised an automated procedure using the test for glutamate decarboxylase as a test for *E. coli* in water. The purpose of this study was to use their procedure, adapted to a Technicon AutoAnalyzer II system, and develop a quantitative test for *E. coli* in milk. To be useful in the quality assurance of milk it is necessary to detect 10 organisms/ml or less and this is below the sensitivity of the automated procedure. Hence, a marriage of the most probable number (MPN) technique with the automated procedure was required. Since the test is specific for *E. coli,* it was possible to use a non-selective medium or a mildly selective medium in the MPN tubes and this allowed for the enumeration of process injured cells.

MATERIALS & METHODS

Organisms

The culture of *Escherichia coli* K-12, *Bacillus cereus* strain T, *Bacillus megaterium* KM (ATCC 13632), and *Bacillus thuringiensis* NRRL B-2172 used in this study were obtained from the departmental stock culture collection. The enteropathogenic *E. coli* strains were furnished by Dr. R.C. Meyer, Dept. of Veterinary Pathology & Hygiene, University of Illinois, Urbana. They were originally isolated from cases of colibacillosis in swine and have proven virulence. The coliforms used in this study were isolated from raw hamburger, milk, and stream water. All cultures were maintained on slants of Trypticase Soy Agar (TSA) at 4°C and revived for use by growth to late logarithmic phase in Trypticase Soy Broth (TSB) at 37°C. Such revived cultures were used as the inoculum in all experiments.

Description of automated systems

A Technicon AutoAnalyzer II system was used to assay for glutamate decarboxylase. The enzyme attacks L-glutamic acid to give carbon dioxide and γ-aminobutyric acid. The decarboxylation proceeds quantitatively and the amount of carbon dioxide formed is measured. Pyridoxal phosphate is added as a required cofactor for maximum activity of the enzyme. A schematic diagram of the system is given in Figure 1.

The sampler automatically takes up 2-ml samples, containing whole bacterial cells, and separates them with a wash of distilled water. The pyridoxal phosphate, buffered at pH 3.8, is mixed with the sample and the stream passes through a 50°C heating bath for 2 min. The combination of low pH and elevated temperature is designed to remove the CO_2 produced by the cells during growth through a debubbler. All of the air introduced into the system and the headspace air in all reagent bottles feeding into the system was kept CO_2-free by passing it through absorbers. The glutamic acid substrate solution, buffered at pH 3.8, is then added, and the stream passes into a 45°C heating bath (42 ml capacity) for 25 min where the reaction takes place. After emerging from the heating bath, sulfuric acid is mixed into the stream to release all dissolved CO_2 to the gaseous form. The CO_2 passes through a CO_2 gas dialysis membrane and decolorizes a buffered phenolphthalein solution. The decrease in the absorbance of the phenolphthalein is measured at 550 nm on a colorimeter and is directly proportional to the amount of CO_2 present and therefore also to the amount of enzyme present. This decrease in absorbance is converted to peaks on a

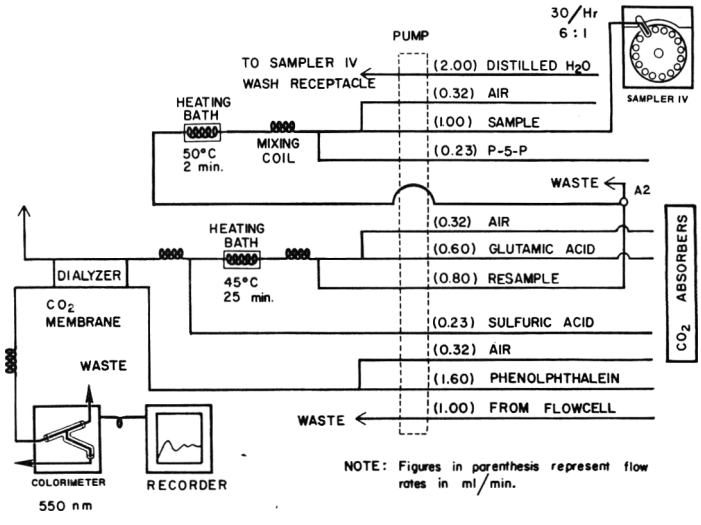

Fig. 1—Automated system for the detection of Escherichia coli in milk by the determination of glutamate decarboxylase.

recorder. The sample stream and all reagent streams are segmented by air bubbles scrubbed free of CO_2 as the system operates by continuous flow analysis. A proportioning pump is the driving force for the whole system, which can handle 30 samples per hour. The total elapse time from sample cup to result was 30 min.

MPN procedure

The MPN procedure recommended by the American Public Health Association (APHA, 1972) was used. Each of ten tubes was inoculated from the appropriate dilutions of the *E. coli* K-12 inoculum or milk sample to be tested and incubated at 37°C which was the temperature for optimal enzyme production. No gas collecting vials were included in the tubes. The media used for the MPN procedure were TSB and Lauryl Tryptose Broth (LTB). After incubation, the recorder of the AutoAnalyzer II was set to zero using sterile medium and a 2-ml sample from each tube was tested for *E. coli*. Various incubation times were tried to determine the minimum amount of time necessary to detect all of the positive tubes.

When the minimum amount of time for detecting all positive MPN tubes was used the activity peaks were small and returned to the baseline with the 6:1 sample to wash ratio employed. With an extended incubation of 24 hr the large activity peaks did not return to the baseline on successively positive samples but were clearly separated by deep valleys. From the positive and negative tubes, the MPN was determined (APHA, 1972). The MPN was then compared to a plate count on triplicate surface-streaked plates of TSA incubated at 37°C for 24 hr.

Testing of other cultures for glutamate decarboxylase

The coliform isolates, the enteropathogenic *E. coli* strains, and the Bacillus spp. were all grown in TSB at 37°C for 18 hr. A sample was withdrawn and diluted one to four with 0.1% peptone-distilled water. This was then assayed for the presence of glutamate decarboxylase with the AutoAnalyzer II system.

Injury procedure

For injury, a 1-ml inoculum of *E. coli* cells was added to 99 ml of pretempered TSB and heated with constant agitation at 55°C for 20 min. Samples were withdrawn from the heating vessel at zero time and after 20 min; dilutions were made in 0.1% peptone-distilled water and surface plated in triplicate on TSA and Violet Red Bile Agar (VRBA). The plates were incubated at 37°C for 48 hr. MPN tubes of TSB and LTB were also inoculated at the same times and then tested with the AutoAnalyzer II after incubation at 37°C until all of the injured cells were recovered.

Milk samples procedure

Commercial samples of pasteurized milk were obtained and inoculated with *E. coli* K-12 to the level of 2.6–6.0/ml. The level of inoculum was determined from plate counts on TSA. Standard plate counts (SPC) and coliform counts using VRBA were then made on the milk samples according to Standard Methods (APHA, 1972). The milk samples were also inoculated into MPN tubes of TSB and LTB. The tubes were incubated at 37°C for 10 hr, and then tested with the AutoAnalyzer II for *E. coli* as previously described.

RESULTS & DISCUSSION

TO CHECK the feasibility of using the glutamate decarboxylase assay system as a measure of *E. coli*, the minimum number of *E. coli* that might give a positive test with the AutoAnalyzer II system was determined. Using *E. coli* K-12 growing in pure culture in TSB, a minimum of approximately 50,000 cells/ml was required to give a positive detectable reading on the recorder. Consequently, a single *E. coli* cell in a MPN tube would need to go through about 16 generations before the tube could be judged positive for growth, but this is still considerably less than required to detect growth by visible turbidity. If the *E. coli* culture in the MPN tube had an average generation time of 1/2 hr then it would take about 8 hr to detect positive tubes and this is substantially faster than present tests. Using *E. coli* K-12 in pure culture we confirmed that only 8–9 hr incubation at 37°C was necessary to detect all of the positive MPN tubes with the AutoAnalyzer II. Further experiments using milk samples showed that it was necessary to incubate the tubes for 10 hr. This was to provide larger peaks on the recorder over a certain baseline level of CO_2 formed during growth of the cells and not removed in the 50°C heating bath. The TSB and LTB were equally effective as media for the MPN tubes.

The results of a survey of coliform isolates for glutamate decarboxylase are presented in Table 1, along with their IMViC (35°C) patterns, EC Broth (44.5°C) results, and rough identification according to Edwards and Ewing (1972). No further tests were run to differentiate *Enterobacter* from *Klebsiella*. Only *E. coli* cultures were positive for glutamate decarboxylase; *Enterobacter*, *Citrobacter*, and the other intermediate types were negative. Leclerc (1967) surveyed 230 bacterial cultures of different species and strains and using the AutoAnalyzer found glutamate decarboxylase to be present only in *E. coli* and in certain species or strains of *Shigella*, *Clostridium* and *Proteus*. Earlier King and Fletcher (1950) and Proom and Woiwod (1951) had shown that only *E. coli* of the coliforms tested was positive for glutamic acid decarboxylase. Glutamate decarboxylase has also been reported in germinating spores of certain *Bacillus* species (Foerster and Foerster, 1973; Akers and Aronson, 1971). However, we were unable to demonstrate glutamate decarboxylase activity in actively growing cultures of *B. megaterium* KM, *B. thuringensis*, or *B.*

Table 1—Survey of coliform isolates for glutamate decarboxylase

Isolates	I	M	Vi	C	EC Broth (44.5°C)	Identification[a]	Glutamate decarboxylase
8	+	+	–	–	–	*E. coli*	+
1	+	+	–	–	–	*Escherichia*	–
2	–	+	–	–	–	*Escherichia*	–
7	–	–	+	+	–	*Enterobacter*	–
2	–	–	+	+	–	*Enterobacter*	–
8	–	+	+	+	–	*Enterobacter*	–
3	–	–	+	–	–	*Enterobacter*	–
4	–	+	–	+	–	*Citrobacter*	–

[a] Tentatively identified (Edwards and Ewing, 1972).

Table 2—Recovery of thermally injured E. coli K-12 with the AutoAnalyzer II system

Method-Medium	Count (cells/ml) Before heating	After heating[a]
PLATE-TSA	5.5×10^6	2.2×10^5
PLATE-VRBA	4.0×10^6	5.3×10^3
MPN-TSB	4.9×10^6	1.3×10^5
MPN-LTB	3.3×10^6	1.3×10^5

[a] Cells were heated at 55°C for 20 min.

Table 3—Detection of E. coli in milk using the AutoAnalyzer II system

Milk sample	Bacterial counts[a] SPC	Inoculum E. coli	Coliforms VRBA	MPN[b] TSB	MPN[b] LTB
1	40×10^2	60	47	79	49
2	45×10^2	26	20	33	33
3	50×10^2	33	30	33	23

[a] All counts expressed per 10 ml

[b] The MPN counts were obtained using the AutoAnalyzer II system.

cereus T. We tested three isolates of enteropathogenic *E. coli* and all were positive for glutamate decarboxylase. The outbreak of food poisoning in a dairy product caused by enteropathogenic *E. coli* (Marier et al., 1973) makes the above outcome of interest.

Using the MPN procedure for counting and the AutoAnalyzer II system for detecting positive tubes we were able to recover thermally stressed cells of *E. coli* K-12 (Table 2). The difference between the TSA count and the VRBA count indicated that 98% of the survivors were injured and would not have been counted using the standard procedure. These injured cells were able to recover in both the nonselective TSB and the mildly selective LTB used in the MPN tubes.

The results of the examination of some pasteurized commercial milk samples for *E. coli* are given in Table 3. Using the AutoAnalyzer II system we were able to enumerate the inoculated *E. coli* with both TSB and LTB, although there was a small problem with dispersed coagulated casein using the LTB. The inoculum count, the coliform count, and the MPN for both media were in very close agreement. The differences between the two media are only one tube differences and also are not significant. Although the SPC in all of these samples was quite low, the competing organisms present did not hinder the AutoAnalyzer method even when using the nonselective TSB.

With larger laboratories testing more and more samples, the trend toward automation of microbiological analysis is becoming increasing popular. We have shown that a combination of the MPN technique and the Technicon AutoAnalyzer II system by measuring glutamate decarboxylase can detect, enumerate, and is quite specific for *E. coli* in milk. Further, this test is rapid (less than 10 hr) and, perhaps most important, is capable of detecting process-injured organisms.

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CHANGES IN TRYPSIN INHIBITORY ACTIVITY IN SOME SOYBEAN VARIETIES DURING MATURATION AND GERMINATION

ABSTRACT

Trypsin inhibitory activity (TIA) of 4 varieties (var) of soybeans was measured. Values (mg/g soybean) represent TIA extracted under conditions employed and are equivalent to a weighed amount of crystallized soybean trypsin inhibitors (TI). As the beans matured, the amount of TIA in the extract increased. Dare variety had the sharpest increase, 13.4–27.5 mg/g. Mean mg/g of TI for Kanrich was 22.5; Verde, 21.7; Soylima, 17.5; and Dare, 21.8. Dehulled beans held 2.5 min in boiling water had 97–98% of the extractable TIA destroyed. Rinsing beans in water removed up to 10.2% TIA. Removing the testa from unrinsed beans reduced TIA up to 4.3%. Decorticated, rinsed cotyledons retained 50.9% (Soylima) to 80.6% (Verde) of the extractable TIA. Beans which were soaked, germinated 3 days and rinsed twice daily lost up to 13.2% TIA.

INTRODUCTION

THE SOYBEAN (*Glycine max* (L.) Merrill) provides dietary protein for a large segment of the world's population, and its potential for the future is practically incalculable. Yet, the soybean is not without its limitation. One of the most studied limitations regards the presence of protease inhibitors, primarily trypsin inhibitors (TI). These substances are responsible for reducing digestibility of protein by inhibiting tryptic activity. Other abnormalities may occur as a result of the presence of TI in the diet. The literature abounds with articles on various aspects of TI, and excellent reviews are available on this topic (Liener, 1972; Liener and Kakade, 1969; Rackis, 1972, 1974).

The purpose of this study was to determine the influence of maturity, immersion heating, location within the bean and germination on trypsin inhibitory activity (TIA) of some varieties (var) of soybeans.

EXPERIMENTAL

Kanrich, Verde, Soylima (vegetable type) and Dare (field type) var of soybeans were grown in 1974 on the University Plant Science Farm at Knoxville by personnel of the Department of Plant and Soil Science. The beans were planted in mid-June (Table 1). Plantings ranged from 60m of row for Verde var to about 2 ha for Dare var.

The effect of maturity on TIA was determined on all varieties. Harvesting was begun early in the development of the bean and was discontinued when the mature beans began to dry. Criteria of maturity levels are presented in Table 1 (Rackis et al., 1972). Sufficient plants of each variety were gathered at each harvest period to yield at least 450g of dehulled beans. At each period, plants were taken from different areas within the planting and transported to the laboratory. All pods were removed from the plants by hand and combined.

The pods were prepared for shelling by holding them in water at 70°C for 3 min and cooling in tap water. The beans were removed from the pods by passing them through a roller-type sheller (Collins et al., 1971). From the 450g lot of beans, two 30-g samples were freeze dried (FD) and ground to pass a N.B.S. No. 30 sieve. Each sample was placed into a 177 ml sterilized plastic bag, closed and held at −18°C for assay of TIA.

Table 1—Planting dates in 1974 and moisture content at different harvest periods for soybeans of four varieties for determination of trypsin inhibitory activity[a]

Planting dates							
Kanrich		Verde		Soylima		Dare	
June 12		June 12		June 12		June 14	
Criteria for expressing soybean maturity							
Kanrich		Verde		Soylima		Dare	
Harvest date	Moisture %	Harvest date	Moisture %	Harvest date	Moisture %	Harvest date	Moisture %
Aug. 29	69.3	Aug. 29	72.6	Sept. 26	67.5	Sept. 19	70.0
				30	69.5	23	65.8
Sept. 3	68.1	Sept 3	69.7			26	62.2
6	64.7	6	66.8	Oct. 3	65.2	30	62.8
9	66.2	9	66.0	7	64.5		
12	62.1	12	64.7	10	60.8	Oct. 3	59.2
16	60.7	16	63.7	14	59.0	7	58.4
19	55.4	19	60.1	17	58.6	10	55.2
		23	34.4	21	54.4	14	43.9
				24	55.5	17	32.7
				28	25.2		

[a] Moisture means are derived from six observations.

The effect of immersion heating on TIA was determined for immature beans of Kanrich, Soylima and Dare var. The yield of Verde var was insufficient to provide enough beans for this and the germination studies. Sufficient plants of each variety were taken on the date indicated to yield at least 2000g of dehulled beans: Kanrich, Sept. 12; Soylima, Oct. 14; and Dare, Oct. 12 (Table 1). These dates were selected because the beans had reached a stage of development for eating as a green vegetable. Beans were removed from the pods in a manner similar to that used in the maturity study.

200g of beans were placed into a wire mesh basket of about 500 ml capacity, closed with a mesh top and immersed in boiling water for 0, 0.5, 1, 1.5, 2, 2.5, 3.75, 5, 7.5 and 10 min. Ice water was used for cooling the beans. Two 30-g samples were taken from each 200-g lot, FD, ground and stored in accordance with the procedures used in the maturity study.

Moisture content of the beans was determined, and six observations were made for each lot by drying 10-g samples for 24 hr at 200 torr (AOAC, 1965).

Immature beans were utilized to determine the relative location of TI within the bean. Enough plants were taken to yield at least 500g of dehulled beans for each of the four varieties. Harvest dates were: Kanrich, Sept. 16; Soylima and Dare, Oct. 7; and Verde, Oct. 3. Beans of the first three varieties were comparable to the beans harvested for maturity studies on the dates indicated in Table 1. Beans of Verde var were taken from later maturing plants of a separate planting. All beans had developed to a stage for eating as a green vegetable. The beans were removed from the pods by the procedure given previously.

Measurements for TIA were made on (a) washed beans, (b) unwashed testae, (c) unwashed cotyledons, (d) washed testae, (e) washed cotyledons, (f) the bean wash water, (g) testa wash water and (h) the cotyledon wash water. The testa was removed from the cotyledons by hand. Group (a) was prepared by covering 50g of beans with 400 ml of distilled water and placing the mixture on a magnetic stirrer for 1 hr at 26°C. The water was decanted and retained (group f). The beans were rinsed with distilled water, drained and retained. The water was discarded. For groups (b) and (c) the testae were removed from 50g of beans; the testae and cotyledons were retained. To prepare groups (d) and (e) 50g of beans were decorticated and the testae and cotyledons were covered separately with 200 ml of distilled water and stirred 1 hr by a magnetic stirrer. The water from the wash of each part was decanted and retained (groups g and h). The bean parts were rinsed, drained and retained. The beans and bean parts of all groups were FD, ground and stored until assayed for TIA as previously stated. The wash water (groups f, g and h) was frozen and held for assay of TIA.

Mature (dry) beans of Kanrich, Soylima and Dare var were studied for the effect of germination on TIA. Enough plants were taken to yield at least 1800g of beans of each variety. Beans were prepared by placing 600g in excess tap water at about 26°C for 24 hr. Weight of the rehydrated beans was approximately 1450g. The beans were rinsed with tap water and 390g were placed in a perforated 1 qt (0.95 liter) milk carton and held up to 3 days at 22°C. Twice daily the beans were rinsed copiously with tap water in the carton and allowed to drain thoroughly before incubation was resumed. After 24-hr intervals, a 90-g sample was taken. The samples were FD, ground and stored for assay of TIA as reported previously.

TI was extracted from 0.05–1.25g of FD ground bean samples by covering with 50 ml of 0.25N H_2SO_4 and placing the mixture on a stirrer for 1 hr at 26°C (Kunitz, 1946). The larger samples were used for extraction when the beans were heated at the longer periods of time. The mixture was centrifuged at 39,000 × G for 15 min. The supernatant containing the TI was transferred to a test tube, frozen and held for assay of TIA. The extraction time of 1 hr was sufficient to remove virtually all of the extractable TI from the samples. TIA values represent only the amount of activity extracted under conditions employed and may not represent total TIA in the intact bean (Kakade et al., 1974; Rackis et al., 1974).

Assay of TIA was performed by a modification of the procedure by Hummel (1959). This indirect method measures the rate of substrate hydrolyzed by trypsin. The greater the amount of TIA present, the lower the hydrolytic rate of trypsin. p-Toluenesulfonyl-L-arginine methyl ester (TAME) served as substrate for trypsin. A solution was prepared to contain 0.01M TAME in distilled water. Tris buffer (0.04M, pH 8.1) containing 0.0115M $CaCl_2 \cdot H_2O$ was used. A stock solution of bovine trypsin (twice crystallized, salt free, lyophilized, 180 trypsin units/mg protein, Worthington Biochemical Corp., Freehold, N.J.) was prepared to contain 40 μg/ml in 0.001N HCl. Solutions of crystalline soybean TI were prepared to contain 0.0–3.5 μg/ml in 0.25N H_2SO_4. The soybean TI (Worthington Biochemical Corp.) had the following specifications: chromatographically prepared, lyophilized, salt-free and 1 mg inhibited 1.4 mg trypsin (bovine) by weight.

A standard curve (Fig. 1) was prepared by regressing μg of commercial soybean TI against the percentage inhibition of trypsin. Standard solutions were prepared to contain 2.5 ml of buffer, 0.3 ml of TAME solution, 0.1 ml of trypsin solution and 0.1 ml of solutions containing different amounts of the commercial soybean TI. The final concentration of inhibitor ranged from 0.0–3.5 μg/3 ml.

The reference solution contained 0.1 ml of 0.001N HCl and 0.1 ml of 0.25N H_2SO_4 as substitutes for the 0.1 ml of trypsin and 0.1 ml of commercial soybean TI solutions of the standard solution.

In the test solution 0.1 ml of the extract from the soybean material or wash water containing the TI was substituted for 0.1 ml of commercial soybean inhibitor solution of the reference solution.

The rate of tryptic hydrolysis of TAME was measured by recording at 247 nm the change per min in absorbance of the test mixture. A double beam Hatachi (Coleman Model 124) recording spectrophotometer with a temperature control cell was used. All solutions were equilibrated and measurements made at 25°C. Absorbance change was linear during the first 3.5 min, and this was used to calculate the rate of tryptic hydrolysis.

TIA values (mg/g) are expressed on a dry weight basis of soybean material for all studies except those including the bean parts, which are expressed on the wet weight basis. The values represent TIA of soybeans that is equivalent to that produced by a weighed amount of the TI standard. The linear regression equation (Fig. 1) was utilized to calculate the TIA values for the bean samples.

Tests for the effect of maturity, heating and removal of testa and washing of bean parts on TIA were analyzed in two replications with two observations each. Three replications were made for the germination study with two observations each.

Values for the effect of maturity are presented graphically; a polynomial of the second or third order was used to fit the curve. The multiple regression equation was calculated where X = No. of days from initial day of the study. Values reported later were calculated rather than read from the curve of the figures. Values for the effect of heating time are presented graphically for up to 2.5 min. A curve was constructed by transforming the data for the dependent variable (trypsin inhibitor) to natural logarithms and regressing them on the independent variable (time). TIA values for the effects of removing the testa and washing the bean parts and for germination are presented in tabular form (Sanders, 1975).

RESULTS & DISCUSSION

TIA per g of beans for 4 varieties of soybeans was compared with TIA of a weighed amount of a commercial preparation of soybean TI. The standard curve (Fig. 1) is presented with μg of

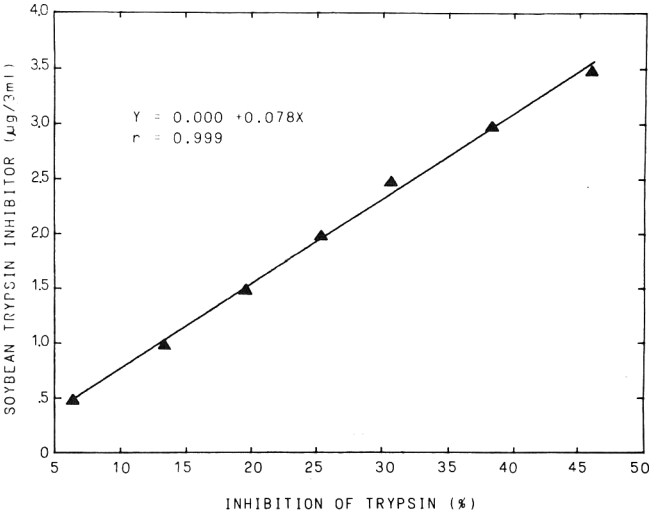

Fig. 1—Standard curve for commercial soybean trypsin inhibitor regressed against percentage inhibition of bovine trypsin.

commercial TI regressed against percentage inhibition of bovine trypsin. The linear correlation coefficient, 0.999, and the regression equation, μg TI = 0.000 + 0.076x, were calculated.

Testing of TIA was begun before the beans reached a stage of development at which they might ordinarily be eaten as a green vegetable. The criteria for different stages of maturity are presented in Table 1 where planting dates and moisture content of beans of different harvest periods are given. As the beans continued to mature, TIA generally increased (Fig. 2 and 3). Kanrich beans (Fig. 2) had the highest TIA at the beginning of the study. This may be relative, however, since there was no procedure for initiating the study at a time when beans of all varieties were precisely at comparable maturities. Initial TIA was 21.7 mg/g of bean tissue and activity increased almost linearly to 23.6 mg/g after 21 days. The coefficient of determination, R^2 = 0.916, and the multiple regression equation, TIA (mg/g) = 21.6636 + 0.1323x − 0.0076x^2 + 0.00027x^3, were calculated.

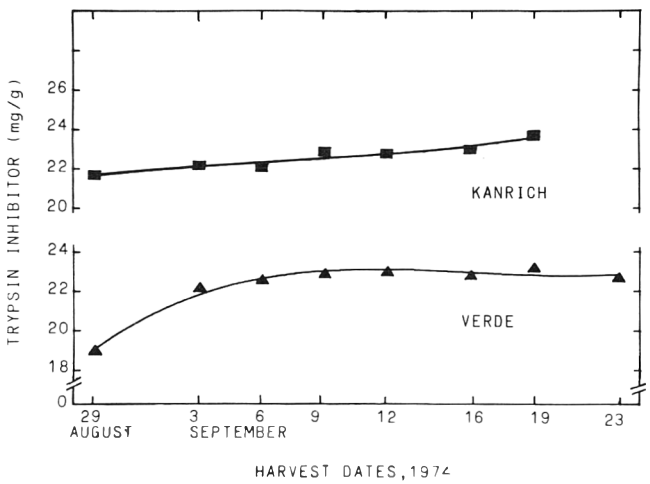

Fig. 2—Trypsin inhibitor content of Kanrich and Verde varieties of soybeans during maturation. Values represent activity extracted under conditions employed and may not represent total activity of the intact bean.

TIA of Verde beans (Fig. 2) increased from 19.1 mg/g at the earliest sampling date to 23.0 mg/g on the 11th day with no appreciable change thereafter. The R^2 was 0.916. The regression equation was TIA (mg/g) = 19.1118 + 0.7556x − 0.0455x^2 + 0.0008x^3.

Soylima beans had the lowest overall TIA (Fig. 3). Values increased from 14.0 mg/g on the first day to 18.5 mg/g at 18 days. As the beans continued to mature to 32 days, a small decrease in TIA was indicated. However, with a R^2 of 0.777, TIA data toward the end of the season exhibited a high degree of variability. TIA (mg/g) (as shown by the regression equation) = 14.0243 + 0.4513x −0.0114x^2. The term "Soylima" is not the official variety name for this soybean; however, the correct name is unknown. Growers over the years have applied this term since it connotes the lima bean-like characteristics of the bean. The immature beans have a green coloration and the following approximate dimensions just prior to becoming yellow at maturity: 6 mm thickness x 13 mm width x 20 mm length. The testa surrounding the hilum has no pigmentation different from that of the whole testa. The plant had indeterminate growth, and the pods are produced on the lateral branches as well as the central stem.

Dare beans had 14.4 mg/g TIA at the earliest maturity sampled and activity increased almost linearly for the 28-day duration (Fig. 3). Final TIA was 27.4 mg/g. The R^2 was 0.966 and the regression equation was TIA (mg/g) = 14.3650 + 0.5931x − 0.0045x^2.

When dehulled beans were held in boiling water, TIA in the extract decreased rapidly. After heating 2.5 min, TIA of the extract of Kanrich beans decreased 97%; Soylima, 98%; and Dare, 97%. Since differences among the three varieties are so small, only the curve for the Soylima var is presented (Fig. 4). These percentage values seem to be high; however, they represent the destruction of the extracted TI only. Whether TIA was not extracted from the soybean tissue was not determined. Albrecht et al. (1966) reported when soybeans of at least 60% moisture were heated by immersion in boiling water, no TIA was detected in the beans. According to Rackis' (1974) review, total destruction of TI is not essential to obtain maximum PER for some soybeans products.

Moisture content for beans used for immersion heating was 62.4% for Kanrich beans; 58.5%, Soylima; and 54.4%, Dare.

A portion of the TIA of dehulled, unwashed beans was

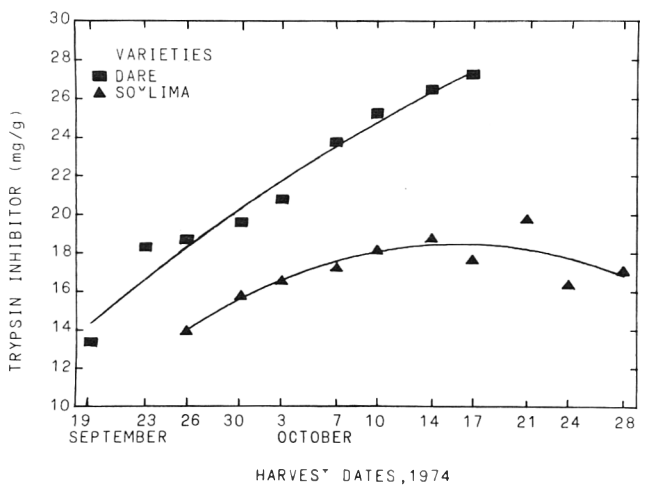

Fig. 3—Trypsin inhibitor content of Soylima and Dare varieties of soybeans during maturation. Values represent activity extracted under conditions employed and may not represent total activity of the intact bean.

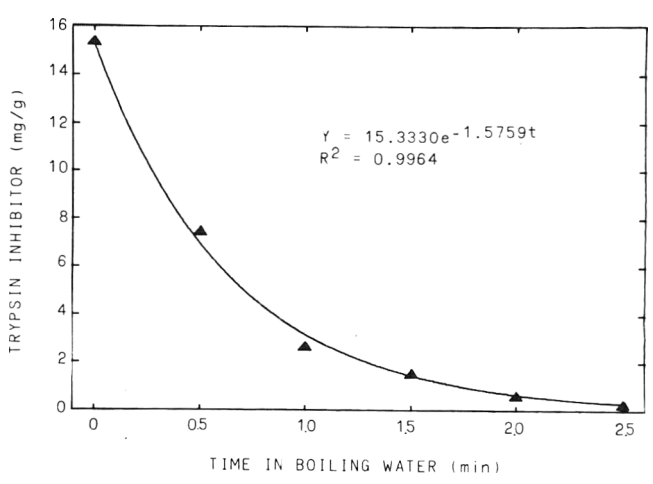

Fig. 4—Inactivation of trypsin inhibitor in Soylima variety of soybeans by immersion in boiling water. Values represent activity extracted under conditions employed and may not represent total activity of the intact bean.

Table 2—Trypsin inhibitory activity for beans, bean parts of immature soybeans and water in which beans and parts were stirred 1 hr[a, b, c]

	Washed whole bean		Unwashed bean parts		Washed bean parts			
					Testa		Cotyledon	
Variety	Water	Tissue	Testa	Cotyledon	Water	Tissue	Water	Tissue
Kanrich	10.2	89.8	4.3	95.7	4.0	0.8	44.3	50.9
	0.79 ± 0.01	6.99 ± 0.12	0.30 ± 0.03	6.66 + 0.07	0.28 ± 0.01	0.06 ± 0.00	3.11 ± 0.23	3.6 ± 0.24
Verde	6.1	93.9	0.7	99.3	0.7	0.2	18.5	80.6
	0.56 ± 0.15	8.67 ± 0.29	0.06 ± 0.01	9.12 ± 0.24	0.07 ± 0.01	0.02 ± 0.01	1.79 ± 0.08	7.82 ± 0.22
Soylima	4.2	95.8	2.4	97.6	2.3	0.6	30.3	66.8
	0.28 ± 0.03	6.34 ± 0.34	0.17 ± 0.05	6.86 ± 0.21	0.16 ± 0.06	0.04 ± 0.01	2.09 ± 0.17	4.61 ± 0.08
Dare	3.4	96.6	0.5	99.5	0.8	0.0	22.6	76.6
	0.32 ± 0.03	9.10 ± 0.42	0.05 ± 0.01	9.70 ± 0.21	0.08 ± 0.01	0.00 ± 0.00	2.36 ± 0.23	8.01 ± 0.17

[a] Top value in each pair is percentage of TIA; bottom value is TIA (mg/g) of beans on wet weight basis.
[b] Means of four observations with ± one standard deviation
[c] Values represent activity extracted under conditions employed and may not represent total activity of the intact bean.

removed by stirring the beans in water for 1 hr (Table 2). TIA of Kanrich beans was reduced by 10.2%; Verde, 6.1%; Soylima, 4.2%; and Dare, 3.4%. This indicates TI was removed from the outer portion of the bean and/or was leached from within or beneath the testa.

Removal of the testa from the unwashed beans reduced TIA by a small amount: Kanrich, 4.3%; Verde, 0.7%; Soylima, 2.4%; and Dare, 0.5%. Thus, the decorticated cotyledons of the different varieties possessed 95.7% or more of the original amount of TIA. By comparison, one can ascertain that the unwashed decorticated cotyledons had a slightly higher TIA than washed beans of a respective variety with the testa intact. This substantiates the earlier hypothesis that TIA was leached from beneath the testa when beans were stirred in water.

When the testa and decorticated cotyledons were stirred separately in water, TIA was found to be associated with both bean parts and the wash water of each. Most all of the extractable TIA associated with the testa was removed by the stirring action in water. All extractable TIA was removed from the testa of Dare beans, while 83, 78 and 79% was removed from the testa of Kanrich, Verde and Soylima beans, respectively. It is likely that all of the extractable TIA would have been washed from the testa of each variety had the washing been more extensive.

Extractable TIA associated with the unwashed cotyledons was reduced 46.5% for Kanrich, 18.7% for Verde, 31.2% for Soylima, and 22.8% for Dare beans by stirring 1 hr in water. Since it is the cotyledons that are useful as food, the reduction of TIA by decortication and washing should be emphasized. Cotyledons treated in this manner retained the following percentages of the initial TIA extracted from unwashed whole beans: Kanrich, 50.9; Verde, 80.6; Soylima, 66.8 and Dare, 76.6. These general findings might have been anticipated since Zimmermann et al. (1967) found a greater concentration of TIA in the outer part of the soybean cotyledon.

It is possible to speculate on the reason for the fairly rapid reduction of extractable TIA of the whole bean in boiling water. A considerable portion of the TI existed on or near the testa thereby allowing it to be subjected rapidly to the temperature or boiling water. Had a greater portion of the TI been located in the more inner tissues, the rise in temperature at the loci of the TI would have been slower and consequently, the rate of TIA destruction undoubtedly would have been lower.

There was a slightly higher amount of extractable TIA in the dry, mature Soylima beans compared to that in similar beans after soaking. Evidently, some of the TIA was removed during soaking. Soaking did not alter TIA of Kanrich and Dare beans (Table 3).

The germination process, changes that occurred following the 24-hr soaking period, had only a slight effect at most on altering TIA of soybeans (Table 3). Sprouting began during the second 24-hr germination period. At each 24-hr period Kanrich and Dare beans exhibited a gradual reduction in TIA; Soylima beans did not show such a change. Since the beans

Table 3—Trypsin inhibitory activity of mature, soaked and germinating soybeans[a,b,c]

Treatment	Time period (hr)	Variety		
		Kanrich	Soylima	Dare
Soaked	0 – 24	22.6 ± 0.9	16.5 ± 0.8	26.9 ± 0.6
Germinating	24 – 48	21.4 ± 0.7	16.8 ± 0.6	26.4 ± 0.9
	48 – 72	20.5 ± 0.6	16.2 ± 0.3	25.2 ± 0.8
	72 – 96	19.8 ± 0.6	16.9 ± 0.5	24.7 ± 0.4
		Mature dry beans, not soaked or germinated		
		22.8 ± 0.5	17.2 ± 0.5	26.9 ± 0.8

[a] TIA (mg/g) of beans on dry weight basis taken at end of the time periods
[b] Means of six observations with ± one standard observation
[c] Values represent activity extracted under conditions employed and may not represent total activity of the intact bean.

were thoroughly washed in clean water twice daily, it follows that a portion of the TIA was removed by the water as this was the finding in another part of this study with immature beans. No plausible explanation is available to account for the finding that TIA for Soylima beans did not change.

The following conclusions may be made from this study. TIA increased in the four varieties of soybeans during maturation. As the period of time was extended in which the immature beans were held in boiling water, TIA in the extract decreased rapidly until at 2.5 min 97–98% was destroyed. A portion of TIA was removed by washing the beans and by decortication. Soaking and germination did not cause TIA to change appreciably in any of the varieties studied.

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DEGRADATION OF RAFFINOSE AND STACHYOSE IN SOYBEAN MILK BY α-GALACTOSIDASE FROM Mortierella vinacea. Entrapment of α-galactosidase within polyacrylamide gel

ABSTRACT

The use of α-galactosidase from *Mortierella vinacea* to remove raffinose and stachyose in soybean milk was studied. Disrupted *M. vinacea* mycelium was further entrapped within 7.5% polyacrylamide gel and its general properties were determined. It was observed that there were only slight changes in optimal pH and temperature after the α-galactosidase was immobilized, while the recovery of α-galactosidase activity was approximately 65%. Three different forms (undisrupted, disrupted, and entrapped) of *M. vinacea* mycelium were used to hydrolyze the oligosaccharides in soybean milk. Disrupted mycelium gave the highest hydrolysis ratio in these three different forms; however, it was found that after long-term usage at 50°C, the thermal stability of the disrupted mycelium was relatively poor. A fluidized reactor was chosen to be used in this study.

INTRODUCTION

THE POTENTIAL of soybean milk as a substitute for cow or human milk has been emphasized over the years, especially for infants and children who are allergic to cow's milk or for adults who have a low level of lactase in their intestine. Moreover, soybean milk can be used as an economical protein beverage where cow's milk is not available or expensive. Because of its low content of fiber, the nutritional value of soybean milk is quite comparable to that of milk (Kay, 1960). However, soybean milk is deficient in the sulfur-containing amino acids and contains considerable amounts of oligosaccharides which have been implicated as factors responsible for the flatulence (Cristofaro et al., 1970; Rackis et al., 1970).

Flatulence is a common complaint even among healthy individuals and is one of the most common causes of abdominal discomfort. It is also associated with dyspepsia, constipation and diarrhea. It has been suggested that flatus is caused by microbial fermentation of oligosaccharides such as raffinose and stachyose which are not digested because humans do not have α-galactosidase (E.C. 3.2.1.22) in their digestive tract. By using rats, Cristofaro et al. (1970) showed a correlation between the flatus production and the oligosaccharide level. Commercial preparations of α-galactosidase have been used to hydrolyze the oligosaccharides in soybean milk (Sugimoto and Van Buren, 1970); however, the effectiveness and economical feasibility of such treatment in reducing the flatulence remains to be determined.

On the other hand, a mold *Mortierella vinacea* which produces α-galactosidase mainly in the mycelium was used to reduce the content of raffinose in beet molasses (Suzuki et al., 1969). The crystalline α-galactosidase free from protease and other glycosidases was obtained and its general properties were studied (Suzuki et al., 1970). In the present paper, *M. vinacea* was used as the source of α-galactosidase to remove oligosaccharides from soybean milk. General properties of entrapped mycelium of *M. vinacea* in polyacrylamide gel and its possible application have been studied.

EXPERIMENTAL

Microorganism

M. vinacea was obtained from the American Type Culture Collection (Rockville, Md.) and grown on a malt extract agar medium at 30°C. Inocula of 50 ml of the seed incubated for 24 hr in the shaking flask were inoculated in 10 liters of medium and cultured at 30°C for 3 days in a 14 liter jar fermentor (Microferm Fermentor, New Brunswick Scientific Co., New Brunswick, N.J.) with agitation of 200 rpm and at an aeration rate of 5 liters/min. The culture medium was 0.1M phosphate buffer (pH 6.0) containing 1% of lactose, 0.3% of yeast extract, 0.3% of peptone, 0.05% of KCl, 0.05% of $MgSO_4 \cdot 7H_2O$ and trace of $FeSO_4 \cdot 7H_2O$.

Preparation of α-galactosidase

The mycelium was harvested by filtration, thoroughly washed with distilled water and stored at −20°C until used. The mycelium was suspended in distilled water and disrupted by the Sonifier Cell Disruptor (Model W-350, Branson Sonic Power Comp., Danbury, Conn.) in an ice bath. The homogenate was used as the enzyme source.

Entrapment of *M. vinacea* within 7.5% polyacrylamide gel

To 10 ml of the homogenate of mycelium prepared as mentioned above were added 712.5 mg of acrylamide and 37.5 mg of N,N'-methylene-bisacrylamide (crosslinking reagent). The solution was deaerated by bubbling nitrogen and the catalyst system consisting of 20 mg of ammonium persulfate and 50 μl of β-dimethylaminopropionitrile was added (Mosbach and Mosbach, 1966). After polymerization the obtained gel containing disrupted mycelium of *M. vinacea* was passed through a 32-mesh sieve. The granules formed were then washed several times by decantation with distilled water.

Assay of α-galactosidase activity

The assay of α-galactosidase activity was conducted according to the method used by Sugimoto and Van Buren (1970) at an incubation temperature of 50°C. One unit of enzyme is defined as the amount of the enzyme which liberates 1 μg of glucose in 60 min. The concentration of protein in the enzyme solution was determined by Lowry's method (Lowry et al., 1951) and bovine albumin was used as a standard protein.

Soybean milk

Dried soybeans (Ransom variety, supplied by the Georgia Seed Development Commission of Athens, Ga.) were ground and the fat was removed with hexane. Fat-free soybean powder was suspended in 10 volume of water and heated to boiling. Soybean milk was separated from the undissolved residues by centrifugation for 5 min at 5,000 rpm.

Treatment of soybean milk by enzyme

M. vinacea mycelium was added to soybean milk in one of three different forms: undisrupted, disrupted (mycelial homogenate) and entrapped within gel. The soybean milk was incubated at 50°C with a reciprocal shaking (200 rpm, Environment Incubator Shaker, New Brunswick Scientific Co., New Brunswick, N.J.). The enzyme reaction was stopped by placing the mixture in a boiling water bath for 10 min.

Fluidized reactor

The granules of acrylamide gel containing disrupted *M. vinacea* were placed in a jacketed glass column (45 cm × 2.8 cm i.d., Pharmacia Fine Chemicals, Uppsala, Sweden). The jacket temperature was controlled at 50°C and the soybean milk preheated to 50°C was pumped into the column upwards at various flow rates. Effluents were collected at each flow rate to analyze the contents of raffinose and stachyose.

Estimation of oligosaccharides in soybean milk

The soybean milk (50 ml) was poured into 120 ml of absolute ethanol and filtered. The filtrate was concentrated to 50 ml under vacuum at 50°C. The amount of raffinose and stachyose in the concentrated filtrate was estimated by the method established by Tanaka et al. (1975).

RESULTS & DISCUSSION

General properties of entrapped enzyme

A series of polyacrylamide gels with the same enzyme concentration but with different compositions were prepared and a total gel concentration of 7.5% (95% acrylamide and 5% crosslinking reagent) showed the best recovery of α-galactosidase activity with good mechanical rigidity. At any concentration, an increase in the relative amounts of crosslinking reagent decreased the mechanical rigidity of the gel. The relative activity of enzymes entrapped in the polyacrylamide gels is usually low. The maximum yield of enzyme activity trapped was reported to be about 60–65% (Zaborsky, 1973), which was the range of recovery obtained in this study for entrapped α-galactosidase.

Optimal pH for the gel-entrapped α-galactosidase was 4.0 whereas the α-galactosidase of the mycelium exhibited its highest activity at pH 4.5 (Fig. 1). However, there was only a slight change in the shape of the pH-activity curve of the immobilized enzyme compared with the curve of the mycelial homogenate. Immobilization of enzymes in the polyacrylamide gel does not cause much shift of the pH-activity curve since polyacrylamide is electrically neutral. A relatively higher value of the Michaelis constant for melibiose was observed for α-galactosidase entrapped in a 7.5% polyacrylamide gel (2.5 mM, compared with 1.14 mM for the enzyme in mycelial homogenate). The entrapment of the enzyme did not alter the maximum velocities.

Enhanced thermal stability of α-galactosidase was observed after the immobilization of enzyme in the polyacrylamide gel (Fig. 2). A high temperature optimum (50–55°C) of α-galactosidase would be a great advantage for removing oligosaccharides from soybean milk since the contaminants during the incubation would be limited to thermophiles. Moreover, there would be slight thermal inactivation of the enzyme during polymerization of acrylamide which is an exothermic reaction.

Table 1 shows the stability of α-galactosidase at 50°C. The enzyme in mycelial homogenate had better stability in the first 24 hr incubation at 50°C, whereas the immobilized enzyme was more stable after 24 hr. The loss of the entrapped enzyme activity in the early period of incubation was probably due to the leakage of the enzyme from within the crosslinked polymeric network. Since the relative amount of crosslinking reagent used in this study was small (5%), some leakage was expected. The leakage might have stopped after 24 hr of incubation so that the entrapped enzyme showed a steady activity thereafter, while the α-galactosidase activity in the mycelial homogenate decreased continuously after 1 day.

Table 1—Stability of α-galactosidase at 50°C[a]

Time	Relative activity (%)[b] in mycelial homogenate	Relative activity (%)[b] in 7.5% polyacrylamide gel
0	100	100
1 hr	100	100
3	95.0	92.1
6	94.0	87.0
12	84.5	83.7
1 day	56.7	50.7
2	36.8	42.2
3	29.4	43.0
4	30.4	38.2
5	21.9	40.8

[a] Both disrupted and immobilized mycelia were suspended in 0.1M acetate buffer (pH 5.0) and incubated at 50°C with shaking (200 rpm).

[b] The remaining activity of α-galactosidase was determined at 50°C using 1% melibiose as the substrate.

Enzymatic hydrolysis of oligosaccharides in soybean milk

The pH of the soybean milk was between 6.2 and 6.4, which was off the optimum range for the enzyme α-galactosidase (Fig. 1). However, the pH of the soybean milk was not lowered for the following experiments because it caused the soybean proteins to precipitate out as well as a sour taste. *M. vinacea* mycelium was added to 200 ml of soybean milk as the enzyme source in three different forms (undisrupted, disrupted and entrapped) and then incubated at 50°C on a shaker (200 rpm) for 3, 6 and 12 hr. Each form of the mycelium had a total α-galactosidase activity of 6×10^4 units. Disrupted mycelium (mycelial homogenate) showed the highest ratio of hydrolysis for every incubation period (Fig. 3), while undisrupted mycelium had the lowest efficiency toward the hy-

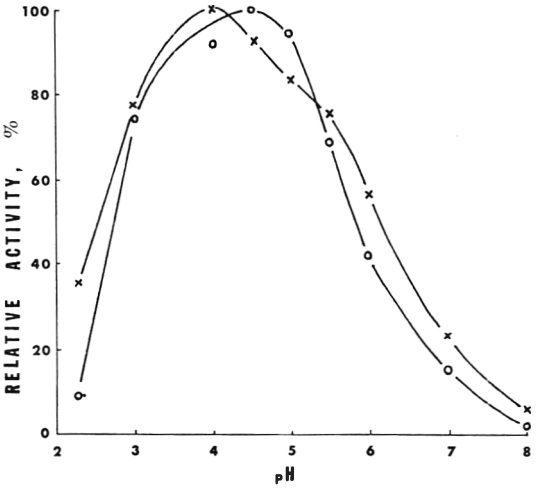

Fig. 1—Effect of pH on α-galactosidase activity. McIlvaine buffer (0.1M) was used. Enzyme activity was determined at 50°C using 1% melibiose as a substrate. (o—o α-galactosidase activity in mycelial homogenate; x—x α-galactosidase activity in 7.5% polyacrylamide gel.)

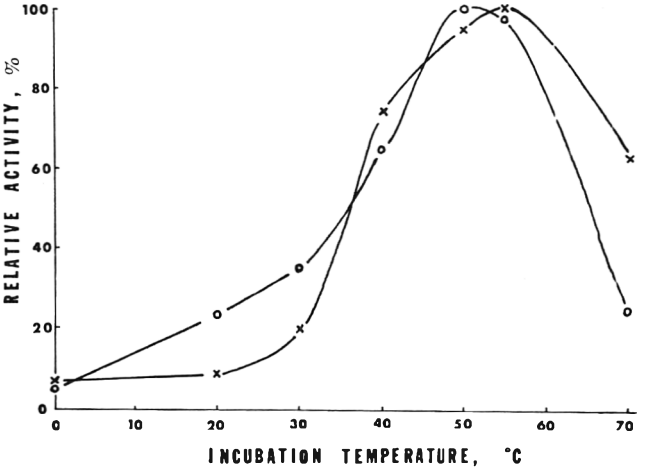

Fig. 2—Effect of temperature on α-galactosidase activity. Enzyme activity was determined using 1% melibiose as a substrate. The incubation time was set to 60 min and 0.1M acetate buffer, pH 5.0 was used. (o—o α-galactosidase activity in mycelial homogenate; x—x α-galactosidase activity in 7.5% polyacrylamide gel.)

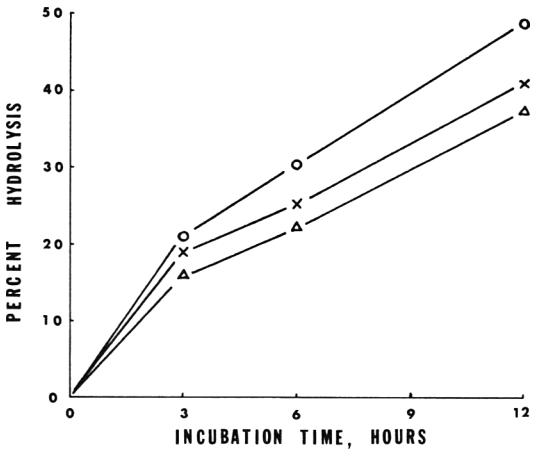

Fig. 3–Hydrolysis of raffinose and stachyose in soybean milk by α-galactosidase in three different forms of M. vinacea mycelium. 2g of mycelium (wet weight, total α-galactosidase activity = 6 × 10⁴ units) in each form was added to 200 ml of soybean milk (prepared with 15 vol of water, solid content = 2.71%) and incubated at 50°C on a shaker (200 rpm) for 3.6 and 12 hr. (△–△ undisrupted; o–o disrupted; x–x immobilized.)

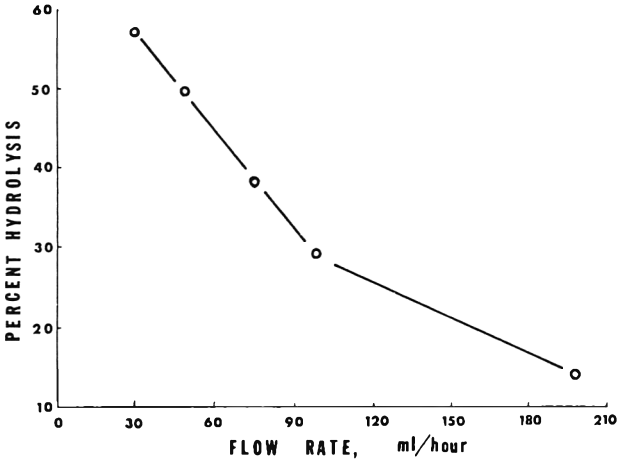

Fig. 4–Effect of flow rate in a fluidized reactor on the hydrolysis of raffinose and stachyose in soybean milk by entrapped α-galactosidase.

Table 2–Continual use of M. vinacea mycelium (undisrupted) for hydrolysis of raffinose and stachyose in soybean milk[a]

No. of incubation	Raffinose[b]	Stachyose[b]	Total[c]	% Hydrolysis
0	65.0	220.0	285.0	0
1	47.0	50.5	97.5	65.8
2	54.5	54.5	109.0	61.8
3	67.5	54.5	122.0	57.2
4	70.0	80.5	150.5	47.2

[a] 10g of undisrupted mycelium (wet weight, total α-galactosidase activity = 3 × 10^5 units) was used repeatedly for the enzyme reaction. 100 ml of soybean milk (solid content = 4.88%) was incubated with washed mycelium at 50°C for 6 hr on a shaker (200 rpm).
[b] mg/100 ml of soybean milk
[c] The total amount of raffinose and stachyose (mg/100 ml of soybean milk)

drolysis of oligosaccharides in soybean milk. Since the amount of mycelium used in this batch process experiment was small, the hydrolysis ratio of oligosaccharides was 48.8% at best among them after 12 hr incubation. There was a substantial increase in raffinose as the result of hydrolysis of stachyose in soybean milk. However, the decrease in the concentration of stachyose is more important than the increase in that of raffinose because stachyose is present almost three times the concentration of raffinose in soybean milk and also because stachyose induces more significant increases in flatus than raffinose does (Cristofaro et al., 1970). Before attempting further investigations of a continuous process with the entrapped mycelium in the column, the possibility was studied for the continual use of the undisrupted mycelium. The undisrupted mycelium (total α-galactosidase activity = 3 × 10^5 units) was used repeatedly as the enzyme source for 100 ml of soybean milk. Soybean milk with the mycelium was incubated at 50°C for 6 hr each time on a shaker (200 rpm). After each incubation period the mycelium was recovered by the filtration and washed thoroughly with water. The washed mycelium was added back to another batch of soybean milk (100 ml) for another 6-hr incubation. Even after triple use, α-galactosidase in the mycelium hydrolyzed 47.2% of the oligosaccharides in soybean milk (Table 2).

It was found that a column packed with polyacrylamide gel granules (32 meshes) containing disrupted *M. vinacea* mycelium could not be used as a continuous reactor because it was easily clogged and could not maintain a constant flow rate as designed. Therefore, a fluidized reactor was used to hydrolyze raffinose and stachyose in soybean milk continuously. Twenty grams of *M. vinacea* mycelium (net weight, total α-galactosidase activity = 2.52 × 10^6 units), entrapped within the 100 ml of the polyacrylamide gel, was placed in a glass column and the soybean milk (preheated to 50°C, solid content = 4.10%) was pumped upwards through the column. The inverse relationship between the rate of raffinose and stachyose hydrolysis and the flow rate was shown in Figure 4. At a flow rate of 30 ml/hr, approximately 60% of the raffinose and the stachyose was hydrolyzed; however, this flow rate is too low for practical use. Thus, in order to increase the flow rate, it would be worthwhile to develop another method of immobilizing the enzyme. It is also essential to optimize the conditions of cultivation for *M. vinacea* in large scale to have more production of α-galactosidase per gram of mycelium.

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EFFECT OF SODIUM CHLORIDE ON REHYDRATION OF FREEZE-DRIED CARROTS

ABSTRACT

The incorporation of NaCl into freeze-dried carrot slices was found to have a marked effect on rehydration. Highest rehydration ratios were obtained at the 0.2M NaCl treatment. After 5 min of rehydration, the 0.2M NaCl treatment resulted in a 54% increase in rehydration ratio over nontreated control samples. Microstructure of the freeze-dried carrot tissue was characterized utilizing the scanning electron microscope. Location of crystalline and amorphous salt deposition was observed and related to rehydration characteristics.

INTRODUCTION

A BASIC OBJECTIVE in food preservation is that the product be easily prepared and indistinguishable from a fresh sample. One major problem with many freeze-dried foods is long rehydration times (Hanson, 1961). Adequate rehydration is essential in order for the foodstuff to have satisfactory eating quality. Macphearson (1973) found that freeze-dried carrot bars exhibited rehydration periods as long as 45 min. Gane and Wager (1958) found in vegetable tissue that the elasticity of cells and the swelling power are important for good rehydration. Both are reduced by substantial heat treatment and drying. Van Arsdel and Copley (1963) observed that drying can diminish the osmotic properties of cell walls.

Early workers demonstrated the superiority of freeze-dried food over the more conventional air-dried counterparts (King, 1971; Karel and Goldblith, 1964). Structural rigidity is maintained and tissue collapse is avoided. However, in some cases, complete structural rigidity may hamper rehydration due to the absence of pathways for the entrance of water. Recent investigations have employed processing modifications to aid in water uptake.

The rate of freezing affects the rehydration characteristics of freeze-dried vegetables. With rapid freezing, ice crystals are small; however, as speed of freezing is reduced the size of ice crystals increases and results in severe mechanical damage to the cell structure (Brown, 1967; Litwiller and Pettit, 1957). Longan (1973) determined that slowly frozen carrots had the highest rehydration ratios because of greater cell disruption which facilitates water uptake.

Cooking procedures have been another method employed to aid in rehydration of freeze-dried foods. Rahman (1972) determined that increased cooking times could improve rehydration via textural and composition changes in cell walls. Individual cell walls lose rigidity upon heating and separation of cells may take place when intercellular pectic materials are softened (Sterling, 1955; Anderson, 1935).

The present work examines the incorporation of sodium chloride into freeze-dried carrots in relation to rehydration. Optimum molar concentrations can be an important processing variable. Microstructure of tissues and adjuncts are necessarily related to rehydration mechanisms.

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EXPERIMENTAL

Processing

Carrots used in this study were of the Imperator 58 Cultivar, obtained from the Texas Agricultural Experiment Station. The carrots were graded to remove roots less than 3/4 in. and larger than 1 1/2 in. in diameter. Carrots were hand peeled, washed and 3/8-in. slices were prepared. The slices were cooked in distilled water at 212°F for 8 min. In order to determine the effect of NaCl on rehydration, aliquots of cooked carrots were soaked for 24 hr in increasing molar concentrations which included: 0.0, 0.1, 0.2, 0.5, 1.0 and 2.0M. In addition to the above concentrations, a no-soak control was included. Samples were frozen at 0°F and freeze drying was accomplished.

A REPP model FDD 15 Sublimator Freeze-dryer equipped to continuously measure and record product weight and temperature was used for all experiments. A shelf temperature of 80°F was utilized and the drying time was 44 hr. Longan (1973) showed these conditions to be optimum in that tissue destruction is minimized. The vacuum was maintained at 10 microns. The final moisture content was 3.0%. The freeze-dried samples were stored in desiccators for evaluation.

Rehydration was determined by placing samples in distilled water at 75°F and calculating rehydration ratios at 30 sec, 5 and 10 min intervals. After each time period, the samples were removed, allowed to drain on a #16 screen for 30 sec without blotting and weighed. Rehydration ratios were calculated by dividing the rehydrated weight by the initial freeze-dried weight. Carrots used in this study contained 91% initial moisture and were freeze dried to 3% moisture. Thus a 12-g sample of freeze-dried carrots which rehydrated to 100g would have a rehydration ratio of 8.33 indicating that all moisture lost during freeze drying had been reabsorbed. Differences in processing variables may cause slight variations in this ratio.

Histology

Rehydration of freeze-dried foods can be observed by the unaided eye. However, since rehydration takes place on a cellular level, a scanning electron microscope was employed to investigate the mechanisms involved. The scanning electron microscope included two areas of investigation: (1) developmental techniques for sample preparation of freeze-dried carrot tissue; and (2) observation of parenchyma and tracheid tissue of freeze-dried carrot slices precooked and soaked for 24 hr in varying molar concentrations of NaCl.

The best method of sample preparation involved simply notching the sides of a rectangular block of freeze-dried tissue with a razor blade and breaking the sample between the thumbs. This resulted in a surface free from artifacts which proved excellent for viewing. Plastic tape provided a good mount.

Once quality tissue mounts were obtained, they were placed on a rotary stage of a high vacuum evaporator. After evacuation, a 50–100 Å coating of carbon was deposited on the tissue surface followed by a 150–200 Å coating of 40% paladium/60% gold alloy. This preparation was necessary to inhibit surface charging of the freeze-dried tissue so that a quality picture could be obtained.

Observation of parenchyma tissue concerned freeze-dried carrot slices pre-cooked and soaked for 24 hr in varying molar concentrations of 0.0, 0.1, 0.2, 0.5, 1.0 and 2.0M NaCl. Photographs were taken at a power of 50×. In addition, a no-soak control was examined. Next, observations of tracheid tissue from the same freeze-dried carrot slices were performed. Molar concentrations of 0.0, 0.1, 0.2, 0.5, 1.0 and 2.0M were examined and photographs of 0.2 and 2.0M were taken at 2000 and 1500×.

RESULTS & DISCUSSION

GRAPHIC RESULTS for this experiment are presented in Figure 1. Rehydration ratios were highly significantly different between NaCl treatments (Table 1). It was found that significant increases in rehydration, based on Duncan's Multiple Range Test, occurred between the no-soak and the 24 hr distilled water soak for all rehydration times (Table 2). After rehydration, for 30 sec for example, the 24 hr distilled water soak gave a 42.6% increase in rehydration. Likewise, at 5 and 10 min rehydration times, increases of 37.5 and 37.0% were observed. Therefore, it can be stated that simple soaking in distilled water for 24 hr prior to freeze drying can have a dramatic effect on rehydration characteristics. This phenomena can be explained by the fact that a 24-hr soak would cause cells to become turgid with a maximum uptake of water. Consequently, freezing would cause greater tissue disruption since much more water would be available for ice crystal formation. Another possible explanation would be that sugars and cell constituents were partially leached during soaking. This would tend to confirm previous research that sucrose has a negative effect on rehydration (Longan, 1973; Macphearson, 1973; Curry, 1974).

Another very interesting result was that soaking in 0.1 and 0.2M NaCl gave significant increases above all other treatments for all rehydration times. For 30 sec, 5 and 10 min rehydration times, corresponding increases in rehydration ratios for the 0.2M treatment were 21.2, 15.9 and 14.0% greater than the 0.0M control. It appears that 0.2M NaCl gave maximum rehydration in all cases. Above this concentration, rehydration ratios began to decline rapidly. For example, 0.5M and 1.0M NaCl treatments showed no significant increase from the distilled water control. Even more noticeable was that in the 2.0M treatment, significant decreases in rehydration below the 0.0M control were observed for all rehydration times. For 30 sec, 5 and 10 min corresponding decreases of the 2.0M NaCl treatment below the control were 48.6, 37.0 and 35.6% (Table 2). This effect was probably due to a physical blockage resulting from excess crystal deposition (Fig. 2D). Another important aspect was that supporting statistical analysis indicated a lack of significant interactions between soak and time. This would imply that regardless of time of rehydration, all main effects are behaving in the same manner; therefore, the 0.2M NaCl treatment gave maximum rehydration ratios.

The results with NaCl are dramatic but questions remain as to the mechanisms involved. In theory, NaCl is a dipolar molecule and will readily ionize into sodium and chloride ions when placed in water. These ions could permeate carrot tissue during a 24-hr soak period. During freeze drying the ions would reassociate to form NaCl crystals inside cellular components such as parenchyma cells and vascular tissue. Based on physical chemistry phenomena, the NaCl crystals could have a profound effect when reassociated with water (Hamill et al., 1966; Eggars and Gregory, 1964; Morris, 1965).

Water, being a polar compound, will show strong attraction toward positive and negative charges of the sodium chloride. According to O'Conner (1974), the initial direction of reaction would be almost totally toward the NaCl crystals due to the many exposed surface charges. Once these were neutralized, the molecule of NaCl would immediately dissociate to form concentrated areas of Na and Cl ions which would also have an attraction effect on water. These aspects would certainly have an effect on rehydration.

Another possible explanation as to mechanisms involved centers around the osmosis concept. Interestingly, maximum rehydration was observed at 0.2M, which is very close to being isotonic in relation to cellular constituents.

The scanning electron microscope is a highly useful tool for studying rehydration in freeze-dried foods. One main advantage exists in the fact that tissue can be observed in the freeze-dried state. Thus, crystal substances, such as NaCl, can be readily observed. With the light microscope, crystals would be destroyed during sample preparation because of contact with liquids. The sample preparation developed for this study resulted in a high quality specimen with excellent depth of field.

Observation of parenchyma and tracheid tissue from freeze-dried carrot slices treated with NaCl for 24 hr were very interesting and differences in tissue composition were observed between treatments. Actual NaCl crystals were observed in

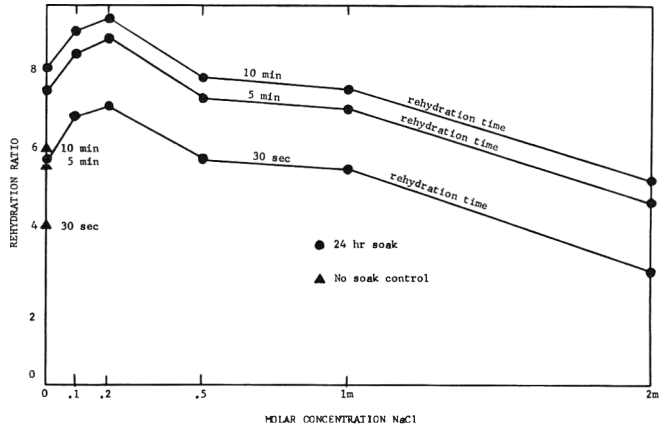


Fig. 1—Rehydration of freeze-dried 3/8 in. pre-cooked carrot slices soaked in NaCl for 24 hr.

Table 1—Analysis of variance of the effects of NaCl on rehydration ratios of freeze-dried 3/8 in. carrot slices

Source	df	Mean square for rehydration ratios
Total	62	
Treatments	6	18.40**
Treatment samples	14	0.93**
Time	2	24.82**
Treatments X Time	12	0.02
Residual	28	0.02

** Significant at the 1% level of probability

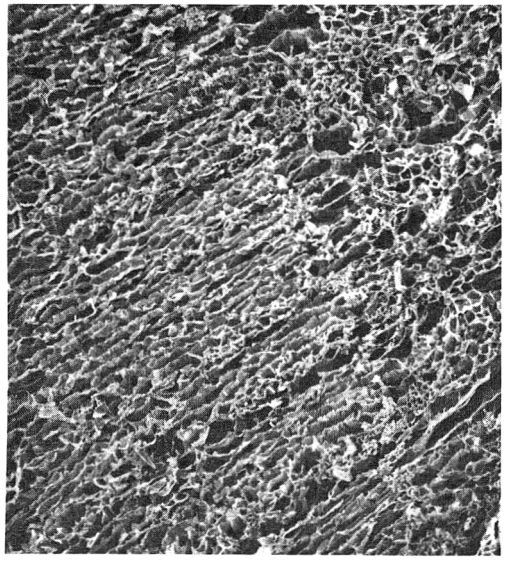
Table 2—Rehydration ratios of freeze-dried 3/8 in. pre-cooked carrot slices soaked in NaCl for 24 hr

NaCl Treatment 24-hr soak	Rehydration ratios[a] 30-sec soak	5-min soak	10-min soak
No-soak control	3.98 a	5.36 a	5.80 a
0.0 M	5.68 b	7.37 bc	7.95 bc
0.1 M	6.66 c	8.20 cd	8.77 cd
0.2 M	6.89 c	8.55 d	9.06 d
0.5 M	5.66 b	7.14 bc	7.68 bc
1.0 M	5.40 b	6.92 bc	7.42 b
2.0 M	2.92 d	4.65 a	5.12 a

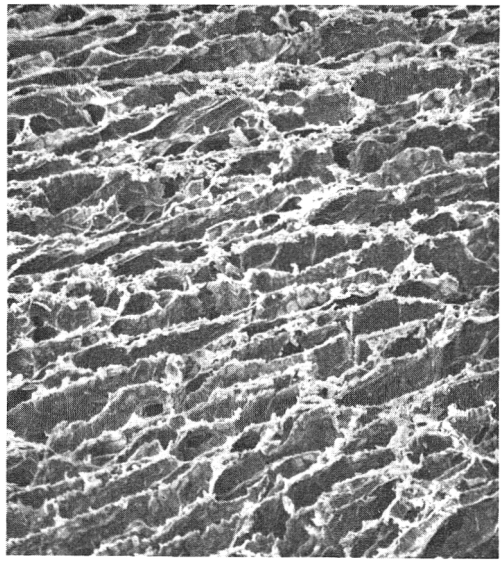
[a] Means followed by different letters are significantly different at the 5% level according to Duncan's Multiple Range Test.

cellular components. It appeared that, as molar concentration of NaCl increased past the 0.5M level, greater tissue disruption occurred. However, past the 0.5M level, cell walls were clustered with amorphous salt crystals. This may be a basis for decreased rehydration. Certainly excess salt would plug portals of entry for water. Photomicrographs depicting the salt treatments are presented in Figure 2. Examination of photomicrograph B reveals greater cell disruption than A; however, rehydration was much less. This confirms the theory that excess salt can inhibit rehydration regardless of cell disruption. Very little difference in cell structure was observed between the 0.0M and 0.2M NaCl soak. It was interesting that 0.2M concentration was very close to physiological saline and thus approximated an isotonic solution. Although salt did penetrate tissue, plasmolysis did not occur as with higher concentrations. The deposition of NaCl crystals in tracheid cells of tissue soaked for 24 hr in 0.2 and 2.0M solutions prior to freeze drying is shown in Figures C and D. The crystals formed at 2.0M were larger than those crystals formed at 0.2M and this increase in size may have a blockage effect on water uptake. Also, the noncrystalline amorphous deposition is much greater at the 2.0M concentration.

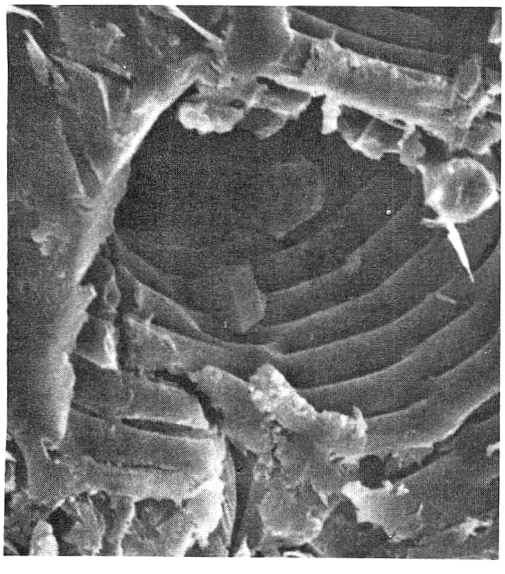
A possible mechanism is illustrated in Figure 2 for increased rehydration in freeze-dried carrots pre-treated with NaCl. Each crystal contains many surface charges which would have an affinitive action on a polar water molecule. Since the crystals are small in size, ionization would take place easily and form concentrated areas of positive and negative ions. This in turn,



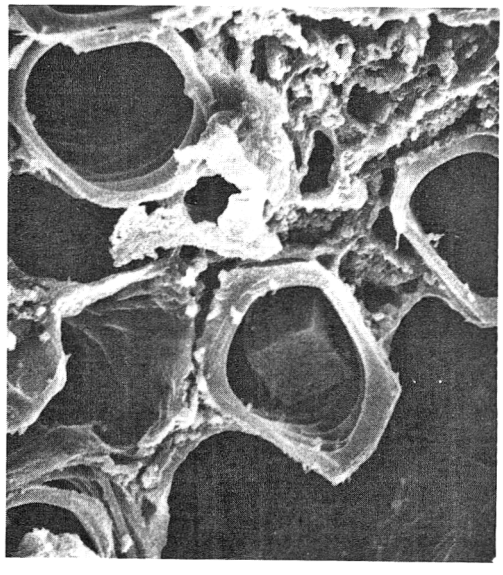
A. Precooked tissue soaked in 0.2M NaCl for 24-hr (50X)



B. Precooked tissue soaked in 2.0M NaCl for 24-hr (50X)



C. Tracheid cell with NaCl crystal deposition; 0.2M 24-hr soak (1500X)



D. Tracheid cell with NaCl crystal deposition; 2.0M 24-hr soak (1000X)

Fig. 2—Scanning electron photomicrograph of freeze-dried carrot tissue.

might further attract water setting up a current flow. This process would proceed as a chain-type reaction; whereby, water attracted to one crystal would be brought into close proximity with other crystals causing an increase in flow rate. Although purely speculative, this theory does offer a reasonable explanation of how NaCl affects rehydration.

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PARTICULATE PROPERTIES AND RHEOLOGY OF PREGELLED YAM (Dioscorea rotundata) PRODUCTS

ABSTRACT

Yam was processed and roller dried. Processing variables included tissue cooking and comminution conditions. Water related characteristics of these pregelled flours were obtained. Increasing the degree of starch damage changed flour specific surface, crystallinity, monolayer adsorption, heat of adsorption, wetting temperature, water-holding capacity and swelling power of the flour. Rheological properties of doughs were determined by simple compressive and cyclic tests with an Instron Universal Testing Machine. Modulus of elasticity, ultimate deformation, recoverable elasticity and energy loss ratio of the various samples were related to the degree of starch damage.

INTRODUCTION

THE YAMS of the genus *Dioscorea* L. Poir contain many species and cultivars and form a group of very important staples in the Tropics. In recent years attempts have been made to apply modern food technology to the processing and food utilization of the yams. However, there is little scientific and technological data on the *Dioscorea* yams. Rasper (1969) reported on the gelatinization and pasting viscosity of yam starches.

Rodriguez-Sosa and Gonzalez (1972) studied a flaking process of the species *Dioscorea alata*. A study of starch retrogradation in reconstituted yam products was reported by Brennan and Ayernor (1973). An approach to the development of foods based on yams for areas where the yam plays a major role in the food economy is reported elsewhere (Ayernor et al., 1974). This paper is concerned with the study of physicochemical properties of pregelled yam powder systems which can be utilized in various forms such as composite flours, thick pastes and doughs in accord with food culture patterns in the Tropics.

EXPERIMENTAL

THE YAM used in this work was *Dioscorea rotundata*. Its proximate composition is shown in Table 1; for comparison, composition of Irish and sweet potato (Coursey, 1967) are included. A standard method for the processing of pregelled yam flour and reconstitution of a thick paste is summarized in Table 2. Two of the steps were varied to effect differences in powder characteristics. Preheating was done at 0, 5 and 10 psig steam pressure. Three different intensities of tissue comminution were applied; (1) 'mashing' with a Kenwood mixer (15 min) (2) 'standard' comminution with a bowl clapper (15 min); and (3) colloid milling of the standard slurry from (2).

Powder characteristics

Starch damage. The method of Mullins et al. (1955) for the iodine blue value index (BVI) was used.

Water sorptions. Sorption isotherms were constructed using the flask method (Karel and Nickerson, 1964). Monolayer values were calculated from a BET plot and specific surface areas and heats of sorption were derived as described by Labuza (1968).

Relative crystallinity of powders. This was determined from water adsorption by the gain in weight method (Sterling, 1960) at relative humidity 75%, using cellulose powder "D300" as a standard at 70% crystallinity.

Swelling and water-holding capacity. The method of Leach et al. (1959) for starch was adopted.

Temperature of wetting. Five cm^3 of distilled water at 25°C was measured into a test tube containing an "Ellab" thermocouple. The thermocouple was moved into various positions in the forming paste. Temperatures were noted and the highest temperature reached was recorded. The means of the highest temperature increase from ten replicates is reported here.

Rheological tests

Simple compressive and two-cycle tests on short cylinders of thick pastes were carried out using an Instron Universal Testing Machine according to Ayernor et al. (1974); Brennan and Ayernor (1973). The

Table 2—Steps in the processing of pregelled yam flour and its reconstitution into a dough

Processing stages	Conditions
Peeling and dicing	1/2" × 1" × 1"
Washing	0.2% $Na_2S_2O_5$ water
Preheating	Steam: 10 min
Comminution	Slurry: 15% solids
Dehydration	Drum: 80 psig:20–30 sec.
Milling	Hammermill
Screening	100 mesh (150μ)
Reconstitution to dough	2 min:30 g_s:50 cm^3 water

Table 1—Proximate composition of yam (Dioscorea rotundata) used here as compared to Irish potato and sweet potato

Tuber	Water (%)	Carb. (%)	Fat (%)	Crude Prot. (%)	Fiber (%)	Ash (%)
Yam	62	34	0.09	1.23	0.42	0.97
Potato	68–82	14–27	0.02–0.18	1.14–2.28	0.28–0.55	0.78–1.16
Sweet potato	58–81	17–43	0.18–0.68	0.45–4.37	0.60–4.54	0.66–1.98

parameters (1) modulus of elasticity; (2) ultimate (yield) deformation; (3) recoverable elasticity; and (4) energy loss ratio were determined.

RESULTS & DISCUSSION

TABLE 3 shows an increase in starch damage in the pregelled yam powders with intensity of tissue heating. Swelling power and solubility also increased. Bulk density of the powders showed a decrease with increase in cooking of tissues. Irregularity of particle shape was observed to increase with severity of cooking. Further tests with particles in excess water showed that water-holding capacity and temperature of wetting also increased with intensity of tissue cooking (Table 4) and starch damage (Table 3). These trends suggest an increase in the water-binding component of the starch system. This assumption is confirmed by a decrease in the crystalline component (Table 4) meaning an increase in the amorphous component which is the effective fraction for water-binding (Collison, 1968).

Water sorption characteristics of the three differently heat-treated samples are shown in Figure 1. These were used to obtain BET plots, Figure 2. The monolayer capacity was obtained from the BET plots and are given in Table 5. These showed an increase with intensity of tissue cooking. Increase in specific surface of powders and heat of water vapor sorption paralleled the increase in starch damage that resulted from increased tissue cooking.

The specific surface values obtained here are very high as compared to values reported for other foods (Berlin et al:, 1966). This is probaly due to differences in adsorbate: nitrogen is usually used for this determination while water was used here. Specific surface values of processed foods determined with water, generally show higher values than those determined with nitrogen (Berlin et al., 1966). This is so because water molecules are capable of plasticizing the various long chain polymers that make up the structural matrix, thus exposing all the interior sites for water adsorption (Stitt, 1958).

In addition, in these highly processed pregelled powder systems where the degree of disintegration of the starch granule is so high, the relation of water to specific surface may be expected to be highly increased.

The highly pregelled powder has a high affinity for water (Fig. 1). The wettability rate of all three powders was so high

Table 3—Influence of steam pressure used to cook yam dice for ten minutes on four powder characteristics

Steam pressure (psig)	Powder bulk density (g_s/cm^3)	Starch damage BVI	Swelling power (cm^3/g Solids)	Solubility (g_s/cm^3 water)
0	0.31 ± 0.05	70 ± 2	11.7	2.4
5	0.30 ± 0.07	100 ± 3	11.7	2.6
10	0.28 ± 0.03	110 ± 1	13.2	3.1

Table 4—Relation of crystallinity, water-holding and wetting properties of powders to the steam pressure used to cook the tissue

Steam pressure (psig)	Powder crystal-linity %	Water–holding capacity (g water/ g solids)	Wetting temp (T)°C
0	27.7	10.60	2.30 ± 0.10
5	24.2	12.96	2.90 ± 0.21
10	18.8	15.37	3.11 ± 0.23

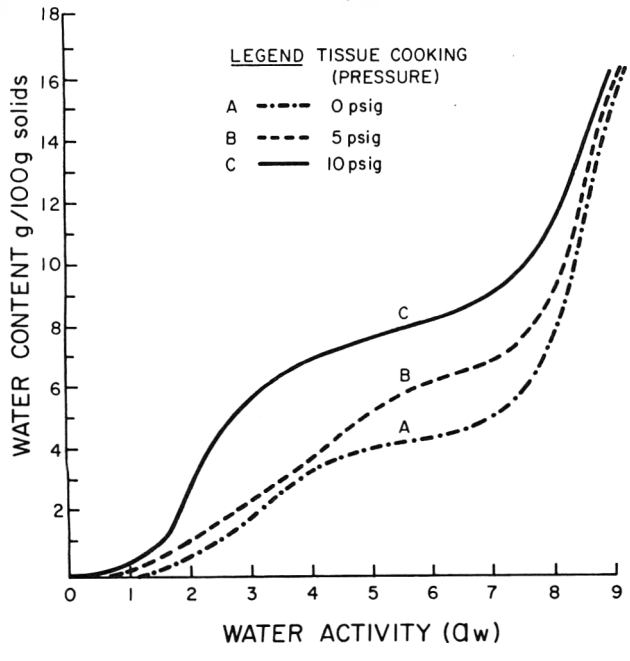

Fig. 1—Water sorption isotherms of pregelled yam flours.

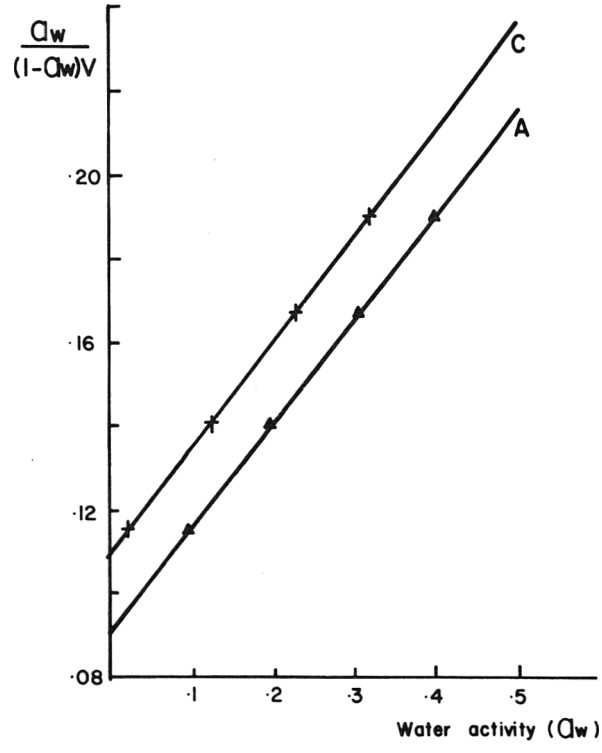

Fig. 2—A B.E.T. plot from Figure 1.

Table 5—Water sorption characteristics of powders

Pressure cooking (psig)	Monolayer capacity (g water/ 100g solids)	Heat of sorption ($\times 10^{3\,2}$ kcals)/g solids	Specific surface powders ($\times 10^3$ m^2/g)
0	4.54	9.476	10.641
5	5.18	14.007	18.301
10	7.57	32.072	23.989

Table 6—Rheological properties of yam dough at 15 min after reconstitution

Steam pressure (psig)	Modulus of elasticity ($\times 10^5$ dynes/cm^2)	Ultimate (yield) deformation	Recoverable elasticity %	Energy ratio
0	5.2	0.50	66	0.77
5	4.9	0.48	73	0.71
10	4.4	0.45	81	0.55

Table 7—Influence of tissue comminution on powder and dough characteristics. Tissue was cooked for 10 min at atmospheric pressure

	Powder	Dough
Tissue comminution	Starch damage BVI	Modulus of elasticity ($\times 10^5$ dynes/cm^2)
Mash (Kenwood mixer)	61	2.10
Standard (Bowl chopper)	72	1.52
Colloid (Colloid milled)	97	1.35

that they formed a thick paste in only two minutes under the standard mixing operation (Table 2). Under the specified conditions (Table 2) for reconstitution, the resulting dough contains 64% moisture (wet basis); the powder thus absorbs more than twice as much its own weight of water.

The rheological characteristics of the reconstituted doughs are shown in Table 6. Modulus of elasticity, which measures rigidity, decreased with increase in heat treatment and starch damage (Tables 3 and 6). Ultimate or yield deformation, which is the strain at yield force, also followed a similar trend. However, recoverable elasticity or "springiness" increased with degree of tissue cooking and starch damage. Table 6 also shows a decline in energy ratio with increased cooking. Energy ratio is a useful rheological parameter which is dimensionless (Ferry, 1970). The ratio reported here (ratio of area under irreversible deformation to that under reversible deformation) gives an indication of energy loss in a cyclic deformation exercise. The decrease in this energy ratio means there is an increase in resilience with increase in the starch-water interaction. This is supported by the parallel increase in monolayer capacity, specific surface and heat of water sorption found above.

Since reconstitution conditions and moisture contents in the dough samples were the same, differences in rheolcgical properties reflect differences in starch-water binding. The latter differences are due to starch damage. Up to this point, starch damage has been attributed to the steam cooking. However, Table 7 shows that the degree of starch damage is also affected by tissue comminution; increased energy input increased starch damage. Rheologically, this variable also affected the rigidity of the reconstituted dough; the modulus of elasticity (Table 7) decreased, meaning that rigidity decreased, with increased energy input.

The traditional method for making tuber or root doughs calls for cooking followed by a strenuous pounding of the boiled tissue. The end product should be highly elastic. Therefore, the practical significance of this work is that it makes possible the development of a process for making a commercial yam powder which will reconstitute into a paste having the traditional rheological properties of resilience and elasticity as is mandatory for consumer acceptance.

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CAROTENE OXIDIZING FACTORS IN RED PEPPER FRUITS (Capsicum annuum L.). Ascorbic Acid

ABSTRACT

Two active carotene-bleaching fractions were separated from aqueous pepper extract, one a protein and the other a low-molecular weight compound. The low-molecular weight compound was identified as ascorbic acid, accompanied by dehydroascorbic acid according to the rate of migration upon TLC and specific chemical reactions. The behavior of this low-molecular fraction also fitted ascorbic acid and dehydroascorbic acid with regard to the effect on carotene oxidation and the changes in bleaching activity brought about by the addition of EDTA, changes in pH and in the concentration of the fraction.

INTRODUCTION

RED PEPPER FRUITS (*Capsicum annuum* L.) are used for preparing various dehydrated products, such as ground pepper (paprika), red pepper flakes and oleoresin, i.e., a solvent extracted fraction from the fruit which is used especially as a food colorant.

The most important quality criterion of red pepper products is their color which is due to carotenoids, especially capsanthin and capsorubin (Vinkler and Kizel-Richter, 1972).

During processing and storage the carotenoids are oxidized and the color fades (Lease and Lease, 1956, 1962; Chen and Gutmanis, 1968). The glycerides of the pericarp are rich in linoleic acid (Szabo, 1970; Philip et al., 1971) which is itself subject to peroxidation and may induce the bleaching of carotenoids by a coupled process (Budowski and Bondi, 1960).

The aim of this research was to separate and characterize water-soluble factors present in pepper fruits which affect the rate of carotenoids bleaching in aqueous carotene-linoleate solutions.

MATERIALS & METHODS

RED PEPPERS (*Capsicum annuum* L.) var. Wander California, were used for the separation of active water-soluble factors.

Linoleic acid and dehydroascorbic acids were obtained from Fluka AG, Buchs, SG, Switzerland. Ascorbic acid was from E. Merck AG, Darmstadt; all trans β-carotene from Eastman Kodak Co., Rochester, N.Y.; Tween-20 from Nutritional Biochemical, Cleveland, Ohio; Na_2 EDTA and Tris (hydroxymethyl) aminomethan were obtained from Fisher Scientific Co., N.J.; and Sephadex G-50 from Pharmacia Fine Chemicals Inc., N.J.

The aqueous pepper extract was prepared by homogenizing 100g pepper pericarp with 100 ml distilled water or 0.05M Tris buffer, pH 7.0. This was done in a Waring Blendor operated at top speed for three 2-min periods at 15-min intervals. The blending and subsequent preparative operations were carried out in a cold room maintained at 4°C. The homogenate was filtered through four gauze layers and centrifuged for 10 min at 20,000 × G. In some experiments the supernatant was dialyzed against 0.01M Tris buffer at pH 7.0 for 24 hr.

Fractionation by Sephadex G-50 was conducted as follows: The aqueous pepper extract from 100g fruit was dehydrated by lyophilization. The powder was dissolved in 25 ml acetate buffer 0.05M, pH 5.5. 1 ml of this extract, representing 4g pericarp, was absorbed onto a Sephadex G-50 column, 2 × 30 cm, and the column was washed with acetate buffer 0.05M, pH 5.5. 6-ml fractions were collected and tested for protein (Lowry et al., 1951) method and carotene-bleaching activity, as described below.

The low-molecular weight fraction separated by Sephadex G-50 was lyophilized and dissolved in 2 ml ethanol 50%. This solution was submitted to thin-layer chromatography on 20 × 40 cm glass plates coated with 0.5 mm silica gel-G. The developing solvent was chloroform-methanol (3:1, v/v) containing 2.5% (w/v) oxalic acid (Hasselquist and Jaarma, 1963). The separated compounds were located and identified by viewing the plates under long-wave UV light, and by spraying with sulfuric acid, phosphomolybolic acid or phenylhydrazine (Stahl, 1969). The corresponding areas of adsorbent which had not been sprayed were scraped into 10 ml 0.1M Tris buffer pH 7.0 and after mixing and centrifuging, the carotene bleaching activity was measured in the supernatant. Ascorbic acid (110 μg) and dehydroascorbic acid (250 μg) were used as references.

The assay of carotene-oxidizing activity was carried out according to the method of Ben-Aziz et al. (1971). Briefly, the technique consists in following the decrease in absorbance at 460 nm in the cuvette of a double-beam recording spectrophotometer. The test sample contained 1.5 ml buffered carotene-linoleate, 0.1–0.4 pepper extract, and distilled water to a final volume of 2.0 mL Concentrations in the initial reaction mixture were as follows: β-carotene 1.4×10^{-5}M, linoleate 2×10^{-3}M, Tween-20 0.05%, Tris buffer, pH 7.0, 0.1M. The initial rate of decrease in absorbance was computed from the recorded graph and converted into rate of carotene decrease in concentration (μM).

RESULTS

Effect of dilution

Carotene-bleaching activity was found in the crude extract only after dilution, with maximal activity appearing at a 40-fold dilution (Fig. 1A). Similar results were obtained whether

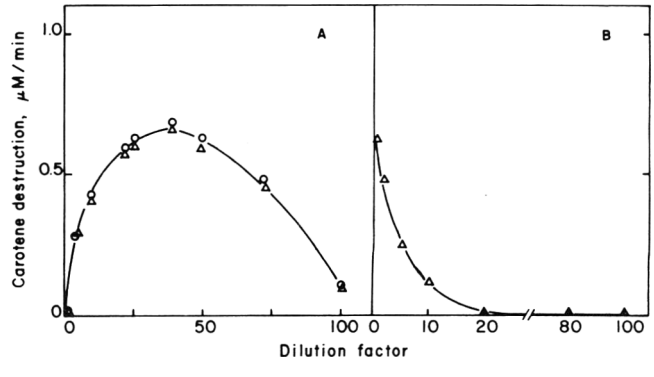

Fig. 1—Effect of dilution on carotene destruction in the presence of pepper extract before (A) and after dialysis (B). (△, dilution by buffer Tris 0.1M, pH 7.0; ○, dilution by distilled water.)

the blending and subsequent dilutions were carried out with 0.05M Tris buffer, pH 7.0, or with distilled water. In all subsequent experiments, distilled water was used for extraction and dilution. Unlike the crude extract, the dialyzed extract exhibited carotene-bleaching activity without dilution. Upon dilution, the bleaching activity of the dialyzed extract progressively declined (Fig. 1B).

Effect of heat and addition of EDTA

The activity of the crude diluted (1:40) extract was heat resistant but strongly inhibited by EDTA (Fig. 2). The dialyzed extract was not affected by EDTA but lost its activity upon boiling.

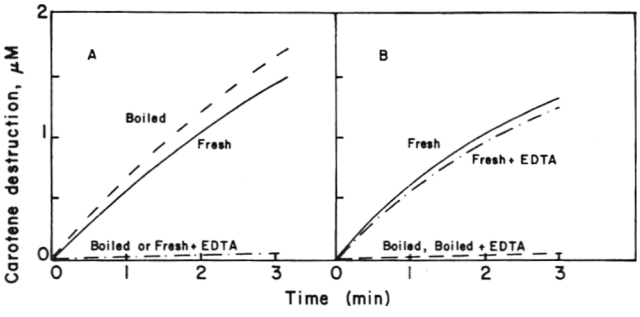

Fig. 2—Effect of heat treatment and EDTA on carotene destruction in the presence of pepper extract before (A) and after dialysis (B). (A, diluted 1/40 with distilled water; B, dialyzed undiluted.)

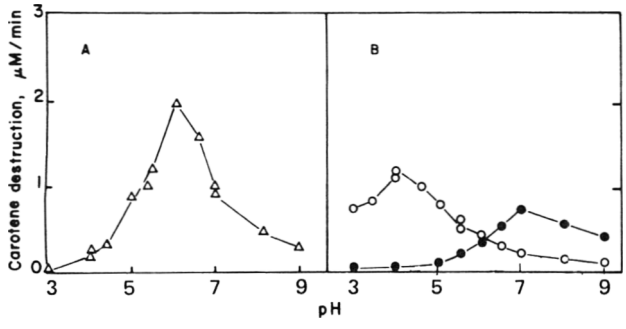

Fig. 3—pH profiles of pepper extract before (A) and after dialysis (B). (△, pepper extract diluted 1/40; ●, dialyzed pepper extract; ○, dialyzed pepper extract diluted 1/5.)

Table 1—Thin-layer chromatography of low-molecular weight fraction[a]

Fraction	R_f	PMA[a]	Compounds identified by PHZ Before[b]	PHZ After[b]	Fluorescence (long-wave UV)	Carotene destruction (μM/min)
1	0	–[c]	–	–	–	0.2
2	0.32	+	–	–	–	0.2
3	0.45	+	–	+	+	1.7
4	0.50	–	+	+	–	0.8
5	1.00	–	–	–	–	0.2
AA	0.46	+	–	+	+	1.2
DHAA	0.49	–	+	+	–	0.6

[a] Abbreviations: PMA, phosphomolybdic acid; PHZ, phenylhydrazine; AA, ascorbic acid; DHAA, dehydroascorbic acid.
[b] Before or after exposure to Br_2 vapor.
[c] –, negative; +, positive

Effect of pH

The crude diluted extract showed greatest activity at pH 6.0 (Fig. 3A). The optimal pH of the dialyzed extract was 6.0, but shifted to 4.0 upon dilution (Fig 3B).

Fractionation on Sephadex G-50

Two carotene-bleaching fractions were obtained by passage of the crude extract through Sephadex G-50 (Fig. 4). Only the first fraction, A, contained protein. The second, low-molecular fraction, B, was protein-free.

Identification of fraction B

Thin-layer chromatography on silica gel-G yielded five fractions, two of which (2 and 3, Table 1) exhibited strong carotene-bleaching activities. These were identified as ascorbic and dehydroascorbic acids, respectively, on the basis of R_f, reduction of phosphomolybdate, and reaction with phenylhydrazine, before and after exposure of the plates to bromine vapors.

Bleaching activities of ascorbic and dehydroascorbic acids

Both compounds were tested in 0.1M Tris buffer at pH 7.0. The carotene-bleaching activity of ascorbic acid (○——○, Fig. 5) increased with concentration, reached a maximum at 10^{-5} M and decreased again at higher concentrations, while the optimal concentration of dehydroascorbic acid (△——△, Fig. 5) was about 10^{-3} M. At concentrations of 10^{-3} M, ascorbic acid was about twice as active as dehydroascorbic acid. The bleaching activity of ascorbic acid resembled that of the dilute pepper extract with respect to the optimal pH, inhibition by EDTA and lack of sensitivity to heat.

DISCUSSION

OUR WORK has revealed the presence of two carotene-bleaching factors in aqueous pepper extracts, one of which is a low molecular nonprotein substance, identified as ascorbic

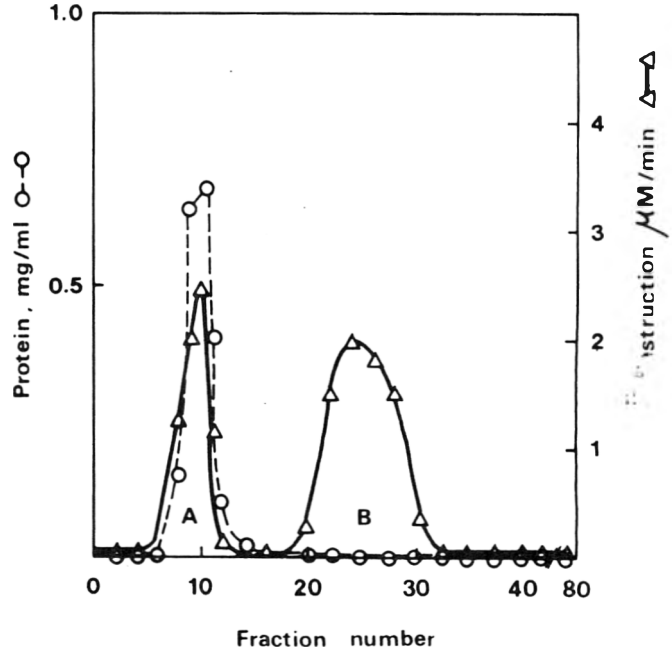

Fig. 4—Separation of pepper extract by Sephadex G-50.

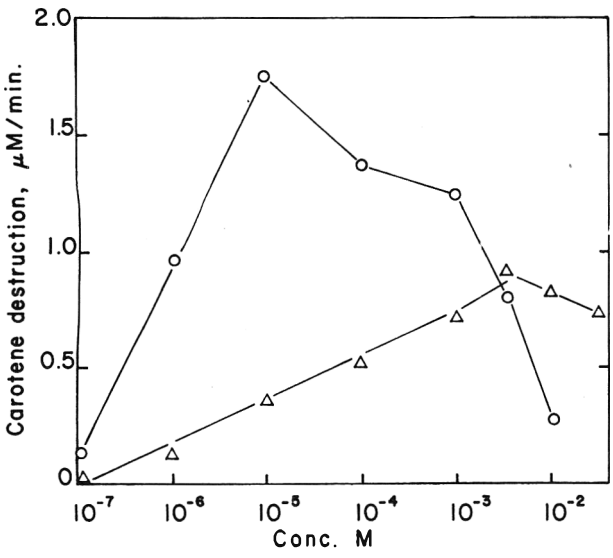

Fig. 5—Effect of ascorbic acid and dehydroascorbic acid concentrations on carotene destruction. (○, ascorbic acid; △, dehydroascorbic acid.)

acid, accompanied by dehydroascorbic acid. Pepper fruit is known as a rich source of vitamin C, hence the presence of ascorbic acid in the aqueous extract is not unexpected. The carotene-bleaching properties of this reducing compound become understandable if the following facts are considered:

(a) In the particular assay system used carotene-bleaching is coupled to the peroxidation of linoleate, so that any factor influencing linoleate oxidation will also be expected to affect the rate of bleaching (Budowski and Bondi, 1960).

(b) Ascorbic acid is known to exhibit pro-oxidant properties toward linoleate in the presence of cupric or ferric ions (Barber, 1966; Hasse and Dunkley, 1969; Allan and Wood, 1970).

That metal ions are involved in carotene-bleaching under the conditions of the test is seen from the strong inhibitory effect of EDTA toward the bleaching activity of pure ascorbic acid, as well as that of dilute crude pepper extract. It appears therefore that carotene bleaching proceeds via the enhanced oxidation of linoleate. Moreover, ascorbic acid, in the presence of cupric or ferric ions, has been shown to catalyze the breakdown of lipid peroxides (O'Brien and Little, 1969). Since the homolytic scission of hydroperoxides is responsible for the autocatalytic character of linoleate autoxidation (Labuza, 1971), it is probable that the primary action of ascorbic acid also involves peroxide decomposition in the present bleaching system. In this respect it will be noted that the optimal pH for carotene-bleaching in the presence of ascorbic acid or dilute crude pepper extract is similar to the values at which maximal activities were reported for the ascorbic acid-catalyzed peroxidation of tissue lipids (Barber, 1966) and for the ascorbic acid-catalyzed decomposition of lipid peroxides (O'Brien and Little, 1968).

The peculiar dilution effect, whereby carotene-bleaching activity in the crude pepper extract appears only upon dilution, is paralleled to a large extent by the bleaching activity of ascorbic acid itself. In fact, the natural ascorbic acid content of crude pepper extract is about 10^{-2} M (Bernice and Merrill, 1963) a value sufficiently high to account for its lack of bleaching activity.

Dehydroascorbic acid, which was present alongside ascorbic acid in the crude pepper extract, also exhibited carotene-bleaching activity. This compound may be an artifact, formed from ascorbic acid in the course of the extraction and separation procedures.

It is clear from the results reported here that the bleaching activity of ascorbic acid will depend on many factors, such as the concentrations of ascorbic acid, metal ions, lipid peroxides, the presence of water, and others. Some of these interacting factors are being further investigated (Kanner, 1974).

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PEROXIDASE REACTIONS AND ORANGE JUICE QUALITY

ABSTRACT

Representative samples of fresh orange juice (OJ) with various flavor scores were assayed for peroxidase activity. In the assays, p-phenylenediamine (PPDA), the hydrogen donor, was oxidized by H_2O_2 after a short time lag (about 1 min) caused by interaction of ascorbic acid in the juice with a PPDA-oxidation intermediate. A search was made for compounds that are native substrates of OJ peroxidase. Thus, the peroxidase activity of a protein fraction precipitated from neutralized OJ with ammonium sulfate and dialyzed free of ascorbic acid was tested with H_2O_2 and a number of hydrogen donor compounds that are normal juice constituents. Ascorbic acid was very reactive, as were the phenolic acids (caffeic, gentisic and coumaric). The flavonoids, eriodictyol, hesperidin and naringin were unreactive. Reduced nicotinamide adenine dinucleotide (NADH) was also reactive in the presence of hydroquinone and other compounds that mediate electron transfer through intermediate states. The dialyzed protein fraction also catalyzed the oxidation of pyridoxal-PO_4, indoleacetic acid, dihydroxymaleic acid, and NADH + p-cresol by O_2 plus Mn^{++}. Although OJ appears to contain many compounds that are reactive with peroxidase, their reactivities in OJ are apparently very slow due to the level of H_2O_2. Concentrations of ascorbic and caffeic acids did not change in OJ incubated at 30°C for 4 hr. Processing conditions that increased peroxidase activity and pulp content in OJ decreased quality of the juice.

INTRODUCTION

CONVENTIONAL juice handling practices in the citrus processing plant were recently described (Veldhuis, 1971). Juice from the extractor-finisher is held in large blending tanks until checked for acidity and soluble solids, blended with orange or grapefruit juice, and sweetened with sugar if needed. From the tanks, the juice is piped through deoilerdeaerators to the flash pasteurizer, where it is stabilized against microbial and enzymic deterioration.

Before the juice is pasteurized, products of enzyme-catalyzed reactions could accumulate and produce off-flavor compounds during storage. For example, acetaldehyde, which accumulates in unpasteurized orange juice [as result of the decarboxylation of pyruvic acid by α-ketocarboxy-lyase (Roe and Bruemmer, 1974a)], is probably a source of the diacetyl produced during storage.

Peroxidase in a large number of stored fruits and vegetables has been studied because its rate of destruction during heat treatment parallels that of unknown enzymes actually involved in off-flavor formation in the stored product (Joslyn, 1949). Also, the enzyme is relatively stable to heat inactivation and can be regenerated, if inactivated under certain conditions (Lu and Whitaker; 1974). Orange juice has not been tested for peroxidase activity, although citrus fruits contain many phenolic compounds (Kefford and Chandler, 1970) that are substrates of the enzyme (Balls and Hale, 1934).

We report a study to assay orange juice for peroxidase activity and to determine the significance of peroxidase reactions to quality of commercially processed juice.

MATERIALS & METHODS

Fruit extraction

Juice for assay of peroxidase was extracted from commercially mature, locally grown oranges (Valencia cultivar). They were chilled to 4°C and hand reamed the day of harvest or after storage at 4°C for up to 2 wk. The pulpy juice was strained through two layers of cheesecloth to remove seeds and heavy pulp, and peroxidase extracts were prepared immediately.

For the juice quality study, the orange cultivars, Hamlin, Pineapple and Valencia, were each harvested periodically during their respective producing seasons. Juice was extracted by commercial extractors set to give a hard or light squeeze to the fruit. Details of the extraction and processing procedures were described by Attaway et al. (1972). A total of 96 juice samples was prepared (eight replicates of three cultivars and four setting combinations). A 100 ml aliquot of each sample was removed from the blending tank and quickly frozen with liquid nitrogen (−196°C) for later assay of peroxidase. The mean juice residence time in the tank before freezing was about 15 min. The remaining juice was pasteurized, canned and stored frozen for flavor tests.

Juice serum

Sera were prepared from hand-reamed and commercially extracted juices. Strained juice was immediately neutralized to pH 7 and centrifuged at 10,000 × G for 10 min at 4°C. The supernatant was decanted and assayed for peroxidase by use of p-phenylenediamine (PPDA).

Sera from frozen juice sacs were prepared as described by Bruemmer and Roe (1971) for peroxidase fractionation with ammonium sulfate.

Sephadex-G25 chromatography

Centrifuged, neutralized, juice serum (500 ml) was chromatographed on a 5 × 100 cm Sephadex-G25, medium (Pharmacia Fine Chemicals) column equilibrated with 0.015M citric acid–0.03M sodium phosphate buffer, pH 7.2. Serum was added at the rate of 300 ml/hr, and the column eluted at the same rate with the equilibrating buffer. Eluates were collected in 15 ml portions.

Extract fractionation with ammonium sulfate

Solid ammonium sulfate (AS) was added to juice sacs serum to 30% saturation at pH 7.0. After 1 hr equilibration at 4°C, the supernatant was recovered by centrifugation and made 60% saturated with AS. After 1 hr, the supernatant from this mixture was recovered by centrifugation and made 90% saturated. Each of the three residues recovered from the serum at 0–30%, 30–60% and 60–90% saturation was dissolved in cold 0.005M phosphate buffer, pH 7.0, and dialyzed overnight at 4°C against 4 liter of the buffer.

Peroxidase assays with H_2O_2

p-Phenylenediamine (PPDA) oxidation. Peroxidase activities in orange juice sera and fractions were assayed by the PPDA method described by Lück (1965). Activities were calculated as PO units. One PO unit (U) represents an increase at 485 nm of 0.1 absorbance unit per min of the following solution: 1.8 ml 0.067M potassium phosphate buffer (pH 7.0) 1.0 ml 0.003M H_2O_2, 0.1 ml enzyme prep, and 0.1 ml 1% PPDA. Absorbance was measured with a Beckman DB Spectrophotometer (Beckman Instrument Company).

NADH oxidation. Compounds were tested as mediators in the oxidation of NADH by orange juice peroxidase from the 60–90% AS fractionation. The overall reaction rate was followed by use of a Gilford modified Beckman DU spectrophotometer, which measured the decrease in NADH absorbance at 340 nm. The cuvette contained 135 μmoles of mono- and di-hydrogen potassium phosphates (to buffer the reaction at 7.0), 0.1 μmole test substrate, 3 μmoles H_2O_2, and 0.5 μmole NADH, in 2.9 ml glass-distilled water. The reaction was initiated by adding 0.1 ml enzyme fraction. Relative reactivity was expressed as μmoles NADH oxidized per min per mg protein. Absorbance was proportional to time and to volume of enzyme fraction.

Residual H_2O_2. Compounds were tested for reactivity with orange juice peroxidase from the 60–90% AS fractionation by measuring rate

of H_2O_2 disappearance. The peroxidase-H_2O_2 assay of Altschul and Karon (1947) was modified so that O_2 released from H_2O_2 by catalase was measured polarographically. Catalase activity in preparations of orange juice peroxidase did not interfere with the assay, so they were not preincubated with pyrogallol as recommended by Altschul and Karon (1947). The reaction mixture (2.85 ml) in the chamber of the Biological Oxygen Monitor (Yellow Spring Instrument Co.) contained 135 μmoles of mono- and di-hydrogen potassium phosphate (pH 7.0), 5 μmoles of test substrate, and an aliquot of the enzyme prep. The reaction was initiated with 0.05 ml of 6×10^{-2} M H_2O_2 delivered by a syringe. After 2 min, for determination of unreacted H_2O_2, 0.1 ml of catalase (1.0 mg protein/ml) was injected into the chamber. Activity of the catalase (333 Sigma Units, Sigma Chemical Co.) was sufficient to completely degrade the H_2O_2 to O_2 in 10 sec. The O_2 liberated was measured and converted to μmoles residual H_2O_2 which was subtracted from initial H_2O_2 to give the amount utilized in the reaction. Rate of H_2O_2 utilization was proportional to time and volume of the enzyme prep.

Peroxidase assays with O_2 plus Mn^{++}

NADH oxidation. Compounds were tested as mediators of NADH oxidation by O_2 plus Mn^{++} in presence of orange juice peroxidase from the 69–90% AS fractionation. The method was similar to that described for NADH oxidation by H_2O_2-peroxidase (above) except that air (O_2) and 3×10^{-4} M $MnCl_2$ replaced H_2O_2.

O_2-uptake. The rate of O_2-uptake was measured with a Biological Oxygen Monitor (Yellow Springs Instrument Co.) connected to a Model-SR Recorder (Sargent Instrument Co.). The reaction chamber at 30°C contained substrate and cofactors in 2.9 ml 0.1M potassium phosphate buffer, pH 7.0. The reaction was initiated by adding 0.1 ml enzyme prep. O_2-uptake was immediate, and proportional to time and volume of enzyme prep. The rate was calculated from initial 5 min uptake.

Other methods

Protein contents of the preps were determined by the turbidimetric method of Layne (1957) or by the colorimetric method of Potty (1969). Phenolic contents of eluate fractions from the Sephadex column were estimated by the method of Folin-Ciocalteau (1927). Ascorbic acid was determined by the AOAC method (Horowitz, 1970). Oxidation products of caffeic acid which was added to orange juice, were examined by thin-layer chromatography. Ethyl acetate extracts of fresh orange juice incubated at 30°C for 2 hr with 20 μmoles of caffeic acid per ml were spotted on Silica Gel (with fluorescent indicator) (Eastman Kodak Chromagram 6060, Eastman Kodak Co.). The chromatograms were developed in benzene-methanol-acetic acid (45:8:4), and examined under short wavelength UV light. In the juice quality test, 12 experienced tasters evaluated orange juice flavor using the nine point Hedonic scale (Peryam, 1964). Taste panel scores were obtained as described by Attaway et al. (1972) and analyzed statistically (analysis of variance, multiple regression analyses).

Chemicals

NADH, NADPH, and catalase were from Sigma Chemical Co.; all other chemicals were from Fisher Scientific Co.

RESULTS & DISCUSSION

Latent PPDA-peroxidase activity

Orange juice serum contained a heat-labile, latent PPDA-peroxidase activity (Table 1). After a time lag of 65 sec, PPDA was oxidized at the rate corresponding to 9.6 U/ml. Formation rate of the 485 nm absorbing dye was proportional to time and serum volume. The substance(s) causing the lag were removed from the serum by dialysis or by chromatography on Sephadex-G25. Figure 1 shows the distributions of peroxidase activity, proteins and phenolic compounds in eluates from the Sephadex column. PPDA was oxidized immediately by fraction A of the void-volume eluate, but after a time lag by fractions collected from 800–1000 ml which also contained the first phenolic eluates from the column. Fraction B, a portion of the "inner volume" eluate containing substances of molecular weights less than 5000, had no activity itself, but delayed, without otherwise affecting, appearance of color in the assay of fraction A (Table 1).

Ascorbic acid was probably the major cause of the time lag of color formation; fraction B contained about 0.2 μmoles of ascorbic acid per ml. Ascorbic acid added to orange juice extracts increased the lag without affecting the reaction rate (Table 2). Time lag was proportional to concentration of ascorbic acid. When added after start of color formation, ascorbic acid did not reverse but did interrupt color development. Dehydroascorbic acid had no effect on either the lag or rate of color formation. PPDA is oxidized to the dye compound via a reversible free-radical semiquinoid, in a two-step reaction involving two electrons (Michaelis et al., 1939). In the assay of orange juice serum, ascorbic acid interrupted oxidation of PPDA to the stable dye by chemically reducing the semiquinoid. After all the ascorbic acid was oxidized, then oxidation of the semiquinoid proceeded. Ascorbic acid was also enzymically oxidized by peroxidase-H_2O_2, but much

Table 1–Latent PPDA-peroxidase activity of orange juice

	PPDA activity	Time lag[a]
	U/ml	sec
Extract from hand-reamed juice	9.6	65
Heated juice extract[b]	0	0
Dialyzed juice extract	9.4	0
Sephadex fraction A[c]	4.1	0
Sephadex fraction B[c]	0	0
Sephadex fraction A + B; 1:1	4.0	45
Sephadex fraction A + B; 1:2	4.1	62
Sephadex fraction A + B; 1:4	4.0	90

[a] Time lag was measured from addition of PPDA to start of linear absorbance increase.
[b] Neutralized juice heated to 90°C for 5 min before centrifugation
[c] Fractions from Sephadex-G25 chromatography of juice extract are identified in Fig. 1.

Table 2–Effect of ascorbic acid on PPDA-oxidation time lag in juice serum

Ascorbic acid	Total lag[a]	PPDA activity
μmoles/tube	sec	U/ml
0	82	7.2
1.5	135	7.3
3.0	180	7.2
4.5	210	7.0
6.0	232	7.0

[a] Time lag was measured from addition of PPDA to start of linear absorbance increase.

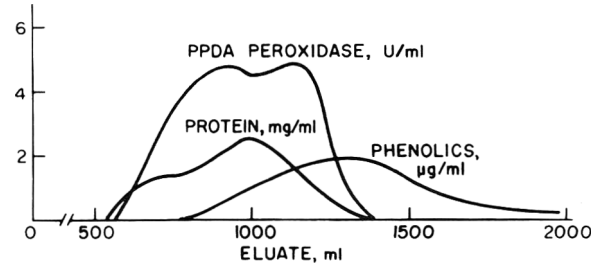

Fig. 1–Sephadex-G25 chromatography of orange juice extract: distributions of peroxidase activity, proteins and phenolic compounds. Fraction A–Eluate collected between 700 and 800 ml; Fraction B–Eluate collected between 1400 and 1500 ml.

more slowly than PPDA was; hence, practically all of the ascorbic acid was oxidized chemically by the semiquinoid. The ascorbic acid redox couple with PPDA semiquinoid is similar to that with o-toluidine semiquinone in the peroxidase assay described by Purr (1950) and with benzidine semiquinone in the chronometric method for peroxidase described by Gregory (1966). Redox coupling is the basis of a chronometric assay of ascorbic acid in citrus juices (Roe and Bruemmer, 1974b).

Solibuility. Orange juice peroxidase was soluble (not sedimented at 300,000 X G for 120 min) and was extracted from pulpy juice that had been stored frozen, then milled to a fine powder before thawing. PPDA-peroxidase activity was more than 100% greater in such a serum than in one prepared without freezing (Table 3).

Table 3—Extraction of peroxidase from orange juice

	Centrifugation conditions		PPDA-peroxidase activity	Lag time
Treatment	XG	min	U/ml	sec
Liquid blended	10,000	20	7.3	78
	300,000	120	7.5	80
Frozen milled	10,000	20	16.2	52
	300,000	120	15.8	54

Table 4—Substrates of orange juice peroxidase-H_2O_2 [a]

	Peroxidase-H_2O_2 activity
Substrates	Δmoles H_2O_2/min/mg protein
Ascorbic acid	1.14
Caffeic acid	0.75
Gentisic acid	0.65
p-Coumaric acid	0.49
Oxaloacetic acid	0.55
Oxaloacetic acid with 7×10^{-4} M $MnCl_2$	0.96
Pyridoxal-5-PO_4 with 7×10^{-4} M $MnCl_2$	0.55

[a] Assay conditions are described under Methods subsection, "Residual H_2O_2." Rates are corrected for nonenzymic activity. The following compounds were unreactive: quercetin, hesperidin, naringin, eriodictyol, pyridoxal-5-PO_4, tyrosine and phloroglucinol.

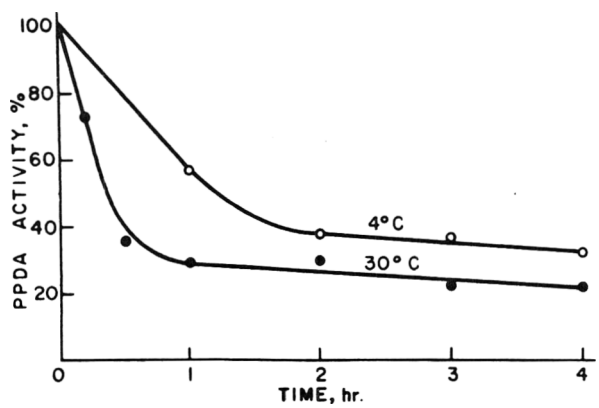

Fig. 2—Time course of peroxidase inactivation in orange juice (pH 3.4) at 4 and 30°C. Peroxidase (PPDA activity) is plotted as % of zero time value.

Stability. Orange juice lost about 45% of its peroxidase activity when stored at 4°C and about 70% when stored at 30°C for 1 hr (Figure 2). Juice retained from 20 to 35% of its original peroxidase activity after 4 hr. The loss in activity was probably pH dependent. Extracts of peroxidase at pH 7.0 were stable for several hours at 30°C.

Peroxidase and juice quality

All orange cultivars, maturing early-, mid- and late-season, yielded 5–10% more finished juice from the commercial extractors at "hard" than at "light" settings. As compared to low-yield juices, high-yield juices contained higher peroxidase activity and were judged by the taste panel to be of poorer quality. The negative correlation coefficient between the inverse logs of peroxidase activity and flavor scores for Hamlin, the early season cultivar was 0.990. The composite correlation coefficient for all 96 samples of the three cultivars was −0.730.

The significant negative correlation between peroxidase activity and flavor score indicates that conditions that increased peroxidase activity also lowered flavor quality but should not be interpreted that peroxidase caused the lower quality. The "hard extraction" of the fruit apparently liberated flavor detracting substances, as well as peroxidase. Release of peroxidase from pulp particulates by fine milling of frozen pulpy juice (Table 3) would explain, in part, the high peroxidase activity. The larger shearing force of the extractors at the "hard" as compared to the "light" setting, could have liberated more peroxidase from the pulp. "Hard" settings could also have increased the contribution of peel and seed peroxidase to the juice. The peroxidase content of seeds and peel is about 10-fold that of the endocarp (Davis, 1942).

Substrates of citrus peroxidase

Activity of peroxidase in orange juice was assessed with compounds reported to be normal constituents of the juice.

Purification. Dialyzates of proteins fractionated by ammonium sulfate had more peroxidase activity per unit volume than orange juice serum, but less than 60% of the protein was recovered. About 80% of the recoverable activity was in the proteins from the 60–90% AS fractionation; and the dialyzate of these proteins had a specific activity only twice that of the serum. Sephadex-G25 chromatography of juice extract increased specific activity of PPDA-peroxidase more than four-fold in several fractions without actually separating the proteins. Apparently the extract contained peroxidase inhibitors that were removed by the G25. Attempts to increase specific activity with DEAE- and CM-cellulose chromatography were not successful.

Peroxidase-H_2O_2. A number of juice constituents were reactive in the peroxidase-H_2O_2 assay with the dialyzed proteins from the 60–90% AS fractionation (Table 4). Ascorbic acid was the most reactive, followed by caffeic and gentisic acids. Mn^{++} stimulated oxaloacetic acid reactivity and was required for reactivity of pyridoxal-5-PO_4. Quercetin was unreactive in this assay, although reported to be oxidized by other peroxidases (Sizer, 1953).

Peroxidase as an oxidase with O_2. Mn^{++} was required for activity of orange juice peroxidase as an oxidase with O_2 (Table 5). Oxaloacetic acid and pyridoxal-PO_4 were reactive, but the latter did not support the oxidation of amino acids as reported for pea-seedling extracts by Hill and Mann (1966). Resorcinol, p-coumaric acid and p-cresol mediated the oxidations of NADH and NADPH with Mn^{++} and orange juice peroxidase. Horseradish peroxidase also catalyzes the oxidation of NADH and NADPH with Mn^{++} and these phenols (Akazawa and Conn, 1958). Catechol, ascorbic acid, and PPDA were not reactive with peroxidases of either orange juice or horseradish (Akazawa and Conn, 1958).

Table 5—Orange juice peroxidase activities in oxidations with O_2 + Mn^{++}[a]

Substrate[b]	O_2-uptake $\mu l\ O_2$/min/mg protein
Pyridoxal PO_4	0.3
Pyridoxal PO_4 + amino acid[c]	0.3
Oxaloacetic acid	0.3
Indoleacetic acid	0.5
Dihydroxymaleic acid	1.3
NADH + p-resorcinol[d]	0.5
NADH + p-coumaric acid[d]	1.3
NADH + p-cresol[d]	3.8

[a] No activity without Mn^{++} which was present as 3×10^{-4} M $MnCl_2$. Conditions are described under Methods subsection "Peroxidase assays with O_2 + Mn^{++}."
[b] Substrates were present at 3×10^{-3} M; oxalic and ketomalonic were unreactive.
[c] Methionine, aspartic acid, alanine or glutamic acids at 3×10^{-3} M.
[d] NADPH was also reactive with these phenols; however, catechol, caffeic acid, ferulic acid, phloroglucinol, ascorbic acid and p-phenylenediamine were not reactive with either NADH or NADPH.

Table 6—Mediators of NADH oxidation by orange juice peroxidase-H_2O_2

Mediator[a]	NADH oxidation[b] μmoles/min/mg protein
None	0
Hydroquinone	0.490
p-Cresol	0.241
Catechol	0.196
p-Phenylenediamine	0.163
4-Amino-2-methyl-1-naphthol (Vit. K_5)	0.163
Ascorbic acid	0.114
p-Coumaric acid	0.087

[a] Inactive: tyrosine, inositol, phloroglucinol, ferulic acid, quercetin, eriodictyol, hesperidin, dihydroxyphenylalanine, naringenin, 2-methyl-1,4-napthoquinone (Vit. k_3), ubiquinones (coenzymes Q_6 and Q_{10}).
[b] Conditions are described under Methods subsection, "NADH oxidation." Rates are corrected for nonenzymic oxidation.

Mediators of NADH oxidation by H_2O_2 with peroxidase. Hydroquinone was the most effective mediator of NADH oxidation by orange juice peroxidase-H_2O_2 (Table 6). Each mediator tested was capable of forming a one electron-less, reversible state. The inactive phenolic derivatives, such as ferulic acid and the flavonoids, are either poor substrates for peroxidase or do not form reversible intermediates.

Products of peroxidase reaction in orange juice

Peroxidase precipitated from orange juice catalyzed the oxidations of vitamins or their derivatives (ascorbic acid, pyridoxal-PO_4, 4-amino-2-methyl-1-napthol, NADH and NADPH), growth regulators (indoleacetic acid), and phenolic acids. Reaction rates with these substrates in juice are probably very slow. When juice was held at 30°C for 4 hr, concentration of ascorbic acid, the most reactive of the substrates tested with peroxidase-H_2O_2, did not change. Phenolic acids were also reactive substrates of orange juice peroxidase; but when orange juice was incubated for 2 hr at 30°C with 2 μmoles/ml of caffeic acid, its oxidation products were not detected. The level of H_2O_2 is probably limiting the rate of the reaction even though juice sacs contain flavoprotein oxidases (Bean et al., 1961; Bruemmer and Roe, 1975) that are a source of H_2O_2. Orange juice peroxidase probably reacts with other substrates in juice; but unless the reaction products are easily tasted, their concentration build-up in the holding tanks would probably not adversely affect juice quality.

CONCLUSION

FRESH ORANGE JUICE contains a latent peroxidase which catalyzes the oxidations of a number of juice constituents by H_2O_2 and by O_2 + Mn^{++}. However, enzyme activity is insufficient to produce detectable changes in ascorbic acid or caffeic acid content in juice held 2 hr at 30°C. Thus, during the few hours that juice is held before pasteurization, its flavor would probably not be adversely changed by peroxidase-catalyzed reactions.

The significant negative correlation between peroxidase activities and flavor scores of high- and low-yield orange juices, obtained from differently-set extractors, suggests the potential use of peroxidase activity as an index of adverse flavor of high-yield juice.

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Mention of brand names is for identification only and does not imply recommendation by the U.S. Dept. of Agriculture.

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CASHEW NUT UNSAPONIFIABLE MATTER

ABSTRACT

Unsaponifiable matter (UNS) of lipids from cashew nuts either roasted, unroasted or unroasted in shell was fractionated on a Florisil column. Gas-liquid chromatography (GLC) of the hydrocarbons showed that squalene was the major component of the 21 peaks detected. There were three homologous hydrocarbon series: normal, iso and/or anteiso and branched chain. GLC of the alcohol fraction silyl derivatives indicated the presence of three homologous series: normal, iso and/or anteiso and multiple branched chain alcohols. Beta and/or gamma tocopherol was found to be the major tocopherol. This component decreased during nut shell removal and roasting. Cycloartenol, amyrin (alpha-beta) and 24-methylene cycloartanol were the triterpenoids identified. GLC of the sterol fraction silyl derivatives showed beta-sitosterol to be the major sterol followed by campesterol.

INTRODUCTION

THE CASHEW NUT TREE is native to Brazil (Woodroof, 1967). The tree's "false fruit," the swollen peduncle or hypocarp, is commonly known as cashew apple. This "apple" is actually the receptacle for the true fruit, the cashew nut (Morton, 1961).

While some information is published regarding certain characteristics of cashew nut lipids, only limited information is known about the unsaponifiable fraction (UNS) (Barosso et al., 1973; Jacqmain, 1959; Pereira and Pereira, 1963).

This study was undertaken to determine the components in cashew nut UNS.

EXPERIMENTAL

Lipid extraction

Three kinds of cashew nut material were obtained from a processing plant in Fortaleza-Ceara, Brazil. They were unroasted shelled and unshelled and roasted shelled.

All solvents used in this work were redistilled. Lipid extraction was by the method of Bligh and Dyer (1959) with certain modifications. A 10-g sample was homogenized in a blender for 2 min with 50 ml chloroform, 100 ml methanol and 40 ml water. To this mixture was added 50 ml chloroform and after blending for 30 sec, 50 ml water was added and blending continued for another 30 sec. The homogenate was filtered through Whatman paper Number 1 on a Buchner funnel under suction. The filtrate was transferred to a separatory funnel, allowed to separate into layers and the chloroform layer removed. Chloroform was evaporated to a small volume (about 5 ml) under reduced pressure at 45°C, and the lipid stored in sealed containers under nitrogen atmosphere at 0°C until analyzed.

Saponification

For saponification and extraction a method based on section 28.063 of AOAC (1970) and Eisner and Firestone (1963) was used.

15g of cashew nut oil extracted as previously described were weighed and the following reagents were added: 150 ml absolute ethyl alcohol, 5 ml hydroquinone solution (30 mg hydroquinone in 1 ml absolute ethyl alcohol) and 10 ml potassium hydroxide solution (3g KOH: 2 ml H_2O). The mixture was refluxed for 90 min on a water bath under a stream of nitrogen.

The alcoholic soap mixture was transferred while still warm to a 1000 ml separatory funnel containing 300 ml distilled water. The saponification flask was rinsed with 300 ml ethyl ether, the ethyl ether rinses transferred to the separatory funnel, the funnel shaken vigorously and the phases allowed to separate and clarify. The lower aqueous layer was removed and the ether layer was transferred into another separatory funnel containing 120 ml distilled water. Two more aqueous layer extractions were made using 200 ml ethyl ether each. The combined ethyl ether extracts were gently rotated with 120 ml water (violent shaking at this stage may cause troublesome emulsions). The layers were allowed to separate and the aqueous layer drained. Two more washings using 120 ml water each were made.

The ether solution was washed three times with alternate 120 ml portions of 0.5N aqueous potassium hydroxide and water followed by shaking vigorously each time. After the third potassium hydroxide treatment, the ether solution was successively washed with 120 ml portions of water until the washings were no longer alkaline to phenolphthalein (1% alcoholic).

The ether extract was evaporated under a nitrogen stream on a water bath at 45°C. After cooling, the extract was passed through a funnel containing glass wool and 15g anhydrous sodium sulfate. The extract was then evaporated to dryness on a water bath under a nitrogen stream. The UNS was dried in a vacuum oven at 45°C to constant weight. The dried UNS was stored in sealed vials under nitrogen at −10° C.

UNS fractionation

Fractionation was accomplished by the method of Eisner and Firestone (1963). Florisil (60–100 mesh, Floridin Co., Pittsburgh, Pa.) was activated at 140°C for 4 hr and then cooled to room temperature. A 100-g portion was weighed into a round bottom flask, 10.5 ml water added and the mixture swirled so that water did not collect in one area. The flask was shaken vigorously for about 15 min until it felt cool (exothermic reaction stops). The Florisil was allowed to equilibrate overnight before use. For unsaponifiable matter separation, a glass column (2.5 cm × 30 cm long) was filled three-fourths with hexane and 30g of Florisil was added using a vibrator to insure even packing. After all of the Florisil had been added, the excess hexane was drained through the stopcock until the hexane level was about 4 cm above the packing surface. A 2 cm layer of anhydrous sodium sulfate was added, the inner tube side washed down with hexane and solvent excess was drained until it reached the sodium sulfate layer. The samples (30 mg) were dissolved in 0.5 ml chloroform and transferred to the column top. The flask was rinsed with two 5-ml hexane portions and the elution carried out. The following elution sequence was used: hydrocarbons eluted with 40 ml hexane followed by 120 ml hexane-ethyl ether (95:5); aliphatic alcohols, tocopherols and triterpenoid alcohols eluted with 120 ml hexane-ethyl ether (85:15); sterols eluted with 175 ml hexane-ethyl ether (70:30) followed by 175 ml hexane-ethyl ether (50:50).

Gas-liquid chromatography (GLC)

A gas chromatograph, Micro Tek Model DSS 170 (Tracor, Inc., Austin, Texas), equipped with a dual flame ionization detector, two glass columns (with the dimensions 0.4 cm × 1.65m) was used in this work. The gas chromatographic columns were each packed with 3% SE 52 on chromosorb W, 60–80 mesh (Analabs, North Haven, Conn.), in small amounts using a vibrator and suction. The ends of the columns were filled with glass wool. Column conditioning was by heating for 24 hr at 295°C with 40 ml per minute argon flow. The columns were then silanized with hexamethyldisilazene (HMDS) (Pierce Chemical Co., Rockford, Illinois) by injecting three 0.5 microliter portions at 1-min intervals. The gas flow was turned off and the temperature maintained at 295°C for 24 hr.

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Each Florisil fraction was weighed and diluted in 0.5 ml chloroform. Trimethylsilyl derivatives preparation was accomplished in the following way: 0.5 ml of each fraction (except hydrocarbons) was transferred to a vial and 0.5 ml N,O-Bis-Trimethylsilylacetamide (BSA) (Supelco, Inc., Bellefonte, Pa.) and 20 microliters trimethylchlorosilane (TMCS) (Supelco, Inc.), were added, the vials tightly closed and the mixture allowed to stand for 30 min at room temperature. Aliquots of the samples were then injected directly into the gas chromatograph. Relative component amounts were determined by peak areas comparison calculated by an Infrotonic Model CRS-108 integrator (Infrotonic Corp., Houston, Texas) with an analog slope detection component for baseline sensing and correction.

The following conditions were used for programmed temperature runs: column temperature 180–280°C for alcohols and sterols and 200–280°C for hydrocarbons. The temperature of the detector and injection port was 295°C. The program rate was 5°C per minute. The carrier gas was argon at a flow rate of 60 ml per minute.

Standard curves preparation

Standard solutions were prepared for the following hydrocarbons: eicosane, tetracosane, octacosane, triacontane and dotriacontane; for the following aliphatic alcohols: eicosyl, tetracosyl and hexacosyl (Analabs, North Haven, Conn.); and tocopherols: d-alpha-tocopherol, d-gamma-tocopherol, d-delta-tocopherol and d-beta-tocopherol (Supelco, Inc.). For sterol identification, campesterol, stigmasterol and beta-sitosterol standards were supplied by Dr. W.H. Kircher, Professor of Agricultural Biochemistry, The University of Arizona. Preparation of the TMSE derivatives for alcohols, tocopherols and sterols was done in the way as previously described.

These pure standards were gas chromatographed to obtain the respective retention times. Separate retention time plots versus carbon number were prepared on arithmetic graph paper for hydrocarbons and aliphatic alcohols. Unknown compounds were identified by extrapolation from the respective arithmetic plots. Sterol identification was based on comparison with the chromatographic behavior of pure compounds. Triterpenoid alcohols and squalene were identified by comparison with similar published information.

Thin-layer chromatography (TLC)

An additional analysis of cashew nut tocopherols was performed by TLC. Silica gel G precoated plates 20 × 20 cm, 250 microns thickness were used (Analtech Assoc., Inc., Arlington Heights, Ill.). Fraction 3 was applied at the bottom of the plates with solutions of alpha-tocopherol, beta-tocopherol, gamma-tocopherol and delta-tocopherol being spotted along the plate side as reference standards. The plates were developed in benzene-methanol (98:2 v/v) (Erickson et al., 1973). After development, the plates were sprayed with rhodamine 6G and viewed under UV light.

Table 1—Hydrocarbons in cashew nut unsaponifiable matter expressed in terms of total peak area

Peak no.	RT (min)	Samples: Roasted (%)	Unroasted (%)	Unroasted in shell (%)	Identification
1	1.8	tr*	0.2	tr	C-19
2	2.2	tr	0.2	0.2	C-20 br
3	2.5	2.0	1.5	1.4	C-20
4	3.1	tr	tr	tr	C-21 br
5	3.5	2.9	5.5	7.0	C-21 iso
6	3.8	28.5	29.0	25.0	C-21
7	4.9	1.0	1.3	2.0	C-22
8	5.6	0.2	1.0	0.9	C-23 iso
9	5.9	2.2	1.0	2.3	C-23
10	6.7	tr	tr	tr	C-24 iso
11	7.0	2.7	1.8	3.0	C-24
12	7.8	tr	0.3	0.3	C-25 iso
13	8.2	tr	tr	tr	C-25
14	9.0	tr	tr	tr	C-26 iso
15	9.4	3.0	2.7	3.0	C-26
16	10.6	0.3	0.3	0.4	C-27
17	10.8	49.7	48.0	47.2	Squalene
18	11.7	1.0	1.2	1.2	C-29 br
19	12.9	0.8	1.0	0.8	C-30 br
20	14.0	3.9	4.0	4.3	C-31 br
21	14.6	1.0	0.4	0.5	C-31

* Trace amounts

RESULTS & DISCUSSION

Hydrocarbons

Chromatograms of UNS hydrocarbons from three cashew nut oil samples were similar. A typical chromatogram shows 21 peaks (Fig. 1 and Table 1). By the use of an arithmetic plot constructed with standard compounds these 21 peaks were found to be distributed in three homologous series: first series comprised of straight chain hydrocarbons in the range C-19 to C-31; second series comprised of iso and/or anteiso-hydro-

Table 2—Aliphatic alcohols, tocopherols and triterpenoid alcohols present in the unsaponifiable matter of cashew nut oil, expressed in terms of total peak areas[a]

Peak no.	RT (min)	Samples: Roasted (%)	Unroasted (%)	Unroasted in shell (%)	Identification[b]
1	2.1	0.2	0.2	0.2	C-15 iso OL
2	2.3	0.5	0.5	0.3	C-15 OL
3	2.8	0.1	0.1	0.1	C-16 br OL
4	3.0	0.1	0.1	0.1	C-16 iso OL
5	3.4	0.2	0.2	0.2	C-16 OL
6	4.2	0.1	0.3	0.2	C-17 br OL
7	6.3	1.9	1.3	1.0	C-18 OL
8	6.8	0.5	0.9	0.8	C-19 br OL
9	7.4	0.1	0.3	0.3	C-19 iso OL
10	7.7	1.0	1.5	1.0	C-19 OL
11	8.2	tr	tr	tr	C-20 br OL
12	9.0	0.2	0.2	0.2	C-20 OL
13	9.4	tr	tr	tr	C-21 br OL
14	10.2	16.0	16.0	12.0	C-21 OL
15	12.6	0.1	0.4	0.3	C-23 iso OL
16	13.0	8.5	10.5	8.0	C-23 OL
17	14.7	0.2	0.3	0.4	C-25 br OL
18	15.1	0.6	0.2	0.2	C-25 iso OL
19	16.3	1.2	1.2	1.0	C-26 iso OL and/or delta tocopherol
20	16.8	0.4	0.4	0.4	C-26 OL
21	17.3	16.9	21.0	26.5	gamma and/or beta-tocopherol
22	18.1	0.2	0.2	0.2	C-27 OL
23	19.2	0.2	0.5	0.7	C-28 iso OL and/or alpha tocopherol
24	20.5	0.5	0.7	1.0	C-29 iso OL
25	21.2	1.0	1.0	1.0	C-30 br OL
26	21.6	10.0	8.5	9.0	Amyrin (alpha/beta)
27	22.5	25.0	23.0	25.0	Cycloartenol
28	23.5	14.0	10.0	9.5	24-Me-Cycloartanol
29	24.9	tr	tr	tr	Unknown

[a] Total aliphatic alcohols 32.4, 34.9, 28.0; total tocopherols 18.3, 22.7, 28.2; total triterpenoids 49.0, 41.6, 43.5.
[b] OL = aliphatic alcohols

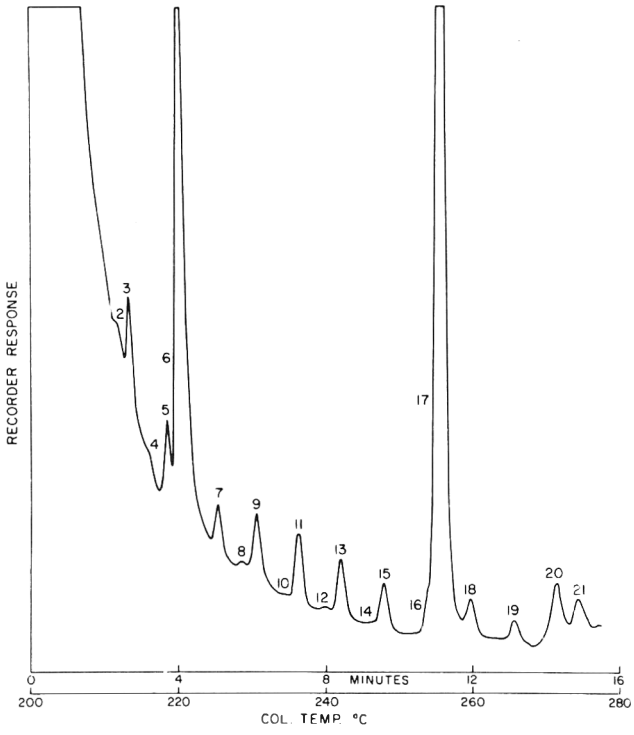

Fig. 1—Programmed temperature gas chromatogram of cashew nut oil hydrocarbons. (Refer to Table 1 for identification of numbered peaks.)

carbons from C-20 to C-31; and a third series comprised of branched chain hydrocarbons from C-20 to C-31. The procedure for the identification of the hydrocarbons in cashew nut oil was similar to that used by Eisner et al. (1965). These authors found normal straight chain hydrocarbons using a plot of retention time of standards versus carbon number, in olive oil.

From the chromatogram (Fig. 1) it can be seen that the major hydrocarbon fraction components are peak 17 tentatively identified as squalene and peak 6 identified as C-21. Squalene was found to occur in the unsaponifiable matter of cashew nut oil, by Jacqmain (1959). This compound was also found to occur in a number of vegetable oils such as olive and coconut (Eisner et al., 1965; Moura Fe, 1971).

In this work, squalene was found to comprise 47.2% of the hydrocarbon fraction calculated as peak area by an integrator with very small differences between the three products tested.

The greatest difference in the hydrocarbon fraction was found in C-21 iso (peak 5). This peak showed the smallest value for the roasted nut and the largest for the unroasted in shell. Peak 6 (C-21) showed a small difference between the samples. The most interesting finding here is that peak 17 is the predominant hydrocarbon in the three types of nut tested. The branched hydrocarbons (except C-31 br) and the iso and/or anteiso (except C-21 iso) appeared in small amounts.

Aliphatic alcohols, tocopherols and triterpenoid alcohols

A typical temperature programmed chromatogram of the alcohol fraction shows aliphatic alcohols, tocopherols and triterpenoid alcohols (Fig. 2 and Table 2). Three homologous series of aliphatic alcohols were found in cashew nut oil samples: (a) normal aliphatic alcohols in the range C-15 to C-31;

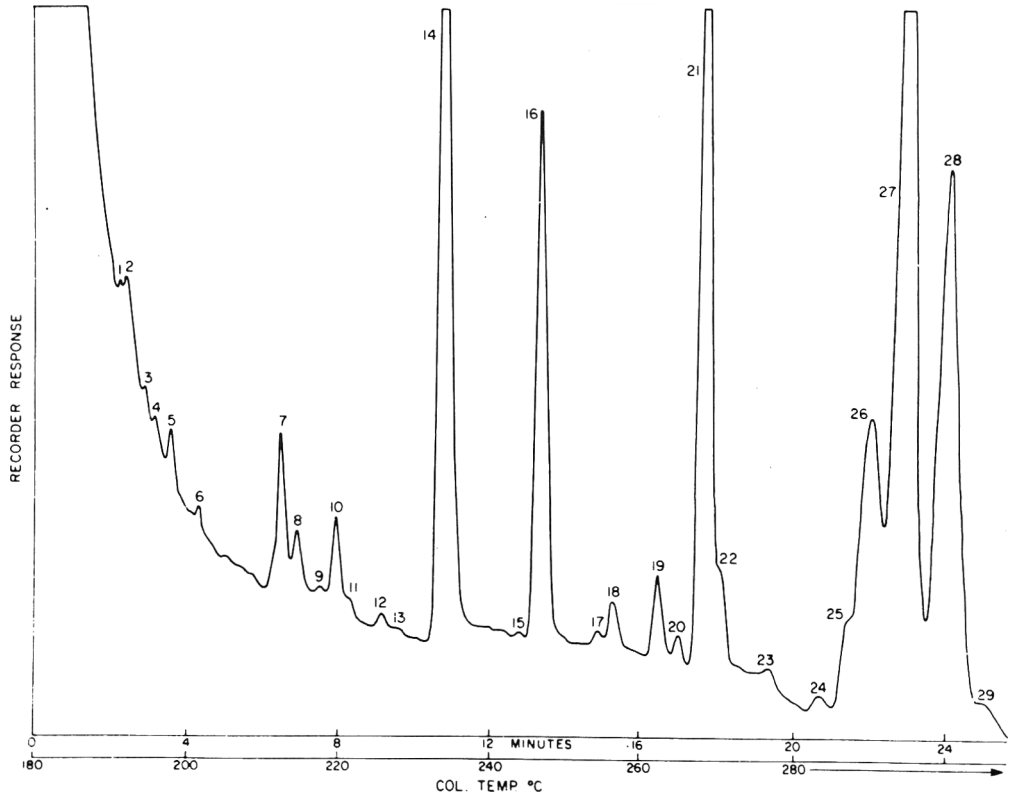

Fig. 2—Programmed temperature gas chromatogram of cashew nut oil aliphatic alcohols, tocopherols and triterpenoid alcohols. (Refer to Table 2 for identification of numbered peaks.)

(b) probably iso and/or anteiso alcohols in the range C-15 to C-30; (c) probably multiple branched chain alcohols in the range C-16 to C-31 (Table 2). Tentative identifications of the latter two series of alcohols were made after examination of arithmetic plots of the gas chromatographic data. Similar alcohols were previously found in olive oil (Eisner et al., 1965) using similar procedures, and in several other oils (Eisner et al., 1966) such as butter fat, corn, cottonseed, linseed, milomaize, peanut, rice bran, safflower, soybean and tung oil. By fractionation of the UNS on a Florisil column, these authors showed that this fraction consisted of three homologous series tentatively identified as normal, iso and/or anteiso and multiple branched chain alcohols.

The major normal aliphatic alcohols found in this fraction were C-21 OL and C-23 OL. These two alcohols showed only minor differences between the three types of nuts, being present in the largest amount in the unroasted sample.

Iso and/or anteiso and multiple branched chain alcohols were present in small amounts. These results are comparable to those found by Eisner et al. (1966) with several vegetable oils.

GLC of pure tocopherol standards showed the following elution order: delta-tocopherol, beta-tocopherol and gamma-tocopherol as one peak; and alpha-tocopherol. Under the conditions of this experiment gamma and beta-tocopherols were not separated.

On the basis of standards retention times, it was observed that peaks 19, 21 and 23 showed the same values as those for delta, gamma, beta and alpha tocopherols, respectively. The largest peak area corresponded to that identified as gamma and/or beta tocopherol. This peak showed a value of 26.5% for unroasted nut in shell, 21.0% for unroasted nut and 16.9% for roasted nut. This variation can be explained by the fact that the tocopherols were protected against oxidation in the nut containing a shell covering. The process of heating the nut to remove the shell was found to promote a decrease in the levels of gamma and/or beta tocopherol and a decrease in the amount of alpha tocopherol. An additional evidence for the presence of gamma and/or beta tocopherol as being the major tocopherol in cashew nut was supplied by TLC. When chromatographed with pure standards a spot with the same R_f as gamma tocopherol was detected.

Tocopherols relative peak areas were: 28.2% of unroasted nuts in shell, 22.7% of unroasted nuts and 18.9% of roasted nuts. Eisner et al. (1966) found that beta and/or gamma was the major component of the tocopherol fraction in the oils of

Table 3—Sterols in the unsaponifiable matter of cashew nut oil expressed in terms of total peak areas

Peak no.	RT (min)	Samples: Roasted (%)	Samples: Unroasted (%)	Samples: Unroasted in shell (%)	Identification
1	7.7	0.6	0.6	0.6	C-19 OL
2	13.5	tr	tr	0.4	C-23 OL
3	18.0	tr	tr	0.3	Unknown
4	18.9	0.1	0.4	0.2	Unknown
5	20.1	6.1	5.6	5.8	Campesterol
6	21.2	91.4	90.7	91.8	beta-sitosterol
7	22.6	1.0	0.8	0.6	Unknown
8	23.4	0.2	1.5	1.0	Unknown
9	24.5	0.3	0.4	0.3	Unknown

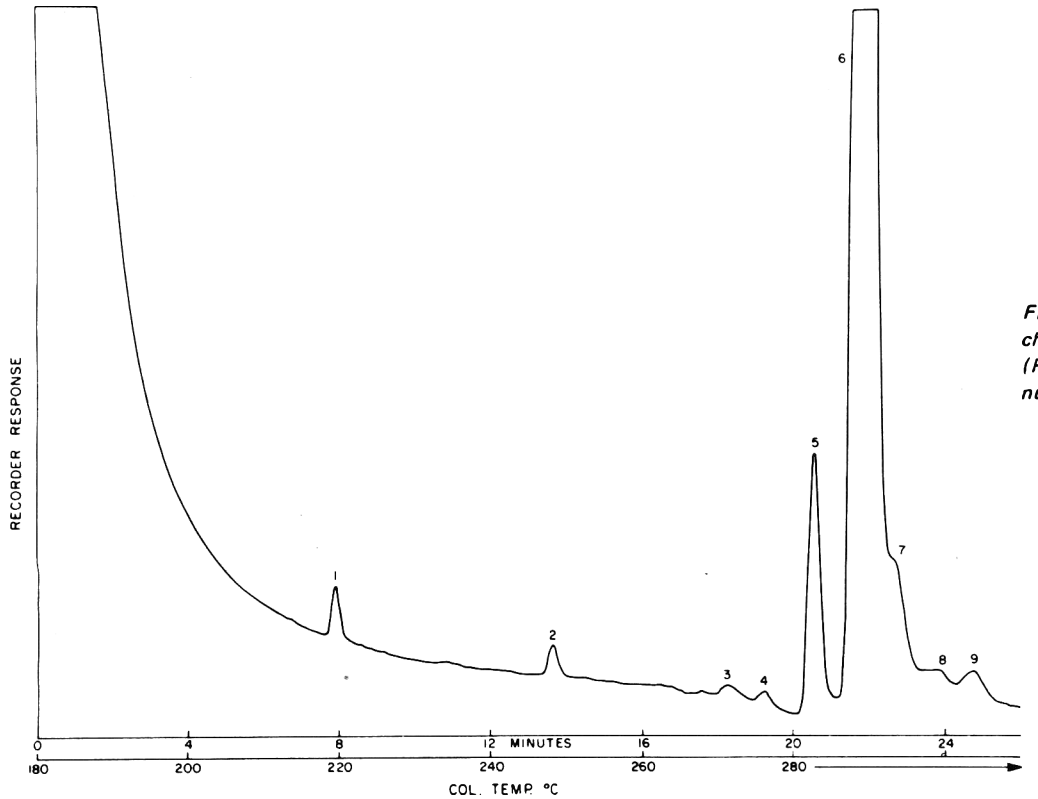

Fig. 3—Programmed temperature gas chromatogram of cashew nut oil sterols. (Refer to Table 3 for identification of numbered peaks.)

corn, peanut, tung, linseed, soybean and cottonseed. Erickson et al. (1973) reported that the beta-gamma isomers comprised the major tocopherol fraction component of cocoa lipids analyzed by TLC and GLC.

Rao et al. (1965) separated alpha, beta, gamma and delta tocopherols by TLC with subsequent colorimetric estimation. They found that gamma tocopherol was the major tocopherol in castor oil, groundnut, neem, sesame and soybean. Alpha tocopherol was the major component in cottonseed and safflower. Lamberstein et al. (1962) studied tocopherols in eight different nuts using spectrophotometry and TLC. Jacqmain (1959) determined the amount of total tocopherol in cashew nut. He found that it comprised 5.3% of the UNS. This author did not identify individual tocopherol isomers.

The components corresponding to triterpenoid alcohols were identified tentatively as: peak 26 as amyrin (alpha-beta), peak 27 as cycloartenol and peak 28 as 24-methylene cycloartanol (Table 2). The identification was based on work done by other investigators (Eisner et al., 1965, 1966; Fedeli et al., 1966). Fedeli et al. (1966) studied 18 vegetable oils such as linseed, peanut, olive, rice bran, palm kernel, corn, sesame oiticica, palm, coconut, rapeseed, grape seed, sunflower, poppy seed, castor, tea seed, cocoa butter and soybean. Two triterpenoid alcohols, cycloartenol and 24-methylene cycloartanol, were found to be present in all oils except soybean oil which contained only cycloartenol. These authors also indicated that beta amyrin eluted before alpha amyrin. Eisner et al. (1966) cited that Shimizu and others studied the gas chromatographic behavior of a number of triterpenoid alcohols.

Sterols

GLC of the sterol fraction indicated the presence of nine peaks (Fig. 3, Table 3). Of these, peak 5 was identified as campesterol and peak 6 as beta-sitosterol. These compounds were identified by comparison with the retention times of pure standards which eluted in the following order: cholesterol, campesterol, stigmasterol, and beta-sitosterol.

Beta-sitosterol was found to be the most abundant sterol comprising a relative peak area of 91.4% for roasted nut, 90.7% for unroasted and 91.8% for unroasted in shell (Table 3). Jacqmain (1959) reported that 89.4% of sterol fraction in cashew nut oil was beta-sitosterol. He did not identify the other sterols. Small amounts of aliphatic alcohols were eluted in the sterol fraction.

Vegetable oils contain variable amounts of phytosterols (sitosterols and stigmasterol are the common phytosterols) which differ from cholesterol, the characteristic sterol of animal fats.

Eisner and Firestone (1963) studied the sterols of several oils such as corn, cottonseed, olive, milomaize, rice bran, safflower, peanut, soybean and cocoa butter. They found that beta-sitosterol was the major sterol found in each of the oils investigated.

Fedeli et al. (1966) showed that the following order of elution was observed for sterols on GLC (glass column 2m × 2 mm i.d. packed with silanized Gas Chrom. P, 100–200 mesh and coated with 1% SE-30. The column was operated at 230°C with nitrogen at 20 ml per minute as carrier gas. Evaporator temperature was 280°C): cholesterol, brassicasterol, campesterol, stigmasterol and beta-sitosterol.

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A Research Note
ISOLATION AND CHARACTERIZATION OF THE LIPIDS FROM THE CHICKEN PREEN GLAND

ABSTRACT

Although the preen gland of chickens contain 39% fat, it is still discarded or sent to rendering plants along with the other waste from the poultry processing plants. This study was conducted to determine some of the physical properties and to characterize the fatty acids of the oil from the preen gland. A low temperature solvent extraction using an isopropyl alcohol-hexane system was used to extract the oil from the preen gland. The values obtained for specific gravity (0.9086), melting point (17–28°C), iodine number (66.04), and saponification value (197.4) were within the range of values reported for chicken fat. The fatty acid distribution of the preen gland oil as determined by gas chromatographic analysis was very similar to the distribution of fatty acids that were reported for abdominal adipose lipids. Oxidized flavors could not be detected after holding the oil at 40°C for 8 days. These results indicate that the oil of the preen gland can be efficiently extracted and the quality of the oil is such that it could be used as a commercial chicken fat in the food industry.

INTRODUCTION

THE PREEN GLANDS of broilers are removed from the carcasses during processing of the birds and either discarded or sent to rendering plants along with the other waste from the processing plant. Toledo (1974) was able to isolate a protein concentrate with good functional properties from the preen gland by using an isopropyl alcohol-hexane (IPA-hexane) solvent extraction procedure. At the same time he reported that the fat could be recovered very easily from the hexane and that this solvent extraction system could be used to extract oil from tissue containing large amounts of lipids. The preen gland has 39% fat (Toledo, 1974) and with the large number of broilers that are processed, the preen glands could be a source of a commercial grade chicken fat. This chicken fat would have a higher market value than the products of a rendering plant. This study was conducted to extract the fat from the preen gland and to determine the properties of the extracted oil.

EXPERIMENTAL

CHICKEN PREEN GLANDS were collected from the processing line of a local processing plant and ground twice with a meat grinder fitted with a plate having 32 mm diameter holes prior to extraction. The fat was extracted from a fresh batch of preen glands and a batch that was stored for 1 yr at 0°C.

The fat was removed from the preen glands using an isopropyl alcohol-hexane (IPA-hexane) extraction procedure which was similar to the one described by Toledo (1974). The solvent was mixed with the ground meat at a solvent to meat ratio of 1.5:2.0:1.0 (IPA:hexane: meat) for 1 hr. After vacuum filtration, the residue was mixed with hexane using a solvent to meat ratio of 2:1, stirred for 1 hr and vacuum filtered. The residue from the second extraction was taken through another hexane extraction stage using the same solvent to meat ratio as in the second stage.

The IPA-hexane solvent from the first extraction stage was mixed with 1.5 parts of water to facilitate the separation of the hexane rich phase from the water-IPA phase. This hexane phase was combined with the hexane extracts of the second and third extraction stages and the hexane was removed from the fat by using reduced pressure steam distillation. After the distillation the water was removed from the fat by freeze drying.

The specific gravity, melting point, Hanus iodine number and saponification value were determined using the methods described by AOAC (1970).

Methyl esters of the fatty acids in the fat were made using the boron trifloride method (Metcalfe et al., 1966). The methyl esters were analyzed using gas liquid chromatography.

The oxidative stability of the extracted lipids was determined by measuring the peroxide value (AOCS, 1971) during storage at 40°C.

RESULTS & DISCUSSION

THE IPA-HEXANE SOLVENT extraction procedure developed by Toledo (1974) proved to be an excellent method for removing the lipid from the preen glands. The partitioning of the fat into the hexane solvent enabled the fat to be easily desolventized. Vacuum steam distillation allowed the removal of the hexane from the lipids without heat degradation occurring. The water trapped in the lipids during steam distillation

Table 1—Physical and chemical properties of preen gland lipids and other chicken fats

Property	Preen gland lipids 1[a]	Preen gland lipids 2[b]	Commercial chicken fat	Literature data[c]
Melting point	18–30° C	15–28° C	18–20° C	21–27° C
Iodine value	68.6	66.1	88.7	66–71.5
Saponification no.	193.0	197.0	204.0	193–204
Specific gravity (22/22° C)	0.9027	0.9087	0.9121	0.924

[a] Preen glands stored at 0° C for 1 yr before extraction
[b] Fresh preen glands
[c] Meyer, 1960

Table 2—Major fatty acids in extracted preen gland lipids and other chicken fat

Fatty acids[a]	Preen gland fat 1[b]	Preen gland fat 2[c]	Commercial chicken fat	Adipose tissue[d]
10:0	1.1	2.1	0	0–1.4
12:0	1.7	3.6	0	0–22.7
12:1	0	0.4	0	0
14:0	2.8	9.1	0.4	0.2–15.4
14:1	0	0.5	0	0–0.5
16:0	23.9	24.4	19.4	23.4–37.9
16:1	4.2	10.9	3.8	0.3–7.8
18:0	8.1	3.5	4.7	2.7–9.5
18:1	37.2	18.1	51.1	23.1–54.3
18:2	17.1	25.0	20.7	3.6–44.1
18:3	0	0.5	0	0

[a] Fatty acid denoted by carbon chain length and number of double bonds. Each fatty acid expressed as percent of total.
[b] Solid fat fraction (mp, 28–30°C; iodine value 65.5)
[c] Oil fraction (mp, 18°C; iodine value 72)
[d] Range found for chickens on different fat rations (Jen et al., 1971)

was removed by freeze drying the lipids.

Extractions using IPA-hexane in the first extraction stage followed by hexane in the second and third stages removed more than 98% of the lipids from the chicken preen glands. The odor and flavor of the lipids after removal of the hexane by vacuum steam distillation and removal of the water by freeze drying was good and very typical of chicken fat. The color of the extracted lipid did not appear to be affected by the extraction procedure.

The measured properties of the preen gland lipids are listed in Table 1 along with data for a commercial chicken fat and published values for these properties. Although the melting point, iodine value, saponification number and specific gravity of the preen gland lipids differ slightly from the commercial chicken fat, they all fall within the range of values reported in the literature. Lipids from fresh preen gland and preen gland stored frozen for 1 yr had essentially the same values for these properties. Also, no difference in color or flavor was detected in the two preen gland extracts.

After storage for 2 days at room temperature, the extracted lipids separated into a solid fat fraction and an oil fraction. Melting points, iodine values and fatty acid analysis were determined on each fraction. The solid fat fraction had a higher melting point and lower iodine value than the oil fraction (Table 2). The lower iodine value for the solid fat indicated that this fraction was more saturated than the oil fraction. Fatty acid analysis revealed, however, that the solid fat contained 58.5% unsaturated fatty acids while the oil contained 55.4%. The solid fat had 17.1%, 18:2; 37.2%, 18:1; and 4.2%, 16:1 and the oil fraction was composed of 25%, 18:2; 18.1%, 18:1; and 10.9%, 16:1 (Table 2). This difference in distribution of unsaturated fatty acids in the solid fat and oil fractions accounts for the different melting points and iodine values. Jen et al. (1971) found that the ratio of solid fat to oil in chicken adipose lipids as well as the distribution of the fatty acids in the two fractions were highly dependent on the type and amount of fatty material in the diet of the chickens. This is perhaps true for the lipids in the preen gland.

The fatty acid distribution of a commercial chicken fat sample and adipose tissue lipids are also listed in Table 2. The range of fatty acid percentages shown for adipose tissue lipids were obtained from the data of Jen et al. (1971) and include chickens fed on different fat diets. The percentage fatty acids found in preen gland lipids fall within the ranges reported for adipose tissue and it is likely that the preen gland lipids are the same as the adipose tissue lipids.

The preen gland lipids fatty acids also differ from the commercial chicken fat. This commercial chicken fat was obtained from a plant that processes laying hens and it would be expected that the lipids from hens are different from boilers in fatty acid composition since the rations of the two types of birds may not be the same.

The peroxide values of the preen gland lipid increased slightly (2.0–10) when held at 40°C for 8 days. However, no detectable oxidized flavor could be detected.

This study indicates that the IPA-hexane solvent extraction procedure can be used to extract the lipids from the preen gland of chickens and that the chemical and physical properties of the oil are the same as other lipid tissue in the birds. Therefore, the preen gland can serve as a new source of chicken fat that could be used in the food industry provided that the cost of collecting and processing the preen glands does not exceed revenue generated by the fat.

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A Research Note

PEAK SHEAR-FORCE VALUES OBTAINED FOR VEAL MUSCLE SAMPLES COOKED AT 50 AND 60°C: INFLUENCE OF AGING

ABSTRACT

Warner-Bratzler peak shear-force values have been obtained for fresh and aged veal muscles cooked at 50 and 60°C. The results have shown that the magnitude of the difference between the shear-force values obtained for veal samples cooked at these temperatures was significantly increased by aging treatment. This suggested that changes in connective tissue alone would not account for the observed effect.

INTRODUCTION

WARNER-BRATZLER (WB) peak shear-force values are generally greater for muscle samples cooked at 50 rather than 60°C. The difference has been ascribed to temperature effects on connective tissue (Machlik and Draudt, 1963; Draudt et al., 1964; Draudt, 1972; Bouton and Harris, 1972a) and to the nature of the connective tissue since the magnitude of the difference varies with animal age (Bouton and Harris, 1972a). This difference in shear-force values has also been shown to vary with myofibrillar contraction state (Bouton et al., 1974). If the difference is a function of myofibrillar as well as connective tissue properties then aging should also affect it since aging is believed to (a) weaken the myofibrillar structure (Davey and Gilbert, 1967, 1968, 1969; Davey and Dickson, 1970; Davey et al., 1967; Stromer and Goll, 1967a, b; Stromer et al., 1967) and (b) have little or no significant effect on the mechanical properties of the connective tissue (de Fremery and Streeter, 1969; Steiner, 1939; Winegarden et al., 1952; Bouton and Harris, 1972b; Kruggel and Field, 1971; Herring et al., 1967; Pfeiffer et al., 1972; McClain et al., 1970).

This note describes an experiment to determine whether aging affected the difference between peak shear-force values of veal muscle samples cooked at 50 and 60°C. Veal was used since the earlier work has shown that the temperature effect was most marked in young animals (Bouton and Harris, 1972a) and the work on effects of myofibrillar contraction state (Bouton et al., 1974) was also on veal.

EXPERIMENTAL

Material

Six calves were slaughtered at a local abattoir, hung by the Achilles tendon within 2 hr of slaughter and conditioned for 24 hr at 10–12°C to avoid cold-shortening effects (Bouton et al., 1973a). The biceps femoris (BF), semimembranosus (SM) and longissimus dorsi (LD) muscles were removed from the carcasses after the initial conditioning period. One of each pair of these muscles was cooked fresh viz 24 hr after slaughter while the remaining muscles were vacuum sealed in a gas impermeable (Cryovac) bag and stored for a further 3 wk at 0–1°C. These aging conditions were chosen since earlier work on mutton (Bouton et al., 1973a) indicated that any period greater than 1 wk was satisfactory. Each BF and SM muscle and the lumbar region of the LD muscles both fresh and after aging, was split transversely into two parts. The parts were assigned to cooking at 50 or 60°C for 1 hr. The allocation of individual muscle parts to the cooking treatments was alternated so that each intramuscular location was represented an equal number of times.

Cooking method

Samples of approximately equal size (130–150g) were cooked in polyethylene bags totally immersed in water maintained at 50 or 60°C (± 0.5°C). Samples were weighed before and after cooking to determine cooking losses. The cooking time was long enough to ensure that all samples had been at the water temperature for about 15–20 min. It is known that quite large differences in cooking time at 50 or 60°C can be tolerated without affecting shear-force values (Bouton and Harris, 1972a).

Oven cooking methods were not employed since it was already known (Schmidt et al., 1971) that samples cooked, in an oven at 135°C, to internal temperatures of 50 and 60°C, did not show the temperature effects on shear force values reported by other workers (Machlik and Draudt, 1963; Draudt et al., 1964; Draudt, 1972; Bouton and Harris, 1972a; Bouton et al., 1974) who cooked in water baths at the selected temperatures.

Sarcomere length, shear force and pH measurements

Sarcomere lengths of cooked samples were measured using the laser method described by Bouton et al., 1973b.

Peak shear-force values were obtained on samples of rectangular cross section (1.5 × 0.66 cm) about 5–8 cm long, and with the fibers lying parallel to the long axis. The shear blade thus cut directly across the meat fibers.

pH values were measured directly on all raw meat to ensure that all samples used had pH values of 5.4–5.8.

RESULTS & DISCUSSION

THE RESULTS obtained are shown in Table 1. Analysis of variance showed there were no significant muscle × treatment interactions so the means of the combined results from the LD, BF and SM muscles have been presented to show the changes due to cooking temperature (50 or 60°C) and aging. There was a significant ($P < 0.001$) aging × cooking temperature interaction for the shear-force values, since the effect of aging was greater for the samples cooked at 60°C. Aging increased cooking losses but neither the aging nor the cooking temperature treatments significantly affected sarcomere length values.

Table 1—Effect of cooking temperature (50 or 60°C) and of aging on sarcomere length, cooking loss and peak WB shear-force values of veal muscles (BF, LD and SM)

	Fresh[b]		Aged		LSD (5%)
Cooking temp (°C)	50	60	50	60	
Peak shear force (kg)	5.99	2.91	6.20	1.43	0.74
Sarcomere length[a] (μm)	1.92	1.86	1.88	1.83	0.12
Cooking loss (%)	2.3	7.3	3.8	15.8	1.0

[a] Of cooked samples
[b] Cooked 24 hr after slaughter

CONCLUSIONS

SINCE AGING has no effect on shear force values for the samples cooked at 50°C while for the samples cooked at 60°C it produces a very significant ($P < 0.001$) reduction, it is clear that aging greatly enhances the temperature effect. This is an important result because it means that aging, a treatment believed to affect primarily the myofibrillar component (Stromer and Goll, 1967a, b; Stromer et al., 1967; Davey and Gilbert, 1967, 1968, 1969; Davey and Dickson, 1970; Davey et al., 1967), has a large influence on an effect which was considered to be due to temperature-induced changes in connective tissue (Machlik and Draudt, 1963; Draudt et al., 1964; Draudt, 1972; Bouton and Harris, 1972a). This result—the effect of aging—plus the influence of myofibrillar contraction state (Bouton et al., 1974) indicates that more could be involved in the effect of cooking at 50 and 60°C than just changes in connective tissue.

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A Research Note

EVALUATION OF ASCORBYL MONOESTERS FOR THE INHIBITION OF NITROSOPYRROLIDINE FORMATION IN A MODEL SYSTEM

ABSTRACT

The effect of a number of ascorbic acid derivatives on the nitrosation of pyrrolidine was determined in a model system developed to simulate the lipid-aqueous-protein composition of bacon. Sodium ascorbate reduced nitrosopyrrolidine formation 43% in the aqueous phase, but had little effect in the lipid layer. The combination of several ascorbyl esters with sodium ascorbate increased the inhibitory effect to a high of 70% in the aqueous phase. In the lipid phase up to 49% reduction in nitrosation was observed.

INTRODUCTION

ASCORBIC ACID (Vitamin C), its isomer erythorbic acid and their salts have been permitted in cured meat products since 1955 (USDA Memorandum No. 217, June 1955). Initially, they were used to accelerate the formation of the stable pink-red color (nitrosyl hemochrome) of cured meats. Recently however, sodium ascorbate (NaAsc) and sodium erythorbate (NaEry) were found to inhibit nitrosamine formation in frankfurters (Fiddler et al., 1973) and bacon prepared with higher than permitted levels of NaAsc (Herring, 1973). It is postulated that NaAsc and NaEry inhibit nitrosamine formation because the rate of reaction of nitrite with the reductants is greater than the rate of nitrosation of the secondary amines, depending on their basicity. However, they do not completely prevent nitrosamine formation in bacon.

We have shown (Fiddler et al., 1974) that nitrosopyrrolidine (NO-Pyr) is formed primarily in the adipose tissue of bacon. The nitrite reducing ability of the reductants in adipose tissue may be limited by their fat solubility. Therefore, experiments with more fat soluble derivatives of ascorbic acid, in combination with NaAsc, were undertaken to determine whether a more effective means of inhibiting the nitrosation of pyrrolidine could be obtained in a model system simulating the composition of bacon. The results of these experiments are reported herein.

EXPERIMENTAL

ASCORBYL PALMITATE, ascorbyl laurate, erythorbyl palmitate, erythorbyl laurate and the potassium salt of ascorbyl-2-sulfate were obtained from Dr. Winifred Cort, Hoffmann-LaRoche. These ascorbyl monoesters were prepared by known procedures and their identities have been established. In addition, these compounds were purified by chromatographic methods (by the suppliers) to a purity in excess of 99%. The magnesium salt of ascorbyl-2-phosphate was obtained through the courtesy of Dr. Paul Seib, Kansas State University (Seib et al., 1974). Ascorbyl oleate was prepared by Dr. Leonard Silbert, Animal Fat Products Laboratory, Eastern Regional Research Center. Pyrrolidine, sodium nitrite, and sodium ascorbate were obtained from commercial suppliers.

The study was carried out in a model system consisting of 60g of safflower oil, 30g of pH 6.00 aqueous buffer (1N NaOH, 1M KH_2PO_4), 8.0g bovine albumin, 1.5g sodium chloride and 0.5g sodium tripolyphosphate. This would correspond to bacon having a composition of 60% fat, 30% H_2O, 8% protein and 2% ash. To this system was added 1×10^{-4} mole of pyrrolidine (71 ppm), 2.52×10^{-4} mole of sodium ascorbate (5.04×10^{-4} mole in the control reaction), 2.52×10^{-4} mole of the compound to be tested, and 2.17×10^{-4} mole of sodium nitrite (150 ppm). The mixtures were stirred and heated at 52°C for 2 hr. The reaction mixtures were cooled to room temperature, then centrifuged at 6000 rpm for 10 min to facilitate separation of the oil and aqueous layers with a separatory funnel. To precipitate the protein, 40 ml of 25% trichloroacetic acid (TCA) was added to the aqueous layer (A) and 10 ml of 25% TCA to the fat layer (B). After centrifuging at 6000 rpm for 5 min, the aqueous layer (A) was decanted into a separatory funnel containing 10 ml of 5N NaOH, mixed, then extracted three times with 100 ml of methylene chloride (CH_2Cl_2). The combined CH_2Cl_2 extracts were washed with 50 ml of 6N HCl, then 50 ml of 5N NaOH and separated. The CH_2Cl_2 extract was dried by passing it through anhydrous sodium sulfate into a Kuderna-Danish apparatus and concentrated to 4 ml in a steam bath prior to gas chromatographic analysis. The remaining oil layer (B) was decanted into a separatory funnel containing 5 ml of 5N NaOH using 60 ml of n-hexane to facilitate the transfer. This mixture was extracted three times with 50 ml of water, the water washes combined, extracted with CH_2Cl_2 and treated in the same manner as described for the aqueous layer (A). An average total recovery of 79% was obtained using a sample to which 5 ppb NO-Pyr had been added and carried through the entire procedure.

The NO-Pyr was determined with a Varian Aerograph model 1740-1 gas chromatograph equipped with an alkali flame ionization detector as described previously (Howard et al., 1970). A 9-1/2 ft × 1/8 in. stainless steel column packed with 15% Carbowax 20M-TPA on 60–80 mesh Gas Chrom P was used with the following operating conditions: column temperature, 170°C; injector port, 185°C; detector, 225°C. The flow rates were: helium-60, air-215, and hydrogen-86 ml/min. Conditions for confirmation of NO-Pyr by GLC-high resolution mass spectrometry have been reported previously (Pensabene et al., 1974).

RESULTS & DISCUSSION

THE EFFECTS of some ascorbate derivatives on the nitrosation of pyrrolidine in a lipid-aqueous-protein system are shown in Table 1. The amount of nitrosopyrrolidine in the lipid layer was considerably less than that in the aqueous-protein portion contrary to reported findings in bacon (Fiddler et al., 1974). This may reflect the greater solubility of pyrrolidine, and its nitrosated form, in the aqueous phase and the possibility that the nitrosopyrrolidine was not forced into the lipid layer by evaporation of the water, which could occur during the frying of bacon. Alternatively, NO-Pyr in the lipid phase may be higher due to the high temperature achieved during frying, which increases the rate of nitrosation. The results, therefore, demonstrate the overall effect of the esters on nitrosation in both the aqueous and lipid phases of the system. Sodium ascorbate at 500 and 1000 ppm inhibited NO-Pyr formation in the aqueous phase 43.0 and 85.5%, respectively. No interference with NO-Pyr formation occurred in the lipid phase. However, this finding cannot be explained at this time. The esters

Table 1—Ascorbyl monoesters as inhibitors of the nitrosation of pyrrolidine in a model system

Compound[a]	Ascorbyl[b] ester (ppm)	Nitrosopyrrolidine[c,d] Aqueous ppb	Aqueous % Reduction	Lipid ppb	Lipid % Reduction
None	–	167	–	10.5	–
NaAsc (1000 ppm)	–	24.2	85.5	11.4	(+ 8.6)
NaAsc (500 ppm)	–	95.2	43.0	14.8	(+41.0)
NaAsc (500 ppm) + Asc palmitate	1046	63.5	62.0	5.4	48.6
Asc laurate	905	72.6	56.5	6.3	40.0
Asc oleate	1110	62.4	62.6	6.6	37.2
Ery palmitate	1046	56.3	66.3	6.8	35.2
Ery laurate	905	123	26.3	7.7	26.7
K_2 Asc-2-sulfate	880	50.4	69.8	5.8	44.8
Mg Asc-2-phosphate	731	82.4	50.7	9.5	9.5

[a] Plus pyrrolidine and $NaNO_2$
[b] Equimolar with NaAsc, 2.52×10^{-4} mole
[c] Confirmed by MS
[d] All values are the average of three experiments.

were tested in the presence of an equimolar concentration of NaAsc to determine whether additional inhibition of NO-Pyr formation occurred in a lipid system. The ascorbyl esters of oleic, palmitic and lauric acids had only a small effect on the nitrosation of pyrrolidine in the aqueous phase, increasing the inhibition due to the NaAsc alone only 13–20%. However, in the lipid phase, ascorbyl palmitate reduced nitrosation 48.6% while the other two esters inhibited the reaction about 40%. The erythorbyl ester of palmitic acid showed a slightly lower inhibitory effect in the lipid phase, reducing nitrosation by 35.2%, whereas erythorbyl laurate showed very little inhibition in either phase. The magnesium salt of ascorbyl-2-phosphate gave only a 9.5% reduction in the lipid phase and the potassium salt of ascorbyl-2-sulfate produced the same effect as the ascorbyl esters, reducing nitrosation by 44.8%. The later compound is of particular interest since it is 18 times more stable than potassium L-ascorbate (Quadri et al., 1973) in a boiling, aerated water system. Recently, Quadri et al. (1975) have shown that the potassium salt of ascorbyl-2-sulfate is generally more stable in bread, in pancake, in an extruded wheat product, and in whole pasteurized milk than L-ascorbate.

The results show that nitrosation can be reduced in both the aqueous and the lipid phases of the model system studied when NaAsc is used in combination with an ascorbyl ester. However, the esters, even though more soluble in the lipid phase than NaAsc, were only slightly fat soluble. It is possible, therefore, that a more fat soluble ascorbyl ester introduced into the adipose tissue might produce a greater reduction of nitrosamine, particularly in the preparation of cured meat products that contain a great deal of adipose tissue. Ascorbyl palmitate and the potassium salt of ascorbyl-2-sulfate are currently being investigated for their NO-Pyr inhibitory activity in bacon.

NOTE: Precaution should be exercised in the handling of nitrosamines since they are potential carcinogens.

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A Research Note

INSTABILITY OF PATULIN IN CHEDDAR CHEESE

ABSTRACT

When 50 μg/g patulin was added to Cheddar cheese and stored at 5° and 25°C for varying time periods, the toxin became undetectable by chemical and biological means. Less than 27% of the added toxin was detected after 3 hr and less than 16% after 48 hr. The reaction was not affected by storage temperature or the presence of microorganisms and thus appeared to be chemical in nature. A patulin producing strain of *Penicillium patulum* grew well on Cheddar cheese at 5° and 25°C, but patulin production was not observed at 5°C, and only small, variable amounts of patulin were noted at 25°C. The patulin formed at 25°C on the cheese was authentic as shown by UV absorption maxima and high pressure liquid chromatography, and it possessed biological activity against *Bacillus megaterium* and *Clostridium perfringens*. Potassium sorbate, vacuum packaging and coating cheese with paraffin wax all prevented growth of *P. patulum* on retail size pieces of cheese stored at 5° and 25°C. A heat-sealed wrapping on large (20 lb) blocks of cheese also prevented growth of this organism on cheese stored at 12°C for 6.5 wk.

INTRODUCTION

PATULIN, a metabolite of numerous *Penicillium* and *Aspergillus* species, is toxic to animals (Abraham and Florey, 1949) and is carcinogenic to rats (Dickens and Jones, 1961). It is stable in food systems, such as apple juice (Pohland and Allen, 1970a; Scott and Somers, 1968), grape juice (Scott and Somers, 1968), and dry corn (Pohland and Allen, 1970a), and has been isolated from commercial apple juices (Scott et al., 1972; Wilson and Nuovo, 1973; Ware et al., 1974).

Patulin is reactive with sulfur containing compounds such as cysteine, glutathione and thioglycollate, resulting in detoxification (Abraham and Florey, 1949). These reactions have been used to explain the instability of patulin in food systems such as orange juice (Scott and Somers, 1968), flour (Timonin, 1946), sausages (Mintzlaff et al., 1972; Hofmann et al., 1971) and breads (Reiss, 1973). The latter two products supported extensive growth of *Penicillium expansum* a known patulin producing mold even though no patulin was found. Also, Harwig et al. (1973) suggested that microbiologically mediated degradation of patulin may occur in apple juice fermented by *Saccharomyces cerevisiae* and *Saccharomyces ellipsoides*.

Mold growth on cheese can occur during ripening, curing and refrigerated storage (Bullerman and Olivigni, 1974; Foster et al., 1957). In a recent study, 82.2% of molds isolated from commercial Cheddar cheeses were *Penicillium* species (Bullerman and Olivigni, 1974); 4% of these isolates produced patulin in laboratory media. One of these isolates was identified as *Penicillium patulum* and was shown to produce patulin on substrates containing lactose and peptonized milk or casein at 5°C (Stott and Bullerman, 1975a).

This study was done to determine the recoverability, by chemical and biological detection methods, of patulin added to sterilized and natural Cheddar cheese. Also, growth and production of patulin by *P. patulum* on Cheddar cheese at 5° and 25°C and the effects of an antifungal agent and several packaging methods on these activities were studied.

MATERIALS & METHODS

A STRAIN of *P. patulum*, isolated from Cheddar cheese and known to produce patulin, was used in this study (Bullerman and Olivigni, 1974). The mold was carried on potato dextrose agar (PDA) slants and stored at 5°C.

The stability of patulin on Cheddar cheese was studied using sterile and nonsterile cheeses. Blocks of 3 month old Cheddar cheese were wrapped in aluminum foil, autoclaved at 121°C for 10 min to kill vegetative cells, and allowed to cool. Sterilized and nonsterilized cheeses were then aseptically shredded and 10-g samples from each group were placed in 125 ml erlenmeyer flasks. Purified patulin in 0.5 ml of ethyl acetate was then added to the cheese and the solvent evaporated under a stream of nitrogen, to give an average concentration of 50 μg patulin/g cheese. Samples were held for 0, 3, 12, 24 and 48 hr at 5° and 25°C before being analyzed. The treatments were done in duplicate and the experiment was replicated three times.

Samples were extracted according to the method of Pohland and Allen (1970b), except that 50 ml of acetonitrile was used and the samples were mixed with the solvent for 15 min. The patulin concentration was estimated by visual comparison of sample extracts to known amounts of patulin on thin-layer chromatography (TLC) plates (20 × 20 cm, coated with a 0.25 mm thick layer of Silica Gel GHR, Brinkmann Instruments). The plates were developed in benzene/methanol/acetic acid (90/5/5) (BMA) (Scott and Somers, 1968). Patulin was observed as a yellow derivative in natural light after spraying the plates with 4% phenylhydrazine in water and heating for 3 min at 110°C (Scott and Somers, 1968). As little as 0.2 μg patulin/g cheese could be detected by this method. All of the samples stored at 5°C were also subjected to a column clean-up procedure described by Scott and Kennedy (1973) and quantitatively tested for biological activity to *Bacillus megaterium* NRRL 1368 (Stott and Bullerman, 1975b).

Patulin production by *P. patulum* on Cheddar cheese was studied using blocks of cheese (8 × 8 × 5 cm) coated with paraffin sealing wax on all but one side. The uncoated side was inoculated with 0.2 ml of spore suspensions, from 7–10 day old PDA slant cultures of *P. patulum*, containing ca. 10^9 spores and 30 μg patulin/ml. The inoculated blocks were incubated in covered beakers over distilled water at 25° and 5°C for 2 and 6 wk, respectively, until a heavy, sporulated growth of mold was obtained.

Blocks of cheese molded by *P. patulum* were extracted and analyzed in the same manner as shredded cheese except that the cheese blocks were sliced into layers ca. 3 mm thick, cut into small pieces, weighed and then extracted. Patulin content of the cheese was verified and determined using internal standards on TLC plates by the method previously described. In addition, several samples found to contain patulin by TLC were also analyzed using high pressure liquid chromatography (HPLC) according to the method of Ware et al. (1974) by the Bureau of Foods, Dept. of Chemistry & Physics, Food & Drug Administration, Washington, D.C. This method had a detection limit of ca. 0.04 μg patulin/g cheese.

To determine if biologically active compounds were present in cheese adjacent to areas of mold growth, an anaerobic microbial assay was developed. Core samples of cheese from moldy blocks were obtained by pushing a No. 4 cork bore through the block toward the mycelia. These cores were then sliced into 3 mm thick disc-like plugs. The plugs were then placed on the surface of 10 ml of solidified 2% agar in a 15 × 100 mm petri dish. A medium consisting of 0.5g tryptone, 0.25g yeast extract, 0.1g glucose, 1g agar and 100 ml distilled water and seeded with 2 ml of a 24-hr meat broth culture of *Clostridium perfringens* NCTC 1054 was added to the plates and allowed to

solidify. This organism was found to be sensitive to as little as 1 μg patulin on assay discs. The plates were incubated in anaerobic jars for 6 hr at 37°C under a 100% N_2 atmosphere. These conditions prevented possible growth of hyphae present in cheese chores and potential patulin production during the assay.

The effects of sorbate and two commercial packaging techniques on the growth of *P. patulum* on Cheddar cheese were examined. Blocks of cheese (8 × 8 × 2 cm) were inoculated with a spore suspension of the mold and either left untreated or dipped in a 14% solution of potassium sorbate, which deposited less than 0.1% sorbate on the surface of the cheese (Pfizer, 1974). Treated and untreated samples were then packaged as follows: (1) packaged in an air atmosphere; (2) vacuum packaged in polyethylene pouches using a Cryovac Pouch Machine, Model 6207 (Cryovac Co., Cambridge, Mass.); (3) coated with paraffin wax. These samples were then incubated at 5° and 25°C for 6 and 2 wk, respectively. In addition, 20-lb blocks of unaged Cheddar cheese were inoculated with mold in a similar manner and were wrapped in Cello-Parakote cheese wrappers (American Can Co., Menasha, Wisc.) and heat sealed at 164–178°C for 30 sec using a Coast Sealing Machine, Model TSO-H (Coast Repair Co., Tillamook, Or.). These large cheese blocks were incubated for 6 wk at 12°C to simulate aging.

RESULTS & DISCUSSION

WHEN PATULIN was added to Cheddar cheese, the quantity recovered by chemical extraction and biological activity decreased with storage time. There was a rapid decrease in the amount of patulin recovered, since up to 50% of the toxin was chemically undetectable when the samples were extracted immediately after addition (Fig. 1). Less than 27% of the added patulin was recovered after 3 hr and less than 16% after 48 hr. Bioassay results with *B. megaterium* followed a similar decreasing pattern of toxicity, indicating that the biological activity of the compound decreased with chemically identifiable patulin. Temperature of storage and sterilization of the cheese had no apparent effect on the rapid disappearance of patulin in contact with Cheddar cheese. Thus, it appears that the disappearance of patulin was due to chemical reaction with components of the cheese and not due to microbial action. The reaction rate did not appear to be affected by the temperature employed in this study. Since cheese is high in casein, it was possible that reactive groups in the protein reacted with the patulin causing its disappearance as has been suggested with other products.

A thick, heavily sporulated mycelium covered the surface of inoculated cheese blocks incubated at 25° and 5°C. Cheese cores taken from these samples stored at 5°C inhibited *C. perfringens* NCTC 1054 at 3 mm from the cheese surface adjacent to the mold growth. Mycelia, possibly containing toxic metabolites, were observed to extend up to a distance of 12 mm into certain areas of porous cheese and may have been responsible for these bioassay results. Also, patulin may have diffused into the cheese.

Chemical analyses of extracts of cheese layers from molded cheese blocks incubated at 25°C revealed patulin to be present in at least two of the samples. In these samples, both mycelia and the top 3 mm of cheese were extracted together. Patulin concentrations of 1.3 μg and 0.4 μg were found by TLC, and 0.6 μg and 0.2 μg were found by HPLC. Also, a UV scan of the first sample showed absorption maxima identical to those of pure standard patulin. Thus, it appears that patulin was produced on Cheddar cheese inoculated with *P. patulin* and incubated at 25°C. A third sample obtained from the first 3 mm of cheese only, yielded 0.2 μg of patulin per g of cheese as determined by TLC, but this was not confirmed by HPLC. No patulin was obtained from mycelia alone, from slices deeper than 3 mm in the cheese, or from samples incubated at 5°C, even though extensive mold was present at 5°C.

Though patulin was found in cheese incubated at 25°C, the amounts found were low. At 5°C, no patulin was found in the cheese even though the mold had grown extensively. These data suggest that cheese is not a good substrate for patulin

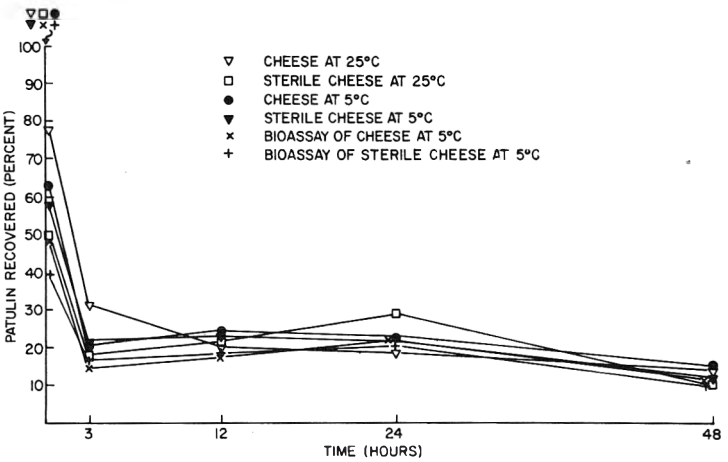

Fig. 1–Disappearance of patulin from sterile and nonsterile Cheddar cheese at 25°C and 5°C. First analysis was within 15 min after addition.

production or retention of the free toxin. This is in agreement with a previous study which indicated that substrates low in carbohydrate and high in casein are probably not good substrates for patulin production (Stott and Bullerman, 1975a). Refrigerated temperatures also appeared to depress toxin formation to a level where no extractable patulin was obtainable though some biological activity was still present.

No growth of *P. patulum* was observed at 25° and 5°C after 2 and 6 wk incubation, respectively, on any cheese treated with potassium sorbate, vacuum packaged or coated with paraffin wax, while samples not treated with sorbate and packaged in air supported heavy mold growth. Cheese blocks wrapped in Cello-Parakote wrappers and heat sealed before aging, for the most part, did not support mold growth, but a small amount of growth occurred on the corners of two of the blocks where heat sealing may have been deficient. Analyses of cheese from these areas of mold growth revealed no chemically detectable patulin when analyzed by TLC.

This study showed that cheese does not appear to be a favorable substrate for patulin production though it is an excellent substrate for mold growth. Furthermore, patulin becomes chemically undetectable when in contact with cheese, and if produced, would be expected to disappear and lose biological activity. Thus, there does not appear to be a great potential health hazard associated with growth of patulin producing molds on cheese stored at refrigerated temperatures. Further, this growth can be prevented through the proper application of existing technology in the form of mycostatic agents, low temperature storage and packaging systems which exclude air from the product.

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A Research Note
SEROLOGICAL AND HEMOLYTIC CHARACTERISTICS OF *Vibrio parahaemolyticus* FROM MARINE SOURCES

ABSTRACT

Of 703 suspect *V. parahaemolyticus* isolates from TCBS plates, representing oyster, water, and sediment samples, 319 (45%) were confirmed as *V. parahaemolyticus*. The most common reason for suspect colonies to fail confirmation was their ability to grow in trypticase peptone broth with 10% NaCl. When 922 confirmed isolates were tested serologically 72 (7.8%) were typable. The most predominant serotype was O5:K17. Of 2218 confirmed isolates from marine sources only 0.18% were Kanagawa positive.

INTRODUCTION

CURRENT INTEREST in *Vibrio parahaemolyticus* is related primarily to outbreaks of gastroenteritis caused by consumption of contaminated seafoods. Procedures for isolating *V. parahaemolyticus* from foods are based on selective enrichment (glucose salt teepol broth for example) and subsequent streaking of the broth onto thiosulfate citrate bile salts sucrose agar (TCBS). Suspect (blue-green) colonies are then confirmed primarily by biochemical and physiological tests. β-hemolysis of *V. parahaemolyticus* on a special medium with fresh human erythrocytes is reported to be highly correlated with pathogenicity (Miyamoto et al., 1969; Sakazaki et al., 1968b). This report provides information on (a) the characteristics of suspect *V. parahaemolyticus* isolates (blue-green colonies) on TCBS agar, and (b) serological characteristics and Kanagawa reaction of confirmed isolates from oysters, water and sediment from the Galveston Bay system.

MATERIALS & METHODS

ISOLATION of *V. parahaemolyticus* from oysters, water and sediment and confirmatory tests are reported in a previous paper (Thompson et al., 1976). Hemolysis was determined with fresh human erythrocytes on the medium proposed by Wagatsuma (1968). Plates were incubated at 37°C for 24 hr. Only β-hemolysis within 24 hr was considered a positive "Kanagawa" reaction. Serological identification was made by slide agglutination with K and O antisera (Nichimen Co., New York), according to the scheme of Sakazaki et al. (1968a). A suspension (0.85% NaCl) of a 24-hr culture from trypticase soy agar (TSA, 3% NaCl) slants was mixed with an equal volume of polyvalent K or monovalent K antiserum. After mixing for 30–60 sec, agglutination was rated from 1+ to 4+ by visual observation. Prior to testing with O antisera, suspensions of isolates in 0.85% NaCl diluent were autoclaved for 1 hr to unmask the O antigen. Cultures which reacted were then re-tested with the monovalent K antisera. Cultures were considered typable when they reacted with one polyvalent O and one monovalent K antiserum.

RESULTS & DISCUSSION

OF 703 SUSPECT (blue-green) colonies from TCBS plates, representing 153 samples of oysters, water and sediment, 45% could be confirmed by biochemical and physiological tests (Thompson et al., 1976). The most common reasons for isolates to fail one or more of the characteristics of *V. parahaemolyticus* were their ability to grow in trypticase peptone broth (TPB) with 10% NaCl, inability to grow in TPB with 3 and 6% NaCl, ability to ferment sucrose, inability to ferment mannitol, or inability to produce amylase. Some of the suspect colonies may have been *V. alginolyticus*, since this species grows in TPB with 10% NaCl and ferments sucrose.

Of 922 confirmed isolates from oysters, water and sediment from Galveston Bay only 72 (7.8%) were typable (Table 1). Of the typable isolates, 54% reacted only with a poly O and a mono K antiserum, 28 were typable with both poly O-mono K as well as poly K-mono K antisera. Five isolates reacted with a poly O and a mono K antiserum, yet when retested with poly K-mono K antisera were agglutinated only by the poly K antiserum. Predominant serotypes were O5:K17 (31.9%), O4:K34 (12.5%), O3:K31 (6.9%), O4:K42 (6.9%), and O3:K54 (6.9%) (Table 2). The most common O groups were O5 (33.3% of typable isolates), O3 (30.6%), and O4 (23.6%). Fishbein et al. (1974) reported a much higher percentage (40% vs 7.8%) of typable isolates from seafoods. Thirteen of the 18 serotypes obtained in the present study were among their isolates from seafoods. In their study, serotypes in order of descending frequency were K17, K30, K37, K32 and K28. Except for serotype K17 which was predominant in both studies, differences existed among the most frequently occurring antigenic K types from seafoods. When seafood isolates were arranged according to antigenic O group frequencies, results were similar. Twelve of the serotypes from marine sources in the Gulf of Mexico were also among 18 types obtained by Wagatsuma (1974) from seawater, mud and oysters in Japan. The most frequently occurring serotypes from seafoods in this study were not the types isolated from patients with gastroenteritis caused by seafoods contaminated with *V. parahaemolyticus*. Similar obser-

Table 1—Comparison of serological reactions of 922 V. parahaemolyticus isolates with "O" and "K" antisera

No. of isolates	Percentage	Type of reaction			
		Poly O-Mono K		Poly K-Mono K	
28	3.0	+	+	+	+
5	0.5	+	+	+	–
39	4.2	+	+	–	–
324	35.1	+	–	–	–
497	53.9	–	–	–	–
12	1.3	+	–	+	–
11	1.2	–	–	+	+
6	0.7	–	–	+	–
922					

Table 2—Antigenic distribution of 72 typable isolates of V. parahaemolyticus from oysters, water and sediment

O Type	K Agglutinins	No. cultures positive
II	3	2
III	5	1
V	15	1
V	17	23
VI	18	3
VIII	20	1
III	29	2
III	30	4
III	31	5
I	32	1
III	33	3
IV	34	9
IV	42	5
VI	46	1
III	54	5
IV	55	3
I	56	1
III	57	2
		72 (7.8%)
Total no. of isolates examined:		922

vations have been reported by others (Kudoh et al., 1974; Sakazaki et al., 1968b; Wagatsuma, 1974). The reason for this phenomenon is unknown.

When 2,218 confirmed isolates from oysters, water and sediment in Galveston Bay were tested for the Kanagawa reaction, only 4 (0.18%) were Kanagawa positive. Sakazaki et al. (1968b), Wagatsuma (1974), and Kudoh et al. (1974) also found few (0.35–2.8%) Kanagawa positive strains among seafood isolates. However, isolates from patients with gastroenteritis were often (88–96%) Kanagawa positive. Sanyal and Sen (1974) reported human volunteer studies in which administration of Kanagawa negative strains did not product gastroenteritis. Nevertheless, occasional outbreaks of food poisoning, due to Kanagawa negative strains, are reported (Teramoto et al., 1971; Zen-Yoji et al., 1970). Teramoto et al. (1971) reported isolating 10 serotypes (Kanagawa negative) from three outbreaks involving 59 cases of gastroenteritis. Although present information indicates a high correlation between β-hemolytic properties of *V. parahaemolyticus* and pathogenicity, no adequate explanation is available why stool isolates from epidemics differ from food isolates with respect to serotype and Kanagawa reaction. It is possible that pathogenic strains exist in the food among large numbers of similar nonpathogenic strains and that present methods of isolation do not favor the detection of Kanagawa positive strains (Barrow and Miller, 1974). Examination of a large number of colonies cultured from incriminated foods has yielded Kanagawa positive strains of the same serotype that was associated with gastroenteritis (Peffers et al., 1973; Wagatsuma, 1974). In addition, the organism may acquire the hemolysin(s) through genetic changes in the intestinal tract mediated either by chemical activities or by bacteriophage.

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A Research Note

TEXTURAL QUALITY PREDICTION OF REHEATED FROZEN FRENCH FRIED POTATOES BY OBJECTIVE TESTING OF RAW STOCK

ABSTRACT

Research is described which evaluates and relates a simple Durometer test of raw potato lots with instrumental texture measurements and sensory panel evaluation of oven-reheated frozen French fried potatoes. Statistical evaluation of the mean data shows no difference between the Idaho and Maine Russet Burbank samples for any of the measurements. Durometer means were significantly correlated with shear press measurements and all shear press values were significantly intercorrelated. The large correlation coefficients between Durometer readings and other measurements indicate that Durometer evaluations can be used to predict from the raw tubers the shear press measurements and overall moistness, crust crispness and internal mealiness of oven-reheated frozen French fried potatoes. Further work is necessary with other source and variety combinations before a general prediction statement can be made.

[1] Retired

INTRODUCTION

A RESEARCH PROGRAM at this laboratory has for some years been directed to examine by instrumental means the

Table 1—Instrument means for Durometer readings on raw tubers and shear press values for oven-reheated frozen French fried potatoes[a]

	Durometer reading[c]	Shear press[b]					
		AUC hot[d]	AUC cold	Peak 3 hot	Peak 3 cold	Peak 2 hot	Peak 2 cold
Idaho Russet Burbank	36.85a[e]	405a	337a	560ab	503ab	237a	340ab
Maine Russet Burbank	36.18a	443a	415a	610a	603a	307a	417a
Red River Valley Kennebec	32.21b	317b	235b	527b	417bc	233b	270bc
Maine Katahdin	25.32d	170c	160b	453c	370bc	127c	153d
Maine Kennebec, high specific gravity[e]	29.03c	293b	225b	457c	390bc	220b	213cd
Maine Kennebec, low specific gravity	27.37cd	225c	182b	450d	320c	133c	200cd

[a] Values followed by the same letter are not significantly different from each other at the 5% level by Duncan's Multiple Range Test (Duncan, 1955).
[b] Reported values are means of three results, each obtained by shearing seven sampled slices in a single layer in a standard shear test cell. The mean range for all the shear measurements is 74.
[c] Each value is a mean of 10 observations on 15 tubers.
[d] AUC is area under the shear press curve (work).
[e] High sp. gr. ≥ 1.090 and low sp. gr. ≤ 1.080.

Table 2—Treatment means for sensory scores and hedonic ratings for oven-reheated frozen French fried potatoes[a]

	Treatment minus standard			
	Overall moistness	Crust crispness	Internal mealiness	Hedonic rating
Idaho Russet Burbank	−0.21a	0.06a	−0.11a	0.07b
Maine Russet Burbank	−0.34a	−0.10a	−0.13a	−0.16b
Red River Valley Kennebec	−0.29a	−0.04a	−0.31a	0.37a
Maine Katahdin	−1.57c	−1.17c	−1.51c	−2.16d
Maine Kennebec, high specific gravity[b]	−0.89b	−0.60b	−0.80b	−0.49c
Maine Kennebec, low specific gravity	−1.03b	−0.83bc	−0.90b	−0.19b

[a] Panel of 14 judges, balanced incomplete block design. Values followed by the same letter are not significantly different from each other at the 5% level by Duncan's Multiple Range Test (Duncan, 1955). Each value is based on 70 observations.
[b] High sp. gr. ≥ 1.090 and low sp. gr. ≤ 1.080.

textural characteristics of frozen and reheated French fried potatoes. During this research a modified shear press, equipped with special electronic equipment, was developed for the precise and accurate evaluation of the various textural components of raw and French fried potatoes (Porter and Ross, 1966; Ross and Porter, 1966, 1968, 1969, 1971).

Since the vast frozen French fried potato industry makes huge purchases of raw tubers, a method of predicting the quality, from the raw tubers in the field, of the frozen and reheated French fried potatoes was sought. In this study we examined the relationships among data from laboratory shear testing of reheated French fries, sensory evaluation, and a simple instrumental test applied to the raw tubers.

MATERIALS & METHODS

RUSSET BURBANK potatoes grown in Idaho and in Maine, Kennebec potatoes grown in the Red River Valley and in Maine and Katahdin potatoes grown in Maine were used for this work. The Maine Kennebecs were brine graded into three specific gravity groups. The highest 25% (above s.g. 1.090) and the lowest 25% (below s.g. 1.080) were used for this work.

The Durometer used for this work was made by the Shore Instrument Co., Jamaica, N.Y. It is a pocket-sized, relatively inexpensive, dial penetrometer. The instrument selected is a type A-2 with a 1/2 in. round foot for irregular surfaces, a maximum reading needle for convenience, and a truncated cone-shaped indentor. Bourne and Mondy, 1967, used a hemispherical Durometer indentor which yielded raw potato readings in the 75–85 range. With the truncated cone-shaped indentor used in these studies, readings were in the 25–35 range. The Durometer reading vs. force per unit area curve is a straight line in this range and, therefore, yields a valid evaluation. For use, the potato is peeled at the point where the reading is to be taken, the Durometer is pressed firmly against the tuber, and the reading is noted. Durometer tests were made on representative samples of each of the six variety lots. Ten evenly spaced tests were made in a line from the bud end to the stem end of 15 tubers from each of the samples.

Six of the Durometer tested sample tubers were cut into 3/8 in. × 3/8 in. × 2-5/8 in. slices and 400g (0.88 lb) lots of slices were taken through the procedure until sufficient sample resulted for both shear and organoleptic evaluations. The slices were blanched in 79.4°C water for 6 min with occasional agitation. After a 1-min drain, the blanched slices were fried for 3 min in a 15 lb (6.8 kg), 4500 watt fryer set at 185°C and filled with hydrogenated vegetable oil type shortening. Occasional agitation was also employed at this point. After frying, the excess fat was shaken off the slices. Next, the French fried slices were quick-frozen for 20 min on a screen over liquid nitrogen inside a covered draft shield. The frozen fries were then stored in double polyethylene bags in a −17.8°C laboratory freezer. Two inches were maintained from all freezing surfaces in the unit by the use of raised shelves of wood and screen. For the evaluations, the frozen French fries were reheated at 218°C in a 14 in. × 14 in. × 14 in., 1500 watt gravity convection oven for 20 min.

Organoleptic evaluations were carried out by a trained 14-member panel in a special taste panel room. A balanced incomplete block design with three treatments (samples) and a hidden standard per block (serving tray) was used. The hidden standard–Idaho Russet Burbank–was included in each block to compare each treatment with the standard. Each panelist evaluated 10 blocks (on 10 different days), each treatment appeared eventually five times and each combination of two treatments appeared twice. The treatments were assigned at random to the treatment numbers (the order in which the 10 organoleptic evaluation series was conducted). Each block was presented to only two judges at a time. Overall moistness was judged with a 5-point scale with 1, soggy and 5, dry. Crust crispness was also judged on a 5-point scale with 1, limp and 5, crisp. Mealiness was judged on a 4-point scale with 1, not mealy and 4, mealy. A 9-point hedonic scale of 1, dislike extremely and 9, like extremely, was used for overall acceptability. Balanced incomplete block analyses of variances were calculated on the actual values and on the differences between the treatments minus the internal standard in that block.

Shear press evaluations were carried out on oven-reheated frozen French fried potatoes to obtain the area under the shear force curve and the heights of peaks 2 and 3 (Ross and Porter, 1968, 1969). Determinations were carried out on hot samples and on samples cooled for

Table 3–Correlation coefficients for all instrumental values, sensory scores and hedonic ratings

	Treatment minus standard				Shear press				
	Crust crispness	Internal mealiness	Hedonic rating	Durometer reading	AUC hot[a]	AUC cold	Peak 3 hot	Peak 3 cold	Peak 2 hot	Peak 2 cold
Overall moistness, treatment minus standard	0.994**[b]	0.989**	0.879*	0.926**	0.897*	0.769	0.813*	0.685	0.912*	0.842*
Crust crispness, treatment minus standard		0.979**	0.826*	0.941**	0.905*	0.783	0.835*	0.719	0.921*	0.847*
Internal mealiness, treatment minus standard			0.864*	0.953**	0.940**	0.839*	0.853*	0.746	0.947**	0.899*
Hedonic rating, treatment minus standard				0.677	0.669	0.493	0.506	0.331	0.693	0.599
Durometer reading					0.975**	0.927**	0.933**	0.875*	0.970**	0.951**
Shear press area under the shear force curve, hot						0.964**	0.923**	0.907*	0.997**	0.971**
Shear press area under the shear force curve, cooled 30 min							0.950**	0.972**	0.944**	0.984**
Shear press peak 3, hot								0.957**	0.901*	0.975**
Shear press peak 3, cooled 30 min									0.885*	0.946**
Shear press peak 2, hot										0.952**

[a] AUC is area under the shear force curve (work).
[b] Correlation coefficient significance: *5%, **1% levels. Correlations are based on six observations of lot means.

30 min. The entire procedure from slicing the potatoes to the shear testing was repeated three times.

RESULTS & DISCUSSION

STATISTICAL CALCULATIONS of the Durometer data indicate that better reproducibility could have been obtained with less effort as the mean of one equatorial Durometer reading for each of 23 representative potatoes rather than the 10 observations of 15 tubers used in this work.

Values for all the variables shown in Tables 1 and 2 rate Maine's one-time standard potato, the Katahdin, significantly inferior to the Russet Burbank. However, for all variables, Maine Russet Burbanks do not differ significantly from the long-accepted Idaho Russet Burbanks. Although the means for the Maine Kennebec high specific gravity samples were greater than those of the low specific gravity tubers (Table 1), the Durometer values and three of the six shear press values show no significant differences.

Table 2 shows the means of the organoleptic evaluations. Using the difference between a treatment minus the hidden standard within the block of the organoleptic data does not improve the evaluations. The Maine Katahdin tubers, as with the instrumental evaluations, yield the lowest organoleptic scores of all 6 treatments.

Table 3 contains all the correlation coefficients between the eight organoleptic and seven objective evaluations. All correlations between the shear press variables exceed 0.885 and are significant with only four degrees of freedom. Durometer and shear press values have significant correlation coefficients.

Organoleptic scores are highly correlated except for the hedonic rating. Durometer readings are highly correlated with overall moistness, crust crispness and internal mealiness scores. However, correlation between the Durometer readings and the hedonic rating scores is 0.638 and not significant. The objectivity of the taste panel in evaluating the hedonic score was probably influenced by taste, color and appearance despite careful instructions to rate only for texture attributes. This problem was not evident when evaluations were made on only one facet of texture at a time.

CONCLUSIONS

IT MAY BE CONCLUDED from Table 3 that for the tubers used in these tests that the Durometer evaluation can readily be used to predict from the raw tubers the texture parameters overall moistness, crust crispness, and internal mealiness in the oven-reheated frozen French fried potatoes. If, for example, the Durometer mean for a lot of tubers is found to be 25, it may be concluded that this lot of potatoes will make French fries with poor textural characteristics. However, tubers with a Durometer mean of 35 will make French fried potatoes with excellent textural quality.

Further work is necessary with potato samples of other source and variety combinations before a general statement can be made about prediction of final textural quality of the reheated product from Durometer values of any raw stock.

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Reference to a brand or firm name does not constitute endorsement by the U.S. Dept. of Agriculture over others of a similar nature not mentioned.

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A Research Note

APPARENT SOLUBILITY AND ALKALI DEGRADATION PATTERNS OF RICE VARIETIES

ABSTRACT

A simple procedure for evaluating cooking quality of rice is desirable because of the vast number of varieties grown and lack of instruments and trained personnel in rice-growing areas. A method measuring the apparent solubility of cooked and parboiled rice indicated solubility may play an important role in determining texture as the least soluble types tend to be less cohesive. The purpose of this work was to investigate the relationship between apparent solubility of 21 rice varieties and their response to the alkali test (alkali score). Results show no significant relationship. However, there was an interesting relationship between apparent solubility and alkali reaction type, falling into groups according to their alkali reaction pattern: generally varieties exhibiting a C-type pattern had an apparent solubility > 40%; A-type between 28–40%; and B-type < 28%.

INTRODUCTION

MUCH EFFORT has been directed towards the development of simple procedures for evaluating the cooking quality of rice. Such tests are desirable because there are large numbers of varieties used for screening purposes and many of the rice-growing areas lack the necessary instruments and trained personnel.

The gelatinization temperature of rice is considered to be an important index of its quality, a low value being associated with the more cohesive short and medium-grain types (Halick and Kelly, 1959). Several test methods have been devised to measure this property, the most popular being the alkali test (Little et al., 1958) which relies on visual estimation of the extent of kernel disintegration in dilute alkali. A more objective result can be obtained by spectrophotometric measurement of the amylose-iodine complex in an extract of the rice flour treated with alkali (Priestley and Birch, 1973). Bhattacharya and Sowbhagya (1972) made several improvements to the visual test procedure and based their scoring system on nine distinct stages of kernel degradation in 1.4% KOH. In addition to the varying extent of degradation they also observed that different patterns of breakdown occurred, which they classified into three types: Reaction type A was characterized by progressive cracking and corrosion; type B by progressive grilling and cottony transformation; and type C by opening and splitting with gradual dense cottony transformation of the kernel. However, they found no relationship between the reaction pattern and any other physicochemical property of the rice.

A method has been developed for measuring the "apparent solubility" of cooked rice after macerating the kernels and centrifuging the extract (Priestley, 1974). Preliminary studies using different rice varieties (Priestley, 1974) and rice subjected to parboiling treatments (Priestley, 1975) have indicated that solubility may play an important role in determining the texture of cooked rice, the least soluble types tending to be less cohesive.

The purpose of the work presented here was to investigate the relationship between apparent solubility of rice varieties and their response to the alkali test.

EXPERIMENTAL

Rice varieties

A series of 21 varieties of rice were used, representing a wide range of alkali score. They were kindly donated by K.R. Bhattacharya, Mysore, India.

Response to alkali

Studies on the same varieties used in this work had been published previously (Bhattacharya et al., 1972) from which details of their alkali score and reaction type were obtained.

Apparent solubility of cooked rice

The apparent solubility of each variety was determined after cooking for 10, 20 and 40 min using the method of Priestley (1974). 1g of rice is added to 20 ml boiling distilled water in a boiling tube fitted with a condenser. After the desired heating period the cooking water is drained, its volume measured, and the grains rinsed with 20 ml cold distilled water. The volume of drained cooking water is replaced by an equal volume of cold distilled water. The kernels are then macerated for 20 sec using a tissue homogenizer (Ultra-Turrax, Janke & Kunkel, W. Germany) operating at 20,000 rpm and the dispersion is transferred to a 50 ml centrifuge tube with 15 ml water used for rinsing. The dispersion is shaken and then centrifuged at 3,000 rpm for 15 min (Minor, M.S.E., England). A 20 ml aliquot of the supernatant is evaporated on a steam bath and dried for 4 hr at 105°C.

The weight of soluble material released after maceration is calculated as a percentage of the sample dry weight. The term "apparent solubility" is used since, under these conditions, starch does not form a true solution.

RESULTS & DISCUSSION

THE RESULTS of these studies are shown in Table 1. The varieties are ranked in order of decreasing apparent solubility after cooking for 40 min, by which time they would be expected to have reached a maximum value (Priestley, 1974). If shorter cooking periods are used the results are subject to the influence of other factors, such as kernel dimensions and protein content, which affect the rate of water absorption (Bhattacharya and Sowbhagya, 1971), although the ranking is not markedly altered by using the results obtained after cooking for 20 min.

There was no significant relationship between apparent solubility and alkali score (a high alkali score corresponds to a low gelatinization temperature). Schoch (1967) believes that the value of gelatinization temperature in rice quality evaluation has been previously overemphasized and other work has shown that it is not significantly related to eating quality (Juliano et al., 1965, 1969).

There was, however, an interesting relationship between apparent solubility and alkali reaction type. When ranked as described the varieties fall into groups according to their alkali reaction pattern. All varieties exhibiting a C-type pattern had an apparent solubility greater than 40%, all the A-type varieties fell between 28 and 40% and all the B-types, with one exception (variety ADT-27) gave an apparent solubility below 28%. Regarding the anomalous behavior of variety ADT-27, Bhattacharya (Personal communication, 1974) has noted that this variety also gives an alkali reaction pattern atypical of, but

Table 1—Apparent solubility and response to alkali of rice varieties

Variety	Alkali score[a]	Alkali reaction type[a]	Apparent solubility after cooking % 10 min	20 min	40 min
Century Patna 231	0.0	C	26.6	49.3	60.7
AC-9800	0.6	C	21.4	43.8	53.2
Rexoro	0.5	C	20.0	47.6	52.9
Tainan 3	6.6	C	18.0	36.1	45.6
Taichung 65	6.0	C	18.1	32.9	40.7
Co-25	8.0	A	19.8	33.7	39.9
S-139	3.1	A	16.3	27.5	39.4
S-701	4.0	A	18.1	26.1	37.9
S-317	3.5	A	16.2	25.1	35.6
S-1092	4.0	A	17.6	26.8	35.4
SR-26B	4.0	A	14.2	27.8	35.4
ADT-27	3.5	B	13.5	24.2	34.2
S-718	3.9	A	14.4	23.5	33.4
Vankasannam	1.6	A	19.3	31.5	32.3
ADT-8	1.0	A	15.3	25.4	31.9
Halubbulu	1.4	A	12.2	25.6	31.7
S-749	2.4	A	13.5	24.9	29.5
S-661	2.5	A	13.4	28.5	28.4
China 2	4.4	B	8.5	17.1	26.4
Taichung(N)1	7.4	B	9.3	16.5	23.1
IR8	7.4	B	9.0	16.2	20.9

[a] Data from Bhattacharya et al. (1972)

more closely resembling the B-type than either of the other patterns. Adding further weight to the above relationship, he also stated that variety Co-25 which, on the basis of the apparent solubility data, lies on the borderline between the C- and A-type patterns, was originally classified as type A? C?, but was placed in group A for convenience. The cause of this relationship is not yet understood, but may be connected with the arrangement of starch granules, cells and other components within the rice kernel. Suppression of starch swelling in rice and the various patterns of disruption observed in different varieties after cooking have been associated with such factors (Little and Dawson, 1960).

Further work is necessary to establish the relationship between apparent solubility and cooked rice texture. Determination of the alkali reaction pattern using the method of Bhattacharya and Sowbhagya (1972) may then prove to be a valuable screening or field test for assessing rice quality since the only requirements are potassium hydroxide and Petri dishes.

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A Research Note

A MICROBIOLOGICAL PROFILE OF COMMERCIALLY PREPARED SALADS

ABSTRACT

Commercially prepared salads from nine different producers were examined for the number of aerobic, psychrotrophic and coliform organisms, the number of yeast and molds, and pH. Samples were examined twice: once when received and once 5 days following the designated expiration date. Colonies from both sets of plates were selected, based on visual differences, for purification and identification in an attempt to determine what bacteria were present in the salads. Organisms recovered included the genera *Bacillus, Lactobacillus, Leuconostoc, Streptococcus, Micrococcus, Staphylococcus, Pseudomonas, Xanthomonas, Escherichia, Citrobacter, Enterobacter* and one *Actinomyces*. The genera found in largest numbers were *Bacillus, Lactobacillus, Leuconostoc,* and *Streptococcus*.

INTRODUCTION

THE MARKET for commercially prepared salads is increasing yearly (Anon., 1969) due to their convenience. Since the late 1960's salads have been investigated for pathogens, the causes of bacterial contamination and a short shelf life. As the producers of salads are generally small businesses and are not well schooled in food safety, concern for the consumer has become evident.

Food poisoning due to contaminated salad has been documented by Hill et al. (1969) but most salads have been found to contain only small numbers of pathogens (Christiansen and King, 1971; Jopke and Riley, 1968). Salads from restaurants and cafeterias were examined by Jopke and Riley (1968) and those from other retail outlets by Christiansen and King (1971). However, pathogens were found only in small numbers. Holtzapffel and Mossel (1968) found that several pathogens died when they were inoculated into prepared salads, even at temperatures conducive to pathogen growth.

By utilizing data from aerobic plate count (APC), coliform count, and oxidase positive plate count obtained from salads, Hankin and Ullman (1969) devised a method for determining the source of the contamination of salads. Hankin and Stephens (1972) tried to correlate various microbiological quality tests with the keeping quality of potato salad and cole slaw. They found that pH was correlated negatively with the quality of potato salad and that oxidase positive plate counts were correlated negatively with the quality of cole slaw.

In the report by Christiansen and King (1971) and Holtzapffel and Mossel (1968), the lactic acid bacteria, yeasts and molds were determined to be the only organisms capable of growth in salads. However, only a limited attempt was made to identify the bacteria which occurred in commercially produced salads.

MATERIALS & METHODS

NINE DIFFERENT BRANDS of salads were collected from the producers or from local retail outlets. The samples were iced and brought to the laboratory for microbiological examination. These included chicken, ham, and potato salads, cole slaw and pimento cheese spread. The samples were examined for aerobic plate count (APC), coliform count, psychrotrophic plate count (PPC), yeast and mold count, and pH within 48 hr of receipt and after storage at 3–6°C until 5 days after the expiration date given to each salad by the producer. Samples that did not have an expiration date (those from delicatessens) were examined the second time after storage at 3–6°C for 15 days. Similar salads produced by all nine producers are included in the following report.

All media used were produced by Baltimore Biological Laboratories. Standard Methods Agar was used for APC and PPC; coliforms were enumerated on Violet Red Bile Agar (VRB); and Mycophil Agar was used for yeast and mold counts. Incubation times and temperatures were those recommended for use by *Standard Methods for the Examination of Dairy Products* (APHA, 1972). Selective and differential agars were used, where applicable, during identification procedures.

A 10-g portion of salad was weighed aseptically into a sanitized, stainless steel, Virtis homogenizer jar and homogenized for 1 min with 10 ml of 2% sterile sodium citrate. The speed of homogenization varied with each type of salad in order to produce a slurry that could be transferred with a 2.2 ml pipette.

pH values were determined by mixing a portion of the salad with an approximately equal portion of distilled water and by measuring with a Beckman pH meter.

From each set of APC, PPC and coliform plates, bacterial colonies were selected for purification and identification. A colony of each separate type based on size, color, location in agar (surface or subsurface), and appearance (smooth, matte, etc.) was selected. Only one of each kind of colony was selected and no constant number of colonies was selected from each plate.

Purification was accomplished by diluting a portion of the selected colony in a 99 ml dilution blank and pour plating a drop of the dilution. Transfers were carried out at least three times on each colony by picking a dilution plate colony most similar to the original. Purified cultures were kept at 3–6°C on Standard Methods Agar slants *until* they were identified.

Identification was carried out in accordance with *Bergey's Manual of Determinative Bacteriology* (1957, 1974) and *Laboratory Methods in Microbiology* (Harrigan and McCance, 1966).

RESULTS & DISCUSSION

THE MICROBIAL PROFILES and pH of the salads of this study are shown in Table 1. Counts on most of the salads were relatively low. Only the pimento cheese had high median counts and this could have resulted from the cheese that was used in its preparation. Although some of the salads had very high individual APC and PPC values, the median values were low. This reflects upon the sanitary production and handling of the salads and the quality of the ingredients used. Also, if some of the producers can manufacture low microbial count salad, improvement by other producers should be feasible.

Table 2 contains a breakdown of the 376 isolates identified in this study by the number of each species or genus. The genus *Bacillus* was represented by 146 isolates and was the most plentiful. The lactic acid bacteria of *Lactobacillus, Leuconostoc* and *Streptococcus* genera were almost as plentiful as the bacilli with 140 isolates. Several *Micrococcus, Pseudomonas* and *Staphylococcus* were found along with a few *Escherichia, Enterobacter, Citrobacter* and *Xanthomonas*. One *Actinomyces* was found but not completely identified. Only

Table 1—Microbiological and pH ranges and medians of salads from nine producers

Salad	APC[a]			PPC[b]		Coliform		Yeast and mold			pH	
	Low	Median (counts/g)	High	Low	Median (counts/g)	Low	High	Low	Median (counts/g)	High	Low	Median	High
Original examination													
Chicken	<100	2,600	>30,000,000	<100	100	<1	7740	<1	800	7,800	4.34	4.80	5.14
Cole Slaw	400	7,100	700,000	<100	1000	<1	1000	<1	180	7,000	3.90	4.48	4.80
Ham	<100	6,900	>30,000,000	<100	300	<1	1000	<1	80	1,400	4.25	4.55	5.01
Potato	300	6,900	180,000	<100	600	<1	900	<1	11	19,000	4.06	4.57	5.06
Pimento Cheese	3000	490,000	8,000,000	<100	1400	<1	40	<1	3100	3,100	4.69	5.24	5.68
Final examination													
Chicken	<100	6,400	1,200,000	<100	1600	<1	1990	2	500	3,000	4.22	4.70	4.91
Cole Slaw	300	3,700	630,000	<100	400	<1	28	<1	38	240,000	3.51	4.24	4.70
Ham	100	5,600	19,000,000	<100	100	<1	30	<1	9	6,700	4.36	4.55	5.40
Potato	500	16,000	40,000,000	200	6300	<1	70	<1	2800	19,000	4.10	4.46	5.04
Pimento Cheese	420	142,000	15,000,000	100	7000	<1	14	<1	18	26,000	4.55	4.96	5.80

[a] APC — Aerobic plate count
[b] PPC — Psychrophilic plate count

11 of the isolates were potential pathogens, of these, 9 were *Bacillus cereus* and two *Staphylococcus* aureus. No *Salmonella* or *Shigella* were found.

The large numbers of *Bacillus* were expected due to the high content of vegetables in salads. *Bacillus* are soil organisms and are often found on vegetables.

The lactic acid bacteria (140) were the most prevalent non-sporulating bacteria in the salads. However, the proportion of rods to cocci (75:65) was much different than that reported by Holtzapffel and Mossel (1968). More than 46% of the lactic acid bacteria isolated were cocci whereas Holtzapffel and Mossel (1968) had only 11% cocci. *Lactobacillus* was the most prevalent lactic acid bacteria and *Leuconostoc* constituted the majority of the cocci. Both of these genera are associated with plants and this could account for their number.

Some *Micrococcus* were present and all three species (Buchanan and Gibbons, 1974) were represented. The largest group of these were *Micrococcus luteus* followed by *Micro-*

Table 2—Quantity of microorganisms isolated and identified from all salads

Microorganism	Quantity	
Bacillus		146
Bacillus brevis	1	
Bacillus megaterium	1	
Bacillus coagulans	4	
Bacillus firmus	5	
Bacillus cereus	9	
Bacillus licheniformis	29	
Bacillus subtilis	45	
Bacillus pumilis	52	
Lactobacillus		75[a]
Lactobacillus lactis	2	
Lactobacillus delbrueckii	2	
Lactobacillus brevis	4	
Lactobacillus casei	10	
Lactobacillus leichmannii	12	
Lactobacillus plantarum	12	
Leuconostoc		50
Leuconostoc dextranicum	5	
Leuconostoc mesenteroides	21	
Leuconostoc paramesenteroides	24	
Streptococcus		15
Streptococcus lactis	1	
Streptococcus uberis	2	
Streptococcus cremoris	3	
Streptococcus faecalis	4	
Streptococcus durans	5	
Micrococcus		41
Micrococcus roseus	1	
Micrococcus varians	17	
Micrococcus luteus	23	
Staphylococcus		12
Staphylococcus epidermidis	10	
Staphylococcus aureus	2	
Enterobacteriaceae	12	12
Escherichia coli	4	
Enterobacter aerogenes	4	
Citrobacter intermedia	4	
Pseudomonas		20
Xanthomonas		4
Actinomyces		1

[a] 33 Lactobacillus were not identified to the species level.

coccus varians. Only one *Micrococcus roseus* was found. Staphylococci were a small minority of the organisms with 12 isolates. The most common were the *Staphylococcus epidermidis* with 10 isolates. Only 2 isolates were *Staphylococcus aureus*. Most of the remaining organisms were gram-negative rods and most of those were *Pseudomonas*. The *Pseudomonas* are well-known psychrotrophs and they appeared to survive in the salads stored at low temperatures.

From the examination of salads from nine producers, results demonstrated that low microbial count salads can be manufactured. In general, when pH values were low and salads were stored at normal to low refrigeration temperatures, very little if any microbial growth occurred before the expiration date of the salads. Although the yeast and mold counts appear fairly low, the observation of just one mold colony on the surface of a newly opened container would cause a housewife to consider the salad spoiled. Awareness of this factor alone points out the necessity of sanitary production and handling of salads. From this survey, good keeping quality for prepared salads in the market place with little microbial multiplication taking place before the expiration date could be expected.

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A Research Note

NUTRITIONAL QUALITIES OF SOME AFRICAN EDIBLE LEAFY VEGETABLES. Effect of Boiling on the Essential Amino Acid Composition of Their Extracted Protein

ABSTRACT

This study was carried out to investigate the effect of the local method of cooking Nigerian edible leaves on the essential amino acid composition of their proteins. Six common edible leaves were boiled in water in covered earthenware pot as a housewife would normally do. Protein concentrates were extracted from both raw and cooked samples, hydrolyzed with 6N HCl and then analyzed for all their amino acids in the Beckmann automatic analyzer. Values for the essential amino acid were calculated. Boiling in water for 20 min at about 150°C increased the valine content of all the samples while their levels of leucine, threonine and tryptophan were unaffected. Isoleucine was decreased by about 10% in one sample and the level of phenylalanine + tyrosine was slightly lowered generally. Lysine and the sulphur-containing amino acids were most adversely affected and decreased by 12–22%, and 6–11%, respectively. This resulted in the general lowering of their total essential amino acids, leading to a decrease of their chemical score by 9–19%. Comparison with the latest provisional amino acid pattern of the FAO showed that the proteins of these edible leaves were of high quality with adequate levels of tryptophan and phenylalanine + tyrosine and that lysine and the sulphur-containing amino acids were their first limiting amino acids.

INTRODUCTION

LEAFY VEGETABLES generally are eaten in many parts of Africa, especially in the western, central and the southern regions. They are eaten at least once daily in these areas and some of them have been found to have a high crude protein content (Oke, 1968; Shanley and Lewis, 1969; Imbamba, 1973). They thus provide an important source of protein for numerous people, and information is therefore needed as to their protein nutritional qualities and as to the conditions affecting these qualities.

Before being consumed, the proteins in these leaves are usually subjected to a variety of processing conditions which may ultimately affect nutritional value. In Nigeria, the process of cooking or parboiling in water is most commonly applied to these leaves. Mitchell et al. (1945) have reported that the most important agency modifying the nutritive value of food proteins during processing was heat.

This study was therefore undertaken to investigate the effect of the parboiling of some of these leaves on their nutritional quality, in terms of the essential amino acid contents of their proteins.

EXPERIMENTAL

The leafy vegetables

The six chosen are some of those widely consumed in the West, Central and some parts of South Africa. They are *Amaranthus hybridus* (TETE), *Corchorus olithorus* (EWEDU), *Solanum africara* (IGBO), *Talinum triangulare* (GBURE), *Solanum nodiflorum* (OGUNMO) and *Vernonia amygdalina* (EWURO). Their local names in Nigeria are put in bracket.

All were grown on our Departmental farm. When 4 wk old they were harvested, thoroughly washed with tap water and portions boiled in about three volumes of water for 20 min at about 150°C, as a housewife would normally do, but without adding other ingredients.

Nitrogen determinations were performed on aliquots of leaves and of the water before and after boiling.

Preparation of protein concentrates

Bulk proteins were extracted from the leaves both in their boiled and unboiled states by pulping them with the IBP minipulper (Davys and Pirie, 1969), and pressing known weights of pulp with the IBP Press (Davys et al., 1969) to obtain juice samples. Proteins were steam-precipitated from the juice samples at 80°C. The protein concentrates were thoroughly washed with tap water and then freeze dried.

Table 1—Essential amino acid composition of protein concentrates prepared from raw and cooked leafy vegetables[a]

	TETE (Amaranthus hybridus)		EWEDU (Corchorus olithorus)		IGBO (Solanum africana)		GBURE (Talinum triangulare)		OGUNMO (Solanum nodiflorum)		EWURO (Vernonia amygdalina)	
Essential amino acid	Raw	Cooked	Raw	Cooked	Raw	Cooked	Raw	Cooked	Raw	Cooked	Raw	Cooked
Isoleucine	32	29	30	30	30	29	31	30	30	29	32	32
Leucine	56	54	55	53	53	51	56	54	58	58	58	57
Lysine	40	32	40	32	39	31	41	32	39	34	42	34
Methionine + cystine	25	22	30	27	27	24	27	25	30	27	26	24
Phenylalanine + tyrosine	64	63	68	66	64	62	69	66	68	65	68	67
Threonine	30	29	30	29	30	30	30	31	31	30	30	29
Tryptophan	14	13	15	14	13	14	13	13	15	14	13	14
Valine	36	39	38	41	36	38	39	42	37	40	38	40
Total essential amino acids	297	281	306	292	292	279	306	293	308	297	307	297
Total protein (N X 6.25, %of dry matter)	58.9	57.0	60.7	58.5	58.9	57.0	61.9	58.8	60.4	57.8	61.8	58.9
Chemical score (%)	71	58	73	58	71	56	77	58	71	62	74	62

[a] Figures are expressed as g amino acid per g protein.

Table 2–Comparison of the essential amino acid composition and chemical score of protein concentrates from raw and cooked Nigerian edible leaves with the latest FAO reference pattern of amino acids[a]

Essential amino acid	Protein[b] raw	Conc cooked	FAO[c] reference pattern
Isoleucine	30–32	29–32	40
Leucine	53–58	51–58	70
Lysine	39–42	31–34	55
Methionine + cystine	25–30	22–27	35
Phenylalanine + tyrosine	64–69	62–67	60
Threonine	30–31	29–31	40
Tryptophan	14–15	13–15	10
Valine	36–39	38–41	50

[a] Figures are expressed as mg amino acid per g protein.
[b] The range of the values for the six selected leaves
[c] FAO (1973)

Table 3–Chemical score and the first limiting amino acid of proteins from six Nigerian edible leaves with those of western vegetables and other common foods

Protein	New chemical score (%)	First limiting amino acid
TETE (*Amaranthus hybridus*)	71	Methionine + cystine
EWEDU (*Corchorus olithorus*)	73	Lysine
IBGO (*Solanum africana*)	71	Lysine
GBURE (*Talinum triangulare*)	75	Lysine
OGUNMO (*Solanum nodiflorum*)	71	Lysine
EWURO (*Vernonia amygadlina*)	74	Methionine + cystine
CABBAGE (*Brassica oleracea Capitata*)[a]	39	Methionine + cystine
SPINACH (*Spinacia oleracea*)[a]	68	Methionine + cystine
Soybean (*Glycine max*)[b]	74	
Ground nut (*Arachis hypogaea*)[b]	65	
Cotton seed (*Gossypium spp.*)[b]	81	
Rice, polished (*Oryza* spp.)[b]	67	
Wheat, whole (*Triticum* spp.)[b]	53	

[a] FAO (1970)
[b] FAO (1973)

Amino acid analysis

Aliquots (about 15–20 mg) of each protein sample were hydrolyzed in vacuo with 6N HCl for 18 hr at 110°C and then analyzed for all their amino acids on Aminex A-4 resin (M/S Bio-RAD Labs., Richmond, Calif.) with a Beckmann Spinco Model 120 automatic analyzer, using the method of Byers (1971). Tryptophan was estimated colorimetrically, after alkaline hydrolysis with barium hydroxide (Miller, 1967).

The chemical score for each protein was calculated according to the new pattern described in FAO (1973): the content of each essential amino acid in test protein was expressed as a percentage of the content of the same amino acid in the latest reference pattern, and the amino acid with the lowest percentage was the limiting one, and the value the chemical score.

RESULTS & DISCUSSION

TABLE 1 shows that differences occurred to varying degrees in the essential amino acid composition of proteins extracted from cooked and uncooked leaves.

No statistical comparisons were made as only one analysis per sample was made.

Heat increased the valine content of all the protein samples by about 7% while the levels of leucine, threonine and tryptophan were unaffected. Isoleucine was unchanged in all samples except in that from *Amaranthus hybridus* where it was lowered by about 10%.

There was a slight decrease of phenylalanine and tyrosine levels in three samples but was constant in all others.

Lysine and the total sulphur-containing amino acids (methionine and cystine) decreased in all the samples; that of the sulphur-containing amino acids was least in *Vernonia amygdalina* (6%) and greatest in *Corchorus olithorus* (11%). Lysine seemed to be the most adversely affected of all the amino acids; decreases ranged from 12% in *Solanum nodiflorum* to 22% in both *Corchorus olithorus* and *Talinum triangulare*. This result is in agreement with that of Adrian (1967) who reported that lysine was the most affected amino acid during the heating of several protein foodstuffs in an oven at about 150°C.

The fall in chemical score of the various samples due to boiling ranged from 9–19% with the greatest fall by proteins from *Talinum triangulare* with the highest loss of lysine.

Varying amounts of proteins were leached from the leaves into the processing water during cooking; the range was found to be 2–5% of their crude protein content which varies between 15–30% on a dry weight basis. The protein content of concentrates obtained from cooked leaves were slightly lower than those from uncooked leaves (Table 1).

Although they are usually harvested for food at various stages of growth, 4-wk-old plants were chosen for study since preliminary studies had shown that their protein content was maximal around this period.

The levels of the aromatic amino acids (phenylalanine + tyrosine) and of tryptophan in the protein samples studied were higher than their corresponding values in the latest FAO reference pattern (Table 2). The other essential amino acids are also about 50–80% of this new pattern which is higher than the adult requirements and meets the needs of children in the pre-school age but not those of older children (FAO, 1973).

Table 3 shows that, based on the new scoring pattern, lysine and the sulphur-containing amino acids are the first limiting in the edible leaves studied, and compares their protein nutritional quality with those of some other edible leaves and cereals.

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A Research Note

EFFECT OF HIGH TEMPERATURE CONDITIONING ON SUBCELLULAR DISTRIBUTION AND LEVELS OF LYSOSOMAL ENZYMES

ABSTRACT

The control (C) side of 23 animals was placed in a 2°C chill room at 1 hr postmortem, while the other side was high temperature conditioned (HT) at approximately 22°C for 4 hr postmortem, at 12°C for an additional 8 hr and was then placed in the 2°C chill room. The activity of cathepsin C and β-glucuronidase was measured on the nuclear, microsomal, and unsedimentable fractions at 12, 18 and 24 hr postmortem in order to determine the amount of sedimentable and free enzyme activity at these postmortem times. High temperature conditioning enhances the disruption of the lysosomal membrane as evidenced by a significant increase in percent of free enzyme activity at 12 hr postmortem for both cathepsin C and β-glucuronidase. There was also a significant decrease in total activity for both enzymes of the HT group at 12 hr postmortem due to autolysis of the free enzyme. These differences were not present at 18 and 24 hr postmortem (except for decreased total activity of cathepsin C at 18 hr), indicating that differences caused by high temperature conditioning take place very early postmortem and that the differences in enzyme activities are not detectable at later postmortem times. These results indicate that some of the differences in tenderness produced by HT treatments are possibly associated with the increased level of free lysosomal enzymes during the first 12 hr postmortem.

INTRODUCTION

MEAT TENDERNESS, a major quality attribute, is greatly affected by postmortem carcass handling. A significant decrease in tenderness associated with cold shortening of muscles from lamb carcasses has been demonstrated by Marsh et al. (1968). Improvements in tenderness may be achieved by methods which minimize shortening either by the use of physical restraint (Locker, 1960; Herring et al., 1965; Hostetler et al., 1970; 1975; Smith et al., 1971) or by the use of high temperatures during the development of rigor mortis (Parrish et al., 1969; Smith et al., 1971; 1974; Fields et al., 1975). These studies attribute improvements in tenderness to the prevention of muscle fiber shortening as shown by increased sarcomere lengths. However, Dutson et al. (1975) and Parrish et al. (1973) have shown that additional factors may contribute to overall meat tenderness as shown by a tenderness improvement of muscles from high temperature conditioned sides over control sides when both were restrained to hold sarcomere length constant. The present study was designed to investigate these additional factors influencing differences in tenderness due to high temperature conditioning.

EXPERIMENTAL

23 ANIMALS of the USDA Good grade weighing 850–1100 lb were slaughtered according to normal procedures. One side, control (C), of each carcass was placed in a chill room (2°C) at 1 hr postmortem while the other side, high temperature conditioned (HT), remained on the kill floor (approximately 22°C) until 4 hr postmortem. The HT side was then held at 12°C for an additional 8 hr and then moved to the 2°C chill room.

Samples of the longissimus dorsi muscle were taken adjacent to the second lumbar vertebrae at 12, 18 and 24 hr postmortem from both the C and HT sides for 5, 8 and 10 animals, respectively. Samples were trimmed of fat and epimysial connective tissue and ground through a 3 mm plate.

10g of each ground muscle sample were placed in 50 ml of 0.25M sucrose containing 0.02M KCl and homogenized for 50 sec at full speed in a Virtis "23" homogenizer. The homogenate was filtered through two layers of cheesecloth and the pH was adjusted to 7.3 with 0.1M KOH. The adjusted filtrate was then centrifuged at 700 × G for 10 min to obtain a nuclear fraction (pellet). The supernatant from the nuclear fraction was centrifuged at 105,000 × G for 2 hr to obtain a microsomal fraction (pellet) and an unsedimentable fraction (supernatant). After each centrifugation, the pellet was resuspended in 25 ml of the homogenizing solution. The activity of cathepsin C and β-glucuronidase was determined on the nuclear, microsomal and the unsedimentable fractions.

Cathepsin C activity was determined fluorometrically using glycyl-phenylalanine-β-naphthylamide (GPNA) as the substrate. The reaction mixture concentration was 0.2 mM GPNA, 3 mM β-mercaptoethylamine, 0.67 mM EDTA and 0.25 M sucrose in 67 mM phosphate buffer (pH 5.8). After 60 min at 37°C, the reaction was terminated by adding sufficient iodoacetate to achieve a 6 mM final concentration. Total activity of the nuclear and microsomal fractions was determined in the presence of 0.01% Triton X-100. Excitation and emission wavelengths were 335 and 410 nm, respectively.

The fluorometric assay medium for β-glucuronidase consisted of 0.5 mM 4-methylumbelliferyl-β-D-glucuronide in 0.1M citrate buffer (pH 5.0) made up in 0.25M sucrose. The reaction was stopped after 20 min at 37°C by the addition of an equal volume of 1.0M Na_2CO_3. Total activity of the nuclear and microsomal fractions was measured in the presence of 0.02% Triton X-100. Excitation and emission wavelengths were 360 and 448 nm, respectively.

Data were statistically analyzed using the students paired t method (Li, 1964).

RESULTS & DISCUSSION

HIGH TEMPERATURE conditioning causes a more rapid pH drop in carcasses (Cassens and Newbold, 1967). This low pH in conjunction with the high carcass temperature enhances the disruption of the lysosomal membrane and the concurrent release of acid hydrolases into the muscle tissue as shown by a rise in the % free enzyme activity (activity in the supernatant) of the HT samples (Table 1). Similar phenomena have been demonstrated in other tissues (Sawant et al., 1964). Holding the carcass at an elevated temperature not only stimulates the release of lysosomal enzymes into the soluble cell fraction, but also increases the activity of these enzymes during the conditioning period because the combined effect of high temperatures and low pH approaches optimal conditions for these enzymes. These increased levels of free enzymes are also sustained over a longer period of time in this optimal environment for the HT sides. Samples taken at 18 and 24 hr postmortem failed to show consistent differences between free (unsedimentable) enzyme levels of treated and control carcass sides (Table 1). This indicates that a similar degree of lyso-

Table 1–Mean differences in free and total enzyme levels between high temperature conditioned and control carcass sides[a]

Postmortem sampling time	Cathepsin C		β-Glucuronidase	
	% Free enzyme[b]	Total enzyme activity[c]	% Free enzyme	Total enzyme activity
	$\bar{\delta} \pm S_{\bar{\delta}}$	$\bar{\delta} \pm S_{\bar{\delta}}$	$\bar{\delta} \pm S_{\bar{\delta}}$	$\bar{\delta} \pm S_{\bar{\delta}}$
12 hour (n = 5)	2.50 ± 1.11[d]	−12.65 ± 2.92[e]	5.54 ± 1.89[d]	−0.475 ± 0.209[d]
18 hour (n = 8)	1.13 ± 2.06	− 6.69 ± 3.47[d]	1.04 ± 1.38	−0.187 ± 0.148
24 hour (n = 10)	−2.00 ± 1.90	3.96 ± 12.64	−0.77 ± 1.95	0.164 ± 1.95

[a] A positive mean difference indicates more activity for the high temperature conditioned sides, while a negative mean difference indicates less activity for these sides.
[b] Differences in % of total enzyme activity not sedimentable by centrifugation at 105,000 X G for 2 hr.
[c] Differences in total enzyme units (total nanomoles product formed/min); no significant differences were detected in enzyme levels of nuclear or microsomal fractions between treated and control sides so they were pooled with the unsedimentable fraction to obtain total activity.
[d] Significant at the 0.05 level.
[e] Significant at the 0.01 level.

somal disruption is finally reached in the control sides at these later postmortem times.

A significant decrease in total enzyme activities (nuclear, microsomal and unsedimentable) of treated vs control samples is shown in Table 1 for 12 hr samples. This is probably due to autolytic degradation of these enzymes during the early holding periods (Snellman, 1969; Otto and Riesenkonig, 1975). The 18 hr samples contained a significant difference in the level of cathepsin C activity. This phenomenon was not present in the 24 hr samples (Table 1), again indicating that the effects of high temperature conditioning on tenderization resulting from enzyme action are more prominent in the early postmortem stages. After 24 hr, the only remaining differences reported between control and high temperature conditioned samples are differences in tenderness (Dutson et al., 1975; Fields et al., 1975). These differences in tenderness are probably brought about by subtle alterations in muscle structure associated with high free enzyme levels immediately postmortem.

Earlier workers, using classical methods of measuring proteolysis, have been unable to correlate differences in tenderness with intracellular proteolytic activity (Parrish, 1971). However, a recent study (Dutson and Lawrie, 1974) has shown a correlation between soluble β-glucuronidase levels and tenderness. The work of Dutson et al. (1975) has shown that improvements in tenderness due to high temperature conditioning involve other factors in addition to sarcomere length. Thus, the data in the present study further suggests that lysosomal hydrolases contribute to meat tenderness.

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A Research Note
ADAPTING AN EXPERIMENTAL BEAN COOKER FOR AUTOMATIC RECORDING

ABSTRACT

The experimental bean cooker devised by Mattson and modified by Morris has been modified electrically so that the operator need not be present during a cooking run. This not only saves time but, with samples that cook relatively rapidly, increases accuracy. When the percentage of beans cooked "done" is plotted against time on log-probability paper, the points fall closely about a straight line. It is believed that the "median cooking time" derived from such a plot represents the cookability of an entire sample somewhat better than does that from the S-shaped curve which results when the data are plotted on rectilinear graph paper.

INTRODUCTION

THE EXPERIMENTAL bean cooker devised by Mattson (1946) and modified by Morris (1963) and Burr et al. (1968) provides a precise and objective measure of the relative cooking rates of dry beans. Essentially, it is a framework holding 100 vertical plungers, each resting on an individual bean. The lower part, holding the beans, is lowered into a steam-jacketed water bath at 99°C. When a bean reaches a certain stage of tenderness, it is penetrated by the plunger, which then drops about 4 cm. The operator counts the number of plungers that have dropped at the end of each minute, and when the cumulative number is plotted against time, an S-shaped curve is obtained. The midpoint of the curve is taken as the "median cooking time."

With beans that have become very slow cooking because of unfavorable storage, a single run may take 3 or 4 hr or longer and the operator's task is very tedious. On the other hand, near the middle of a run with normally cooking beans, plungers are dropping rapidly and it is difficult to get an exact count of the dropped plungers. In order to remedy these two difficulties, the instrument was modified to permit automatic recording of the drop of the plungers.

A piece of micarta was cut to the size of the uppermost stainless steel plate of the cooker and was drilled for the 100 plungers and the four corner posts using the plate as a template. In addition, a 1/32 in. hole was drilled diagonally adjacent to each plunger hole 5/16 in. from its center. One hundred modules were assembled, each consisting of a subminiature magnetic reed switch; a 47-ohm, 1/2-watt, 5%-tolerance resistor connected in parallel with it; and a short wire lead connected to its lower end. The reed switches are normally open but when approached by a magnet, they close.

The lower end of a reed switch was cemented into each of the 1/32 in. holes with laboratory wax and the wire lead from each module was soldered to the top end of an adjacent module, thus putting all the modules in series.

Each plunger, the upper part of which is stainless steel tubing, was fitted internally with an alnico bar magnet 3/16 in. x 3/16 in. x 7/16 in. (Magnet Sales Co., Los Angeles, CA 90037, Package 40210) so that its center was about 2-1/2 in. below the top of the plunger and the total weight was adjusted to 90g with granulated lead. A short length of vinyl laboratory tubing was slipped over the plunger and cemented in place at such a point that with the plunger in the "down" position the center of the magnet is opposite the center of its reed switch.

The micarta panel with the attached modules was placed on top of the uppermost plate of the experimental cooker where it was accurately located by the four corner posts. With all the plungers in the "up" position, their lower ends resting on intact beans, the resistance of the circuit is 4700 ohms. Each time a plunger drops, its magnet closes the adjoining reed switch, shorting out its 47-ohm resistor and reducing the total circuit resistance by an equal amount.

The resistance is converted to a corresponding voltage by the circuit shown in Figure 1. Batteries, rather than an electronic power supply, are used because of their relative freedom from electrical noise. R_1 is used to adjust the output voltage to zero when all plungers are up. R_2 is adjusted to give an output of 1.0 volt when all plungers are down. The output is fed to a Varian Model G-14 recording potentiometer set for a sensitivity of 1 volt full-scale. Connecting the two 9-volt batteries in a bucking relationship permits the fall of each plunger to cause a nearly equal change in output signal across the full width of the chart, approximately 0.01 volt or about 0.05 in.

The record of a cooking run consists of a roughly S-shaped curve made up of steps. The operator chooses a suitable interval, e.g., 1 min on short runs to 15 min on very long ones, and counts the cumulative number of steps at the end of each successive interval. These numbers are then plotted on log-probability paper against the time at the mid-point of each interval. The best straight line is drawn through the points and

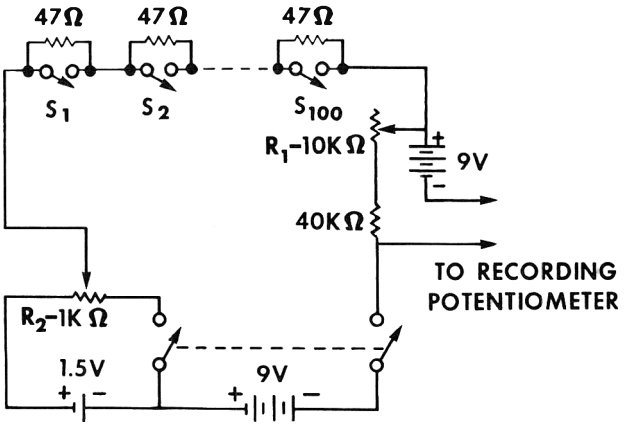

Fig. 1—Circuit diagram for automated bean cooker. S_1, S_2,...S_{100} are magnetic reed switches.

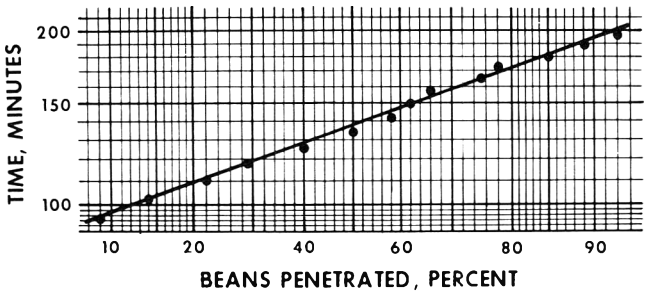

Fig. 2–Log-probability plot of a cooking run on slow-cooking beans. Median cooking time is 138 min.

the "median cooking time" is read off from this line. It is believed that the time derived in this way somewhat better characterizes the cookability of an entire sample than does that read from the S-shaped curve that results when the data are plotted on rectilinear paper.

Figure 2 is a plot of the data from a typical run on slow-cooking beans. 9% of the beans were penetrated by the end of the interval centered at 95 min and 92% had been penetrated in 203 min (199-min interval center). The median cooking time was 138 min.

As part of another study duplicate determinations of median cooking time were run on 23 different samples of beans that had been stored under various conditions. The values ranged from 23.0–201 min and the mean was 76 min. Analysis of the duplicate data gave a coefficient of variation (pooled standard deviation divided by the mean) of 4.3%.

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A Research Note

DEVICES FOR IMPROVING COLOR DEVELOPMENT IN GLYCOALKALOID DETERMINATION

ABSTRACT

An automatic shaking device was made for improving color development in glycoalkaloid determination. The device comprises two basic parts. The stationary part includes two kinds of special constant volume burettes which were calibrated to deliver 5 ml of concentrated sulfuric acid in exactly 3 min and 2.5 ml of 1% formaldehyde in exactly 2 min. The moving part consists of 12 reaction tubes mounted on a rocker plate which was coupled to a shaker with a connecting rod. Optimum speed of shaking was 4/sec. The device worked six times faster than that by conventional manual operation.

INTRODUCTION

SOLANINE, a major glycoalkaloid, occurs in potato tubers in variable amounts depending upon the variety, stage of development and the environmental conditions. The U.S. Standards for Potatoes issued July 15, 1958, defined greening as "damage" if more than 5% of the total weight of the potato must be removed to eliminate the greened tissue, and as "serious damage" if the loss is over 10%. Green potatoes contain an unusual amount of glycoalkaloids. Solanine and other glycoalkaloids are toxic to man and animals. Considerable literature exists citing cases of potato poisoning generally including gastrointestinal and neurological disturbances. Occasional mortality due to solanine or potato poisoning has been reported (Hansen, 1925). In 1970, the U.S. Dept. of Agriculture withdrew the "Lenape" cultivar of potato from commerce because of high glycoalkaloid content in the tubers. Recognition of the potential toxicity of glycoalkaloids in new potato varieties bred for disease resistance, high yield, better processing quality, and monitoring the toxic glycoalkaloids in green or mechanically injured potato tubers have increased the need for a simple, rapid procedure for routine analysis of total glycoalkaloids.

The commonly used method for glycoalkaloid determination was ammonia precipitation of glycoalkaloid, followed by color development of steroid (aglycone) with sulfuric acid and formaldehyde and spectrophotometric quantitation (Pfankuch, 1937; Lampitt et al., 1943; Dabbs and Hilton, 1953; Baker et al., 1955; Gull and Isenberg, 1960). The procedure for color development required drop by drop addition of 5 ml of concentrated sulfuric acid over a period of exactly 3 min, followed by like addition of 2.5 ml of 1% formaldehyde over a period of exactly 2 min with constant shaking in ice water bath. This is a time consuming process especially when there is a large number of samples. In this paper we describe an automatic shaking device with constant volume burettes which works much faster than the conventional manual operation. The device also provides uniform shaking which eliminates the possible error due to nonuniform shaking created by manual operation.

MATERIALS & METHODS

Constant volume burettes

As shown in Figure 1, both constant volume burettes were made by fusing of a top section of Pyrex test tube (16 mm x 60 mm) and a capillary tube (o.d. 7 mm, i.d. 1.5 mm, length 37 mm). Burette A was calibrated by adjusting the orifice of the capillary tube at 25°C, so that 5 ml of concentrated sulfuric acid passed through the burette in exactly 3 min. Burette B was also calibrated the same way, so that 2.5 ml of 1% formaldehyde passed through the burette in exactly 2 min.

Automatic shaking device

Figure 2 shows the automatic shaking and reagent delivering device for color development in total glycoalkaloid determination. The device consists of two basic parts, moving and stationary. The stationary parts consist of a stand for supporting 12 constant volume burettes and a stand for supporting a rocker plate with 12 reaction tubes and an ice

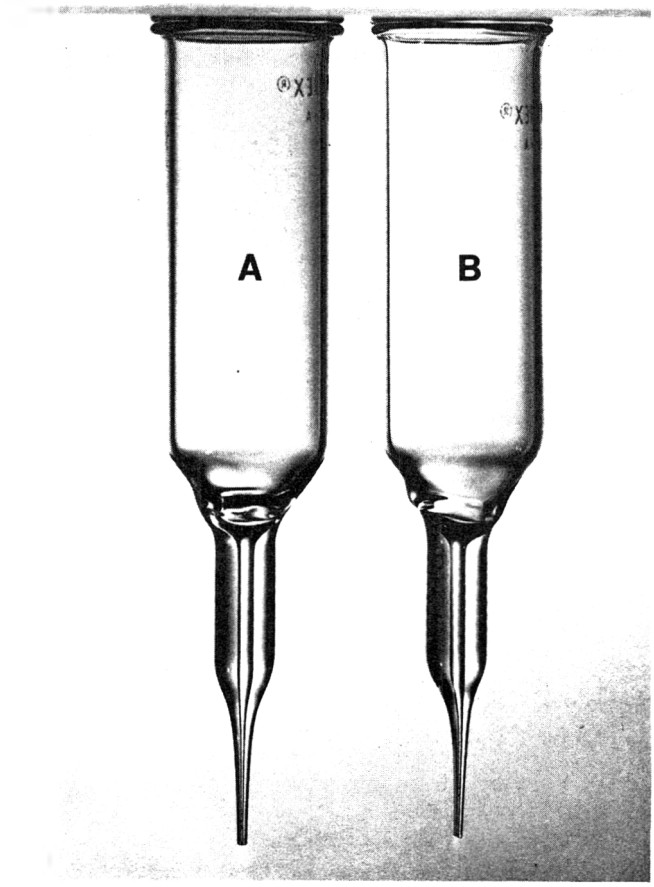

Fig. 1—Photograph of special burettes for delivering (A) 5 ml of conc sulfuric acid in 3 min and (B) 2.5 ml of 1% formaldehyde in 2 min for color reaction in total glycoalkaloid determination.

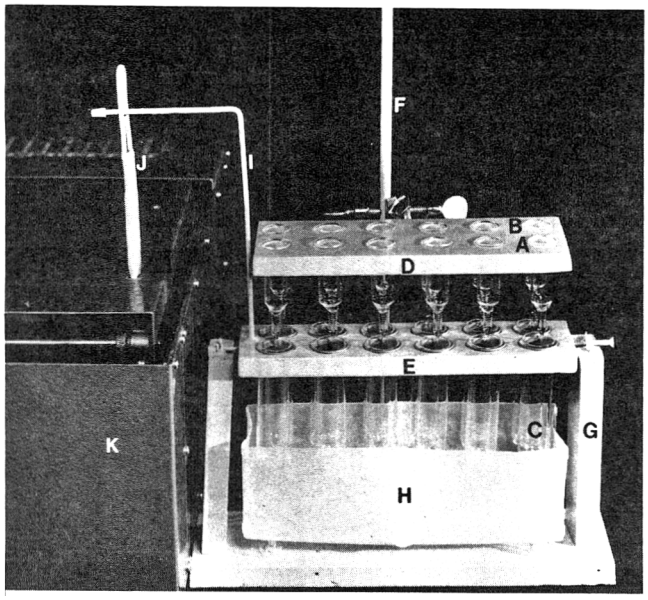

Fig. 2–Photograph of automatic shaking and reagent delivering device for color development in total glycoalkaloid determination. (A) special burette for delivering concentrated sulfuric acid; (B) special burette for delivering 1% formaldehyde; (C) reaction tube; (D) special burette holder; (E) reaction tube holder (rocker plate); (F) supporting stand for special burette holder; (G) supporting stand for rocker plate; (H) ice water bath; (I) connecting rod to shaker; (J) shaker coupler; (K) shaker.

water bath. Six burettes in the front row were used for delivering sulfuric acid, while those in the rear row were used for delivering formaldehyde solution. The plate holding the 12 burettes can be moved to the side when there is a need for changing reaction tubes. 50-ml glass centrifuge tubes with beak were used as reaction tubes. A metal connecting rod mounted on the rocker plate was mechanically coupled to a metal loop of shaker coupler which was mounted on a shaker. An "Eberbach" culture shaker was adopted in this device. The direction of shaking was perpendicular to the axis of the rocker plate. A shaking angle of 25° was applied in this device. The shaker was adjusted so that four shakings per second could be obtained.

Operation procedure

To use this device for color development, each reaction tube on the front row was filled with 2.5 ml of sample solution. The level of the ice water bath was adjusted so that the reaction mixture could be cooled properly. After turning on the shaker, each burette in the front row was filled with 5 ml concentrated sulfuric acid from a 50-ml regular burette. After all the sulfuric acid passed through the constant volume burettes, shaking was stopped. Reaction tubes in the front row were transferred to the rear row, and the front row was filled with six tubes of new samples. After turning on the shaker, each burette in the front row was filled with 5 ml of concentrated sulfuric acid and each burette in the rear row was filled with 2.5 ml of 1% formaldehyde from a 50-ml regular burette. After all the reagents passed through the constant volume burettes, tubes in the rear row were removed. This finished six samples.

RESULTS & DISCUSSION

THIS DEVICE was designed for operation with maximum efficiency by one person. By using this device for a large number of samples, the time required was only one-sixth of that by conventional manual operation. The optimum speed of shaking was four per second. At a speed slower than this, incomplete mixing of the reagent and sample occurred; at a faster speed the mixture spilled. Any shaker which can be adjusted to a speed of four shakings per second can be adopted to this device.

The use of special constant volume burettes had the advantage over that of regular burettes for delivering reagents over a specific time period. Accidental turning of the stopcock of the burettes resulted in changes of the rate of flow. In the case of these special burettes, the rates of flow will not be altered once calibrated and adjusted. Constant shaking of the reaction mixture by this device had the advantage over that of manual shaking which caused some differences in color development due to difference in mixing between samples. An additional advantage is the low cost of the device which can be built easily in the laboratory.

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Erratum Notice

● J. Food Sci. 40(4): 745–746 (1975), O.M. Batcher and P.A. Deary: "Quality characteristics of broiled and roasted beef steaks." On page 745, Materials & Methods, line 8: Change refrigerator temperature "30 ± 1°C" to read "3 ± 1°C." Also, in Table 1, page 746, there is a missing decimal point for electrical energy (kwh). Change "253b, 331c, 115a and 135a" to read: "2.53b, 3.31c, 1.15a and 1.35a." The correct figures make cooking meat much more reasonable costwise.