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Studies on Pasteurized and Commercially Sterilized Poultry Meat Bologna. Effects of Chopping Condition and Type of Meat

RAYMOND F. MAWSON, BYRON F. MILLER and GLENN R. SCHMIDT

ABSTRACT

Poultry meat bologna has been studied under two systems of heat processing, pasteurizing and commercial sterilizing in cans. Mechanically deboned turkey meat was chopped (1) from the tempered state, (2) after thawing, refreezing and tempering, (3) after thawing. Treatments 1 and 2 resulted in reduction in cooking loss, while treatment 1 gave a firmer textured product, as measured by shear value but not by taste panel. Turkey meat was compared with spent Leghorn laying fowl meat, and mechanically deboned meat with manually deboned meat. Bologna made from mechanically deboned laying fowl had the highest cooking loss. Laying fowl bologna was firmer textured than turkey bologna, and manually deboned meat bologna was firmer than MDM bologna by both shear value and taste panel measuremnts.

INTRODUCTION

THE FUNCTIONAL CHARACTERISTICS of whole poultry meat tissues and mechanically deboned poultry meat as they relate to emulsion type meat products have been the subject of three reviews (Cunningham and Froning, 1972; Froning, 1976; Randall, 1977). Many studies have evaluated the emulsifying capacity of uncooked poultry materials, which is of doubtful value in relation to cooked product. In the present studies various aspects of poultry meat bologna processing are studied in relation to properties of the cooked product.

From consideration of inventory control and product quality it would be desirable if frozen mechanically deboned (MDM) poultry could be processed without thawing. The few studies that have been published on the effect of cutting temperature on emulsion type meat products are difficult to interpret as cutter temperature and cutting time effects are inevitably intermingled. The performance of an emulsion product appears to be related to the nature of the fat and the formation of a heat set protein gel matrix which can entrap fat particles (Lee et al., 1981). It is possible that specific surface effects between the fat and protein are also involved (Deng et al., 1981). The formation of the heat set protein gel is believed to depend on the extraction of salt-soluble protein, and the effects of temperature on this have been studied in model systems (Trautman reported by Bard, 1965, Gillett et al., 1977). Trautman measured salt solubilized protein and reported maximal extraction from $-5°C$ to $-2°C$ for beef and $-5°C$ to $0°C$ for pork using a 3.9 percent NaCl extracting solution. Gillet et al. (1977) measured total protein extracted by a 7.5% NaCl solution and found a maximum extraction at $7.2°C$, averaging over three beef and two pork meats. Hargus et al (1970) chopped white and dark turkey meat emulsions to different end point temperatures and measured the solubilized protein. They found maximum solubilization at $1.7°C$, however, the reduced extraction at higher temperatures would have been influenced by a chop-

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ping time effect which they also demonstrated. Schut and Brouwer, cited in Schut (1976) reported a linear relationship between the lean meat precutting temperature and the ultimate emulsion stability when chopped to the optimum end point temperature. The effect seems to be related to the properties of the fat and/or cutting time rather than protein extraction. They found that cooling the precut lean to a fixed temperature before adding the fat and then cutting to the optimum end point resulted in uniform emulsion stability irrespective of the precutting temperature.

Several studies have indicated that cooling overheated emulsions after cutting to temperatures below those required for optimum emulsion stability, then reworking to the optimum end point temperature, restores much if not all of the emulsion stability (Helmer and Saffle, 1963; Brown and Toledo, 1975; Deng et al., 1981). The incomplete restoration of stability, reported in the latter two studies, suggests that some denaturation in the protein due to prolonged mechanical working may have occurred. It is believed the emulsion stability restoration phenomenon is related to the melting and dispersibility characteristics of the fat; when it becomes too fluid the emulsion loses stability (Schut, 1976; Lee et al., 1981). Consequently, softer lower melting point fats require lower end point cutting temperatures. Hargus et al. (1970) reported optimal end points of $1.7°C$ for dark turkey meat and $7.2-12.8°C$ for white turkey meat as giving the most stable emulsions with rendered chicken fat. In the light of these findings the first objective of these studies was to determine whether it was possible to prepare a stable bologna emulsion by cutting turkey MDM from the tempered state at a very low cutting temperatures.

Spent Leghorn laying fowl are a potential source of raw material for the manufacture of poultry bologna. There is some indication from emulsion stability and tensile strength measurements that fowl meat may possess superior functional properties to turkey meat (Baker et al., 1970). Processed turkey meats have become well accepted in the market place so the second objective of the present study was to compare fowl meat with turkey meat.

Cunningham et al. (1971) evaluated MDM from whole fowl carcasses and demonstrated the potential for this material in a variety of processed meat products. However, there is some doubt concerning the functional performance of this material. Froning (1970) concluded on the basis of histological evidence that there was less protein available

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RANDOMIZED COMPLETE              3 REPLICATES
BLOCK DESIGN            MECHANICALLY DEBONED TURKEY MEAT
                                        |
MAIN PLOT                   ┌───────────┼───────────┐
CUTTING CONDITION    TEMPERED  THAWED, REFROZEN  THAWED
                                    TEMPERED
                                        |
SPLIT PLOT _____|_____|_____|_____
                              |         |         |
                              ▼    ┌────┴────┐    ▼
SUB PLOT                           |         |
HEAT PROCESS              PASTEURIZED    STERILIZED
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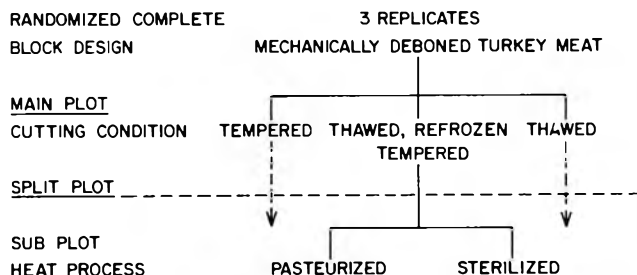
Fig. 1—Design for experiment to determine the effect of chopping condition on poultry meat bologna.

to form a protein matrix in MDM. He also compared the tensile strength and stability of cooked emulsions made from various mechanically deboned raw materials with emulsions made from hand deboned chicken. Using a variety of chopping conditions he found that hand deboned material gave more consistent results and better overall performance. However, he included egg white solids to act as a moisture binder in the products made from MDM so the comparison is questionable. Depending on the source of the MDM there can be a wide variation in the fat, protein and moisture composition (Froning, 1976) which can influence functional performance. The third objective of this study was to compare mechanically deboned meat with manually deboned meat as raw materials for poultry meat bologna under conditions of constant fat composition.

There have been relatively few studies which have evaluated emulsion stability during cooking or the cooked cohesive binding strength (Randall, 1977) and few if any of these studies have evaluated stability in terms of the severity of heat processing.

With the prospect that nitrite may eventually be excluded from use in processed meat products, and with rising energy costs and lack of consumer convenience making freezing an unattractive alternative means of enhancing shelf stability for a product such as bologna there is increasing interest in canning and pouch processing methods for obtaining adequate shelf life. Heat processing to commercial sterility could be expected to have some detrimental effect on product quality. The fourth objective of this study was to compare canned pasteurized poultry meat bologna with canned bologna processed to commercial sterility.

MATERIALS & METHODS

THIS STUDY involved two experiments. The first compared cutting; (1) tempered mechanically deboned turkey meat, (2) thawed mechanically deboned turkey meat, and (3) thawed, refrozen and tempered mechanically deboned turkey meat, as outlined in Fig. 1. The second compared spent Leghorn laying fowl with turkey and manually boned meat with MDM as shown in Fig. 2. The meat was cut from the tempered state. Both experiments compared bologna heat processed to pasteurizing standards with that processed to commercial sterility.

In both experiments the bolognas were made in 10 kg batches to the following specification: 18.2% fat, 10% added water (crushed ice), 1.8% salt, 100 ppm nitrite (as sodium nitrite), 300 ppm sodium erythorbate. Chicken fat was used to bring the fat content up to specification in the fowl meat bologna and turkey pouch skin was used in the turkey bologna.

Materials and formulation

The spent Leghorn fowl meats were obtained from a commercial source as frozen frames, frozen manually deboned dark meat, frozen manually deboned white meat and frozen chicken fat. The
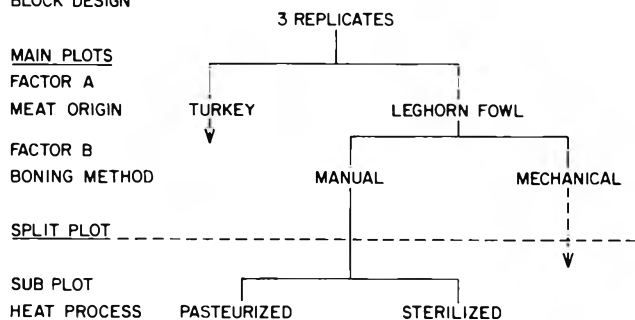
frozen frames were partially thawed then mechanically deboned with a Beehive Mechanical Deboner (Beehive Machinery Inc., Sandy, UT) and the meat product was chilled to 2°C by mixing with CO_2 snow before packing into cartons and freezing.

The turkey meats were obtained from a different commercial source as frozen manually deboned light and dark meats, frozen pouch skin and frozen mechanically deboned meat derived from turkey frames processed through a Yieldmaster deboner (The Kartridg Pak Co., Davenport, IA) and a scraped surface heat exchanger to cool the meat to 1.5°C before freezing.

Random core samples were taken from the frozen blocks using a 1.3 cm electric drill. The samples of each type of meat and fat were analyzed for moisture and fat by vacuum drying and solvent extraction (AOAC, 1970). The analyzes were used to calculate the meat and fat required to bring each type of bologna to the fixed formulation specification.

The frozen blocks were cut into approximately 500g chunks which were randomly assigned to three lots (replicates) and stored at −25°C. The period in frozen storage varied between 1 and 4 weeks depending on when the replicate in question was processed.

The tempered material for both experiments was held in a plastic tote box in a 4°C cold room for 18 hr and tempered to −3°C.

The material to be thawed was placed in a plastic tote box and held 18 hr at room temperature (20°C). The thawed refrozen and tempered material was completely thawed on a wire rack in a fan driven blast of room temperature air, recombined with the thaw drip, frozen in a 2–3 cm thick slab at −25°C overnight, and tempered for 4 hr at room temperature to −3°C. There was little if any weight change during this procedure indicating that evaporative moisture loss was balanced by condensative moisture gain.

Processing

The bologna was chopped in a pilot model Meissner silent cutter (RMF Steel, Kansas City, MO) to the following processing specification: knife speed 4,000 rpm (6 blades, 32 cm diameter), 60 bowl revolutions at 16.67 rpm (3.6 min), vacuum 0.78 bar. New factory sharpened knives were used. The temperature of the tempered material typically fell to −5°C then rose to −3°C during cutting. The temperature of the thawed material rose from 8.5°C to 13.4°C during cutting.

The bologna was stuffed into 301 × 401 cans and closed under vacuum at a gross closing weight of 556 ± 3g. The cans were divided into two batches, one for pasteurizing and the other for sterilizing and placed into two tote boxes filled with cold water in a 4°C cold room for a minimum of five hours to allow the cans to equilibrate to a common temperature.

Sample cans fitted with thermocouples to monitor the center temperature were used to verify the equilibration and to monitor the heat processing. The pasteurized cans were processed in 76.5°C water, stirred by bubbling air through it, to an end point of $F^5_{65.6} = 15$. The sterilized cans were processed in steam at 115.6°C to an end point of $F_o = 6.0$. The sterilizing process used in this study represents a processing extreme as nitrite containing products are usually processed to a shelf stable condition, $F_o = 2.0–2.8$ rather than to commercial sterility, $F_o = 6.0$ (Lechowich et al., 1978). It was of interest to heat process this material fully since if nitrite is ever excluded from use in processed meats, full heat processing would be required to obtain satisfactory shelf life.

The amount of processing was determined by the integral calculation method (Geankoplis, 1978) as processing proceeded using an appropriately programmed calculator. Processing was terminated and cooling started in time to give the desired end point. The sterilized cans were cooled under pressure. The cans were removed to the 4°C cool room once the center temperature dropped below 50°C and were held there for a minimum of 15 hr before analysis.

Product analysis

Cooking loss was calculated from the drained weight of the product after equilibrating to room temperature and slicing into four pieces and from the net weight of product before heat processing. Equilibration of the cooked product to room temperature allowed any expelled gel to melt. Gel pockets within the product were not common in any of the products. Fat expulsion was uncommon and then only in very small amounts. Measurements were duplicated.

Texture was evaluated by shear value and taste panel. The shear value was measured with a Warner Bratzler shear apparatus modified to increase its sensitivity by replacing the spring balance with a

RANDOMIZED COMPLETE
BLOCK DESIGN

3 REPLICATES

MAIN PLOTS
FACTOR A
MEAT ORIGIN   TURKEY   LEGHORN FOWL

FACTOR B
BONING METHOD   MANUAL   MECHANICAL

SPLIT PLOT _

SUB PLOT
HEAT PROCESS   PASTEURIZED   STERILIZED

Fig. 2—Design for experiment to determine the effect of meat type on poultry meat bologna.

strain gauge and recorder. Measurements were made on 2.54 cm diameter core samples of bologna. Eight measurements were taken per can and duplicate cans were sampled. The taste panel had 16 members who had been trained in a preliminary experiment. They were asked to rate texture on a continuous linear scale from mushy to rubbery and were instructed to mark the scale beyond these points if they felt the sample merited it. Intermediate points of soft, firm and chewy were indicated on the scale. The marks on the scale were converted to scores by measuring the distance from a base line a little below mushy (mushy = 0.6 cm, rubbery = 9.6 cm). The continuous open-ended scale was intended to ensure that the scores were always normally distributed.

The raw bologna batter was analyzed for pH, soluble protein, and protein sol. To measure soluble protein and protein sol, 5g of batter were diluted with 15 ml 2% NaCl using a Teckmar SDT homogenizer (18N head, 6 sec, 1,000 rpm, Tekmar, Cincinnati, OH) to disperse and mix the batter into the salt solution. The diluted batter was then centrifuged at 35,000 x g for 35 min to separate it into four layers; fat, solution, sol layer or K layer (Schut, 1976), and residue. The soluble protein in the solution layer was determined by micro-Kjeldahl analysis (AOAC, 1970). The protein sol layer was carefully removed and dried in a vacuum oven to determine dry matter (AOAC, 1970). The protein sol layer dry matter was expressed as a percentage of the whole batter dry matter. Preliminary tests revealed there was little difference in the soluble protein between centrifuging at 3,000 x g for 20 min and 35,000 x g for 35 min. The more vigorous centrifuging was required to compact the protein sol layer and enable separation from the solution layer. Examination of the protein sol layer by light microscopy revealed semiamorphous material, some myofibrillar fragments and a few small pieces of muscle fiber.

Statistical analysis

The experiments were analyzed by appropriate analyses of variance. It was found that the cook loss data differed greatly between the heat processing treatments, also the treatment response and variance differed so the pasteurized and sterilized cooking loss data were analyzed separately. Where interactions were found between subplots and main effects or between main effects the HSD test (Tukey's test) was used to determine whether the treatment effects were significant (Steel and Torrie, 1960). Where the analysis of variance revealed significant effects without interaction the least significant difference test was applied (Steel and Torrie, 1960).

RESULTS & DISCUSSION

TYPICAL heat penetration curves for the pasteurizing and sterilizing processes are given in Fig. 3. The functional performance of the bologna and raw batter data for the chopping condition experiment are illustrated in Fig. 4. The raw batter analysis failed to reveal significant differences between treatments for pH (mean value 6.23, SD 0.04) or soluble protein. The temperd product had more protein sol (P < 0.05) than the other treatments, Fig. 4, so the amount of protein sol formed may be reduced by thawing rather than chopping conditions.

There was significantly more cooking loss (P < 0.01) for the bologna made from thawed turkey MDM than from tempered or refrozen tempered MDM, for both the pasteurizing and sterilizing heat processes. Only a small amount of fat cooked out of the bologna made from thawed MDM and none from the other materials. More liquid was cooked out of the sterilized material than the pasteurized material.

Thawed MDM bologna and refrozen tempered MDM bologna had higher shear values (P < 0.05) than tempered bologna. The apparent shear value interaction in Fig. 4 between chopping treatment and heat process was not significant (0.05 < P < 0.1). The taste panel was unable to detect any textural effect due to chopping treatment, but was able to distinguish between the heat processes (P < 0.01), the pasteurized material being rated a little higher than firm and the sterilized material as soft. There was no interaction between chopping treatment and heat process.

In view of the marked difference in functional performance between chopping treatments in terms of cooking loss

and to a lesser extent, shear value, the lack of difference in soluble protein is surprising as it is generally believed that functionality is related to protein solubilization.

In practical terms the difference in cooking loss between the tempered material and the thawed material is very noticeable and would be of economic significance to the processor. The defects resulting from emulsion preparation procedures become more apparent with increased severity of the heat processing treatment. The pairing of the refrozen tempered cooking loss with the tempered cooking loss implies that the chopping treatment rather than deterioration as a result of thawing is determining the net improvement in cooking loss.

The taste panel results indicate the consumer may not detect the differences between chopping treatments in terms of texture, however, the taste panel data was the only data in this study to show a replicate effect and a larger panel may have been able to detect the differences.

It has been recommended that the end point temperature for processing poultry MDM emulsions should not exceed $7.5-12.8°C$ (Froning, 1970). The end point temperature of $13.4°C$ in the present study is high in relation to this. However, as the selection of temperatures by Froning (1970) and Hargus et al. (1970) does not preclude the possibility that $13.4°C$ would be a satisfactory end point, there seems little basis for concluding that the conditions in the present experiment were unduly harsh for the thawed chopping treatment. It has been reported on the basis of microscopic examination that only $1.5-3$ min chopping at temperatures below $12°C$ are required for complete emulsion development using mechanically deboned poultry (Angel et al., 1974). In the present study, the 3.6 min chopping time under conditions involving considerably more intense chopping action should have been more than adequate for emulsion development.

Applying separate regression analyses to the relationships between cooking loss and shear value for the pasteurized and sterilized products, it was found that shear value was independent of cooking loss for the pasteurized product and for the sterilized product it increases with increasing cooking loss by the relationship.

Shear value = $132 + 5.07x$, $R^2 = 0.65$, where x = cooking loss.

For the milder pasteurizing heat treatment, shear value is not related to the protein's ability to bind water. With the more severe sterilizing heat treatment, shear value is posi-

HEAT PENETRATION: TEMPERATURE HISTORIES AT THERMAL CENTER OF CANS
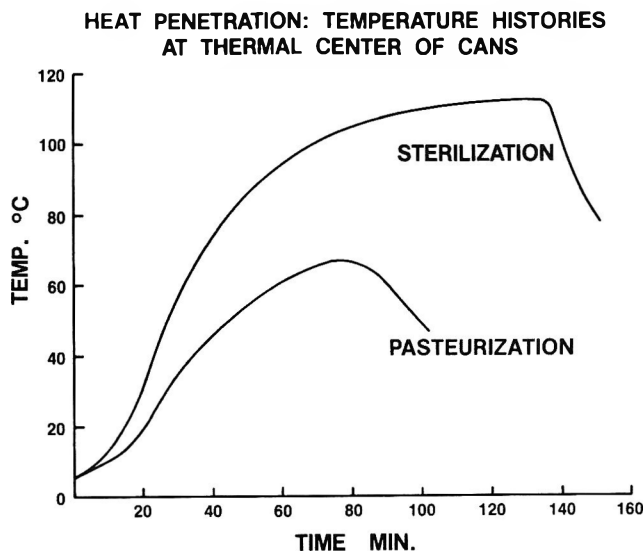
Fig. 3—Heat penetration characteristic for 301 x 401 can filled with poultry meat bologna. Pasteurizing temperature 76.7°C in stirred water. Sterilizing temperature, 115.5°C in steam.

tively related to product dehydration as much of the basic protein functionality has been destroyed.

In conclusion, it seems that not only is processing turkey MDM from the tempered state a practical proposition, it also offers distinct advantages in terms of improved functional performance. The machinery used in this study models larger scale production machinery so that this finding should be true in commercial practice.

The results for the experiment comparing different poultry meats are given in Fig. 5 and 6.

The same amount of protein sol was formed in the fowl bologna batters made from manually or mechanically deboned meat. As shown in Fig. 5, the manually deboned turkey batters formed less protein sol than did fowl (P < 0.05), while the turkey MDM formed more protein sol than did fowl (P < 0.05).

The pH values of the bologna batters made from manually deboned fowl and turkey were similar, while the pH values of the batters made from MDM were higher (P < 0.05). The fowl MDM batter had a higher pH than the turkey MDM batter (P < 0.05) (see Fig. 5). The higher pH of the mechanically deboned materials probably reflects the inclusion of bone marrow (Field and Arasu, 1981). Froning and Janky (1971) reported a small increase in tensile strength and a marked decrease in cooking loss in emulsions made from mechanically deboned turkey frame meat with an increase in pH similar to this.

The cooking losses for the sterilized bologna were generally about ten times higher than the pasteurized bologna [see Fig. 5 (i) & (ii)]. There was no difference in cooking loss between the manually deboned turkey, manually deboned fowl and turkey MDM for either the pasteurized or sterilized bologna. In the sterilizing process fowl MDM had a higher cooking loss than the manually deboned fowl and the turkey meats (P < 0.05). Fowl MDM had a higher cooking loss than turkey MDM in the pasteurizing process.

Fowl meat bologna shear force measurements were superior to turkey meat bologna (P < 0.05) in both the pasteurized and sterilized products [see Fig. 6 (iii)]. Manually deboned meat gave higher shear values (P < 0.05) than mechanically deboned meat. There was, however, more difference in the pasteurized products than the sterilized products (interaction, P < 0.05). The pasteurized manually deboned bologna shear values were equivalent to manually deboned sterilized bologna [see Fig. 6 (iv)].

Taste panel texture assessment could not distinguish between the pasteurized fowl or turkey bologna (very firm) but could distinguish (P < 0.05) between the sterilized fowl bologna (soft-firm) and sterilized turkey bologna (soft) [see Fig. 6(i)]. All pasteurized bolognas were superior to the sterilized bolognas (P < 0.05).

The pasteurized manually deboned bologna had firmer (P < 0.05) texture (firm-tough) than mechanically deboned bologna (firm). Sterilized manually deboned bologna was similar to the sterilized mechanically deboned bologna (slightly firm-soft) [see Fig. 6(ii)]. There was no interaction between poultry species and boning method for taste panel texture evaluation.

In the analysis of variance for taste panel textural evaluation the replicate effect was not significant and the within treatment variation (mean square) was small, indicating that the panel performed consistently with good consensus of evaluation. Replicate effects were absent for all of the other measurements as well, reflecting consistency in the raw material properties and processing procedures.

It appears that the textural firmness of spent laying fowl meat is superior to turkey meat and mechanically deboned fowl loses more moisture on cooking. These results do not appear to be related to pH differences in the manner suggested by Froning and Janky (1971) which indicates that intrinsic properties of the meat are involved.

The relatively inferior functional quality of the mechan-
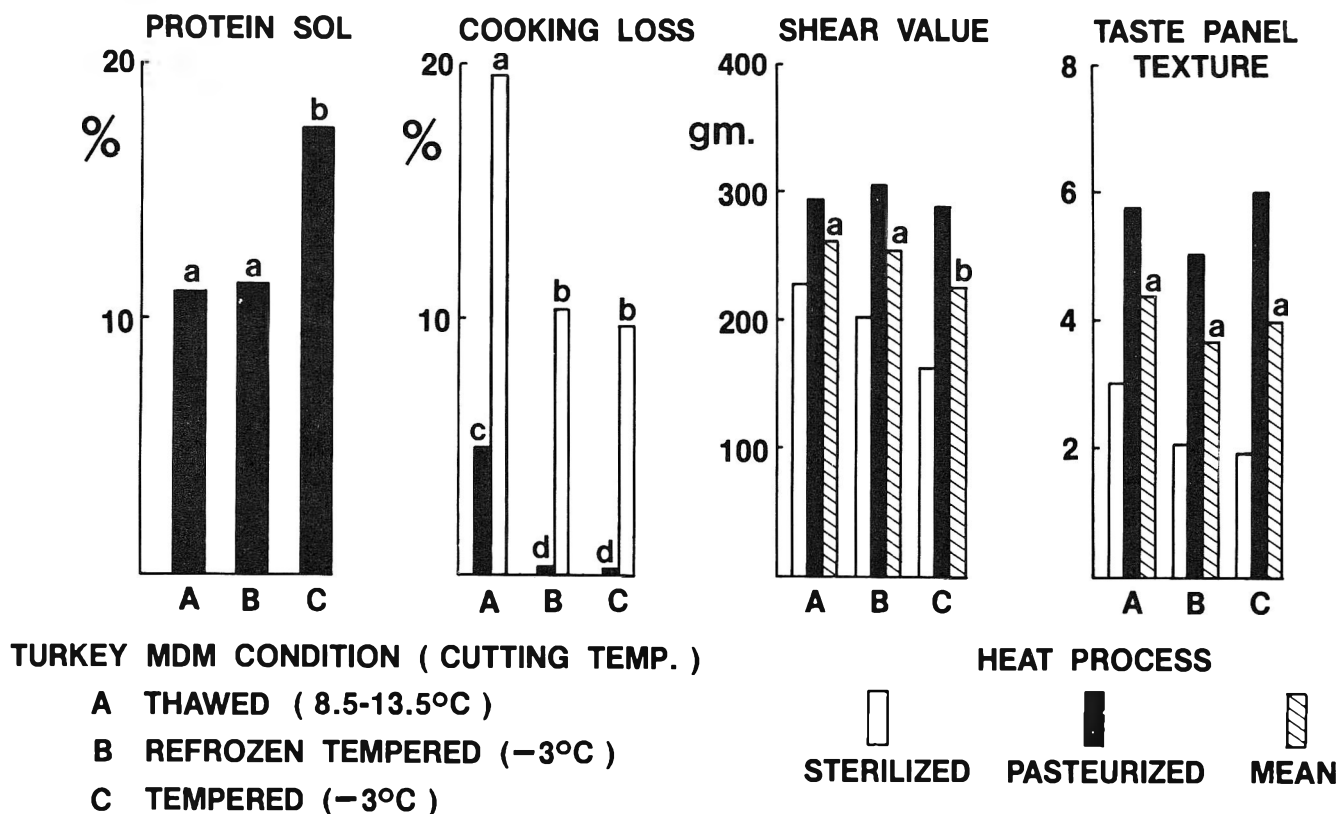
Fig. 4–Effects of meat condition during cutting on the properties of raw turkey MDM bologna batter (protein sol) and cooked turkey MDM bologna (cooking loss, shear value and taste panel texture) for pasteurized and sterilized products. Bars with different superscript letters are significantly different (P < 0.05).

(i) PASTEURIZED COOKING LOSS
(ii) STERILIZED COOKING LOSS
(iii) PROTEIN SOL
(iv) pH
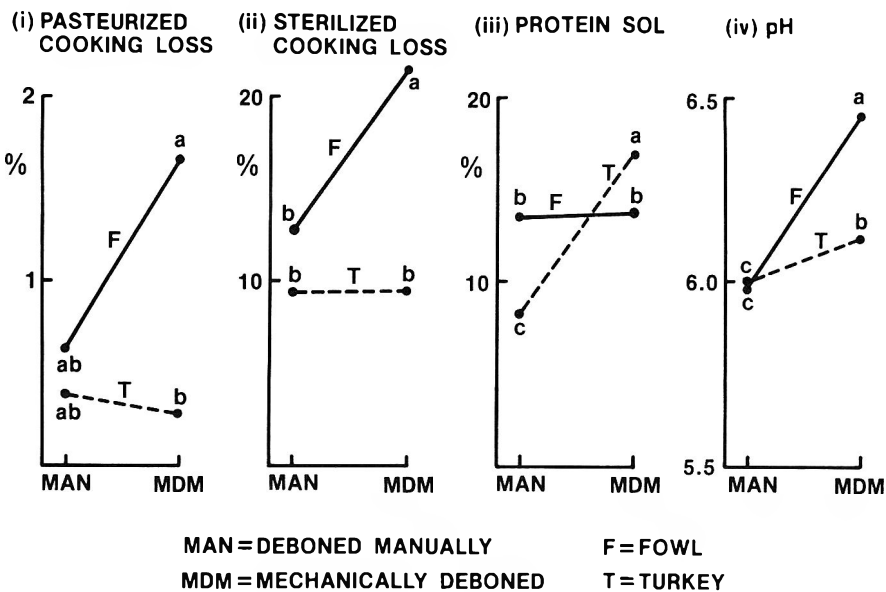
Fig. 5—Interaction diagrams for the effects of meat type and boning method on the cooking loss from pasteurized and sterilized poultry meat bologna, and the properties of the raw bologna batter (protein sol and PH).

MAN = DEBONED MANUALLY   F = FOWL
MDM = MECHANICALLY DEBONED   T = TURKEY

POINTS WITH DIFFERENT LETTERS ARE SIGNIFICANTLY DIFFERENT   P<0.05

(i) TASTE PANEL TEXTURE FOWL / TURKEY
(ii) TASTE PANEL TEXTURE MAN / MDM
(iii) SHEAR VALUE FOWL / TURKEY
(iv) SHEAR VALUE MAN / MDM

MAN = DEBONED MANUALLY   MDM = MECHANICALLY DEBONED
F = FOWL   T = TURKEY
P = PASTEURIZED   S = STERILIZED
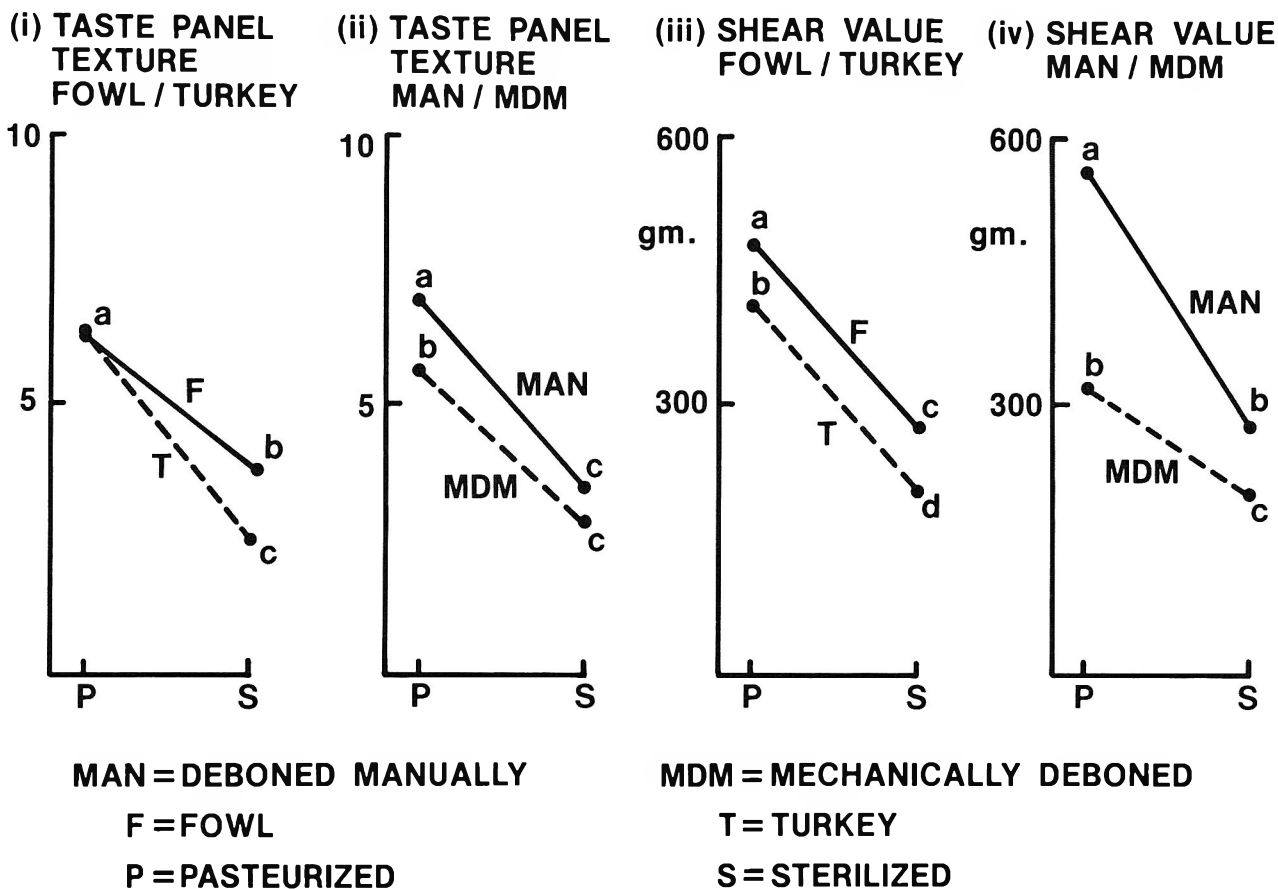
POINTS WITH DIFFERENT LETTERS ARE SIGNIFICANTLY DIFFERENT   P< 0.05

Fig. 6—Interaction diagrams for the effects of meat type on the textural characteristics of poultry meat bologna, measured by shear value and taste panel evaluation.

Studies on Pasteurized and Commercially Sterilized Poultry Meat Bologna. Effects of Nitrite Addition and Vacuum Cutting

RAYMOND F. MAWSON, BYRON F. MILLER, and GLENN R. SCHMIDT

———————————— ABSTRACT ————————————

Turkey bologna was prepared from mixed light and dark turkey meat and heat processed in cans under simulated commercial pasteurizing and sterilizing conditions. The bologna was prepared with and without added sodium nitrite and chopped in air or under vacuum. Nitrite and vacuum chopping had no effect on shear value, texture evaluation, pH, fat layer, soluble protein, residue or salt soluble protein. Nitrite addition increased cooking loss and protein sol. Vacuum chopping increased cooking loss and decreased the amount of protein sol. The pasteurized bologna had firmer texture and lower cooking losses than bologna processed to commercial sterility.

INTRODUCTION

THERE HAVE BEEN few studies on the influence of nitrite addition or vacuum chopping on the functional characteristics of meat in processed meat products. Randall and Voisey (1977) demonstrated that nitrite did not affect the texture, as measured by compression test, of ham and weiners. Swift and Ellis (1957) found that nitrite or nitrate alone had no effect on the tensile strength of a cooked all beef sausage emulsion. Wirth (1978) reported that vacuum chopping results in firmer textured emulsion products and improved emulsion stability.

It has been demonstrated that lipid oxidation may be detrimental to the functional performance of meat proteins (Fretheim and Gumpen, 1981; Miller et al., 1980; Arai, 1980; Shenouda, 1980). Nitrites are known lipid oxidation inhibitors (Waters, 1971; Igene and Pearson, 1979) and vacuum, by limiting the amount of oxygen available, also limits the amount of oxidation. If oxidation during processing is a significant factor in determining functionality then both of these treatments may result in improved functional performance.

In a recent review of the reactions of nitrite in meat (Cassens et al., 1979) it was observed that only a small proportion of the added nitrite (9–12%) was involved in binding to the meat pigment while approximately 25% was bound to the muscle proteins. It has been demonstrated that nitrite readily reacts with sulfhydryl groups in myosin. It may also react with proteins in other ways; by reaction with the ϵ-amino group of lysine, the terminal α-amino groups, the ring nitrogen groups of tryptophane and tyrosine and with the peptide bond itself. As these same protein groups are probably involved in heat set gel formation, nitrite could reasonably be expected to reduce the functionality of the protein by weakening the heat set gel, and may offset any advantage gained from inhibiting free radical attack on these groups as a consequence of lipid autoxidation.

Vacuum has been reported to enhance the extraction of salt soluble protein (Solomon and Schmidt, 1980) and may

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consequently improve functionality. In the practical situation this improvement may be difficult to observe. Chopping in air whips air bubbles into the emulsion which can decrease measurable cooking loss as they entrap exuded liquid (Wiebe and Schmidt, 1982). Generally the strength of rigid and semi rigid foams is related to density and the basic structural properties of the foamed material (Suh and Skochdopole, 1980). As more air is incorporated into the meat foam the textural strength could be expected to decrease. However, Booren et al. (1981) found that vacuum mixing had no effect on the tensile strength of restructured beef steaks.

In the present study the effects of nitrite and vacuum were studied in bologna made from manually deboned turkey meat and processed in cans to pasteurizing commercial and sterility standards (Mawson et al., 1983). Turkey was chosen as it was speculated that it may be very sensitive to lipid oxidation induced functional deterioration. Manually deboned meat rather than mechanically deboned meat (MDM) was used as it has been shown that mechanically deboned turkey is very susceptible to lipid deterioration (Dimick et al, 1972). It was feared that if mechanically deboned turkey was used the meat protein may have already deteriorated before the meat was processed. The meat was chopped from a tempered condition as it has been established that this improves its functional performance (Mawson et al., 1983).

MATERIALS & METHODS

Materials and formulation

Frozen light and dark boneless turkey meat and pouch skin were obtained from a commercial supplier. Random samples were taken with a 1.3 cm electric drill and analyzed for moisture and fat (AOAC, 1970). These analyses were used to determine the quantities of meat and skin required in each batch of bologna to meet the fat specification. The frozen meat and pouch skin were cut into approximately 500g pieces and randomly allocated to three lots (replicates). The light and dark meat was allocated so that each lot contained equal quantities of each type. Each lot was made into four batches of bologna, two of each of the following formulation specifications: (1) 18.2% fat, 10% crushed ice, 1.8% salt; and (2) 18.2% fat, 10% crushed ice, 1.8% salt, 100 ppm nitrite (as sodium nitrite) and 300 ppm sodium erythorbate. Prior to processing, the lot to be processed was tempered by holding in plastic tote boxes for 18 hr in a 4°C cold room.

Processing

The meat was chopped with a Meissner pilot model silent cutter (RMF Steel, Kansas City, MO) to a constant degree of mechanical working end point, as previously described (Mawson et al., 1983), except that one batch from each formulation was chopped without vacuum. The cans filled with bologna chopped under vacuum were closed under vacuum at a closing weight of 556 ± 3 g and the cans filled with bologna chopped without vacuum were closed without vacuum or head space at a closing weight of 552 ± 3 gm. Thermocouples were fitted to monitor the can center temperature during temperature equilibration and heat processing, which were done under the conditions reported previously (Mawson et al., 1983).

Product analysis

The heat processed bologna was analyzed for cooking loss, and shear value, and by taste panel for texture evaluation (Mawson et al., 1983). The raw bologna batters were analysed for pH, and centrifuged to yield the four component fractions; fat layer, protein solution, protein sol and residue (Mawson et al., 1983). Each fraction was separated and vacuum oven dried (AOAC, 1970) to determine dry matter, which was expressed as a percentage of the whole batter dry matter. The solution layer was analyzed for salt soluble protein by dialysing against 0.05M NaCl for 24 hr at room temperature, the insoluble protein was separated by centrifugation at 12,000 x g for 20 min then redissolved in 0.1N NaOH, 2% $NaCO_3$ solution, sampled and analyzed for nitrogen by micro-Kjeldahl analysis (AOAC, 1970). A factor of 6.25 was used to convert nitrogen to protein and the result expressed as a percentage of the total bologna batter dry matter. The specific gravity of the raw batters was also measured.

Statistical analysis

The experimental design is illustrated in Fig. 1. The pasteurized and sterilized cooking loss data were analyzed separately, for the reasons described in the earlier report (Mawson et al., 1983). Where interactions were found between subplots and main effects or between main effects the HSD test (Tukey's test) was used to determine whether the treatment effects were significant (Steel and Torrie, 1960).
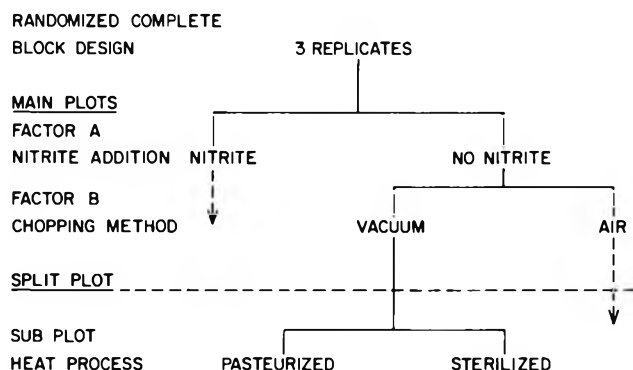
RESULTS & DISCUSSION

CHARACTERISTIC heat penetration curves for the pasteurizing and sterilizing heat processes have been given previously (Mawson et al., 1983).

From the analyses of the raw bologna batters no differences were observed for: pH, mean = 5.94 SD ± 0.08; fat layer, mean = 29% SD ± 4%; soluble protein layer, mean = 26% SD ± 1.4%; residue layer, mean = 26% SD ± 2.2%; or salt soluble protein, mean = 1.08% SD ± 0.06%. There was a difference between the specific gravity of the air chopped batters, mean = 0.939 SD ± 0.005, and the vacuum chopped batters, mean = 1.035 SD ± 0.007. The protein sol layer analysis is given in Fig. 2 (iii). The addition of nitrite increased the amount of protein sol formed (P < 0.05), and chopping under vacuum decreased it (P < 0.05).

The heat processed bologna results are given in Fig. 2 and 3. Nitrite addition and vacuum chopping increased the cooking loss (P < 0.05) in the pasteurized product. Nitrite addition in the sterilized product increased the cooking loss from air chopped bologna (P < 0.05) but had no effect on the vacuum chopped material. Vacuum chopping increased the amount of cooking loss (P < 0.05). Nitrite addition and vacuum chopping had no effect on shear value or taste panel texture evaluation. Sterilizing reduced the shear value and texture evaluation from very firm to soft (P < 0.05).

The taste panel texture evaluation analysis of variance showed no replicate effect and the mean square for variation within treatments was smaller than the error mean square indicating that the panel was consistent and reached a high level of concensus. There was no replicate effects for any of the other measurements except for the fat layer and residue (P < 0.05). This was caused by the first replicate having a lower fat content than the other replicates.

Fig. 1—Experimental design to determine the effects of nitrite addition and vacuum chopping on poultry meat bologna.

Fig. 2—Interaction diagrams for the effects of nitrite addition and vacuum processing on the cooking loss from pasteurized and sterilized turkey meat bologna and the protein sol content of the raw batter.
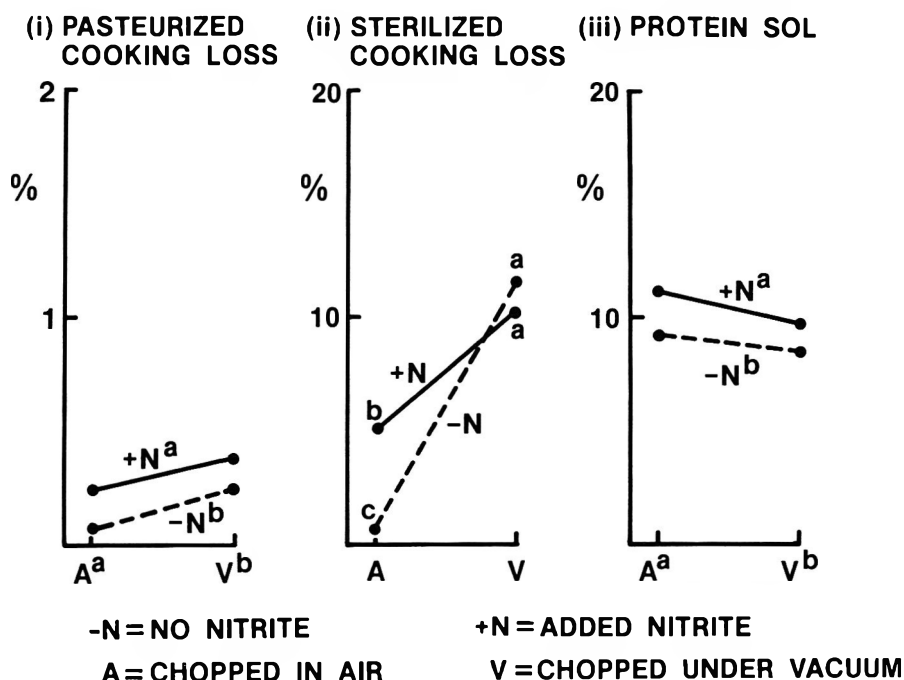
POINTS OR FACTORS WITH DIFFERENT LETTERS ARE SIGNIFICANTLY DIFFERENT   P<0.05

(i) TASTE PANEL TEXTURE NITRITE **(ii) TASTE PANEL TEXTURE VACUUM** **(iii) SHEAR VALUE NITRITE** **(iv) SHEAR VALUE VACUUM**

-N = NO NITRITE   +N = ADDED NITRITE

A = CHOPPED IN AIR   V = CHOPPED UNDER VACUUM

P = PASTEURIZED   S = STERILIZED
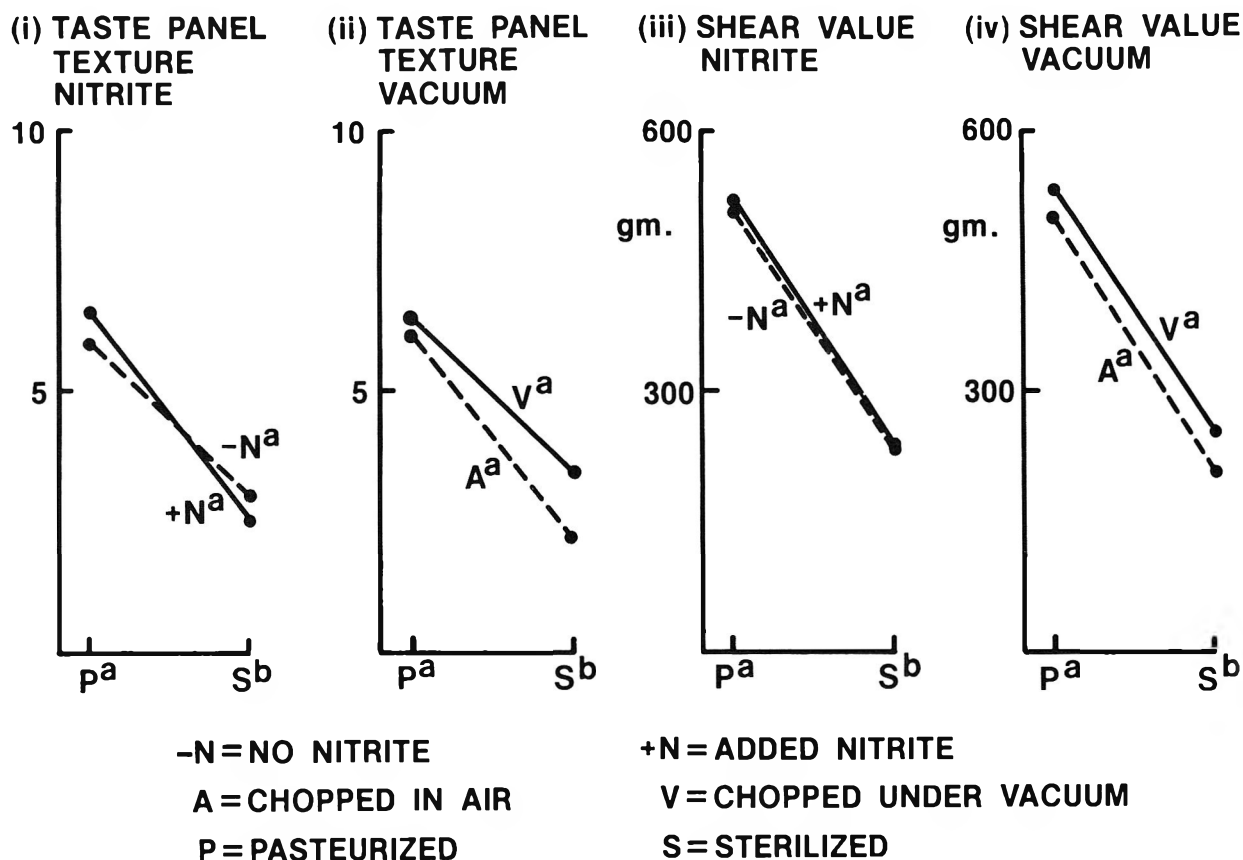
FACTORS WITH DIFFERENT LETTERS ARE SIGNIFICANTLY DIFFERENT $P < 0.05$

Fig. 3—Interaction diagrams for the effects of nitrite addition and vacuum processing on the textural characteristics of turkey meat bologna as measured by shear value and taste panel evaluation.

A difference in texture between the vacuum and air chopped bologna would have been expected from the specific gravity measurements. This difference was not apparent in either of the measurements of texture. In both sets of data there were strong factors by replicate interactions within the error term indicating there were uncontrolled elements in the experiment which strongly influenced the treatment effects. Two possible elements may have been the length of time in frozen storage and the time/temperature history of the meat prior to freezing. Aside from these considerations it appears that either the textural character of the meat emulsion is not greatly changed over the density differences observed in this study or the difference may be offset by an increase in the protein gel strength caused by chopping in air.

The addition of nitrite had no effect on shear value or taste panel evaluation, which agrees with the observation of Randall and Voisey (1977) on the effect of nitrite on the compressive strength of ham and weiners. However, nitrite addition increased cooking loss indicating some loss of functional performance. The increase in protein sol with nitrite addition is probably the result of the weakening of protein structures by reaction with nitrite.

The decrease in cooking loss caused by air chopping reflects the tendency for expelled liquid to become entrapped within the air foam. A similar effect was reported by Wiebe and Schmidt (1982) in comparing vacuum and air mixing.

There was no obvious connection between any of the raw batter measurements and functional performance. This was either because no relationship existed or there was very little difference in functional performance.

Any effect of oxidation due to chopping in air on the cooked bologna measurements has been masked by foam formation and nitrite damage to the proteins.

In conclusion it seems that both nitrite and vacuum have only a small influence on the functional properties of poultry meat which is probably not of significance to the meat processor.

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POULTRY MEAT BOLOGNA — CHOPPING & MEAT TYPE . . .

ically deboned meat may be due to localized heating during bone prebreaking and deboning (both of the systems used in this study use prebreakers and auger and screen type meat separation) or to the inclusion of bone marrow with its high heme pigment concentration.

The reason for the superiority of fowl meat over turkey meat in terms of shear value and texture may be due to inherent differences between the species and/or the spent laying fowl being more mature birds at slaughter than the turkey.

The pasteurized products were better in terms of cooking loss, shear value and texture evaluation than the sterilized products. The cooking loss variability and differences between boning methods for fowl were strongly enhanced by the more severe sterilizing heat process. This effect was not observed with shear value measurement or texture evaluation. The results imply that spent fowl, either manually or mechanically deboned could be a useful functional adjunct when mixed with other meats in processed meat products. However, the high moisture losses from mechanically deboned fowl would have to be reduced by appropriate formulation. A clear relationship between soluble protein or protein sol, and functional performance failed to emerge from these studies.

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Microbial Growth on Fat and Lean Surfaces
of Vacuum-Packaged Chilled Beef

F. H. GRAU

ABSTRACT

Brochothrix thermosphacta, Enterobacteriaceae and the Pseudo-monas-Moraxella group grew more rapidly on the fat than on the lean surfaces of chilled vacuum-packaged beef striploins of pH 5.45–5.85. On the fat, both B. thermosphacta and Enterobacteriaceae grew to outnumber the Pseudomonas-Moraxella. When Enterobacteriaceae and B. thermosphacta were present initially in about equal numbers, Enterobacteriaceae outgrew B. thermosphacta on both the fat and the lean surfaces of packs stored at 5°C. At 1°C the growth rate of the Enterobacteriaceae was greatly reduced. As a result, on the fat at 1°C, B. thermosphacta grew considerably faster than did the Enterobacteriaceae.

INTRODUCTION

WHILE LACTIC ACID BACTERIA dominate the microflora of fresh meat which is vacuum-packaged and stored at chill temperatures, facultative anaerobes such as Brochothrix thermosphacta and fermentative gram-negative bacteria often form a significant proportion of the total population (Seideman et al., 1976; Newton and Rigg, 1979; Shaw et al., 1980). On low pH lean (pH 5.4–5.6) anaerobic growth of these latter organisms is inhibited by the lactic acid content of the tissue (Campbell et al., 1979; Grau, 1980; 1981). However, on adipose tissue removed from such low pH lean, B. thermosphacta and psychrotrophic Enterobacteriaceae are able to grow rapidly, and with little lag, whether incubated aerobically or anaerobically (Egan and Grau, 1981; Grau, 1981).

These results suggest that within packs of vacuum-packaged meat there are likely to be several ecologically different environments, and that B. thermosphacta and Enterobacteriaceae will grow more readily on adipose tissue than on lean tissue of low pH. No information is available in the literature which would allow this postulate to be evaluated.

This study demonstrates that there are significant differences in the pattern of growth of B. thermosphacta and of Enterobacteriaceae on the adipose and lean tissues of vacuum-packaged chilled beef.

MATERIALS & METHODS

Meat packaging

Beef striploins (M. longissimus dorsi) with the overlying fat intact were obtained from a commercial boning room 1–3 days after slaughter, and each striploin was cut into six portions, each 600–700g. In order to spread the naturally acquired contamination as evenly as possible, the surfaces of the pieces were rubbed together, and the pieces were then dipped into 1 liter of distilled water. They were then dried with sterile cloths. Each portion was then placed in a plastic bag (Barrier bag, W gauge; W.R. Grace, Australia) which was evacuated and heat-sealed (Supervac GK170; Kieteubl and Assler OHG, Austria; 40 sec. evacuation cycle). The bags were formed from film composed of a layer of polyvinylidene chloride coated on both sides with ethylene-vinylacetate copolymer. The nominal oxygen transmission rate was about 30 ml/m^2 per 24 hr per 101 kPa

measured at 25°C and 75% relative humidity. Measured thickness of the film was 74 μm mean (range 63–85). The vacuum-packaged portions of meat were stored with the fat side up, and on wire-mesh racks for up to 34 days in a room maintained at 5($\pm$ 0.5)°C, or 1°C. The air temperature was monitored every 4 hr.

Inoculation

In some trials (indicated in the text) the meat pieces were inoculated with Serratia liquefaciens. This organism was grown aerobically in 8 ml of nutrient broth no. 2 (Oxoid) supplemented with 0.3% yeast extract (Oxoid) in a 125-ml side-arm flask incubated in a Gyrotory water bath shaker (New Brunswick Scientific Co., N.J.) at 5°C and 200 rpm. When growth reached a turbidity of 50 Klett units (Klett-Summerson colorimeter, no. 66 filter), 0.25 ml of the culture was added to the 1 liter of distilled water in which the meat pieces were dipped.

Determination of viable count

The outside of the film containing the vacuum-packaged meat was wiped with ethanol which then was allowed to evaporate. With a cork-borer, 4 samples (each 5 cm^2 x ca 4 mm deep) were taken through the film into the fatty tissue, and four samples into the lean. The separate lean samples and fatty tissue samples (including the film) were excised and blended with 90 ml 0.1% peptone water. From appropriate dilutions in 0.1% peptone water, 0.1 or 0.2-ml aliquots were spread on the surface of ATP agar (Difco), MacConkey agar no. 3 (Oxoid), MRS (de Man, Rogosa, Sharpe) agar (Oxoid), streptomycin thallous acetate actidione agar (STAA; Gardner, 1966) and peptone agar. The last medium gave good growth of Enterobacteriaceae, pseudomonads and moraxella-like organisms, while restricting the growth of B. thermosphacta and of the lactic-acid bacteria. It was composed of 0.8% peptone (Oxoid) and 1.2% agar no. 3 (Oxoid) dissolved in a mineral-salts medium (half strength 56 medium, Monod et al., 1951).

Plates were incubated at 25°C for 3 days (4 days for MRS agar) and counted daily. Plates of APT and peptone agars were also incubated at 5°C and counted at both 14 and 28 days. From each medium 5–10 colonies of each colonial type were isolated onto tryptone soya agar (Oxoid) supplemented with 0.5% yeast extract (Oxoid) and 0.2% glucose (TYSG). These isolates were examined for their growth responses on MRS, STAA, and MacConkey agars at 25°C, and on TYSG and APT at 5°C. The isolates were further tested for catalase, oxidase (Collins and Taylor, 1967), motility, oxidative-fermentative utilization of glucose, and their morphology was examined. Some isolates identified as members of the Enterobacteriaceae family were further examined using the API 20E Enterobacteriaceae kit, with incubation at 25°C. After colonies had been isolated from peptone agar plates, the plates were flooded with oxidase reagent (Edwards and Ewing, 1972) to obtain an estimate of the count of oxidase-positive bacteria.

pH measurement

Ten grams of lean tissue was blended with 90 ml of distilled water and the pH of the macerate measured with a Radiometer TTT 1C pH meter fitted with a GK 2302 C Radiometer combination electrode.

RESULTS

WHEN PORTIONS of a naturally contaminated striploin (pH 5.5–5.6) were vacuum-packaged and stored at 5°C, there were significant differences in the composition of the microflora which grew on the lean and fat surfaces (Fig. 1). Throughout the storage time, there were more lactic-acid bacteria on the lean than on the fat. In contrast, while

Formation of N-Nitrosamines in Gid-deed

Y. E. EL-MABSOUT, J. I. GRAY, B. ZYLEMA, and A. K. MANDAGERE

────────────ABSTRACT────────────

Gid-deed is a traditional Libyan food product which is prepared in most households by heavily salting lamb meat and then sun-drying. Nitrates and nitrites are not intentionally added to the product, although nitrate may be present as an impurity in the salt and thus incidentally added in the salting step. In this study, presumptive N-nitrosamine levels in gid-deed samples prepared with rock salt, refined salt, refined salt plus nitrite, and refined salt plus nitrate are reported. Similarly, four gid-deed samples prepared in Libya were also analyzed. The content of free proline and sarcosine was also determined since these amino acids have been implicated as precursors of N-nitrosopyrrolidine and N-nitrosodimethylamine, respectively.

INTRODUCTION

GID-DEED, a delicacy food product, is prepared traditionally in almost every household in Libya, especially on Aid Al-akbar (a religious day which coincides with the day Ibrahim, the prophet, was asked to sacrifice his son). Sheep, preferably yearlings are slaughtered, skinned and eviscerated. Part of the meat is used fresh on the same or the next day, while that remaining is deboned and heavily salted. The salted meat is sun-dried for 2–3 days, depending on the season. The sun-dried product is either stored in glazed clay containers at room temperature, or fried in olive oil, stored and used as a delicacy, when fresh meat is not available. The method was apparently developed to preserve the meat for later consumption. Today, refrigerators are available for the majority of households, but gid-deed is still used in the same manner.

Nitrates and nitrites are not intentionally added to the product; however, nitrate may be present as an impurity in the salt (Rubin, 1977) and is accidentally added in the salting step. This is supported by personal observations (Y.E.M.) of some gid-deed samples which have a bright red color when cooked or after oil-frying. No data are available regarding the nitrate content of the local salt or N-nitrosamine levels in the final product.

The major objective of this study was to investigate possible N-nitrosamine formation in fried and nonfried gid-deed samples produced under conditions similar to those encountered in the domestic preparation of gid-deed. In addition, four commercial samples were analyzed for N-nitrosamine content.

MATERIALS & METHODS

Gid-deed processing

Half of a lamb carcass weighing approximately 8 kg was obtained from a commercial meat distributor in the East Lansing area within 10 hr of slaughter. The carcass was cut into three sections, front leg, middle section and hind leg, and deboned. Each section was divided longitudinally into four equal pieces. Four representative samples (A, B, C, and D) were pooled from the three sections (1350g each). The meat in each sample was further sliced to about 2 cm in thick-

ness. The first sample (A) was heavily salted by rubbing an unknown amount of salt (Alberger Fine Flake, Diamond Crystal Salt Co., St. Clair, MI) on the meat slices until a satisfactory product was obtained. The amount of salt used (126g) was calculated by weighing the remainder of a preweighed stock. Samples B, C and D were treated as follows:

B, 126g refined salt + 120 mg/kg of nitrite;
C, 126g refined salt + 500 mg/kg of nitrate;
D, 126 g crude salt

The samples were hung on a rope in the open air in the sun for 3 days. Representative samples from each treatment were fried in olive oil to a medium "doneness" on a slow flame. The temperature of the frying oil was allowed to increase from room temperature to 190°C in approximately 7 min, after which the frying was discontinued. The fried gid-deed was removed from the oil and placed on a filter paper to remove excess oil. Both the salted-sundried and the fried samples were analyzed for the presence of N-nitrosamines.

Commercial gid-deed samples

Four gid-deed samples prepared under normal processing conditions in Libya were analyzed for the presence of N-nitrosamines.

N-Nitrosamine analysis

N-Nitrosamines were determined using the gas chromatography-thermal energy analyzer (TEA) method of Fine et al. (1975), as modified by Robach et al. (1980), with the exception that ammonium sulfamate (0.5g) was added to the distillation flask immediately before distillation commenced in order to prevent N-nitrosamine formation during the distillation step. The distillate was extracted with 3 x 25 ml aliquots of dichloromethane. The combined dichloromethane extracts were dried over anhydrous sodium sulfate and concentrated in a Kuderna Danish concentrating apparatus fitted with a 4 ml receiver. The concentrated sample (0.5 ml) was transferred to a 1 ml conical-shape vial with a Teflon-lined cap and stored in a freezer (−20°C) until required.

Quantitative determination of N-nitrosamines was carried out using a GC-TEA system comprised of a Varian 3700 gas chromatograph coupled to a TEA (Model LC, Thermo Electron Corp., Waltham, MA) via a 1/8″ glass-lined stainless steel transfer line. The GC column was a 2 m x 2 mm i.d. stainless steel column packed with 10% Carbowax 20M + 5% KOH on 80/100 mesh Chromosorb W (Supelco Inc., Bellefonte, PA). Operating conditions for the system were: GC carrier gas and flow rate, nitrogen at 30 ml/min; GC injection port temperature, 150°C; GC column temperature, 180°C isothermal; TEA pyrolyzer furnace temperature, 425°C; TEA reaction chamber pressure, 1.5 Torr; TEA attenuation, as appropriate; ice bath temperature, −160°C (isopentane/liquid nitrogen slush bath); GC-TEA heated transfer line, 175°C. The linearity of the response of the GC-TEA was established by injecting standard N-nitrosamines (Aldrich Chemical Co., Milwaukee, WI) over a wide range of 0.2–20 ng injected material. Raw data were collected and processed by a Hewlett Packard Model 3390A reporting integrator.

Percent recoveries of the N-nitrosamines from the gid-deed samples were determined by spiking known amounts of N-nitrosopyrrolidine (NPYR) and N-nitrosodimethylamine (NDMA) into the distillation flask containing 25g of fried fresh lamb. The fried lamb had been previously analyzed and found to contain no NPYR and NDMA. Average recoveries following distillation for the spiked samples were 82 and 92% for NPYR and NDMA, respectively.

Free amino acid analysis of the meat samples

Samples from the fresh salted-sundried lamb meat were analyzed for their free sarcosine and proline contents according to the procedure of Clark et al. (1966), as modified by Gray and Collins (1977). The meat samples were homogenized in a Waring Blendor with 1000

ml of 3% 5-sulfosalicylic acid solution and were immediately centrifuged for 10 min in Sorval ll super refrigerated centrifuge at 4000 rpm. The supernatant was removed and the residue was again extracted with 500 ml of the sulfosalicylic acid solution and centrifuged as before. The combined supernatants were freeze-dried and the residue dissolved in 100 ml of citric acid buffer (0.2M) as a pH of 2.1 The sample solution was extracted with 100 ml of n-hexane to remove residual lipids, filtered through a Millipore filter (0.2 μ) and stored at $-20°C$ until analysis. A Dionex amino acid analyzer fitted with a DC-4 resin (Durrum) cation exchange column (column bed 26 cm x 3 mm) was used for amino acid determination. Amino acids in the samples and reference amino acids (including proline) were eluted using a lithium citrate buffer system and reacted with ninhydrin to produce the colored chromogen for quantitation.

Nitrate analysis

Nitrite analyses were carried out according to the standard Association of Official Analytical Chemists (AOAC) procedure (1975), with the following modification: N-1-naphthylethylenediamine dihydrochloride was used to produce the colored chromogen instead of α-naphthylamine since the latter is a recognized carcinogen.

RESULTS & DISCUSSION

THE TRADIATIONAL METHOD of Gid-deed preparation and processing is somewhat like dry curing of ham and bacon. In both cases, dry salt is rubbed on the meat surface, and the final gid-deed product is cooked or fried in a similar manner to bacon.

N-Nitrosamine analysis of the salted sun-dried gid-deed samples and fried sample (Table 1) indicated that NPYR and NDMA were not present in the salted-sundried samples. However, oil frying of samples B and D (in which 120 mg/kg of nitrite and refined salt or rock salt were used, repectively) resulted in detectable levels of both NPYR and NDMA. The presence of these N-nitrosamines was not confirmed by mass spectrometry and should be reported with a qualification, i.e., presumptive or apparent N-nitrosamines based on retention data of unknown and standard N-nitrosamines (Havery et al., 1978). These preliminary results indicated that N-nitrosamine formation can be avoided by using refined salt in gid-deed preparation. Similar observations were made by Fong and Chen (1976) who reported that, when crude salt was replaced by refined salt and benzoic acid was added to control microbial growth, lower N-nitrosamine levels were detected in salted and dried marine fish.

The detection of N-nitrosamines in the rock salt-treated samples and their absence in the samples treated with refined salt and nitrate could be due to a number of factors including: ingoing nitrate level may have been higher in the rock salt-treated samples (unfortunately nitrate analysis of the rock salt was not carried out); the bacterial load of the rock salt may have been higher or more active in reducing nitrate to nitrite; and finally rock salt may contain other impurities that catalyze N-nitrosamine formation.

Nitrite analysis of the salted-sundried samples indicated that N-nitrosamine formation in the fried samples is correlated to the residual nitrite content. Both the nitrite and the rock salt-treated samples (B and D) had residual nitrite contents of 25 mg/kg (Table 2). The nitrate-treated sample (sample C) contained slightly less (18 mg/kg) residual nitrite after sun drying than either sample B (salt and nitrite) or sample D (crude salt). Neither N-nitrosamines nor residual nitrite were detected in sample (A) in which refined salt was used. Recent studies have indicated that it is the residual and not the initial nitrite level that influences N-nitrosamine formation in bacon (Dudley, 1979; Sebranek, 1979). It has also been noted that the higher the residual nitrite in bacon, the higher the possibility of N-nitrosamine formation (Sebranek, 1979). The present study indicated similar trends, i.e., when residual nitrite was detected N-

nitrosamines were produced, although the presence of 18 mg/kg of residual nitrite in the nitrate-treated sample did not seem to result in any detectable N-nitrosamines after frying.

The fact that N-nitrosamines were not detected in the unfried sundried samples indicated that their formation is associated with the high temperature of frying. In this respect, N-nitrosamine formation in gid-deed is quite similar to their formation in bacon, i.e. they are consistently detected in fried bacon but rarely in the raw product (Gray, 1981). However, the quantities of N-nitrosamines formed in gid-deed during oil frying are much less than those encountered in fried bacon. In bacon, the precursors of NPYR are located in the adipose tissue (Fiddler et al., 1974). Fat in the adipose tissue being an excellent heat transfer medium causes the internal temperature of bacon to increase rapidly towards the end of the frying period, and thus accelerates N-nitrosamine formation (Bharucha et al., 1979). On the other hand, lamb or mutton which is used in gid-deed, is much leaner than pork bellies, therefore a frying medium (usually olive oil) is used.

In this respect, gid-deed frying is similar to the frying of other cured meats such as ham. In these products, the internal temperature increases at a slower rate during the course of frying. This is also reflected in the levels of N-nitrosamine which are formed. The Nitrite Safety Council (1980) concluded that cured meat products such as cooked sausages, semi-dry and dry sausages, as well as fried slices of most dry cured hams and shoulders were essentially free of N-nitrosamines.

In order to substantiate the levels of presumptive N-nitrosamines in the gid-deed samples, four samples obtained directly from Libya were also examined for their N-nitrosamine content (Table 3). Results indicated the presence of small amounts of "presumptive" N-nitrosamines, particu-

Table 1—Presumptive N-nitrosamine levels (µg/kg) in raw (salted-sundried) and oil fried gid-deed

Treatment	Salted-sundried		Oil fried	
	NDMA	NPYR	NDMA	NPYR
A, refined salt	ND	ND	ND	ND
B, refined salt + nitrite	ND	ND	0.43	0.55
C, refined salt + nitrate	ND	ND	ND	ND
D, rock salt	ND	ND	0.30	0.33

ND = not detected; limit of detection, 1ng

Table 2—Residual nitrite (mg/kg) in raw (salted-sundried) gid-deed

Treatment	Nitrite level (mg/kg)[a]
A, refined salt	ND[b]
B, refined salt + nitrite	25
C, refined salt + nitrate	18
D, rock salt	25

[a] Average of triplicate determinations
[b] ND, not detected

Table 3—Presumptive N-nitrosamine levels in gid-deed samples prepared in Libya

Sample	N-Nitrosamine (µg/kg)[a]	
	NDMA	NPYR
1	0.14	—
2	0.10	—
3	0.86	3.1
4	1.70	0.65

[a] Average of two determinations

B. thermosphacta was somewhat more numerous on the lean than on the fat at the time of vacuum-packaging, within 3 days of storage the *B. thermosphacta* count on the fat was more than 100 times that on the lean. Over the remaining storage time, *B. thermosphacta* remained more numerous on the fat. On the fat surface, pseudomonads increased rapidly from an initial count of $10^2/cm^2$ to about $10^4/cm^2$ in 3 days, and then remained at about $10^4/cm^2$ for a further 31 days. On the other hand, pseudomonads grew more slowly on the lean from an initial count of $10^3/cm^2$ to about $3 \times 10^4/cm^2$. There is also evidence that *Enterobacteriaceae* grew more rapidly on the fat during the early stages of storage. At 15 days of storage, the count of *Enterobacteriaceae* was about $10^5/cm^2$ on the fat, but only $3 \times 10^2/cm^2$ on the lean. Subsequently, there was a rapid increase in the numbers of *Enterobacteriaceae* on the lean, so that by 34 days of storage the count was slightly higher

than on the fat. *Serratia liquefaciens* was identified as the predominant member of the *Enterobacteriaceae* on both lean and fat.

The numbers of *Enterobacteriaceae* naturally contaminating the meat at the time of vacuum-packaging were below detection ($< 20/cm^2$). In order to monitor the growth of such organisms during the early stages of storage, portions of a striploin (pH 5.45–5.55) were inoculated with *S. liquefaciens*, and then vacuum-packaged and stored at 5°C. The development of the microflora on lean and fat is shown in Fig. 2. Again, lactic-acid bacteria grew better on lean than on fat, although by the end of storage (32 days) the difference in count of lactic-acid bacteria on the two sufaces was not marked. On the fat there was a rapid initial growth of *B. thermosphacta*, *Enterobacteriaceae* and oxidase-positive bacteria (predominantly pseudomonads but including moraxella-like organisms). The count of *Entero-*
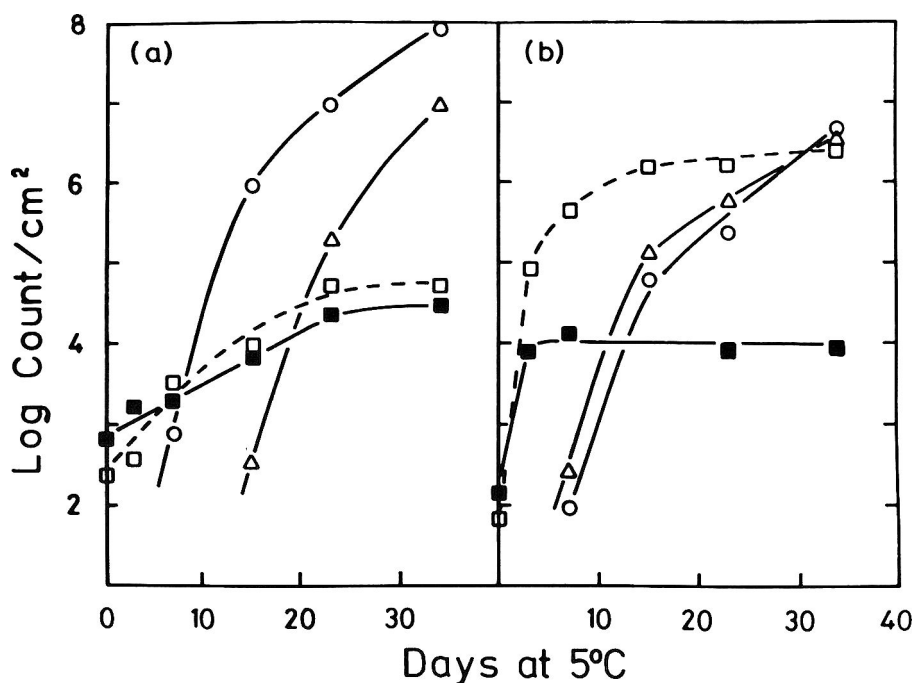
Fig. 1—Microbial growth on the lean (a) and fat (b) surfaces of a naturally contaminated striploin (pH 5.5–5.6) stored at 5°C: o——o lactic-acid bacteria; △——△ Enterobacteriaceae; □---□ B. thermosphacta; ■——■ pseudomonads.
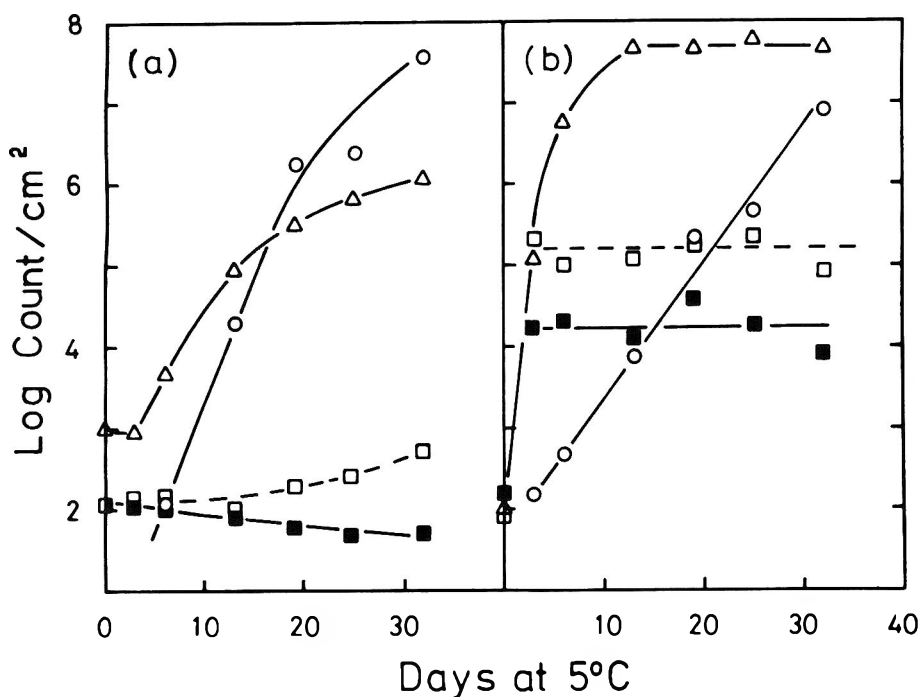
Fig. 2—Microbial growth on the lean (a) and fat (b) surfaces of a striploin (pH 5.45–5.55) inoculated with S. liquefaciens and stored at 5°C: o——o lactic-acid bacteria; △——△ Enterobacteriaceae; □---□ B. thermosphacta; ■——■ oxidase-positive bacteria.

bacteriaceae reached almost $10^8/cm^2$ after 13 days, while the numbers of *B. thermosphacta* and oxidase-positive organsims did not increase after 3 days of storage. On the lean, the growth of these three groups of organisms was much more restricted. There was no increase in the count of oxidase-positive organisms. *B. thermosphacta* grew only slightly during 32 days of storage. The *Enterobacteriaceae*, after a short lag, grew to about $10^6/cm^2$. From both lean and fat samples, two different colonial types of *Enterobacteriaceae* could be distinguished on peptone agar. Both types were identified as *S. liquefaciens*. At least one of these was probably a strain on the meat before inoculation.

In another trial, portions of a striploin of higher pH (5.75–5.85) were inoculated with *S. liquefaciens* and vacuum-packaged. To compare the effect of storage temperature on the development of the microflora, half the portions were stored at 1°C, and the remainder at 5°C (Fig. 3). At both storage temperatures, *B. thermosphacta*, *Enterobacteriaceae* and pseudomonads grew faster on the fat than on the lean. While the lower storage temperature reduced microbial growth rates on both surfaces, the greatest effect was on the growth of *Enterobacteriaceae*. When the count of lactic-acid bacteria on the lean reached about $10^7/cm^2$, there were about $10^7/cm^2$ *Enterobacteriaceae* at 5°C but only about $3 \times 10^4/cm^2$ at 1°C. Similarly, although not as dramatic, the slower growth of *Enterobacteriaceae* on fat at 1°C resulted in *B. thermosphacta* and lactic-acid bacteria forming a larger proportion of the population than at 5°C. Compared to growth obtained on lean of pH 5.5–5.6, growth of *Enterobacteriaceae* at 5°C was faster in this experiment where the pH of the lean was 5.75–5.85.

DISCUSSION

THE RESULTS presented here show that during the initial stages of chilled storage of vacuum-packaged beef, *B. thermosphacta* and *Enterobacteriaceae* grow more rapidly on the fat surfaces than on lean.

More oxygen would be expected to be available for microbial growth at the fat surface since lean tissue has a greater oxygen reducing ability than fat tissue (Ball and

Jungas, 1965). This is seen in the growth patterns of the strictly aerobic pseudomonas-moraxella group. In all experiments, this group grew very rapidly on the fat surface to reach a count of $10^4–10^5/cm^2$. When pseudomonads grew on the lean surface, growth was much slower. Since *Enterobacteriaceae* and *B. thermosphacta* have been shown to be able to grow anaerobically on fatty tissue (Grau, 1981; Egan and Grau, 1981), growth of these organisms can continue on the fat when the rate of oxygen diffusion through the film becomes insufficient for aerobic growth.

B. thermosphacta cannot grow anaerobically on lean when the pH is below about 5.8 (Campbell et al., 1979). The restricted growth of *B. thermosphacta* and of the pseudomonad group on the lean is therefore a reflection of limiting oxygen availability. The *Enterobacteriaceae*, however, grew to $10^6–10^7/cm^2$. This amount of growth suggests that these organisms were growing anaerobically on the lean. This differs from previous results (Grau, 1981) in which aerobically grown broth cultures of *Enterobacteriaceae* were unable to grow on lean (pH 5.4–5.6) when the lean was exposed to anaerobic conditions immediately after inoculation. Here the *Enterobacteriaceae* had the opportunity to grow in the presence of some oxygen and presumably adapt to the low pH and high lactate content of the lean before conditions in the vacuum-pack became essentially anaerobic. Indeed preliminary experiments have shown that, when *S. liquefaciens* is grown aerobically at 5°C on lean of pH 5.5–5.6 for 5 days and the incubation conditions are then made anaerobic, this organism will grow anaerobically. On the other hand, a change from aerobic to anaerobic conditions stops the growth of *B. thermosphacta* on lean of pH 5.4–5.6 (Campbell et al., 1979).

It is not clear what role the different growth pattern on fat and lean tissues in vacuum packages may have in spoilage. In these experiments, meat was not presented to a taste panel for evaluation of off-flavor. However, there was no obvious off-odor, or discoloration, at the time of microbiological sampling even when the count of *Enterobacteriaceae* approached $10^8/cm^2$ on the fat. *S. liquefaciens* appeared to be the major member of the *Enterobacteriaceae* on the fat. Patterson and Gibbs (1977) reported that *S.*
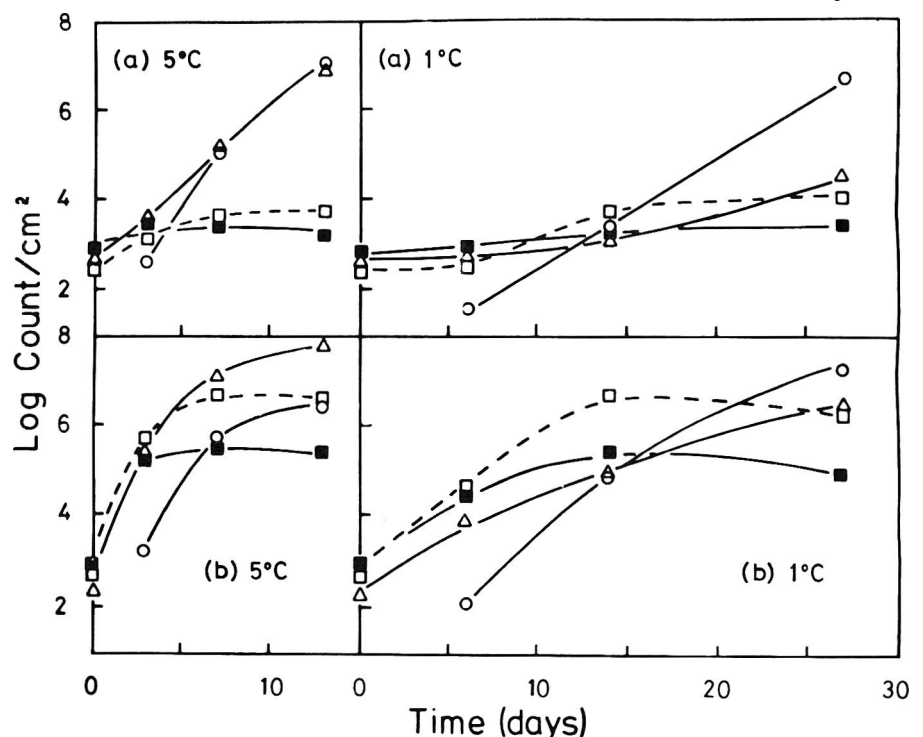
Fig. 3—Effect of storage temperature on microbial growth on the lean (a) and fat (b) surfaces of a striploin (pH 5.75–5.85) inoculated with S. liquefaciens: o——o lactic-acid bacteria; △——△ Enterobacteriaceae; □---□ B. thermosphacta; ■——■ pseudomonads.

Table 4—Free proline and sarcosine in fresh lamb and raw (salted-sundried) gid-deed

Sample	Free proline (μ moles/100g)	Free sarcosine (relative increase)
Fresh lamb	93.0	X
Raw gid-deed	265.6	4X

larly in samples 3 and 4, and generally confirmed the data obtained for the gid-deed samples prepared in our laboratory.

Precursors of NPYR and NDMA in fried gid-deed

Recent evidence suggests that N-nitrosation of proline to N-nitrosoproline followed by decarboxylation to NPYR is the most probable mechanism for NPYR formation in bacon (Gray, 1976; Lee, 1981). Results of the free amino analysis of the fresh lamb samples (Table 4) indicated that free proline was present at a level of 93.0 μmoles/100g of wet tissue. Baldwin et al. (1976) reported that the mean free proline content for 15 raw, deboned lamb legs was 0.28 mg/g (243.5 μmoles/100g) on a dry, fat-free basis or 0.077 mg/g (67.2 μmoles/100g) on a wet basis. They also did not detect free proline in either raw beef or pork longissimus muscles. Contrary to these findings, measurable quantities of free proline have been reported in pork bellies, ham and bovine muscles (Field et al., 1971; Lakritz et al., 1976; Gray and Collins, 1977). Salting and sun drying of the lamb samples for three days resulted in a three-fold increase in the free proline content (Table 4). Part of the increase in the free amino acid content is attributed to loss of moisture during the addition of salt and sundrying (approximately 40% of the fresh weight). Based on the original moisture content of fresh lamb, the free proline content was calculated to be 159.4 μmoles/100g of gid-deed. The remaining increase is probably due to protein hydrolysis caused by the proteolytic enzymes of the muscle or microbial degradation of proteins (Iodice et al., 1966; Parrish et al., 1969; Ayres et al., 1980). Aging of ham and refrigerated storage of pork bellies and beef muscles have been reported to increase the free proline content in these products (McCain et al., 1968; Bowers, 1969; Field et al., 1971; Lakritz et al., 1976).

Similar trends were observed for sarcosine, although no specific values for this amino acid in fresh lamb or gid-deed can be reported. This is due to the fact that in the ion exchange chromatographic analysis of free amino acids, no quantitative data were generated for sarcosine. However, relative peak areas of free sarcosine in fresh lamb apparently increased four-fold during salting and sun drying (Table 4). The role of sarcosine as a precursor of NDMA in cured meats has yet to be established.

This study has shown that gid-deed prepared with rock salt may contain small amounts of nitrosamines due to nitrate impurities in the rock salt. The levels present are approximately the same as those normally encountered in hams and other cured meat products such as frankfurters and bologna.

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Effects of Beef Carcass Electrical Stimulation, Hot Boning, and Aging on Unfrozen and Frozen Longissimus dorsi and Semimembranosus Steaks

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————————ABSTRACT————————

Ninety-six sides from 48 beef carcasses were used to study the effects of hot boning (HB), electrical stimulation (ES), a combination of electrical stimulation and hot boning (ESHB), and steak storage treatments on longissimus dorsi and semimembranosus steaks. Steak storage treatments were: unfrozen (aged until 6 days postmortem) or frozen (ES, HB, and ESHB frozen 24 hr and controls 48 hr postmortem). ES did not improve taste panel ratings or consistently lower shear force values when compared with control counterparts. Storing carcasses at 5°C for the first 24 hr postmortem and freezing ES steaks at 24 hr versus 48 hr postmortem for the control possibly diluted the effectiveness of ES. However, ES did eliminate any toughening due to HB.

INTRODUCTION

REMOVING BEEF MUSCLE before the onset of rigor mortis and chilling or freezing pre-rigor muscle too rapidly may result in cold-induced muscle toughening, "cold shortening," which may be accentuated in prerigor excised muscle (Locker and Hagyard, 1963; Chrystall, 1976). Because of this potential toughening effect, specific processing techniques must be utilized to provide a high quality hot-boned product.

Schmidt and Gilbert (1970) and Schmidt and Keman (1974) noted that the potential detrimental tenderness effects of hot boning at 1–2 hr postmortem can be overcome by conditioning (15°C for 24 to 48 hr) or conditioning (7°C for 4 hr) followed by aging (1°C for 8 days) of the hot-boned muscles. Follett et al. (1974) hot boned muscles at 1 hr postmortem and found that conditioning at 5°C for 24 hr followed by aging at 1°C for 3–13 day postmortem insured tenderness. However, Buchter (1977) found the storage of hot-boned cow muscles (excised at 1.5 hr postmortem) at 5–10°C for 24 hr to give unsatisfactory tenderness results. This was true even though the muscles were subsequently aged at 2°C for 2 wk. In a later study, Kastner et al. (1980) observed that triceps brachii and psoas major muscles excised 2 hr postmortem and stored at 1–5°C until 6 days postmortem were equal or better in tenderness than cold-boned controls but cautioned that this hot-boning methodology may not be equally applicable for all muscles. These studies indicate that the optimum conditioning or chilling temperature(s) and aging time(s) for the total spectrum of hot-boned muscles has not been fully investigated.

To increase processing efficiencies and facilitate continuous product flow, electrical stimulation (ES) can be incorporated into a hot-boning (HB) system. ES speeds the onset of rigor mortis (Will et al., 1979) so that carcasses may be hot boned and chilled soon postmortem without the need for muscle conditioning or aging to avoid prerigor excision and cold shortening effects.

Besides facilitating HB, ES also has been shown to increase tenderness characteristics of longissimus dorsi steaks from conventionally chilled carcasses (Savell et al., 1977, 1978a, b, 1979; Smith et al., 1977).

Our objective was to evaluate the effects of HB, ES, a combination of ES and HB techniques (ESHB), and steak storage treatments on the sensory characteristics and percentage thaw and cooking losses of longissimus dorsi and semimembranosus steaks. Because the chilling (5°C) of HB cuts might result in reduced tenderness, ES was combined with HB. Recognizing the potential of marketing centralized processed retail cuts in the frozen or unfrozen form, both steak storage treatments were also evaluated. HB and ES have been credited with allowing earlier processing of carcasses. Therefore, ES, HB, and ESHB steaks in the frozen storage treatment were frozen at 24 hr postmortem, whereas control counterparts were frozen at 48 hr postmortem.

MATERIALS & METHODS

FORTY-EIGHT STEERS were obtained from the Roman L. Hruska U.S. Meat Animal Research Center at Clay Center, NE. The steers were slaughtered in four groups (12 animals per group). Weight endpoints for slaughter groups 1, 2, 3, and 4 were 441, 494, 560, and 596 kg, respectively. The average quality grade was USDA high Good.

Postmortem treatments of sides and steaks

After slaughter, sides were randomly assigned to one of four carcass treatments:

Control (C). Sides were chilled at approximately 5°C until 48 hr postmortem. Longissimus dorsi (LD) from the anterior tip of the ilium through the 13th rib and semimembranosus (SM) muscles were excised 48 hr postmortem and cut into steaks.

Electrical stimulation (ES). ES was applied to sides 45 min postmortem. One stainless steel probe was inserted on the inside of the rear leg approximately 8 cm below the attachment of the achilles tendon and another was inserted laterally along the humerus. ES consisted of a pulsed (1.6 sec on, 0.8 sec off), 400 volt, 60 Hertz, and approximately 1 amp alternating current for 2 min. Sides were chilled at approximately 5°C until 24 hr postmortem, at which time LD and SM muscles were excised and cut into steaks.

Hot boning (HB). HB was performed on the LD and SM muscles 2 hr postmortem. Hot-boned muscles were loosely wrapped in oxygen-impermeable bags and chilled in wire trays at approximately 5°C until 24 hr postmortem at which time steaks were cut from the muscles.

Electrical stimulation and hot boning (ESHB). Sides were electrically stimulated as were ES sides and the LD and SM muscles were hot boned, chilled, and cut into steaks as previously described for HB.

Four steaks, 2.5 cm thick, were excised from each of the LD (anterior end) and SM (proximal end) muscles for taste panel and Warner-Bratzler shear force evaluations. Paired steaks used for taste panel and shear force analyses were assigned within an unfrozen or a frozen storage treatment. Frozen storage consisted of vacuum packaging the steaks and freezing them at −26°C immediately after removal (24 or 48 hr postmortem). Steaks assigned to the unfrozen storage treatment were vacuum packaged immediately after excision, aged at 2°C until 6 days postmortem, and then frozen and stored at −26°C. Thaw and cooking losses were obtained from steaks used for shear force evaluations.

Temperature declines

Temperature readings were taken at 2, 4, 6, 8, 10, and 24 hr postmortem. Thermistors were inserted approximately 5.0 cm into the center of the SM muscle (20.0 cm above the pubic bone) and into the center of the LD opposite the fourth lumbar vertebrae.

pH Declines

Core (1.27 cm) samples for pH determinations were excised from the LD (opposite the 5th lumbar vertebrae) and SM (2.5 cm above the aitch bone) muscles at 45 min (before stimulation) and at 2, 4, 6, 8, and 24 hr postmortem. One to two grams of muscle were blended with 10 ml of 5 mM NaIAc in 150 mM KCl (Bendall, 1973).

Taste panel, shear force, and thaw and cooking loss analyses

Maximum frozen storage period for the shear force steaks obtained from sides in the four slaughter groups was 50 days.

Steaks for taste panel evaluation were thawed at 2°C for 18 hr, trimmed to 0.6 cm subcutaneous fat thickness, and modified oven broiled in a gas rotary oven. Steaks were cooked at 163°C until an internal temperature of 70°C was reached. Temperatures were monitored by a Honeywell potentiometer according to the recommendations of AMSA (1978). After cooking, samples for taste panel analyses were removed by using a drill press unit equipped with a 1.27 cm coring device (Kastner and Henrickson, 1969). Cores were taken perpendicular to the steak surface and kept warm in egg poaching pans partially filled with warm water.

Taste panel evaluations for flavor, juiciness, myofibrillar tenderness, connective tissue amount, and overall tenderness on the LD and SM muscles were requested from an 8-member trained panel using an 8-point scale (8 = extremely intense flavor, juicy, tender, or no connective tissue; 1 = extremely bland flavor, dry, tough, or abundant connective tissue) for each response. Panelists were selected and trained according to procedures outlined in the AMSA Guidelines for Cookery and Sensory Evaluation of Meat (AMSA, 1978). Panelists were positioned randomly in individual booths equipped with red fluorescent lighting, served a sample, instructed to expectorate each sample, and rinse their mouth with water between samples. Eight samples were presented in a statistically randomized order, and no more than two sessions were conducted in a day.

Steaks for Warner-Bratzler shear force analyses were prepared in a slightly different manner. Before thawing at 2°C for 18 hr, steaks were removed from the vacuum package, trimmed to 0.6 cm subcutaneous fat thickness, and weighed. After thawing, the steaks were lightly blotted, reweighed, and cooked. Those weights were used in calculating the percentages of thaw, cooking, and combined losses. Cooking procedures were the same as previously described. Cooked steaks were allowed to cool at 21°C for 2 hr before coring and shearing (AMSA, 1978).

The steaks were cored as previously described. Six cores were removed from each steak and each was sheared once using the Warner-Bratzler shear apparatus.

Statistical analysis

The experimental design was a completely randomized block design with respect to assigning sides to carcass treatments. Data were analyzed by analysis of variance and means were compared by using the least significance difference method (Snedecor and Cochran, 1978). The analysis was performed by using the General Linear Model procedures in the Statistical Analysis Systems (Barr et al., 1979).

RESULTS & DISCUSSION

Taste panel and Warner-Bratzler shear force

Taste panel results for longissimus dorsi (LD) steaks (Table 1) from C, HB, and ESHB carcass treatments showed improved myofibrillar tenderness due to the unfrozen steak storage treatment (aged at 2°C until 6 days postmortem) when compared with the frozen storage treatment (HB and ESHB steaks frozen 24 hr and C steaks 48 hr postmortem). Unfrozen, aged HB steaks had less (P < 0.05) taste panel detectable connective tissue and significantly higher (more tender) myofibrillar and overall tenderness ratings than their frozen counterparts. Unfrozen, aged ESHB steaks responded similarly with the exception of taste panel ratings

for connective tissue amount. Unfrozen, aged steaks from all treatments had significantly smaller (more tender) shear force values than did frozen steaks. When significant differences were observed between the unfrozen and frozen storage treatments, the unfrozen treatment was favored. Such differences occurred most often in the HB and ESHB carcass treatments. Those various improvements can be explained by the increased aging period for the unfrozen steaks.

For both the unfrozen and frozen systems, LD steaks from the ES carcass treatment were generally comparable with those in the C treatment for all traits listed in Table 1. However, storing carcasses at 5°C for the first 24 hr postmortem and freezing ES steaks (frozen storage system) at 24 hr versus 48 hr postmortem for C steaks may have diluted the effectiveness of ES relative to C.

For unfrozen, aged steaks, less desirable taste panel detectable connective tissue amount ratings and shear force values (P < 0.05) were observed for the HB carcass treatment when compared with C counterparts. Within the frozen storage treatment, HB exhibited less desirable (P < 0.05) taste panel mean ratings for flavor intensity, myofibrillar tenderness, connective tissue amount, and overall tenderness and larger (P < 0.05) shear force values than did C counterparts. This indicates that aging during the unfrozen storage treatment alleviated some of the detrimental effects of HB at 2 hr postmortem.

Considering the tenderness indicating attributes in Table 1, ESHB was equal or superior to C within both the unfrozen and frozen storage treatments. ES alleviated any tenderness differences that existed between C and HB. This was particularly true for the frozen storage treatment. In addition to facilitating the hot-boning technique, ES exerted no detrimental effects on LD flavor and juiciness, which agrees with Gilbert and Davey (1976), Gilbert et al. (1977), Taylor (1979), Corte et al. (1980), and Kastner et al. (1980). Regardless of the steak storage treatment, ESHB gave equal

Table 1—Taste panel[d] and Warner-Bratzler shear force (kg) means for longissimus dorsi muscle by carcass and steak storage treatments

Steak storage treatments[e]	Carcass treatments				Std. dev. of means
	C	ES	HB	ESHB	
Flavor intensity					
Unfrozen	6.3	6.3	6.2	6.2	±0.07
Frozen	6.4[b]	6.2[ab]	6.0[a]*	6.2[ab]	±0.06
Juiciness					
Unfrozen	6.1	5.9	6.3	6.2	±0.10
Frozen	6.3	6.1	6.1	6.0	±0.10
Myofibrillar tenderness					
Unfrozen	6.5	6.6	6.2	6.4	±0.10
Frozen	6.1[bc]*	6.5[c]	4.9[a]*	5.9[b]*	±0.16
Connective tissue amount					
Unfrozen	6.9[b]	6.9[b]	6.5[a]	6.8[ab]	±0.08
Frozen	6.8[b]	6.9[b]	6.1[a]*	6.7[b]	±0.09
Overall tenderness					
Unfrozen	6.5[ab]	6.7[b]	6.1[a]	6.5[ab]	±0.09
Frozen	6.3[bc]	6.6[c]	5.0[a]*	6.0[b]*	±0.14
Warner-Bratzler shear force (kg)					
Unfrozen	3.3[a]	3.0[a]	3.8[b]	3.0[a]	±0.09
Frozen	4.0[b]*	3.3[a]*	4.6[c]*	3.5[a]*	±0.15

* Means within the same column for the same trait differ (P < 0.05).
[abc] Means within the same row with the same or no superscript do not differ (P > 0.05).
[d] Flavor intensity, juiciness, myofibrillar tenderness, connective tissue amount, and overall tenderness were evaluated on an 8-point scale (8 = extremely intense flavor, juicy, tender, or no connective tissue; 1 = extremely bland, dry, tough, or abundant connective tissue).
[e] Unfrozen = aged until 6 days postmortem; frozen = ES, HB, and ESHB frozen at 24 and C at 48 hr postmortem.

cr superior results when compared with HB for all the traits in Table 1.

Taste panel results for the semimembranosus (SM) steaks (Table 2) indicate no differences (P > 0.05) between the unfrozen and frozen storage treatments for all carcass treatments for flavor intensity and juiciness. When taste panel myofibrillar tenderness, amount of detectable connective tissue, and overall tenderness were considered, differences (P < 0.05) generally favored the frozen storage treatment. However, these differences were not supported by Warner-Bratzler shear force comparisons as no statistical differences were observed for shear force means between the unfrozen and frozen storage treatments for each carcass treatment.

Comparing C with ES within the unfrozen and frozen storage treatments revealed no differences (P > 0.05) for any of the traits listed in Table 2. The same trend was observed for the LD steaks (Table 1).

HB was equal (P > 0.05) to C for all traits when the unfrozen storage treatment was considered. These results agree with Schmidt and Keman (1974) and Follett et al. (1974) who observed that SM muscles hot boned as soon as 1 hr postmortem were as tender as control counterparts if conditioned at 5–7°C after excision then aged. However, for the same comparison within the frozen storage treatment, HB was scored significantly lower (less tender) for myofibrillar and overall tenderness than was C. As generally observed with the LD (Table 1), aging SM steaks at 2°C until 6 days postmortem minimized the undesirable effects of HB. These observations, along with shear force results make it difficult to rationalize why taste panel connective tissue amount, myofibrillar tenderness, and overall tenderness ratings occasionally favored the frozen storage treatment. However, Wu et al. (1981) observed that SM connective tissue solubility decreased with increased aging at 2°C.

Regardless of the SM steak storage treatment, ESHB was equal (P > 0.05) to C, agreeing with results of Gilbert and Davey (1976), Gilbert et al. (1977), Taylor (1979), and

Corte et al. (1980). In addition, the ESHB treatment within the unfrozen storage treatment improved upon the method of Kastner et al. (1980), who indicated that SM muscles from carcasses electrically stimulated at 1 hr postmortem, hot boned at 2 hr postmortem, and aged at 1–5°C until 6 days postmortem were less tender than control counterparts. This could have been due to our use of pulsed versus continuous stimulation and stimulating at 45 min rather than 1 hr postmortem. The differences between C and HB within the frozen storage treatment were negated by ES. Regardless of the steak storage treatment, ESHB gave equal or superior results when compared to HB for all traits given in Table 2.

pH and temperature declines

Fig. 1, 2, 3, and 4 illustrate pH and temperature declines for the LD and SM muscles by carcass treatments. It should be noted that ES was effective in speeding the rate of pH declines in ES and ESHB relative to C and HB muscles. C and ES exhibited similar chilling rates as did HB and ESHB muscles. C and ES muscles chilled somewhat slower than HB and ESHB counterparts. None of the muscles reached the cold shortening conditions of 10°C in less than 10 hr postmortem (Locker and Hagyard, 1963) or before pH 6.0 was reached (Chrystall, 1976). However, HB muscles excised at 2 hr postmortem and stored at 5°C were apparently responsive enough to cold-induced toughening to account for the general results of HB being less tender than C. Acceleration of the pH decline rates due to ES (Fig. 1 and 3) was apparently sufficient to alleviate any differences that existed between C and HB because of cold-induced toughening. Regardless of the muscle or steak storage treatment, ESHB was similar in tenderness to C. Even though the pH decline rate for ES muscles was faster than C (Fig. 1 and 3) both chilled at similar rates (Fig. 2 and 4) which should not have caused cold-induced toughening in either (Locker and Hagyard, 1963; Chrystall, 1976). This is particularly true for muscles restrained in the carcass until the ultimate pH is reached, as was the case for C and ES muscles. This may partially account for ES and C being similar for those traits evaluated in Tables 1 and 2.

Table 2—Taste panel[c] and Warner-Bratzler shear force (kg) means for semimembranosus muscle by carcass and steak storage treatments

Steak storage treatments[d]	Carcass treatments				Std. dev. of means
	C	ES	HB	ESHB	
Flavor intensity					
Unfrozen	6.1	6.0	6.0	6.1	±0.05
Frozen	6.1	6.1	6.0	6.1	±0.05
Juiciness					
Unfrozen	5.9	5.8	5.9	5.9	±0.07
Frozen	6.0	5.9	5.9	5.9	±0.09
Myofibrillar tenderness					
Unfrozen	5.8	5.8	5.6	5.8	±0.07
Frozen	6.3*[b]	6.2*[b]	5.6[a]	6.1*[b]	±0.08
Connective tissue amount					
Unfrozen	5.4	5.5	5.4	5.5	±0.07
Frozen	5.9*[ab]	6.1*[b]	5.8*[a]	5.9*[ab]	±0.05
Overall tenderness					
Unfrozen	5.5[ab]	5.6[b]	5.3[a]	5.6[b]	±0.07
Frozen	6.0*[b]	6.1*[b]	5.6*[a]	6.0*[b]	±0.07
Warner-Bratzler shear force (kg)					
Unfrozen	4.7[ab]	4.6[ab]	5.1[b]	4.5[a]	±0.12
Frozen	4.6[a]	4.9[ab]	5.4[b]	4.5[a]	±0.17

* Means within the same column for the same trait differ (P < 0.05).
[ab] Means within the same row with the same or no superscript do not differ (P > 0.05).
[c] Flavor intensity, juiciness, myofibrillar tenderness, connective tissue amount, and overall tenderness were evaluated on an 8-point scale (8 = extremely intense flavor, juicy, tender, or no connective tissue; 1 = extremely bland, dry, tough, or abundant connective tissue).
[d] Unfrozen = aged until 6 days postmortem; frozen = ES, HB, and ESHB frozen at 24 and C at 48 hr postmortem.
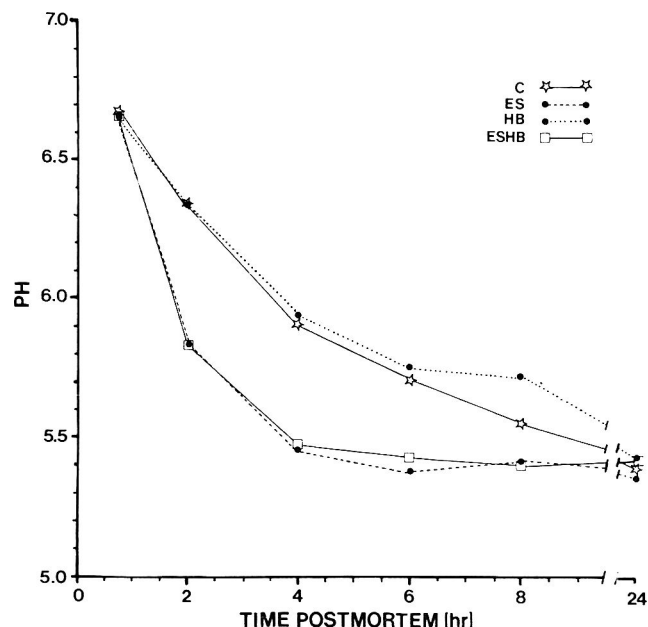
Fig. 1—Postmortem pH declines for the longissimus dorsi by carcass treatment.
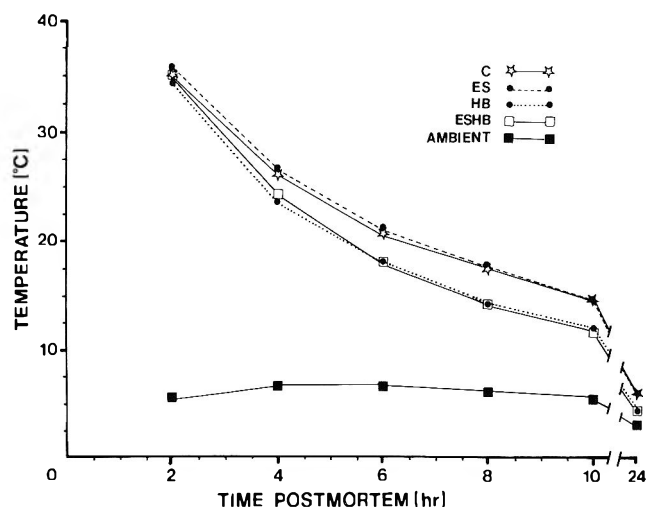
Fig. 2—Postmortem temperature declines for the longissimus dorsi by carcass treatment.

Fig. 4—Postmortem temperature declines for the semimembranosus by carcass treatment.
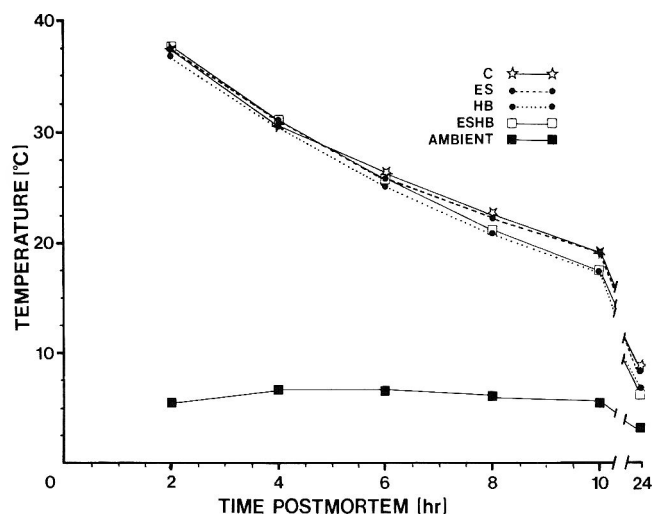
Fig. 3—Postmortem pH declines for the semimembranosus by carcass treatment.

Thaw and cooking losses

Data (Table 3) involving percentage thaw, cooking, and combined losses show few differences (P < 0.05). However, when statistical differences for percentage thaw, cooking, and combined loss means between LD and SM steaks stored unfrozen and frozen were observed, the unfrozen system was favored, particularly for the ESHB carcass treatment for both muscles.

CONCLUSIONS

NEITHER STEAK storage treatment had a distinct advantage over the other, but the unfrozen storage treatment may be somewhat superior, particularly when ES and HB are combined (ESHB).

ES neither improved taste panel tenderness ratings nor consistently lowered shear force values relative to C for either the LD or SM. However, had carcasses been chilled at temperatures lower than 5°C, ES might have been more beneficial as ES has been shown to alleviate the effects of cold-induced toughening.

Storage of HB muscles at 5°C until 24 hr postmortem followed by aging at 2°C until 6 days or freezing at 24 hr

Table 3—Percentage thaw, cooking, and combined loss means for longissimus dorsi and semimembranosus muscles by carcass and steak storage treatments

Steak storage treatments[f]	Carcass treatments				Std. dev. of means
	C	ES	HB	ESHB	
Thaw loss (%)[c]					
Longissimus dorsi					
Unfrozen	2.4	2.4	2.7	2.8	±0.25
Frozen	4.3*b	3.4ab	3.1a	4.1*b	±0.27
Semimembranosus					
Unfrozen	2.6	2.7	3.1	2.6	±0.22
Frozen	2.9ab	2.5ab	3.3b	2.4a	±0.27
Cooking loss (%)[d]					
Longissimus dorsi					
Unfrozen	27.9	28.6	27.4	25.8	±0.97
Frozen	30.4*	28.3	28.7	29.6*	±0.87
Semimembranosus					
Unfrozen	34.6b	33.9ab	35.6b	31.1a	±0.92
Frozen	33.4	35.2	34.3	34.7*	±0.81
Combined loss (%)[e]					
Longissimus dorsi					
Unfrozen	29.7	30.3	29.4	27.9	±0.88
Frozen	33.4*	30.7	30.9	32.5*	±0.83
Semimembranosus					
Unfrozen	36.2ab	35.6ab	37.6b	34.1a	±0.82
Frozen	35.3	36.8	36.4	36.2*	±0.80

* Means within same column for the same trait differ (P < 0.05).
ab Means within the same row with the same or no superscript do not differ (P > 0.05).
c Thaw loss (%) = thaw loss weight ÷ frozen steak weight x 100.
d Cooking loss (%) = cooking loss weight ÷ thawed steak weight x 100.
e Combined loss (%) = weight lost during thawing and cooking ÷ frozen steak weight x 100.
f Unfrozen = aged until 6 days postmortem; frozen = ES, HB, and ESHB frozen at 24 and C at 48 hr postmortem.

postmortem did not consistently prove desirable for both the LD and SM samples. However, when ES was combined with HB, both ESHB muscles were comparable to control counterparts regardless of the steak storage treatment.

Because of the apparent utility of ES in combination with HB, additional studies are warranted. Optimum combinations of ES conditions, hot boning times and subsequent chilling and/or freezing practices for the spectrum of beef muscles and muscle systems need to be determined.

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liquefaciens produced H_2S and off-odor from vacuum-packaged lean beef of pH 6.15 stored at 5°C. Similarly, Gill and Newton (1979) also found that *S. liquefaciens*, when it reached about $10^8/cm^2$, produced spoilage odors from beef longissimus dorsi muscle of pH 6.5 and 5.7 when the meat was vacuum-packaged using film with an oxygen permeability at 25°C of about 300 ml/m^2 per 24 hr per 101 kPa. The strain of *S. liquefaciens* used to inoculate the meat in the experiments reported here also produced obvious off-odors from beef semitendinosus muscle of pH 5.5–5.6 when lean was vacuum packed with film of an oxygen permeability of 200–300 ml/m^2 per 24 hr per 101 kPa, and the organism grew to almost $10^8/cm^2$ (unpublished data). The failure to observe off-odors when the count of *Enterobacteriaceae* was high on the fat may be a result of less volatile off-odors being produced from fatty tissue when the packaging film has a relatively low gas permeability.

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Roasting vs Cooking in a Model System: Tenderness of Bull Adductor Muscle, Conventionally Chilled or Electrically Stimulated—Hot Boned

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──────── ABSTRACT ────────

Conventionally chilled (CC) and electrically stimulated-hot boned (ESHB) bull adductor muscles roasted to 70°C, or muscle strips cooked in a model system (waterbath) to 70°C were compared. Sensory tenderness and texture (mealiness) and Instron texture characteristics were not affected significantly by the cooking system. Differences between carcass treatments (CC, ESHB) for sensory tenderness and texture were significant, but small. Percentage solubilized hydroxyproline did not have a major influence on sensory tenderness or texture, or on Instron texture characteristics of the muscle. Sensory tenderness and texture were related more to Instron cohesiveness and firmness than they were to Instron penetration measurements.

INTRODUCTION

SELECTING A COOKING (heating) method is important in planning and conducting research on effects of production and processing on the characteristics of cooked meat. To obtain reproducible results and conclusive information, model systems have been used to heat small pieces of muscle in a waterbath (Machlik and Draudt, 1963; Paul et al., 1973; Penfield and Meyer, 1975; Penfield et al., 1976; Hearne et al., 1978a, b). In those studies controlled time-temperature programs were used for the waterbath so that cores or strips of muscle cooked at a rate similar to a rate that occurs with cooking roasts by conventional methods.

We need economical, yet still reliable, cooking methods for the scientific study of meat "quality." We need to know whether there are significant effects on the meat that are attributable to the cooking system (conventional cooking methods vs a model system). One way of reducing the cost of materials is by cooking (heating) small samples in a model system. We found only two research studies in which roasts cooked by conventional methods were compared with small samples of muscle cooked in a model system. Brady and Penfield (1981) compared the effects of conventional dry heat roasting of bovine semitendinosus (ST) roasts with those of heating small cores of ST muscle in test tubes in a waterbath on Instron textural characteristics and solubilization of hydroxyproline for two rates of heating (waterbath, 93 or 149°C) and two end point temperatures (60 or 70°C). They concluded that the cooking system had some influence on the "quality" of the cooked meat. McDowell et al. (1982) compared bovine top round roasts cooked by conventional methods (dry heat roasting or moist heat braising) with semimembranosus muscle strips cooked in test tubes in a waterbath. The waterbath was programmed so that the internal temperature of the muscle strips reproduced an average heating curve similar to that of a 1.2–1.4 kg top round roast cooked by dry heat at 177°C. They concluded that their model system may be substituted for oven roasting or braising to evaluate treatment effects on sensory charac-

teristics and related objective measurements, except, possibly Instron texture measurements.

Electrical stimulation and hot boning have been studied extensively as methods of improving tenderness of skeletal muscle and of reducing processing time and cost (Bouton et al., 1971; Henrickson et al., 1974; Bendall et al., 1976; Davey et al., 1976; Gilbert et al., 1977; Shaw and Walker, 1977; Savell et al., 1978a, b, c; Smith et al., 1978; Bouton et al., 1980a, b; Erickson et al., 1980; Elgasim et al., 1981; Ray et al., 1982). Electrical stimulation followed by hot boning, reduced chilling space and aging time from 10–20 days to 2 days (Gilbert et al., 1977).

Research has indicated that generally bull meat is coarser, less tender, and drier than steer meat, but differences between the two classes of animals are small (Albaugh et al., 1975; Hurst et al., 1975; Arthaud et al., 1977; Jacobs et al., 1977; Calkins and Davis, 1980). We found no information on the "quality" of cooked bull adductor (AD) muscle.

We used conventional roasting and a model system to study effects of those cooking systems on the tenderness, texture, and cooking properties of electrically stimulated-hot boned bull AD muscle. Relationships between sensory tenderness and texture attributes and Instron texture characteristics and relationships between those measurements and solubilized hydroxyproline were studied.

MATERIALS & METHODS

Meat used

Forty AD muscles from 20 bull carcasses were purchased from the Kansas State Univ. Dept. of Animal Science & Industry. The bulls were on grass until about 10 months old, then they were fed a ration of 56.1% milo, 40.2% forage sorghum silage. and 3.7% vitamin and mineral supplement until slaughtered (approximately 190 days) at an average weight of 510 kg. Carcass processing treatments, slaughter weights, and USDA quality grades are given in Table 1. One side of each carcass was conventionally chilled (CC); the other side was electrically stimulated and hot boned (ESHB).

Cooking

The AD muscles from the CC side of each carcass and the paired AD muscle from the ESHB side of the carcass were assigned randomly to one of two cooking methods, oven roasting (OR) or cooking muscle strips in a model system (S). Either two roasts or two sets of strips (Fig. 1) were cooked at each of 20 evaluation periods.

Muscles assigned to OR were thawed 4 hr at approximately 25°C, then for 16 hr at approximately 4°C. Thawed muscles were trimmed to provide roasts of similar size and shape (avg, 700g; 10 x 11 x5-8 cm), Fig. 1. A short bulb (1.3 cm long) thermometer (−20 to 105°C, 15 cm long) was inserted into the geometric center of each trimmed roast, which was placed on a low rack in a shallow pan and roasted in a rotary hearth oven at 177°C to 70°C.

The rate heat penetrated the roast was determined by noting the temperature changes for the roasts from the initial temperature to 70°C at 5 min intervals. Total cooking time (min) was recorded; total, volatile, and drip cooking losses were calculated as percentages of the weight of the thawed raw roast. Roasts were sampled by a fixed position plan (Fig. 2).

One week before cooking, AD muscles assigned to S were conditioned 4 hr at approximately 25°C, then cut into strips (2.3 x

Table 1—Carcass processing treatments, slaughter weights, and USDA quality grades

Processing treatment	Treatment conditions	Carcass weight, hot, kg		USDA quality grade 24 hr postmortem
		Average	Range	
Conventionally chilled (CC), one side of each carcass	3–8°C, 48 hr, adductor muscles excised, vacuum packed, stored at 4-5°C until 7 days postmortem, and frozen at −26°C	300	245-340	High Standard to Low Choice
Electrically stimulated-hot boned (ESHB), one side of each carcass	420 volts, A.C., 6.8 sec current on, 3.2 sec current off for 2 min at 45 min postmortem, hot boned at 2 hr postmortem	Same as for CC		Not graded

2.3 x 8 cm) with the muscle fibers running parallel to the length of the strips (Fig. 1) to obtain strips of uniform dimensions. Individual strips were wrapped tightly in household plastic wrap to make them cylindrical in shape. The group of strips from each muscle was wrapped in aluminum foil, frozen, and stored at −22°C until used.

At the time of cooking, 16 strips (eight from each of the two muscles cooked at one evaluation period) were thawed for 25 min at approximately 25°C, unwrapped, and placed in 50-ml centrifuge tubes with thermometers (the same as those used for the roasts) inserted lengthwise into the centers of 12 strips. Because thermometer holes affect the thickness of the samples, and ultimately, the values obtained for Instron penetration and shear measurements, no thermometers were inserted in two strips of each of the muscles that were used for Instron texture measurements. Strip portions with thermometer holes and the trimmings from samples for sensory and Instron texture measuements were ground and used for moisture, hydroxyproline, ether extract, and pH measurements. Test tubes were placed in an 18-liter circulating waterbath in which strips were cooked to 70°C at a rate comparable to oven roasting an 890-g AD roast at 177°C. The temperature of the waterbath rose approximately 1°C/min until the temperature of the strips was between 45 and 55°C, after that the waterbath temperature rose about 0.6°C/min until the strips reached 70°C'

Sensory evaluation

Tenderness, juiciness, mealiness, and softness of 1.3-cm cubes of cooked muscle were evaluated by an 8-member experienced laboratory panel using a 7 to 1-point intensity scale. Training was given to the panel during preliminary work. Each panel member standardized his tenderness scores by counting the number of chews necessary to masticate completely a cube of meat. Cubes were presented to panelists in the top of half-pint enamel double boilers set over hot water (approx 60°C) with the entire system on an electric hot tray set at low heat (approx 35°C). Immediately after samples were prepared for evaluation, each panelist randomly selected two cubes of muscle from samples representing each treatment (CC or ESHB). One sample was used to evaluate tenderness; the other cube was used to score juiciness and textural components.

Instron texture measurements

The Instron Universal Testing Machine, Model 1122, was used for penetration and shear measurements on strips of cooked muscle (1.3 x 1.3 x 7 cm).

Penetration measurements. Hardness, cohesiveness, elasticity, and chewiness were derived from compression curves that were obtained when a flat-ended cylindrical puncture probe (0.63 cm diam) was driven vertically about 80% of the way through a strip (1.3 x 1.3 x 7cm) that was positioned with the fibers perpendicular to the direction of penetration (Bouton et al., 1971). The probe was driven into the strip twice with two compression curves at each of three locations (center and about 1 cm from each end). A 50-kg load with a crosshead speed of 50 mm/min and a chart speed of 100 mm/min was used to record a force-distance curve.

"Hardness," the force (kg) necessary to achieve the first penetration was recorded as the peak height of the first penetration. "Cohesiveness" was the ratio of the work done during the second penetration to that done during the first penetration. Work was estimated as the area (sq cm) of the first or the second penetration, measured with a compensating polar planimeter (Friedman et al., 1963). "Elasticity," the height (mm) that the muscle recovered during the time that elapsed between the end of the first penetration and the beginning of the second, was measured as the horizontal distance (mm) from the origin of the second penetration to the perpendicular line dropped from the peak of the first penetration curve multiplied by (crosshead speed/chart speed). "Chewiness" was derived as the product of "hardness" x "cohesiveness" x "elasticity" and was measured in kg-mm (Friedman et al., 1963; Bourne, 1978).

Shear measurements. Muscle strips (1.3 x 1.3 x 7 cm) were sheared with a Warner-Bratzler shear attachment (D372-26) for the Instron, using the same load, crosshead speed, and chart speed used for penetration measurements. Muscle strips were sheared in the center and approximately 1 cm from each end. The shear force-distance curve was recorded and used to evaluate "shear cohesive-

Fig. 1—Adductor muscles. A-muscle to be cut into 2.3 x 2.3 x 8 cm strips, shown at left are sample strips; B-muscle cut into 10 x 11 x 8 cm roast.
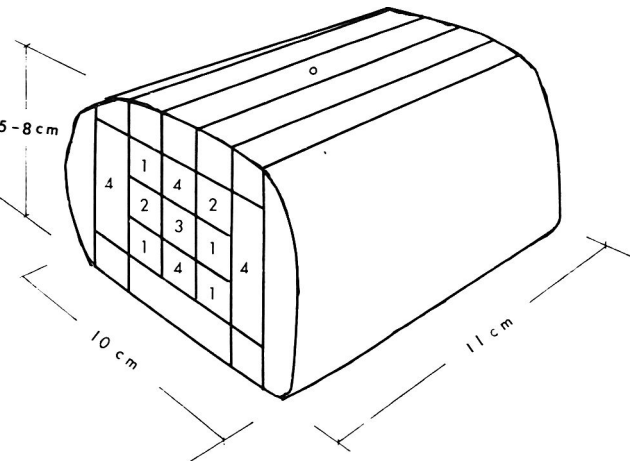
Fig. 2—Sampling plan for cooked roasts: (1) sensory evaluation samples, (2) Instron texture samples, (3) water-holding capacity samples, (4) total moisture, ether extract, pH, and hydroxyoroline samples.

ness" and "firmness." "Shear cohesiveness" was the peak force (kg) on the shear deformation curve. "Firmness" was measured as the slope of the line drawn from the origin of the curve to the peak, and expressed in kg/min (Larmond and Petrosavits, 1972).

Total moisture (TM), ether extract (EE), water-holding capacity (WHC), and pH

Percentage TM in raw and cooked muscles were measured by drying duplicate 10-g samples of ground muscles for 120 min (raw muscle) or for 60 min (cooked muscle) at 121°C in a C.W. Brabender Semi-Automatic Rapid Moisture Tester. Triplicate measurements of percentage dry matter and EE were determined (AOAC, 1975) for samples of ground raw or cooked muscle. Percentage TM was calculated by subtracting the percentage dry matter from 100.

Triplicate measurements of WHC were made on samples (300 mg) of cooked meat using the press method of Miller and Harrison (1965). The ratio of the pressed meat area to the juice area was designated as the expressible liquid index (ELI). WHC values were obtained by subtracting the ELI from 1.0, arbitrarily chosen as the maximum ELI. The ELI is inversely related to the amount of liquid expressed from the sample; the larger the WHC value, the more liquid expressed.

Duplicate pH readings were made on slurries of 5-g ground muscle and distilled, deionized water. The slurry was stirred 30 sec with a magnetic stirrer on an electric stirring table, the pH was measured, the beaker was turned 180°, the slurry was stirred an additional 30 sec, and a second pH reading was taken. The pH meter was standardized against a buffer of pH 6.86 (Rogers et al., 1967).

Hydroxyproline measurement

Duplicate 2-g ground, raw or cooked muscle samples were used to measure hydroxyproline. The amount of hydroxyproline that solubilized during the cooking process was determined by calculating the difference between the total amount of hydroxyproline in the raw meat sample and the amount of hydroxyproline in the water-washed, ground, cooked sample.

Each raw sample was homogenized with 10 ml distilled, deionized water using a Brinkman Unitron homogenizer for 1 min at speed 13. The meat homogenate and the washings from the homogenizer were combined and made up to 20 ml with distilled, deionized water and transferred to a 50-ml ampoule. Concentrated hydrochloric acid (HCl, 20 ml) was added to make a final concentration of 6N HCl. Each cooked sample was washed with 20 ml of warm (40°C) distilled, deionized water, homogenized for 30 sec in the Brinkman Unitron homogenizer, centrifuged at 4,000 rpm for 10 min, and decanted to remove the water soluble proteins (Paul et al., 1973). The washing process was repeated for complete removal of solubilized proteins and amino acids before a final homogenate of 20 ml was transferred to a 50-ml ampoule and acidified with 12N HCl to provide a final concentration of 6N HCl. The ampoules containing the acidified samples were sealed using a propane jet torch and incubated in an oven at 107°C for 20 hr to complete hydrolysis. Preliminary work showed that the amount of hydroxyproline reached a plateau after 18–30 hr of incubation. The resulting hydrolyzate was neutralized to a pH of 6.5–7.0 with 2.5N NaOH and made up to 250 ml in a volumetric flask with distilled, deionized water before the final assay. The assay for hydroxyproline was done using one-half the volume of the sample and reagents suggested by Bergman and Loxley (1963) to increase the sensitivity of their method, and to obtain an accurate measurement of the low hydroxyproline concentration in the meat sample. A standard curve for hydroxyproline with concentrations ranging from 0–10 μg was used to calculate the amount of hydroxyproline in the sample.

Statistical analyses of data

A split plot design with 10 replications was used to evaluate effects of treatment combinations on the measurements made on bull AD muscle. The main plots were the cooking systems (OR, S); the subplots were the carcass treatments (CC, ESHB). The treatment structure was a 2 X 2 factorial in the split plot design. Analysis of the four treatment combinations was orthogonal, i.e., two main effects each with one degree of freedom (df) and the interaction with 1 df.

An F-test was used to compare sample variances to determine if precision in measurements differed between cooking systems.

Correlation coefficients were calculated for selected paired variates on the basis of overall observations, the cooking system, and the carcass treatment.

RESULTS & DISCUSSION

Effects of the cooking system

Heating time. Time that heat penetrated OR and S and the heating curve that OR and S were expected to follow ("estimated") are shown in Fig. 3. Heat penetration curves were plotted between 10 and 70°C, because below 10°C, differences in heating time among S, OR, and the "estimated" roasts were large, resulting from their initial temperature differences. The average time required for the "estimated" roasts, S, or OR to increase from their initial temperatures of −1.0, −1.2, or 2.5°C to 10°C was 40.9, 24.6, or 15.9 min, respectively.

At any point from 10 to 65°C, the time heat penetrated S was not significantly different from that of "estimated" roasts. Also, S and OR did not differ significantly in the time required to increase in internal temperature from 10 to 50°C, but OR required a longer (P < 0.05) time than did strips to increase from 50 to 55−65°C. From 65 to 70°C, no significant differences in heating time were observed between S and OR.

Measurements affected significantly by the cooking system. Measurements affected significantly by the cooking system were cooking time, drip loss, volatile loss, percentage total moisture, and percentage ether extract (Table 2). Cooking time is the time from the initial temperature to 70°C. Because the interaction between cooking systems and carcass treatments was significant for cooking time (Table 4), we did not test main effect means for cooking time.

S and OR did not differ significantly in percentage total cooking losses, but volatile loss was higher and drip loss
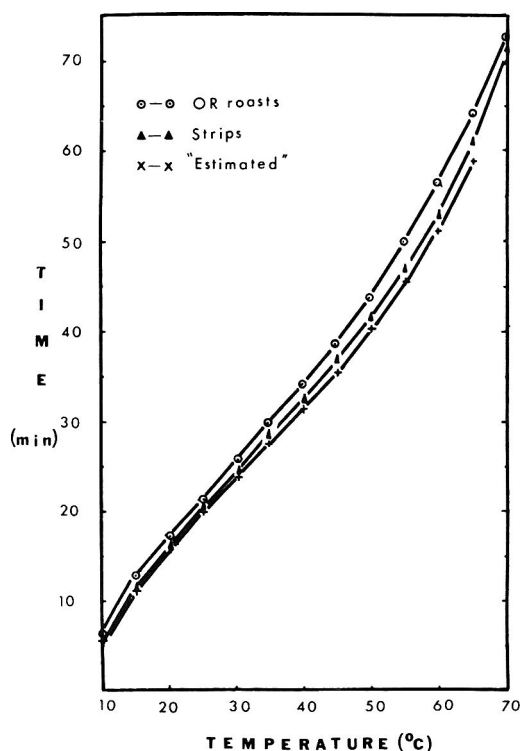
Fig. 3—The time heat penetrated the AD muscle from 10° to 70°C for OR roasts, muscle strips, and roasts "estimated" (average heat penetration curve that strips and roasts were expected to follow).

was lower for OR than for S. Those results were expected, because with OR, most liquids that exuded from the meat during cooking were evaporated, which accounted for greater volatile loss. In the waterbath, strips were enclosed in glass centrifuge tubes and most of the liquid remained in the tubes as drip.

Total moisture, analyzed either by using the Brabender Rapid Moisture Tester or by the AOAC method was greater for S than for OR. The lower moisture content of OR may be attributed, partially, to the moisture gradient in roasts, the central portion being moister than the portion near the edges. About 75% of the ground muscle samples for total moisture, ether extract, pH, and hydroxyproline determinations were taken near the edges of the roast (Fig. 2), and about 25% were from the central portion. Water-holding capacity, measured in samples taken from the center of both the roasts (0.66) and strips (0.64), did not differ significantly between the two cooking systems. That also suggests that differences in total moisture between strips and roasts were attributable to sampling locations in roasts. Bengtsson et al. (1976), in their study of mass and heat transfer in roasted beef, found maximum moisture and minimum temperature near the center of an 800–900-g roast. Similarly, higher (P < 0.05) temperature and the appearance of more done and drier edges than centers of top round steaks cooked to 65°C at 177°C were observed by Moore et al. (1980). Funk et al. (1966) reported similar

observations for beef loin roasts. Also, higher volatile losses for OR than for S may have contributed, partially, to the higher moisture content of S samples than for OR samples.

In our experiment OR yielded more ether extract than did S. Again, greater volatile loss for OR tended to increase ether extract in cooked muscle.

Despite the fact that roasts took a significantly longer heating time to reach an internal temperature of 55–65°C, differences between samples cooked by S or OR were not significant for sensory tenderness and texture, Instron texture, and solubilized hydroxyproline. McDowell et al. (1982) found no significant differences in sensory characteristics between oven roasts cooked by dry heat at 177°C and muscle strips heated in a model system at a rate similar to that of roasts, but they obtained significantly lower Instron hardness, chewiness, firmness, and shear cohesiveness values for strips than for roasts, which they attributed to a significantly slower rate of heat penetration in strips at the 50–60°C range. Our results for texture characteristics and solubilized hydroxyproline were in agreement with those obtained by Brady and Penfield (1981), who reported no significant differences in those measurements between roasts cooked at 149°C to 60 or 70°C and strips heated at rates simulating those of the roasts.

Effects of the carcass processing treatments

CC vs. ESHB. Measurements for which significant differences occurred between CC and ESHB samples were cooking time, sensory tenderness and mealiness, and solubilized hydroxyproline (Table 3). Because the interaction between cooking systems and carcass treatments was significant for cooking time and solubilized hydroxyproline, we did not test main effect means for those measurements. Although, ESHB samples were scored significantly less tender and mealy than CC samples, the respective mean scores differed by only 0.3 or 0.4 point, and standard errors were only 0.09 and 0.17 (Table 3).

Significant cooking system x carcass treatment interactions

Measurements affected significantly by interaction between cooking systems and carcass treatments were cooking time, Instron hardness, and solubilized hydroxyproline (Table 4). The LSD for cooking systems indicated that differences in cooking time between S and OR were attributable to the effects of both CC and ESHB; the differences between carcass treatments were attributable to the effects of OR. Longer cooking time was required for S than for OR with a greater difference for CC than for ESHB samples.

Although neither the cooking system nor the carcass treatment per se significantly affected Instron hardness, interactions (Table 4) indicated that significant differences in Instron hardness occurred between cooking systems for CC samples. CC samples cooked in the model system were significantly less hard than those cooked by OR.

Differences in solubilized hydroxyproline observed between carcass treatments (Table 3) were attributable to the effects of OR (Table 4). Significantly more solubilized hydroxyproline was found in CC samples cooked in the model system than in those cooked by OR. Similarly, Brady and Penfield (1981) reported greater (P < 0.05) solubilized hydroxyproline for strips cooked in a model system at a rate simulating that of roasts cooked at 93°C to either 60 or 70°C than for oven roasts.

Our solubilized hydroxyproline values ranged from 11.6–28.3%, and were higher than values reported by other researchers, who also used water to extract hydroxyproline that solubilized during cooking. Paul et al. (1973) reported that ST and BF muscles cooked to 58, 67, 75, or 82°C

Table 2—Means, standard errors,[a] F-values, and probability levels for measurements affected significantly by the cooking system

Measurement	Strips[b]	Roasts[b]	F-value	P
Cooking measurements				
Cooking time, min[c]	89.7 (±1.68)	81.4 (±1.68)	–	–
Drip loss, %	23.6 (±0.71)	3.7 (±0.71)	366.2	0.0001
Volatile loss, %	2.9 (±0.64)	21.1 (±0.64)	392.4	0.0001
Total moisture, %				
Cooked, Brabender	66.4 (±0.29)	64.1 (±0.29)	30.7	0.0001
Cooked, AOAC	67.3 (±0.89)	63.5 (±0.89)	10.5	0.006
Ether Extract, %	1.7 (±0.34)	3.8 (±0.34)	17.5	0.008

[a] Values in parentheses
[b] Data for conventionally chilled (CC) and electrically stimulated - hot boned (ESHB) were combined
[c] Includes time from initial temperature to 70°C

Table 3—Means, standard errors,[a] F-values, and probability levels for measurements affected significantly by the carcass treatment

Measurement	CC[b]	ESHB[b]	F-value	P
Cooking time, min	83.9 (±0.79)	87.2 (±0.79)	–	–
Sensory scores,[c] 7-1				
Tenderness	5.6 (±0.09)	5.3 (±0.09)	4.8	0.04
Mealiness	4.7 (±0.17)	4.3 (±0.17)	7.9	0.01
Solubilized hydroxyproline, %	17.6 (±1.10)	26.7 (±1.10)	–	–

[a] Values in parentheses
[b] Data for oven roasts (OR) and strips cooked in the model system (S) were combined, CC, conventionally chilled; ESHB, electrically stimulated-hot boned
[c] Range, 7 (tender, mealy) to 1 (tough, chewy)

Table 4—Means, probability levels and LSDs for significant, cooking system x carcass treatment interactions

| Measurement | Cooking system | Carcass treatment | | P | LSD[a] | |
		CC[b]	ESHB[b]		CC vs ESHB	S vs OR
Cooking time, min	S	89.4	90.1	0.043	5.57	3.34
	OR	78.5	84.2			
Instron hardness, kg	S	7.6	8.6	0.045	1.29	0.90
	OR	8.6	7.9			
Solubilized hydroxyproline, %	S	23.7	25.2	0.0002	7.40	4.67
	OR	11.6	28.3			

[a] Least significant difference (P<0.05)
[b] CC, conventionally chilled; ESHB, electrically stimulated-hot boned

at 163°C yielded collagen solubilization of 4.3 to 13.4%. Penfield and Meyer (1975) and Brady and Penfield (1981) showed that ST muscles cooked to 40, 50, 60, or 70°C and to 60 or 70°C at 93 or 149°C had 1.3 to 13.6% and 4.3 to 10.0% solubilized hydroxyproline, respectively. Williams and Harison (1978) reported a range of 1.5–1.8% solubilized hydroxyproline for top round steaks cooked to 70 or 80°C at 94 or 149°C. All the above authors analyzed the water extracts from cooked muscles and their drippings. In our study, drippings from OR formed a hard coagulum in the roasting pan, so we could not measure the solubilized hydroxyproline accurately using the approach of previous workers. Our approach to estimating the amount of hydroxyproline that solubilized during cooking was based on the assumptions that (1) water extractable hydroxyproline in cooked muscle came mainly from the solubilization of collagenous tissue, and (2) the assay used as highly specific for hydroxyproline. The high values obtained in our study may be attributed to the possibility that some partially solubilized collagen was extracted by warm water. Ideally, the amount of hydroxyproline in the hydrolyzed raw sample is equivalent to the summation of hydroxyproline in the hydrolyzed raw sample is equivalent to the summation of hydroxyproline in the hydrolyzed water extracted cooked sample, the water extract, and the drippings. Percentage solubilized hydroxyproline (OHProline) was calculated using the equation:

$$\% \text{ Solubilized OHProline} = \frac{\left(\begin{array}{c}\text{mg OHProline in} \\ \text{raw sample} \\ \text{(dry wt)}\end{array}\right) - \left(\begin{array}{c}\text{mg OHProline in} \\ \text{extracted cooked} \\ \text{sample (dry wt)}\end{array}\right)}{\left(\begin{array}{c}\text{mg OHProline in} \\ \text{raw sample (dry wt)}\end{array}\right)} \times 100.$$

Therefore, less hydroxyproline remaining in the water-washed, acid-hydrolyzed cooked sample resulted in a higher percentage of solubilized hydroxyproline than when percentage solubilized hydroxyproline was calculated as the ratio of the sum of free hydroxyproline in the water extract and in that of the drippings to the amount of hydroxyproline in the raw sample multiplied by 100.

Relationships between tenderness and texture measurements

Simple correlation coefficients were calculated to study the degree of relationships between selected tenderness and texture measurements (variates). Relationships between paired measurements from our data were similar whether r-values were calculated on the basis of the cooking system (df = 18), on the basis of carcass treatments (df = 18), or from data where all treatment combinations were combined (df = 38). Based on Falkner's (1962) classification, correlation coefficients indicated that generally sensory scores for tenderness, softness, and mealiness were related moderately (0.40–0.79) to each other and to Instron values for shear cohesiveness and shear firmness. Correlations were low (0–0.39) between Instron penetration measurements

Table 5—Sample variances, F-values, and the probability that variances between cooking systems are equal

| Measurement | Sample variance[a] | | F | P |
	OR	S		
Cooking time	66.4500	6.6816	9.94	0.000
Cooking losses				
Volatile	5.0659	4.8308	1.05	0.458
Drip	0.6962	9.8863	14.20	0.000
Total	5.9341	4.5925	1.17	0.361
Total moisture				
Brabender	0.9298	1.0589	1.14	0.387
AOAC	31.5940	3.1950	9.89	0.000
Water-holding capacity	0.0020	0.0012	1.71	0.121
pH	0.0042	0.0056	1.34	0.258
Ether extract	2.3884	1.3637	1.75	0.112
Sensory scores				
Tenderness	0.2602	0.3877	1.49	0.190
Softness	0.3867	0.4894	1.27	0.302
Mealiness	0.4973	0.2940	1.69	0.124
Juiciness	0.8037	0.4978	1.61	0.146
Instron measurements				
Hardness	2.3717	2.0015	1.18	0.354
Firmness	55.1665	56.5675	1.03	0.478
Chewiness[b]	69.3514	66.5144	1.04	0.463
Chewiness[c]	52.9116	67.8372	1.28	0.292
Elasticity	1.1012	1.875	1.69	0.126
Cohesiveness[b]	0.0048	0.0023	2.07	0.056
Cohesiveness[c]	0.0023	0.0035	1.53	1.76
Shear cohesiveness	4.4791	4.0953	1.09	0.422
Solubilized hydroxyproline	126.9780	64.7494	1.96	0.070

[a] OR, oven roasting; S, model system
[b] Friedman et al. (1963)
[c] Bourne (1978)

(hardness, chewiness) and sensory tenderness and texture scores. Instron penetration measurements for hardness and chewiness were correlated moderately with Instron shear cohesiveness and shear firmness. Correlation coefficients showed little relationship between percentage solubilized hydroxyproline and any of the sensory tenderness or texture scores, or the Instron texture measurements. For 18 degrees of freedom (df), a coefficient of 0.444 is required for a significant (P < 0.05) relationship between two measurements, a coefficient of 0.561 is required for P < 0.01. For 38 df, coefficients between those required for 35 df (0.375, P < 0.05; 0.418, P < 0.01) and 40 df (0.304, P < 0.05; 0.393, P < 0.01) is required for a significant relationship.

Precision between cooking systems

To study differences in precision between OR and S, for each measurement on cooked muscle, we used the F-test to test sample variances for equality between the cooking systems. Generally, sample variances for a given measure-

ment were similar in size for OR and S. Variances for only four of 21 measurements differed (P < 0.05) between cooking systems (Table 5). Measurements for which OR had significantly larger variances than those for S were cooking time, total moisture (AOAC), and Instron cohesiveness. For drip cooking losses, the variance for S was significantly larger than that for OR.

CONCLUSIONS

UNDER THE CONDITIONS of this study, we concluded: (1) the model system of cooking may be substituted for oven roasting when evaluating processing treatment effects on sensory tenderness and texture or Instron texture measurements, (2) the percentage of solubilized hydroxyproline is not a major influence on sensory tenderness and texture or on Instron texture attributes of bull AD muscle cooked to 70°C, (3) sensory tenderness and texture are related more to Instron shear cohesiveness and firmness than they are to Instron penetration measurements, and (4) CC and ESHB bull AD muscles cooked to 70°C are comparable in sensory tenderness and texture and in Instron texture characteristics.

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Influence of Rate and Length of Cookery upon Product Attributes of Pre- and Post-Rigor Beef

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─────────ABSTRACT─────────

Paired sides of 15 steer carcasses were used to determine the effects of low temperature, long duration cooking upon muscle (semimembranosus, SM; semitendinosus, ST) shortening, cooking and tenderness attributes of beef roasts that were removed 1 hr (hot-boned, HB) and 48 hr postmortem (cold-boned, CB). The cooking treatments were: (1) 1st hr at 47°C then raised 5.6°C/hr through the 5th hr (69°C); (2) 1st hr at 52°C then raised 5.6°C/hr through the 4th hr (69°C); and (3) 1st hr at 58°C then raised 5.6°C/hr through the 3rd hr (69°C) and thereafter at 80°C until an internal temperature of 66°C was reached. Shear values and panel ratings showed HB roasts to be slightly less tender than CB roasts. Cooking yields were higher for HB than CB roasts.

INTRODUCTION

A TREMENDOUS AMOUNT of energy is used to refrigerate and cook beef before it is consumed. Kastner and Henrickson (1977) estimated that hot-boning could save beef packers 50% in refrigeration energy and 80% in cooler space requirements. Besides these substantial savings, hot-boning could reduce shrink, improve the raw materials for sausage emulsions, and reduce labor and equipment costs. Ray et al. (1982) reported that the process of pre-rigor excision of beef roasts followed by immediate cooking of the hot-boned roasts appears to offer several advantages (reduced cooking time of 23–33% and higher cooking yields of 7%) over the conventional process of chilling the carcass, removing the roasts and subsequent cooking. Problems with hot-boning are reduced tenderness and an obvious distortion in the shape of the cooked roasts upon cooking (Weiner et al., 1966; Kastner and Russell, 1975; Ray et al., 1979; Berry et al., 1981; Griffin et al., 1981; Ray et al., 1982). In some instances (Cia and Marsh, 1976; Marsh, 1977), improved tenderness occurred with pre-rigor cooking. This study was designed to determine if muscle shortening, and subsequent toughening could be reduced in pre-rigor roasts from beef carcasses by using low temperature and a long cooking time.

MATERIALS & METHODS

Muscle removal and measurement

Thirty sides (Table 1) of beef (15 carcasses) that were A maturity, USDA Good, and averaged yield grade 3.2 and approximately 338 kg were utilized in this study. The intact semitendinosus (ST) and semimembranosus (SM) muscle were removed from the left sides within one hour post-exsanguination (hot-boned, HB). The ST and SM muscles from the right sides were removed following a 48-hr chilling period (cold-boned, CB) at 2°C. The SM muscles from the left and right sides were divided longitudinally so that the cut portions from each muscle were subjected to the cooking treatments. Prior to cooking, the ST muscles and the SM muscle portions were weighed and measured for length, width (25, 50, and 75% of the length) and depth at the deepest point of the muscle, and tempera-

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tures were recorded. Similar measurements were recorded for the cooked muscles after they were chilled.

Cooking procedure

Hot-boned muscle portions were cooked immediately (20 min post-excision) after the roasts were rubbed with a commercial roast beef preparation (B. Heller® and Co.) and placed in a Cryovac® bag (L600). They were cooked by one of three cooking schedules to an internal temperature of 66°C in a hot water vat. The schedules (Table 2) were: (1) 1st hr at 47°C then raised 5.6°C/hr through the 5th hr (69°C) and thereafter at 80°C (low temperature); (2) 1st hr at 52°C then raised 5.6°C/hr through the 4th hr (69°C) and thereafter at 80°C (medium temperature); (3) 1st hr at 58°C then raised 5.6°C/hr through the 3rd hr (69°C) and thereafter at 80°C (high temperature) until the internal temperature of 66°C was reached. Internal roast temperature was monitored during cooking with temperature probes (Omega®) that were 0.63 cm in diameter and 4.7 cm in length and a Honeywell® potentiometer. The wires attached to the probe passed through the open end of the cooking bag. A string was utilized to close the bag around the wire to inhibit leakage during cooking. Cooking time and yield were recorded and the product was chilled to 3.4°C over a 12-hr period. Subsequently, a 3.8 cm thick sample was removed for Warner-Bratzler shear (WBS) determination. Three cores (1.27 cm in diameter) were removed from each sample parallel to the muscle fibers and all cores were sheared twice. Adjacent to the WBS samples, a 10 cm thick sample of each roast was removed from the center portion, placed in a bag with about 150 ml of juices, vacuumized and frozen (−29°C). Following 30 days of storage, the samples were thawed, reheated and evaluated for tenderness, at the USDA Meat Science Research Lab. (Beltsville, MD) by a nine-member trained panel according to procedures outlined by AMSA (1978). Six samples

Table 1—Carcass traits and measurements

Trait	Cooking treatment			
	1	2	3	SEM[b]
Hot carcass weight, kg	348	330	337	±20
Ribeye area, cm^2	83.9	76.1	70.3	± 1.0
Fat thickness, cm	1.02	1.27	1.52	± .007
Quality grade[a]	10	10	10	± 1.57
Yield grade	2.7	3.3	3.7	± .96
No. of roasts	20	20	20	

[a] Scores based on 11 = USDA High Good; 10 = USDA Average Good; 9 = USDA Low Good.
[b] Standard error of treatment means.

Table 2—Cooking treatment schedule

Hours of cooking	Cooking treatments[a]		
	1	2	3
1st	47[b]	52	58
2nd	52	58	63
3rd	58	63	69
4th	63	69	80
5th	69	80	80
6th until done	80	80	80
No. of roasts	20	20	20

[a] All roasts were cooked to an internal temperature of 66°C.
[b] Temperature of water bath (°C).

were evaluated at each session: paired hot- and cold-boned samples from each of the three cooking methods.

Sarcomere measurement

Sarcomere length for cooked muscle was determined by homogenizing (Virtis Macro 45®) a 3-g sample in 35 ml of a 0.25M sucrose solution for 40 sec. The length of 10 sarcomeres each from 25 myofibrils was measured using an ocular micrometer and a Zeiss® phase contrast microscope (1600 X with oil).

Statistical analyses

A split-split plot design with treatments arranged in a 2 x 3 x 2 factorial was assumed for statistical analyses. Main plots were hot versus cold-boned with 30 roasts each. Within main plots, roasts were assigned to three subplot cooking treatments (i.e. 10 roast in each cooking treatment). Within cooking treatment, roasts were assigned to sub-sub plot treatments which included the ST and SM (i.e. 5 roasts from each muscle). Paired roasts from sides (HB or CB) of the same carcass were assigned to the same cooking treatment. Analyses of variance were computed to test for treatment effects and interactions. If interactions were significant (P < 0.05), effects were evaluated using an LSD test (Snedecor and Cochran, 1967).

RESULTS & DISCUSSION

Physical characteristics

Changes in the ST and SM with regards to length, width and depth are presented in Table 3. Cooking CB, ST roasts at a low temperature resulted in 8% more shortening than the medium and high temperature cooking. Similar reduction in length was observed for HB roasts (cooking treatment 1 and 2) of the SM muscle. Cooking at medium treatment 2 resulted in the greatest change in length, width and depth of HB, ST roasts. The HB, ST roasts became wider on cooking, whereas the CB, ST roasts became narrower. It should be noted that the roasts (HB) which displayed the greatest reduction in length also had the largest increase in depth. In the SM roasts, width changes were different from those observed with the ST roasts in that the HB and CB roasts exhibited a similar and marked reduction (P < 0.05) in their width upon cooking. Changes in depth were most intense for the HB roasts cooked at medium treatment while the CB roasts had a small but similar change in depth for medium and high temperature but a much larger change for low temperature, across the cooking treatment. These dimensional changes are comparable to the length changes reported by Cia and Marsh (1976) and Ray et al. (1980) but are larger than the results reported by Ray et al. (1982) for depth changes.

Cooking time and losses

Time required to reach 66°C internally was less (P < 0.05) for the HB, SM roasts than for CB, SM roasts regardless of the cooking temperature (Table 4). Cooking time at the low temperature was similar (P < 0.05) in the CB and HB, ST roasts. Time required (overall mean) per unit weight (Table 4) to cook HB roasts from the ST and SM was 146.7, and 100.7 min/kg and for the CB roasts (ST and SM) 167.3 and 116.4 min/kg, respectively. The advantage of using HB muscles to reduce time of cooking was minimized (8.7%) when using low temperature, long duration cooking compared with studies (28–33%) that utilized a faster cooking method (Ray et al., 1980, 1982).

Cooking loss (Table 5) was determined as the difference in weight between the raw muscle plus seasonings and cooked weight of the roast. Cooking loss was influenced (P < 0.05) by method of boning, with the roasts from HB, ST muscles having a 7% lower cooking loss than the CB, ST muscle and the HB, SM muscles having a 4.3% lower cooking loss than CB, SM muscle. Kastner et al. (1973), Falk et al. (1975), Ray et al. (1980), Griffin et al. (1981) and Ray et al. (1982) have shown HB to decrease cooking losses when compared with boning after conventional chilling. Furthermore, the results from these studies indicate the magnitude of the decrease in cooking loss for HB roasts is directly related to the excision time postmortem. The cooking treatments did not (P > 0.05) influence the cooking losses of ST and SM roasts (Table 5).

Tenderness evaluation

Tenderness of the pre-rigor (cooked within 20 min post-excision) roasts (SM and ST) was greatly enhanced with the low temperature, long duration cooking (Tables 6 and 7). Other studies (Weiner et al., 1966; Montgomery et al., 1977; Ray et al., 1980, 1982; Griffin et al., 1981; Berry et al., 1981) have reported decreased tenderness of pre-rigor cooked roasts when compared with their CB counterparts. However, Cia and Marsh (1976) found that microwave cooking improved the tenderness of pre-rigor muscles compared with boiling. West et al. (1980) removed muscles 1 hr after stimulation of cow carcasses and found the cooked product to be comparable to the post-rigor cooked product if a low (58°C) temperature-long time cooking method was used. Our data (Table 6) indicate that with a low cooking temperature (cooking tmt 1) initially, the tenderness (WBS and taste panel) was very acceptable and there was no difference (P > 0.05) between the HB, SM roasts and the

Table 3—Changes in dimensions of semitendinosus and semimembranosus beef roasts as influenced by cooking and postmortem boning

| | Cooking treatment | | | | | |
| | 1 (low) | | 2 (medium) | | 3 (high) | |
Item	HB	CB	HB	CB	HB	CB
Semitendinosus						
Change in (cooked vs fresh):						
Length %	−38.4 ± 0.8	−38.6 ± 0.7	−42.0[a] ± 0.8	−30.6[b] ± 0.6	−34.9[a] ± 0.8	−30.4[b] ± 0.7
Width, %	+0.5 ± 0.1	−2.1 ± 0.1	+10.9[a] ± 0.3	−10.7[b] ± 0.3	+5.6[a] ± 0.3	−8.0[b] ± 0.3
Depth, %	+23.7[a] ± 0.5	+19.5[b] ± 0.4	+38.9[a] ± 0.7	+2.2[b] ± 0.1	+23.6[a] ± 0.6	+2.0[b] ± 0.2
Semimembranosus						
Change in (cooked vs fresh):						
Length, %	−39.9 ± 0.6	−39.5 ± 0.6	−40.7[a] ± 0.8	−37.5[b] ± 0.6	−27.3[a] ± 0.5	−33.5[b] ± 0.6
Width, %	−11.9 ± 0.3	−10.2 ± 0.3	−14.4 ± 0.3	−12.9 ± 0.3	−15.5 ± 0.4	−18.4 ± 0.4
Depth, %	+51.3[a] ± 0.8	+22.7[b] ± 0.4	+53.4[a] ± 0.9	+26.7[b] ± 0.5	+36.8[a] ± 0.6	+29.2[b] ± 0.5

[a,b,c] Means in the same row, within the same cooking treatment, with different superscripts are different (P<0.05). (Comparison of cold vs hot-boning.)

Table 4—Influence of cooking treatment and postmortem boning on cooking time of beef roasts

Cooking treatment	Method of boning	Muscle	
		ST	SM
1. Low temp, min/kg	HB	185.63 ± 7.8	127.14^a ± 5.2
	CB	182.00 ± 8.2	141.67^b ± 6.0
2. Medium temp, min/kg	HB	145.04^a ± 5.7	92.60^a ± 4.0
	CB	158.12^b ± 6.1	110.80^b ± 4.4
3. High temp, min/kg	HB	109.43^a ± 4.5	82.45^a ± 3.2
	CB	161.83^b ± 6.1	96.61^b ± 4.1
Combined temp, min/kg	HB	146.70	100.73
	CB	167.32	116.36

^{a,b} Means in the same column, within cooking treatment and muscle, with different superscripts are different (P<0.05). (Comparison of hot vs cold-boning.)

CB, SM muscle. Elevating the initial temperature from 47°C to 52°C (tmt 2) did result (P < 0.05) in a decrease in tenderness of the product, but the WBS and the trained panel values are similar (P > 0.05) for roasts from HB and CB muscles. Further increasing of the initial cooking temperature (58°C, tmt 3) resulted in a marked reduction in tenderness (P < 0.05) for the HB roasts but had no effect upon the CB samples. Sarcomeres of HB cooked SM samples were shorter (P < 0.05) than for CB roasts. The sarcomere lengths (1.98μ) reported for HB roasts are longer than those reported by West et al. (1980) for HB roasts (1.5μ). These differences in sarcomere length could be attributed to the higher temperature (57.2°C) of the water in the cooking vat (West et al., 1980), which may have resulted in extreme shortening of the sarcomeres if heat induced rigor was initiated. Evaluation of tenderness by the trained panel was not performed, because the ST samples in this study thawed while in storage. The WBS values (Table 7) indicate that the ST muscles which were HB were not as tender, even though they are in an acceptable range, as the paired CB roasts (P < 0.05) over all cooking treatments. However, the WBS values for HB roasts from the ST are comparable to those values reported by Ray et al. (1982) for roasts from CB bull carcasses. The values for WBS reported in this study are lower (3.71 vs 7.45, HB and 3.00 vs 3.92, CB) than the values reported for the ST from an earlier study in which 68°C temperature was used for cooking (Ray et al., 1980).

CONCLUSIONS

LOW TEMPERATURE-LONG DURATION cooking of hot-boned SM beef roasts resulted in a product that was comparable to CB cooked product in tenderness, even though muscle shortening and shape distortion were noted in the HB product. HB roasts from the ST exhibited a marked improvement in tenderness over the values (WBS) from previous studies, but the magnitude of improvement (HB vs CB) was not as great as for the SM roasts. Shortening during cooking for the HB, ST was greater than for the HB, SM roasts and may have resulted in reduced tenderness for the HB, ST roasts. The slow, low temperature cooking may have permitted the muscle to have less severe rigor development during cooking, rather than the more severe rigor induced by subjecting the muscle to an initial high cooking temperature (68°C), as was done in earlier studies (Ray et al., 1980, 1982; Berry et al., 1981; Griffin et al., 1981). Cooking time and cooking losses were reduced (P < 0.05) for HB, ST and SM roasts when compared with their CB counterparts in this study.

Table 5—Influence of cooking treatment and postmortem boning on cooking losses of beef roasts

Cooking treatment	Method of boning	Muscle	
		ST	SM
1. Low temp, Cooking loss, %	HB	9.98^a ± 0.11	13.28^a ± 0.08
	CB	17.04^b ± 0.18	18.67^b ± 0.23
2. Medium temp, Cooking loss, %	HB	11.06^a ± 0.10	14.40^a ± 0.12
	CB	16.28^b ± 0.14	17.84^b ± 0.23
3. High temp, Cooking loss, %	HB	10.62^a ± 0.09	14.84^a ± 0.13
	CB	19.50^b ± 0.23	18.91^b ± 0.22
Combined temp, Cooking loss, %	HB	10.55	14.17
	CB	17.61	18.47

^{a,b} Means in the same column, within cooking treatment and muscle, with different superscripts are different (P<0.05). (Comparison of hot vs cold-boning)

Table 6 — Influence of cooking treatment and type of boning on tenderness of semimembranosus roasts

Cooking treatment	Cooking time^f	Type of boning	
		HB	CB
1. Low temp, WBS^g	6.9 hr	2.52^a ± 0.20	2.45^a ± 0.33
Trained panel^h		6.90^a ± 0.30	7.10^a ± 0.35
2. Medium temp, WBS^g	5.5 hr	3.61^b ± 0.34	3.51^b ± 0.39
Trained panel		6.30^b ± 0.45	6.40^b ± 0.36
3. High temp, WBS^g	5 hr	4.99^{c,d} ± 0.48	3.06^{c,e} ± 0.31
Trained panel		5.10^{c,d} ± 0.25	6.00^{c,e} ± 0.30
Cooked product, sarcomere length, μ		1.98^d ± 0.10	2.20^e ± 0.10

^{a,b,c} Means in the same column with different superscripts are different (P<0.05).
^{d,e} Means in the same row with different superscripts are different (P<0.05).
^{f} Mean length of cooking period.
^{g} Warner-Bratzler shear force, kg/1.27 cm.
^{h} Nine member trained panel. Scoring system based on 8 = extremely tender; 1 = extremely tough.

Table 7 — Influence of type of boning and cooking treatment on WBS values of semitendinosus roasts

Cooking treatment	Cooking time^f	Type of boning	
		HB	CB
1. Low temp, WBS^g	7.2 hr	3.50^{a,d} ± 0.18	2.1^{a,e} ± 0.10
2. Medium temp, WBS^g	5.9 hr	5.59^{b,d} ± 0.45	3.27^{b,e} ± 0.17
3. High temp, WBS^g	5.2 hr	5.86^{b,d} ± 0.48	3.94^{c,e} ± 0.35

^{a,b,c} Means in the same column, within type of boning, with different superscripts are different (P<0.05).
^{d,e} Means in the same row with different superscripts are different (P<0.05).
^{f} Mean length of cooking period.
^{g} Warner-Bratzler shear force, kg 1.27 cm

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Characteristics of Pre-Rigor Pressurized Versus Conventionally Processed Beef Cooked by Microwaves and by Broiling

L. M. RIFFERO, and Z. A. HOLMES

─────────ABSTRACT─────────

Eighty-six grams paired beef semitendinosus portions processed either conventionally or by pre-rigor pressure were broiled or microwave cooked. Pre-rigor pressure-treated cooked beef portions were higher (P < 0.05) than untreated portions in total moisture, pH, exterior color a+ values and subjective tenderness and ease of fiber separation scores than did the untreated portions. Total moisture, drip cooking loss, interior a+ (redness) color value, and exterior L (lightness) and b+ (yellowness) color values were significantly higher in the microwaved beef portions as compared to the broiled portions. Neither juiciness nor flavor of samples were influenced (P < 0.05) by treatment or by cooking method.

INTRODUCTION

A PORTION of the price increase seen in beef can be attributed to the cost of energy consumption during production, processing, and preparation. An accelerated processing system such as pre-rigor pressure (PRP) treatment has the advantage of decreased energy consumption for refrigeration, refrigerated space, labor, transportation and inventory costs (West, 1982). This process involves hot-boning of muscles soon after slaughter, vacuum packaging, pressure treatment and chilling of those portions to be consumed (Macfarlane, 1973; Kennick et al., 1980). The use of microwave ovens during the preparation of meat is an energy saving processing technique which could reduce utility costs for the food processor, the foodservice industry, and the consumer (Mandigo and Janssen, 1982). The combination of marketing of pre-rigor pressurized beef for the institutional and/or retail markets with recommendations for microwave oven cooking presents a feasible potential for energy savings. The development of data on the influence of traditional and new cooking methods on pre-rigor pressurized muscle may thus further the use of this product.

The objective of this research was to investigate the influence of microwave and broiling cooking methods on the quality characteristics of portion cuts of pre-rigor pressurized (PRP) beef semitendinosus muscle. The pre-rigor pressurized beef was compared to conventional processed portion sized beef cuts.

MATERIALS & METHODS

Sample preparation

Samples were obtained from six beef cattle (approximate weight 510 kg) slaughtered at Oregon State University Meat Science Laboratory. Eye-of-round muscle (semitendinosus) from one side of each carcass was excised immediately after washing the beef carcasses (approximately 25 min after slaughtering), vacuum packed in Cry-O-Vac bags, inserted into a pressure chamber (10.2 cm in diameter and 30.5 cm long) which was then tightly closed and a pressure of 103.5 MNm-2 (15,000 lb/sq in) was applied for 2 min, removed, and stored at −18°C (Frigidaire Food Freezer Model 191, General Motors, Dayton, Ohio). Matching muscles on the opposite sides

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were left on the carcasses and chilled at 0 ± 1°C according to commercial practices. On the 7th day the matching muscles from the control sides were removed from the carcasses, vacuum packed, and stored at −18°C along with the treated samples until required for further study.

Samples for the microwave and broiling treatments were obtained by cutting each frozen semitendinosus muscle into a 6 x 5 x 3 cm piece (approximately 86g). These were individually wrapped and frozen until use. Prior to cooking all frozen samples (18°C) were defrosted (Precision Scientific Incubator, Freas 815, Precision Scientific Subsidary of GCA Corp.) 1 hr at 25°C and 17 hr at 5°C.

Cooking methods

The defrosted pre-rigor pressurized and control samples (5°C) for each treatment were randomly assigned to microwave or broiling treatments. Duplicate samples of each replication were prepared. Each sample was individually cooked.

Defrosted pre-rigor pressurized sample and control sample were placed on a Pyrex casserole dish and microwave cooked 1.08 min (endpoint temperature approximately 80 ± 6°C). A Sharp Carousel Microwave Oven R-6770, 2450 MHz, 455 Watts (Sharp Electronics Corp., Paramus, NJ) was used with the variable cooking control on ROAST. Final internal temperature was recorded in the mid-portion of the sample.

Defrosted pre-rigor pressurized and control sample was broiled in a preheated self-cleaning electric oven (Magic Chef, Model X876-40X, Cleveland, TN) set on broil. Samples were placed on a wire rack in an aluminum pan 25 cm from the boiling unit. Each sample was cooked to an internal endpoint temperature of 80 ± 2°C for approximately 24 and 25 min for pre-rigor pressurized and control samples, respectively.

Objective testing methods

Water-holding capacity, total moisture, pH, nitrogen content, color, and tenderness were determined on all cooked and raw meat. Lipid content from selected samples was also determined on the raw meat. Cooking losses, temperature/time data and micrographs were collected.

Samples for water-holding capacity, tenderness and electron microscopy were obtained from the excised intact muscle sample. Total moisture, pH, total nitrogen, and total lipids were determined on the defrosted powdered muscle. The raw or cooked samples were frozen in liquid nitrogen and ground (Osterizer Cycle Blender, John Oster Manufacturing Co., Milwaukee, WI) into powder for 30 sec at "liquify speed."

The method of Wierbicki and Deatherage (1958) was used to measure the water-holding capacity of the meat. The areas of the pressed meat were measured with a Licor Area Meter (Model 3100) and the expressible moisture index (EMI) was calculated. Moisture content was determined according to the AOAC vacuum oven method (Horwitz, 1980). Duplicate 5-g samples from each replication and treatment of liquid nitrogen-powdered beef meat were dried. Nitrogen content on duplicate samples was determined by the micro-Kjeldahl method (Horwitz, 1980). Nitrogen was expressed as percent of wet weight and dry weight. Total lipids of the duplicate raw beef samples were analyzed using a modification of the chloroform-methanol procedure (Folch et al., 1957). Total lipid content was expressed on a wet weight and dry weight basis. The uniformity observed in percent lipid and percent nitrogen of paired muscles substantiated the accuracy of the protein, moisture and lipid measurements and consistency of replication uniformity.

Total, drip and evaporation cooking losses were calculated using cooking weight loss of meat and drip weight. Internal temperatures during heating of the broiled samples were monitored with a Leeds and Northrup W12 Temperature Recorder (Leeds and Northrup,

Portland, OR). Endpoint temperatures of the microwaved samples were obtained by inserting a sabre thermocouple in the mid-portion of the steak immediately after removal from the oven.

Color difference values were measured on the interior and exterior surfaces of the cooked and raw beef samples using a Hunter Color Difference Meter (Model D25P-2, Hunter Associates Laboratories, 11495 Sunset Hills Road, Reston, VA 22090) standardized against a white ceramic tile with calculated values of L (lightness), 94.0; a+ (redness), −0.9; and b+ (yellowness), 1.2. Samples were placed in a plexiglass cell and duplicate measurements were made for each color difference factor. A 0.5 cm slice was cut from the cooked meat surface to obtain interior color values.

Dupliate 2-g powdered meat samples were mixed with 10 mL deionized water and pH determined (Orion Research Microprocessor Ionanalyzer/901, Orion Research Inc., Cambridge, MA; calibration buffer pH 6.84 and pH 4.00).

Tenderness was evaluated using a 1.27 cm core meat sample on a Warner-Bratzler Shear Apparatus (25 kg × 50g dynamometer scale, G.R. Electric Mfg. Co., 1317 Collins Lane, Manhattan, KS).

Excised muscle pieces were removed from the raw and cooked beef meat and placed in glutaraldehyde and refrigerated until prepared for scanning electron microscopy. Samples for SEM's were critical point dried and fastened to aluminum planchets with colloidal silver paint. Prepared planchets were fastened in a rotation tilting device (Fullam #1253, Ernest Fullam, Inc., Schenectady, NY) in a vacuum evaporator. Scanning electron micrographs were obtained using an AMRAY 1000A scanning electron microscope. Magnification of 300x was recorded.

Subjective testing methods

Panelists were selected by their performance during screening sessions, and then trained for sensory evaluation of the treatment samples. The trained panel of six Oregon State University staff members evaluated the tenderness (6=extremely tender; 1=extremely tough), fiber separaration (6=extremely easily separated fibers; 1= no separable fibers), juiciness (6=extremely juicy; 1=extremely dry), and flavor (6=extremely pronounced meaty flavor; 1=no meaty flavor) of one 1.5 cm cubed sample from each treatment per replication. The samples were evaluated at room temperature (21°C) using established procedures (ASTM, 1977).

Experimental design and data analysis

A block experimental design consisting of all treatments at each replication period was used. A one-way analysis of variance (Anonymous, 1978) for raw samples and a two-way analysis of variance (Rowe and Brenne, 1981) for the cooked samples were used to evaluate all data. Correlations (Rowe and Brenne, 1981) were evaluated for selected parameters. Significance was determined at the 5% level of probability.

RESULTS & DISCUSSION

EXPRESSIBLE MOISTURE INDEX (EMI) measurements for the cooked and raw beef semitendinosus portions are presented in Tables 1 and 2. Lower EMI values indicate higher water-holding capacity (WHC). The beef prepared by microwave and broiling cooking methods were not significantly different in EMI values. EMI measurements were not significantly lower ($P < 0.05$) for pressure-treated than untreated beef portions regardless of cooking method (Tables 1 and 3). However, no significant ($P < 0.05$) differences due to type of treatment were found in the raw beef portions (Table 2), although the pressure-treated meat had slightly lower EMI values. These higher values for water-holding capacity in the current study do not agree with work reported by Kennick et al. (1980). They found lower water-holding capacity in raw and cooked pressurized beef semitendinosus muscle. The higher water-holding capacity observed for the cooked and raw beef portions in this study may be due to differences in animal characteristics, techniques in determining water-holding capacity, or improvement in the sample pressurization process. The significantly greater ($P < 0.05$) pH of the cooked pressure-treated beef semitendinosus portions in the current study in comparison to the untreated samples may account for the increased WHC (Table 1).

Total moisture values were significantly greater ($P < 0.05$) for the pressure-treated samples when either broiled or microwave cooked (Tables 1 and 3). Microwave cooking

Table 1—Mean values of proximate composition and quality characteristics of microwaved and broiled untreated and pressure-treated beef semitendinosus portions

Parameters	Microwaved		Broiled	
	Untreated	Pressure treated	Untreated	Pressure treated
Expressible moisture Index (EMI)	0.342 ± 0.05	0.250 ± 0.02	0.353 ± 0.08	0.262 ± 0.04
Total moisture (%)	62.24 ± 2.2	66.22 ± 1.5	60.31 ± 1.9	61.32 ± 4.5
Total nitrogen[b] (%)	4.8 ± 0.4[a]	4.5 ± 0.3	5.2 ± 0.3	4.7 ± 0.2
pH	5.8 ± 0.0	5.8 ± 0.1	5.7 ± 0.0	5.9 ± 0.1
Cooking losses (%)				
Total	26.2 ± 6.1	18.0 ± 1.4	36.9 ± 3.0	32.3 ± 2.3
Drip	15.4 ± 5.9	7.9 ± 1.2	3.3 ± 0.8	2.1 ± 0.7
Evaporation	10.8 ± 0.7	10.1 ± 0.8	33.5 ± 3.1	30.2 ± 2.7
Color evaluation				
Exterior				
L	44.2 ± 1.7	43.7 ± 2.9	31.4 ± 2.7	31.2 ± 2.6
a+	4.4 ± 0.0	5.2 ± 0.8	4.7 ± 0.9	5.1 ± 0.2
b+	10.1 ± 0.4	9.9 ± 0.5	8.1 ± 1.4	8.1 ± 1.4
Interior				
L	48.8 ± 1.9	47.5 ± 3.7	49.5 ± 2.7	46.1 ± 2.6
a+	3.7 ± 1.0	4.3 ± 0.8	2.4 ± 0.5	2.5 ± 0.3
b+	9.2 ± 0.4	9.1 ± 0.5	9.6 ± 0.2	9.3 ± 0.5
Sheat value (kg/1.27 cm)	3.6 ± 0.5	2.5 ± 0.4	3.9 ± 1.2	2.1 ± 0.6
Sensory evaluation[c]				
Tenderness	2.8 ± 0.6	3.7 ± 0.5	2.6 ± 0.6	3.3 ± 0.4
Fiber separation	2.6 ± 0.5	2.8 ± 0.6	2.3 ± 0.5	3.0 ± 0.4
Juiciness	3.3 ± 0.6	3.4 ± 0.4	3.2 ± 0.7	2.8 ± 0.5
Flavor	3.4 ± 0.6	3.2 ± 0.5	3.6 ± 0.6	3.2 ± 0.4

[a] Values represent mean ± S.D.
[b] Wet weight basis.
[c] Sensory evaluation: tenderness (6:extremely tender, 1:extremely tough); fiber separation (6:extremely easily separated fibers, 1:no separable fibers); juiciness (6:extremely juicy, 1:extremely dry); flavor (6:extremely pronounced meaty flavor; 1:no meaty flavor).

of the meat retained a significantly greater (P < 0.05) amount of moisture than broiling in both the untreated and pressure-treated samples (Tables 1 and 3). Janicki and Appledorf (1974) found greater total moisture in broiled beef patties than in microwave cooked patties. The difference in water content and size between beef patties and beef portions may account for variations in total moisture. Microwave cooking may be the preferred method over broiling for cooking the pressurized beef portions to attain greater total moisture content. Total moisture was not significantly different (P < 0.05) for the two treatments in the raw samples (Table 3).

Total, drip, and evaporation cooking loss values (Table 1) were affected by both treatment and cooking method. Total cooking losses were significantly greater (P < 0.05) for the cooked untreated broiled beef portions (Table 3). Drip cooking losses were significantly greater (P < 0.05) for the untreated microwave cooked beef portions. Evaporation cooking losses were significantly greater for cooked untreated beef portions. Broiling resulted in significantly greater evaporation cooking losses in the beef portions than microwave cooking. The lower cooking losses in the pressure-treated beef portions may indicate greater water retention upon heating. This is supported by increased water-holding capacity and total moisture values. Kennick et al. (1980) found lower cooking losses and water-holding capacity for pressure-treated beef semitendinosus muscles as compared to untreated controls. Macfarlane (1973) attributed decreasing cooking losses in pre-rigor pressurized ox muscle to exposure of hydrophilic groups in the myofibrillar protein resulting in greater hydrogen bonding of water.

The higher drip cooking loss with microwave cooking is consistent with results by other investigators (Korschgen et al., 1976; Moody et al., 1978; Moore et al., 1980). Increased drip cooking loss with microwave cooking may be due to reduced evaporation from the cooler container surface. Moore et al. (1980) and McCrae and Paul (1974) also found lower evaporation cooking loss in microwave cooked beef steaks as compared to conventional methods of cookery. These investigators attributed lower volatile losses with microwave cooking to decreased cooking time and the surrounding low oven temperature in the microwave oven. Microwave cooking of the pressure-treated beef portions resulted in lower cooking losses than the broiled untreated or the pressure treated portions.

Although a positive correlation (r = +0.92; P < 0.05) exists between juiciness and total moisture, the greater total moisture content of microwave cooked beef portions

was not reflected in panelists' juiciness evaluations. Panelists did not detect any significant differences in juiciness due to treatment or cooking method (Tables 1 and 3). In contrast, other investigators have reported decreased juiciness and moisture content in beef meat subjected to pressure treatment or microwave cooking (Kennick and Elgasim, 1981; Kylen et al., 1964; Moore et al., 1980).

Hunter color difference values for the interior and exterior surface of the cooked and raw beef portions are presented in Tables 1 and 2. Interior color measurements for L (lightness) in the cooked beef portions were not significantly different (P < 0.05) between the untreated and pressure-treated beef portions or the microwave cooked and broiled beef samples (Table 3). Type of treatment or cooking method also did not significantly influence interior b+ (yellowness) values in the cooked samples. The interior a+ (redness) values of the cooked beef portions were not significantly different (P < 0.05) between the untreated and pressure-treated samples (Table 3). However, the microwave cooked beef portions had significantly greater (P < 0.05) a+ values than the broiled samples. The higher a+ values in the microwave beef portions indicates less oxidation or heat denaturation of the myoglobin pigment. Subjective observations of the microwave beef portions revealed uneven heating which may account for variations in a+ values between microwaved and broiled samples. There was also considerable replication variation.

Pressure treatment did not significantly (P < 0.05) affect the exterior L and b; values of the cooked beef portions (Table 3). Exterior surface color measurements for the cooked beef portions were significantly different between cooking methods for the L and b+ color values. Broiling resulted in lower L and b+ values than microwave cooking. The a+ color values were not significantly different between the microwaved and broiled beef portions. However, the a+ values in the cooked pressure-treated portions were significantly higher (P < 0.05) than for the untreated portions. The lower color values of the broiled beef portions indicate greater browning due to denaturation of the meat pigments and from the carbonyl-amine brown-

Table 2—Mean and F-values of proximate composition and quality characteristics of raw untreated and pressure treated beef semitendinosus portions

| Parameters | Treatment | | F-value[a] |
	Untreated	Pressure treated	
Expressible moisture			
Index (EMI)	0.419 ± 0.06	0.364 ± 0.06	2.741
Total moisture (%)	72.26 ± 0.8	72.70 ± 1.2	0.563
Total nitrogen[b] (%)	3.4 ± 0.3[c]	3.2 ± 0.3	1.160
Total lipids[b] (%)	3.9 ± 0.4	3.3 ± 1.3	1.072
pH	5.5 ± 0.0	5.5 ± 0.0	0.309
Color evaluation			
Exterior			
L	32.4 ± 1.7	31.0 ± 1.7	1.946
a+	10.3 ± 1.3	9.7 ± 1.3	0.611
b+	8.1 ± 0.9	6.6 ± 0.4	12.577*

[a] F-values were significant if F-value was ≥ 4.96 (*p < 0.05).
[b] Wet weight basis.
[c] Values represent mean ± S.D.

Table 3—Treatment, cooking method and interaction F-values used to evaluate microwaved and broiled untreated and pressure treated beef semitendinosus portions

| Parameters | F-value[a] | | |
	Treatment	Cooking method	Interaction
Expressible moisture			
Index (EMI)	19.205*	0.281	0.000
Total moisture (%)	4.869*	9.122*	1.716
Total nitrogen[b] (%)	11.691*	4.774*	0.802
pH	16.643*	0.222	9.118*
Cooking losses (%)			
Total	18.099*	69.671*	1.437
Drip	12.050*	50.724*	6.137
Evaporation	5.239*	594.921*	2.228*
Color evaluation			
Exterior			
L	0.100	152.326*	0.019
a+	4.465*	0.023	0.586
b+	0.088	19.889*	0.066
Interior			
L	3.985	0.068	0.828
a+	2.050	26.979*	0.663
b+	1.295	1.763	0.324
Shear value (kg/1.27 cm)	23.015*	0.019	1.161
Sensory evaluation			
Tenderness	13.436*	2.125	0.256
Fiber separation	4.600*	0.224	1.473
Juiciness	0.290	1.527	1.103
Flavor	2.490	0.285	0.245

[a] F-values were statistically significant if F-value was ≥ 4.35 (*p < 0.05).
[b] Wet weight basis.

ing reaction. The different principles of heating for each cooking method may account for variations in the extent of browning. The exterior b+ color value for the raw untreated beef portions was significantly higher (P < 0.05) than that of the raw pressure-treated portions (Table 3).

The pressure-treated cooked beef portions showed a significantly higher (P < 0.05) pH than the untreated samples regardless of cooking method (Tables 1 and 3). The pH was not significantly different between the microwave cooked and broiled beef portions. However, there was a significant (P < 0.05) interaction between type of treatment and cooking method. No significant differences in pH were found between raw untreated and pressure-treated meat (Table 2). Kennick and Elgasim (1981) also found that pressure treatment had no significant effect on the ultimate pH of beef semitendinosus muscle. Macfarlane and McKenzie (1976) stated that pressure treatment favors the release of imidazolium groups of histidine. This may account for the significantly higher pH of the pressure-treated beef portions upon cooking.
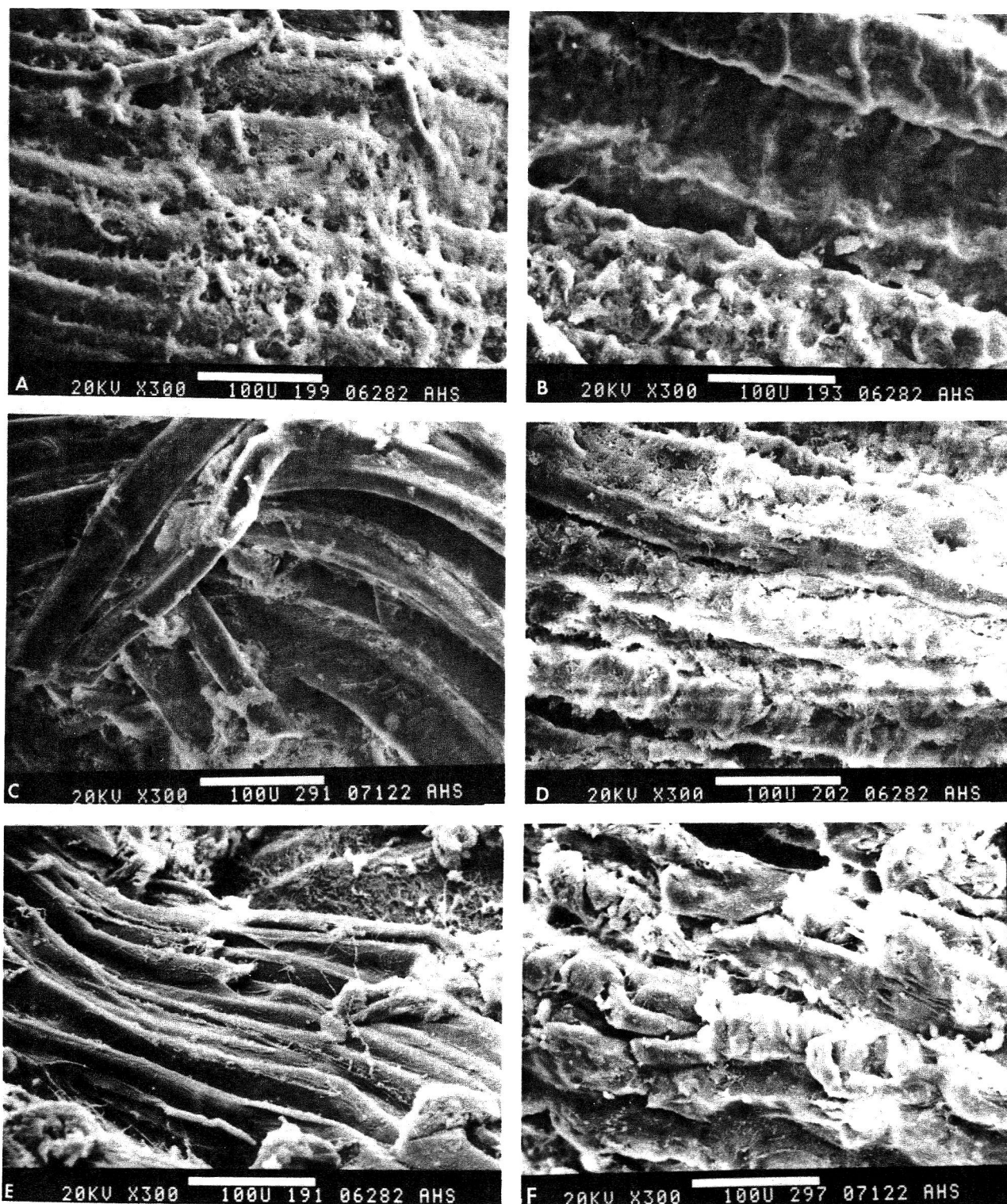
Fig. 1—Scanning electron microscope micrograph (300x) of beef semitendinosus muscle: (A) microwave untreated portion; (B) microwave pressure-treated portion; (C) broiled untreated portion; (D) broiled pressure-treated portion; (E) raw untreated beef muscle; (F) raw pressure-treated beef muscle.

Warner-Bratzler shear force values for the raw and cooked beef portions are presented in Tables 1 and 2. Shear values were significantly lower ($P < 0.05$) for the cooked pressure-treated beef portions than the untreated portions regardless of cooking method (Tables 1 and 3). Lower Warner-Bratzler shear values reflect an increase in tenderness for the cooked pressure-treated beef portions. Shear values tended to be lower in raw pressure-treated than for untreated muscle, although the differences was not significant. Other investigators also reported lower Warner-Bratzler shear values with pressure-treated versus untreated raw and cooked beef semitendinosus muscle (Bouton et al., 1977a, b; Kennick and Elgasim, 1981). Bouton et al. (1977a) attributed the lower Warner-Bratzler shear values with pressure treatment primarily to changes in the myofibrillar component of toughness. Warner-Bratzler shear values were not significantly ($P < 0.05$) different between the microwave cooked and broiled beef portions (Table 3). Hostetler and Dutson (1978) have also reported no significant differences in average shear force values between microwave cooked and broiled beef semimembranosus muscle.

Panelists evaluation of tenderness indicated that pressure-treated beef portions were significantly ($P < 0.05$) more tender than corresponding untreated portions (Tables 1 and 3). No significant differences in tenderness values were found between the microwave cooked and broiled beef portions. Other investigators have also reported greater tenderness in beef semitendinosus muscle subjected to pre-rigor pressure treatment (Bouton et al., 1977b; Kennick and Elgasim, 1981). The tenderizing effect from pre-rigor pressure treatment may be attributed to breakdown of the myofibrillar structure, early release of lysosomal enzymes, creation of breaks in fiber structure as a result of massive contractions, and/or F-G transformation of actin (Kennick and Elgasim, 1981). Tenderness scores and Warner-Bratzler shear values in the current study were significantly correlated ($r = -0.94$; $P < 0.05$) for the broiled untreated beef portions. The results indicate the potential use of pre-rigor pressure treatment to improve the tenderness of tougher cuts of meat.

Panel scores for ease of fiber separation in the cooked beef portions are presented in Table 1. Ease of fiber separation was a measurement of one aspect of tenderness as indicated by the significant correlation ($r = -0.82$; $P < 0.05$) between Warner-Bratzler shear values and ease of fiber separation in the broiled treated beef portions. The pressure-treated cooked beef portions received significantly higher ($P < 0.05$) scores for ease of fiber separation than the untreated portions (Tables 1 and 3). No significant differences in the ease of fiber separation were found between the microwave cooked and broiled beef portions regardless of type of treatment. Panelists' tenderness and ease of fiber separation scores agreed with the Warner-Bratzler shear values in showing a significant ($P < 0.05$) effect from pressure treatment but no significant influence from cooking method on tenderness.

Panel flavor evaluation scores of the beef portions were not significantly ($P < 0.05$) different for treatment or cooking method (Table 3). Kennick and Elgasim (1981) also reported no significant differences in flavor scores between control and pressure treated eye-of-round cuts. The nonsignificant differences in flavor scores of beef cooked by microwave and broiling methods in the current study is in agreement with work reported by Baldwin et al. (1979). Since samples in the current study were obtained from the interior of the beef portions, the amine-sugar (Maillard) reaction likely had a smaller role in flavor development.

Preliminary investigation using the scanning electron microscope (SEM) indicated differences in ultrastructure due to cooking method and pressure treatment (Fig. 1). Observation of the raw pressure-treated beef semitendinosus muscle reveals extensive fraying of fibers in contrast to the untreated muscle (Fig. 1). Kennick et al. (1980) also reported extensive fiber fraying and disruption of the sarcolemma with pre-rigor pressure treatment of beef semitendinosus muscle. Through examination of the SEM micrographs in Fig. 1 it appears that pressure treatment resulted in greater myofibrillar fragmentation of the cooked beef portions. More granular material which may be a mixture of heat denatured collagen and coagulated sarcoplasmic protein was observed in the broiled pressure-treated than untreated beef portions. Microwave cooked beef portions showed more fiber fragmentation and granulated material than the broiled beef portions. Hutton et al. (1981) also found greater fiber fragmentation and coagulated material in microwaved beef semitendinosus muscle cooked to a 70°C endpoint temperature as compared to conventionally heated muscle. The analysis of the SEM's in Fig. 1 and others, was supported by preliminary examination of the samples under TEM. Microwave cooking of the pressure treated beef portions appeared to result in greater changes in myofibril structure than broiling. Further work is needed due to the nature of sampling.

This study investigated the influence of microwave and broiling cooking methods on quality parameters of portion size cuts of beef semitendinosus muscle subjected to pre-rigor pressure treatment. Although total moisture, cooking losses, and color were significantly different between microwave cooked and broiled beef portions, in general microwave and broiling cooking methods gave comparable results for quality parameters in pre-rigor pressurized beef portions. The results of this study indicate the feasibility of pre-rigor pressurization for use by the meat industry.

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Retardation by Glandless Cottonseed Flour of Lipid Oxidation and Discoloration in Raw Ground Beef Containing Salt

KI SOON RHEE, GARY C. SMITH, and KHEE CHOON RHEE

─────────ABSTRACT─────────

Salt (NaCl) was added to raw ground beef at levels of 0, 0.5, 1, 2 or 3% of the weight of the meat and defatted glandless cottonseed flour (GCF) was added at 0 or 3% levels. Patties were made with these mixes and stored for 3 or 6 days at 4°C or for 30 or 60 days at −20°C. Lipid oxidation (TBA values) in samples stored at 4° and −20°C and discoloration in samples stored at 4°C were determined. In samples made with salt only, TBA values within each storage period for each storage temperature increased with increasing salt levels up to 2%; increasing salt from 2 to 3% decreased TBA values. GCF markedly reduced TBA values at each salt level. Discoloration of samples stored at 4°C was also decreased by GCF.

INTRODUCTION

OXIDATION OF LIPIDS and pigments are two major nonmicrobial causes of quality deterioration in raw meats. Meat pigments (hemes) provide catalysts for lipid oxidation, and free radical intermediates therefrom can in turn decompose hemes, resulting in discoloration. Propyl gallate or butylated hydroxyanisole, alone or in combination with ascorbic acid, is known to inhibit both lipid and pigment oxidation in raw meats (Greene, 1969; Greene et al., 1971). Lipid oxidation occurs much more readily in cooked meat than in raw meat. However, when meat is ground, exposed to air, and salt is added, lipid oxidation can progress rapidly in raw meat (Rhee et al., 1983).

Food ingredients (defatted flour and protein concentrate and isolate) prepared from glandless cottonseeds were reported to retard lipid oxidation in cooked ground beef patties (Ziprin et al., 1981). When all-beef patties were stored in gravy containing these cottonseed ingredients, lipid oxidation was also decreased (Rhee and Ziprin, 1981). In addition, alcoholic extracts of the glandless cottonseed ingredients showed uniquely and consistently higher antioxidant activities in model lipid oxidation systems than did extracts of other oilseed ingredients (Rhee et al., 1981). The nature of antioxidant compounds in glandless cottonseeds is not clearly known, but phenolic compounds (flavonoids and phenolic acids) may be of major importance (Rhee et al., 1981).

The present paper reports on the effects of addition of defatted glandless cottonseed flour to raw ground beef containing different levels of salt on lipid oxidation during refrigerated and frozen storage and discoloration during refrigerated storage of the raw meat samples.

MATERIALS & METHODS

DEFATTED GLANDLESS COTTONSEED FLOUR (GCF) was prepared by hexane extraction on pilot-plant scale at the Food Protein Research and Development Center, Texas A&M University. Lean ground beef was purchased locally and mixed thoroughly by hand with additives (sodium chloride and/or GCF). Sodium chloride

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was added at levels of 0, 0.5, 1, 2 or 3% of the weight of the meat and GCF was added at 0 or 3% levels. Patties (115g, 9.2 cm diameter, 1.7 cm thickness) were formed using a hamburger press, packaged in Whirl-Pak bags (transparent) with no vacuum and stored for 3 or 6 days at 4°C or for 30 or 60 days at −20°C.

Fat and moisture contents were determined by the AOAC (1975) methods. pH was determined by blending a 10-g portion of each sample for 1 min with 100 ml of distilled-deionized water and reading the pH of the slurry in duplicate.

Lipid oxidation was determined by a modified distillation thiobarbituric acid (TBA) procedure described by Rhee (1978); 5 ml of a 0.5% solution of propyl gallate and EDTA were added for each 10-g sample during the blending process. To minimize variation due to sampling, 60g of sample were blended with 90ml of distilled deionized water and 30ml of propyl gallate-EDTA solution, and 30g of this slurry (equivalent to 10g sample) were used for each distillation. Results are expressed as TBA number (mg malonaldehyde/kg sample).

Distinctive color differences were noticed among samples of different treatments when they were removed from refrigeration for TBA test at day 3. Hence, objective and subjective color evaluations were made on samples stored for 6 days at 4°C.

Objective color values for samples stored at 4°C for 6 days were determined by a Hunter color difference meter (Model D25D2) calibrated with Standard Plate No. C2-857. One-half of each patty was mixed thoroughly before packing into the sample vessel. Values for redness ("a" values) were determined.

Subjective (visual) color scores for samples stored at 4°C for 6 days were determined using a panel of 8 experienced judges who had frequently served as sensory judges for a variety of meat products including fresh (raw) beef. Color evaluations were made after mixing one-half of each patty in the storage bag. Samples were presented in the storage bags to the panel and were scored for color desirability (as raw ground beef) on a scale of 1 to 8 (1 = extremely undesirable; 8 = extremely desirable).

Data were analyzed by analysis of variance and means were separated by using the multiple range test of Duncan (1955).

RESULTS & DISCUSSION

THE MEAN MOISTURE CONTENT of samples was 68.03% (standard error of the mean, SEM: 0.39) and the mean fat content was 8.40% (SEM: 0.17). Although moisture and fat contents varied among different treatments, the variations were not proportional to the amounts of additives in samples. The addition of GCF to ground beef, however, significantly affected pH (Table 1); samples containing GCF were consistently higher in pH than those without GCF (pH of GCF alone: 6.31).

TBA values of refrigerated and frozen samples are shown in Tables 2 and 3, respectively. In samples made with salt only, TBA values within each storage period increased with increasing salt levels up to 2%; increasing salt from 2 to 3% decreased TBA values. This was true whether samples were stored at 4°C (Table 2) or −20°C (Table 3). However, when GCF was added to samples containing these same levels of salt, there was no consistent trend in TBA values for increasing salt levels as shown when samples were made with salt only. The addition of GCF to raw ground beef markedly reduced TBA values at each level of salt. Reduction of TBA values by GCF was about 50% for each salt level when samples were stored at 4°C for either 3 or 6 days; the reduction was less (i.e., ≤ 40%) when samples were stored at −20°C for 30 or 60 days. A smaller reduction of TBA

Table 1—pH values of raw ground beef as affected by different treatments

Treatment[a]	pH[b]
None	5.80e,f
0.5% NaCl	5.82d,e
1.0% NaCl	5.79f
2.0% NaCl	5.83d
3.0% NaCl	5.83d
0.5% NaCl + 3% GCF	5.99c
1.0% NaCl + 3% GCF	6.03b
2.0% NaCl + 3% GCF	6.08a
3.0% NaCl + 3% GCF	6.10a

[a] GCF: defatted glandless cottonseed flour.
[b] Two observations for each mean. Means in a column which are not followed by a common letter are significantly different (P < 0.05).

Table 3—TBA values of raw ground beef samples stored at −20°C

Treatment	TBA number[a] at day		% Reduction[b] of TBA value by GCF at day	
	30	60	30	60
None	2.46d	2.58c	—	—
0.5% NaCl	2.68c	2.76b	—	—
1.0% NaCl	2.75b	2.84a	—	—
2.0% NaCl	2.96a	2.87a	—	—
3.0% NaCl	2.49d	2.60c	—	—
0.5% NaCl + 3% GCF	1.70g	1.93d	37	30
1.0% NaCl + 3% GCF	1.88e,f	1.71f	32	40
2.0% NaCl + 3% GCF	1.82f	1.82e	39	37
3.0% NaCl + 3% GCF	1.94e	1.92d	22	26

[a] Two observations for each mean. Means in a column which are not followed by a common letter are significantly different (P < 0.05).
[b] 100 − (TBA value of sample with 3% GCF at a given NaCl level divided by TBA value of sample with no GCF at the same NaCl level × 100).

Table 2—TBA values of raw ground beef samples stored at 4°C

Treatment	TBA number[a] at day		% Reduction[b] of TBA value by GCF at day	
	3	6	3	6
None	2.44d	3.93e	—	—
0.5% NaCl	2.61c	4.37d	—	—
1.0% NaCl	2.76b	4.45c	—	—
2.0% NaCl	2.95a	4.72a	—	—
3.0% NaCl	2.62c	4.60b	—	—
0.5% NaCl + 3% GCF	1.33e,f	2.16g	49	51
1.0% NaCl + 3% GCF	1.26f	2.12g	54	52
2.0% NaCl + 3% GCF	1.29e,f	2.06h	56	56
3.0% NaCl + 3% GCF	1.40e	2.20f	47	52

[a] Two observations for each mean. Means in a column which are not followed by a common letter are significantly different (P < 0.05).
[b] 100 − (TBA value of sample with 3% GCF at a given NaCl level divided by TBA value of sample with no GCF at the same NaCl level × 100).

Table 4—Visual color scores and Hunter color "a" values of raw ground beef samples stored at 4°C for 6 days[a]

Treatment	Visual color score[b]		Hunter "a" value[c]	
None	4.6c	(± 0.9)	15.1b,c	(± 2.2)
0.5% NaCl	3.6d	(± 0.9)	13.1c,d	(± 0.5)
1.0% NaCl	3.4d	(± 0.9)	12.9d	(± 0.7)
2.0% NaCl	2.2e	(± 0.7)	11.1d,e	(± 0.6)
3.0% NaCl	1.9e	(± 0.7)	10.2e	(± 0.2)
0.5% NaCl + 3% GCF	7.1a	(± 1.1)	17.4a	(± 0.9)
1.0% NaCl + 3% GCF	6.8a,b	(± 1.0)	17.0a,b	(± 0.4)
2.0% NaCl + 3% GCF	6.3a	(± 1.1)	16.5a,b	(± 0.4)
3.0% NaCl + 3% GCF	3.4d	(± 1.1)	12.2d,e	(± 0)

[a] Means in a column which are not followed by a common letter are significantly different (P < 0.05). Values in parenthesis are standard deviations.
[b] Scored on an 8-point scale (8 = extremely desirable; 1 = extremely undesirable). Eight observations for each mean.
[c] Two observations for each mean.

values by GCF for frozen samples was due to low TBA values of samples without GCF at the storage temperature of −20°C.

Visual color scores and Hunter color "a" values of samples stored at 4°C for 6 days are shown in Table 4. Within samples with or without GCF, color values (both visual and Hunter color "a" values) decreased with increasing salt levels although not all comparisons between salt levels were significantly different. The addition of GCF greatly decreased discoloration of refrigerated raw ground beef at each level of salt. Even ground beef with 2% salt plus GCF had significantly higher visual color scores than did ground beef with no salt and no GCF. However, ground beef with 3% salt plus GCF had lower color values (both visual color and Hunter color "a" values) than did ground beef without any salt and GCF. The results suggest that the adverse effect of salt on raw beef color during refrigerated storage, and also possibly during a long-term frozen storage, may be alleviated by adding a small amount (3%) of GCF, up to the salt level of 2%. Mean visual color scores and mean Hunter color "a" values shown in Table 4 were highly correlated (r = 0.99; p < 0.001).

This study has demonstrated for the first time that salt-catalyzed lipid oxidation and discoloration in a fresh (raw) meat product can be inhibited to a great extent by an oilseed ingredient, i.e., defatted glandless cottonseed flour. Although phenolic antioxidants, when used alone or in combination with a synergist of the antioxidants, effectively retarded lipid oxidation in raw meat without salt (Greene, 1969; Greene et al., 1971), these compounds were less effective or not effective at all for inhibiting lipid oxidation in meat products containing high levels of salt

(Wiesman and Ziemba, 1946; Zipser et al., 1964).

Currently, increasing attention is given to the control of salt-catalyzed lipid oxidation in raw meat products because of restructured meat technology (Breidenstein, 1982). Restructuring requires a particle size reduction or modification, followed by blending with ingredients and reforming. The addition of salt (NaCl) to restructured (sectioned/formed or flaked/formed) meat products is widely practiced as a means of enhancing and expediting the extraction of intracellular (muscle) proteins which are the binding agents of choice at this time. However, oxidation of lipids and meat pigments in such restructured products containing salt is of particular concern (Breidenstein, 1982; Secrist, 1982). Since the concentration of salt required for restructuring meat is rather low (less than 1%), it may be possible to effectively control the development of off-flavor (from lipid oxidation) and off-color (from oxidation of meat pigments associated with lipid oxidation) by incorporating a small amount of a natural antioxidant, such as GCF. Use of GCF in ground beef patties at a level of 3.3% of the raw meat weight did not significantly affect the sensory quality of unseasoned, cooked patties (Ziprin et al., 1981).

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Characterization and Utilization of Ocean Quahog (*Arctica islandica*) Clam Juice as a Liquid and Dehydrated Flavoring Agent

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────────── ABSTRACT ──────────

Ocean quahog (*Arctica islandica*) juice produced from mechanical shucking operations was vacuum evaporated, freeze-dried, or spray-dried for use as a natural clam flavoring agent. The flavorings were judged equal or superior to several commercially available flavors when used in prepared dishes. Gas chromatographic profiles indicated high volatile concentrations in the effluent from the heat shocking and first washing process. Almost no volatiles were present in successive washings. The use of dextrins was not required since the natural chloride salts of potassium and sodium aided in obtaining powders of acceptable quality.

INTRODUCTION

SUBJECTING ATLANTIC SURF and ocean quahog clams to a retort heat shocking process to facilitate mechanical shucking produces a juice by-product which has presented serious problems to clam processors. State and federal statutes prohibit the juice from being discharged directly overboard and reliance upon private and municipal waste disposal systems is economically prohibitive or unreliable. In some instances, municipalities levy high surcharges for the use of their facilities because of high soluble solids content of the juice and, in other cases, the juice can not be handled because of already overloaded waste disposal systems. Although this by-product presents a serious disposal problem, there is also an opportunity to find some revenue-producing use for it.

Wash water obtained from processing surf clam (*Spisula solidissima*) meats has been successfully converted into a marketable clam juice (Hood et al., 1976) and a dehydrated clam flavor ingredient (Joh and Hood, 1979). The clams used in the studies by Hood and associates were subjected to a heat shock procedure in which the clams were immersed in 88°C water for 1 min prior to being manually shucked.

Due to the decreasing supply of surf clams, the ocean quahog (*Arctica islandica*) or mahogany clam has increased in popularity and importance (Bakal et al., 1978). The main objective of this study was to analyze and evaluate ocean quahog clam juice obtained from a mechanized shucking process as a possible food flavoring agent. Vacuum evaporation, freeze-drying and spray-drying were compared as methods for concentrating the juice. The resulting products were evaluated objectively and subjectively to determine if an acceptable natural clam flavoring agent could be developed for further processed foods.

MATERIALS & METHODS

Samples and reagents

Surf clams, ocean quahogs and ocean quahog juice used in this study were supplied by J.H. Miles & Company, Inc., Norfolk, VA.

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Clams were harvested several miles off the Virginia and Maryland coasts and transferred to the processing plant within 24 hr.

A diagram of the mechanized process is shown in Fig. 1. Ocean quahogs received a preliminary cool water wash to remove sand and debris from the outside of the shells and were then subjected to a brief heat treatment in a steam cooker. This heat shock procedure opened the shells and resulted in a liquid referred to as "retort juice." A portion of the retort juice was retained for direct analysis, freeze-drying and spray-drying. The remainder was concentrated ten-fold in a double effect vacuum evaporator. This product is referred to as "concentrated juice."

Juice obtained along the processing line from washing the clam meats was collected to determine the effects of washing on meat volatile profiles. Samples were also collected from the skimmer table and mincing operation for comparison purposes. All samples were held at −17°C until used.

Approximately four dozen each of fresh surf clams and ocean quahogs were hand-shucked after a preliminary wash. The clam meats were pressed in a Carver Laboratory Press (Fred S. Carver, Inc., Menomonee Falls, WI), utilizing up to 5000 psi pressure, and the fresh juice from each species was collected for analysis.

The commercially prepared products utilized were Doxsee Natural Clam Juice (Doxsee Food Corp., Baltimore, MD) and Campbell's V-8 Cocktail Vegetable Juice which were purchased in a local retail grocery store. Clam-flavored powders were obtained from Haarman and Reimer Corp. (Springfield, NJ) (natural and artificial clam flavor R-6780 and natural flavor component-taste of clam R-6964), Nitek International Food Products Company (Lake Bluff, IL) (Baby clam-flavored extract powder No. 8482), Fidco (New York, NY) (Spectra-SN flavor No. 7815), and Synfleur-Scientific Laboratories Co. (Monticello, NY) (natural and artificial clam flavor W.S. #1361). These products were reconstituted to manufacturers' specifications for use. Reagents for SDS gel electrophoresis were purchased from BioRad (Richmond, CA). Chemicals used in gas chromatographic analyses were obtained from Supelco, Inc. (Bellefonte, PA).

Freeze- and spray-drying

Concentrated ocean quahog clam juice was diluted 1:2 (juice: distilled water) to prevent foaming during freeze-drying. Samples
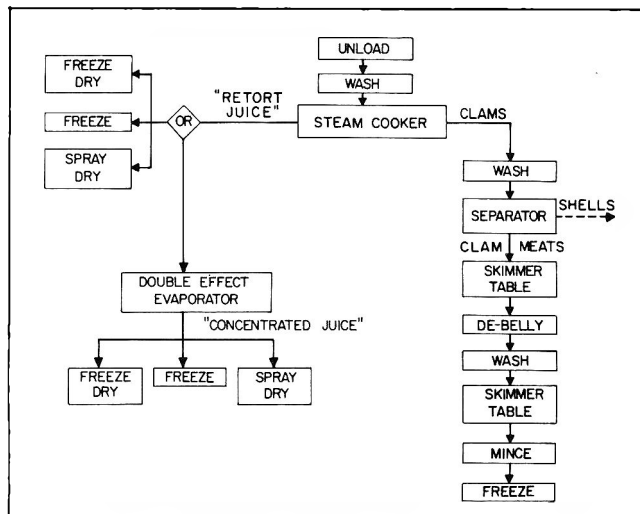
Fig. 1—Flow diagram of mechanized clam process.

of retort and concentrated juice were shell-frozen and dried overnight with a Virtis (The Virtis Company, Gardiner, NY) freeze-dryer under 0.05–0.1 mm Hg vacuum.

Retort and concentrated quahog juice were heated to 60°C and spray-dried separately in a C.E. Rogers, Co. spray-dryer (Detroit, MI). Inlet air temperature was 127°C and exit air, 82°C. A fine powder was produced from both freeze- and spray-drying.

Ultra-high temperature (UHT) pasteurization

Samples of retort and concentrated quahog juice were preheated to 77°C and pasteurized for 3.26 sec at 149°C in an indirect UHT pasteurizer. The pasteurized juice was evaluated by an experienced taste panel.

Chemical analysis

Proximate analyses (nitrogen, 18.026; ether extract, 7.056; and ash, 18.025) were determined on concentrated quahog juice, fresh pressed quahog juice, and commercially prepared clam juice by procedures outlined in AOAC methods (1975). Moisture was determined from freeze-dried samples.

Elemental analysis

About 1g of freeze-dried clam juice was weighed into a clear polyethylene vial (1.5 cm i.d. × 2 cm high) and examined for 34 elements by nondestructive neutron activation analysis. Samples were irradiated twice, once for approximately 1 min and again for approximately 4 hr. The neutron flux was about 10^{12} neutrons/cm^2/sec (1.2×10^{12} for the short irradiation and 1.3×10^{12} for the long irradiation). After the short irradiations, samples were counted within a few min on a Ge(Li) counting system for 8 min. An Intertechnique SA-44 4000 channel analyzer was used and the data stored on magnetic tape for later processing. For the long irradiations, samples were counted as soon as practicable considering the level of activity due to sodium. The time interval varied from 2–5 days after irradiation. Samples were recounted after a minimum of 10 days decay.

Amino acid contents

Freeze-dried clam juice was analyzed on a Beckman autoanalyzer using operating procedures recommended in the Beckman Instruments Manual (1972), according to Spackman et al. (1958). Samples were hydrolyzed for 24 hr with constant-boiling hydrochloric acid in a nitrogen atmosphere in a sealed tube at 110°C.

Direct gas chromatographic (GC) analysis

Analysis of volatiles was performed by the rapid, direct GC method of Legendre et al. (1979).

A Tracor Model 220 gas chromatograph was used in the analyses. The column was 3.2 mm × 183 cm nickel tubing with Poropak Q packing. Flow rates were: helium carrier gas, 35 ml/min; hydrogen 50 ml/min; and air, 660 ml/min. Inlet temperature was 130°C.

Approximately 30 mg clam juice were applied directly to a sample liner which was inserted into the inlet assembly and attached to the carrier gas source. Volatiles were swept from the sample for 15 min. The column oven temperature was increased at this point from 35°C to 100°C and then programmed to 200°C at 4° per min.

SDS-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed by the procedure of Weber et al. (1972). Gels were made 10 cm in length with a final acrylamide concentration of 5%. For a run of 18 gels, the solution was a mixture of 13.5 ml acrylamide solution A (22.2g of acrylamide, 1.6g of methylenebisacrylamide, water to 100 ml), 13.5 ml water, 30.0 ml gel buffer, 0.2M, pH 7.2 (7.8g of $NaH_2PO_4 \cdot H_2O$, 38.6g of $Na_2HPO_4 \cdot 7H_2O$, 2.0g of SDS, water to 1 liter), 3.0 ml ammonium persulfate (15 mg/ml in water) and 0.09 ml TEMED.

After polymerization, the gels were placed in an electrophoresis unit and immersed in a 0.2M, pH 7.2 reservoir buffer (1 part gel buffer, 1 part water).

Samples were prepared by mixing 1 ml of the liquid samples or 100 mg of the powdered samples with 9 ml of 0.01M sodium phosphate, pH 7.0, containing 0.1% SDS and 0.1% 2-mercaptoethanol. The marker protein (a mixture of albumin, beta-lactoglobulin, lysozyme, ovalbumin, pepsin, trypsinogen, and bromphenol blue) was prepared by mixing 5 mg with 9.0 ml of the sodium phosphate

solution. Each mixture was placed in a tube, capped, and incubated in a 100°C water bath for 2 min.

For each gel, one drop of tracking dye solution (0.05% bromphenol blue in 0.01M phosphate buffer, pH 7.0), 1 drop of glycerin and 5 μl of 2-mercaptoethanol were mixed with 100 μl of the sample solution. This mixture was carefully pipetted onto a gel.

Electrophoresis was carried out using a current of 5.0 mA per gel until the marker dye was approximately 1.0 cm from the bottom of the gel (about 4 hr). After electrophoresis, gels were removed from the tubes and stained overnight in test tubes at room temperature with a protein stain (1.25g of Coomassie Brilliant Blue, 227 ml of methanol, 46 ml of glacial acetic acid, water to 500 ml). The gels were rinsed and placed in a destainer filled with 7% acetic acid and destained until distinct blue bands could be seen (about 24 hr).

Sensory evaluation

Several products were tested for sensory qualities using a 10-judge taste panel. Panel judges were staff members, faculty and graduate students in the Dept. of Food Science & Technology, who were familiar with taste panel methods. Judges were pre-tested by triangular taste tests to determine their ability to detect differences in clam flavor.

Products used were a clam-tomato drink, New England and Manhattan clam chowders, clam dip, and spaghetti sauce, containing various amounts of concentrated or retort liquid, freeze-dried, spray-dried, or UHT pasteurized ocean quahog juice. Commercial clam flavorings were compared to the various quahog juice samples.

Procedures outlined by the ASTM (1968) for conducting taste panels were followed. Approximately 50 ml of the liquid samples were served in plastic foam cups to panel members in random order within products. The clam dip was served in 1-oz paper cups. Panelists were seated in partitioned booths under yellow lights to eliminate prejudices from color differences. Each panel member was supplied with water to rinse his mouth between samples and was asked to judge no more than five samples per session.

Samples were evaluated using a ten-point scale adapted from Kramer and Twigg (1970) in which 10 = extremely good and 1 = extremely poor. The data for three replicates were analyzed for significance by analysis of variance and Duncan's multiple range test (Snedecor and Cochran, 1967; Steel and Torrie, 1960) using the Statistical Analysis System package (Barr et al., 1979).

RESULTS & DISCUSSION

THE PROXIMATE COMPOSITIONS of juice from fresh pressed ocean quahogs, concentrated quahog juice and commercially prepared surf clam juice are listed in Table 1. The concentrated quahog juice exhibits a lower moisture and higher nitrogen and ash content than both the fresh and commercially prepared juices. The ash consists largely of sodium, potassium and chloride (Table 2). When expressed in terms of both sodium and potassium chloride, only a small quantity of sodium and potassium remains. It is highly probable that the two metals are present as their chloride salts. The magnesium and iron contents are high when compared to other elements.

None of the juices contains high levels of toxic metals frequently reported in shellfish. Since the juice will be used in small quantities as a flavoring agent, the reported levels of arsenic, zinc, and chromium do not present any potential problems. Mercury and cadmium either occurred in concentrations too low to be detected or were not present in the samples.

Amino acids and other nitrogenous compounds present in concentrated quahog juice, juice pressed from fresh quahog meats, and commercially prepared juice are shown in Table 3. Glutamine and ammonia were the only nitrogenous compounds not exhibiting highest values in the concentrated quahog juice and lowest in the commercially prepared juice. The higher contents of glutamine and ammonia in the pressed clams indicate some decomposition occurred during the time required to transport the clams from the coast to the laboratory. With the exception of taurine the concentration of compounds in the condensed juice did not represent a ten-fold increase over the fresh

Component	Conc juice	Juice pressed from ocean Quahog meats	Commercially prepared juice
		%	
Moisture	65.8	88.8	97.7
Ether extract (dry wt basis)	0.5	6.3	0.7
Nitrogen (dry wt basis)	6.3	8.4	2.6
Ash (dry wt basis)	41.5	20.2	51.3

Table 2—Elements in clam juices (dry wt basis)

Element	Conc juice	Juice pressed from ocean Quahog meats	Commercially prepared juice
		ppm	
Al	24.3	468	170
Ag	4.4	2.6	—[a]
As	89.4	27.5	12.0
Ba	71.6	124	454
Br	836	258	66.8
Ca	—[a]	10950	5475
Ce	1.0	2.3	—[a]
Cl	158000	54250	158250
Co	5.8	3.6	1.2
Cr	6.0	2.9	—[a]
Cs	0.3	0.4	0.3
Cu	—[a]	87	169
Dy	0.6	41.1	40.4
Fe	379	672	—[a]
Hf	0.4	0.4	—[a]
I	15.4	6.1	7.9
K	23450	20400	—[a]
La	1.2	0.6	0.6
Lu	—[b]	0.1	—[a]
Mg	9645	4970	4605
Mn	4.8	8.6	32.2
Mo	5.8	1.4	—[a]
Na	86350	33450	93500
Rb	9.7	14.1	—[a]
Sc	—[b]	0.1	—[b]
Se	3.9	2.4	0.1
Sm	0.2	0.3	—[a]
Sn	279	166	240
Sr	—[a]	543	697
Ta	0.2	—[a]	—[a]
Ti	—[a]	108	213
V	2.8	1.0	1.3
Yb	0.1	0.9	0.2
Zn	75.4	108	39.8

[a] Background interference prevented the detection of these elements
[b] Element was detected in trace amounts

juice. This nonproportional relationship was anticipated due to variables existing in the production of the juice. The steam shucking process produced juice as a consequence of a thermal rather than mechanical procedure and an unknown quantity of the juice was produced from condensed steam.

The taste of taurine has been described as being slightly bitter by Konosu and Hashimoto (1965). However, omission of taurine from a synthetic extract of boiled snow crab meat resulted in little effect on overall flavor (Hayashi et al., 1981). The amino acids with particular importance to overall flavor in seafoods are thought to be glycine, valine, alanine, proline, methionine and especially glutamic acid (Hashimoto, 1965). However, amino acids as a group are necessary for complete flavor as seen in omission tests using synthetic seafood extracts. Removal of amino acids resulted in a much weaker taste and an almost complete disappearance of characteristic flavor (Hayashi et al., 1981; Konosu and Hashimoto, 1965).

Dehydrated products

Both retort and concentrated ocean quahog juices resulted in powders which could be easily reconstituted for use in products following freeze- or spray-drying. Joh and Hood (1979) found that wash water which was collected from the third washing of surf clam meats and concentrated to 2% solids by boiling did not freeze-dry, spray-dry, or drum-dry satisfactorily without the addition of dextrin. They reported that the wash water to be spray-dried or drum-dried required a larger amount of dextrin in order to obtain a product acceptable in color and physical properties. Taste panel tests, however, showed that the addition of dextrin had an adverse effect on the flavor of the powders, diluting the clam flavor and resulting in a "grain-like" flavor in the spray-dried and drum-dried products.

The use of dextrins was not necessary to produce an acceptable product from concentrated quahog juice. The high salt (NaCl and KCl) content of the juice possibly served to prevent some of the problems for which dextrins are often used, such as promoting product stability and enhancing flavor.

Protein study

Sodium dodecyl sulfate electrophoresis has been used in several studies on the identification of fish species (Childs, 1973; Seki, 1976; Kokuryo, 1978). In our study, species differences, as well as the effect of processing on protein patterns, were evaluated by this method. Previous reports on the use of clam juice as a potential flavoring agent employed surf clams as the flavor source. Since ocean quahogs were used in this study, electrophoretic protein patterns were obtained to determine how the clams differed from a biochemical perspective. Also, changes caused in protein banding patterns by processing may relate to sensory evaluations.

The electrophoretic mobility of SDS-treated proteins in polyacrylamide gels is known to be closely correlated to their molecular weight (Weber and Osborn, 1969). Poly-

acrylamide SDS-electrophoresis was used to separate different proteins in the clam juice samples and characterize these proteins by their molecular weights and relative mobilities.

Differences between surf clam and ocean quahog juices are apparent in their protein patterns (Fig. 2). There are only two major bands from the surf clam juice with eleven minor bands. From the quahog juice, there are seven major bands and thirteen minor ones.

Some similarities are also apparent between the two species. Both species have protein bands with relative mobilities of 0.11, 0.33, 0.64, 0.71 and 0.77. The molecular weights for these bands were determined by comparison to marker proteins to be greater than 100,000 Daltons for the 0.11 and 0.33 mobilities. Those for the other common mobilities were 37,000, 28,000 and 23,000 Daltons. Surf clam juice had five components with molecular weights above 100,000 Daltons and quahog juice had ten bands in that region. The lowest molecular weight protein observed in the ocean quahog juice was 15,000 Daltons and the lowest for surf clam juice was 18,500 Daltons. The most intense band for the ocean quahog juice had a mobility of 0.56 which corresponds to an approximate molecular weight of 49,000 Daltons.

Retort, concentrated, spray-dried and freeze-dried quahog juices all exhibited identical gel patterns. It is obvious that differences exist between the fresh pressed and processed juices. The processed juices have five major bands and four minor ones compared to seven major and thirteen minor bands from the fresh pressed quahog juice.

The most intense protein band for the processed juices has a relative mobility of 0.65 and molecular weight of approximately 35,500 Daltons. There are three components with molecular weights above 100,000 Daltons in the processed juices. The 15,000 Dalton protein was retained after processing.

Sensory evaluation

Five products were developed for testing the clam juice samples. These were a clam-tomato cocktail, Manhattan clam chowder, New England clam chowder, sour cream clam dip, and spaghetti sauce. Preliminary tests were performed to determine the formulations to be used in making these products and the concentration of clam juice most preferred in each product. It was determined that the commercially prepared tomato cocktail juice mixed with the clam juice samples and 1.5% added sugar resulted in the most acceptable clam-tomato drink. Only the freeze-dried and the spray-dried clam juices were suitable for use in the sour cream clam dips because of the high water content in the liquid samples. The purpose of the taste panels was to determine if the quahog juice samples could result in products as acceptable as commercially available flavoring agents and if any of the processing methods would result in an objectionable product.

Table 4 contains the results of taste panel studies performed on the various products. Since the samples containing ocean quahog juice were generally rated as high as or higher than the commercial products, some tests were made without their use. This allowed comparisons to be made among the various processed juices and controls without overloading taste panel members with samples.

Taste panel results indicate that the various processing methods used do not adversely affect the flavor of ocean quahog juice. In some instances, the processed samples were preferred. The concentration methods may possibly remove undesirable volatile compounds or cause a chemical

Table 3—Amino acids and other nitrogenous compounds found in clam juices (dry wt basis)

Compound	Conc juice	Juice pressed from ocean Quahog meats	Commercially prepared juice
		mg/g	
Lysine	0.38	0.26	0.01
Histidine	0.21	0.09	0.01
Arginine	0.76	0.42	0.05
Ornithine	0.04	0.03	—[b]
Aspartic Acid	2.72	0.24	0.04
Threonine	0.43	0.20	0.01
Serine	1.10	0.22	0.02
Glutamic Acid	4.82	0.86	0.79
Proline	0.12	0.10	—[b]
Glycine	3.44	0.77	0.02
Alanine	8.37	1.44	0.20
Valine	0.25	0.11	0.01
Methionine	0.16	0.05	—[b]
Isoleucine	0.24	0.08	—[b]
Leucine	0.42	0.11	0.01
Tyrosine	0.36	0.08	0.01
Phenylalanine	0.31	0.07	0.01
Taurine	38.65	3.06	0.33
Asparagine	0.02	—[a]	—[a]
Glutamine	0.02	0.10	—[a]
Ammonia	0.04	0.07	0.01
Urea	1.96	0.05	—[b]
Cystathionine	0.13	—[a]	—[a]
α-aminobutyric	0.04	0.01	—[a]
Sarcosine	0.04	—[a]	—[a]
Phosphoserine	0.45	0.22	0.01

[a] Compound was not present or appeared in concentrations too low to be detected.
[b] Compound was detected in trace amounts

Fig. 2—Diagrams of gel patterns for clam juice proteins. (a) Fresh pressed surf clams; (b) Fresh pressed ocean quahogs; (c) Processed juice (retort, concentrated, freeze- and spray-dried).

Table 4—Sensory results for flavor acceptability of processed clam juice in various products[a]

Sample	Clam-tomato cocktail	Manhattan clam chowder	New England clam chowder	Spaghetti sauce	Sour cream clam dip
			Hedonic Score[b]		
Concentrated juice	6.8 ± 1.8ab	6.9 ± 1.3a	6.1 ± 2.1b	7.3 ± 0.9a	
Retort juice	6.3 ± 2.2b	6.2 ± 1.2a			
Concentrated UHT[c]	7.6 ± 1.1ab	7.6 ± 1.3a			
Retort UHT	6.3 ± 1.3b	6.3 ± 1.6a			
Concentrated freeze-dried	6.0 ± 2.0b	6.3 ± 1.6a	7.0 ± 1.6ab	7.5 ± 1.3a	7.8 ± 1.3a
Retort freeze-dried	6.6 ± 2.0ab	6.6 ± 1.8a	7.7 ± 0.9a	7.1 ± 0.9a	7.2 ± 1.3a
Concentrated spray-dried	7.1 ± 1.2ab	6.7 ± 1.9a	7.9 ± 0.9a	7.0 ± 1.3a	7.7 ± 1.4a
Retort spray-dried					7.5 ± 1.4a
Fidco	6.9 ± 0.6ab				6.5 ± 2.3ab
Synfleur	3.2 ± 1.2c				
H&R — nat. & art. clam	4.0 ± 1.9c				
H&R — nat. flavor comp.	5.9 ± 2.3b	4.4 ± 2.2b			
Nitek baby clam extract	3.8 ± 2.6c	4.7 ± 2.0b			5.8 ± 2.1b
Control	8.2 ± 1.5a	6.5 ± 1.6a	4.5 ± 2.1c	7.0 ± 0.9a	4.0 ± 1.1c

[a] Clam-tomato cocktail contained 15% clam juice; Manhattan clam chowder contained 60% clam juice; New England chowder and spaghetti sauce contained 33% clam juice; sour cream clam dip contained 10% clam juice; controls contained no clam juice.
[b] Samples were rated using a 10-point Hedonic scale in which 10 = extremely good and 1 = extremely poor; means ± standard deviations are the result of three replicates; means followed by the same letter are not significantly different (p < 0.05).
[c] See Materials & Methods section for explanation of abbreviations

change in one or more components of the clam juice. Spray-drying and freeze-drying were considered more practical means of preservation than UHT pasteurization since both processes greatly reduced bulk resulting in a more convenient and easily handled final product.

Gas chromatography

Chromatograms for fresh pressed ocean quahog clam juice, surf clam juice, and retort juice (ocean quahog juice from the retort) have been plotted together in Fig. 3 to illustrate differences between the samples. Noticeable differences appear in peaks at retention times of 9, 21, 24—30, 33, 39 and 44 min. Ocean quahog juice exhibited the largest peaks in every case except at 9 min. Peaks for retort juice were generally smaller or disappeared entirely, except at 9 min, where a very large peak appeared. The compound was most probably produced as a result of thermal decomposition.

Fig. 4 shows the chromatograms obtained from retort and concentrated ocean quahog clam juice. A major dif-

Fig. 3—Gas chromatogram of volatiles obtained from ocean quahogs, surf clams, and retort juice.
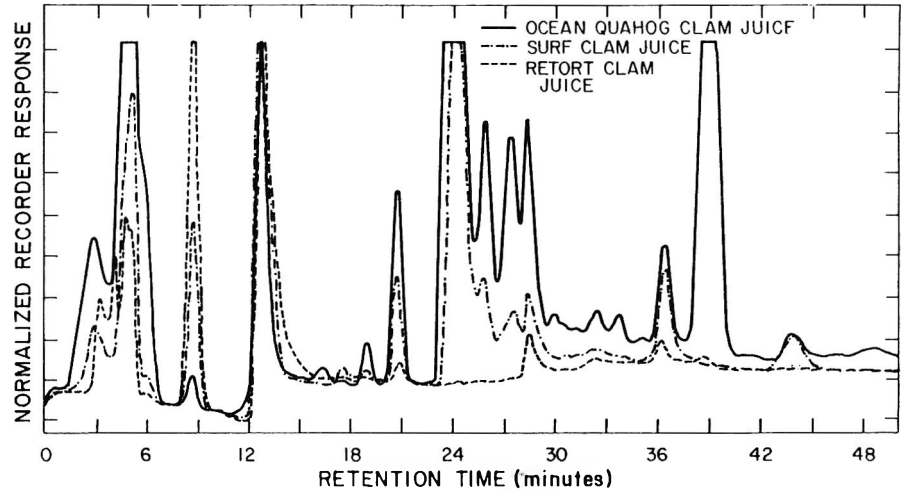
Fig. 4—Gas chromatogram of volatiles obtained from retort and concentrated juice.
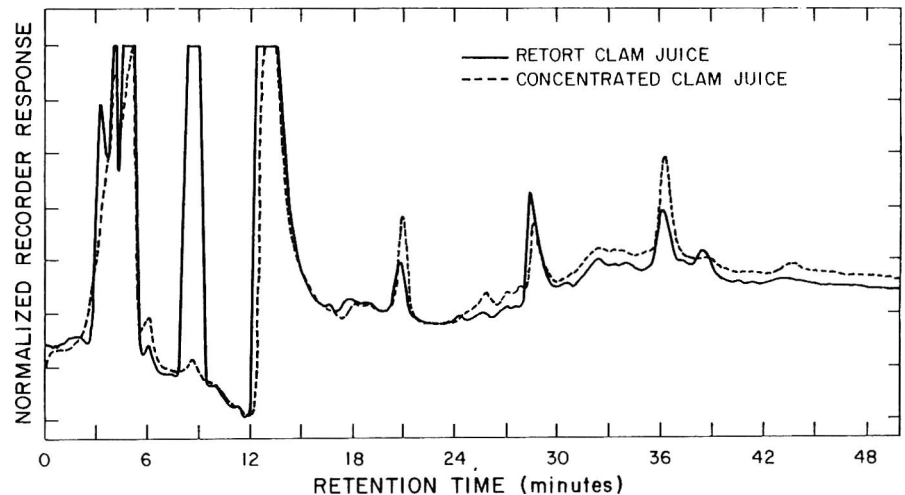
Fig. 5—Gas chromatogram of volatiles obtained from freeze- and spray-dried concentrated juice.
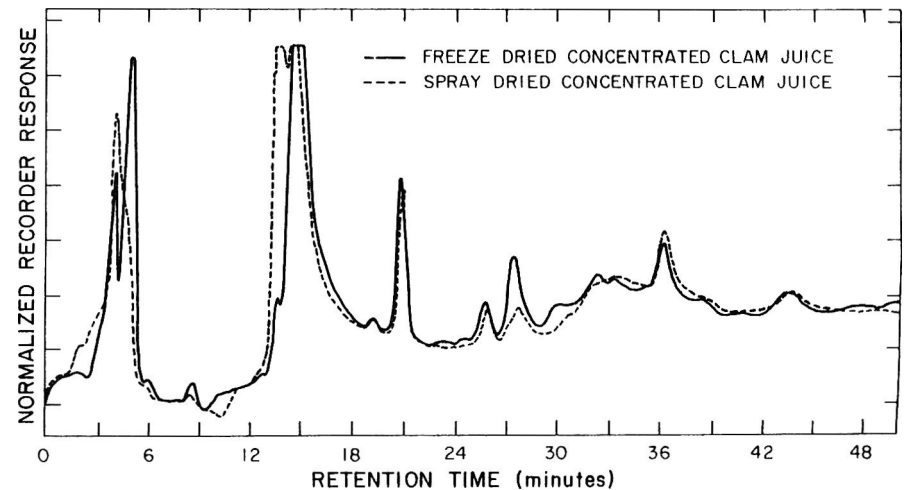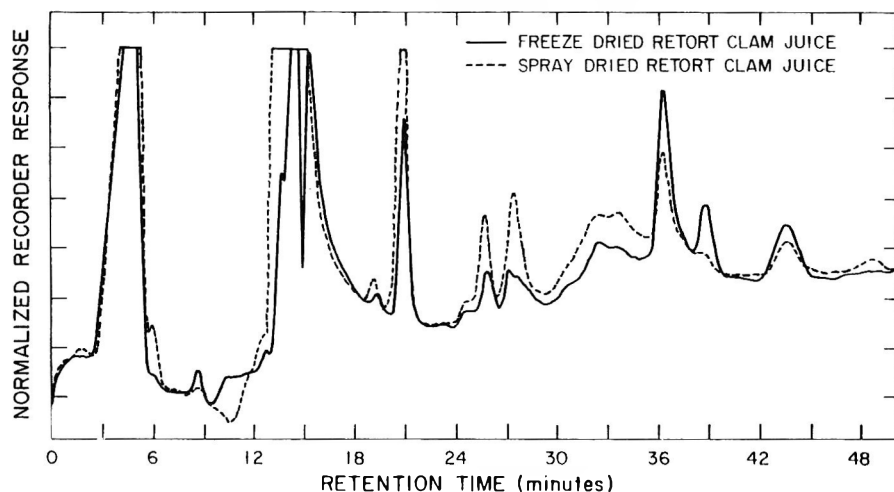
Fig. 6—Gas chromatogram of volatiles obtained from freeze- and spray-dried retort juice.
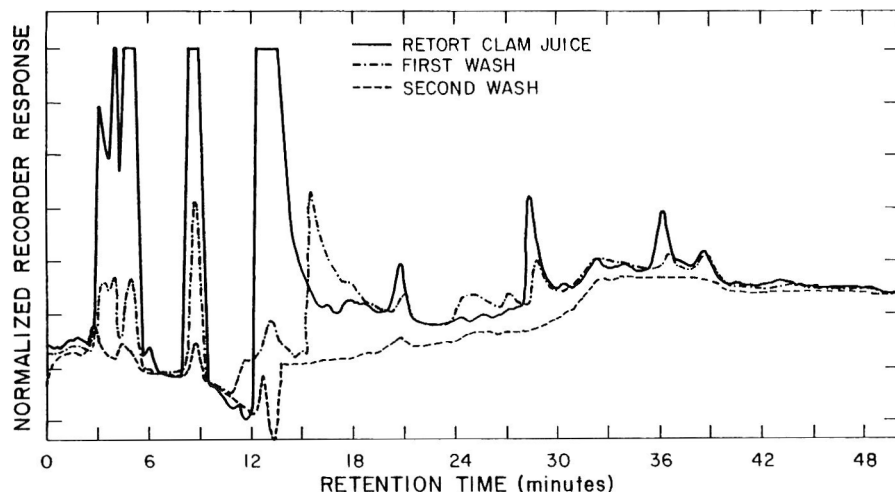
Fig. 7—Gas chromatogram of volatiles obtained from retort juice and the first two washes.

ference is noticed at 9 min. The concentrated juice loses most of the large peak present in the retort sample. Retorting appears to increase the amount of this compound whereas vacuum evaporation causes a decrease.

Fig. 5 indicates that there is little difference between the freeze- or spray-dried concentrated juices. In Fig. 6, freeze-drying and spray-drying both caused a decrease in the 9 min peak in the retort juice. Generally, freeze-drying resulted in lower volatile concentrations.

Chromatograms from the first five collection points were compared to determine the amount of volatiles extracted from clam meats into the wash water. Chromatographic patterns from the first two washes of the quahog clam meats are plotted with the retort juice in Fig. 7. The first wash contains a smaller amount of volatiles than the retort juice. Almost no volatiles are present in the second washing. The third, fourth, and fifth washings produced chromatograms which were almost a straight line. These findings are in contrast to that of Hood and associates (1976, 1979) who found that the wash water from the third washing contained substantially more clam solids and a more distinctive clam flavor than that from the preceding two. The clams in their studies were immersed in 88°C water for approximately 1 min prior to manual shucking. It is apparent that the use of retort juice as a clam flavoring is preferred over the use of wash water since many of the volatiles are water soluble and easily removed by retort heating for a very short time.

It can be concluded from this study that ocean quahog juice obtained from a mechanical shucking operation is a good source for a natural clam flavoring agent. The concen-

tration methods tested have no adverse effects on flavor and resulted in products which were generally scored as high as or higher than commercially available products. Taste panel ratings in the good range show that the use of the flavoring agent in several products is quite feasible.

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Canned Shrimp Texture as a Function of Its Heat History

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ABSTRACT

Texture changes of canned shrimp were determined by sensory and instrumental methods. A direct relationship was found between sensory perception of toughness and instrumental shear forces measurements in canned shrimp packed in 2.6% brine in 307x113 cans, and processed at 124°C. Shrimp muscle toughened during the initial stages of heating and softened during the latter stages of processing. The softening of shrimp texture (shear values) was determined to follow apparent first order behavior. Regression analyses were used to establish apparent reaction rate constants, an apparent activation energy (24 Kcal/mole), textural D values and textural Z values (30°C).

INTRODUCTION

THE INFLUENCE of thermal processing on the sensory quality of foods has been studied by many researchers (Lund, 1975). However, no authors have reported the effect of treatments at retort temperatures on texture quality of meats in terms of kinetic parameters. Draudt (1972) has summarized the effects of heating beef muscle at temperatures up to 90°C and Deng (1981) has reported shear force values for mullet muscle heated to temperatures ranging from 35–100°C. Texture kinetic parameters during the canning of dry legumes have been determined as a function of hydration times (Quast and da Silva, 1977a, b) and of hydration solution compositions (Silva et al., 1981). Both of these latter two authors related their shear force values to perceived sensory texture evaluation as described by Hayakawa et al. (1977).

Shrimp is one of the most popular sea food products available; consequently, it is in demand throughout the year. In order to meet the requirements for supplying the market, either canned or frozen shrimp with a good quality is necessary. The convenience provided by the canned product is, however, offset by the greater sensory degradation incurred during processing when compared to a frozen product. The characteristic canned shrimp flavor is developed relatively early in a process and it does not change substantially after prolonged heating, while the texture of shrimp may have a more dramatic change during extended thermal processing. Therefore, in considering shrimp sensory quality, texture is usually considered the most important factor. Commercial canned shrimp are usually processed at temperatures of 115–121°C which may produce soft shrimp of less than optimum texture. However, the current trend to use higher retort temperatures (127°C) may provide a viable means for improving the quality of canned shrimp. The objectives of this study were to determine the texture changes of shrimp as a function of their heat history, to relate shear force values to perceived texture quality as determined by a sensory panel, and to relate the texture changes to kinetic parameters.

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MATERIALS & METHODS

Correlation of sensory and instrumental values for texture measurement

Frozen peeled and deveined raw pink shrimp (*Penaeus di orarum*), (110–130 count per pound) were used in this study. The shrimp were thawed and packed (120 ± 0.05g) in 307 × 113 cans. Sufficient brine (2.6% NaCl w/w at ambient temperature) was added to each can to prevent the formation of a headspace. The ratio of shrimp to brine was approximately 1.1:1 w/w. The cans were sealed and processed immediately in a vertical miniature retort (Schmidt et al., 1955). Processing times were 3, 6, 9, 15, 20, and 25 min heating at 124°C.

A randomized block design was employed to test for the correlation between sensory and instrumental texture measurement methods. Shrimp were retorted at a single temperature for all processing times and within a day evaluated by the sensory and instrumental methods. Six processing loads of the miniature retort at a given temperature and time were necessary to obtain sufficient shrimp samples for evaluation. Variation between progressive loads was assumed negligible due to the rapid (10–15 sec) heating and cooling of the retort.

The sensory panel was composed of 10 graduate students and faculty of the Food Science & Human Nutrition Dept. of the Univ. of Florida. The panel members were selected from participants of preliminary screening tests. The criterion for selection was consistency in three trials of equivalently processed product.

Prior to a panel session, the cans were opened, and intact, medium-sized shrimp were chosed for evaluation. Two shrimp at room temperature and without added flavoring were used as a sample. The panelists were asked to rate the shrimp's toughness on a 9 point scale (1 = extremely soft; 5 = just right; 9 = extremely tough). Evaluation sessions were conducted in the taste panel laboratory with participants in partitioned booths having subdued fluorescent lighting. During each panel session six randomly coded (3 digit) samples processed at a temperature and different times were provided, served with water for rinsing and evaluated. Later in the day a replicate panel session was held.

The variation in textural quality of the shrimp was also determined by a shearing test using an Instron universal testing machine (Ahmed et al., 1972). A shrimp piece was placed on a flat plate and the maximum force (Newtons, N) required for a 0.5 cm penetration between segment joints by a knife blade was measured. Twenty shear tests were completed for each processing time within each processing temperature trial.

The experimental design included replicate processing time trials which resulted in 40 data sets of sensory and shear values for each combination of process temperature and time. The correlation between sensory and instrumental texture measurement methods was determined by a general linear regression model on the Statistical Analysis System (SAS) (Barr et al., 1979) which did not account for additional sources of variation.

Kinetics of texture changes

Relatively high temperatures were chosen for shrimp processing, beginning with 115°C (a commercial value) up to 140°C. This range of temperatures was necessary to provide an estimate for the E_a and Z values. The process times for each retort temperature are listed in Table 1. sample preparations, retorting procedure and instrumental texture measurement were the same as those discussed previously. Raw samples were used as controls for the instrumental texture measurements. There were three replicates for each treatment and 20 individual determinations in each replicate.

In a separate series of experiments a needle-type copper-constantan thermocouple (O.F. Ecklund, Inc., Cape Coral, FL) was inserted

into the center of a shrimp in each 307 x 113 (8.7 x 4.6 cm) can processed in order to estimate temperature histories. Since these histories may vary in different positions in a can nine lengths of thermocouples were utilized which placed the pierced shrimp in different iso-regions of the can and minimized heat conduction down their length (Cowell et al., 1959). These lengths ranged from 7.8 (outer edge) to 4.4 cm (center). A 4-point recording potentiometer (Speedomax H, Leeds and Northrup Co., North Wales, PA) monitored internal can and the retort temperatures. There were seven replicates completed for each temperature, and position. The f_h (slope of heating curve) and j_h (heating curve lag factor) values of each heating curve (Stumbo, 1973) were calculated using a computer program based on linear regression. Correlation coefficients between f_h, j_h, retort temperature, internal thermocouple positions, and initial temperatures, were calculated by covariance analysis using SAS.

Toughening/softening model

Relationships of texture values versus time for each processing temperature were established. The toughening model was approximated by linear regression of shear values versus process time prior to the maximum shear value. The softening model was approximated by linear regression of the natural logarithm of shear value versus process time. The slope for the semi-logarithmic curve was used to estimate the apparent reaction rate constant (k) for the softening reaction. The apparent activation energy (E_a) for the softening reaction was obtained from the slope of an Arrhenius plot log(k) vs 1/T. The textural D value (D_T) was defined as the time for a 90% reduction in shear value and calculated from the relationship:

$$D_T = 2.303/k$$

When log (D_T) values were plotted versus the retort temperature, the Z_T value was calculated as the negative reciprocal of the slope of the regression line obtained (Stumbo, 1973). All regression calculations were completed by using general linear model procedures on SAS.

RESULTS & DISCUSSION

Sensory/instrument correlation

The regression calculation of sensory scores versus shear values demonstrated that the shear force of cooked shrimp correlated well with the sensory score ($p < 0.0001$; $r = 0.97$). Thus, the sensory responses could be confidently predicted based on the instrumental data for shrimp shear measurements using the equation

$$S = 0.26 + 1.38 F$$

where S = sensory score and F = shear force [in Newtons (N)]. Further experiments were completed using only the shear force measurements of the shrimp samples as a response variable. Since a sensory score of 5 was the rating for optimum texture of canned shrimp, the corresponding shear force was calculated to be approximately 3.4N (Fig. 1).

Heating effects

The texture change of shrimp was found to have two phases for all four temperatures (115, 124, 133, 140°C) as illustrated in Fig. 2. During the initial stages of heating, shear forces increased rapidly and reached peak values in the range of 4.6–4.7N for all temperatures. As cooking time increased, shear forces began to decrease relatively slowly as compared with toughening rate. After prolonged heating, all samples reached a minimum value (1.2–1.4N) where no significant change in texture was apparent. Since all products exhibited similar trends of texture change and maximum and minimum values achieved were equal, it was assumed that chemical reactions involved in texture change were identical for all processing temperatures utilized.

It is generally accepted that the major components in muscle which can affect meat tenderness are muscle fibers

and connective tissues. Therefore, heat-denaturation of the myofibrillar proteins and shrinkage of collagen experienced in early stages of retort processing might result in a tightening and stiffening of structure. The softening during prolonged cooking was probably caused by the conversion of collagen to gelatin and dissociation of muscle proteins (Bouton and Harris, 1972; Draudt, 1972; Dube et al., 1972; Deng, 1981).

Table 1—Processing time/temperature combination

Retort temp (°C)	Time (min) Treatment									
	I	II	III	IV	V	VI	VII	VIII	IX	X
115	3	5	10	20	30	40	50	60		
124	2	3	4	6	9	15	20	25	30	40
133	1	2	3	5	7	8	10	12	15	30
140	0.5	1.5	2	3	5	6	8	9	10	25

$$S = 0.26 + 1.38 F \quad (r^2 = 0.94)$$
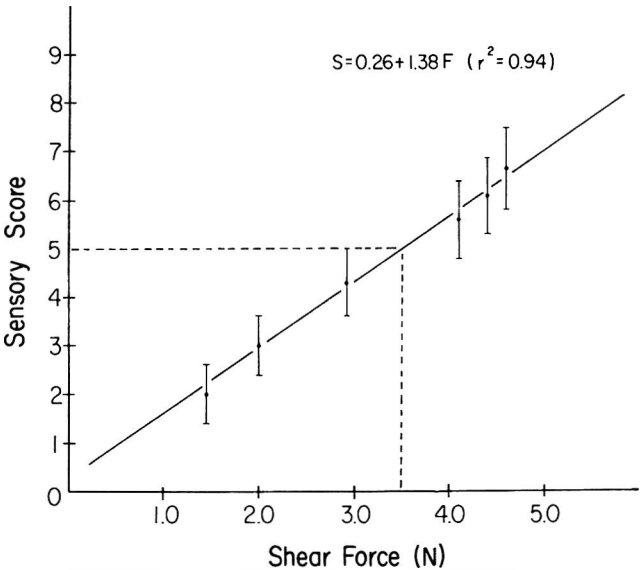
Fig. 1—Correlation line of shrimp toughness for sensory score (S) vs shear force (F), in Newtons (N).

O — 115°C
△ — 124°C
◇ — 133°C
□ — 140°C
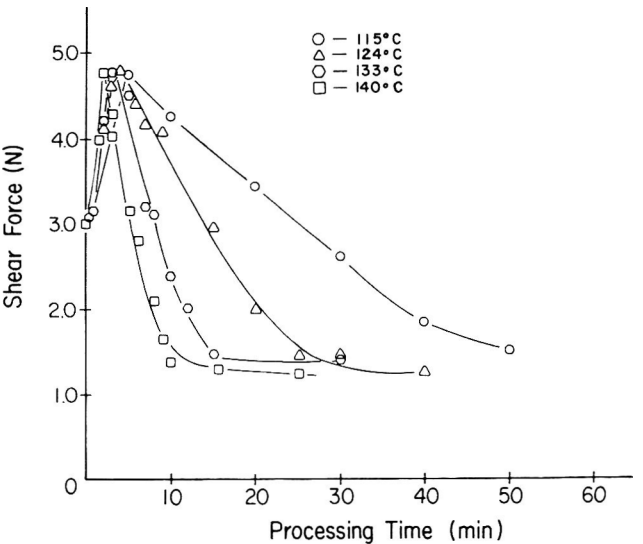
Fig. 2—Shrimp shear force vs processing time at different retort temperatures.

Determination of f_h and j_h values

In order to determine the kinetic parameters of shrimp texture change, the temperature distribution within a wet-packed product was evaluated. Individual f_h and j_h values were calculated from the temperature history of each of nine positions in each replicate for all four temperatures. Correlation coefficients among various variables were obtained from SAS. The results indicated a correlation of f_h with j_h ($p < 0.0001$), retort temperature ($p < 0.0001$) and positions inside the can ($p < 0.003$) but not with initial temperature ($p < 0.73$). The j_h values also demonstrated a correlation with retort temperature ($p < 0.0001$) and position ($p < 0.0015$) but not with initial temperature ($p < 0.069$). Since the initial temperature did not influence f_h and j_h values, an average initial temperature of 23°C was used in further calculations. The j_h values had a negative correlation ($r = -0.55$) with f_h values, which was probably an artifact of the extremely fast heating rates and the somewhat imprecise temperature measurements during initial heating.

Although correlation coefficients of -0.19 and 0.20 existed with probabilities of 0.003 and 0.0015, respectively, for f_h and j_h values versus position, the GLM analysis revealed extremely high error of estimate values for the slope values. These error of estimate values provided 95% confidence limits equal or greater than the slope values themselves. For this reason, it was assumed that the normal variation in the f_h and j_h values was too large to distinguish differences between the heating characteristics and shrimp positions. Further justification for this assumption was based on the extremely short heating times of the shrimp due to the convective nature of the heating mode. In addition, the kinetic modeling calculations were conducted on data from the heat history at much greater process times than the initial heating periods.

The f_h and j_h values did correlate with retort temperatures. The f_h values had a negative correlation with retort temperature ($p < 0.0001$) and j_h had a positive correlation with retort temperature ($p < 0.0001$). The decrease in f_h values with increasing retort temperature was probably due to the increase in convective turbulence with increasing temperature differences between the retort and initial product temperatures. The increase in j_h values was probably an artifact of extremely short come-up times and the limited data per run used to determine the heating parameters. Therefore, predicted f_h and j_h values at each retort temperature were used in further calculations (Table 2).

Prediction of first order reaction model

The cooking of shrimp involved two kinds of reactions (Fig. 2); initially, there was a toughening reaction and, secondly, there was a softening reaction. Subjectively estimated times at which the shear value reached a maximum value are listed in Table 3. In addition the length of time (zero-reaction time) from when steam was turned on to the time the shrimp's internal temperature reached 3°C below the retort temperature was calculated by the following equation (Stumbo, 1973):

$$B = f_h \log (j_h I_h/g)$$

where B = zero-reaction time, g = 3°C and I_h = retort − initial product temperature. The minimum processing time at each retort temperature to reach $F_{121}^{10} = 6.0$ min was calculated (Stumbo, 1973) and is presented in Table 3.

The data in Table 3 indicated that both the zero-reaction times and minimum processing times were greater than the time necessary for the shear force to reach the maximum values. Therefore, in the time region of concern for commercial sterility, the toughening reaction had apparently ceased and the softening reaction was occuring. Thus, essentially after the zero-reaction time, products were held at a constant retort temperature and, therefore, quality degradation became only a factor of time.

The mathematical models derived for the toughening and softening reactions at different retort temperatures are

Table 2—Predicted f_h and j_h values at different retort temperatures

Retort temp (°C)	f_h (min)	j_h
115	4.8 ± 0.4[a]	1.0 ± 0.2
124	3.9 ± 0.3	1.2 ± 0.1
133	3.1 ± 0.3	1.4 ± 0.1
140	2.4 ± 0.4	1.6 ± 0.2

[a] ± values indicates 95% confidence interval (t-test).

Table 3—Comparison of process times for maximum shear force, zero-reaction time and minimum process time at each retort temperature

	Retort temperature (°C)			
	115	124	113	140
Max shear force (min)	5.0	4.0	3.0	2.0
Zero reaction time (min)	7.0	6.8	5.7	4.6
Minimum processing time (min)	31	9.4	4.9	3.3

Table 4—Toughening/softening models

	Retort temperature (°C)			
	115	124	133	140
Toughening model*, 95% Conf. Int. r	F = 4.6 + 0.34 t ±0.4 ± 0.09 0.68 (p<0.0001)	= 5.0 + 0.52 t ±0.2 ±0.07 0.82 (p<0.0001)	= 4.3 + 0.5 t ±0.4 ±0.2 0.57 (p<0.0001)	= 4.2 + 0.6 t ±0.3 ±0.2 0.63 (p<0.0001)
Softening Model 95% Conf. Int. r	Ln(F) = 1.54 − 0.025 t ±0.04 ±0.001 −0.93 (p<0.0001)	= 1.53 − 0.048 t ±0.05 ±0.003 −0.89 (p<0.0001)	= 1.58 − 0.10 t ±0.08 ±0.01 −0.83 (p<0.001)	= 1.65 − 0.17 t ±0.08 ±0.01 −0.87 (p<0.0001)
Predicted Peak Shear Value (N)	4.6	4.7	4.6	4.6
Time at Predicted Peak Shear Value (min)	5.0	3.4	2.5	2.7
D_T (min)	100	48	25	14

*F = Force (N), t = time (min)

presented in Table 4. A linear model was used for the toughening reaction because of the short time span of this reaction and of the lack of data at numerous times. The softening part of the texture profile was approximated with a first-order reaction model because of the reasonably good fit of the data to this model and because this model is required for the calculation of D_T and Z_T values. The intersections of the toughening and softening models were used as predicted maximum shear forces at a specific time (Table 4). The results indicated that maximum shear values for all four temperatures resided in the narrow range of 4.6–4.7N.

For a clearer representation of these mathematical manipulations, Fig. 3 represents the data for 115°C retort temperature. Each point on the graph is the mean value of 60 measurements. The 95% confidence limits are presented by small bars extending vertically through the points. The dotted lines indicate the zero-reaction time of the cans. The data for the softening model was taken for times after this zero-reaction time.

Determination of textural kinetic parameters

The apparent reaction rate constants were the slope of logarithmic lines of best fit (Table 4). For temperatures of 115, 124, 133, and 140°C, the k values were 0.025, 0.048, 0.10, 0.17 min^{-1}, respectively. These values in an Arrhenius plot yielded by linear regression calculation an E_a value of 24 ± 2 kcal/mole (r = 0.999). Likewise, linear regression of the D_T values (Table 4) of 100, 48, 25, 14 min at 115, 124, 133, 140°C, respectively, versus retort temperature yielded at Z_T value of 30 ± 2°C (r = 0.999).

The calculated E_a value of 24 Kcal/mole describes the rate of texture softening dependence on the retort temperature. The physical significance of this E_a value is obscure since it is a description of the summed effect of numerous chemical reactions which are occurring simultaneously. The utility of this E_a value as a predictor in the thermal processing of shrimp is limited to situations in which the tissue texture has proceeded beyond its maximum shear value (i.e., softening of blanched shrimp). Additional experimentation under different processing conditions will be necessary to characterize the toughening reaction. With k and E_a values for both toughening and softening reactions, it may be possible to predict texture changes during the thermal processing of the shrimp.

In this experiment, all the texture determinations including sensory and instrumental methods were completed within two days of processing. However, no consideration of quality change during storage was accounted for. Further experiments were conducted in order to determine if significant changes in texture occurred as a function of ambient temperature storage time. After 11 months statistically significant changes in shear values were found for shrimp processed at 121°C for 14 min. Using linear regression procedures the following equation was calculated:

$$F = 3.6 - 0.10\,t \qquad (r = 0.57,\ p < 0.0001)$$

where F = shear force (Newtons) and t = storage time (months). This equation predicts substantial decreases in shrimp toughness over a year's storage. Certainly this soft-
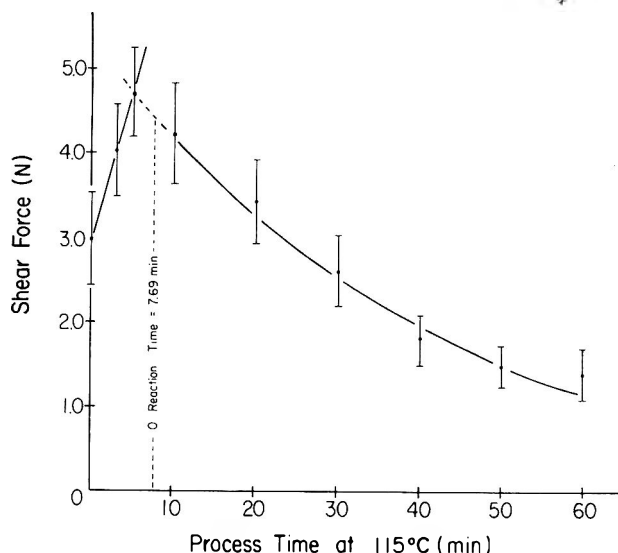
Fig. 3—Comparison of mathematical models and mean experimental values for a 115°C retort temperature.

ening of shrimp during ambient storage is important in modeling texture changes and in the current marketing of commercially canned shrimp.

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High Vacuum Flame Sterilization of Canned Diced Tuna: Preliminary Process Development and Quality Evaluation

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————————ABSTRACT————————

A preliminary study of the potential of high vacuum flame sterilizing canned diced tuna was carried out. The investigation included inoculated experimental sighting packs study at five process levels, and comparative assessment of the physical, chemical and nutritional property of the raw, precooked, high vacuum flame sterilized (HVFS) and still-retorted canned tuna. The vacuum achieved in the HVFS packs was significantly higher (P < 0.05) than the control packs. Fatty acid and amino acid composition, in-vitro protein digestibility, and the Computed Protein Efficiency Ratio (C-PER) were similar for the raw, precooked and canned tuna. Available lysine was marginally decreased by precooking, and HVFS processing caused no measurable change. However, available lysine, thiamin, riboflavin, niacin and mineral concentrations were generally lower in the retort process control canned tuna.

INTRODUCTION

THE GOAL of the canned food industry is to produce safe, high-quality, wholesome food that is available to the consumer year-round at a low price. However, heat sterilization requirements for safety may affect quality. The longer the sterilization process, the greater will be the degradation of food quality, in terms of sensory properties (color, flavor, and texture) and the nutrient content.

Growing consumer awareness and the introduction of mandatory nutritional labeling regulations have created a strong commercial interest in the nutritive quality of foods, and its preservation during processing and distribution. The conventional sterilization process for foodstuffs is an in-container process and the still-retort is one of the standard sterilizers. In recent years, there has been a trend towards higher sterilization temperatures and shorter processing times. Chemical and biochemical reactions such as thiamin loss, enzyme inactivation, color change, and arbitrary measures of flavor and texture are significantly less temperature-dependent than is microbial spore inactivation. Thus, high processing temperatures, with correspondingly reduced time, will lead to smaller amounts of chemical changes for equivalent sterilizing value of microbial spore inactivation (Burton, 1977). Flame sterilization, in which a can is rapidly rotated over a series of gas flame burners is claimed to have advantages for certain types of products. Beauvais et al. (1961) and Leonard et al. (1975a) discussed the advantages of the flame sterilization method for canning foods. Further work by Leonard et al. (1975b, c; 1976a, b) showed that the use of the flame sterilizer for sterilizing canned fruit cocktail, whole-peeled tomatoes, apricot halves, and peaches gave excellent products which are superior to that of the conventionally processed products. Casimir and Lewis (1972) and Carroad et al. (1980) introduced the concept of the high vacuum flame sterilized (HVFS) packs in which the volume of covering liquids normally used with canned foods is reduced or eliminated, yielding high quality product.

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All the work with the flame sterilizer, to date, deals only with sterilizing conventionally packed canned fruits and vegetables. Therefore, this pilot plant study was initiated to explore the possibility of using the high vacuum flame sterilization method for sterilizing canned diced fish. Tests were also undertaken to compare the physical, chemical and nutritional quality of the fish processed by the high vacuum flame sterilization process with that of the conventional still-retort process.

MATERIALS & METHODS

Raw material handling

Approximately 233 kg of the fresh albacore (*Thunnus alalunga*) ranging from 3–10 kg each were purchased from Paladini Seafood Company (San Francisco, CA) in Nov. 1981. The fish were transported on ice to the lab. Upon arrival, the fish were kept in boxes and stored at $-26°C$. The frozen fish were withdrawn randomly over a 2 month period for preliminary experimentation and for the purpose of developing a suitable canned product that could be commercially sterilized by using either the still-retort or the high vacuum flame sterilizer. Heat penetration data were obtained and lethality of the various experimental trials were calculated from these data.

Preparation for canning

The frozen fish were thawed in a tank of running water for approximately 4 hr, dressed and precooked in a steam chest at atmospheric pressure (average temperature 100°C) for 2½ to 3 hr. After precooking, the fish were held at 2°C overnight so that the flesh would cool and firm up enough to handle. Various sizes of flakes or cubes of the precooked tuna were packed in 303 X 406 cans and sterilized. After numerous trials, 0.5 in. cubes were found to produce a satisfactory pack in terms of raw material utilization and heat transfer characteristics.

Conventional (control) packs. The packs (303 X 406 cans) consisted of 300g of 0.5 in cubes of precooked tuna light meat, packed in distilled water, with a headspace of 10 mm. The lethality achieved in the control packs processed in a vertical still-retort was determined as follows: Nonprojecting "plug-in" copper-constantan thermocouples (¾ in.) were mounted in three 303 X 406 cans before filling the tuna. The cubes of tuna were placed around the thermocouple in each can. Spores of *Bacillus stearothermophilus* NCA 1518) were inoculated into each of three cans and into three other similarly packed cans without thermocouple. The concentration of spores in the inoculum was 5×10^7/ml. Two ml of the inoculum were sealed in glass ampoules and placed among the cubes of tuna in the center of each can. The cans were vacuum sealed under 15–20 in. vacuum, and processed at 115°C for 120 min in the still-retort, as recommended by NCA (1976).

Heat penetration data were also obtained using a Hewlett Packard 85 computer equipped with a HP3497A Data Acquisition/Control unit. The sterilization value ($F_{115°C}^{10°C}$) of the process was determined using 115°C as the reference temperature and a z value of 10 C°. To confirm the sterilization value of the process for the control packs, the cans were opened and the suspension of NCA 1518 spores was plated on Dextrose Tryptone Agar. The plates were incubated at 55°C for 48 hr, after which the number of survivors were counted, and decimal reductions (dr) in the process calculated where $dr = \log a - \log b$; a = initial number of spores; and b = number of surviving spores.

High vacuum flame sterilized packs. Preliminary runs were made to establish the equivalent flame sterilization process needed for sterilizing HVFS diced tuna using the F and dr values achieved in

the control packs. Four other process levels were established by varying the flame intensities and the holding times of the cans in each section of the flame sterilizer. Cans (303 × 406) were filled with 300g of 0.5 in. cubes of precooked tuna light meat. As discussed later, in some packs, pieces of tuna adhered together. We speculated that the "glue" causing clumping of the fish pieces might be proteinaceous in nature and on that basis added a preparation of bromelain to determine if this were so. Fifty ml distilled water containing 50 mg of crude bromelain was then volumetrically measured into each can. Lids were clinched on the filled cans. After clinching, the cans were transfered to an angular deaerator.

Deaeration. On the angular deaerator, the cans were slanted at a 30° angle from the vertical and rotated at 25 rpm over three individually controlled 6 in. long burners positioned beneath the cans. The total residence time of each can on the angular deaerator was 78 sec, and the flame intensities of the three burners were regulated at 1½, 1, and 1 psig with a pressure regulator. The loosely clinched lids allowed the discharge of air and steam but were tight enough to withstand some internal pressure associated with the generation of steam. At the end of the angular deaerator, cans dropped 12 in. onto a low-intensity vertical deaerator, where the flame was controlled with a flow meter (Fisher & Porter Co.). The cans travelled in an upright position without rotation into the steam flow closing machine. The vertical deaerator was covered with a housing which adjoined the closing machine. Can temperature was maintained through double seaming by the heat generated in the vertical deaerator, thus avoiding condensation during the transfer between deaeration and closing.

Flame sterilization. Following deaeration and double seaming, the cans were conveyed to the flame sterilizer. Sterilization was accomplished with the cans horizontally rotating over gas flames. The sterilization sequence consisted of the high flame section(s) where the cans were heated rapidly to sterilization temperature. Thereafter, the cans were passed on to the holding section(s) where either low flame or no flame was employed to achieve the desired sterilization values. The cans were rotated at a rate of 45 rpm over all the four sections, but the residence times were proportionately varied for the different process levels. The gas flow rate to each burner was controlled with a pressure regulator. At the end of the four sections, the cans dropped onto the cooling section, which was used as a no flame holding section. Cans were cooled to 25°C in a tank of running water. The lethality achieved at each process level was determined as described above. The copper-constantan thermocouple mounted on one end of the can was wired to the temperature recorder through a rotating thermocouple disk. Due to the problem of accessibility in the cooling section, the internal temperatures were measured in the flame sterilizer only when the cans were in the heating and holding sections. The cans were then held for a time equivalent to the time spent in the cooling section, then cooled. The five process levels were designed to give almost 100% spoilage at the lowest process level, and no spoilage at the higher process level.

Test packs

The test packs consisted of HVFS tuna packed in 303 × 406 cans, processed at five different process levels using the flame sterilizer, and control packs processed by the procedures described earlier using the still-retort. The control pack consisted of 15 cans designed solely for laboratory analysis. These cans were not inoculated. For the high vacuum flame sterilization studies, at each process level, a total of 35 cans were processed and sterilized. The first 20 cans were inoculated with 7.6×10^4 spores of *Clostridium sporogenes* (PA3679), while the remaining 15 were not inoculated. The uninoculated cans were intended for quality analysis. The cans were deaerated and flame sterilized according to the schedules given in Table 1.

Incubation

The inoculated packs were incubated at 35°C and observed for swelling of the cans. Uninoculated packs designed for subsequent chemical analysis were stored at room temperature (20–25°C).

Laboratory analysis

Drained weight was determined by weighing the contents of a can after opening and draining off the liquid portion through a number 8 mesh screen for 2 min (NCA, 1976). Vacuum in the cans was measured by piercing the cans with a vacuum gauge prior to opening. The percent solids in the drained liquid portions of canned tuna was determined by using a microwave moisture/solids analyzer (CEM Corp.). The textural character of the tuna meat was determined by using an Instron model 1122, equipped with a Kramer shear cell and a 500 kg load cell.

The drained meat was lyophilized, homogenized to a fine powder, and stored at −20°C for subsequent analysis. Samples were analyzed for crude protein and ash content by the AOAC method (1975). The fat content was determined by the procedure of Bligh and Dyer (1959).

The amino acid composition was determined using an automated amino acid analyzer equipped with a Durrum chromatography column and an Autolab integrator. The fish samples were hydrolyzed for 24 hr at 110°C with 6N HCl under vacuum. Tryptophan was determined by an alkaline hydrolysis method (Hugli and Moore, 1972), and the sulfur-containing amino acids were analyzed by using a performic acid pretreatment of samples followed by acid hydrolysis with 6N HCl (Moore, 1963).

The in-vitro protein digestibilities of the samples and ANRC sodium caseinate were determined by the method of Satterlee et al. (1979) using a multi-enzyme mixture of trypsin, chymotrypsin, peptidase and a bacterial protease. The Computed Protein Efficiency Ratio (C-PER) values for the fish samples were calculated using the C-PER procedure programmed on the Institute of Marine Resources computer (Satterlee et al., 1977, 1979, 1982).

Table 1—Heat treatment schedules for high vacuum flame sterilized and still-retorted canned tuna

Angular deaeration			Vertical deaeration		Flame sterilization			Sterilization value	
Sections & gas pressure[a] (psig)	Time (min)	Rotation (rpm)	Relative gas flow	Time (min)	Sections & relative gas flow[b]	Time/ section (min)	Speed (rpm)	F115°C	F126°C
1.5–1.0–1.0	1.30	25	10	6.50	12–5–5–0	2.25	45	268	83
1.5–1.0–1.0	1.30	25	10	6.50	10–5–5–0	2.50	45	226	63
1.5–1.0–1.0	1.30	25	10	6.50	12–5–0–0	2.25	45	144	45
1.5–1.0–1.0	1.30	25	10	6.50	10–5–0–0	2.50	45	135	38
1.5–1.0–1.0	1.30	25	10	6.50	10–0–0–0	2.50	45	9	2

Still-retort process

Retort	Come-up time (min)	Steam sterilization[c] Temperature (°C)	Time (min)	Sterilization value F115°C	F126°C
Vertical	7	115	120	100	29

[a] Three sections each with an independently controlled burner.
[b] Four sections each with an independently controlled burner.
[c] Process recommended by Lerke (1981).

Table 2—Comparative physical evaluation of flame-sterilized and still-retorted canned diced tuna

Sample	Drained wt (g)[a]	Vol. of drained liquid (ml)	Vacuum (in. Hg)	Total solids in drained liquid (%)[b]
Flame-sterilized	273.0 ± 23.0[d]	76.3 ± 18.5	27.3 ± 1.6[d]	23.9 ± 4.7
Still-retorted	297.4 ± 8.5[d]	143.6 ± 12.3	16.8 ± 1.3[e]	10.0 ± 1.9

[a] Mean ± Standard Deviation (N = 6).
[b] Mean ± Standard Deviation (N = 18).
[c] Means not sharing common letters are significantly different at P < 0.05.

Chemical estimates of available lysine of the samples were made by the 1-fluoro-2,4-dinitrobenzene (FDNB) extractive method of Carpenter (1960). Thiamin, riboflavin and niacin contents were determined by the AOAC procedures (1975). Samples for atomic absorption spectrophotometric analysis of minerals were prepared by wet digestion using 5 ml of concentrated nitric acid and 2 ml of 70% perchloric acid per g of lyophilized sample for 1.5 hr at 150°C and at 210°C for another 1.25 hr.

The compositions of fatty acids of the samples were determined by gas chromatography. The lipid, extracted by the Bligh and Dyer procedure (1959) was used for preparation of fatty acid methyl esters by the saponification-methylation procedure of Metcalfe et al. (1966). The automated Hewlett-Packard chromatographic system used included a stainless steel column packed with 10% Supelco-2330 on 100–120 mesh Chromosorb W AW (Supelco), and was operated isothermally at 180°C with nitrogen as the carrier gas at a flow rate of 20 ml/min.

Most analyses were carried out in duplicate on six cans or the corresponding lyophilized and homogenized samples each of the control and the HVFS processes. For the HVFS process, the pack above the lowest process level which did not spoil was used for comparative quality analysis.

Statistical analysis

Data were analyzed by analysis of variance and significance between means was determined using Duncan's multiple range test at the 5% level (Steel and Torrie, 1960).

RESULTS & DISCUSSION

Product and process development

Flame Sterilization, a high temperature short-time process, is an ideal method for sterilizing particulate products packed in liquid. The high temperature of the flame coupled with rapid rotation of the cans result in rapid heat penetration and destruction of microorganisms. The conventional method of solid packing tuna would not be suitable for flame sterilization as heat penetration into the tuna would be slow compared to the availability of heat (flame) and scorching of the surface of the product would likely occur. Flaking or cutting the precooked tuna into cubes was experimentally tried. It was found that flaking the precooked tuna was a difficult and time consuming process, an the idea was abandoned after running a few cans of tuna flakes through the flame sterilizer. One-half in. cubes were found to be ideal due to the ease of cutting cubes and it was believed that cubes could move freely in the rotating cans resulting in rapid heat penetration into the products. The immediate problem that was encountered was the adhesion of the pieces of tuna together to form a solid cylinder. Certainly, the cylindrical solid mass of tuna was not ideal in terms of heat transfer and did not have the expected appearance. After numerous trials, by incorporating 50 mg of crude bromelain enzyme (Sigma Grade II) into 50 ml distilled water (covering liquid) and pouring the solution onto the cubes of tuna in each can, an acceptable HVFS product was produced. Occasionally, packs of solid mass of tuna or "mushy" tuna resulted. This was attributed to an uneven distribution of enzyme incorporated into each enidividual pack. Although bromelain is not used in commercial practice, it is a GRAS substance.

Table 3—Mean (n = 18) Instron texture values of thermally processed canned tuna

Treatment	Fish:Liquid ratio	Instron energy[a] (kg cm/g)
Flame-sterilized	3.58	7.41 ± 1.07
Still-retorted	2.07	4.84 ± 0.96
Commercial[b]	3.32	6.64 ± 0.70

[a] All means are significantly different at P < 0.05.
[b] Commercially canned tuna (303 × 113, standard packs).

The sterilization value ($F_{115°C}^{10°C}$) achieved in the control packs was 100 min. As the process schedule used is recommended for all styles of tuna packs (Lerke, 1981), including the usual commercial solid packs, the sterilization value attained for the diced tuna packs would far exceed the required sterilization value for such packs. Nevertheless, this value was used as a basis for developing the equivalent high vacuum flame sterilization process. At the time of processing it was not feasible to use thermocouples in the cans, thus the temperature of the pack in each can was estimated from surface temperatures measured by infrared devices located at the end of the heating and holding sections. Previous studies have shown that internal temperature measurements obtained with copper-constantan thermocouples had excellent correlation (r = 0.9997) to can surface temperatures measured by the infrared sensors (Heil, 1982).

Table 1 shows the heat treatment schedules for the conventional and high vacuum flame sterilized canned fish study. The lethalities achieved in the HVFS packs were higher than expected because in the final run, the flame sterilizer was operated to full capacity. Leonard et al. (1977) reported that the proximity of cans causes significant increases in internal can temperatures by 2–4°C. Also, the effect of bromelain on bacterial spores has not been explored. As it turned out, all the inoculated packs from the five process levels, including the lowest process, which was expected to produce some spoilage, were found to be safe after 6 months of incubation at 35°C.

Analytical results

The pack from the HVFS process ($F_{115°C}^{10°C}$ = 135 min) which was closest to the control was used for comparative quality studies. Although the lethality achieved in the HVFS process was higher than the control process, for the purpose of comparative quality study, the products were assumed to be comparable in terms of thermal degradation. This assumption considers the large differences in z values which characterize the destruction of microorganisms (z = 10 C°) and the thermal degradation of nutrients and quality attributes (z = 30 C°).

No significant differences were shown in the drained weights (Table 2). However, there were significant differences in the volume of the drained liquid and the vacuums achieved in the HFVS and control packs.

The total solids content of the drained liquid in the HVFS pack was 23.87% as compared to 10.03% in the control pack. This was due in part to the reduced volume of

Sample	Moisture content (%)	Protein content (%)	Fat content (%)	Ash (content (%)
Raw	64.47 ± 0.91[c]	25.29 ± 0.47[c]	9.55 ± 0.28[c]	1.37 ± 0.02[c]
Precooked	57.07 ± 0.14[d]	27.87 ± 0.33[d]	13.06 ± 2.69[d]	1.49 ± 0.03[d]
Flame-sterilized	61.52 ± 1.94[e]	29.73 ± 1.14[e]	7.19 ± 1.59[c]	1.05 ± 0.02[e]
Still-retorted	64.78 ± 1.72[c]	23.74 ± 0.99[f]	10.05 ± 2.14[c,d]	0.79 ± 0.05[f]

[a] Each mean ± standard deviation represents four determinations for the raw and precooked samples and six determinations for the flame-sterilized and still-retorted samples.
[b] Means followed by the same letter are not significantly different (P < 0.05) from each other.

Table 5—Amino acids (g/16g N) of raw, precooked, flame-sterilized and still-retorted canned tuna

Amino acids	Raw[a]	Precooked[a]	Flame-sterilized[b]	Still-retorted[b]
Essential				
His	6.32	5.88	5.34	4.96
Ile	4.29	4.44	4.55	4.78
Leu	7.94	8.30	8.31	8.81
Lys	9.50	9.35	10.41	9.55
Met	2.99	2.99	3.08	3.20
Cys	1.17	1.09	1.19	1.04
Phe	3.88	3.91	4.08	4.23
Thr	4.89	5.03	4.81	5.34
Trp	1.25	1.21	1.42	1.35
Val	5.38	5.05	5.30	5.35
Nonessential				
Ala	5.76	5.99	6.10	5.99
Arg	6.58	6.61	6.60	6.79
Asp	9.75	9.69	9.85	10.11
Glu	13.71	13.52	13.43	13.93
Gly	4.24	4.93	4.30	4.46
Pro	3.70	3.98	3.58	3.50
Ser	3.84	3.80	4.14	4.06
Try	3.67	3.71	3.94	3.97
Amm	1.02	0.96	0.90	0.89

[a] Mean of two analyses
[b] Mean of three analyses

Table 6—In vitro protein digestibility of raw, precooked, flame-sterilized and still-retorted canned tuna[a]

Sample	% digestibility	% decrease[b]
Casein (ANRC)	90.27 ± 0.30	—
Raw	82.62 ± 0.22[c]	—
Precooked	81.38 ± 0.28[c,d]	1.50
Flame-sterilized	81.09 ± 0.85[d]	1.85
Still-retorted	81.62 ± 0.84[c,d]	1.21

[a] Means followed by the same letter are not significantly different (P < 0.05) from each other. Each mean ± standard deviation represents four determinations for the casein, raw and precooked samples and six determinations for the flame-sterilized and still-retorted samples.
[b] Compared to digestibility of raw fish.

liquid needed in the HVFS processing. Statistical analysis shows that there is no significant difference (P < 0.05) between the total solids content of the drained liquid of the HVFS and control packs.

The average vacuum achieved in the HVFS packs was 27.31 in. Hg as compared to 16.75 in. Hg for the control packs. The high vacuum resulted from the deaeration steps employed in the high vacuum flame sterilization process. During the deaeration steps, the flame vaporized some of the liquid packed with the product and the steam generated in the can caused air to be vented past the loosely clinched lid. The residual water/steam in the sealed can acted as the heat transfer medium during flame sterilization. High vacuum was achieved as the air was displaced by steam which condensed during cooling, after flame sterilization.

Removal of oxygen has been shown to prevent peach color oxidation and maintain the stability of vitamin C in fruits and vegetables (Leonard et al., 1975b, c). In meats and fish, several undesired reactions and changes can take place in the presence of oxygen. During heating, a large proportion of the denatured muscle pigment reacts with oxygen to form brown ferri-hemochrome, which would affect the visual appearance of the sterilized products (Wirth, 1977). Also of importance is the oxidation of unsaturated fatty acids during the sterilization process and duing storage of the canned products, causing an undesirable change in the flavor of the product. When HVFS tuna was freshly opened, the color of the canned product closely resembled the color of fresh precooked tuna. No undesirable discoloration was observed.

The Instron texture values of the HVFS, still-retorted and commercially canned tuna were significantly different (Table 3). Likewise, casual evaluation of the products revealed that the flame sterilized tuna had firmer but not objectionable texture. However, sensory panel experiments must be performed to ascertain the acceptability of the texture of high vacuum flame sterilized canned tuna. Decreasing the fish to liquid ratio by increasing the volume of covering liquid used, may yield a more desirable textural quality in the flame sterilized products. However, greater dilution of soluble attributes would result.

Table 4 shows the composition of the raw, precooked, HVFS, and still-retorted canned tuna. The moisture content of the still-retorted canned tuna was significantly higher than that of the HVFS product. Consequently, the protein and ash contents were significantly lower than in the HVFS samples. There were no significant differences in the mean fat content of the raw, HVFS, and still-retorted canned tuna.

Table 5 shows the amino acid composition of the fish samples. Glutamic acid, aspartic acid, lysine, leucine and arginine were the major amino acids, and their amounts, considering inherent sample variability, were comparable in all samples.

In-vitro protein digestibility of the heat processed fish samples was not altered drastically (Table 6). The small decrease in the in-vitro digestibility would not be expected to affect overall quality of the fish samples (Table 7). Previous studies as reported by Satterlee et al. (1977, 1979) have proven that the C-PER procedure can reliably estimate the quality of a food protein. Excellent correlation was obtained between the values derived by the C-PER procedure with that of the various bioassay techniques (Satterlee et al., 1977, 1979). The simplicity, reliability and the added advantage of being a rapid assay technique bespeaks the practicality of the C-PER procedure for evaluating the quality of food protein at various stages of processing.

Chemical estimates of available lysine show that precooking marginally (2%) decreased the amount of available lysine (Table 8). A further heat treatment whereby the fish were high vacuum flame sterilized did not result in any further loss of available lysine. However, when conventionally processed, the canned fish lost 10% more of the total available lysine. This loss is expected as the conventional

method of sterilizing canned products involves a lengthy heat-treatment period in order to ensure the achievement of commercial sterility in the products. Lysine, being a heat-labile amino acid, is a useful index of thermal destruction during heat treatment of the food. The results indicate that the high vacuum flame sterilized products which received a high temperature short-time process lost only an unmeasurable amount of the heat-labile nutrient. A further point of interest is that the lysine contents of the heat-treated samples as determined by the conventional acid hydrolysis procedure (Table 5) only indicate the potential, but not the actual, nutritional availability of lysine. Therefore, notwithstanding the evidence of the total amino acid analysis, the biologically available amino acid composition of the heated and the unheated protein may be widely different. During heat processing, the epsilon-amino group of lysine can crosslink with reducing substances, and these bonds are resistant to the hydrolytic activity of digestive enzymes (nonavailable lysine). Reactions can also take place within proteins themselves between the free amino group of lysine and arginine and the free acid groups of aspartic and glutamic acids, or amide groups of asparagine and glutamine. In this case, the Carpenter's method (1960) actually measures the available lysine content of the fish samples. Although the loss of available lysine is not of dietary significance since lysine is not a limiting amino acid in the fish samples, it can be used as an index to measure the thermal degradation of similarly heat labile essential nutrients.

Other amino acids such as cysteine and methionine can also be affected. However, currently there are no suitable chemical techniques for determining available cystine and methionine in processed foods. Miller et al. (1965) and Donoso et al. (1962) have demonstrated that when meat or fish were given severe heat treatment under different conditions, the value of these products as sources of the sulfur-containing amino acids or lysine was similarly reduced. Whether the same phenomena would occur under the present processing condition was not investigated.

The mean thiamin contents of all the thermally processed fish samples were significantly lower than the raw samples (Table 9). Precooking resulted in a loss of about 40% of the original thiamin content. High vacuum flame sterilized fish had a four fold higher retention of thiamin compared to the canned fish sterilized by the conventional method. Thiamin loss seems to be generally characterized by a low activation energy for destruction, although McBee and Marshall (1977) indicated that the destruction rates can be markedly affected by components often present in foods. The result in this study show that thiamin loss was minimized by the use of HVFS process. Similar findings have also been reported for thiamin retention in HTST processed vegetables as compared with conventional processing methods (Feaster et al., 1948). The workers further found that

increasing the rate of heat penetration through agitation of the cans during processing further reduced the amount of thiamin loss by one-third.

The loss of riboflavin and niacin followed a similar trend, although such losses were not as drastic as that of thiamin. Smaller losses of these vitamins could be attributed to their greater heat stability. The leaching of the vitamins into the liquid portions could be an important contributory cause of the losses, although this was not assessed in the present study. Niacin has been implicated to be less heat sensitive but more oxygen-sensitive. Therefore, for preservation of niacin, it is important to exclude oxygen from the can. Thus, high vacuum in canning (HVFS) may help preserve this vitamin.

The mineral content of the samples is given in Table 10. It has been shown that many of the minerals of nutritive value are more abundant in the nonedible portions of the fish body, which are removed along with the removal of the head, bones, dark meat and skin at an early stage of processing. Nevertheless, it can be seen from the table that the light meat of the tuna fish is also a rich source of various minerals required in human nutrition. The data show that, in general, the still-retorted canned tuna had a lower concentration of each of the minerals except for copper. This could be due to the dilution (leaching) of the minerals by the liquid portion of the canned fish.

The fatty acids in the raw tuna samples consisted of 37.28%, 33.95% and 28.78% of saturated, monounsaturated

Table 7—C-PER of raw, precooked, flame-sterilized and still-retorted canned tuna[a]

Sample	C-PER
Casein (ANRC)	2.5
Raw	2.5
Precooked	2.6
Flame-sterilized	2.6
Still-retorted	2.6

[a] Mean of three analyses.

Table 8—FDNB-available lysine[a,b]

Sample	FDNB-available lysine (g/16g N)	% of total lysine[c]
Raw	8.21 ± 0.40[d]	100
Precooked	8.04 ± 0.66[d,e]	98
Flame-sterilized	8.07 ± 0.41[d,e]	98
Still-retorted	7.21 ± 0.69[e]	88

[a] Each mean ± standard deviation represents four determinations for the raw and precooked samples and six determinations for the flame-sterilized and still-retorted samples.
[b] Means followed by the same letter are not significantly different (P < 0.05) from each other.
[c] Compared to total lysine of raw fish.

Table 9—Mean contents and percent retention of vitamins[c]

Vitamins and % Retention	Raw[a]	Precooked[a]	Flame-sterilized[b]	Still-retorted[b]
Thiamin (g/100g)	84.68 ± 2.35[d]	48.78 ± 0.00[e]	27.24 ± 7.20[f]	7.82 ± 0.98[g]
% retention	—	58	32	9
Riboflavin (g/100g)	85.74 ± 6.58[d]	75.09 ± 3.22[d,e]	63.85 ± 8.72[d,e]	51.70 ± 20.38[e]
% retention	—	88	74	60
Niacin (mg/100g)	9.38 ± 0.61[d]	9.02 ± 0.12[d]	7.82 ± 0.31[e]	6.35 ± 0.72[f]
% retention	—	96	83	68

[a] Mean ± standard deviation of at least two analyses.
[b] Mean ± standard deviation of at least six analyses.
[c] Means followed by the same letter are not significantly different (P < 0.05) from each other.

Table 10—Mineral content as determined by atomic absorption spectrophotometry[a,b]

Sample	Na	K	Mg	Ca	Cu	Fe	Zn
	g/100g			mg/100g			
Raw	0.420 ± 0.127[c]	0.994 ± 0.069[c]	0.067 ± 0.000[c]	23.563 ± 15.822[c]	0.225 ± 0.053[c]	1.675 ± 0.230[c]	0.975 ± 0.000[c]
Precooked	0.718 ± 0.021[d]	0.695 ± 0.000[d]	0.065 ± 0.006[c,d]	64.788 ± 6.240[d]	0.247 ± 0.000[c]	1.763 ± 0.035[c]	1.350 ± 0.050[d]
Flame-sterilized	0.418 ± 0.071[c]	0.699 ± 0.065[d]	0.058 ± 0.006[d]	24.383 ± 5.030[c]	1.363 ± 0.606[c,d]	1.871 ± 0.365[c]	1.355 ± 0.089[d]
Still-retorted	0.434 ± 0.116[c]	0.525 ± 0.038[e]	0.042 ± 0.003[e]	20.908 ± 8.489[c]	1.879 ± 0.841[d]	1.583 ± 0.438[c]	1.421 ± 0.183[d]

[a] Each mean ± standard deviation represents six determinations for the raw and precooked samples and eighteen determinations for the flame-sterilized and still-retorted samples. Values are expressed on a dry weight basis.
[b] Means followed by the same letter are not significantly different (P < 0.05) from each other.

Table 11—Percent fatty acid composition of muscle tissues from raw, precooked, flame-sterilized and still-retorted canned tuna

Fatty acid Carbon no:Double bond	Raw[a]	Precooked[a]	Flame-sterilized[b]	Still-retorted[b]
Saturates				
C14:0	4.07	3.95	3.82	3.82
C16:0	25.92	26.19	25.86	25.51
C18:0	7.29	7.43	7.55	7.73
Total	37.28	37.57	37.24	37.06
Monounsaturates				
C16:1	5.25	5.39	5.51	5.23
C18:1	24.36	25.42	25.08	25.73
C20:1	4.34	3.57	3.53	4.14
Total	33.95	34.38	33.74	35.09
Polyunsaturates				
C18:3ω6	0.11	0.10	0.09	0.11
C18:3	1.34	1.30	1.31	1.31
C20:2ω6	0.63	0.48	0.48	0.46
C20:3ω6	0.10	0.10	0.09	0.10
C20:4ω6	1.55	1.56	1.51	1.45
C20:5ω3	7.90	7.56	7.21	7.22
C22:4ω6	0.25	0.24	0.22	0.23
C22:4ω9	—	—	1.25	—
C22:5ω3	0.71	0.68	0.62	0.74
C22:6ω3	14.40	14.26	15.04	13.80
Total	28.78	34.38	29.84	27.84

[a] Mean of two analyses.
[b] Mean of three analyses.

and polyunsaturated fatty acids respectively (Table 11). The saturated fatty acids consisted mainly of palmitic acid (C16:0), while oleic acid (C18:1) predominated in the monounsaturated fatty acids group. Among the polyunsaturated fatty acids, eicosapentaenoic acid (C20:5) and docosahexaenoic acid (C22:6) prevailed. The ratios of unsaturated to saturated fatty acids did not change measurably in any of the fish samples which underwent the various thermal processes. The results show that neither high vacuum flame sterilization nor processing in the still-retort caused any apparent oxidative deterioration of the polyunsaturated fatty acids. The data are further supported by comparable relative thiobarbituric acid value (TBA) for both the high vacuum flame sterilized and the still-retorted canned tuna.

Additional work will be needed to evaluate and compare the effects of storage on canned tuna processed by the conventional and the HVFS procedures.

CONCLUSION

HIGH VACUUM FLAME STERILIZED canned diced fish was superior to the conventionally processed products in terms of a number of physical, chemical and nutritional parameters. The vacuum levels achieved in the HVFS packs were significantly higher (P < 0.05) than in the control packs. Amino acid composition, in-vitro protein digestibility and the Computed Protein Efficiency Ratio (C-PER) of both the raw and the heat processed tuna meat were essentially the same. Chemical estimates of available lysine show that precooking marginally decreased the available lysine content. A further HVFS heat treatment of the canned fish did not result in any measurable loss of available lysine.

However, the control process caused an additional 10% loss of the available lysine content. Thiamin contents of all the heat processed samples were markedly lower than the raw samples. Again, the HVFS samples had a higher retention of thiamin than the control samples. The loss of riboflavin and niacin followed a similar trend, although these losses were not as drastic as the loss of thiamin. Control samples contained lower concentrations of each of the mineral determined. However, the fatty acid compositions of the raw and both heat processed tuna meat samples were essentially the same.

This preliminary study shows that by following a new approach to product formulation, it is possible to high vacuum flame sterilize canned fish. However, many more trials, complete cost analysis, and appropriate surveys should be carried out before the HVFS process can be commercially utilized. Investigations of the potential use of the HVFS process for sterilizing canned shellfish such as shrimp, oyster, crayfish, lobster, and crabmeat deserve similar attention.

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Modified Atmosphere Storage of Dungeness Crab (*Cancer magister*)

KIRK L. PARKIN & W. DUANE BROWN

—————————————ABSTRACT—————————————

A modified atmosphere (MA) of 80% CO_2 and 20% air coupled with refrigerated storage was effective in extending shelf life of cooked whole Dungeness crab. Aerobic plate counts were lower over the storage period tested (25 days) for samples held in MA compared to controls held in air. Tissue pH decreased in MA samples, but increased in samples held as air controls (AC). There were significant differences in redox potential between groups, but these did not appear large enough to affect bacterial growth. Trimethylamine and ammonia values were similar for 14 days, then became higher in controls. There were no differences between treatment groups in thiobarbituric acid values, in textural properties as determined by Instron or in reflectance measurements. After removal from MA storage, the crab had a microbial shelf life that mimicked that of a fresh product.

INTRODUCTION

INTEREST in modified atmosphere (MA) technology as applied to muscle foods has experienced a resurgence in the past decade. Efforts to prolong the shelf life of meats (Jurdi et al., 1980; Silliker et al., 1977) and fish (Brown et al., 1980; Mitsuda et al., 1980; Banks et al., 1980) have intensified and have recently been reviewed (Wolfe, 1980; Parkin and Brown, 1982). The preponderance of these investigations have focused on CO_2 levels of 60% and below. The bacteriostatic effect of MA storage increases with increasing CO_2 concentrations (King and Nagel, 1967; Enfors and Molin, 1980; Gill and Tan, 1980). High CO_2 concentrations might be used for many seafoods with little risk of heme protein discoloration because of the low pigment concentration. Our previous study (Parkin et al., 1982) confirmed that trained panelists could not detect discoloration on rockfish fillets held in an 80% CO_2 atmosphere for 13 days. Little work has been reported on MA storage of shellfish although studies have been done on a combined refrigerated seawater-CO_2 treatment of raw pink shrimp (Branett et al., 1978). We had been informed by industry that effective MA storage of cooked crab meat should be very beneficial (Ghio, 1979). Therefore, it was our purpose to explore the effectiveness of MA storage of whole cooked crab at 1.7°C using an 80% CO_2:10% O_2: 10% N_2 atmosphere. The effectiveness of MA storage of whole cooked crab was judged by microbial analysis and common chemical indices of spoilage.

MATERIALS & METHODS

Storage and handling

Live Dungeness crabs (*Cancer magister*) were obtained from a distributor in Sacramento, CA and were transported to the lab. The crabs were boiled in a 30° salometer brine for 21–23 min with the boiling time beginning at the point where the brine returned to a full, rolling boil after immersion of the crabs. After the boiling time had expired, the brine was drained and the cooked crabs were sorted on racks in cylinders identical to those used in previous studies

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(Brown et al., 1980; Parkin et al., 1982). Ice was added to the bottom of each container to maintain a high humidity.

Filled containers were charged by evacuations to 19, 20 and finally 2 in. with each evacuation being followed by release of the vacuum to atmospheric pressure with an inflow of 100% CO_2, 100% CO_2 and 100% O_2, respectively. This resulted in an initial charge of 80 ± 5% CO_2, 10 ± 3% O_2, balance N_2 as verified by a Carle Model 8000 gas chromatograph. Air control (AC) samples of crab were treated similarly and all cylinders were held at 1.7 ± 1°C in the dark until sampling at 0, 4, 7, 11, 14, 19 and 25 days. At each sampling interval, four crabs from each treatment group were removed and subjected to each analytical test. Each MA cylinder that was opened was recharged to the initial MA composition. Through the course of the study each MA cylinder was recharged every 4 to 7 days. We realize that this does not provide a "modified atmosphere" in the usual sense since the containers were recharged during the course of the study. However, this seems to be the most commonly used experimental procedure.

At intervals of 7 and 14 days from the start of the study, appropriate amounts of whole crab were removed from the MA, transferred to and subsequently held in cylinders under a normal air atmosphere. This secondary storage period is referred to as the post treatment (PT) stage and was intended to simulate the marketing procedure of the product after MA storage. Sampling and analysis of materials transferred at 7 (PT_1) and 14 (PT_2) days were carried out for periods of 7 and 11 days, respectively, after the onset of the PT stages.

Aerobic plate counts (APC)

At each sampling interval, 5.0 or 10.0g of the intestinal tissue were aseptically obtained with a twice flame sterilized spatula after removal of the carapace. Each sample was then homogenized in 40 ml (for 10g samples) or 245 ml (for 5g samples) of sterile 0.9% NaCl in a Waring Blendor for 1 min. After the appropriate serial dilutions in sterile 0.9% NaCl, duplicate 0.5 ml aliquots from several consecutive dilutions were spread-plated on standard methods agar with 0.5% NaCl as suggested by Liston and Matches (1976). Plates were incubated at 20°C and counts were expressed as the number of bacteria per g of tissue. Due to the considerable daily variation in counts during the course of the study, mean log APC's and their standard deviations were calculated by the statistical procedure of Hansen (1962).

Surface pH

Surface pH measurements were obtained on crab leg meat portions with a surface combination electrode using a time response format described previously (Parkin et al., 1982).

Surface oxidation reduction potential (E_h)

E_h on the surfaces of the crab leg meat samples were measured with a platimun inlay electrode and a reference electrode, using the time response format cited above in the pH measurement section.

2-Thiobarbituric acid (TBA)

TBA analysis was carried out according to the method of Vyncke (1970). Roughly 5g of body cavity meat and 15g of leg meat composed the 20g sample.

Trimethylamine and ammonia

Twenty five gram samples (roughly half each of body and leg meat) were extracted with 50 ml of 0.6N perchloric acid for 1 min in a Waring Blendor and the extract filtered through Whatman #2 filter paper. The cleared extract was then neutralized to a pH of 6.5–7.0 with 5N KOH, and then diluted to 75 ml with deionized, distilled water and held on ice to precipate the perchlorate. Tri-

methylamine content of the extracts was measured according to the method of Chang et al. (1976) as modified by Brown et al. (1980) using the time response format.

Ammonia determinations were performed using the Orion ammonia ion electrode following the instructions of the manufacturer.

Weight loss

The weight of each whole crab was measured at the beginning of the study and at the time of analysis and/or transfer to the PT stages. Weight loss was calculated as the difference between the weights of each individual sample at each point.

Instron texture measurement

Textural characterization of the crab meat was performed with an Instron model 1122, using a Kramer shear cell and a 500 kg load cell. Weighed portions (5–15g) of crab leg meats were mounted in the holding compartment of the cell with the muscle fibers aligned perpendicular to the plane of the shear plates. A single compression of the sample was applied. The area inside each trace was determined and the Instron energy parameter (kg × cm/g) was calculated.

Reflectance colorimetry

Reflectance tristimulus colorimetric analysis was performed on crabmeat originating from the claw appendage, encased by the mersus/carpus portions of the exoskeleton. X, Y and Z reflectance tristimulus values were measured for each sample using the Hunterlab C/D Meter with reference to the standard white tile. After standardization of the instrument, the samples were placed on a glass plate to obtain a flat reflecting surface and were measured through a ½ in. aperture. For each individual sample, x, y and z trichromatic coefficients were calculated and characteristic brightness, dominant wavelength and purity values were obtained according to Francis and Clydesdale (1975).

Statistical analysis

Statistical differences between treatment groups were determined by analysis of variance for all of the chemical and physical tests. The APC data were examined by a one-tailed t-test using the mean data for each day. Comparison groups treated by statistical analysis included AC vs MA, PT_1 vs AC and PT_2 vs AC. Since the PT periods were designed to mimic the marketing phase of a product, (i.e. initiation of post-MA storage), the parameters measured during each PT period were compared to the parameters measured during the first 7 or 11 days of the AC period. That is, PT_1 was compared to AC values for 0–7 days, while PT_2 was compared to AC values for 0–11 days.

RESULTS & DISCUSSION

Aerobic plate count (APC)

The results of the APC determinations from the intestinal region of the whole crabs are presented in Fig. 1. After a relatively long lag phase, typical of a cooked product, a rapid bacterial growth rate was observed in the AC crab after approximately 11 days. By 14 days, there was pronounced build-up of ammoniacal and putrid odors. Further spoilage was evident from the appearance of slime on the exoskeleton of the crab by 19 days, and from the onset of exterior mold growth on the 25th day storage period. On the other hand, crabs held in MA displayed negligible microbial growth throughout the storage period. This difference in APC between treatments was verified to be statistically significant at the 5% level by using the mean log APC data and subjecting it to a one-tailed t-test (Table 1). This bacteriostatic effect of CO_2, with respect to whole crabs, implies that CO_2 has the ability to penetrate deeply into biological tissues, since sampling was from the interior of the product. Interestingly there were no significant differences observed for the APC trends between the AC and either PT period (Table 1). This indicates that the microbial shelf life of the product upon removal from MA storage would be similar to that of the fresh product. Similar behavior has been noted by others (Coyne, 1933;

Silliker et al., 1977). Although it was not investigated, it would be interesting to look at the limits of PT shelf life after short exposures (less than 1 week) of the product to MA. It could then be determined if the bacteriostatic effect of CO_2 is cumulative depending on length of exposure to MA or if maximal benefits are derived from minimal exposure times.

Surface pH

The results of the tissue surface pH measurements are presented in Fig. 2. The pH of the tissues of crab held as controls (AC) underwent a rapid, considerable rise in pH over the first 4 days. This high pH was maintained throughout the remainder of the storage period. A rising pH in a fresh product usually indicates amine production by bacteria but APC (Fig. 1) and ammonia (Fig. 5) measurements do not correlate well with the pH data. Thus, it does not appear that pH can be utilized as an adequate index of crabmeat quality, as was found earlier for cooked blue crabmeat (Webb et al., 1976).

The pH of the tissue held under MA reflects the apparent absorption of CO_2 and its hydration to carbonic acid. The result was a decrease in pH over the first 11 days of storage with little further change. The difference in pH between the MA and AC groups was statistically significant at the 0.1% level as indicated by analysis of variance (Table 1).
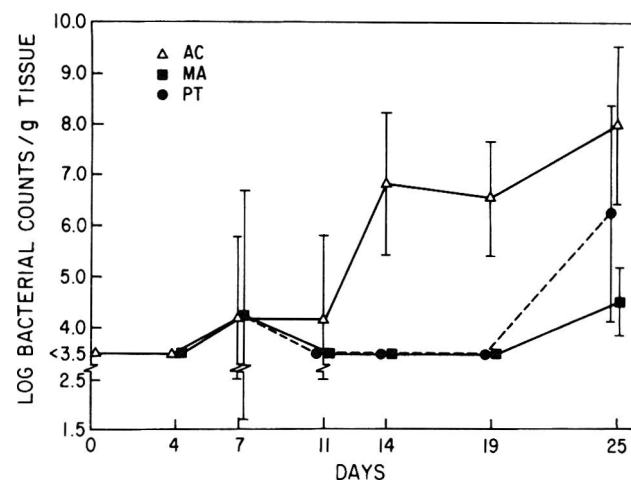
Fig. 1—Aerobic plate counts. Bars denote standard deviations. AC, Air control; MA, Modified atmosphere; PT, Post-treatment.

Table 1—Statistical analyses of data among comparison groups[a]

Parameter	Comparison groups		
	MA vs AC	PT_1 vs AC	PT_2 vs AC
Bacterial counts	5.0%*	NS	NS
Surface pH	0.1%	0.1%	0.1%
Surface E_h	5.0%	NS	1.0%
TMA content	0.1%	0.1%	0.1%
Ammonia content	0.1%	0.1%	0.1%
TBA	NS	NS	NS
Weight loss	NS	NS	NS
Instron energy	NS	NS	NS
Reflectance colorimetry:			
Brightness	NS	NS	NS
Dominant	NS	NS	NS
Purity	NS	NS	NS

[a] Statistical differences determined by analysis of variance, with the exception of those determined by the t-test (*) using the mean data for each day. Numerical values in table refer to the level of significance of difference; NS = Not significant; MA, modified atmosphere; AC, air controls, PT_1 and PT_2, post-treatment groups.

As the product is removed from MA to the PT stages, a corresponding rise in pH is observed. However, this pH rise is not nearly as sharp as that seen in the AC crabs between 0–4 days. Perhaps some CO_2 retained in the tissue accounts for the residual bacteriostatic effect noted during the PT stages.
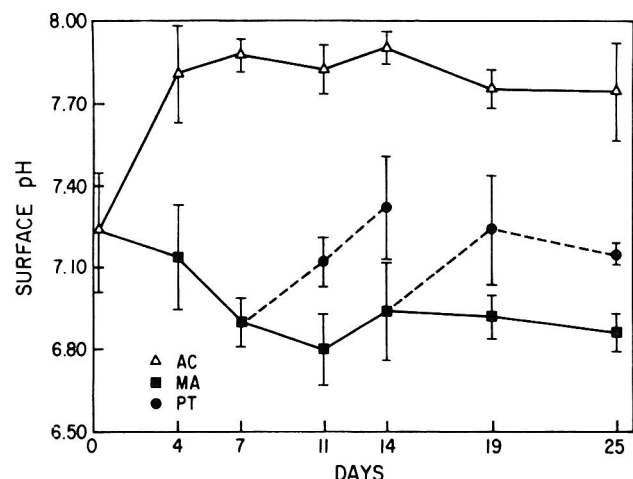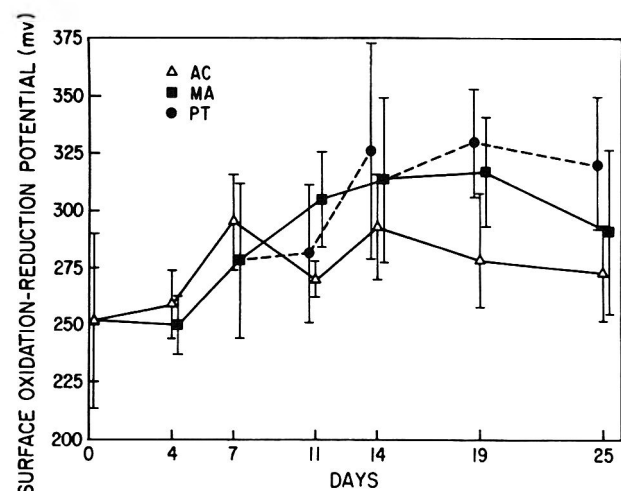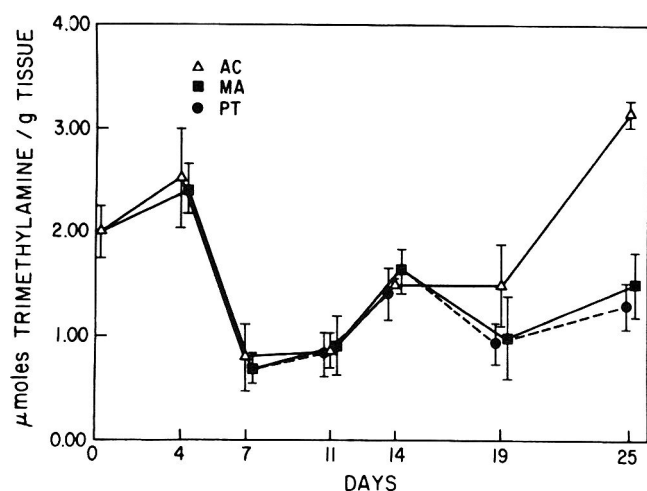
Oxidation-reduction potential (E_h)

The results of the tissue surface E_h measurements are presented in Fig. 3. As is evident from these results, aerobic conditions existed on the crab meat under all of the treatment and control atmospheres. Analysis of variance (Table 1) indicates that there are significant differences in E_h of tissues among the AC/MA and AC/PT$_2$ comparison groups at the 5.0 and 1.0% levels, respectively. However, in an absolute sense these differences are insignificant, since they represent differences in E_h of less than 50 mv which would have little bearing on bacterial growth in this range (−250 to +300 mv) of potentials. Most important is the fact that the tissues of crab held under the MA and PT periods consistently maintained E_h's of above +250 mv, preventing the likelihood of strict anaerobic growth. Nevertheless, due attention must be paid to proper refrigeration.

Trimethylamine (TMA)

The results of the TMA analysis are shown in Fig. 4. TMA production was observed to be unaffected by the type of storage condition over the first 14–19 days. Subsequently, there was a sharp increase in tissue TMA for the crabs held as air controls, whereas those held as MA samples remained low. The TMA levels for crab meat during both PT periods also remained low. Analysis of variance indicates that the difference among the treatments is statistically significant at the 0.1% level for the AC/MA, AC/PT$_1$ and AC/PT$_2$ comparison groups (Table 1). However, all values are well below the upper limit of 10 mg TMA nitrogen/100g suggested by Connell (1980) for chilled fish.

Ammonia

The results of tissue ammonia determinations are presented in Fig. 5. No differences between treatments were observed over the first 14 days of storage after which the AC samples displayed a sharp increase in tissue ammonia content. In contrast, ammonia accumulation in the meat of crab held under MA and PT conditions remained static at about 30 μmoles/g throughout the entire storage period. Analysis of variance indicates significant differences among the AC/MA, AC/PT$_1$ and AC/PT$_2$ comparison groups at the 0.1%, 0.1% and 1.0% levels, respectively (Table 1). The

Fig. 2—Surface pH values. Bars denote standard deviations. AC, Air control; MA, modified atmosphere; PT, Post-treatment.

Fig. 3—Surface oxidation-reduction potentials. Bars denote standard deviations. AC, Air control; MA, Modified atmosphere; PT, Post-treatment.

Fig. 4—trimethylamine levels. Bars denote standard deviations. AC, Air control; MA, Modified atmosphere; PT, Post-treatment.

Fig. 5—Ammonia levels. Bars denote standard deviations. AC, Air control; MA, Modified atmosphere; PT, Post-treatment.
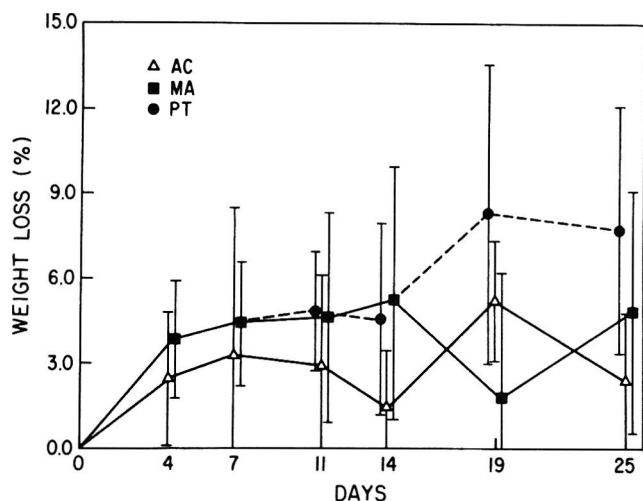
Fig. 6—Weight losses. Bars denote standard deviations. AC, Air control; MA, Modified atmosphere; PT, Post-treatment.

Fig. 7—Thiobarbituric acid values. Bars denote standard deviations. AC, Air control; MA, Modified atmosphere; PT, Post-treatment.

tissue ammonia contents show some correlation with bacterial counts, and may provide a suitable quality index.

Weight loss

The weight loss data are provided in Fig. 6. Most of the weight loss was suffered during the first 4 days of storage and, for the most part, amounted to less than 5%. Analysis of variance (Table 1) indicated no significant differences among treatments.

2-Thiobarbituric acid (TBA) analysis

The results of the TBA analysis are shown in Fig. 7. Overall, there is little variation in the TBA values for the products stored under the various conditions, as verified by analysis of variance (Table 1). Moreover, the actual values obtained indicate that there is negligible lipid oxidation taking place in this product under the various conditions. This might be expected, since it has been shown that the muscle tissues of Dungeness crab contain only 0.8% lipid (Gordon and Roberts, 1977), and the cooking process would be expected to eliminate any enzymatic lipid oxidation.

Instron texture analysis

The results of the Instron texture characterization are depicted in Fig. 8. As indicated by analysis of variance (Table 1), there are no significant differences in this textural parameter among treatment groups. Although not included in Table 1, analysis of variance also indicated that there were no significant changes in this parameter as a function of time during the course of the study. Therefore, one must conclude that holding this product under either of the conditions employed in the study maintains a textural character resembling a fresh product during all stages of storage as judged by this Instron parameter. However, sensory panel experiments must first be performed before this conclusion can be justified. Most important is the fact that, even though there was a decrease in the tissue pH, there were no textural changes (as measured by this method) associated with this subnormal pH condition. From this standpoint, MA systems would appear to offer an advantage over conventional frozen storage where significant muscle fiber deterioration is observed with crustacean food products (Giddings and Hill, 1976).
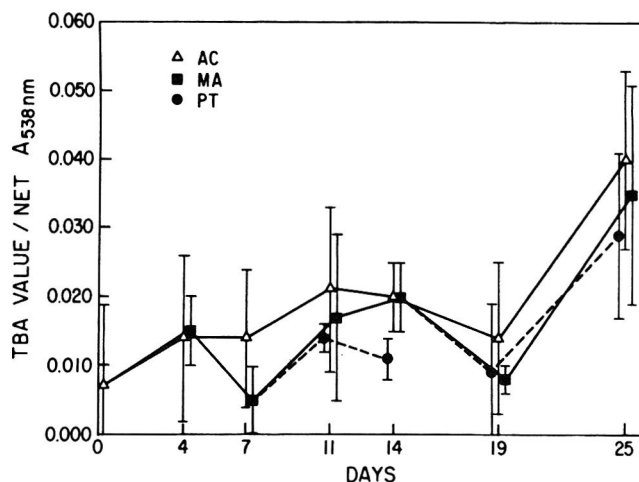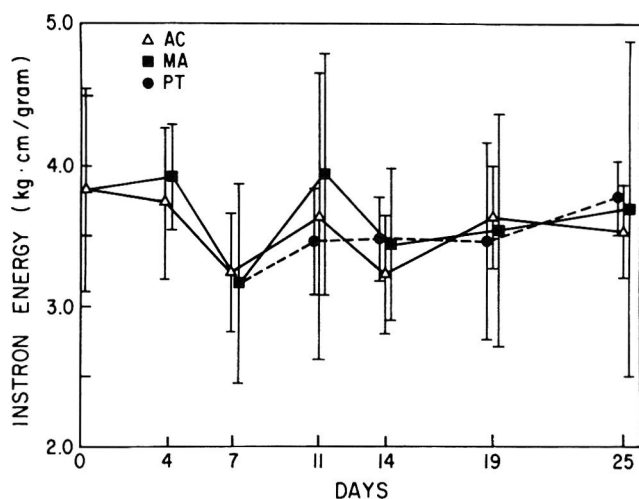
Fig. 8—Instron energy parameter. Bars denote standard deviations. AC, Air control; MA, Modified atmosphere; PT, Post-treatment.

Reflectance colorimetry

The results of the colorimetric parameters obtained from the reflectance measurements showed no differences among treatments with respect to brightness, dominant wavelength and purity for crab muscle (data not shown). This was verified by analysis of variance (Table 1). There were also no changes in the brightness parameter that would indicate problems of blue discoloration sometimes seen in crab (Babbitt et al., 1973).

CONCLUSIONS

MODIFIED ATMOSPHERE STORAGE of refrigerated cooked crab meat shows promise. Bacterial growth was retarded in the lag phase for the MA samples whereas prolific growth was observed under an air atmosphere after 11 days. Tissue ammonia contents reflected the trend observed for bacterial counts. As was seen in a previous study with rockfish fillets (Parkin et al., 1982), the 80% CO_2 atmosphere served to lower the muscle pH, indicating that the bacteriostatic effect may be due in part to a pH effect. Another important aspect of this study of cooked crab was that, after removal from MA storage, the product had a microbial shelf life that mimicked that of a fresh product. This is attributed to a residual bacteriostatic effect of CO_2 treatment, previously noted with other commodities (Coyne, 1933; Silliker et al., 1977). —Continued on next page

These studies show good potential for the application of MA technology to shellfish. However, before conclusive interpretations can be drawn, further studies must be conducted involving sensory evaluation of shellfish products held under MA. Preliminary findings suggest that absorption of carbon dioxide by crab tissues during long term storage may impart an off-flavor to the product. Off-flavor development in fruit products held under elevated levels of CO_2 has been reported (Ulrich, 1975). This is an area of future research.

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Preparation of High Protein Curd from Field Peas

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―――――――――――ABSTRACT―――――――――――

Field peas were investigated as an alternative to soybeans to produce a high protein curd which resembles tofu. Yield, texture, color, proximate composition and sensory evaluation of both curds were compared. The yields of total curd from pea flour and soybeans were 13.6% and 39.8% respectively with protein yields of 43.0% and 55.5%. Amino acid composition of the pea curd compared quite closely to that of the soybean curd. Addition of gluten improved the sulfur amino acid profile but reduced the lysine content. Flavor of the pea and soybean curds was rated similar but texture and color of the pea curd was scored lower (p < 0.05). Gluten modified the texture and the color of the curds.

INTRODUCTION

EXPANDING world food requirements have increased interest in the use of leguminous seeds for high protein foods. Soybeans and their products have played an important role for many centuries as a source of protein in the diet of Oriental people (Smith and Circle, 1972). Field peas (*Pisum sativum*) are being evaluated as a high protein crop for foods and feed in some areas where soybeans cannot be grown.

The market for field peas is being expanded by the development of new products such as fortified bread, meat extenders, snacks and beverages (Youngs, 1975; Nielsen et al., 1980; Sumner et al., 1981). It is possible that field peas may be suitable for the production of high protein food curd similar to tofu.

In recent years soy foods in general and tofu in particular have been receiving considerable attention in North America. Tofu is one of the most important soy foods which consists of a bland, cheese-like curd. It is prepared by adding a precipitant to the water extract of soybeans and molding the resulting high-protein curd into cakes (Smith et al., 1960; Lu et al., 1980). Tofu is highly digestible and relatively inexpensive and is used in a variety of Chinese dishes (Miller et al., 1952). Fresh tofu contains about 6−8% protein, 3.5% oil, 1.9% carbohydrate, 0.6% ash and 88.0% water (Smith and Circle, 1972).

The objective of this study was to investigate whether field peas could produce a curd similar to tofu. Also, the effect of wheat gluten on soybean and pea curds was investigated on the basis of nutritional and physical properties. All products were compared on the basis of yield, composition, texture, color and sensory evaluation.

MATERIALS & METHODS

Materials

Pea flour and pea protein concentrate were prepared by the Prairie Regional Laboratory of the National Research Council, from

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field peas (*Pisum sativum* var. Trapper) by the procedure of Youngs (1975). Dry whole soybeans (Grade No. 1) were purchased from Early Seed and Feed Ltd., Saskatoon, Canada. Vicrum vital gluten containing 80.0% protein was provided by J.R. Short Canadian Mills Ltd., Toronto, Canada.

Preparation of curds

To determine the effect of coagulant type and concentration on pea curd properties, preliminary trials were carried out at coagulant concentrations in the extract of 0.15−0.54%. The coagulants evaluated included reagent grade calcium sulfate, calcium chloride and acetic acid obtained from Fisher Scientific Co.

Preparation of the pea flour extract was carried out as described by Sumner et al. (1981) and consisted of extracting 100g of flour with 500 ml of distilled water adjusted between pH 8.8−9.0 with 0.2% calcium oxide while stirring for 20 min. The extract was separated by centrifuging at 1000 x g for 20 min. The resulting solution was heated for 20 min at 95−100°C and then filtered through a double layer of cheesecloth. The pea extract was cooled to 75−80°C, stirred vigorously for about 20 sec; agitation was stopped and the curd was then precipitated by 0.54% coagulant concentration using the procedure described by Lu et al. (1980). When formed, the curd was then transferred into Tyler 20-mesh screens (12.5 cm diam and 6.5 cm deep) lined with cheesecloth. A circular plywood disc was placed on the top of the curd and pressed with a 275g weight for 1−2 hr until draining almost stopped.

When preparing the soybean curd, 50g of dry whole soybeans were washed and soaked in water at room temperature overnight. Next day the water was discarded and the soaked beans were transferred to a Waring blendor and water was added in the ratio of 10:1 (water:dry soybeans). The curd was then precipitated, separated and drained using the procedure previously described.

Yield

Curd yield was expressed as the percentage of the original pea flour or soybeans recovered in the final curd (dry basis). The protein yield was expressed as the percentage of the original pea flour or soybean protein recovered in the curd (dry basis).

Proximate analysis

Moisture content was determined by drying a 5−10g sample of curd at 100 ± 1°C to constant weight.

The proximate composition was determined by standard AACC (1976) procedures for crude protein (method 46-11), crude fat (method 30-25), crude fiber (method 32-15) and ash (method 08-11). Protein calculation was based on nitrogen factors of 5.7 for wheat and 6.25 for pea flour and soybeans. Nitrogen factors for blends were weighted on the basis of the relative proportion of the proteins in the ingredients. All analyses were carried out in duplicate.

Amino acid analysis of the curds was carried out on a Beckman Model 120c analyzer using the procedure described by Sosulski and Sarwar (1973). Results were corrected to 100% nitrogen recovery and expressed as grams amino acid per 16 g nitrogen. The chemical score for these products reflects the nutritional value and was calculated as described by Block and Mitchell (1946). The FAO/WHO (1973) provisional pattern of essential amino acids was used as the reference protein.

Texture

Textural quality was measured by a Texturecorder Model T-2100 using the CE-1 Universal cell with a parallel extrusion grid base and the following conditions: 300 lb ring, ram speed 0.7 cm/sec, and ram force 100 lb. Replicate curd samples were 5 cm diam x 1 cm thick. Maximum shear stress is reported in Newtons (N)/cm². *―Continued on next page*

The color of the curd was measured with the Hunterlab Model D25 D2M Color and Color Difference Meter. The L a b values were an average of four readings obtained while rotating samples 90° between readings.

Sensory evaluation

The sensory evaluation panel was composed of 25 adult males and females who were not familiar with tofu. Curd samples measuring 2.5 x 2.5 x 1 cm were fried on each side for 2 min in an all purpose, deep frying vegetable oil, in a Hoover stainless steel frying pan set at 375°F (190.5°C). Panel members evaluated the flavor, texture, color and acceptability of the pea and soybean curds alone and in combination with gluten using a 7-point scale in which 1 = extremely good, 2 = very good, 3 = slightly good, 4 = average, 5 = slightly poor, 6 = poor, 7 = extremely poor. Scores were subjected to analysis of variance and Tukey's least significant difference test (Larmond, 1977).

RESULTS & DISCUSSION

Curd coagulants

Preliminary trials were carried out to determine the most suitable coagulant and concentration for producing pea curd. Calcium sulfate, calcium chloride and acetic acid in concentrations ranging from 0.15–0.54% were used to precipitate pea flour protein. Curd produced by acetic acid had a firmer texture than when calcium salt coagulants were used, but it was easily broken and had a sour taste when the coagulant concentration exceeded 0.27%. For subsequent trials, calcium sulfate was used to precipitate the protein curd because it appeared to be the most commonly used salt in the production of traditional tofu (Wang, 1967; Schroder et al., 1973; Tsai et al., 1981).

Table 1 summarized the relationship between the concentration of calcium sulfate used and the characteristics of the resulting pea curd. The moisture content of the curd decreased from 86.4% to 83.8% with increasing concentration of the coagulant from 0.15% to 0.54%. On the other hand, protein yield increased from 36.8% to 43.0% and curd yield increased from 9.8 to 13.6% as the concentration of the coagulant increased over the range investigated. The lower yield and the turbid filtrate that appeared, when 0.15% calcium sulfate was used, indicated incomplete coagulation of the protein. The protein yield was slightly lower than yields reported for soybean curds by Hang and Jackson (1967).

The highest moisture curd resulted in a soft texture. An increase in the amount of coagulant concentration from 0.15% to 0.54% led to an increase in curd shear stress from 1.11 to 2.09 N/cm^2. Lu et al. (1980) in their studies of soybeans, reported that calcium salt concentrations ranging from 0.10–0.50% were suitable for soybean curd preparation.

From the trial data, it was found that best curd on the basis of curd yield, protein yield, firmness and smooth texture, was formed when the pea protein was coagulated at 75–80°C by the addition of a 2% calcium sulfate solution until it reached a concentration of 0.54% in the extract. These conditions were used to evaluate field pea and soybean curds in subsequent studies.

Comparison of pea and soybean curds

Pea flour containing 24% protein was compared to pea protein concentrate with about 60% protein for producing high protein curd. As expected, the curd yield from pea protein concentrate was about three times greater than from pea flour and was quite similar to the soybean curd yield. Protein yield, texture and other properties of the two pea curds were similar. Remaining trials were carried out with pea flour rather than pea protein concentrate which is more expensive to produce.

Table 2 compares the yield and properties of curds prepared from the pea flour and soybeans. The moisture contents of the pea and soybean curds were 83.8% and 79.5%, respectively. Curd made from peas was softer in texture than soybean curd as shown by shear stress values of 2.09 and 5.40 N/cm^2 respectively.

Although there is no recognized standard of identity for tofu, generally commercially available hard tofu contains from 79–75% moisture while the water content of soft tofu ranges from 88–82% (Shurtleff and Aoyagi, 1979). Moisture contents of 84.7% and 85.9% for tofu products reported by Tsai et al. (1981) and Smith et al. (1960), were similar to the pea curd moisture.

The yields of curds from the pea flour and soybeans were 13.6% and 39.8% respectively with protein yields of 43.0% and 55.5%. The lower curd yield from pea flour was due to the lower protein content and also to the large amount of carbohydrate residue removed during the curd preparation. Schroder and Jackson (1972), in their study of soybeans, obtained a curd yield of 31.7%.

Results of the curd color measurements (Table 2) showed that soybean curd had a light yellow color whereas the pea curd was gray in color. The undesirable gray color may have been caused by polyphenols which oxidize readily at a high pH. A better color might be achieved at a lower pH.

Effect of gluten on curd properties

Preliminary trials were carried out to determine the best concentration of gluten to be added for texture modification by incorporating 7.5, 15.0 and 30.0g of gluten into the pea and soybean extracts equivalent to concentrations of 2.0, 4.0 and 8.0% (w/v). The data showed that firmer and smoother textured curd was formed when 4.0% gluten was

Table 1—Effect of calcium sulfate concentration on pea flour curd properties

Coagulant conc (%)	Moisture (%)	Protein yield (%)	Curd yield (%)	Shear stress (N/cm^2)
0.15	86.4 ± 1.0[a]	36.8 ± 1.4	9.8 ± 2.1	1.11 ± 0.15
0.27	85.8 ± 0.3	37.1 ± 1.8	11.8 ± 1.8	1.48 ± 0.25
0.40	85.0 ± 0.4	40.0 ± 2.0	13.3 ± 2.5	1.74 ± 0.19
0.54	83.8 ± 0.8	43.0 ± 1.4	13.6 ± 1.3	2.09 ± 0.08

[a] Mean ± standard deviation of four batches.

Table 2—Comparison of curds prepared from pea flour and soybean alone and in combination with wheat gluten

Sample	Moisture (%)	Curd yield (%)	Shear stress (N/cm^2)	Color[a] L	a	b
Pea curd	83.8 ± 0.8[b]	13.6 ± 0.5	2.09 ± 0.08	51.9	−1.3	8.0
Pea/gluten curd	78.7 ± 1.0	27.7 ± 2.8	3.79 ± 0.32	61.3	−1.5	12.2
Soybean curd	79.5 ± 0.9	39.8 ± 1.0	5.40 ± 0.18	70.6	−0.4	18.0
Soybean/gluten curd	72.7 ± 0.5	50.5 ± 2.1	5.66 ± 0.41	71.0	−0.3	13.1

[a] L (100 white, 0 black); a (+ red, − green); b (+ yellow, − blue).
[b] Mean ± standard deviation of four batches.

Table 3—Compositions of pea flour, soybeans and curds

Constituents (dry basis)	Pea flour	Pea curd	Soybeans (dry, whole)	Soybean curd
Crude protein %	23.9	81.4	41.3	57.3
Crude fat %	1.5	3.7	19.6	29.0
Crude fiber %	3.8	0.3	5.1	0.0
Ash %	2.7	5.3	4.6	5.6
Nitrogen free extract %	68.1	9.3	29.4	8.1

Table 4—Essential amino acid distribution and chemical score of curds

Sample	Amino acids (g/16 g N)[b]							Chem. score
	Lys	Met + Cys	Thr	Iso	Leu	Tyr + Phe	Val	
Reference[a] protein	5.5	3.5	4.0	4.0	7.0	6.0	5.0	100
Pea curd	7.2	2.1	3.7	4.5	8.2	9.5	5.0	60
Pea/gluten curd	3.5	3.0	2.7	4.0	7.0	8.4	4.3	64
Soybean curd	6.3	2.6	4.1	4.8	8.0	9.1	5.1	74
Soybean/gluten curd	3.9	3.1	3.0	4.0	7.3	9.1	4.3	71

[a] FAO/WHO (1973).
[b] First limiting amino acid underlined.

Table 5—Sensory evaluation of curds prepared from pea flour and soybean alone and in combination with gluten

Sensory property	Rating scores[a][b]			
	Pea curd	Pea/gluten curd	Soybean curd	Soybean/gluten curd
Flavor	2.72a	2.64a	2.12a	2.12a
Texture	3.20b	3.12b	2.00a	1.96a
Color	2.96b	2.76b	1.44a	1.76a
Ranking score	3.08b	3.04b	1.88a	2.00a

[a] 1 = Extremely good, 7 = Extremely poor.
[b] Means in the same row followed by different postscripts differ significantly (p < 0.05).

added to the soybean and pea extracts. When 2.0 and 8.0% gluten were incorporated, the resulting curds were softer and lacked elasticity.

Table 2 also compares some properties of the pea and soybean curds when 4.0% gluten was incorporated into the extracts. Gluten decreased the moisture in these curds by 5.1 and 6.8 percentage points respectively. This increased the firmness of pea curd from 2.09 to 3.79 N/cm² but had little effect on the soybean curd. The higher gluten recovery compared to pea flour increased the pea/gluten curd yield to 27.7% compared to 13.6% for pea curd. The corresponding increase for soybean/gluten curd was from 39.8% to 50.5%. Gluten increased the lightness and yellow appearance of pea curd so that it resembled more closely the soybean curd.

Chemical composition

Proximate analysis of pea flour, soybeans and the curds produced from them are shown in Table 3. There was a concentration of protein in the pea curd to 81.4% compared to 23.9% in pea flour because the carbohydrates and crude fiber were largely removed during the preparation of the curd. Pea curd contained 3.7% crude fat which indicated most of the lipids remained with the protein. The soybean curd, on a moisture-free basis, contained 57.8% crude protein, 29.0% crude fat and no crude fiber. These values were similar to those reported by Smith et al. (1960) and Schroder and Jackson (1972). The major differences between the pea and soybean curd were the higher protein and lower crude fat content in the pea curd. However, it was found that the crude fat content of pea curd could be increased to about the level in soybean curd, with a corresponding decrease in protein, by the addition of Canola (rapeseed) oil to the extract prior to coagulation.

The high ash content in both curds resulted from the calcium sulfate used for the precipitation. Soybean curd prepared in this way is considered to be a good source of calcium in countries where the milk supply is low or expensive (Miller et al., 1952; Chiu and Van Duyne, 1961).

Table 4 presents the essential amino acid composition and chemical scores of the curds prepared from pea flour and soybeans alone and in combination with gluten. The essential amino acid profile of the FAO/WHO reference protein is also shown. In general, the amino acid distribution of the pea curd was quite similar to soybean curd. The combined sulfur amino acids methionine and cystine were the first limiting amino acids for the curds which did not contain gluten. Pea curd was the most deficient with a sulfur amino acid content of 2.1 g/16g nitrogen and soybean curd contained 2.6 g/16g nitrogen compared to the recommended reference protein at 3.5 g/16g nitrogen. This resulted in chemical scores of 60 and 75 respectively for the two curds. Threonine was limiting also in the pea curd. Unlike cereal proteins, the pea and soybean curds with lysine contents of 7.2 and 6.3 g/16g nitrogen respectively, surpassed the reference protein requirements. The data on amino acid analysis of the soybean curds was in general agreement with those reported by Hackler and Stillings (1967) and Schroder and Jackson (1972).

Amino acid analysis of curds containing gluten was also carried out to determine if a better essential amino acid balance was obtained. These curds were prepared by the addition of 8.0% (30g) gluten to the pea and soybean extracts which provided an initial gluten protein concentration similar to the protein contents in the legume ingredients. Gluten improved the sulfur amino acid balance from 2.1 to 3.0 g/16g nitrogen for the pea/gluten blend and from 2.6 to 3.1 g/16g nitrogen for the soybean blend (Table 4). However, because of the low lysine content in gluten, lysine became the limiting amino acid for both of the blended curds while the chemical scores remained almost unchanged. The essential amino acid profiles and chemical scores would be improved if less gluten was added. On the basis of the values in Table 4, it was estimated that 4% (15g) gluten added to the extracts would increase the chemical score of the pea/gluten curd to 73% and the soybean/gluten curd to 81%.

Sensory evaluation

Sensory evaluations of freshly prepared pea and soybean curds, with and without gluten, are summarized in Table 5. In general, judgements for the sensory properties of all samples ranged from a mean of about 2 (very good) to 3 (slightly good). Addition of gluten did not result in any significant (p < 0.05) change. The panelists did not detect any significant (p < 0.05) difference in the flavor of any of the four samples, but the soybean curds received a slightly better mean score. Texture and color of both soybean curds were judged to be significantly (p < 0.05) better than the corresponding pea curds. When the 25 panel members were asked if any of the curd samples were unacceptable, three judged the pea and two judged the soybean curds to be unacceptable, but this was not significant.

Changes in the Starch Fraction During Extrusion-cooking of Corn

M.H. GOMEZ and J.M. AGUILERA

──────────── ABSTRACT ────────────

Whole ground corn was extruded at 23.7, 18.5, 15.4, 13.9 and 7.6% moisture contents (EMC). Decreasing EMC resulted in increases in water solubility index (WSI), enzyme susceptibility (ES), degree of gelatinization and blue values, while water absorption index and water insoluble carbohydrates decreased. ES and WSI of several blends prepared by combining raw (R), gelatinized (G), and dextrinized (D) corn were compared to those of extruded products. Corn extrudates had properties similar to blends containing G and D corn only. The relative proportion of D corn increased from about 10 to 60%, as EMC decreased. "Dextrinization" appears to become the predominant mechanism of starch degradation during low-moisture, high-shear extrusion. Viscoamylographs, scanning electron and light photomicrographs support these findings.

INTRODUCTION

LIMITED INFORMATION is available on changes occurring in the starch fraction of foods during extrusion-cooking although most physical and sensory properties of extrudates depend on the extent of starch degradation.

Initial studies on extrusion-cooking of corn were done by Conway et al. (1968), Anderson et al. (1969) and Conway (1971a, b). The authors felt that low-moisture extrusion, which provided high temperatures and shear rates, enhanced degradation of starch and the formation of dextrins. Mercier and Feillet (1975) extruded several cereal starches and concluded that the amount of soluble starch increased as feed moisture decreased, without any formation of maltodextrins. Williams et al. (1977), in discussing extrusion of yellow corn grits, indicated that maximum WAI was obtained when the moisture content was 27% and the discharge temperature 135°C. At higher temperatures and under drier conditions dextrinization occurred which increased WSI and decreased WAI. None of these studies have quantified the extent of starch degradation. One of the reasons for this is that there is no single method which fully characterizes changes in the starch fraction during extrusion-cooking. However, several analytical techniques have been used to determine the extent of starch cooking in high-moisture, medium temperature (<100°C), shearless systems.

The objectives of this study were to characterize corn products extruded under various moisture contents and to quantify the extent of starch degradation.

MATERIALS & METHODS

Preparation of samples

Commercial yellow corn was ground in a swinging blade Model D6 Fitzmill (W. J. Fitzpatrick Co., Chicago, IL) provided with a screen having 0.3 mm holes. Proximate analysis of the corn flour was as follows: moisture, 14%; protein, 10.5%; fat, 3.4%; ash, 1.3% and crude fiber, 1.4%. Portions of the flour were equilibrated overnight to five moisture levels: 23.7, 18.5, 15.4, 13.9 and 7.6%.

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Extrusion was performed in a Wenger X-5 laboratory extruder (Wenger Mfg. Co., Sabetha, KA) under the following conditions: Screw speed, 750 rpm; feed rate setting, 6; heads, 8; die opening, 4.0 mm. The last head before the die was heated with 40 psig steam. Extruded samples were air dried at 50°C for 24 hours, ground in a Wiley laboratory mill and sieved through a 60 mesh screen.

Gelatinized corn was obtained by autoclaving a corn flour-water suspension (2.5%, w/w) at 120°C for 1 hr and freeze-drying. Dextrinized corn was prepared by treating a gelatinized suspension with 0.15% (w/w) based on dry starch content) α-amylase from Bacillus licheniformis, Takatherm, (Miles Laboratories, Elkhart, IN) at pH 6.3 and 90°C for 2 hr and then freeze-drying.

Blends containing different proportions of raw, gelatinized and dextrinized corn as well as the five extrudates were evaluated for physicochemical properties.

Analytical methods

Moisture content was determined according to AOAC (1975). Proximate analyses were performed by AACC methods (1969).

Enzymatic susceptibility (ES) was assayed by digesting 1g of sample in 45 ml of distilled water containing 1 mg α-amylase from Aspergillus oryzae (Sigma Labs, St. Louis, MO) at pH 6.9 and 20°C. After 30 min acetic-sulfuric acid buffer and a 12% sodium tungstate solution were added. The suspension was filtered, and aliquots were assayed for reducing sugars by Method 80-60 (AACC, 1969).

Water absorption (WAI) and water solubility (WSI) indexes were determined as described by Anderson et al. (1969).

Degree of gelatinization (DG), defined as the ratio of gelatinized starch to total starch, was calculated from spectrophotometric measurements of the starch-iodine complex formed in an aqueous suspension of sample before and after complete solubilization of the starch by alkali, (Wootton et al., 1971).

Amylose extracted in an aqueous solution was estimated by the absorbance value at 680 mμ upon addition of tri-iodide. The reading is defined as blue value, BV, (Gilbert and Spragg, 1964) and expressed as BV = (Absorption x 4)/C, where C is the concentration of the carbohydrate solution (mg/dl).

Water insoluble carbohydrates (WIC) were determined by extracting 2.5 – 3.0g of sample with 50 ml of cold water for 1 hr. The extract was centrifuged at 1,085 x g for 10 min, and the residue re-extracted similarly twice. The final insoluble residue was hydrolyzed with 20 ml of HCl (sp gr 1.19) and 200 ml of water for 2.5 hr in a round bottom flask provided with a reflux condenser. The hydrolyzate was cooled, neutralized with NaOH, filtered and assayed for dextrose as described in Handbook of Sugars (Pancoast and Junk, 1930). Weight of dextrose times 0.9 is equivalent to the insoluble carbohydrate fraction.

Viscosity changes with time and temperature were measured using a Brabender viscoamylograph. A 15% (w/w, dry basis) aqueous suspension of sample was heated from 25 – 95°C for 16 min and then cooled to 50°C in 30 min.

Raw, gelatinized and dextrinized corn, as well as extruded samples cleaved at a plane perpendicular to the main axis, were mounted on specimen holders with silver conducting paint. Samples were coated with gold-palladium in a Hummer coater and examined with a JEOL ISM-25 scanning electron microscope at 15KV. Loss of birefringence was followed by examining a 0.2% (w/w) sample dispersion in water under polarized light with a Zeiss research microscope.

RESULTS & DISCUSSION

EXTRUDATES PROCESSED at 23.7, 18.5, 15.4, 13.9 and 7.6% moisture content are referred to as E_1, E_2, E_3, E_4 and E_5, respectively. As expected, decreasing extrusion

Sample	EMB[b] (%)	ES[c] (g maltose/100g)	DG[d] %	BV[e]	WIC[f] (%)	WSI[g] (%)	WAI[h] (g/g)
Raw	—	0.43 ± 0.1	0.00	0.00	74.62 ± 2.0	4.23 ± 0.1	1.73 ± 0.00
E_1	23.7	7.68 ± 0.2	33.96 ± 2.0	8.60 ± 0.4	51.69 ± 1.5	16.17 ± 2.1	7.22 ± 0.06
E_2	18.5	8.39 ± 0.2	48.00 ± 2.0	10.10 ± 0.3	37.65 ± 2.0	27.07 ± 1.2	6.57 ± 0.10
E_3	15.4	9.07 ± 0.2	68.63 ± 1.5	9.73 ± 0.3	31.78 ± 2.0	30.20 ± 2.3	5.34 ± 0.12
E_4	13.9	9.01 ± 0.1	70.37 ± 2.0	10.64 ± 0.4	26.70 ± 1.5	35.94 ± 0.3	5.79 ± 0.11
E_5	7.6	10.13 ± 0.2	76.36 ± 2.0	11.03 ± 0.4	21.43 ± 1.5	46.00 ± 1.5	42.5 ± 0.10

[a]The data are averages of two determinations. Values are listed as mean ± confidence interval with confidence coefficient 95%.
[b]Moisture content before extrusion.
[c]Enzyme susceptibility.
[d]Degree of gelatinization.
[e]Blue value.
[f]Water insoluble carbohydrate.
[g]Water solubility index.
[h]Water absorption index.
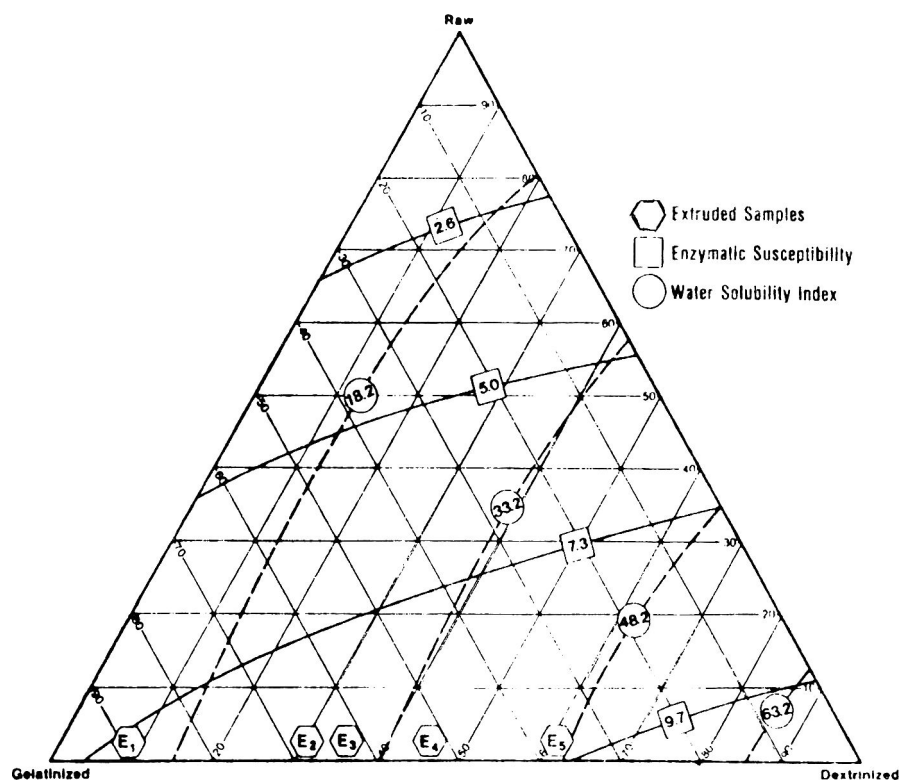
Fig. 1—Response surfaces of raw, gelatinized and dextrinized corn blends and extruded corn products.

moisture contents (EMC) increased the temperature at the die plate from 90 to approximately 130°C. Although product temperature is a desirable response to analyze, most extruders are not designed to accurately measure it. This paper defines products in terms of actual moisture contents before extrusion, eliminating the uncertainties related to the definition of "product" temperature.

Results of several analytical procedures applied to raw corn and extrudates are presented in Table 1. Raw corn flour had very low ES (0.43g maltose/100g sample), WSI (4.23%) and WAI (1.73 g/g), zero DG and BV and almost 75% water insoluble carbohydrates. These values reflect that almost intact starch granules are present in the raw flour, making enzymatic attack, leaching of components and water absorption, very difficult.

All extruded samples show physiochemical properties significantly different from those of the raw material, and denoted changes in the state of the main chemical components. Progressive degradation of starch is suggested due to increases in susceptibility to enzyme attack, higher "degree of gelatinization" and BV, and increases in water solubility with corresponding reductions in the amount of insoluble carbohydrates and water absorption capacity.

Further analysis of the data revealed interesting relationships among different tests. BV, (which is based on the affinity of iodine and linear carbohydrates) increased as EMC decreased, thus indicating higher concentration of linear polysaccharides. WIC values had a highly significant negative correlation with WSI ($r = -0.979$), suggesting that breakdown of the insoluble carbohydrate fraction is a major source for water soluble materials. DG, which increases continuously as EMC decreased, correlated very well with WSI ($r = 0.963$), indicating that the case of extruded products DG should be considered as an indicator of the extent of starch degradation rather than as an index of gelatinization.

WAI was low for raw corn flour (1.73 g/g), maximum for E_1 (7.22 g/g) and decreased for successive extrudates. Water absorption depends on the availability of hydrophilic groups which bind water molecules and on the gel-forming capacity of macromolecules. This was confirmed for gelatinized and dextrinized corn flours which had WAI's of 9.85 and 2.36 g/g, respectively.

Data at this point suggested that the extent of starch degradation in extruded products could be estimated by comparing and matching the values of their physicochemical

properties with those of ternary blends made up of raw, gelatinized and dextrinized corn flours. After some initial trials, ES and WSI were chosen as response variables because they gradually increased as starch broke down, yet both are based on different physiochemical principles.

The ES and WSI values of various blends of raw, gelatinized and dextrinized corn, were analyzed by the General Lineal Model (GLM) in the area of main interest for extruded products (high proportion of gelatinized and dextrinized material) giving the following response surfaces:

Water solubility index:

$$WSI = 0.05029*R + 0.09473*G + 0.71193*D + 0.00071*R*G + 0.00020*R*D - 0.00132*G*D$$

$$\alpha = 0.01 \qquad r = 0.999$$

Enzyme susceptibility:

$$ES = 0.00156*R + 0.07230*G + 0.10867*D - 0.00048*R*G + 0.00055*R*D + 0.00026*G*D$$

$$\alpha = 0.01 \qquad r = 0.994$$

where R = % raw corn in blend/100; G = % gelatinized corn in blend/100; D = % dextrinized corn in blend/100; α = significant level; r = correlation coefficient; and R + G + D = 100.

Fig. 1 presents the response surfaces in a ternary diagram. The diagram is derived from a simple model which assumes that breakdown products formed during extrusion of starchy materials can be grouped into one of three pure states: "raw", "gelatinized" or "dextrinized." Extruded samples were positioned inside the triangle according to their ES and WSI values. A line tying points E_1 through E_5 can be regarded as the operating line for the Wenger X-5 extruder under the specified working conditions. Variations in screw design, shaft rpm, die size, etc. would change the shape of the operation line, and hence, the physiochemical properties of the extrudates.

Fig. 2—Viscosity of extruded corn pastes.
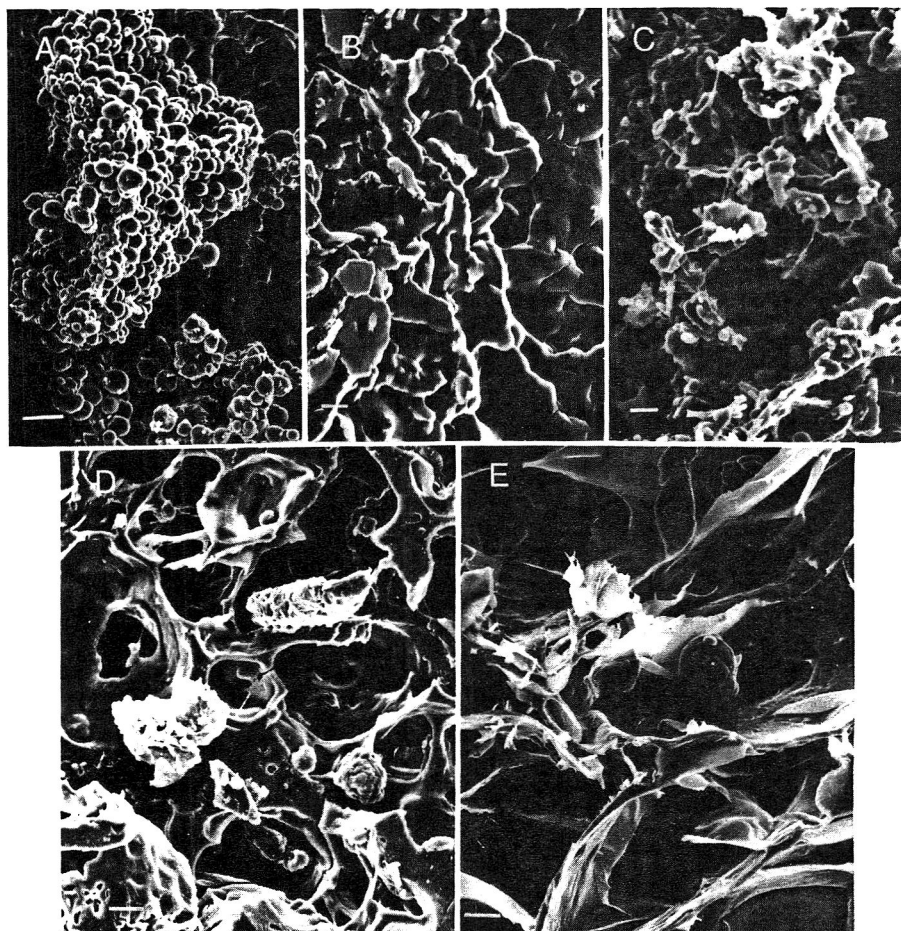
Fig. 3—Scanning electron micrographs of corn samples: (A) Raw; (B) Gelatinized; (C) Dextrinized; (D) Extruded at 23.7% moisture content; and (E) Extruded at 7.6% moisture content. Markers: (A) 30 μm; (B) and (C) 10 μm; (D) and (E) 50 μm.
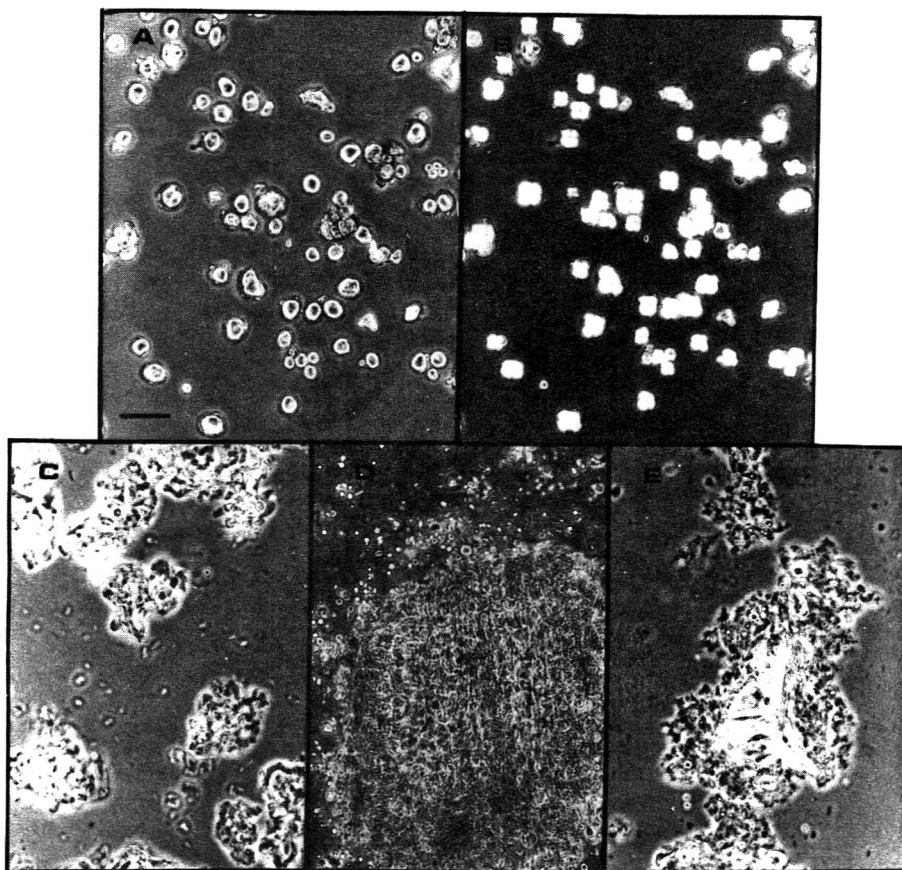
Fig. 4—Light micrographs of corn samples: (A) Raw; (B) Raw under polarized light showing birefringence of starch granules; (C) Gelatinized; (D) Dextrinized; and (E) Corn extrudate at 15.4% moisture content. Markers 20 μm.

Extrudates E_1 and E_5 were equivalent in ES and WSI to blends having no raw corn and increasing proportion of dextrinized material. Products with specific functional properties lying within the feasible range can be tailor made simply by varying the amount of water added before extrusion.

Further evidence of the breakdown of carbohydrates is presented in Fig. 2 through 4. The initial viscosity of extrudates decreased as EMC was reduced (Fig. 2). The high initial viscosity of E_1 is typical of gelatinized products (Anderson et al., 1969), while lower viscosities indicate breakdown of polymers. The absence of a peak at $60-70°C$ reveals that no intact starch granules were present in the extrudates, otherwise they would have gelatinized increasing the paste viscosity.

Fig. 3 shows scanning electron micrographs of raw, gelatinized and dextrinized corn, as well as of E_1 and E_5. In Fig. 3A, intact starch granules can be observed in the raw flour, but they have been fused into a coarse mass after gelatinization (Fig. 3B) and further transformed into a finer structure by dextrinization (Fig. 3C). Similar changes can be observed between samples extruded at 23.7% and 7.6% moisture (Fig. 3D and 3E).

Fig. 4 illustrates the presence of birefringence in raw corn (Figure 4A and 4B) which could not be detected in any other sample, confirming that all extrudates were well beyond the initiation of gelatinization. Fig. 4C, 4D and 4E suggest that the morphology of extruded samples can be visualized as a composite of gelatinized and dextrinized material.

Future work along these lines will include the study of similar phenomena in pure starch systems.

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Changes in Amino Acid Content
of Acidified Sweet Potato Puree

GLENN CREAMER, CLYDE T. YOUNG, and DONALD D. HAMANN

─────────────── ABSTRACT ───────────────

The stability of nitrogenous substances in processed sweet potato puree was examined. Protein and nonprotein nitrogen (NPN) fractions were extracted from sweet potato puree adjusted to pH 4.2, treated with amylolytic enzymes and processed at 88°C to determine changes in amino acid concentrations over a 6-month period. Amino acid concentrations in the protein fraction were generally found to remain stable over the storage period. NPN amino acid concentration either remained stable or increased slightly with storage time. The results of this work indicate that there would be no appreciable loss of nitrogenous substances in sweet potato puree subjected to the conditions of the experiment.

INTRODUCTION

SWEET POTATOES have the potential of fulfilling an important role in the food supply and chemical industry of the future (Stacey, 1973; Kohn, 1977; Williams, 1977). For further utilization of sweet potatoes, two goals worthy of investigation are (1) the manner of securing a year-round supply of sweet potato material, and (2) the most energy-efficient method of doing so. Staggered plantings have been tried in Australia to provide a year-round supply of roots (Wood, 1976). An attractive method for preserving sweet potatoes indefinitely until they are needed is aseptic bulk processing, which is already commercially used for tomato puree, grape juice, and banana puree (Anonymous, 1976; Wilson, 1978). This system receives the produce immediately from the field, comminutes the produce into puree, thermally processes the puree, and stores the puree in bulk under aseptic conditions. It can provide a supply of product year-round. Storage costs are minimal as there is no energy input after the initial thermal processing. Bulk storage is accomplished with reusable containers (e.g., glass-lined silos and bulk tanks). The puree form is conducive to pumping.

Because tomatoes may vary in their acidity around the 4.5 pH outgrowth limit of *C. botulinum* spores, a process was developed to standardize the acid content of tomato juice for pasteurization in the field as the tomatoes were harvested (Anonymous, 1970). Benefits of this process include reduced product loss and minimal waste disposal. Ice (1978) showed a similar process could be developed for sweet potatoes by adjusting puree to pH 4.2 with phosphoric acid and using a 90°C water bath process. A pH of 4.2 was selected since even incorporation of the acid in the puree is somewhat difficult and to serve as a precaution against dilution by steam injection which might cause puree pH to rise above pH 4.5. Sweet potatoes processed in this manner would still be classified as a low-acid food (NCA, 1973). Desirable features of this process are (1) whole unpeeled roots are utilized, (2) minimal waste treatment facilities are needed, (3) hand-grading of roots for size or damage is not necessary, (4) use of amylolytic enzymes facilitates

pumping of product, and (5) storage volume is reduced compared to storing whole roots (Harris and Karmas, 1975). Work with canned sweet potatoes has shown that canning syrups acidified to pH 4.2 with citric acid for pectin firming impart no off-flavor to the roots (Edmond and Ammerman, 1971). This should be encouraging if the processed puree is to be used for human food products.

Crude protein content in sweet potatoes ranges from 1.73–11.8% on a dry weight basis and compares closely with cereal grains which average 10% crude protein. Though sweet potatoes are recognized nutritionally mainly for their caloric and provitamin A contents, the crude protein content can assist in maintaining nitrogen balance in humans (Purcell et al., 1972; Scrimshaw et al., 1975). As much as 30% of the crude protein is present as nonprotein nitrogen (NPN) (nitrogen-containing compounds that are not precipitated by either 13% trichloracetic acid or 70% methanol). Nonprotein nitrogen is classified as free amino acids, small peptides, and other low molecular weight compounds. They do not contribute significantly to the total essential amino acids, but represent a source of nitrogen that may be reclaimed for animal protein production by ruminants. NPN is presently lost in most processing operations (IAEA, 1970; Scrimshaw et al., 1975; Purcell et al., 1978; Purcell and Walter, 1980).

It was the goal of this research project to develop an aseptic process for the long-term storage of sweet potatoes in puree form by adding phosphoric acid to lower puree pH below 4.5 and processing at 88°C. Changes in protein and NPN amino acid components during storage over a 6-month period were determined.

MATERIALS & METHODS

Processing method

Cured Jewel cultivar sweet potatoes (*Ipomoea batatas*) were procured from the North Carolina State University research farm at Clayton, NC and a commercial grower near Wake Forest, NC. Unpeeled roots were washed in a jet spray tumbler and then comminuted in a Fitz Mill (Fitzpatrick Co., Chicago, IL) fitted with a 0.762-mm mesh screen. Water (8% by weight) was added to the puree from the rinsing of potato material out of the Fitz Mill. Puree and water were thoroughly mixed in a large container. Control samples were removed at this time and put into standard canning glass jars (0.946-L capacity) with screw cap lids. Three 10-kg batches of puree were set aside for processing.

Method 1 (Fig. 1) involved lowering puree pH to 4.2 ± 0.05 with concentrated phosphoric acid and heating to 88°C by direct steam injection while mixing. Prewarmed glass containers were used for collecting the puree as it was pumped from the steam injector. The sealed containers were immediately placed into an 88°C water bath for 30 min. Upon completion of thermal processing, the containers were cooled quickly to 29°C in a water bath, dried and stored in the dark at room temperature. The process constituted the no-enzyme method.

Method 2 (Fig. 1) was the alpha-amylase method where 0.6 mL alpha-amylase, liquid, activity 170,000 MWV/g (Taka-Therm, Miles Laboratories, Inc., Elkhart, IN) per kg puree was added at native pH 5.7. Heating to 88°C by steam injection occurred next followed by a 30-min interval during which the heated puree was regularly agitated to facilitate thorough enzymatic action. The puree was covered to prevent contamination and temperature drop was ob-

served to be less than 3°C. Upon completion of enzymatic incubation, the puree was cooled to 29°C via a tubular heat exchanger before the addition of phosphoric acid. The adjustment to pH 4.2 inactivated the alpha-amylase. All remaining processing steps were the same as in the no-enzyme method.

Method 3 (Fig. 1) was the amyloglucosidase method. The puree was adjusted to pH 4.2 and amyloglucosidase (Diazyme L-100, Miles Laboratories, Inc.) was added in the ratio of 0.6 mL per kg puree. The puree was heated by steam injection to 77°C and incubated for 30 min in the same manner as the alpha-amylase method. Containers of puree were then promptly placed in a water bath and brought to 88°C. Process timing started when the puree temperature in the center of a container reached 88°C. Amyloglucosidase was inactivated at this temperature. The remaining processing steps were performed as in the no-enzyme method.

The three processing methods were selected to show the difference in puree consistencies which would affect pumping performance. The alpha-amylase under the conditions described here would virtually deplete the starch content by converting it to limit dextrin, maltose, and small quantities of glucose and would result in a large decrease of apparent viscosity. Hydrolysis of gelatinized starch of the puree would occur to a much smaller extent with amyloglucosidase used under the described conditions.

Statistical design

The statistical design involved a three by four factorial with an additional treatment combination designated as the control (Fig. 2) (Snedecor and Cochran, 1967). The two factors involved were enzyme method and storage time. Enzyme method had three qualitative levels which were the no-enzyme method, alpha-amylase method and amyloglucosidase method. Storage time had four quantitative levels spaced orthogonally from 0 through 6 months. Each treatment combination contained duplicates of the two replicates for a total of four observations. Response variables were the amino acid analysis data calculated as g amino acid/100g dry solids and percent of total amino acids present. Analysis of variance (AOV) was performed on the pellet fraction and supernatant fraction response variables. Analysis of covariance (ANOCOV) was performed on the response variable of the supernatant fraction (Helwig and Council, 1979).
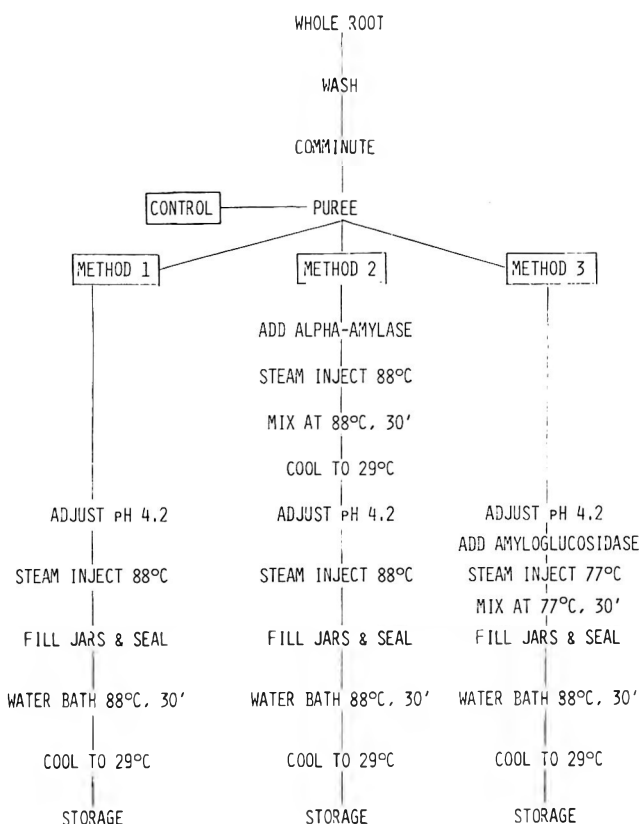
Spoilage determination

Samples were analyzed for preservation by four indicators which were (1) general appearance, (2) container pressure, (3) odor, and (4) puree pH. Container contents were examined for gas pockets, digested appearance and visible cultures. Container pressure was determined with a pressure gauge fitted with a needle puncture. Final pressure of 3.38–5.06 N/m^2 (10–15 inches Hg vacuum) is standard. Pressures above this range would be an indication of gas production by spoilage organisms. Any off-odor of acidic or putrefactive nature noticed immediately after a lid was removed was considered an indication of microbiological spoilage. A pH electrode was inserted into the container contents to a 30-mm depth where pH was measured. Then the puree was mixed thoroughly for another pH measurement.

Moisture analysis

Moisture determination for the puree was performed according to the method of the AOAC (AOAC, 1970). Twenty-gram samples, weighed to 0.01g, were dried in Petri dishes at 90°C for 18 hr in a forced-air oven. Water content of the extracted solids from the pellet fraction was determined with 2-g samples weighed to 0.0001g.

Rheology

Apparent viscosity measurements were determined for the alpha-amylase and amyloglucosidase methods with the Haake Rotovisco, Model RVI rotational viscometer using the MV1 fluted rotor (Van Wazer et al., 1963). Measurements were recorded only for the 0 and 2-month periods of storage as Ice (1978) showed no major change in apparent viscosity over storage.

Extraction procedure

Processed puree was separated by centrifugation into the pellet fraction containing the protein, and supernatant fraction containing the NPN. A 50-g sample was weighed to 0.01g, rinsed into a Waring Blendor (Waring Products Div., New Hartford, CT) with 100 mL methanol, and blended at high speed for 3 min (Purcell and Walter, 1980). The slurry was transferred to centrifuge bottles. Residue was rinsed from the blender with 50 mL 70% methanol and poured into the centrifuge bottles. Centrifugation was for 10 min at 15,000 × g.

After centrifugation, the supernatant was filtered through Whatman #1 filter paper into a graduated cylinder. A final volume of 190 mL was obtained by pouring methanol through the filter paper. The NPN fractions were then stored at −7°C until analyzed. Any solids (mostly peel) from the damp filter paper were transferred back into the centrifuge bottle. Bottles containing the pellet fraction were aspirated to facilitate removal of the extracted solids. Removal of the remaining methanol necessitated heating the solids in a vacuum dryer. Solids were then ground to a fine powder using a Micro-Mill (Lab Apparatus Co., Cleveland, OH). Powdered pellet fractions were stored at −7°C until analyzed.

Nitrogen determination

Kjeldahl analyses were performed on the processed purees, pellet fraction solids and supernatant fractions. Total nitrogen determination was carried out on 10g of wet sample (weighed to 0.0001g) of the puree, a 25-mL aliquot of the supernatant, and 0.5–1.0g of the pellet solids (weighed to 0.0001g). Weight of the pellet samples depended upon enzyme method due to the varying amounts of carbohydrates left insolubilized from the extraction procedure. Digestion was aided with copper and selenium catalysts. A correc-

Fig. 1—Sweet potato puree processing methods.
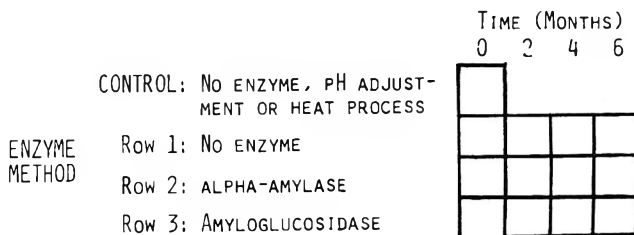
Fig. 2—3 x 4 factorial design.

tion factor was needed to convert nitrogen concentration of the pellet fraction from an extracted solids basis to the raw solids basis of the supernatant fraction for the ANOCOV.

Amino acid analysis

Sample size for pellet fraction amino acid analysis depended upon the enzyme method. Best chromatographic results were obtained from samples containing 5.5 mg nitrogen. This corresponded to sample sizes of 0.15–0.40g. Supernatant fraction sample sizes also varied with enzyme method. Best results were obtained by samples containing 8 mg nitrogen (30–40 mL filtrate). Supernatant fraction aliquots were evaporated in a rotary evaporator at 45°C in preparation for acid hydrolysis. The methanol-water-free residue was transferred to the hydrolysis tube in four 5-mL aliquots of 6N hydrocholic acid. Acid hydrolysis and amino acid analysis were done in accordance with the methods of Young (1978). Amino acid analyses were performed on the Durrum D-500 (Durrum Instrument Corp., Pal Alto, CA) automated amino acid analyzer. Tryptophan was not analyzed as it was destroyed by the 6N acid hydrolysis procedure.

RESULTS & DISCUSSION

Rheology

The alpha-amylase method of puree processing achieved the greatest reduction in apparent viscosity of the three methods employed. Rotational viscometer readings produced an average apparent viscosity of 2.05 Pa.s at a shear rate of $20s^{-1}$ (90% sweet potato material by weight and 81–86% water). The puree flowed readily. The amyloglucosidase-treated puree had an apparent viscosity of 6.68 Pa.s. This puree also flowed readily but was not as fluid as the alpha-amylase-treated puree. Ice's procedure at pH 4.2 employed three different amylolytic enzymes which required an incubation time of 24 hr (compared to 30 min). His average apparent viscosity for a puree containing 91% sweet potato material was 0.87 Pa.s. For pumping purposes there is little difference between the purees. However, carbohydrates from the alpha-amylase process would contain a far greater percentage of limit dextrin. The no-enzyme method produced a puree that was solid due to gelation as was also experienced by Ice (1978).

Statistical analysis

Because seven samples spoiled and one sample was lost during processing, destroying the balance in the experiment, some adjustments were required. The duplicate of the sample lost during processing spoiled at 6 months of storage and consequently an average of the 2 and 4-month replicates was used for one of the replicate's 6-month storage data since little change was expected to occur during this interval. The degrees of freedom for the error term (see Fig. 3, REP x TIM-MET) were consequently reduced from 12 to 11. Also to maintain balance, spoiled samples were replaced by a second analysis from their duplicates.

In order to clarify the ensuing statistical discussion, the following terms will be defined: (1) method, (2) treatment, and (3) processing effect. Method is one of the two factors tested in the experimental design (Fig. 2) with the other factor being time. The method factor consists of three enzymatic applications which are the no-enzyme, alpha-amylase and amyloglucosidase methods (Fig. 2). Treatment is used in the strict statistical sense which in this case is a unit of puree subjected to a specific enzyme method and storage time (see Fig. 2). Processing effect refers to the difference between the control and the average of all of the other treatments. Processing involves the enzymatic action applied to the puree, the pH adjustment from pH 5.7 to 4.2 and the thermal process at 88°C for 30 min. The control was not processed.

Average differences between the amino acid concentra-

tions of the different methods were calculated to supplement the F tests conducted under the experimental design (see Table 1). A processing effect involved the average of the 48 treatment observations versus the treatment average of the four control observations (see CNTRL, Fig. 3). Percentage difference was determined with the average concentration of amino acid in the control in the denominator of the ratio. Comparison of the two enzyme methods involved the ratio of the row average concentration of the amyloglucosidase method over the row average concentration of the alpha-amylase method (see T23, Fig. 3). The difference between amino acid concentrations for the two enzyme methods and the no-enzyme method was determined with amino acid concentration of the no-enzyme method in the denominator of the ratio (see T123, Fig. 3).

Lines on the graphs (Fig. 4 and 5) showing amino acid concentration over storage time indicate at least the 0.05 significance level between enzyme methods for the cor-

Fig. 3—Definition of statistical terms.

1.	R^2	Coefficient of determination = % of variability explained by model.
2.	REP	(1 df) replication.
3.	TIM-MET	(12 df) AOV only. Time by enzyme method treatment combination = one square unit of Figure 2. Values analyzed are g amino acid/100 g solids.
4.	PELLET	(1 df) ANOCOV only. Covariate term = supernatant TIM-MET value − pellet TIM-MET value.
5.	CNTRL	(1 df) Processing effect, i.e. control (no processing or storage) vs overall treatment average effect of TIM-MET (average of all TIM-MET treatment combinations).
6.	TL	(1 df) Linear term time effect on average of all methods (column averages excluding control TIM-MET of Figure 2).
7.	TQ	(1 df) Quadratic term time effect on average of all methods.
8.	TC	(1 df) Cubic term time effect on average of all methods.
9.	T123	(1 df) Storage time average effect of no-enzyme method (average of all TIM-MET in Row 1 of Figure 2) vs storage time average effect of α-amylase and amyloglucosidase methods (average of all TIM-MET in Rows 2 and 2 in Figure 2).
10.	T23	(1 df) Storage time average effect of α-amylase method (average of all TIM-MET in Row 2 of Figure 2) vs storage time average effect of amyloglucosidase method (average of all TIM-MET in Row 3 of Figure 2).
11.	TIM x MET	(6 df) AOV only. Time by enzyme method interaction effect.
12.	TL x T123	(1 df) ANOCOV only. Time by enzyme method interaction effect.
13.	TZ x T123	(1 df) ANOCOV only. Time by enzyme method interaction effect.
14.	TC x T123	(1 df) ANOCOV only. Time by enzyme method interaction effect.
15.	TL x T23	(1 df) ANOCOV only. Time by enzyme method interaction effect.
16.	TQ x T23	(1 df) (ANOCOV only. Time by enzyme method interaction effect.
17.	TC x T23	(1 df) ANOCOV only. Time by enzyme method interaction effect.
18.	ERROR	(11 df) REP x TIM-MET interaction effect.

responding F tests. The average supernatant fraction amino acid concentratons are unadjusted for pellet fraction amino acid levels.

In cases where all three methods are present on a graph, the amino acid concentrations are significantly different with respect to enzyme method over storage time. When the average of the alpha-amylase and amyloglucosidase methods is used, the TIM × MET interaction effect has not contributed significantly to the model (see Table 1). This means that the amino acid concentrations for the two enzyme methods are not significantly different from each other but their combined average is significantly different with respect to the no-enzyme method over time. Where the average of all three methods is given, amino acid concentrations are not significantly different with respect to method over storage time. There are several cases where the two enzyme methods are significantly different from each other, but their combined average is not significantly different from the no-enzyme method. For these cases all three methods are presented.

Aspartic acid (ASP). Replicate sweet potato samples differed in their ASP contents. Differences in ASP among pellet fractions were significant, while differences among the supernatant of nonprotein nitrogen (NPN) fractions were highly significant (Table 2). ASP was the most abundant amino acid, accounting for nearly 20% of the total amino acids (g amino acid/100g amino acids) in the pellet fraction and nearly 60% of the amino acid content of the NPN fraction. Most of the ASP, at least in the NPN, was probably present as asparagine before 6N acid hydrolysis

Table 1—TIM-MET average values, g amino acid/100 g solids

Amino acid	Control	Time (mo)			
		0	2	4	6
Pellet					
ASP	1.506	1.337	1.536	1.387	1.420
		1.216	1.224	1.247	1.257
		1.157	1.309	1.311	1.218
THR	0.340	0.348	0.382	0.360	0.369
		0.333	0.347	0.348	0.358
		0.295	0.371	0.368	0.335
SER	0.374	0.384	0.422	0.399	0.407
		0.371	0.379	0.392	0.391
		0.325	0.397	0.402	0.363
GLU	0.680	0.714	0.768	0.729	0.746
		0.689	0.691	0.718	0.723
		0.588	0.734	0.718	0.657
TOTAL	6.641	6.721	7.577	7.139	7.282
		6.359	6.742	7.112	6.935
		5.567	7.125	7.144	6.479
Supernatant					
ASP	0.596	0.679	0.668	0.675	0.678
		0.713	0.724	0.727	0.708
		0.725	0.705	0.749	0.726
THR	0.0459	0.0444	0.0438	0.0442	0.0449
		0.0428	0.0447	0.0447	0.0447
		0.0483	0.0475	0.0507	0.0520
SER	0.0425	0.0366	0.0400	0.0402	0.0411
		0.0404	0.0417	0.0407	0.0412
		0.0447	0.0442	0.0485	0.0492
GLU	0.0715	0.0773	0.0640	0.0660	0.0660
		0.0827	0.0830	0.0835	0.0817
		0.0718	0.0695	0.0750	0.0772
TOTAL	1.061	1.064	1.042	1.067	1.067
		1.094	1.088	1.114	1.074
		1.190	1.136	1.231	1.196

(Purcell and Walter, 1980). The NPN contained 35% of the total ASP (g amino acid/100g solids). The overall treatment average of the pellet fracton showed ASP depletion of 13.6% (g amino acid/100g solids) when compared with the control (Table 2). At the same time the NPN concentration incrased by 18.5% due to processing. The pellet fraction lost roughly twice as much ASP as the NPN fraction gained (0.20g vs. 0.11g). ASP was stable in both fraction method averages over the storage period. The NPN aspartic acid levels from the enzyme methods had a 7% (0.05g) average increase over the no-enzyme method. More than three times the amount of ASP was lost from the average of the enzyme method pellet fractions than was gained by its corresponding average NPN fraction when compared to the no-enzyme method.

Covariate analysis (Table 3) indicated that the ASP content of the NPN fraction was dependent on that of the pellet fraction. There was no difference between fractions with regard to the two enzyme methods employed; however, the enzyme methods lost on the average 12.5% (0.18 g) of their pellet fraction ASP when compared with the no-enzyme method.

Analysis of variance suggests a time by method interaction effect on the NPN fractions, whereas the ANOCOV does not. If such an interaction exists, the fate of ASP cannot be determined from strictly processing method or time equation fit.

Threonine (THR). There was no difference in the pellet fraction THR concentrations between the root replicates, but there was a highly significant difference between the NPN fractions. THR accounted for about 5% of the pellet fraction's amino acids and 4% of the NPN amino acids. The NPN contained 11% of the total THR. The AOV showed that processing had not affected the THR content of either fraction's overall treatment average when compared to the control. Pellet fraction THR was stable over the storage period, whereas NPN THR concentration increased linearly as a function of time at a slight rate. This apparent anomaly might be explained in terms of the ratio of NPN THR to pellet fraction THR. A nonsignificant decrease in the THR concentration of the pellet fraction could be reflected as a significant gain the THR cover of the NPN fraction. This approach may be considered in the comparison of the two enzyme methods. THR concentration did not differ significantly in the pellet fractions, but the amyloglucosidase processed samples contained about 1.2% (0.004g) less THR than the alpha-amylase samples. The differences between NPN concentrations were highly significant with the amyloglucosidase processed samples containing 12% (0.005g) more THR than the alpha-amylase samples. While these figures did not match, it might be argued that the increase in NPN concentration of the amyloglucosidase method came from its pellet fraction counterpart, and that the remaining nonsignificant amino acid difference of the pellet fraction was destroyed. The pellet fraction content of the no-enzyme method was the same as the average of the enzyme methods, but their average NPN content was nearly 6% (0.003g) greater than that of the no-enzyme method.

Serine (SER). As in the case of THR, root replicates were not different in pellet fraction SER content but showed highly significant differences for the NPN SER content. SER made up 5.7% of the amino acids in the pellet fraction and close to 4% of the NPN amino acids. Ten percent of the total SER was in the NPN. There was no processing effect on the overall treatment average for either fraction. No change was evident in the average pellet fraction content over storage for any method, but NPN SER values increased slightly in a linear fashion over storage. Samples from both enzyme methods contained equivalent concentrations of SER in their pellet fractions, while their

SER concentrations of the NPN fractions differed. The amyloglucosidase method was almost 14% greater than that of the alpha-amylase method. There were differences in the SER content of the pellet and NPN fractions of enzyme treated and untreated samples. On a percent basis, the pellet fractions of the enzyme method samples had 6.4% less SER than those of the no-enzyme method, while the NPN fractions had 11.1% more SER for the enzyme method.

Glutamic acid (GLU). GLU, some of which was probably glutamine, appeared in the same amount for both root replicates. GLU made up about 10% of the amino acids in the pellet fraction and 7% of the NPN's amino acids. NPN GLU contributed 9.5% of the total GLU. Processing had no significant effect on the concentration of GLU in either fraction. Storage time did not alter the concentration of the methods' average pellet fraction, but while there appeared to be no NPN change, there was an interaction effect between method and storage time (Table 2). The GLU content of the pellet fractions of the enzyme methods were the same, whereas the amyloglucosidase method's NPN level was 11% (0.009g) less than the alpha-amylases. This significant difference is easily within the range of the nonsignificant pellet fraction difference of 0.03g. There was no difference in GLU content of the pellet fraction of the no-enzyme and the average of the enzyme methods. The NPN content of GLU of the average of the enzyme methods surpassed the no-enzyme method's by 14% (0.01g). This difference followed the same established pattern concerning nonsignificant and significant differences in the pellet and NPN fraction contents. Interestingly, the statistical covariate term (Table 3) hinted that the NPN GLU concentration could be dependent upon the pellet fraction.

Other individual amino acids

The results and discussion of the above four amino acids are typical of that for the remaining amino acids that were measured. Thus, the details and graphs are not presented here, but are discussed in a thesis by Creamer (1981).

Total amino acids

Processing had no effect on the overall average of the total amino acids in the pellet fraction (Table 2) which contained 86% of the total amino acids. Storage stability analysis indicated significant quadratic trends (Table 2). These same general trends were seen in 13 of the 17 pellet fraction amino acids analyzed (including ammonia-NH_3).

Percent amino acid recovery yields averaged over 81% which closely coincided with a report of 83% in which it was concluded that the compounds recovered from the amino acid analyzer represented almost all of the nitrogen in the supernatant fraction (Purcell and Walter, 1980). Details are discussed by Creamer (1981).

Spoilage

Evidence of spoilage was based upon the following criteria: (1) pH change from 4.2, (2) container pressure, (3) odor, and (4) general appearance. Those samples deemed preserved registered pH readings of 4.20–4.48 upon the initial probe into the jars. Upon mixing of contents, the pH range was determined to be 4.23–4.37. Corresponding container pressures were in the range $3.04-5.23 \times 10^4$ N/m^2 (9–15.5 inches of Hg vacuum). There was no noticeable etching of the lids, no detectable off-odor from the puree or unusual appearance of the pasteurized puree samples. Spoiled samples registered pH readings from
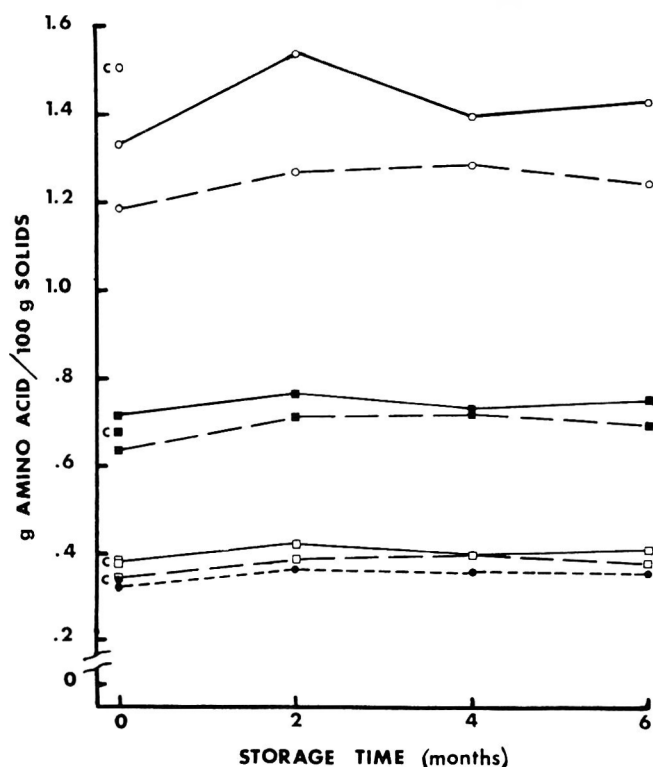
Fig. 4—Acidic amino acids from pellet fraction. (○) ASP, (●) THR, (□) SER, (■) GLU, (C) control, (—) no-enzyme method, (— —) average of α-amylase and amyloglucosidase methods and (- - -) average of all three methods.
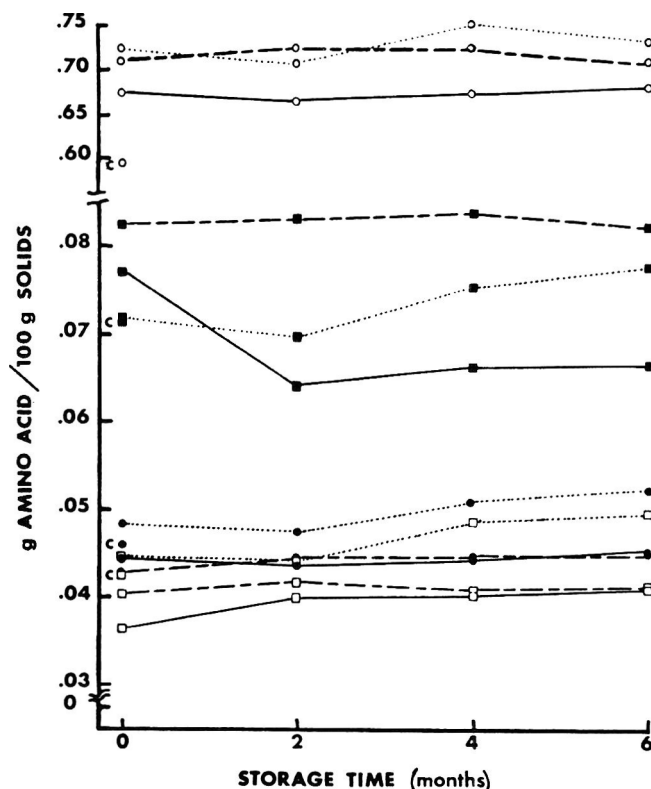
Fig. 5—Acidic amino acids from supernatant fraction. (○) ASP, (●) THR, (□) SER, (■) GLU, (C) control, (—) no-enzyme method, (— - —) α-amylase method and (· · ·) amyloglucosidase method.

initial probes of 4.58–5.07 and 4.56–5.04 after mixing. Spoiled containers had bulging lids with pressures of $1.08–1.67 \times 10^5$ N/m² (1–9.5 psig). A putrid odor and digested appearance was noted in these spoiled samples. It was thought that vegetative cells of a putrefactive anaerobe survived the thermal processing to cause the spoilage because the puree's initial pH of 4.2 was too low to permit outgrowth of spores (Stumbo, 1973; Speck, 1976). Contents of containers showing spoilage were not analyzed for amino acids. The degree of spoilage clearly showed a more severe thermal process would be required for commercial operations. Ice's work (1978) showed that by using the same criteria for determining spoilage, a water bath process at 90°C achieved pasteurization.

CONCLUSIONS

ESSENTIALLY no change due to processing or storage was observed in the amino acid content of the protein (pellet) fractions of all three methods. Nonprotein nitrogen content remained stable and increased in some instances, but to a small extent.

Enzyme methodology would be an essential part of a bulk pasteurization process for sweet potato puree in order to facilitate pumping. As for the two enzyme methods studied, a decision would have to be made concerning the carbohydrate consistency of the final storage product. Of the processes used there, the alpha-amylase method accomplished starch breakdown into limit dextrin, maltose and glucose in 30 min. However, the incubated puree re-

Table 2—Mean squares, g amino acid/100g solids

Source[a]	d.f.	ASP	THR	SER	GLU	TOT[b]
Analysis of Variance — Pellet						
REP	1	1.15 E-1*	6.10 E-7	5.48 E-4	4.58 E-3	8.02 E-1
TIM-MET	12	5.40 E-2*	1.97 E-3	2.34 E-3	8.27 E-3	1.04 E-0 (0.07)
CNTRL	1	1.54 E-1**	4.70 E-4	5.49 E-4	2.57 E-3	1.60 E-1
TL	1	1.25 E-2	3.71 E-3	3.78 E-3	9.51 E-3	2.48 E-0*
TQ	1	5.55 E-2	6.27 E-3 (.06)	7.41 E-3*	1.92 E-2	4.07 E-0**
TC	1	2.08 E-2	1.68 E-3	6.52 E-4	3.24 E-3	3.21 E-1
T123	1	3.36 E-1**	4.22 E-3	7.02 E-3*	2.59 E-2 (.07)	2.63 E-0*
T23	1	1.21 E-3	1.33 E-4	1.11 E-3	7.72 E-3	3.46 E-1
TIM × MET	6	1.15 E-2	1.19 E-3	1.26 E-3	5.20 E-3	4.17 E-1
Error	11	1.78 E-2	1.46 E-3	1.44 E-3	6.48 E-3	4.26 E-1
R^2		0.80	0.60	0.64	0.59	0.74
Analysis of Variance — Supernatant						
REP	1	6.34 E-1**	4.91 E-5**	2.44 E-3**	3.31 E-5	1.14 E-0**
TIM-MET	12	6.19 E-3**	3.10 E-5**	4.91 E-5**	1.95 E-4**	1.47 E-2**
CNTRL	1	4.48 E-2**	1.40 E-7	7.00 E-8	3.97 E-5	1.02 E-2 (0.06)
TL	1	1.08 E-4	3.24 E-5*	7.43 E-5**	1.02 E-5	8.71 E-4
TQ	1	1.16 E-4	7.40 E-7	1.73 E-6	8.42 E-5 (.06)	8.54 E-6
TC	1	1.87 E-3	1.77 E-6	2.00 E-8	6.32 E-5 (.10)	1.34 E-2*
T123	1	2.38 E-2**	7.29 E-5**	2.04 E-4**	1.01 E-3**	6.94 E-2**
T23	1	5.45 E-4	2.32 E-4**	2.54 E-4**	7.00 E-4**	7.26 E-2**
TIM × MET	6	5.08 E-4*	5.33 E-6	9.23 E-6	7.13 E-5*	1.54 E-3
Error	11	7.38 E-4	4.87 E-6	6.22 E-6	1.98 E-5	2.29 E-3
R^2		0.99	0.89	0.98	0.92	0.98

[a] Mean squares divided by error mean square to determine F value. Analysis of variance component is nonsignificant and significant at 5 and 1% of probability, respectively.
[b] Total of amino acids.

Table 3—Mean squares, g amino acid/100g solids

Source[a]	d.f.	ASP	THR	SER	GLU	TOT[b]
Analysis of Covariance — Supernatant Adjusting for Pellet						
REP	1	6.34 E-1**	4.91 E-5**	2.44 E-3**	3.31 E-5	1.14 E-0**
PELLET	1	4.46 E-2**	1.30 E-5	3.16 E-5 (.06)	9.38 E-5*	2.71 E-2**
CNTRL	1	1.89 E-2**	5.60 E-7	1.00 E-7	5.54 E-5	1.35 E-2*
TL	1	7.84 E-4	4.93 E-5**	1.11 E-4**	8.60 E-7	9.76 E-3 (.07)
TQ	1	2.53 E-3 (.07)	1.90 E-6	2.51 E-5 (.08)	4.00 E-5	1.05 E-2 (.06)
TC	1	3.29 E-4	1.00 E-8	1.21 E-6	4.88 E-5	6.14 E-3
T123	1	5.82 E-3**	5.00 E-5**	1.27 E-4**	1.01 E-3**	3.68 E-2**
T23	1	6.88 E-4	2.28 E-4**	2.39 E-4**	6.57 E-4**	6.72 E-2**
TL × T123	1	3.49 E-5	1.01 E-5	1.89 E-6	2.29 E-4**	1.42 E-4
TQ × T123	1	4.40 E-4	1.88 E-6	4.58 E-6	1.03 E-4*	1.24 E-3
TC × T123	1	1.26 E-3	3.90 E-7	5.38 E-6	1.25 E-5	3.27 E-3
TL × T23	1	4.17 E-4	7.98 E-6	2.82 E-5 (.07)	6.08 E-5	2.66 E-3
TQ × T23	1	4.70 E-7	3.98 E-6	7.80 E-7	1.53 E-5	6.03 E-5
TC × T23	1	1.22 E-3	6.14 E-6	1.39 E-5	6.89 E-6	2.21 E-3
Error	1	6.12 E-4	5.54 E-6	6.85 E-6	1.99 E-5	2.46 E-3
R^2		0.99	0.87	0.98	0.92	0.98

[a] Mean squares divided by error mean square to determine F value. Analysis of variance component is nonsignificant and significant at 5 and 1% of probability, respectively.
[b] Total amino acids.

quired cooling from 88°C to 29°C before final thermal processing to lower the pH to 4.2 with phosphoric acid. Should the carbohydrate structure not be of paramount concern, the amyloglucosidase method would be preferable as it does not include the cooling step in the alpha-amylase method. Though the amyloglucosidase method did not achieve the degree of liquefaction of the alpha-amylase method because of insufficient incubation (apparent viscosity 2.05 Pa.s vs 6.68 Pa.s), the puree flowed readily enough for pumping. Should this process be pursued, a microbiological study would seem to be in order, particularly since Ice (1978) achieved pasteurization with no spoilage and the process discussed here had an unacceptable level of spoilage.

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CONCLUSION

THIS STUDY has shown that field peas can be used to produce a curd similar to tofu but with a higher crude protein and lower crude oil content. The low curd yield from pea flour could be improved by using more expensive pea protein concentrate. Although the sensory properties of pea curds were judged inferior to tofu, they were generally acceptable. Addition of gluten can improve the color and texture of pea curd and may also be used to improve nutritional quality.

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Role of Gas Diffusion in Bloater Formation of Brined Cucumbers

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ABSTRACT

A method was developed to measure diffusion rates of CO_2 and N_2 through fresh and brined cucumber tissue to examine the role of gas diffusion in bloater formation. The diffusion rate of CO_2 was 3.2 times greater than N_2 through fresh and 2.4 times greater than N_2 through brined cucumber tissue. The ratio of the diffusion rates of CO_2 to N_2 through brined cucumbers was not significantly altered from that of fresh fruit. Solubility of gases was determined to be a factor governing the rate and extent of bloater damage. Maximum rates of increase in expansion volume of N_2-exchanged cucumbers was 0.42, 1.30, and 11.90% hr^{-1} for brines purged with Ar, CO_2, and SO_2, respectively, and 0.89% hr^{-1} for Ar-exchanged fruit following purge of the brine with CO_2.

INTRODUCTION

THE PRODUCTION of carbon dioxide by fermentative microorganisms has long been implicated as the cause of bloater damage in pickling cucumbers (Veldhuis and Etchells, 1939; Etchells et al., 1945). However, only recently has a detailed mechanism for bloater formation of pickling cucumbers been proposed (Fleming and Pharr, 1980). It was hypothesized that a liquid clogged layer of tissue develops in brine-stored cucumbers due to the entrance of brine. This liquid-clogged region of tissue was further postulated to serve as a differentially permeable barrier to the diffusion of N_2 and CO_2. Since a large portion of the internal gas atmosphere of a cucumber prior to brining is N_2, and the CO_2 concentration in the brine increases rapidly upon fermentation (Fleming et al., 1973b), a diffusion gradient for CO_2 arises toward the fruit interior. It was further hypothesized that considering the much greater aqueous solubility of CO_2 than N_2 (Hodgman et al., 1958), there is a greater inward transport of CO_2 than outward transport of N_2, resulting in an internal gas pressure in excess of atmospheric pressure. This may then cause the tissue to rupture with resultant formation of a gas pocket (bloater).

Diffusion of gases through apples and other fruits has been found to be governed by Fick's first law of diffusion (Burg and Burg, 1965). This relationship describes the amount of some diffusing species i, crossing a certain area per unit time, e.g. moles of a solute per cm^2 in a second. For one dimension, the law may be written as:

$$J_i = -D_i \partial C_i / \partial x \qquad (1)$$

where J_i = flux (moles/cm^2–s), D_i = diffusion coefficient (cm^2/s), and $\partial C_i / \partial x$ = concentration gradient in the x – direction (moles/cm^4). Frequently in the study of gas transfer through biological tissue, gas diffusion through different phases must be considered. For this reason a diffusion coefficient expressed in terms of a pressure gradient is more convenient than the use of concentration gradient as described in Eq (1). In addition, the solubility of a gas is a major factor governing its transport rate through a liquid (Krogh, 1919; Guyton, 1971). Fenn and Rahn (1964) incorporated this solubility factor, replaced concentration with partial pressure, and isolated area from flux to yield the following form of Fick's first law:

$$dQ_i / dt = -A \alpha_i D_i \partial P_i / \partial x \qquad (2)$$

The expression dQ_i / dt is the instantaneous rate of movement of a gas perpendicularly across an area A driven by a partial pressure gradient $\partial P_i / \partial x$, where P_i is the partial pressure of the gas species and x is the distance. The diffusion coefficient D, frequently expressed in cm^2/s, is a property of both the medium and the diffusing substance. The two constants α D represent an analog to Krogh's diffusion coefficient where α is the Bunsen solubility coefficient. When applied to gas diffusion through fruits, the surface area term in the equation is in actuality the effective surface area. For apples, this represents the surface area of lenticels (Burg and Burg, 1965) and for cucumbers may be the total surface of the stomatal pores. Total stomatal pore area has been estimated by Smith et al. (1979) to be about 0.062% of the total surface area for large 'GY14' cucumbers.

According to the hypotheses of Fleming and Pharr (1980) the diffusion rates of CO_2 and N_2 for a given partial pressure gradient through fresh cucumbers should be nearly the same for both gases due to the relatively continuous nature of the intercellular spaces. However, if a liquid-clogged layer of tissue develops in fruit upon brine storage as hypothesized, the diffusion rate of CO_2 should be greater than for N_2 due to the presence of a liquid barrier to diffusion. In addition, the rate of increase in expansion volume of brine to the exterior of cucumbers, could be expected to be dependent on the difference in solubilities of gases inside the fruit and in the brine.

This study was conducted to examine the role of gas diffusion on bloater damage in pickling cucumbers. Specific objectives were to: (1) develop a method to measure and compare diffusion rates of CO_2 and N_2 through fresh and brined cucumbers and (2) test the hypothesis that gas solubility is a major factor governing the rate and extent of bloater damage in pickling cucumbers due to the effect of solubility on the diffusion rate of a gas in a liquid system.

MATERIALS & METHODS

Cucumbers

Size no. 3 pickling cucumbers (3.8–5.1 cm diam), cv. Calypso, were obtained from a local grower and from field plots at University Research Unit 4 at Raleigh. Following harvest, cucumbers were washed, humidified with moist cloths and held in 13.0 ± 1.0°C storage for not longer than 7 days. Fruit were equilibrated at 22.0 ± 1.0°C prior to experimental use.

Gas diffusion apparatus

Ends of cucumbers were removed perpendicular to the longitudinal axis of the fruit, and a 5.0 cm length, approximately cylindrical shaped section was taken. A 2.0 cm diam cavity was made

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through the center of each fruit with a cork borer, which removed most of the seed region. All cut surfaces were blotted to remove liquid expressed from cut cells. Individual cucumber segments were then placed in the diffusion apparatus (Fig. 1). Gas tight seals were made at the junctions of: (1) each end of the segment and the two rubber gaskets, (2) the bottom rubber gasket and the apparatus platform, and (3) the beaker and bottom rubber gasket. The seals were made with a stiff, nonmelting, silicone, high vacuum grease (Cat. No. 970 V; Dow Corning Corp., Midland, MI).

Gas diffusion measurements

An experiment was conducted to test if dQ_i/dt was linearly related to ΔP_i for diffusion of CO_2 and N_2 through fresh cucumber segments using the diffusion apparatus (Fig. 1). This was achieved by determining the steady state diffusion rates of both CO_2 and N_2 for different partial pressure gradients of the respective gases. Various humidified (bubbled gases through water) mixtures of CO_2 and N_2 were flowed into the internal gas cavity of the cucumber segments through the gas cavity inlet at a rate of 250 ml/min. Measurement of CO_2 diffusion from the internal gas cavity through the surface of the cucumber segment was achieved by flowing pure N_2 through the chamber at 25 ml/min and analyzing the gas composition of a 0.5 cm^3 sample of gas taken from the chamber sampling port with a gas chromatograph.

Diffusion of N_2 was measured by using CO_2 as the chamber gas. Attainment of a steady state diffusion rate was presumed when there was no change in composition of the chamber outlet gas over time. Measurements of CO_2 and N_2 diffusion rates were made on separate cucumber segments.

An additional experiment was designed to compare the diffusion rates of CO_2 and N_2 through fresh and brine-stored (72 hr) cucumbers. Diffusion rates for both CO_2 and N_2 were measured on the same cucumber segments for a partial pressure difference (ΔP_i) of about 1 atmosphere (i.e. pure CO_2 in the gas cavity and pure N_2 in the chamber; and the converse). The ratio of the diffusion rates of CO_2 to N_2 was calculated for each segment.

Gas exchange of cucumbers and brine

The effect of differences in the aqueous solubility of gases inside the fruit and in the exterior brine on bloater damage was tested using three paired combinations of CO_2, N_2, and Ar. These gases differ appreciably with respect to their solubilities in water and aqueous NaCl solutions (Table 1). Data for the same concentration of NaCl solution were not found in the literature. However, the solubility of each gas is decreased markedly in aqueous NaCl solutions of concentrations comparable to that used in this study. In water (25°C), CO_2 is 53.1 times more soluble than N_2 and 24.2 times more soluble than Ar; and Ar is 2.2 times more soluble than

N_2. These three comparisons of aqueous solubilities formed the basis of three experimental treatments.

Cucumbers (1.7 kg) were packed into 3.8 liter jars to give a 45:55 (w/v) pack-out ratio of fruit to brine. Each jar lid was equipped with a glass gas dispersion tube, graduated reservoir and glass rod to support the reservoir as described previously by Fleming and Pharr (1980). For each gas pair treatment, the internal atmospheres of fruit were exchanged with the less soluble gas of a gas pair at a flow rate of 300 ml/min. Acidified, aqueous NaCl solution was added to the jars, while maintaining gas flow to exclude air from the system. The brining medium was 10.6% (w/w) NaCl, 0.32% (v/v) glacial acetic acid, and 0.20% (w/w) sodium benzoate. The acidified, aqueous NaCl solution containing sodium benzoate was used to suppress microbial growth during storage (Fleming et al., 1980). Following addition of brine, a flow of the less soluble gas was continued at a rate of 50 ml/min for 48 hr. This length of time in brine storage was shown previously to bring about a bloater susceptible condition in artificially carbonated cucumbers (Fleming et al., 1978). After 48 hr brine storage, flow of the more soluble gas of a gas pair was initiated at a high flow rate (300 ml/min) for 20 min in order to bring about a rapid saturation of the exterior brine. The flow rate was adjusted to 50 ml/min thereafter. A continuous N_2-purge served as the control. "Expansion volume" of the brined cucumbers was determined by the rise in brine level in the graduated reservoir and was expressed as a percentage of the volume of cucumbers (Fleming et al., 1973a). Cucumbers were evaluated for bloater damage according to Etchells et al. (1974) and bloater indices calculated according to Fleming et al. (1977).

An additional treatment with SO_2 as the gas in the exterior brine and N_2 as the exchange gas was used to determine the effect of an extreme solubility difference on the rate of expansion. The Bunsen solubility coefficient of SO_2 in water at 25°C is 30.0287 cm^3 gas/cm^3 H_2O (Hodgman et al., 1958), which is 2100 times greater than the value for nitrogen. Due to the extremely high aqueous solubility of SO_2, a flow of 300 ml/min would have been too low to bring about a rapid near saturation of the brine. Therefore, the flow of SO_2 through the brine was made sufficiently rapid to produce free gas bubbles at a rate that was visually estimated as comparable to the other gas treatments.

Regression analysis

Second-order polynomials, forced through the origin, were fit to the percentage expansion volume data. A comparison of the rates of increase in expansion volume for the gas pair treatments was of interest, since this response was presumably governed by the rates at which the various gases diffused into and out of the cucumbers. Therefore, the coefficients of the first-order terms in the second-order regression equations were compared. This coefficient represents the maximum rate of expansion and is equivalent to the slope of the tangent to the regression curve through the origin.

RESULTS

Gas diffusion

The relationship of diffusion rate (dQ_i/dt) to partial pressure difference (ΔP_i) for both CO_2 and N_2 through fresh cucumber segments was approximately linear (Fig.
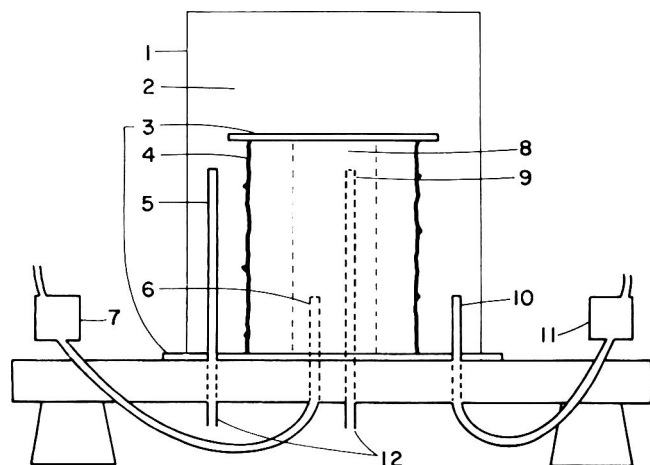
Fig. 1—Schematic diagram of the diffusion apparatus: (1) glass beaker; (2) chamber; (3) rubber gasket; (4) cucumber segment; (5) chamber inlet; (6) gas cavity outlet; (7) gas cavity sampling port; (8) internal gas cavity; (9) gas cavity inlet; (10) chamber outlet; (11) chamber sampling port; (12) flow meter leads.

Table 1—Solubility of gases in water and aqueous NaCl solutions at 25°C

Gas	α^a (cm^3 gas/cm^3 solution)	
	H_2O	Aqueous NaCl solution[d]
CO_2	0.7590[b]	0.4585 (10.9)[e]
N_2	0.0143[b]	0.0052 (11.9)[f]
Ar	0.0314[c]	0.0216 (15.9)[g]

[a] α denotes the Bunsen solubility coefficient; the volume of gas when reduced to 0°C and 1 atmosphere, absorbed by one volume of solution when the pressure of the gas itself is 1 atmosphere.
[b] From Hodgman et al. (1958)
[c] From Lannung (1930)
[d] Parenthetical values are the % NaCl (w/w) as calculated from other concentration units given in the references cited in this table.
[e] From Fleming et al. (1975)
[f] From International Critical Tables (1928)
[g] From Åkerlof (1935)

2). This was expected and verified that Fick's first law of diffusion applied to gas diffusion through cucumbers as measured with the apparatus diagrammed in Fig. 1.

The diffusion rate of CO_2 was significantly greater than N_2 through both fresh and brine-stored cucumber segments (Table 2). Also, the diffusion rate of CO_2 was significantly decreased upon brine storage. A trend toward a reduction in the diffusion rate of N_2 in brine-stored compared to fresh cucumbers was measured. The ratio of CO_2 to N_2 diffusion rates did not change significantly upon brine storage.

Expansion volume and bloater damage

The largest increase in expansion volume was obtained for N_2 (in)-CO_2 (out) (Fig. 3); the gas pair having the greatest difference in aqueous solubility (Table 1). Gas pairs with less difference in solubility resulted in correspondingly less increase in expansion volume. This trend was the same for both experiments (Fig. 3). The maximum rates of increase in expansion volume (average of estimates of linear term coefficients) for the different gas pairs were all significantly different and increased with increasing difference in the solubilities of the gases in a pair (Table 3). The same trend existed for the bloater index values. However, a statistically significant difference was detected only between N_2(in)-CO_2(out) and the control.

When SO_2 was charged rapidly through the brine surrounding cucumbers stored for 48 hrs in brine under a continuous N_2-purge, expansion volume increased to over 5% within 1 hr (Fig. 4). The maximum rate obtained from

the second-order polynomial was 11.9% hr^{-1}, which was over nine times the maximum rate estimated for the N_2-CO_2 gas pair.

DISCUSSION

FLEMING AND PHARR (1980) proposed that there is a differential flux of CO_2 and N_2 through brined cucumbers, leading to the development of internal gas pressure upon carbonation of the brine, with subsequent bloater damage. The observation that bloater damage is minimal in cucumbers carbonated immediately following brine addition (Fleming et al., 1978; Corey et al., 1983) was proposed to be due to the following: " . . . N_2 is displaced from the cucumbers by CO_2 before intercellular gas spaces become clogged and restrict the rate of N_2 removal from the
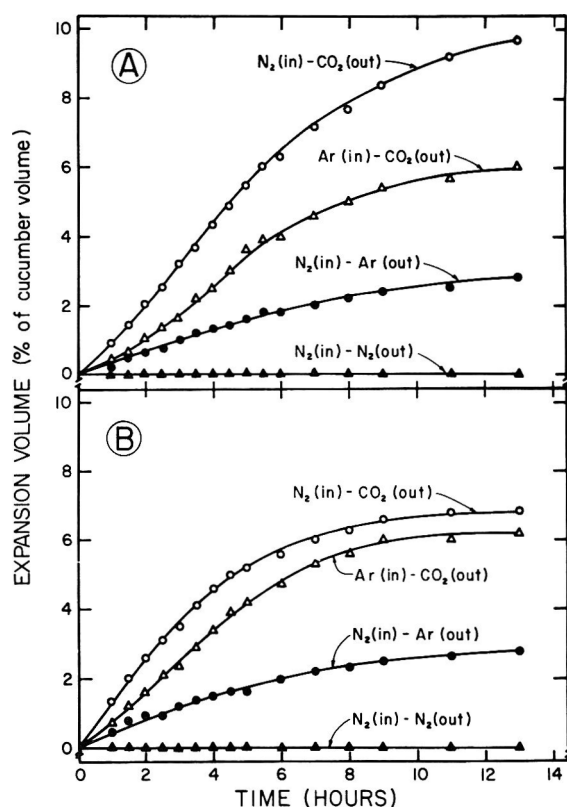
Fig. 3—Expansion volume changes of brined cucumbers as influenced by various gas pairs, used to exchange the internal atmospheres of fresh pickling cucumbers and to saturate the exterior brine following 48 hr brine storage. The first gas given for a gas pair (in) denotes the gas used to exchange the internal atmosphere of fruit before brining, and the second (out) denotes the gas used to saturate the brine after the fruit was gas-exchanged and then brined. The A and B denote the same experiment conducted with two different cucumber harvests at two separate times. See Table 3 for related data.

Fig. 2—Steady state diffusion rates (dQ/dt) of CO_2 and N_2 through fresh cucumber segments as influenced by the partial pressure difference ΔP_i, where ΔP_i = the partial pressure of the ith gas in the internal gas cavity less the partial pressure of the same gas in the chamber. Each point represents the mean of three replications. Vertical bars represent ± one standard error of the mean.

Table 2—Diffusion rates (dQ/dt) of CO_2 and N_2 through fresh and brine-stored cucumber segments

| Cucumbers | dQ/dt ($\mu l\ min^{-1}$ – segment)[a] | | Row comparison | CO_2/N_2[a] |
	CO_2	N_2		
Fresh	655.4 ± 15.5	219.1 ± 43.6	**	3.20 ± 0.52
Brine-stored (72 hr)	360.2 ± 63.6	158.5 ± 20.1	*	2.40 ± 0.67
Column comparison	**	NS[b]		NS

[a] Mean of three replications ± standard error of mean
[b] NS = Not significant
* = significant at P = 0.05
** = significant at P = 0.01

tissue." Their hypothesis was supported by the demonstration of a reduced rate of CO_2 removal from brined compared to fresh cucumbers that were CO_2-exchanged prior to brine storage, with a simultaneous reduced rate of N_2 entrance in brined cucumbers upon N_2-purging. Hence, fresh cucumbers were proposed to exchange more rapidly with the ambient gaseous environment than brine-stored fruit due to the formation of a "continuous liquid-clogged outer layer" of tissue in brined fruit. Considering the much greater solubility of CO_2 than N_2, this implied that the ratio of the diffusion rates of CO_2 to N_2 for brined cucumbers would be considerably higher than for fresh cucumbers. Results of the present study are not in agreement with this idea (Table 2). Differential diffusion rates of CO_2 and N_2 were measured through both fresh and brine-stored cucumbers. However, a significant change in the ratio of dQ_{CO_2}/dt to DQ_{N_2}/dt upon brining was not detected.

A reduction in the internal gas volume of cucumbers held in brine storage has been measured previously (Corey et al., 1983). This suggests that the number of barriers to gas exchange increases in brine-stored cucumbers and hence the resistance to gas diffusion should increase accordingly.

Table 3—Effects of different gas pair combinations used to exchange the internal atmospheres of cucumbers and to saturate the brine on maximum rate of increase in expansion of exterior brine volume and bloater damage[a]

Gas pair (in-out)[b]	Δ solubility of gases (cm^3 gas/cm^3 H_2O)[c]	Max rate of increase in expansion vol (% hr^{-1})[d]	Bloater index[d]
$N_2 - N_2$ (control)	0	0	0
$N_2 - Ar$	0.017	0.42	9.3
$Ar - CO_2$	0.728	0.89	11.1
$N_2 - CO_2$	0.745	1.30	26.4
LSD (0.05)		0.23	18.4

[a] Values are means of duplicate 3.8 liter jars each containing 12–14 cucumbers
[b] Internal atmospheres of cucumbers were exchanged with the less soluble gas in a pair (first gas), followed by a purge of the same gas for 48 hr following addition of brine. Flow of the more soluble gas in a pair (second gas) was begun after 48 hr brine storage.
[c] See also Table 1.
[d] Coefficient of correlation between max rate of increase in expansion volume and bloater index was 0.87 (P = 0.01).
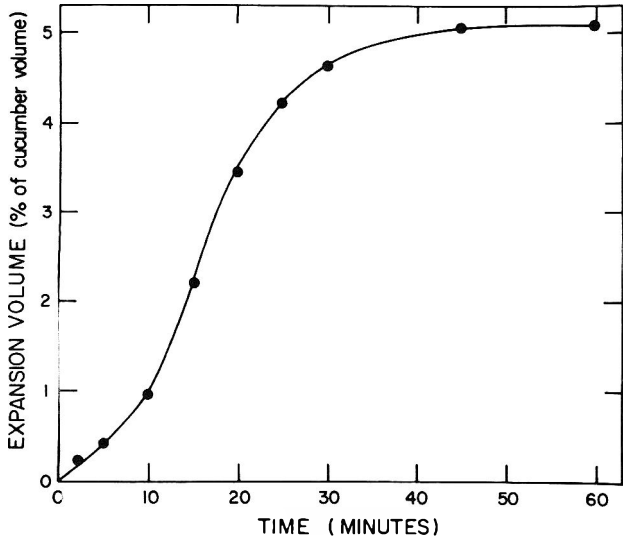
Fig. 4—Expansion volume changes of brined cucumbers charged with SO_2 following 48 hr brining under a continuous N_2-purge. Cucumbers were N_2-exchanged before brine storage.

Although a reduction in the diffusion rates of both CO_2 and N_2 was measured in brined compared to fresh fruit (Table 2), most important was the fact that the ratio of dQ_{CO_2}/dt to dQ_{N_2}/dt was not altered substantially upon brine storage of cucumbers. If a 'continuous' liquid-clogged barrier to gas diffusion develops in brine-stored cucumbers, then the ratio of dQ_{CO_2}/dt to dQ_{N_2}/dt should be substantially higher than for the same ratio in fresh cucumbers.

Interpretation of diffusion rates

A possible explanation for the lack of difference in the ratio of dQ_{CO_2}/dt to dQ_{N_2}/dt in brined compared to fresh cucumbers may be due to the absence of a continuous liquid-clogged region of tissue in brined cucumbers. A simplified model will aid in the interpretation of the diffusion rates of CO_2 and N_2 measured experimentally.

The intercellular gas channels may be conceived to be analogous to small capillary tubes. In both fresh and brined cucumbers, some of these passages may be blocked with continuous liquid barriers (Fig. 5A). Other gas channels may be relatively unblocked, containing a liquid film along the sides of the channel, but possessing a continuous gas phase channel throughout the length of the tube (Fig. 5B). For a continuous gas phase channel, the diffusion rates of CO_2 and N_2 would be expected to be equal. The binary diffusion coefficient for the CO_2-N_2 gas pair in the gas phase, denoted by $D_{CO_2-N_2}$, is 0.16 cm^2/s-atm (Fenn and Rahn, 1964). Since $D_{CO_2-N_2} = D_{N_2-CO_2}$, the same value may be used to describe the gas phase diffusion of both CO_2 and N_2. Therefore, dQ_{CO_2}/dt from a to c should be equal to dQ_{N_2}/dt from c to a in Fig. 5B.

Suppose, that a relatively thin continuous liquid barrier (e.g. 5 cell layers thick) develops in cucumbers upon brining. Assuming an average cell diameter of approximately 24μ, the thickness of this barrier would be 1.2×10^{-2} cm. The average path length from the cavity-tissue interface to the surface of the cucumber was measured to be 8.0×10^{-1} cm. The simplified model of Fig. 5A may be applied to this situation. Let the distances $\overline{ac} = 8.0 \times 10^{-1}$ cm and $\overline{ab} = 1.2 \times 10^{-2}$ cm, the thicknesses of the liquid barrier and the remaining gas phase distance respectively. Further, assume that the liquid barrier behaves essentially as an aqueous barrier. The absolute diffusion coefficient of CO_2 in H_2O at $22°C$ is 1.91×10^{-5} cm^2/s and for N_2 is 2.02×10^{-5} cm^2/s (International Critical Tables, 1929). However, if these values are multiplied by their respective Bunsen
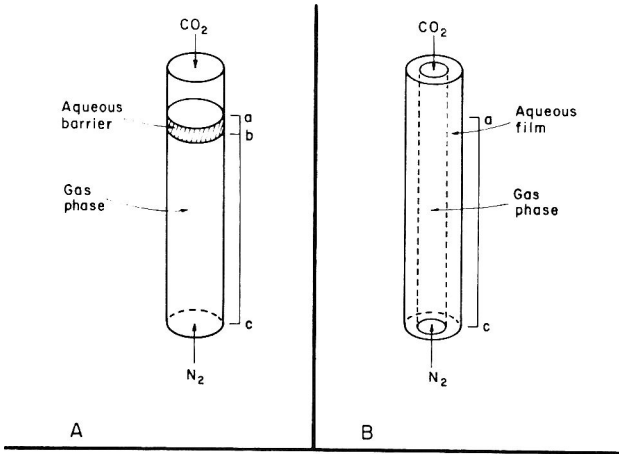
Fig. 5—Schematic representation of: (A) capillary tube with a continuous aqueous barrier to diffusion of CO_2 and N_2 and (B) capillary tube lined with an aqueous film containing a continuous gas channel for diffusion of CO_2 and N_2. See text for discussion.

solubility coefficients at 22°C (Hodgman et al., 1958), the diffusion coefficient $D' = \alpha_i D_i$ is defined, where D_i is the absolute diffusion coefficient in water. Hence, D'_{CO_2} = 1.45 × 10⁻⁵ cm²/s-atm and D'_{N_2} = 3.0 × 10⁻⁷ cm²/s-atm. Experimentally, dQ_i/dt was determined in the steady state. Applied to the capillary tube model this means that dQ_i/dt is the same for any point along the path $\overline{ac}$. In addition, for pure CO_2 at one end of the tube and pure N_2 at the other in a flowing system, the partial pressures of the gases at points a and c are given as follows:

$$P_a(CO_2) = 1, P_c(CO_2) = 0 \text{ and}$$
$$P_a(N_2) = 0, P_c(N_2) = 1$$

Letting $X_1 = \overline{ab}$ and $X_2 = \overline{bc}$, substituting D_i' for $\alpha_i D_i$, assuming steady state conditions, and applying Eq (2) yields the following relationship to describe diffusion of CO_2 from point a to point c in the capillary of Fig. 5A.

$$dQ_{CO_2}/dt = \frac{AD'_{CO_2}}{X_1}(P_a - P_b) = \frac{AD_{CO_2 - N_2}}{X_2}(P_b - P_c) \quad (3)$$

Similarly, for diffusion of N_2 from point c to point a;

$$dQ_{N_2}/dt = \frac{AD_{N_2 - CO_2}}{X_2}(P_c - P_b) = \frac{AD'_{N_2}}{X_1}(P_b - P_a) \quad (4)$$

Substituting the appropriate values and solving for P_b for each gas yields, $P_b(CO_2) = 5.9 \times 10^{-3}$ atm and $P_b(N_2)$ = 9.9 × 10⁻¹ atm. Substituting the respective values for P_b into Eq (3) and (4) gives dQ_{CO_2}/dt = (1.2 × 10⁻³ cm/s) (A) and dQ_{N_2}/dt = (2.5 × 10⁻⁵ cm/s) (A). Taking the ratio of dQ_{CO_2}/dt to dQ_{N_2}/dt gives a value of 48, which is nearly equal to $\alpha_{CO_2}/\alpha_{N_2}$. Thus, a barrier of a continuous nature must not develop in cucumbers stored in brine for short periods of time (e.g. 72 hr) since the ratio of dQ_{CO_2}/dt to dQ_{N_2}/dt was only 3.2 (Table 2). This finding is in contrast to the 'continuous' liquid clogged region of tissue proposed previously to occur in brined cucumbers (Fleming and Pharr, 1980). Reduction in the absolute values of dQ_{CO_2}/dt and dQ_{N_2}/dt upon brining implies that the number of capillaries analogous to Fig. 5A must increase in brine-stored fruit. However, because the ratio of diffusion rates of CO_2 to N_2 more nearly approaches the expected value for pure gas phase diffusion, there must be some proportion of intercellular avenues analogous to the capillary of Fig. 5B. In actuality, the continuous gas channels present in brined cucumbers are probably extremely tortuous. The proportion of the total gas avenues analogous to the capillary of Fig. 5B, denoted by p, may be approximated using the experimentally determined value for the ratio of dQ_{CO_2}/dt to dQ_{N_2}/dt through brined fruit by the following equation.

$$\frac{dQ_{CO_2}/dt}{dQ_{N_2}/dt} = \frac{pD_{CO_2 - N_2} + (1-p)D'_{CO_2}}{pD_{N_2 - CO_2} + (1-p)D'_{N_2}} \quad (5)$$

Substituting values and solving for p gives p = 6.15 × 10⁻⁵. This calculation demonstrates that the presence of an extremely small number of continuous gas channels may result in an overriding effect on the diffusion rates of 2 gases (e.g. CO_2 and N_2) differing greatly in solubility.

Internal gas pressure development in freshly brined cucumbers upon carbonation of the brine is an expectation based on the difference in CO_2 and N_2 diffusion rates measured through cucumber tissue in this work and has been experimentally measured (Corey et al., 1983). Since the ratio of dQ_{CO_2}/dt to dQ_{N_2}/dt was not found to be different for fresh and brined cucumbers, the lower internal gas pressure measured in freshly brined compared to brine-stored cucumbers (Corey et al., 1983) may be attributed to

mass exit of gases through fresh cucumbers upon internal pressurization. This is consistent with the evidence of Corey et al., (1983) for the occurrence of mass flow through fresh cucumbers in brine. Resistance to mass flow of gases in fresh cucumbers is much less than in brined cucumbers. Therefore, both diffusion and mass flow of gases are important processes regulating the internal gas pressure of freshly brined cucumbers upon carbonation of the brine, whereas transport of gases in brine-stored cucumbers is probably limited to diffusion.

Influence of gas solubility on bloater damage

The rate of increase in expansion volume and bloater damage in brined cucumbers was shown to be strongly dependent on the difference in aqueous solubility of gases inside the fruit and in the exterior brine (Fig. 3, 4 and Table 3). The differences in the maximum expansion rates for the gas pairs was presumably due to differential rates of diffusion of the two gases in a given gas pair treatment. Since solubility is a factor governing the diffusion rates of gases through both the fruit tissue and the brine; a greater amount of the more soluble gas diffuses into the fruit than the less soluble gas out of the fruit in a given time as proposed by Fleming and Pharr (1980). This results in an internal gas pressure in excess of atmospheric pressure and was measured by Corey et al. (1983) for the gas pair N_2-CO_2. The extent of boater damage for the different gas pairs (Table 3) may vary due to variations in the rate and extent of internal gas pressure development. In addition, the relationships of maximum rate of increase in expansion volume and bloater index to Δ solubility as determined are not clearly defined, though a definite trend is evident (Table 3). The role of gas solubility as it governs diffusion of gases through the brine as compared to through the cucumber tissue has not been evaluated. Preservative concentration (e.g. sodium benzoate) and pH also influence gas solubility. Their effects on transfer rates of different gases also needs evaluation. Although solubility of gases has a strong influence on their relative diffusion rates, other factors may influence gas transport through fresh and brined cucumbers. Changes in the effective surface area for diffusion of gases, permeability of the cuticle, molecular sieve effects, and pH are a few such factors that may have an effect on the differential diffusion rates of gases through cucumbers. These may be important additional considerations in studying the mechanism of bloater formation in brined cucumbers.

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Influence of Cultivars, Soak Solution, Blanch Method, and Brine Composition on Canned Dry Pea Quality

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————————ABSTRACT————————

The composition of two commercially grown cultivars of dry peas (Pisum sativum L.), 'Alaska' and 'Garfield', and their physical and sensory qualities after rehydration and thermal processing under various conditions were evaluated. Raw Garfield peas were larger and higher in lipid content ($P<0.05$) than Alaska peas, but no differences between cultivars were observed for moisture, protein, fiber, insoluble solids, alcohol insoluble solids, or starch contents. Sensory and physical assessment of the pea cultivars when canned revealed differences for a number of variables, but the results were complicated by interaction with the variety of processing conditions employed. The data indicate that appropriate control of processing conditions can minimize the difference in sensory characteristics among peas of different cultivars.

INTRODUCTION

DRY PEA (*Pisum sativum* L.) cultivars with improved agronomic characteristics have been developed with little attention paid to processing quality or consumer acceptance. Recently, there has been concern over differences in the processed quality of two dry pea cultivars. Specifically, a new cultivar (Garfield) did not produce an acceptable product when the standard industry rehydration and thermal process was used in the United Kingdom. This is of concern to farmers and scientists in eastern Washington and northern Idaho because approximately 30% of the dry peas grown in this region are shipped to England for further processing into canned products and maintenance or expansion of that market for dry peas is a primary interest (Blaine, 1979).

Dry peas are grown in rotation with wheat in a relatively small region (approximately 60,000 ha annually) along the border between Washington and Idaho. This region is responsible for approximately 95% of the U.S. production of dry peas (Washington and Idaho Dry Pea and Lentil Commission, undated); the exported portion of the crop is worth approximately $24 million to the Pacific Northwest states annually (O'Rourke, 1981). A switch by farmers to newer, higher yielding cultivars is of concern if the new cultivars do not process into a product consistent with accepted standards.

Although research on the effects of varying processing methods on the quality of canned dry peas has been quite limited, considerable research has been conducted on the canning of dry beans (*Phaseolus vulgaris* L.) which may be applicable to dry peas (Nordstrom and Sistrunk, 1977, 1979; Junek et al., 1980; Davis et al., 1980; Quenzer et al., 1978; Luh et al., 1975; Sevilla and Luh, 1974). Factors frequently studied in the processing of canned, dried

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legumes include soak medium, soak time, blanch treatment (steam vs hot water, length of blanch time, and post blanch cooling) and brine composition. It has been observed that addition of organic acids to the soak water improved color scores, decreased drained weight and increased firmness of canned, rehydrated dry beans (Sevilla and Luh, 1974; Junek et al., 1980); calcium chloride had similar effects (Luh et al., 1975). Steam blanching produced a canned bean product equivalent to water blanching (Davis et al., 1980), while others reported that steam blanching produced higher drained weights and firmer beans than water blanching (Sevilla and Luh, 1974; Nordstrom and Sistrunk, 1979). Nordstrom and Sistrunk (1977) noted that an acidic brine (tomato sauce) resulted in firmer canned beans than those canned in water.

The objective of this research was to compare the raw and processed characteristics of the standard 'Alaska' and higher-yielding 'Garfield' dry peas when thermally processed by several methods which involved alterations in soak, blanch and brine treatments.

MATERIALS & METHODS

ALASKA AND GARFIELD peas grown in 1978 were obtained from the USDA, ARS Dry Pea and Lentil Program, Pullman, WA. The dry peas (9–12% moisture) were held at ambient temperature and humidity for 90 days and sorted for cracks and splits before analysis and thermal processing.

Raw characteristics. Moisture, fat, Kjeldahl nitrogen, crude fiber, starch, alcohol insoluble solids, and insoluble solids contents of the dry peas were determined according to standard procedures (AOAC, 1975). Physical characteristics of size (number of peas required to fill a 100 ml volume), specific gravity (Casimir et al., 1967) and rehydration rate (g of water absorbed at 22°C after 8 hr per g of pea) were also measured.

Processing. Dry peas were soaked for 12 hr at 22°C in an equal volume of one of the following six solutions: 0.25 or 0.50% calcium chloride, 0.25 or 0.50% citric acid, 2% sodium chloride or deionized water. Soaked peas were drained and after either water (82°C for 8 min) or steam (steam used to maintain peas at 82°C for 8 min) blanching, 220g of blanched peas were added to 303 x 406 cans. Two hundred twenty milliliters of one of three brine solutions were added to each can: 2% sodium chloride, 2% NaCl plus 0.5% calcium chloride, or 2% NaCl plus 0.5% citric acid. The combinations of two cultivars, and six soak, two blanch and three brine conditions resulted in 72 treatments. The filled cans were sealed and processed for 27 min at 118°C. The processed cans were held for 90 days prior to evaluation.

Processed characteristics. The processed peas were evaluated for drained weight, shear force, color and sensory characteristics; and the brine for volume, turbidity, soluble solids and starch content.

Drained weights were assessed by AOAC procedures (1975). Force required to shear a 100g sample of peas was evaluated using a Food Technology Corporation shear press equipped with a multiple blade cell. Green and yellow colors were measured with an Agtron Model 500 A reflectance spectrophotometer; Rd, a and b color values were obtained with a Hunter Color Difference Meter standardized against a green tile (Rd = 34.7, a = −19.3, b = 13.7). Brine turbidity was determined as transmittance at 660 nm of a sample diluted 1:25. Soluble solids content of brine was assessed by use of an Abbé refractometer. Starch content was determined by the dinitrophenol method (AOAC, 1975). Each assay was replicated twice.

For sensory evaluation, the 72 treatments were randomized and tested twice in two 5½ wk periods with two evaluation sessions per

week and six samples per session. Samples were served warm (37°C), in foil covered 50 ml beakers placed in trays of hot water. Order of presentation was different for each panelist. The 10 panelists were staff, faculty and graduate students. A 30-min training period was used to instruct panelists on use of the scorecard and to expose panelists to the range of variation which could be expected among samples. A multiple paired comparison test was used in which each of the six coded samples was compared to a clearly marked control. The control was standard Alaska peas processed with a water soak, water blanch and NaCl brine. Panelists were asked to score the degree of difference from the control using a nine-point scale (1 = no difference from control; 9 = extreme difference) for each of three characteristics: flavor, texture and appearance. If a difference was perceived, panelists were asked to indicate the type of difference, selected from a list of terms on the score sheet (Fig. 1). A coded "hidden" control was periodically substituted for one of the treatment samples to check panel performance.

Statistical analysis. The experiment was designed as a four-way factorial. Analysis of variance and Duncan's new multiple range test were calculated for sensory and objective data to determine significantly different treatment means (Steel and Torrie, 1980). Correlation coefficients were calculated between appropriate variables.

RESULTS

Raw characteristics

Garfield peas were larger and had a higher lipid concentration (P<0.05) than Alaska peas (Table 1). The difference in lipid composition, although small, may be of importance in the flavor of dry peas. Flavor and off-flavor compounds derived from lipids are important in many legume products (Sessa, 1979).

Processed characteristics

The analysis of variance of both objective and sensory data revealed a number of significant interactions, which

Fig. 1—Score card used to assess sensory characteristics of rehydrated, canned dry peas.

Table 1—Means and standard deviations for compositional and physical characteristics of raw Alaska and Garfield dry peas[a]

Pea characteristic	Alaska	Garfield	S[b]
Composition			
Moisture (%)	7.52a	7.32a	1.64
Lipid (%)	1.13a	1.29b	0.08
Protein[c] (%)	20.9a	21.7a	1.6
Crude Fiber (%)	5.8a	5.6a	0.6
Starch (%)	65.8a	65.3a	2.4
Alcohol Insoluble Solids (%)	80.3a	80.3a	0.9
Insoluble Solids (%)	64.6a	61.5a	2.9
Physical measurements			
Size (number of peas/100ml)	330b	243a	46.4
Specific gravity	1.33a	1.36a	0.01
Rehydration (g H_2O/g pea after 8 hr)	1.09a	1.06a	0.04

[a] Means within a row followed by different letters are significantly different (P<0.05).
[b] Standard deviation of 9 observations.
[c] Protein content calculated as % nitrogen x 6.25.

made it difficult in some cases to generalize about the primary variety and processing variables (Table 2). Mean values for sensory and objective data observed for the main effects of cultivar, soak treatment, blanch method and brine composition are presented in Tables 3 and 4. Significant treatment effects will be discussed for each primary variable which showed consistent trends.

Pea cultivar. The sensory panel found significant (P< 0.05) differences between the two cultivars for flavor, texture and appearance, with Garfield being more dissimilar in comparison to the control (Table 3). This result was expected, since the control was the Alaska cultivar. Examination of data means for the various processing treatments revealed that significant interactions (Table 2) do not alter this result, and panelists were able to differentiate the flavor, texture and appearance between Alaska and Garfield peas. However, when statistical analysis was done separately for each soak treatment, the analysis revealed panelists could not differentiate between the flavor of Alaska and Garfield peas for soaks of H_2O or NaCl.

Examination of descriptive terms (Table 5) indicated panelists found Garfield peas to be "less sweet" and "more bitter;" to be "less firm" ("more mushy") and appear less visually "intact," but to have tougher seed coats ("more tough skins"); and to be "more yellow" (and "less green") and larger than Alaska peas.

A significantly higher shear value for Alaska peas was observed (Table 3). Instrumental measurement of color did not verify the descriptive color terms chosen by panelists for yellowness and greenness (Tables 3 and 5). In addition, the degree of greenness of the peas as measured by the Agtron and Hunter instruments was also not in agreement. Although differences between the instrumentally measured color means were not large (Table 3), Garfield peas were consistently less yellow and green by Agtron measurement (higher numbers), but more green (smaller 'a' value) and lighter in value (larger 'Rd' value) by Hunter measurement than Alaska peas.

Garfield peas had consistently higher drained weights and lower brine volumes (except for the 2% NaCl soak) than Alaska peas, but no cultivar differences were observed for brine starch content, soluble solids or turbidity (Table 4). Thus, Garfield peas appear to absorb more of the brine, producing a canned pea product with a higher ratio of peas to brine.

Soak treatment. Soak treatment produced significant effects for every variable measured. Soak treatment significantly interacted with a number of other treatments,

Table 2—Significant treatment effects for objective and sensory data of rehydrated, canned dry peas considering variety, and soak, blanch and brine conditions and their interactions

| | | | | | | | | | | | | | | Brine | |
| Source | Sensory data | | | Agtron color | | Hunter color | | | Shear force | Drained weight | Brine volume | Brine starch | Brine soluble solids | Brine turbidity |
	Flavor	Texture	Appearance	Green	Yellow	Rd	a	b						
Variety	*	*	*	***	***	*	*	–	*	**	**	–	–	–
Soak	***	***	**	***	***	**	*	*	***	***	***	***	***	**
Variety x Soak	–	***	*	***	**	–	–	–	**	***	***	***	***	*
Blanch	***	–	–	**	*	–	**	**	***	***	***	***	***	***
Variety x Blanch	–	–	**	*	**	*	–	–	–	*	***	–	**	***
Soak x Blanch	**	–	–	–	–	–	–	–	*	–	–	–	**	–
Variety x Soak x Blanch	–	–	*	–	–	–	*	*	–	–	*	**	***	–
Brine	***	***	***	***	***	***	***	***	***	***	***	***	***	***
Variety x Brine	***	***	***	**	**	–	–	–	***	**	***	–	–	–
Soak x Brine	***	***	***	*	**	–	–	**	***	***	***	***	***	***
Variety x Soak x Brine	–	***	***	**	***	–	–	*	–	***	***	***	***	***
Blanch x Brine	**	***	***	–	–	–	–	–	**	**	***	***	–	***
Variety x Blanch x Brine	–	*	–	–	–	–	–	–	*	**	***	*	*	***
Soak x Blanch x Brine	***	***	***	–	–	–	–	–	**	–	***	**	***	***
Variety x Soak x Blanch x Brine	–	–	**	–	–	–	–	–	**	–	***	***	**	***

*Indicates significance at the 5% level.
**Indicates significance at the 1% level.
***Indicates significance at the 0.1% level.

and these interactions have been taken into consideration in discussion of the data.

The H_2O, NaCl and both of the $CaCl_2$ soaks did not cause much variation in pea flavor from the control, but the soak treatment containing the higher level of citric acid altered pea flavor as would be expected. Descriptive flavor terms and an examination of the flavor means separated by soak, blanch and brine treatments further verified this result.

Although soak treatment had an effect on canned pea texture, the effect of brine was much more pronounced (Table 6). Shear press data verified this result. Descriptive terms and shear values revealed the effect of soak treatment on texture generally to be (from most firm to least): 0.50% $CaCl_2$ > 0.25% $CaCl_2$ > 0.50% citric acid > 0.25% citric acid > H_2O > 2% NaCl.

Soak treatments containing the higher levels of calcium chloride or citric acid were significantly more different in appearance from the control than peas soaked in the other four solutions (Table 3). Descriptive terms indicated citric acid soak peas were "less green" and "more yellow" than the others, and calcium chloride soaked peas were "more green." Agtron measurements indicated that peas soaked in either concentration of calcium chloride were darker green and yellow than peas receiving the other four soak treatments. Hunter 'a' values did not differ from each other for peas soaked in calcium chloride or citric acid, but 'a' values were higher (peas less green) for H_2O and NaCl soaks.

Calcium chloride at the 0.50% level in soak consistently produced the lowest drained weights; followed by 0.25% $CaCl_2$ and 0.50% citric acid which produced similar values for drained weight (Table 4). The drained weights of peas

Table 3—Means for sensory and objective data from rehydrated, canned dry peas considering cultivar, soak treatment, blanch method and brine composition[a]

| | | | | Objective data | | | | | |
| Main effect | Sensory data[b] | | | Agtron color | | Hunter color | | | Shear force |
	Flavor	Texture	Appearance	Green (%)	Yellow (%)	Rd	a	b	(kg)
Cultivar									
Alaska	4.8a	4.6a	4.6a	50a	74a	16.5a	1.8b	20.5a	71b
Garfield	5.7b	5.5b	6.0b	55b	78b	18.0b	1.3a	20.7a	65a
Soak treatment									
H_2O	4.7a	4.3a	5.1a	54c	77c	18.1b	1.8b	20.6ab	51b
2% NaCl	5.2ab	4.6b	5.2c	55c	78cd	17.3b	2.0b	20.4a	41a
0.25% Citric Acid	5.4b	4.6b	5.2ab	55c	79d	17.5b	1.4a	20.9bc	63c
0.50% Citric Acid	6.7c	5.3c	5.7c	54c	78cd	18.1b	1.4a	21.0c	77d
0.25% $CaCl_2$	4.7a	5.4c	5.3ab	49b	72b	16.5a	1.5a	20.5a	81e
0.50 $CaCl_2$	5.0ab	6.0d	5.5bc	48a	70a	16.1a	1.4a	20.4a	97f
Blanch method									
Water	5.1a	5.1a	5.4a	52a	75a	17.2a	1.7b	20.7b	72b
Steam	5.4b	5.0a	5.3a	53b	76b	17.4a	1.5a	20.5a	65a
Brine composition									
NaCl	4.2a	3.9a	4.4a	56b	80b	17.8b	1.8b	21.0b	38a
Citric Acid	6.4c	4.4b	5.1b	56b	81c	18.6c	1.7b	21.0b	52b
$CaCl_2$	5.3b	6.8c	6.4c	45a	67a	15.4a	1.3a	20.0a	116c

[a] Means within a column and main effect heading followed by different letters are significantly different (P < 0.05).
[b] Scores based on a nine-point scale; values closer to one (1) are most similar to the control sample.

Table 4—Mean values for canned characteristics of rehydrated, canned dry peas considering cultivar, soak treatment, blanch method and brine composition[a]

Main effect	Drained weight (g)	Brine volume (ml)	Brine starch (%)	Brine soluble solids (%)	Brine turbidity (%T)
Cultivar					
Alaska	322a	99b	24a	7.9a	79a
Garfield	333b	89a	28a	7.7a	80a
Soak treatment					
H_2O	341d	76b	41c	7.5b	78a
2% NaCl	357e	55a	48d	8.0d	79ab
0.25% Citric Acid	331c	91c	23b	7.7c	79ab
0.50% Citric Acid	317b	112d	12a	7.4a	81bc
0.25% $CaCl_2$	315b	109d	18ab	8.1de	80ab
0.50% $CaCl_2$	304a	120e	14a	8.1e	82c
Blanch method					
Water	314a	107b	16a	7.5a	83b
Steam	341b	81a	36b	8.1b	76a
Brine composition					
NaCl	368c	44a	50c	7.6a	76b
Citric Acid	339b	81b	24b	8.1b	73a
$CaCl_2$	275a	156c	4a	7.7a	90c

[a] Means within a column and main effect heading followed by different letters are significantly different (P < 0.05).

soaked in 0.25% citric acid were intermediate and followed by soaks of water and then sodium chloride which produced the highest drained weights.

The citric acid and water soaks produced lower values for brine soluble solids than did soaks of sodium chloride or calcium chloride (Table 4). For brine volume, brine starch content and brine turbidity, the effect of brine composition was more pronounced than the effects of soaking medium.

Blanch treatment. Although the effect of type of blanch on canned pea flavor appeared to be significant, further analysis of the data revealed that this significance was complicated by treatment interactions and no consistent trend emerged. Blanch did not significantly effect sensory texture or appearance of the canned peas (Table 3).

Although the effect of blanch appeared to be significant for the instrumental color readings of Agtron green and yellow and Hunter 'a', and 'b', examination of the analysis of variance by soak indicated that blanch type did not significantly or consistently affect instrumental measurement of pea color.

Table 5—Number of times descriptive terms were marked by sensory panelists for rehydrated, canned Alaska and Garfield peas[a]

	Cultivar	
	Alaska	Garfield
Descriptive flavor terms		
Less sweet	49	129
More sour	229	292
More salty	64	56
More bitter	7	67
More bland	33	65
Descriptive textural terms		
More firm	357	260
More mushy	31	172
More tough skins	189	239
More grainy	153	156
Descriptive appearance terms		
Less intact	52	134
More yellow	74	399
Less green	6	68
More large	0	564

[a] Only data for terms marked by six or more panelists is included. Total possible number of judgements is 720 (6 soaks x 2 blanches x 3 brines x 2 replications x 10 panelists).

The water blanch treatment produced significantly higher shear values (firmer peas).

The type of blanch treatment also produced highly significant differences among the canning characteristics measured. Water blanching produced lower drained weights and higher brine volumes along with lower brine starch contents, soluble solids and turbidity (Table 4).

Brine composition. Of the experimental variables tested, brine composition had the greatest effect on canned pea quality.

Brine composition had a highly significant effect on flavor and this effect was not obscured by the significant interactions. In all cases, peas receiving a NaCl brine were rated as being most like the control in flavor and peas receiving a citric acid brine were least like the control, regardless of other treatments. Descriptive terms indicated differences from the control peas were primarily due to sourness and lack of sweetness.

Peas in the calcium chloride brine were the most different from the control in texture and were observed to be more firm and grainy and had tougher seedcoats as compared to control peas. Peas in the sodium chloride brine were most like the control and those in the citric acid brine were intermediate in texture. The observations regarding pea firmness were verified by measurement of shear force (Table 3).

Brine composition also tended to affect appearance. The brine effect tended to vary with pea cultivar, with brine composition affecting the appearance of Garfield peas more than it did for Alaska peas. Calcium chloride brine had the greatest effect on appearance. Panelists indicated peas in a calcium chloride brine were more green and less yellow

Table 6—Interactive effects of soak treatment and brine on sensory texture scores[a]

	Soak treatment					
		2.0%	Calcium chloride		Citric acid	
Brine	H_2O	NaCl	0.25%	0.50%	0.25%	0.50%
NaCl	3.8	4.9	3.3	4.4	3.0	3.8
$CaCl_2$	6.0	5.0	7.7	8.0	6.9	7.4
Citric acid	3.3	3.8	5.2	5.7	3.9	4.7

[a] Scores based on a nine-point scale; values closer to one (1) are most similar to the control.

Table 7—Selected correlation coefficients among sensory scores and objective data[a]

	Drained weight	Brine				Instrumental Color					Shear force	Sensory scores	
		Turbidity	Soluble solids	Starch content	Volume	Agtron yellow	Agtron green	Hunter Rd	Hunter b	Hunter a		Appearance	Texture
Sensory scores													
Flavor	−0.15	−0.13	0.10	−0.24*	0.19	−	−	−	−	−	−	0.45***	0.23
Texture	−0.72***	0.70***	−0.17	−0.38***	0.73***	−	−	−	−	−	0.81***	0.86***	
Appearance	−0.51***	0.55***	−0.25*	−0.25*	0.55***	−0.33**	−0.29*	−0.19	−0.33***	−0.55**	0.54***		
Shear Force	−0.92***	0.67***	−0.05	−0.63***	0.93***	−							
Instrumental color													
Hunter a	0.43***	−0.27*	−0.04	0.37**	−0.50***	0.26*	0.26*	0.25*	0.20				
Hunter b	0.54***	−0.55***	0.17	0.23	−0.53***	0.69***	0.64***	0.69***					
Hunter Rd	0.61***	−0.61***	0.05	0.32**	−0.59***	0.87***	0.85***						
Brine													
Volume	−0.97***	0.62***	−0.07	−0.77***									
Starch	0.81***	−0.31**	−0.21										
Soluble solids	−0.43***	−0.42***											
Turbidity	−0.62***												

[a] Correlation coefficients are omitted when relationships between parameters would be meaningless.
*Indicates significance at the 5% level.
**Indicates significance at the 1% level.
***Indicates significance at the 0.1% level.

than those in citric acid or sodium chloride brine. The peas in calcium chloride also appeared more intact and the canning media was less cloudy and viscous.

Brine composition also affected instrumental measurement of color values. Hunter Color Difference meter 'Rd' values and Agtron values indicated the calcium chloride brine produced the darkest peas. However, no consistent trends were noted for Hunter 'a' values except within the 0.50% $CaCl_2$ soak; there the $CaCl_2$ brine produced significantly greener peas than the other brines.

Drained weight was significantly lower for peas packed in a calcium chloride brine, intermediate for citric acid brine and highest for sodium chloride brine (Table 4). Brine volume followed an inverse pattern to drained weight. The turbidity and brine starch content of peas canned with the calcium chloride brine were significantly lower than for the sodium chloride and citric acid brines. Although the effect of brine composition on brine-soluble solids was highly significant, the influence of other variables obscured the influence of brine composition and no consistent trends for soluble solids were observed.

Correlation. It is apparent that several dependent variables varied together as treatment was altered. A correlation matrix is presented in Table 7. Kramer and Twigg (1970) have suggested that a correlation coefficient smaller than ± 0.80 is not acceptable for concluding that the objective measurement sufficiently corresponds with sensory data. In these experiments flavor and appearance were not correlated with any objective measurements with an r ≥ ± 0.8. There was a high bimodal correlation (r = 0.9 and r = −0.8) between Food Technology Corporation shear press readings and sensory texture scores (Fig. 2).

Among instrumental analyses, the Hunter 'Rd' and both Agtron values were highly correlated (r = 0.87 and 0.85). Drained weight, brine volume and shear press values were also highly related, as would be expected.

Practical application. The means for flavor, texture and appearance from the 36 processing treatments of Garfield peas were compared to identify the treatments which produced peas most similar (i.e., sensory scores closest to 1) to standard Alaska canned dry peas processed using the control method (i.e., water soak, water blanch and NaCl brine). The processing treatments so identified were 0.25% $CaCl_2$ soak, steam blanch and NaCl brine.

DISCUSSION

BRINE COMPOSITION had the greatest effect on canned pea quality characteristics, followed by soak treatment, then blanch treatment of the processing treatments studied. The effect of various treatments on canned pea character-

istics are generally in agreement with those found by other researchers in work with canning of dry beans. Uebersax and Bedford (1980) reported soak water calcium had a greater effect on bean firmness than did brine calcium, however, most of their research was carried out at elevated temperatures.

Soak. Using a citric acid soak in this study altered pea flavor, increased pea firmness, decreased green color, and lowered drained weights (as compared to water and sodium chloride soaks). Junek et al. (1980) reported increased shear force and decreased drained weights for beans soaked in citric or malic acids as opposed to a water soak, although the effect varied with type of bean. An effect of the acid solution on carbohydrate hydration was suggested (Junek and Sistrunk, 1980). Sevilla and Luh (1974) observed a decrease of water imbibition by red kidney beans as citric acid concentration was increased in the soaking water.

Use of a calcium chloride soak in this study generally produced firmer, darker canned peas with lower drained
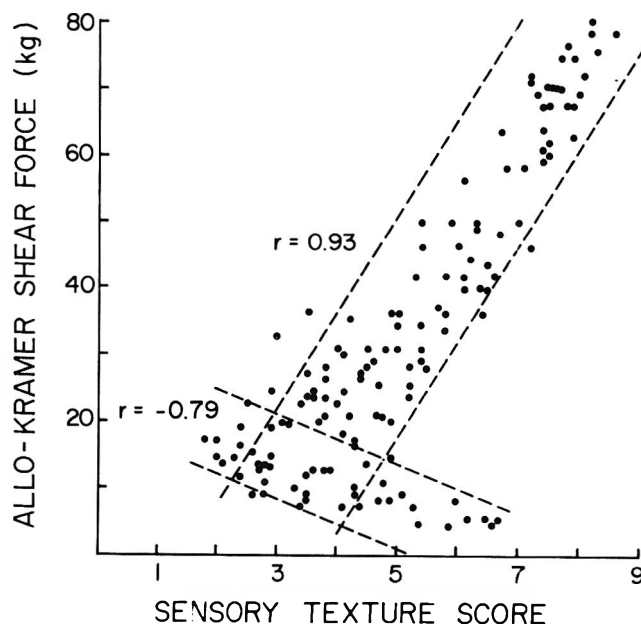
Fig. 2—Instrumental measurement of shear force plotted against sensory assessment of texture of rehydrated canned dry peas resulted in a bimodal distribution, since peas that were softer than the control were rated as dissimilar. (Sensory scores based on a nine-point scale; values closer to one are most similar to the control.)

weights, higher brine volume and soluble solids, and lower brine starch contents. Luh et al. (1975) have similarly found firmer canned lima beans when 0.1 or 0.3% CaCl$_2$ · 2H$_2$O was added to the soak, although no effect on color was noted.

Blanch. Use of a steam blanch (as opposed to a water blanch) in this study produced less firm peas, higher drained weight, lower brine volume, and higher brine starch, soluble solids and turbidity values, but had minimal effect on other characteristics. Steam blanching (as opposed to water blanching) of beans has also been observed to produce higher drained weights (Sevilla and Luh, 1974; Nordstrom and Sistrunk, 1979). Steam-blanching may leach less of the soluble components than water blanching (Sevilla and Luh, 1974). Steam blanching has also been observed to produce firmer beans (Nordstrom and Sistrunk, 1979), but in this study the opposite effect was observed. The reason for this is not known.

Brine. Acidification of the brine in canning of beans generally has produced firmer beans and lower drained weights due to inhibition of swelling of the beans due to decreased hydration of starch and protein in the presence of acid (Nordstrom and Sistrunk, 1977; Luh et al., 1975). This was observed in the present study; however, calcium chloride produced these effects to a greater degree. The firming effects of calcium ion on the texture of certain canned foods are well known (Lindsay, 1976).

CONCLUSION

SENSORY AND OBJECTIVE DATA reveal that variations in thermal processing parameters of soak composition, blanch type and brine composition result in differences in the canned dry pea product. The differences between a steam or water blanch were small. Varying brine composition produced the greatest differences in the final product; soak composition also had a considerable effect on product characteristics. Calcium chloride added to either soak or brine produced firmer, more intact, lighter color canned peas than those soaked or canned in citric acid, sodium chloride or water. Citric acid in the soak or brine solutions produced firmer peas than either use of sodium chloride or water; however, surprisingly, little effect of acid on pea color was noted. The effects of acid on pea flavor were well noticed by the panel, and may prove to be unacceptable; Sevilla and Luh (1974) reported that the flavor scores of red kidney beans soaked in citric acid decreased as the levels of citric acid in the soaking solution increased from 0.25 to 0.75%. The effects of processing variables were

affected by pea cultivar. It appears that an acceptable canned dry pea product can be produced from different pea cultivars if care is given to composition of soak and/or brine in processing.

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Production and Regeneration of Principal Volatiles in Apples Stored in Modified Atmospheres and Air

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————————ABSTRACT————————

In a series of exploratory experiments, storage of McIntosh apples (*Malus domestica* Borkh.) in modified atmospheres (MA) (5% CO_2 + 3% O_2 at 2.8°C) suppressed the development of headspace ethanol and acetaldehyde from that in apples stored in air at 0°C (RA). Acetaldehyde, ethanol, ethyl butyrate and hexanal production from intact fruit was further suppressed when the apples were stored in 1.5% CO_2 + 1.5% O_2 or 1.5% CO_2 + 1.0% O_2 at 2.8°C. Placement of fruit in RA following MA storage initially regenerated ethyl butyrate and hexanal in preference to ethanol and acetaldehyde. However storage of fruit in 1.5% CO_2 + 1.0% O_2 for 320 days completely suppressed the principal headspace volatiles and blocked their subsequent regeneration in RA.

INTRODUCTION

TEXTURE and titratable acidity are retained when McIntosh apples (*Malus domestica*, Borkh.) are stored in 1.5% CO_2 + 1.0% O_2 rather than the present MA storage recommendations of 5% CO_2 + 3% O_2 (Lidster et al., 1980, 1981). Sensory panelists determined that the juiciness and overall acceptability of the apples stored in 1.0% O_2 are higher than for apples stored in 3% O_2, but lack intensity of the characteristic McIntosh flavor. Although the McIntosh flavor results from a complex mixture of esters, alcohols and aldehydes (MacGregor et al., 1964; Sapers et al., 1977), Fargas (1966) identifies the main volatile component of the McIntosh flavor to be ethyl-n-butyrate. Acetaldehyde and ethanol which are minor flavor components often appear as indicators of advancing fruit senescence and contribute to off-flavors and atypical fruit taste (Thomas, 1929; Fidler, 1933). Consequently, storage and marketing life of apples are terminated when excessive levels of ethanol and acetaldehyde accumulate (Anderson, 1967; Workman, 1963; Blanpied et al., 1968). Storage of apples in modified atmospheres (MA) suppresses production of alcohols and esters over that found in apples stored in air (RA) (Meigh, 1956). Other researchers subsequently determined that apple volatiles can be partially regenerated after MA storage by exposing the fruit to air at 20°C (Patterson et al., 1974).

The present study investigated the effects of conventional MA and very low oxygen atmospheres on headspace volatile production and the subsequent regeneration in RA.

MATERIALS & METHODS

IN 1978, three 20-kg samples of preclimacteric [as determined by starch-iodine test and ethylene evolution (Lidster et al., 1981)] McIntosh apples were harvested from each of two mature trees (replicates) within an orchard located in Kentville, Nova Scotia. The three sets of replicates were cooled to 2.8°C within 24 hr of harvest and each set of replicates was immediately sealed in individual air-tight chambers, in which the chamber O_2 level was reduced to the appropriate level by N_2 flushing. Each of three chamber atmospheres were maintained at 5.0% CO_2 + 3.0% O_2, 1.5% CO_2 + 1.5% O_2 and 1.5% CO_2 + 1.0% O_2 by controlled dry lime scrubbing (CO_2) and

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adjustable venting to ambient air (O_2), as previously described by Lidster et al. (1980). All fruits were removed after 210 days of modified atmosphere (MA) storage and each replicate placed in a 38 μm perforated polyethylene liner to prevent moisture loss (approximately 94–96% RH) and placed in 0° air storage (RA). Ten fruit per replication for each MA treatment were removed from RA after 0, 30, 60, 90 and 120 days from MA storage opening for volatile assessment.

The 1979 study consisted of nine 20-kg samples of preclimacteric McIntosh apples harvested from each of five 40-year old trees (replicates) within a single orchard. The experimental factors consisted of two storage atmospheres (1.5% CO_2 + 1.0% O_2 and 5.0% CO_2 + 3.0% O_2 at 2.8°C), four removal dates from MA storage (40, 80, 160 and 320 days) and three subsequent holding durations in RA (40, 80 and 120 days). Each group of five replicates was stored within a single experimental chamber in which CO_2, O_2 and relative humidity levels was maintained as in the 1978 study. A control series of fruit were stored in 38 μm perforated polyethylene liners in RA and ten fruit were removed for volatile assessments at 0, 40, 80, 120, 160 and 200 days. At each removal from MA or RA, headspace volatiles were determined.

For the 1980 study seven 20-kg samples of preclimatic McIntosh apples were harvested from each of five commercial orchards (one replicate from the same source used in 1978 and 1979 studies). The experimental factors consisted of two MA (1.5% CO_2 + 1.0% O_2 and 5.0% CO_2 + 3.0% O_2 at 2.8°C), three removals from MA (90, 180, and 270 days) and one subsequent holding duration of 90 days in RA. A control series of five 20-kg samples was held in RA and fruit removed for volatile determination after 0, 90, 180 and 270 days of storage. Storage conditions and volatile assessments were identical to the procedures followed in the 1978 and 1979 studies.

Volatile assessment

Ten fruit per replication were removed from storage and conditioned for 3 days at 20°C and 94% RH. The fruit were then weighed and sealed in airtight 4.5L glass jars for 3 hr at 20°C after which 1 mL of headspace was sampled and injected into a GLC (Forsyth and Webster, 1971).

In 1978 and 1979, a Varian Aerograph 204B equipped with a FID detector was used to analyze the volatile constituents. Volatile separation was performed using a 3.2m stainless steel (SS) column packed with 20% Hallcomid M18 on 60/80 mesh Chromosorb W (AW/DMCS treated). An oven temperature of 85°C and injector temperature of 135°C was used with a He carrier gas flow of 33.3 cc/min, H_2 flow 8 cc/min and O_2 flow 400 cc/min.

In 1980, a Varian 3700 GLC equipped with a FID detector was employed to separate the volatile components using a 5-foot SS column packed with 20% Hallcomid M18 on 60/80 mesh Chromosorb W (AW/DMCS treated). Gas flows were: He 41 cc/min, H_2 30 cc/min, and O_2 300 cc/min. Instrument temperatures were maintained as: oven, 60°C; injector, 110°C; and detector, 90°C.

Concentration and tentative identity of volatile components were determined by comparison to standard curves prepared and analyzed at each fruit sampling and expressed as μg of volatile produced per kg of fruit per hr.

RESULTS

REPLICATION in these experimental series included replication among trees or orchards within each year but not among chambers. Replication of MA regimens was provided among years by the repetition of similar experiments. These studies were of an exploratory nature. There is no established model for describing volatile production in MA and subsequent RA. Moreover, volatile production is com-
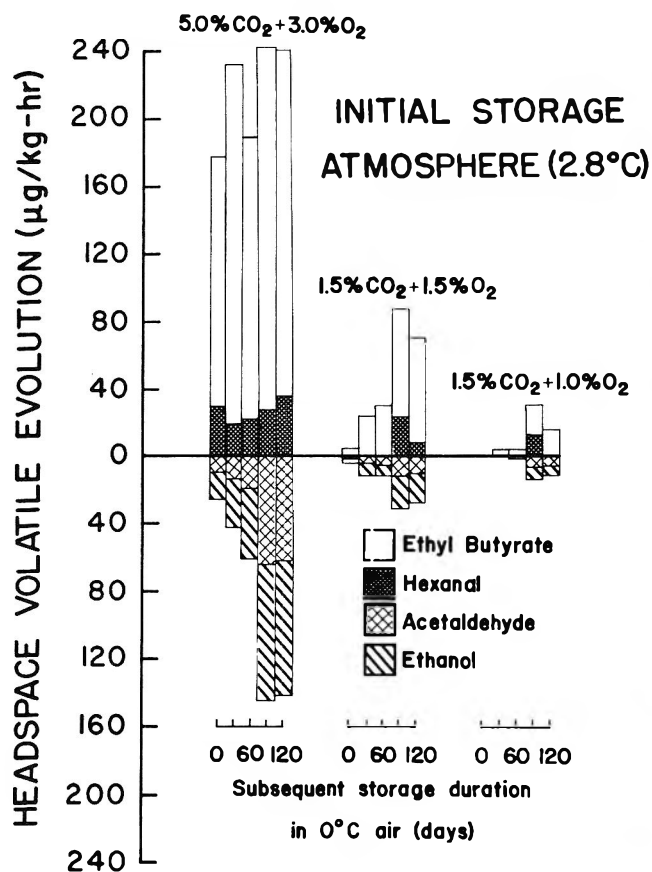
Fig. 1—Regeneration of headspace volatiles by McIntosh apples at 30 day intervals in RA following 210 days of MA storage (n = 2).

Fig. 2—Headspace volatile production from McIntosh apple immediately after 40, 80, 160 and 320 days of MA storage and subsequent regeneration at 40 day intervals in RA storage and volatile development at 40 day intervals in RA, 1979 (n = 5).

Fig. 3—Headspace volatile production from McIntosh apples immediately after MA storage and subsequent regeneration after 90 days in RA storage, and volatile development at 90 day intervals in RA, 1980 (n = 5).

posed of several components each with their own variability and interrelationships. A simple presentation of experimental results is provided in Fig. 1, 2, and 3. Compound bar graphs describe the production rates of volatiles, ethyl butyrate and hexanal, above the center line, and that of acetaldehyde and ethanol below. Trends in volatile production over time in subsequent air storage can be assessed readily for: each principal component, the combined components above and below the center line, their ratio and total production. Comparison among time periods within a MA storage regimen, and among MA regimens at fixed storage periods can be made readily. Levels of volatile production ranged from 0–8000 $\mu g/kg$-hr and affected the precision of the estimate. For example, the fruit removed after 160 days of air storage from replicate storage units in the 1979 study had means (and standard errors) for the volatile components ($\mu g/kg$-hr) of: acetaldehyde 100.8 (32.51), methyl acetate 2.61 (0.575), ethyl acetate 7.22 (1.859), ethyl alcohol 123.1 (26.01), ethyl butyrate 12.3 (4.09) and hexanal 15.38 (6.20).

Of the volatiles detected and tentatively identified, (Fig. 4) ethyl butyrate, hexanal, ethanol and acetaldehyde were found to be the major constituents accounting for 96.5, 96.1, 98.5% of the total volatiles measured in 1978, 1979 and 1980, respectively. Methyl acetate, ethyl acetate and ethyl propionate also tentatively identified, tended to increase during the early storage and then decline to very low levels in patterns similar to those observed by Forsyth et al. (1971) for cold stored apples. Storage of fruit in both MA regimens suppressed the production of methyl acetate, ethyl acetate and ethyl propionate at all removal dates. These volatiles were found in very small but variable quantities and although they may contribute to the overall flavor, their inclusion was not considered necessary in a general description of volatile suppression in MA and subsequent regeneration.
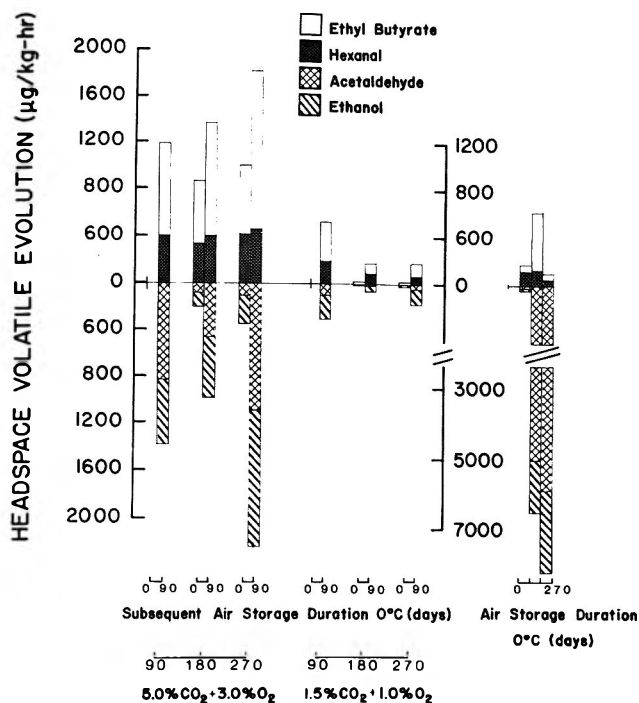
Storage of apples in 1.5 or 1.0% O_2 atmospheres suppressed the production of ethyl butyrate, hexanal, ethanol and acetaldehyde in comparison with similar fruit stored in either 5.0% CO_2 + 3.0% O_2 or RA, in each of the three years (Fig. 1, 2, 3). Suppression of these volatiles by very low O_2 atmospheres increased with extended exposure beyond 40 days of storage in 1.0% O_2 in 1979 (Fig. 2). No detectable volatile production was found immediately
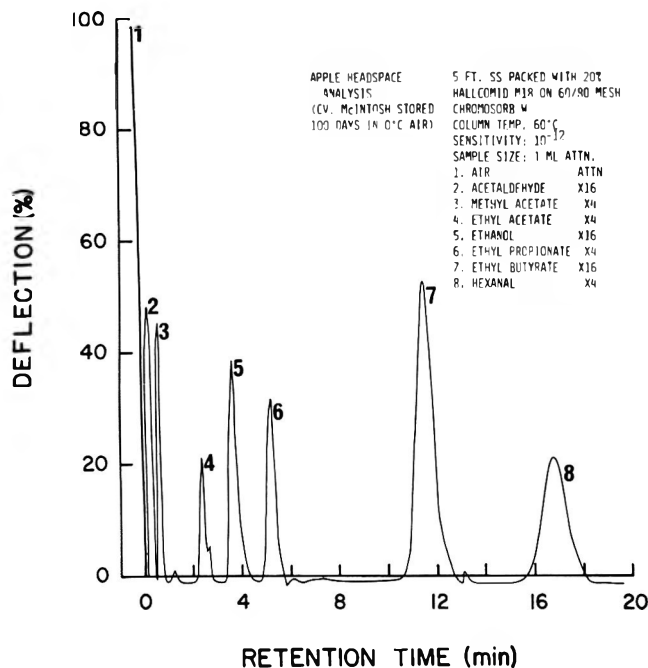
Fig. 4—Sample chromatogram of headspace volatile separation for McIntosh apples stored in 0°C air for 100 days.

after 1.0% O_2 storage durations of: 210 days in 1978, 40, 80, 160 or 320 days in 1979 or at 90 days in 1980.

Storage of apples at 5.0% CO_2 + 3.0% O_2 and subsequent RA stimulated ethyl butyrate and hexanal production in comparison with similar apples held continuously in RA Fig. 2, 3). Also, apples stored in 5.0% CO_2 + 3.0% O_2 produced less acetaldehyde and ethanol than similar apples in RA except for fruit stored for 80 days at 5.0% CO_2 + 3.0% O_2 plus 120 days in RA. Fruit stored in 1.5% CO_2 + 1.0% O_2 produced less of each major volatile than fruit stored in either 5.0% CO_2 + 3.0% O_2 or RA. The capacity of the fruit to regenerate these volatiles in RA subsequent to 1.0% O_2 storage decreased with length of exposure to very low O_2 atmospheres. In 1979, the storage of apples for 320 days at 1.0% O_2 completely inhibited volatile production in subsequent RA (Fig. 3). The volatile regenerative capacity of fruit stored in 1.0% O_2 for 270 days was reduced to a very low level but was not completely suppressed in 1980 (Fig. 3).

Considerable yearly variations in volatile production capacity by apples was observed. The apples in the 1980 study produced about 10X the amount of ethyl butyrate, hexanal, ethanol and acetaldehyde than the fruit used in the 1979 study. In addition the fruit used in the 1980 study retained the ability to produce ethyl butyrate and ethanol immediately after removal at 180 and 270 days from 1.0% O_2 atmosphere (Fig. 3).

DISCUSSION

PREVIOUS STUDIES (Fidler, 1933; Thomas, 1929) report the accumulation of ethanol and acetaldehyde in response to anaerobic atmospheres and advancing apple senescence. Other workers (Anderson, 1967; Workman, 1963) report alcoholic flavors in apples stored in 1.0% O_2, while Blanpied et al. (1968) employed O_2 atmospheres of 1.0% or less to enhance ethanol development in McIntosh apples.

The present study indicated that storage of McIntosh apples in both 3.0% and 1.0% O_2 atmospheres suppressed the ability of the fruit to produce ethanol and acetaldehyde in a headspace volume, which is consistent with the observation of Furlong (1961) who reports that ethyl alcohol

levels in Cox's Orange Pippin apples declines in 3.0% O_2 atmospheres. However, fruit deprived of O_2 will generate ethanol and acetaldehyde rapidly (Thomas, 1929) and ethanol accumulation can be reversed by immediate aeration (North and Cockburn, 1975). The stimulation of ethanol production observed by Blanpied et al. (1968) in low O_2 atmospheres may have resulted from injured tissue and may not be directly attributable to the low O_2 atmospheres. The present evidence supported the view of Thomas (1929) and Smagula and Bramlage (1977) that acetaldehyde and ethanol accumulation may result from tissue disorganization and needs not be a causative agent to cellular disruption.

The suppression of volatile evolution by very low O_2 atmospheres was not as great in 1980 as that observed in the presvious two years. The fruit in 1980 demonstrated a 10-fold increase in their capacity to produce the measured volatiles. The apples used in the 1980 study were from various sources but one replicate was from the same source as that used in the 1978 and 1979 studies. This replicate was similar in most respects to the other four replicates in the 1980 study.

McIntosh apples were shown to be capable of regenerating the ethyl butyrate and hexanal in RA following storage in MA without excessive production of ethanol or acetaldehyde observed by Workman (1963), Anderson (1967), Blanpied et al. (1968). Conventional MA was shown to stimulate ethyl butyrate and hexanal production in subsequent RA storage in comparison with continuous RA, whereas very low O_2 atmospheres suppressed development of all volatiles in subsequent RA. The residual suppression of volatile evolution by very low O_2 atmospheres paralleled the metabolic suppression observed by Lidster et al. (1983) and suggested that a metabolic block is imposed by storage in 1.0% O_2 atmospheres. The residual metabolic inhibition imposed by 1.0% O_2 atmospheres may inhibit alcohol formation in particular which Knee an Hatfield (1981) consider to be the precursor to the esters and aldehydes responsible for apple flavors.

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Influence of Calcium Treatment on 'Golden Delicious' Apple Quality

S. R. DRAKE and S. E. SPAYD

―――――――――ABSTRACT―――――――――

The use of a $CaCl_2$ treatment in the storage of apples improved the keeping quality of fresh apples in prolonged cold storage and did not deter from the quality of the processed product. The fresh market apples in controlled atmosphere storage were firmer with more ascorbic acid than $CaCl_2$ treated apples in standard cold storage. However, fresh market $CaCl_2$ treated apples had less green color, were firmer, with a high titratable acidity and ascorbic acid content, than untreated apples in standard cold storage. The processed products made from $CaCl_2$ treated apples had better color and firmness than those made with apples from standard cold storage and better consistency and less weep than those made with apples from controlled atmosphere storage.

INTRODUCTION

IN RECENT YEARS the production of 'Golden Delicious' apples has increased steadily and production has surpassed early seasonal demand. To sell this crop 'Golden Delicious' apples must be stored for extended periods of several months. 'Golden Delicious' apples are not adapted to prolonged cold storage due to rapid deterioration (Tukey, 1976). To overcome this storage problem, most 'Golden Delicious' apples are subjected to controlled atmosphere (CA) storage. The effects of CA storage on fresh and processed apple quality have been documented (Anderson and Penny, 1973; Drake et al., 1979; Liu, 1971). There are some problems associated with CA storage with regard to flavor, aroma and color (Tukey, 1976).

The use of calcium dips to overcome the deterioration of apples in storage has been suggested (Batts and Bramlage, 1977; Blanpied, 1981; Lee and Dewey, 1981; Poovaiah and Shekhar, 1978). The use of calcium has been shown (Poovaiah and Shekhar, 1978; Poovaiah et al., 1978) to increase fruit firmness and delay senescence. Calcium treatment of 'Golden Delicious' apples prior to cold storage may become a standard procedure in the future.

Apple growers in the Pacific Northwest presently sell most of their crop on the fresh market. Increased use of Washington apples through processing could result in a higher total net return to the fruit industry (O'Rourke et al., 1972). Presently there are few commercially calcium-treated apples available. If calcium treatment of apples prior to storage becomes an accepted procedure information on how calcium influences apple quality will be required. The purpose of this study was to determine how calcium treated apples in standard cold storage compare with untreated apples in standard cold storage and CA storage.

MATERIALS & METHODS

THIS STUDY was conducted in 1980 and 1981 using 'Golden Delicious' apples grown at the Washington State University Irrigated Agriculture Research and Extension Center (Prosser, WA). Four single tree plots were used in two successive years. The trees were uniform in size and vigor and bore similar crops loads. Immediately

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after harvest flesh color, firmness, soluble solids, pH, titratable acidity, moisture content and vitamin C were determined. Each replication was randomly divided into four lots. One lot was used immediately to manufacture applesauce and canned apple slices. The second lot was placed in commercial controlled atmosphere (CA) storage at 2–3% O_2 and 1–2% CO_2 at 1–2°C. The third lot was placed in standard cold storage at 1°C. The fourth lot of apples was placed in a pressure chamber and was covered with 3% $CaCl_2$. The chamber was sealed and pressurized at 5 p.s.i. for 8 min. The apples were removed from the pressure chamber, rinsed in H_2O, dried and placed in standard cold storage at 1°C. After 5 months the apples lots 2, 3, and 4 were removed from storage and manufactured into applesauce and canned apple slices.

Apples for sauce were sliced and immediately cooked for 5 min at 93.5°C. The cooked apple slices were then manufactured into sauce with a Langsenkamps pulper-finisher using a 1.25-cm screen. Soluble solids were adjusted to 17° Brix with dry sucrose. The sauce was heated to 83°C and filled hot into 303 x 406 cans. The sealed cans were processed for 10 min at 98°C and rapidly cooled. Apples for slices were peeled, sliced, and blanched at 93.5°C for 5 min. A weighed amount of fruit was placed into 303 x 406 cans or 1.5 mil polyethylene bags. The canned slices were covered with 20% sucrose syrup, sealed, processed for 10 min at 98°C and rapidly cooled. The bagged slices were placed in a blast freezer operating at −20°C. The canned and frozen products were stored 30 days before analysis.

Color of the whole apples was determined using an Agtron model E-5W. The color of the applesauce and the canned apple slices was determined with an Agtron model 300A and a Hunter Color and Color Difference Meter model D25-2 using a reference standard with values of "L" = 78.4, "a" = −2.3, and "b" = 22.8. A Food Technology Corp. Texture Test System equipped with a PTI penetration test set and a 1.1-cm probe was used to measure firmness of the whole apples. A CE-1 universal cell was used to measure the shear values for the sliced products. Shear values are reported as the amount of force required to shear 100g of sliced product. Soluble solids were determined using a digital refractometer. A glass electrode pH meter with an expanded scale was used to determine pH. Titratable acidity was determined by titrating to an end point of pH 8.2 with 0.1N NaOH and expressed as percent malic acid. Consistency of the applesauce was measured on a USDA flow sheet using prescribed procedures (USDA, 1974). Free liquor (weep) was determined by allowing 100g of sauce to drip through one layer of cheesecloth for 30 min. Drip for frozen apple slices was measured after slices had been allowed to thaw at ambient temperature (20°C) for 12 hr. Moisture and drained wts were determined by (AOAC, 1970) methods.

Polyphenoloxidase (PPO) was measured (Harel et al., 1965) and enzyme activity was calculated as the change in absorbance/min/ml and one unit of enzyme activity equals 0.001 absorbance untis/min/ml. Total browning of the stored apples was measured by noting the decrease of reflectance in the yellow mode on an Agtron model 500A reflectance spectrophotometer calibrated with 00 and 90 discs immediately after blending a portion of apple tissue. Agtron readings were taken at 2-min intervals. In all cases, readings had ceased to change after 12 min, so the 12 min value was taken as the end point and used as an indication of total browning. A randomized block design, consisting of four treatments and four replications over two years, was used for data analysis.

RESULTS & DISCUSSION

TYPE OF STORAGE influenced the quality of 'Golden Delicious' apples (Table 1). After 5 months in storage, regardless of the storage method, all apples were higher in Agtron E5W color than the fresh controls. Both the CA and $CaCl_2$ treated apples were similar and neither lost as much

green color as the untreated cold storage apples. After 5 months of storage the firmness of CA apples was similar to the firmness of fresh controls and both were much firmer than CaCl$_2$ treated apples, which were firmer than the untreated cold storage apples.

The pH of CA apples was lower than the pH of fresh apples and higher than that of CaCl$_2$ treated apples. Untreated apples stored for 5 months in standard cold storage had the highest pH and the lowest titratable acidity. CaCl$_2$ treated and CA apples were equal in acidity level and lower in acidity than fresh apples. Ascorbic acid content decreased as much as 26% for untreated apples in cold storage, 24% for CaCl$_2$ treated apples and 18% for CA apples.

Soluble solids and moisture content were similar for all treatments, regardless of storage treatment.

Hunter 'L' values for applesauce were similar for sauce made from CA, CaCl$_2$ treated and fresh control apples but were lower for sauce from untreated cold storage apples indicating, a darker, less desirable product (Table 2). Sauce from CA and CaCl$_2$ treated apples were redder (higher Hunter 'a' values), than the sauces from the fresh control apples or untreated cold storage apples, which were similar in red color. Less yellow color (lower Hunter 'b' values) was evident for the sauce from the control, untreated cold stored and CA apples, when compared to the CaCl$_2$ treated apples, which had the highest yellow colored applesauce.

The USDA flow rate or consistency was best for the sauce made from the fresh control apples. The flow rates for the sauces from the untreated cold storage and CaCl$_2$ treated apples were significantly higher than the flow rate for the sauce from fresh apples, but were still within an acceptable grade A level. The flow rate for the sauce from CA apples (6.6) was in excess of the amount (6.5) allowed in USDA standards for grade A sauce. This would limit the sauce from CA apples to a maximum of USDA grade B. The free liquor for the sauce from the CA apples was also in excess of the amount (0.7 cm) in USDA standards for grade A, limiting the sauce from CA apples to a maximum of USDA grade B. This excess free liquor can readily be seen in the excessive weep (105 ml) from the sauce of CA apples as compared to the weep from other treatments. The weep for the sauce from untreated cold storage (70.8 ml) and CaCl$_2$ treated (83.6 ml) apples was similar, but still higher

than the weep (23.8 ml) for sauce from the fresh control apples. The amounts of water and soluble solids present in the sauces from the stored apples were similar, so excess free liquor and weep displayed by the sauce from CA apples was not related to these factors. The water content of the sauce from fresh control apples was lower than the water content of the sauces from the stored treatments and this may partly explain the very low weep of the sauce from fresh control apples. The pH and titratable acidity of the sauces from fresh control and CA apples were similar. The pH and titratable acidity of the sauces from the untreated cold storage and CaCl$_2$ treated apples were similar, but higher in pH and lower in acidity, than the sauces from fresh control and CA apples.

Apple slices produced from fresh control apples displayed the lightest color, i.e. the highest Hunter 'L' value (Table 3). This high Hunter 'L' value was not significantly different from the values for slices from CA or CaCl$_2$ treated apples, but was significantly higher than the value for slices made from untreated cold storage apples. Apple slices from fresh control apples and CA apples were much redder (higher Hunter 'a' values) than either the slices from the untreated cold storage apples or CaCl$_2$ treated apples, which were similar in red color. Apple slices from the fresh control, CA and CaCl$_2$ treated apples displayed a higher yellow color (increased Hunter 'b' values) than the slices from untreated cold stored apples.

Increases in drained weight were evident for all the treatments. Apple slices from untreated cold stored and CA apples had a larger increase in drained weight than did apple slices from fresh control or the CaCl$_2$ treated apples, which were similar. Shear values (firmness), in Newtons, were highest for apple slices from CA stored apples. CaCl$_2$ treated apple slices displayed a shear value similar to that for fresh control apples, and higher than the values for apple slices from the untreated cold stored apples. The CA, CaCl$_2$ treated or fresh control apples produced firm apple slices with little sloughing of the product. The minimal sloughing of the canned apple slices from the CA apples was evident in the low turbidity values obtained. The turbidity of the apple slices from the CaCl$_2$ treated or fresh control apples was much higher than either the CA or cold stored apples. This was evident even though the firmness or shear values

Table 1—Quality attributes of fresh 'Golden Delicious' apples as influenced by storage treatment

Storage treatment	Agtron color E-5W	Firmness	pH	Titratable acidity @ % malic	Vit. C mg/100g	Soluble solids %	H$_2$O %
Control[z]	46.8 c[v]	14.9 a	3.63 c	0.45 a	7.58 a	12.4 a	84.2 a
Cold stored[y]	55.9 a	12.0 c	4.20 a	0.23 c	5.60 c	12.9 a	84.4 a
CA[x]	51.0 b	15.7 a	3.70 bc	0.32 b	6.23 b	13.2 a	84.2 a
CaCl$_2$[w]	52.1 b	13.0 b	3.79 b	0.31 b	5.75 c	12.9 a	84.2 a

[z] Evaluated 1–2 days after harvest.
[y] Evaluated after 5 months in cold storage @ 1°C.
[x] Evaluated after 5 months in controlled atmosphere storage.
[w] Treated with CaCl$_2$ and evaluated after 5 months in cold storage @ 1°C.
[v] Means not sharing the same letter are significantly (P < 0.05) different from each other.

Table 2—Quality attributes of canned applesauce produced from 'Golden Delicious' apples as influenced by storage treatment

Storage treatment	Hunter color			Consistency USDA flow (cm)	Weep (ml)	Soluble solids %	H$_2$O %	pH	Titratable acidity @ % malic
	'L'	'a'	'b'						
Control[z]	53.5 a[v]	4.6 b	20.0 bc	3.9 c	23.8 c	17.3 a	80.0 b	3.6 b	0.26 a
Cold stored[y]	49.5 b	5.1 b	18.9 c	4.9 b	70.8 b	16.8 a	80.9 a	4.2 a	0.15 b
CA[x]	52.3 a	6.6 a	20.5 b	6.6 a	105.0 a	17.6 a	80.8 a	3.6 b	0.23 a
CaCl$_2$[w]	52.7 a	5.9 a	21.6 a	5.2 b	83.6 b	16.9 a	81.0 a	4.1 a	0.18 b

[z] Applesauce from apples after 1 wk storage @ 1°C.
[y] Applesauce from apples after 5 months in cold storage @ 1°C.
[x] Applesauce from apples after 5 months in controlled atmosphere storage.
[w] Applesauce from apples treated with CaCl$_2$ and stored for 5 months @ 1°C.
[v] Means not sharing the same letter are significantly (P < 0.05) different from each other.

Storage treatment	Hunter color			Increase in drained wt (%)	Shear (N)	Turbidity (NTU)
	'L'	'a'	'b'			
Control[z]	66.6 a[v]	5.1 a	24.3 a	11.2 b	117.4 b	52.5 a
Cold Stored[y]	63.2 b	2.6 b	21.2 b	14.8 a	64.9 c	34.3 b
CA[x]	64.6 ab	4.1 a	23.7 a	14.2 a	177.0 a	36.4 b
CaCl₂[w]	65.0 ab	2.3 b	23.6 a	10.2 b	110.8 b	57.3 a

[z] Apple slices from apples after 1 wk in storage @ 1°C.
[y] Apple slices from apples after 5 months in cold storage @ 1°C.
[x] Apple slices from apples after 5 months in controlled atmosphere storage.
[w] Apple slices from apples treated with $CaCl_2$ and stored for 5 months @ 1°C.
[v] Means not sharing the same letter are significantly ($P < 0.05$) different from each other.

for the cold stored apples was much lower than all other treatments. Soluble PPO activity and Agtron color correlated extremely well (0.975**). The use of calcium in this study may have retarded PPO activity which reduced browning (determined as change in Agtron color of macerated fruit with time) when $CaCl_2$ treated apples were compared with untreated cold storage apples.

The advantages and disadvantages of CA storage on the quality of fresh (Anderson and Penny, 1973) and processed (Drake et al., 1979) apples have been documented. There is little doubt that CA storage of apples greatly improves the keeping quality of the fresh product over standard cold storage. With regard to processed products CA stored apples produce a product with more yellow color and acid than products from standard cold stored apples, but CA apples yield less processed product. The use of calcium on fresh market apples prior to cold storage in the form of sprays, dips, etc., has been documented by several researchers. Batts and Bramlage (1977) determined that a $CaCl_2$ dip treatment reduced softening and delayed breakdown of 'McIntosh', but did not influence the quality of 'Baldwin' or 'Cortland' apples. Hydrocooling of 'Jonathan' apples with refrigerated $CaCl_2$ has offered a practical solution to the control of storage disorders (Lee and Dewey, 1981). Poovaiah et al., (1978) found that pressure or vacuum infiltration of 'Golden Delicious' apples with $CaCl_2$ solution was effective in controlling fruit firmness, bitter pit and senescence.

In this study, as in previous work, the use of $CaCl_2$ helped to maintain the firmness of 'Golden Delicious' apples when compared to apples from standard cold storage. The calcium content of the $CaCl_2$ treated apples averaged 154.8 ppm greater than that of apples from untreated cold storage after 5 months. It may be possible with more uniform calcium infusion, by the use of vacuum, that firmness of $CaCl_2$ treated apples would be similar in firmness to CA apples. The $CaCl_2$ treated fresh apples did not respire at the same rate as the untreated cold storage apples as indicated by lower pH and higher titratable acidity.

The use of calcium in the storage of apples improves the keeping quality of the fresh product and does not deter from the quality the processed products manufactured from apples after prolonged storage. The quality of fresh market apples in CA storage was superior to that of $CaCl_2$ treated apples. However, the fresh market quality of $CaCl_2$ treated apples was much improved over untreated apples. Processed product quality of $CaCl_2$ treated apples was better than that of CA or cold stored apples with better color and shear than cold stored apples and better consistency and less weep than CA stored apples.

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Sweetened Mango Purees Preserved by Canning and Freezing

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ABSTRACT

Mango fruit (*Mangifera indica* L., Kent variety) at canning ripeness was processed as sweetened puree of 42–43° Brix soluble solids by canning and freezing. Sucrose and high fructose corn syrups in various combinations were used as sweeteners. Processed purees, diluted with three parts of water, produced mango nectars of 13% soluble solids for evaluation of chemical and sensory quality. The frozen mango purees contained slightly more ascorbic acid than the canned products. Sucrose and high fructose corn syrups may be used interchangeably as sweeteners. Panel members were not able to distinguish differences in preference for nectars even though some difference in sugar components among them were detected by high performance liquid chromatography.

INTRODUCTION

MANGO (*Mangifera indica* L.) is the second largest tropical crop next only to banana, in terms of production and acreage. It is relished for its succulence, exotic flavor and delicious taste (Subramanyam et al., 1975).

In spite of its excellence, mango has not been developed as a commercial and export crop because of its poor stability during storage and irregular productivity. It still lacks recognition in the marketplace and tends to lose its flavor during thermal processing (Doughtery, 1971; Brekke et al., 1968; Varshney and Barhate, 1978).

Preservation of mango fruit remains at the "home canning" stage in most countries because of difficulty in removing the skin and seed. Industrial utilization of mangoes has been limited due to lack of adequate procedures for extracting the pulp. Peeling of the fruit and removal of the pulp by hand is not commercially feasible because of the high labor requirements, resulting in high production costs (Sanchez-Nieva et al., 1959). Ripen mangoes may be peeled by freezing and then scraping away the disintegrated skin (Czyhrinciw, 1969). They can also be peeled by use of lye and steam Brekke et al., 1975) and by a combination of mechanical devices such as brush pulpers (Benero and Rodriguez, 1971), special centrifuges (Angara et al., 1969) and padale pulpers (Brekke et al., 1975).

Although there are many ways to convert mango into nonperishable products, mango puree offers definite advantage because it requires less labor and it can be used for remanufacture of other mango products (Luh, 1980). The use of sucrose and high fructose corn syrups as sweeteners in foods has been reported by Cantor (1975) and in kiwi nectar by Wildman and Luh (1981).

Mango puree can be used for remanufacture into jellies, jams, beverages, dairy and bakery products (Brekke et al., 1975). Nanjundaswamy et al., (1976) recommended a processing time of 5 min at 100°C for canning mango pulp with an initial temperature of 76.7–79.4°C. The pulp was filled into cans, sealed, inverted for 5 min to sterilize the lids, and then cooled in a water bath. Shrikhande et al., (1976) reported on heat processing of acidified mango puree in high-density polyethylene containers of 6 kg

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capacity. For frozen mango puree, it is necessary to raise the temperature of the puree to 90.6–93°C in a plate heat exchanger, hold at that temperature for 2 min, and then rapidly cool to 32.2– 37.8°C. The heat treatment serves to inactivate catalase and other enzymes (Brekke et al., 1975).

This paper reports the evaluation of two processing methods, hot-fill canning and freezing of mango puree and mango puree sweetened with various combinations of sucrose and high fructose corn syrup.

MATERIALS & METHODS

Mango

Two hundred pounds of Kent mango grown in Sinaloa, Mexico, in the summer of 1979 were supplied by a local fruit dealer. The mangoes were uniform in size and maturity. The magnoes were stored for 4 days at 15.6°C and 90% relative humidity and ripened for 3 days at 26.7°C and 90% relative humidity before processing (Hatton et al., 1965).

Processing

The ripened mangoes were washed with 10 ppm chlorinated water and hand-peeled. The pulp was scraped from the peel and cheeks and pieces were dipped into a cold 20% sucrose syrup containing 0.5% ascorbic acid, drained and passed through a Brown Screw finisher with a 0.033 in screen. For hot-fill canning, the puree was mixed with 80° Brix syrups made of various sweetener types at 20°C. The puree mixture was then passed through an APV junior paraflow heat exchanger to raise the temperature to 90.6–93.3°C and then held for 2 min. After that, the hot puree was canned in 6 oz cans, sealed under steam injection, held for 5 min and then cooled in an ice bath. The canned products were stored at 1°C.

For freezing, the mango puree was passed through a heat exchanger at 90.6–93.3°C, held for 2 min and then rapidly cooled to 32.3–37.8°C. The cooled puree was formulated with various sweetener types to <2.5° Brix. The puree mixture was canned, sealed and frozen in a Conrad blast freezer using Freon-22 and Freon-13 at –67.8°C for 3 hr. The frozen puree was stored at –17.8°C.

Sweetener types

The types of 80° Brix sweeteners used in this investigation are shown in Table 1.

Sugar analyses by high performance liquid chromatography

Sugars in fresh mango were determined according to the method described by Palmer and Brandes (1974). A 5-g sample of mango pulp was weighed into an Erlenmeyer flask and extracted with occasional stirring under reflux at 75–80°C for 1 hr with 75 ml of 80% ethanol and a small quantity of $CaCO_3$. The mixture was cooled, filtered and evaporated to dryness in a rotary vacuum evaporator at 40°C. The residue was washed repeatedly with hot water to dissolve the sugars and the combined washings were made up to 100 ml. A small portion of this solution was filtered through 0.5 µm celotate filter. Unless stated otherwise, the volume of injection was 20 µl.

For the sweetened mango puree, the puree was diluted with 4 volumes of deionized water. The product was centrifuged for 15 min at 15,000 rpm after neutralizing with $CaCO_3$. A small portion of the clear solution was filtered through a 0.5 µm celotate filter. Samples of 3–5 µl were injected for HPLC analyses.

For the processed plain mango puree, the same procedure was followed but without dilution. Standard solutions of 1, 2, 3, 4 and 5% were prepared for glucose, fructose and sucrose. A calibration curve was obtained for each of the three sugars.

Sucrose, glucose and fructose were determined by high performance liquid chromatography (HPLC) using a Waters Associates Model 6000A solvent delivery system with a differential refractometer Model R401. A 30 cm x 4 mm i.d. stainless steel μ-Bondapak-carbohydrate column (Water Associates) and precolumn packed with Co:Pell Pac (Whatman) were used. The flow rate was 1.5 ml/min and the solvent was acetonitrile (Burdick and Jackson) and water *85:15. The acetonitrile was previously filtered through 0.5 μm floropore and the water filtered through a Millipore Q system. The solvent mixture was degassed under vacuum for 2 min.

Total ascorbic acid

The method for the determination of reduced, dehydro- and total ascorbic acid as reported by Schaffert and Kingsley (1955) was used with slight modifications. Twenty grams of mango puree were mixed with 980 ml of 0.5% oxalic acid. To 20 ml of this solution was added 2 ml of 10% thiourea and 3g active carbon. The mixture was shaken for 1 min and then filtered through a Whatman No. 42 filter paper. Four portions of the filtrate were transferred into three test tubes. One tube was set aside as blank. To each of the remaining tubes was added 1 ml of 2%, 2,4-dinitrophenylhydrazine (DNPH) solution. All the tubes were placed in a water bath at 37°C for exactly 3 hr. The tubes were then cooled in an ice bath. While the tubes were kept in the ice bath, 5 ml of 85% H_2SO_4 were added slowly to each one. Then 1 ml of DNPH solution was added to the blank. The content of the tubes was mixed thoroughly and allowed to stand for 30 min at r.t. Percent transmission was read at 515 nm with the respective blank in place using a Perkin-Elmer Coleman 575 spectrophotometer. Results were expressed as mg total ascorbic acid/100g of mango puree after referring each individual reading to a standard curve. The same standard curve was used for dehydro-ascorbic acid because the method was the same except for Norit treatment. The difference between the total ascorbic acid and dehydro-ascorbic acid is expressed as reduced ascorbic acid.

Color

The color of the mango puree was measured with a Hunter automatic color difference meter, Model D25D2. A light yellow tile with the following parameters: L = 78.2; a = −2.2 and b = 21.7 was used as a reference plate.

Total pectins

The Versene-pectinase-carbazole method described by McCready and McComb (1952) was used.

Viscosity

The consistency of the mango puree was determined at 20°C using a Brookfield Model RVT viscometer. A No. 3 spindle was used at speeds of 5, 10, 20, 50 and 100 rpm. The torque readings were converted into Brookfield dial readings and plotted against the speed in rpm on a log-log scale graph paper.

Total solids

The AOAC (1975) vacuum oven method for total solids in fresh and canned fruits was utilized.

Soluble solids

The mango puree was centrifuged for 10 min at 10,000 rpm in a Sorvall centrifuge. A drop of the clear solution was placed on the prism of a Zeiss-Opton refractometer. The results are expressed as degrees Brix at 20°C.

Total acidity

Twenty grams of mango puree were mixed with 200 ml of distilled water and titrated with 0.1N NaOH solution to pH 8.0. The results are reported as percent citric acid. A Corning Model 12 research pH meter was used for pH measurement.

Sensory evaluation

Paired comparison test for sweetness and preference was utilized. Twenty-six judges participated in a first session and 28 judges in a second session. For statistical analysis, the judges who tasted once were grouped togehter. The judges were asked to designate the sample within each pair as to which one was more sweet and in another set of samples to designate the sample in each pair that was preferred and to express the reason for its preference. The sweetened mango purees were diluted with three volumes of distilled water and served at room temperature (21–23°C) in 2 oz portions in paper cups. Two separated trays with four pairs of diluted samples were presented to each judge in isolated booths under red light. The first tray was utilized to designate the sweetest sample in each of four pairs and the second one was utilized to designate the most preferred sample in each of another four pairs. The samples were served randomly utilizing a three-digit key number to designate each sample. The tests were performed from 10–12 a.m. and 2–4 p.m. One-tailed binomial table for the sweetness test and two-tailed binomial table (Geng and Hills, 1980) for the preference test were used to establish statistical significance.

RESULTS & DISCUSSION

Sugars

Results of sugar distribution in the fresh mango puree are presented in Table 2. Fructose, glucose and sucrose were found to be present in the fresh mango fruit. Sucrose was the major sugar in fresh mango followed by fructose and glucose. On the contrary, fructose (7.85%) was the major sugar, followed by glucose (4.87%) and sucrose (1.90%) in kiwifruit as was reported by El-Zalaky and Luh (1980–1981). The order of elution of the sugars was fructoe, glucose and sucrose (Fig. 1).

Ascorbic acid

Results of the ascorbic acid content of the mango products are shown in Table 3. Reduced ascorbic acid was esti-

Table 1—Percentage distribution of sucrose, high fructose, corn syrup solids and citric acid in the syrups (80° Brix) used in freezing and canning of mango puree

Syrup type		Sucrose (%)	High fructose corn syrup solids[a] (%)	Citric acid (%)
Freezing	Canning			
F1	C1	100	0	0.6
F2	C2	0	100	0.6
F2	C3	67	22	0.6
F4	C4	33	67	0.6

[a] The high fructose corn syrup contained 42% fructose in the carbohydrate fraction. The syrup contained 71% total solids.

Table 2—Sugar composition of mango products[a]

Sample	Fraction in the total sugars		
	Fructose (%)	Glucose (%)	Sucrose (%)
Fresh mango (20.6% total solids)	23.8	7.2	69.0
Frozen mango purees			
Plain	21.6	5.9	72.5
Syrup type			
F1 Sucrose[b]	4.6	1.8	93.6
F2 High Fructose Corn Syrup	75.0	9.5	15.4
F3 2/3 Sucrose + 1/3 High Fructose corn syrup	28.0	4.0	68.0
F4 1/3 Sucrose + 2/3 High Fructose corn syrup	54.0	6.6	39.4
Canned mango puree			
Plain	23.3	6.3	70.4
Syrup type			
C1 Sucrose	7.9	4.5	87.6
C2 High Fructose Corn Syrup	77.2	9.4	13.4
C3 2/3 Sucrose + 1/3 High Fructose corn syrup	31.2	7.3	61.5
C4 1/3 Sucrose + 2/3 High Fructose corn syrup	55.0	10.0	35.0

[a] Analysed by High Performance Liquid Chromatography
[b] See Table 1 for syrup types

mated by difference. It was found that the frozen and canned samples were significantly different ($p < 0.01$) in total and reduced ascorbic acid content. In general, more ascorbic acid and less dehydroascorbic acid was found in the frozen samples than in the fresh mango because the peeled mangoes were dipped in 0.5% ascorbic acid solution.

Color

Color data of the mango products are shown in Table 4. The L value for products from both processes are significantly different ($p < 0.05$) while a and b values are not significantly different. Fresh mangoes had higher L, a and b

scores than the frozen and canned mango puree. The frozen mango puree is closer in color to the fresh mango than the canned mango puree which showed lower L, a and b values.

Pectin and viscosity

Data showing total pectin of mango products are presented in Table 5. Total pectins in the frozen and canned samples were not significantly different ($p > 0.05$). Total pectin present in the plain puree was higher because it contained more mango pulp than the sweetened mango puree. Viscosity is related to the content and the type of pectins present in the mango puree (Saeed et al., 1975). The viscosity of the mango products is shown in Table 6. The viscosity values at different spindle velocities were not significantly different ($p > 0.05$) for the frozen and canned mango puree.

Total and soluble solids

Results of total and soluble solids of the samples are shown in Table 7. Total and soluble solids contents of the
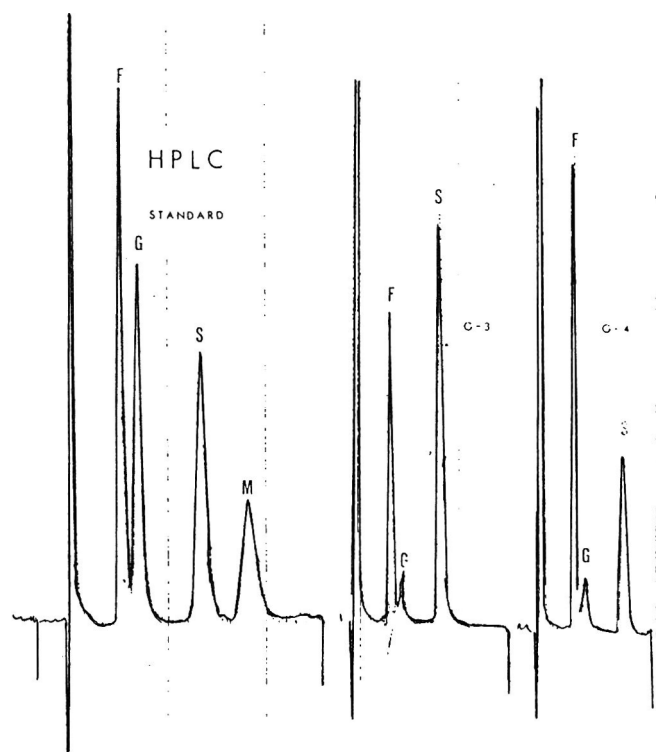
Fig. 1—Elution order of sugars in canned sweetened mango puree by HPLC. Left: authentic sugars, F (fructose), G (glucose), and S (sucrose). Middle: canned mango puree sweetened with syrup C-3 (Sucrose: HFCS solids, 67:33). Right: canned mango puree sweetened with C-4 syrup (sucrose: HFCS solids, 33:67).

Table 4—Color measurement of mango products

| Sample | Hunter color readings | | |
	L	a	b
Fresh mango	52.3	15.6	34.3
Frozen mango puree			
Plain	40.1	2.0	24.8
Syrup type[a]			
F1 Sucrose	22.8	−0.6	13.8
F2 High Fructose Corn Syrup	23.2	−0.8	13.9
F3 2/3 Sucrose + 1/3 High Fructose corn syrup	23.0	−0.7	13.9
F4 1/3 Sucrose + 2/3 High Fructose corn syrup	24.5	−0.7	14.8
Canned mango puree			
Plain	39.4	0.5	24.0
Syrup type			
C1 Sucrose	22.4	−0.8	13.4
C2 High Fructose Corn Syrup	21.8	−0.9	13.0
C3 2/3 Sucrose + 1/3 High Fructose corn syrup	21.1	−0.8	12.6
C4 1/3 Sucrose + 2/3 High Fructose corn syrup	21.0	−0.8	12.5

[a] See Table 1 for details

Table 3—Vitamin C content of mango products

Sample	Total ascorbic acid (mg/100g)	Dehydroascorbic acid (mg/100g)	Reduced ascorbic[a] acid (mg/100g)
Fresh mango	45.6	18.7	26.9
Frozen mango puree			
Plain	106.3	22.9	83.4
Syrup type[b]			
F1 Sucrose	63.4	20.6	42.8
F2 High Fructose Corn Syrup	65.3	18.7	46.6
F3 2/3 Sucrose + 1/3 High Fructose corn syrup	64.9	16.9	43.0
F4 1/3 Sucrose + 2/3 High Fructose corn syrup	70.8	19.4	51.4
Canned mango purees			
Plain	95.6	24.4	71.2
Syrup type			
C1 Sucrose	56.3	21.4	34.9
C2 High Fructose Corn Syrup	54.6	26.6	28.0
C3 2/3 Sucrose + 1/3 High Fructose corn syrup	53.7	20.6	33.1
C4 1/3 Sucrose + 2/3 High Fructose corn syrup	54.6	26.0	28.6

[a] Estimated by difference
[b] See Table 1 for syrup types in making the sweetened mango puree

frozen and canned samples were not significantly different at the 95 probability level (p > 0.05). Less total and soluble solids were found in the unsweetened puree than the fresh mango. This can be explained by the leaking of solids into the dipping solution. More variation was evidenced in total and soluble solid content in the canned samples because the portions treated through the heat exchanger were relatively small as compared with the whole portion of puree passing through the heat exchanger in preparing the frozen samples.

pH and titratable acidity

pH and titratable acidity data relating to the mango products are presented in Table 8. Mango is considered an acid food although pH values of 4.5 or slightly above have been reported (Bruno and Goldberg 1963). The citric acid (0.6%) was added in the processing not to get an acid food but to get an optimum sensorial sugar/acid balance after diluting the sweetened puree with three parts of water. Results indicated that the pH was essentially the same for the frozen and canned samples. The difference

between the pH of the fresh mango and that of the plain processed mango puree may be explained by the formation of pectic acid from pectin by activation of pectin esterase, and the ascorbic acid used in the dipping solution. The differences in pH and titratable acidity between the plain processed puree and the sweetened processed puree can be explained by the citric acid added to the sweetened samples.

Sensory evaluation

Results of paired comparison test for sweetness and preference among mango nectars are shown in Table 9. Only the canned mango puree sweetened with high fructose corn syrup was preferred significantly (p < 0.05) to its counterpart, frozen mango puree. The phenomenon may be explained by enzymic changes occuring which may develop some off flavor during the thawing and serving period in the frozen product. On the other hand, the canned mango

Table 5—Total pectins in mango products

Sample	Total Pectins (mg/100g)
Fresh mango	342
Frozen mango puree	
Plain	205
Syrup type[a]	
F1 Sucrose	151
F2 High Fructose Corn Syrup	134
F3 2/3 Sucrose + 1/3 High Fructose corn syrup	142
F4 1/3 Sucrose + 2/3 High Fructose corn syrup	159
Canned mango puree	
Plain	205
Syrup type[a]	
C1 Sucrose	175
C2 High Fructose Corn Syrup	157
C3 2/3 Sucrose + 1/3 High Fructose corn syrup	150
C4 1/3 Sucrose + 2/3 High Fructose corn syrup	158
L.S.D. (p = 0.05)	25

[a] See Table 1 for details

Table 6—Consistency of mango products in Brookfield dial readings at 20°C[a]

	rpm			
Sample	10	20	50	100
Frozen mango puree				
Plain	3560	1962	950	560
Syrup type[b]				
F1 Sucrose	1525	990	586	388
F2 High Fructose Corn Syrup	1535	990	573	384
F3 2/3 Sucrose + 1/3 High Fructose corn syrup	1225	810	494	335
F4 1/3 Sucrose + 2/3 High Fructose corn syrup	1795	1125	642	423
Canned mango puree				
Plain	3610	2088	1053	639
Syrup type				
C1 Sucrose	1630	1015	581	381
C2 High Fructose Corn Syrup	1760	1112	625	410
C3 2/3 Sucrose + 1/3 High Fructose corn syrup	1510	992	577	380
C4 1/3 Sucrose + 2/3 High Fructose corn syrup	1770	1115	627	410

[a] Viscosity expressed in Brookfield Viscometer RVT dial readings, using Spindle No. 3
[b] See Table 1 for syrup type

Table 7—Total solids and soluble solids in mango products

Sample	Total solids (%)	Soluble solids (%)
Fresh mango	20.6	18.9
Frozen mango puree		
Plain	16.6	15.4
Syrup type[a]		
F1 Sucrose	45.6	42.6
F2 High Fructose Corn Syrup	45.6	42.7
F3 2/3 Sucrose + 1/3 High Fructose corn syrup	46.6	42.7
F4 1/3 Sucrose + 2/3 High Fructose corn syrup	44.2	42.5
Canning mango puree		
Plain	16.7	15.4
Syrup type[a]		
C1 Sucrose	44.9	42.6
C2 High Fructose Corn Syrup	45.3	42.6
C3 2/3 Sucrose + 1/3 High Fructose corn syrup	46.4	42.7
C4 1/3 Sucrose + 2/3 High Fructose corn syrup	44.6	42.6

[a] See Table 1 for details

Table 8—pH and titratable acidity in mango products

Sample	pH	Titratable Acidity as Citric Acid (%)
Fresh mango	3.96	0.447
Frozen mango puree		
Plain	3.51	0.543
Syrup type[a]		
F1 Sucrose	2.71	0.898
F2 High Fructose Corn Syrup	2.71	0.894
F3 2/3 Sucrose + 1/3 High Fructose corn syrup	2.72	0.885
F4 1/3 Sucrose + 2/3 High Fructose corn syrup	2.72	0.890
Canned mango puree		
Plain	3.51	0.545
Syrup type[a]		
C1 Sucrose	2.72	0.888
C2 High Fructose Corn Syrup	2.72	0.879
C3 2/3 Sucrose + 1/3 High Fructose corn syrup	2.73	0.866
C4 1/3 Sucrose + 2/3 High Fructose corn syrup	2.71	0.889

[a] See Table 1 for details

Table 9—Paired comparison test for sweetness and preference of frozen and canned mango nectars[a]

Syrup type	Type of nectar	Sweetness 1[b]	Sweetness 2	Preference 1	Preference 2
Sucrose					
	Frozen Mango Puree	19	12	20	9
	Canned Mango Puree	17	5	16	8
High Fructose Corn Syrup					
	Frozen Mango Puree	15	7	11	2
	Canned Mango Puree	21	10	25*	15*
2/3 Sucrose + 1/3 High Fructose corn syrup					
	Frozen Mango Puree	16	9	16	7
	Canned Mango Puree	20	8	20	10
1/3 Sucrose + 2/3 High Fructose corn syrup					
	Frozen Mango Puree	17	7	18	7
	Canned Mango Puree	19	10	18	10

[a] The sweetened mango purees of 42.6° Brix soluble solids were diluted with three volumes of distilled water to form nectars and served at 21—23°C in paper cups
[b] Set 1: 18 Judges participated in two Sessions
   Set 2: 17 Judges participated in one session
[c] The number represents panel members reporting the relative sweetness and preference for the nectars made from frozen or canned mango puree
* Significant at 95% probability level as evaluated by the two tailed binomial test (Geng and Hills, 1980)

purees were heat processed and were less liable to enzymic changes during preparation and serving. Judges were not able to distinguish differences in sweetness in the frozen and canned mango puree even though different sugar composition was detected by HPLC. Also there was no difference in preference between the frozen and canned mango puree by the tasting panel.

CONCLUSION

RIPE MANGO FRUIT can be processed as sweetened purees of 42—43% soluble solids by canning and freezing. The products were diluted with three volumes of water to form mango nectars prior to the panel test. Sucrose and high fructose corn syrups can be used interchangeably as sweeteners. The panel members showed no difference in preference for the nectars even though some difference in sugar components among them were detected by high performance liquid chromatography.

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Anthocyanin Degradation in the Presence of Furfural and 5-Hydroxymethylfurfural

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ABSTRACT

The influence of furfural (F) and 5-hydroxymethylfurfural (HMF) on degradation of cyanidin-3-glucoside (CG) was investigated in blackberry juice and in a citrate buffer model solution (pH 3,45) at 24°, 50° and 70°C. Presence of 0.012 M F or HMF accelerated pigment degradation. The acceleration was directly temperature-dependent, more pronounced in fruit juice, and considerably decreased in nitrogen. CG and HMF disappearance – but not the interaction between these compounds – followed the first-order reaction kinetics. The influence of formaldehyde (Fa) acetaldehyde (Aa) and benzaldehyde (Ba) on the stability of CG was investigated in blackberry juice. CG degradation effect of non-furane aldehydes (0.012 M) was Fa > Aa > Ba. CG and cyanidine (C) reactivities were studied by condensation with F and examination of molecular electrone properties (cyanidine, keto-pseudobase, anhydrobase, chalcone). Furfural did not react with CG but it did react with C. Electron charge distribution points to anhydrobase as the tautomeric form of C most reactive to F. Possible mechanisms of C decomposition in presence of F are proposed and discussed.

INTRODUCTION

SINCE ANTHOCYANINS are the principal natural colorants in many fruits, stability of these pigments during processing will largely determine the extent of the preservation of the original characteristic color in the final product.

Chemically, anthocyanins (2-phenylbenzopyrrilium or flavylium salts) are unstable compounds, whether in cellular juice or in the aqueous model solutions. Anthocyanins easily break down, yielding undesirable brown-colored substances. Such changes of the characteristic pigment coloration (red and blue shades) then cause change of the product color itself.

The very same conditions favoring anthocyanin degradation during processing (e.g., high temperature, increased concentration) also give rise to the formation of furfural and 5-hydroxymethylfurfural (HMF). Furyl aldehydes can be formed by sugar degradation (furfural mostly from aldopentoses, and HMF from ketohexoses), as well as by the transformation of other compounds, e.g. ascorbic acid (Sloan et al., 1969) or polyuronic acids (Starr and Francis, 1968).

Regarding possible influence of furfural and HMF on the stability of anthocyanin pigments, the researchers' opinions differ. HMF level is often used as a quality indicator in fruit products; however, it does not necessarily follow that its presence affects pigment degradation. Meschter (1953), Markakis et al. (1957), Tinsley and Bockian (1960), Lovrić (1962), Lovrić et al. (1968), Daravingas and Cain (1968) and Segal and Negutz (9169) found that furfural and HMF accelerate the degradation of anthocyanins, even at low HMF concentrations (e.g., 30–50 mg/kg, Segal and Negutz, 1969), while Triffiro (1965), Poretta et al. (1966), Casoli and Dall'Agilio (1967) and Calvi and Francis (1978) all

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found that low HMF concentrations which typically occur in fruit products during processing and storage could not have an effect on the pigment degradation.

Thus, the aspect of anthocyanin degradation in the presence of furfural and HMF remains very much an open research area.

The impetus for our study comes from the work of Tinsley and Bockian (1960). These authors followed the studies of Wizinger and Luthiger (1953) and Blackburn et al. (1957) and pointed to a certain possible degradation mechanism of pelargonidin-3-glucoside in the presence of such active components as furfural and HMF.

The purpose of the present study is to investigate the degradation kinetics of cyanidin-3-glucoside (a principal pigment of blackberries), in the natural blackberry juice and in the model system (an aqueous citrate buffer solution) in the presence of furfural and HMF at various temperatures; and also to establish the pigment reactivity through two different approaches: (a) condensation tests of cyanidin-3-glucoside and cyanidine with furfural; and (b) examination of electron properties of isolated molecules of cyanidin-cation, cyanidin-keto-pseudobase, cyanidin-anhydrobase and chalcone.

In addition, we wanted to test the influence of formaldehyde, acetaldehyde and benzaldehyde on the stability of cyanidin-3-glucoside in blackberry juice.

From the above results, we attempted to examine and propose the possible mechanism(s) of anthocyanin breakdown in presence of furyl aldehydes.

MATERIALS & METHODS

BLACKBERRY JUICE was prepared from thawed frozen fruit using pilot-plant scale equipment. Pure cyanidin-3-glucoside pigment was extracted from the same lot of raw fruit, by the procedure of Willstätter and Zollinger (1916), and used to prepare the model solution. Pigment separation from crude extract was carried by column chromatography on cellulose column (Daravingas and Cain, 1968). Separated glucoside was purified on a 2-mm layer of Silicagel G (Morton, 1967).

The pigment was identified by partition paper chromatography on Whatman No. 1 paper and comparison of measured R_f values with those found in the literature (Daravingas and Cain, 1966; Adams and Woodman, 1973); visible, UV and IR spectra were employed as well. Pigment purity of cyanidin-3-glucoside (formula $C_{21}H_{21}O_{11}Cl$) was confirmed by the elemental analysis yielding 50.12% C and 5.09% H vs. theoretical values of 52.00% and 4.37%, respectively.

For the performance of the experiments, we first prepared a 1.2 mM solution of cyanidin-3-glucoside in citrate buffer pH 3.45.

Three groups of samples were made for each tested system (i.e., blackberry juice and model pigment solution): a control group without aldehyde addition, addition of 12 mM furfural and addition of 12 mM HMF. Thus, molar ratio of cyanidin-3-glucoside/aldehyde used was 1:10. Separate control samples containing 12 mM HMF only (no pigment added) were prepared in the model system only. In tests with formaldehyde, acetaldehyde and benzaldehyde, the same pigment and aldehyde concentrations were employed as above.

Eight milliliters of sample solution were filled into sterile glass test tubes, closed with stoppers and thermostated at 24, 50 and 70°C. At 70°C/model system only, HMF and furfural samples were also prepared in inert nitrogen atmosphere.

Quantitative pigment and HMF assays were carried out in triplicate, initially and after 7, 14 and 20 days (24°C); 6, 24, 48 and 72 hr (50°C) and 2.5, 5.5, 8.5, 18.5 and 24.5 hr (70°C).

Quantitative determination of the anthocyanin pigment was carried out by the pH differential method introduced by Sondheimer and Kertesz (1948) and later modified by Lovrić (1964). The quantities of anthocyanin were expressed as mg cyanidin-3-glucoside per 100g sample using calibration curve. Colorimetric HMF assay was performed by Winkler's (1955) method.

Condensation tests of cyanidin-3-glucoside and cyanidine (the latter obtained by the hydrolysis of the glucoside) with furfural were carried out separately in methanol and $POCl_3$ solvents at the boiling point temperatures. The reaction flask was fitted with a reflux condenser. The microquantities of the reacting substances (2–5 mg) in ratio pigment/aldehyde 1:2 were dissolved in 1 ml methanol, heated for 2 hr in a water bath (100°C) cooled and then treated with an excess of saturated Na-acetate solution (Blackburn et al., 1957). Heating of the reaction mixture in $POCl_3$ at 100°C lasted 10 min and was followed by cooling and additions of 3 ml glacial acettic acid and 30 ml sodium perchlorate (Wizinger, 1953).

The purpose of this test was not to identify the reaction products but rather to find out whether certain reaction conditions bring about the formation and the separation of the condensation compound in the form of a sediment. Condensation tests were only done in air atmosphere. The electronic structures of cyanidine and of its more important tautomeric forms were calculated according to the Hueckel's molecular orbital theory (HMO) (1932) and the compound reactivities predicted on the basis of electron indices.

RESULTS & DISCUSSION

IN THE COURSE of pigment/aldehyde interaction experiments, concentrations of both reactants decreased with time. Changes at three temperatures are shown in Fig. 1 through 3. Compared to controls, degradation of cyanidin-3-glucoside and cyanidine was always more pronounced in presence of either aldehyde, in both examined systems and at all three temperatures. Negative effect of aldehyde presence was consistently compounded further by the direct effect of storage temperature.

The negative influence of furfural and HMF on the pigment stability was more pronounced in blackberry juice than in the model system. The same behavior had been
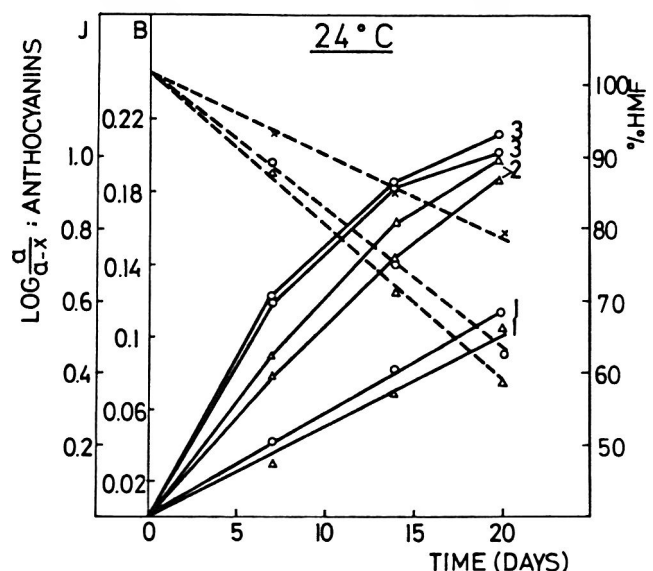
Fig. 1—Degradation of cyanidin-3-glucoside (CG; full lines) and 5-HMF (dashed lines) in a model citrate buffer solution (scale B) and in blackberry juice (scale J): 1 — controls, 2 — addition of furfural, 3 — addition of HMF. Open circles represent CG in buffer; triangles, CG in juice; and crosses, HMF control in buffer. Initial concentrations, 1.2 mM pigment and 12 mM aldehyde, respectively.
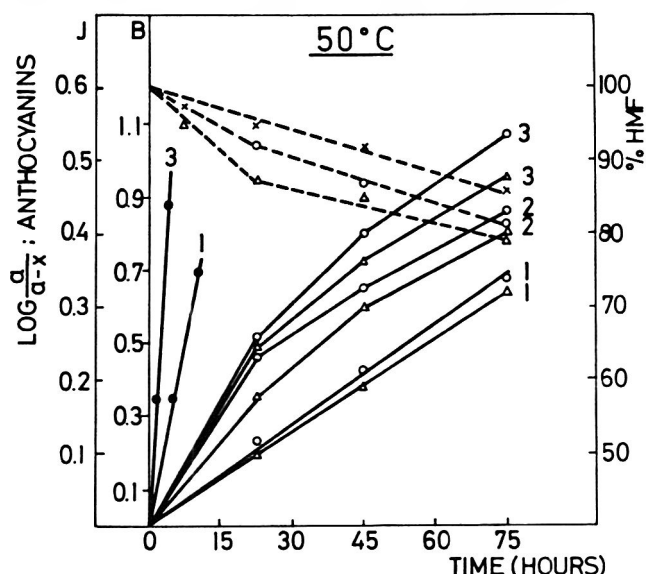
Fig. 2—Degradation of CG and cyanidine, C (full lines) and HMF (dashed lines). Full circles represent C in buffer. All other symbols and experiment conditions are the same as for Fig. 1.
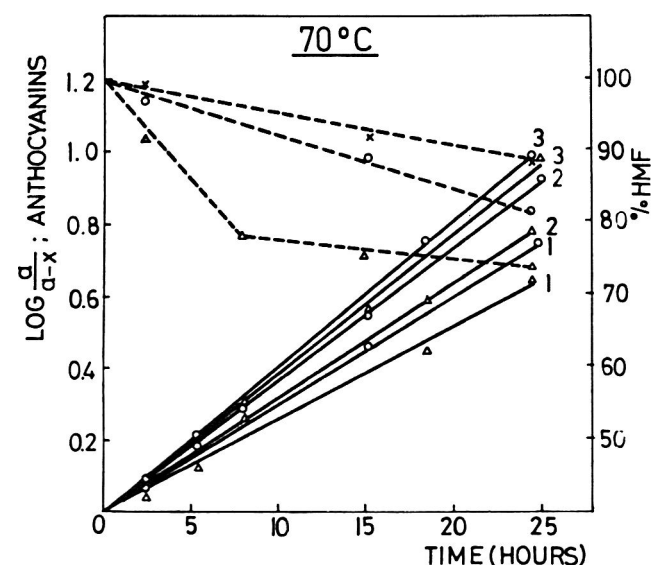
Fig. 3—Degradation of CG (full lines) and HMF (dashed lines) in a model system and in blackberry juice. Except for the uniform left-hand scale, all other symbols and experiment conditions are the same as in Fig. 1.
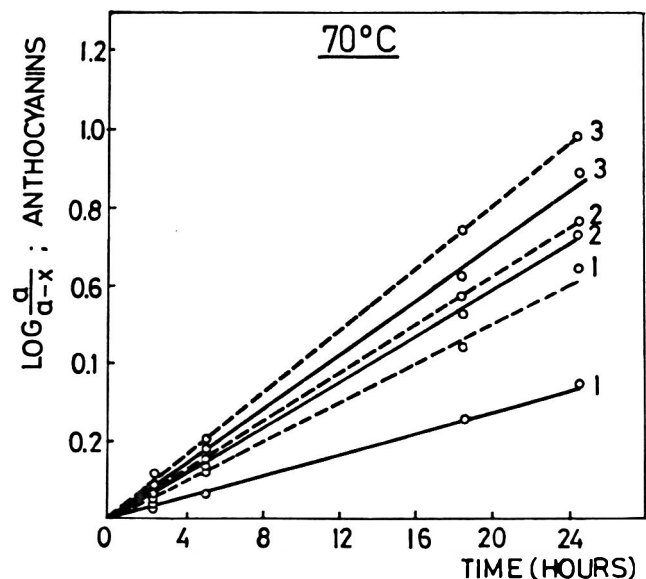
Fig. 4—Degradation of CG in a model citrate buffer solution in different atmospheres: 1 — controls, 2 — addition of furfural, 3 — addition of HMF. Full lines represent air atmosphere; dashed lines, nitrogen atmosphere. Experimental conditions are the same as in Fig. 1.

noted by Daravingas and Cain (1968) in their work on the degradation of the black raspberry anthocyanin in model systems.

At the same molar concentration (12 mM), HMF has in all instances exhibited the stronger negative influence on the pigment retention than furfural.

The aldehyde effect on pigment degradation at 70°C was considerably diminished when the reaction was taking place in the inert nitrogen atmosphere, Fig. 4. Since defradation did proceed even without the presence of oxygen, we concluded that oxygen appeared to accelerate the degradation but was not a necessary prerequisite for the degradation to occur.

Other aliphatic and an aromatic aldehyde had similarly affected the pigment stability. As shown in Fig. 5, at 70°C formaldehyde effect in the juice system was the strongest, followed by acetaldehyde and benzaldehyde. These vations were extended by recording absorption spectra of these samples prepared in citrate buffers at pH 1.5 and pH 4.0 and thermostated for 15 hr at 70°C (Fig. 6). The spectra show the occurrence in all samples of a shift in the absorption maximum, from the characteristic cyanidin-3-glucoside wavelength of 510 nm toward higher wavelenghts. The highest shift (to 538 nm) was observed in the sample of blackberry juice with formaldehyde added.

Pigment disappearance rate in the control samples of pigment without the aldehyde added show the first-order kinetics in all samples, in both systems and at all three temperatures (Fig. 1 through 3). When either aldehyde was added, the system behavior was found temperature-dependent. At 24 and 50°C, rate of anthocyanin degradation is initially higher and then slows down. This seems to indicate that there may be an additional factor which also influences the rate of interaction between the pigment and aldehyde. This factor could most probably have been oxygen, since its

concentration had been limited by the available headspace volume in our experimental conditions. At 70°C, the reaction rate was constant in both systems, indicating that another, different reaction kinetics prevailed. It could be inferred, for example, that the influence of temperature on the reaction rate had prevailed over the other factors.

The occurrence of pigment/aldehyde interaction had been confirmed by the collection of data for HMF disappearance, Fig. 1 through 3, which clearly show that the aldehyde loss was always larger when the pigment was present and at the higher storage temperature. The HMF disappearance kinetics had followed the first-order behavior in all model system tests and in juice at 24°C only. However, the kinetics deviated from the first-order in juice sys-
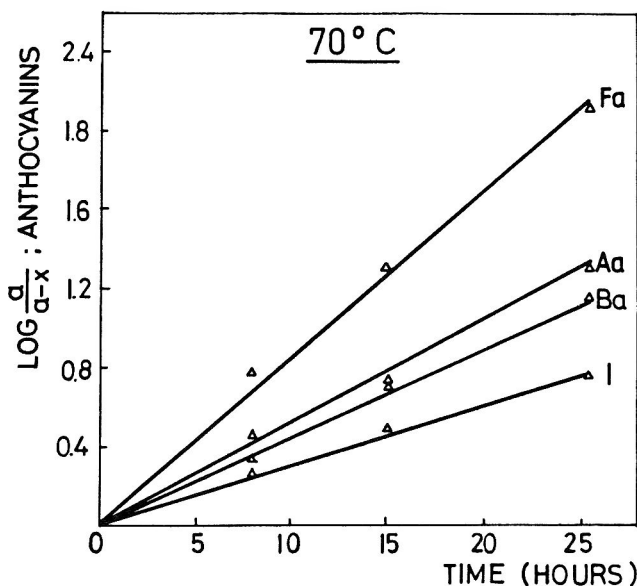
Fig. 5—Degradation of CG in blackberry juice with different aldehydes added: 1 — control; addition of benzaldehyde (Ba), acetaldehyde (Aa) and formaldehyde (Fa). Experimental conditions same as in Fig. 1.
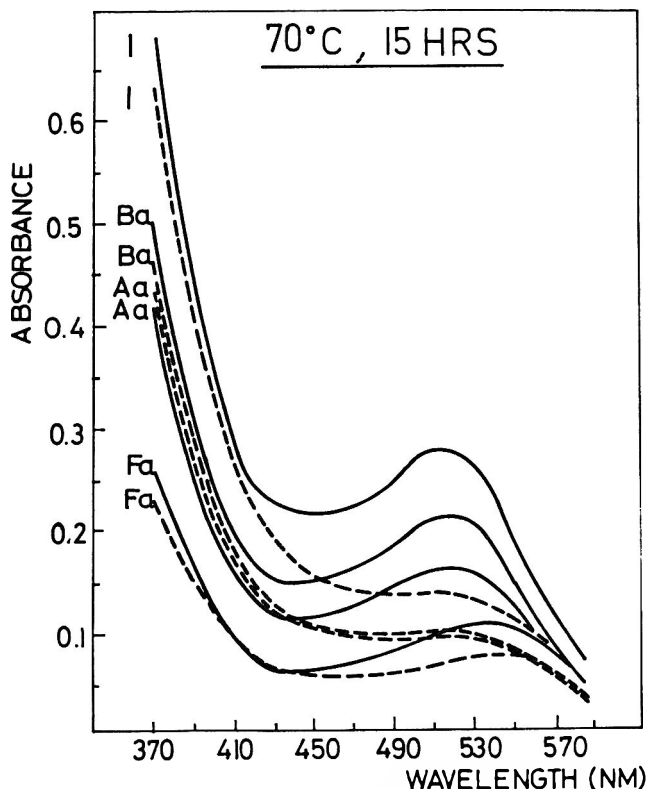
Fig. 6—Absorption spectra of blackberry juice measured in solutions buffered to pH 1.5 (full lines) and pH 4.5 (dashed lines): 1 — control; addition of benzaldehyde (Ba), acetaldehyde (Aa) and formaldehyde (Fa). Pigment/aldehyde molar ratio same as in Fig. 1.
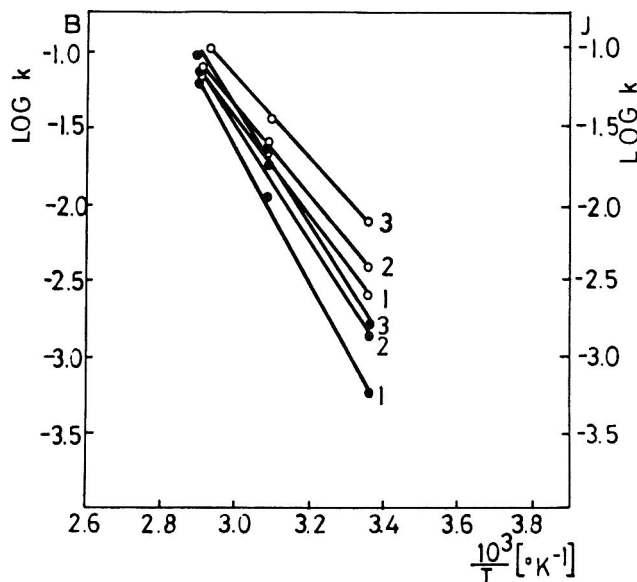
Fig. 7—Arrhenius plot for cyanidin-3-glucoside degradation in a model citrate buffer solution (full circles) and blackberry juice (open circles): 1 — controls, 2 — addition of furfural, 3 — addition of HMF. Initial molar concentration ratio pigment/aldehyde = 1/10 (1.2 mM pigment and 12 mM aldehyde, respectively).

Fig. 8—Structures and diagrams of π-electron charge distribution for the tautomeric forms of cyanidine and for furfural. (Note seoarate different formula numeration for the chalcone form of C.) Positions with minimum values in forms of cyanidine and maximum values in furfural are indicated with asterisks.

tem at 50 and 70°C. The probable reason could be the new simultaneous formation of HMF from the present sugars via nonenzymatic browning reactions.

Fig. 7 is the Arrhenius plot of the cyanidin-3-glucoside degradation in model and juice systems. Over the examined range of temperatures, a linear line was obtained.

Other kinetic data are shown in Table 1. Inspection of Q_{10} data reveals that the temperature effect on the rate of pigment loss had exceeded the effect of aldehyde presence in all instances but one (addition of formaldehyde).

From the condensation tests, we were able to establish that cyanidin-3-glucoside could enter the reaction with furfural only if it had been previously hydrolyzed at C_2, i.e. free cyanidine had been released. The reaction product of cyanidine/furfural interaction in methanol appeared as a dark-brown voluminous seidment only after the prolonged refrigerated storage. By contrast, the reaction in $POCl_3$ was relatively much faster. A black-pinkish precipitate had separated, poorly soluble in methanol or acetone and insoluble in acetic acid. Following partial dissolving in meth-

Table 1—Kinetics of cyanidin-3-glucoside degradation with and without aldehydes. Values for half-time decay $t_{1/2}$ (hours), rate constant k (hours^{-1}), temperature coefficient $Q_{10°C}$ and activation energy E_a (kcal).

System		24°C		50°C		70°C		$Q_{10°C}$*	E_a
		$t_{1/2}$	$k \times 10^{-3}$	$t_{1/2}$	$k \times 10^{-2}$	$t_{1/2}$	$k \times 10^{-2}$		
Model	Control	1,233	0.56	63.0	1.10	12.0	57.6	2.61 —	20.120
(citrate	Furfural	533	1.30	37.7	1.84	9.4	73.0	2.00 (3.35)	17.350
buffer)	HMF	529	1.31	31.4	2.21	7.6	91.5	2.07 (3.75)	14.910
	Control	292	2.37	33.0	2.10	9.8	70.7	1.68 —	14.760
Blackberry	Furfural	161	4.30	25.0	3.04	8.3	83.7	1.09 (1.99)	13.040
Juice	HMF	148	4.68	20.0	3.47	7.8	88.7	1.27 (2.11)	12.650
	Benzaldehyde	—	—	—	—	6.0	115.6	1.76 (2.76)	11.290
	Formaldehyde	—	—	—	—	3.4	202.0	0.87 (4.85)	6.780

* First set of values represents the effect of temperature on the rate of cyanidin-3-glucoside degradation. Values in parentheses represent the effect of temperature and the aldehyde together, on the rate of cyanidin-3-glucoside degradation.

Table 2—Positions in the molecules of cyanidine tautomeric forms and furfural having extreme values of π-electron densities

Molecule (tautomeric form)	π-electron densities		
	Maximum	Minimum	
		On condensed rings	On side ring
Cyanidine	$C_{11} > C_8$ O_{20}, O_3	$C_1 > C_4$	C_{17}
Cyanidin-anhydrobase	$C_{11} > C_8$ O_{20}, O_3	$C_1 > C_4$	C_{17}
Cyanidin-keto-pseudobase	$C_4 > C_1$ O_{22}, O_{20}	$C_2 > C_9$	$C_{19} > C_{17}$
Chalcone	$C_9 > C_3$ O_{18}, O_{16}	$C_{10} > C_8$	C_{15}
Furfural	$C_5 > C_6$	$C_4 > C_1$	

Table 3—Superdelocalization values of different cyanidine tautomeric forms

Position	Cyanidine	Cyanidin-anhydrobase	Cyanidin-keto-pseudobase	Chalcone
1	2.5584	2.2645	0.1414	0.1071
2	0.8124	0.8124	1.4314	1.2315
3	0.0518	0.0523	0.3714	0.7909
4	2.5397	2.2341	0.1356	1.2458
5	0.6913	0.6650	0.6697	0.7700
6	1.2838	1.2095	0.7846	1.2877
7	0.1152	1.1043	0.0474	0.6736
8	0.7248	0.7132	0.6721	2.3805
9	1.2063	1.1380	0.7843	0.9786
10	0.1045	0.0946	0.0471	2.1169
11	0.6903	0.6880	0.6716	1.0994
12	1.2054	1.1383	0.7871	0.6850
13	0.6221	0.5271	0.0474	1.1766
14	0.6770	0.6055	0.7628	0.7706
15	1.2750	1.2128	0.7548	1.1283
16	0.7712	0.7476	0.7754	0.0958
17	1.2201	1.0791	0.7429	0.7501
18	0.1097	0.3807	0.0444	0.0457
19	0.7603	0.7515	0.7647	1.1603
20	0.0456	0.0467	0.0462	1.1603
21	1.2512	1.1284	0.7523	
22			0.0013	

anol, the increased spectral absorption of the solution was observed at wavelengths of 290, 364, 440 and 538 nm.

The observation that the reaction product between cyanidine and furfural in methanol had formed only upon treating the solution with saturated Na-acetate suggests that cyanidine was active in that reaction as a pseudobase (keto- or enol form).

π-electron densities for the studied compounds based on the HMO calculations (Heuckel, 1932), are shown in Fig. 8. Different tautomeric anthocyanin forms have very similar π-electron density distributions. Molecular locations on the condensed ring and on the side ring having extreme electron density values are summarized in Table 2. The positions with the lowest π-electron densities are the most attractive ones to the nucleophylic reagents. These positions are rather similar in the different tautomers studied. In cyanidin-cation and cyanidin-anhydrobase these positions are C_1 or C_4, keto-pseudobase C_2 or C_9 but in chalcone C_8 or C_{10} (Fig. 8).

As far as highest π-electron densities are concerned, oxygen atoms were the primary positions with these properties. Both oxygen and carbon atoms with the highest π-electron densities were more irregularly distributed in the examined molecules. Generally, high π-electron density locations are the potential candidates for the electrophylic addition.

Due to the electron-withdrawing substituent -CHO in the furfural molecule, positions C_4 and C_1 are electron-deficient, particularly in the acidic medium, and can easily react with the nucleophiles. On the other hand, the most suitable positions for the electrophylic attack to this molecule are C_5 or C_6 atoms.

Electron densities are not always reliable as the indicators of relative molecular reactivity. Thus, we additionally calculated the nucleophylic superdelocalizability indices for the studied compounds (Table 3). The indices for cyanidin-keto-pseudobase interchanged positions with those of the highest affinity forward nucleophylic reagents. The most reactive positions are C_2 or C_{12}.

The energy values for the first electron transition, (in β) i.e. the energy difference between the ground state and the first excited state, show that the most unstable tautomeric form is actually the anhydrobase. This agrees with the observation that during the condensation tests it was necessary to treat the cyanidine/furfural mixture with Na-acetate in order to initiate the reaction. (Since this presumably causes the formation of keto- or anhydrobase, it follows that these forms are more reactive than the parent form).

Overall conclusion from the studies of molecular properties of anthycyanin tautomeric forms is that each tested cyanidine form can enter the reaction with furfural or HMF, the most reactive form being the cyanidin-anhydrobase. Further, it is not improbable that the condensation reaction can take place not just on the benzpyrane core but also on the noncondensed side benzene cyaidine ring. In

Fig. 9—Proposed reactions between cyanidine and furfural: (1) mechanism proposed, but not recorded, previously by Tinsley and Bockian (1960); (2) reaction of furfural and the OH groups of noncondensed side benzene cyanidine ring; (3) reaction of furfural with two molecules of cyanidin-anhydrobase.

other words, the mechanism of cyanidine degradation in presence of aldehydes can not be limited to one singular pathway; pigment degradation can apparently occur via several different reactions. Thus the possible mechanism proposed previously by Tinsley and Bockian (1960), which have not been investigated, is shown as (1) in Fig. 9. Beside this, we now also propose two additional possible mechanisms (2) and (3), Fig. 9.

In case (1) cyanidine enters in the reaction with furfural in the form of the keto-pseudobase on the C_4 atom. In case (2) cyanidine reacts with furfural through OH groups of the noncondensed side benzene ring, but in (3) two molecules of cyanidin-anhydrobase (partially ionized), react with one molecule of furfural.

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Study of the Evolution of Tyramine Content During the Vinification Process

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─────────────── ABSTRACT ───────────────

A preliminary study is described of the evolution of tyramine in four different brands of wine, starting from the must and sampling at different times during the vinification process. In the samples of two of the brands, other parameters are also studied (sulphur dioxide and alcoholic contents and density) with the purpose of considering the relationship between the alcoholic fermentation and the appearance of relatively important amounts of tyramine. The relationship between the variations in the content of this amine and its metabolic precursor, tyrosine, is also discussed.

INTRODUCTION

THE PRESENCE and relative content of biogenic amines in general and specifically tyramine in foods and drinks are of great interest from a toxicological point of view [dietary migraine, interaction with monoamine-oxidase inhibiting drugs (IMAO) (Marine-Font, 1978; Rivas-Gonzalo et al., 1978; Hanington, 1980)]. However, the amounts of tyramine in wines are comparatively low when contrasted with those found in other kinds of foods and in most cases are not high enough to cause undesirable effects due to any possible interaction with IMAO drugs. Indeed, the contents quoted in the literature for wines range, in most cases, between 0 and 25 μg/ml (Horwitz et al., 1964; Sen, 1969; Puputti and Soumalainen, 1969; Spettoli, 1971; Cerutti and Remondi, 1972; Zappavigna and Cerutti, 1973; Zappavigna et al., 1974; Rivas et al., 1982). However, Tarjan and Janossy (1978) reported average contents of 38.80 μg/ml in red wines and 110.76 μg/ml in white wines. Furthermore, the figures reported in the literature would probably not be sufficient to cause dietary migraine either, except in the case of the ingestion of huge amounts of wine, in which case the resulting intoxidation would not merely be due to the presence of the amine in question.

Various microorganisms take part in the formation of tyramine in drinks and foods and thus it has been proposed that one of the most important factors related to the amount of this amine in wines is the hygienic status of the vinification process; it has even been suggested that a wine elaborated in optimal conditions, from the point of view of hygiene, should be "practically" free of amines (Cerutti and Remondi, 1972). Nevertheless, we have observed that tyramine is in fact present, to a greater or lesser extent, in all (71) the samples of wine studied (Rivas et al., 1982), suggesting that the amine, at last up to a certain point, is a normal component appearing during the vinification process and not just the consequence of deficient hygiene conditions. Furthermore, other factors are reported to take part in its formation such as pH, the intensity of malolactic fermentation (Zappavigna and Cerutti, 1973), and the presence of certain aminoacids in the must (Cerutti et al., 1978).

Starting from the results of a preliminary study on the evolution of tyramine throughout the vinification process

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in which the samples were taken at relatively spaced out intervals (Rivas et al., 1979), the aim of the present work is to elucidate the evolution of tyramine using a greater number of samples, specially those collected during the first few days after making the must. Using certain suitable parameters, the relationship between alcoholic fermentation and the amounts of tyramine present in the samples will also be considered.

MATERIALS & METHODS

Wine samples

Brand A. Rosé wine from Benavente (Zamora, Spain). Starting from the must, 14 samples were taken on consecutive days.

Brand B. Rosé wine from Alaraz (Salamanca, Spain). Seven samples were taken over a period of 49 days.

Brand C. Red wine from Cañizal (Zamora, Spain). Starting from the must, 31 samples were taken. Initial sampling was daily though later on this was staggered (see below).

Brand D. Hybrid red wine from Canizal (Zamora, Spain). This is made from red grapes of a binary hybrid *Vinifera X Berlandieri* 41^B. Thirty samples were collected.

The exact periodicity of the sampling procedure is shown in Tables 1 and 2. The samples were stabilized by adding NaF (1%) in a maximum period of 2 hr after the sample was collected, and were stored in the absence of light at 4°C. All samples were gifts from the wine-growers concerned, who followed the traditional wine-making procedures of small businesses. In no case was there any possibility of modifying these procedures.

Methods of analysis

Tyramine determination. The method described by Rivas-Gonzalo et al. (1979) is applied.

Density measurement. Direct determination of the density using a Mohr-Westphal hydrostatic balance. It affords a rapid and convenient method for this determination (Lecoq, 1965).

Sulfur dioxide determination. The Rankine procedure is used; this is an aspiration-oxidation method that determines both free and total SO_2 (Moreno and De la Torre, 1977).

Ethyl alcohol determination. The alcohol content was evaluated by using an aerometric measurement after distilling the wine (Moreno and De la Torre, 1977). The alcoholometer is graduated to indicate the percentage of ethyl alcohol by volume (alcoholic degree) at 20°C.

RESULTS & DISCUSSION

TABLE 1 shows the amounts of tyramine in wines A and B. Table 2 shows the same for brands C and D, and also includes sulfur dioxide and alcoholic contents and density. From the information presented in these tables, it may be deduced that the evolution of tyramine content is not always the same and it is noteworthy that in the must, the amounts of the amine are small.

As may be seen from Table 2, it is evident that the increase in tyramine content in the samples coincides directly with the onset of fermentation. This is the moment when ethyl alcohol begins to appear and is accompanied by a concomitant fall in density.

The amount of SO_2 present in the wines determines both the onset and the fermentation process itself. Because of this, in the samples of brand C (particularly in the first days when the SO_2 content is too high) ethyl alcohol only

appears after several days, showing that the yeasts of the must have remained inactive for this time. Thus, as the tyramine content also begins to increase at the end of this period, it would seem reasonable to suppose that its formation is indeed concomitant to alcoholic fermentation. This is corroborated by the fact that in brand D, where the SO_2 content is much lower, and fermentative activity is manifest from the first moments; tyramine is found in appreciable amounts from the first day.

When the amount of free SO_2 in brand C increases again, the tyramine content stabilizes. So in brand D it may be seen that even though the content of this amine stabilizes when the SO_2 increases (about half-way through the period

studied), towards the end of this period, when SO_2 decreases, there is another increase in tyramine content.

It may be thought that SO_2 governs the activity of the microorganisms which are able to form tyramine; in this sense, highly sulphited musts which maintain high levels of SO_2 during their fermentation, will *a priori* have low levels of tyramine. If this is not the case, and the must does contain such tyramine forming microorganisms, the amine will be present in greater proportions.

Bearing in mind that the presence of SO_2 in wines is due to its addition by the wine-grower, it is curious that the evolution followed by tyramine in these wines (brands C and D) is, generally speaking, similar for different years of production for the same kind of wine (Rivas-Gonzalo et al., 1979).

In these samples, the tyrosine content was also determined (Rivas-Gonzalo et al., 1981) and on comparison of the evolution of tyramine with this amino acid, it may be seen that when the latter decreases, a simultaneous increase in the amount of tyramine present takes place. However, these changes are not proportional and it is not possible to attribute the appearance of tyramine directly to the decrease in tyrosine, let alone by a direct transformation of one into the other. Several workers have reported that large amounts of tyrosine have an induction effect on the formation of tyramine (Eitenmiller et al., 1978) which could explain the change in tyramine content once the tumultuous effect of alcoholic fermentation has finished.

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Table 1—Evolution of tyramine content in brands A and B wines

Brand A		Brand B	
Must to final product, days	mg/L	Must to final product, days	mg/L
0 (must)	0.1	1	0.1
1	0.1	3	0.1
2	0.1	5	0.5
3	0.1	12	0.7
4	0.1	19	0.7
5	0.1	34	0.3
6	0.1	49	0.3
7	0.1		
8	0.1		
9	0.1		
10	0.2		
12	0.2		
13	0.3		
14	0.3		

Table 2—Evolution of tyramine, alcohol and sulfur dioxide contents and density in brands C and D wines

Must to final product, days	Tyramine (mg/L)		Density (d^{15}_{15})		Alcoholic degree		Sulfur dioxide (mg/L)			
							free		total	
	C	D	C	D	C	D	C	D	C	D
0 (must)	0.1	0.1	—	—	—	—	—	—	—	—
1	0.1	0.3	1.0910	1.0900	0	0.9	—	—	—	—
2	0.1	0.3	—	1.0768	—	2.0	—	12.8	—	284.8
3	0.1	0.3	—	1.0617	—	4.0	—	19.2	—	257.6
4	0.1	0.4	1.0985	1.0376	0	7.6	153.6	19.2	393.2	262.4
5	0.1	0.4	—	1.0166	—	10.0	—	14.4	—	241.6
6	0.1	0.5	1.0913	1.0118	0	11.1	150.4	12.8	390.4	230.4
7	0.1	0.5	—	—	—	—	—	—	—	—
8	0.1	0.5	—	1.0003	—	12.3	—	11.2	—	190.4
9	0.2	0.5	1.0905	—	0	—	160.0	—	406.4	—
10	0.2	0.6	—	0.9963	—	12.3	—	19.2	—	240.0
11	0.2	0.6	—	—	—	—	—	—	—	—
12	0.2	0.6	1.0896	0.9930	1.1	12.3	80.0	8.0	291.2	208.0
13	0.2	0.6	1.0750	—	2.4	—	32.0	—	368.0	—
14	0.3	0.6	—	0.9958	—	12.4	—	20.8	—	228.8
17	0.3	0.6	1.0187	0.9958	7.5	12.7	14.4	9.6	304.0	131.2
23	0.3	0.6	0.9977	0.9958	12.1	12.7	12.8	6.4	259.2	128.0
27	0.3	0.6	0.9957	0.9938	12.7	12.7	9.6	9.6	259.2	166.4
32	0.4	0.6	0.9950	0.9950	12.7	12.7	3.2	8.0	204.8	129.6
36	0.4	0.7	0.9979	0.9935	12.7	12.7	12.8	9.6	246.4	163.2
42	0.4	0.7	0.9987	0.9951	12.7	12.7	12.8	8.0	249.6	132.8
45	0.4	0.8	0.9990	0.9942	12.7	12.7	17.6	22.4	292.8	204.8
51	0.4	0.8	0.9965	0.9930	12.7	12.7	16.0	22.4	281.6	200.0
57	0.4	0.8	0.9955	0.9935	12.7	12.7	19.2	24.0	291.2	201.6
65	0.4	0.8	0.9971	0.9945	12.7	12.7	20.8	12.8	296.0	163.2
71	0.3	0.8	0.9993	0.9960	12.7	12.7	16.0	22.4	275.2	208.0
78	0.4	0.8	0.9978	0.9950	12.7	12.7	32.0	28.8	323.2	208.0
84	0.3	0.8	0.9999	0.9988	12.7	12.7	32.0	17.6	331.2	187.2
91	0.3	1.0	0.9961	0.9940	12.7	12.7	32.0	19.2	320.0	179.2
120	0.3	1.3	0.9965	0.9920	12.7	12.7	48.0	20.8	374.4	195.2
157	0.3	—	—	—	—	—	—	—	—	—

Kinetics of Malvidin-3-Glucoside Condensation in Wine Model Systems

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ABSTRACT

Wine model systems containing equimolar quantities of malvidin-3-glucoside and d-catechin, with and without acetaldehyde, were stored anaerobically at 22, 32, 42, and 52°C. Malvidin-3-glucoside and d-catechin disappearance and polymer appearance were monitored by HPLC and colorimetric methods. Reaction rates and activation energies were calculated. The malvidin-3-glucoside and d-catechin condensation reactions showed pseudo first order kinetics both with and without the presence of acetaldehyde. However, activation energies were not significantly different.

INTRODUCTION

WINE-MAKING has traditionally been more of an art than a science. Until recently, there has been little attempt to scientifically investigate the process. The chemistry of wine aging has been especially neglected.

As a red wine ages, it changes from a bright red to a reddish-brown color. This corresponds to a decrease in the concentrations of monomeric anthocyanins and other phenolic compounds (Nagel and Wulf, 1979), and an increase in colored polymeric compounds (Somers, 1971). The average molecular weight increases from 500–700 Daltons to 2000–3000 Daltons (Somers, 1971). If aging goes on for long periods, colored precipitates appear which are attributed to polymers too large to remain in solution.

The pigments of aged wine are more stable than are the anthocyanins themselves (Somers, 1971). Thus it was proposed that the anthocyanins were condensing directly with other phenolic compounds such as catechins or procyanidins (Jurd, 1965; 1967). However, in model system mixtures of these compounds, reactions were slower than in the aging of wines (Timberlake and Bridle, 1976).

Acetaldehyde has long been known to cause a more intense red color when present in wines (Berg and Akiyoshi, 1975). Timberlake and Bridle (1976) added acetaldehyde to model systems containing anthocyanins and catechins or procyanidins, and found that the polymerization rate was accelerated. They proposed that polymers, bridged by acetaldehyde, were formed from anthocyanins and other phenolic compounds. Repeated condensations could produce large polymers.

These experiments gave substantial evidence for the existence of anthocyanin polymers formed as a result of wine aging. They did not, however, give any exact information on the polymerization reaction. This research was designed to investigate the kinetics of the polymerization reaction.

MATERIALS & METHODS

Preparation of model systems

Malvidin-3-glucoside was isolated from Royalty grapes using the method of Hrazdina (1970), and further purified by high performance liquid chromatography (HPLC) separation on a Partisil ODS

preparatory column (25 cm x 0.5 in. o.d.). d-Catechin was isolated by Baranowski and Nagel (1981).

Wine model systems consisted of 0.1% potassium bitartrate buffer (pH 3.5), 10% (v/v) ethanol, 280 mg/L malvidin-3-glucoside, and 145 mg/L d-catechin. Redistilled acetaldehyde was added at concentrations of 0, 22, or 110 mg/l. This provided equimolar concentrations of malvidin-3-glucoside, d-catechin, and acetaldehyde (5.0×10^{-4} mole/L) or a fivefold excess of acetaldehyde (2.5×10^{-3} mole/l). All amounts used, as well as the pH, approximated those found in young red wines. Mixtures were made in duplicate.

Solutions were prepared under nitrogen, then put into tubes, flushed with nitrogen and sealed with rubber septa. The sample tubes were placed in jars containing an alkaline pyrogallol reservoir to scavenge oxygen, flushed with nitrogen, and sealed. Storage was at 22, 32, 42, and 52°C for up to 22 wk. Controls were made and stored under identical conditions. Control solutions contained malvidin-3-glucoside alone or d-catechin alone, with none or equimolar quantities of acetaldehyde.

Analyses

Sampling was done periodically by removing tubes from the jars, and using a syringe to withdraw samples through the rubber septa. An equal volume of nitrogen was injected into each tube to replace the sample removed, the tubes were replaced into the jars, the oxygen scavenger was replenished, and the jars were flushed with nitrogen.

HPLC was used to measure amounts of d-catechin and malvidin-3-glucoside. The system consisted of a Waters Associates (Milford, MA) model 6000-A pump, U6K injector, and WISP 710B automatic sampler. The detector was a Micromeritics (Norcross, GA) model 785 variable wavelength spectrophotometer. Peak areas and retention times were measured using a Spectra-Physics (Santa Clara, CA) Minigrator and a Houston Instrument Co. (Austin, TX) OmniScribe recorder. The column used was a 4.6 mm i.d. x 25 cm Zorbax ODS analytical column (Dupont, Inc., Wilmington, DE) preceded by a 4.6 mm i.d. x 4 cm Bondapak C18/Porasil B (Waters Associates, Milford, MA) precolumn. The solvent system was 12% (v/v) acetonitrile, and 88% (v/v) of 2% (w/v) formic acid in water; the flow rate was 2.0 ml/min. Detection was at 280 nm for d-catechin, and 280 or 520 nm for malvidin-3-glucoside and the polymers. Standards of known concentrations of malvidin-3-glucoside and d-catechin (both isolated as described previously) were analyzed periodically to produce response factors.

Colorimetric analyses were done by the method of Somers and Evans (1977) using a Beckman (Fullerton, CA) model 35 UV-visible scanning spectrophotometer. Quartz cuvettes with inserts were used to give a one mm light path. Parameters measured were the absorbance at 520 nm, and the absorbance at 520 nm after treatment with an excess of sodium meta-bisulfite.

Data analysis

Results for duplicate samples were averaged, and converted to concentrations by use of response factors for HPLC data or Beer's Law for spectrophotometric data. The extinction coefficient used for malvidin-3-glucoside was 33,700 at 520 nm (Nagel and Wulf, 1979); this value was also used to estimate the concentrations of the polymeric pigment. To determine the order of reaction, the log of concentration, inverse of concentration, and inverse of concentration squared were also calculated; all were graphed against time. The log of concentration versus time curves were linear, so further calculations were done using first order kinetic laws.

Reaction rates and Arrhenius activation energies were calculated based upon both the disappearance of malvidin-3-glucoside (as shown by the HPLC data), and on the appearance of polymers (as shown by colorimetric data). Statistical analyses were done accord-

ing to the student's t test for comparing sample means (Steel and Torrie, 1960).

RESULTS & DISCUSSION

SEVERAL REACTIONS were expected to occur in the model system mixtures. First, there should be transformations of the malvidin-3-glucoside. In an aqueous medium at pH 3.5 and 25°C, the equilibrium distribution is about 75% carbinol base, 10.4% flavylium ion, 8.3% chalcone, and 2.1% quinoidal base (Brouillard and Delaporte, 1978). These transformations occur rapidly (Brouillard and Delaporte, 1977), and the equilibrium should be reached within an hour at pH 3.5 and 25°C. Thus the reaction rates for the anthocyanin transformations were not considered to contribute to the apparent reaction rate for the condensations. However, the relative amounts of the forms of anthocyanin are of utmost importance in determining the type and extent of condensation reactions.

In the aging of wine, oxidation of all reactants would also be a contributing factor (Ribereau-Gayon, 1974). In this work, however, oxidation was hopefully eliminated by maintaining a nitrogen atmosphere above the reaction mixtures. The reactions to be considered, then, are mainly the condensation reactions between malvidin-3-glucoside and d-catechin, with or without an acetaldehyde bridge.

Fig. 1 to 3 show the production of polymer under the various conditions used. When an excess of acetaldehyde is present, condensation proceeds rapidly, and produces two to three times as much polymeric material as when acetaldehyde is present in amounts equimolar to malvidin-3-glucoside and d-catechin. The equimolar system in turn produces about two times as much polymeric material as does the mixture of malvidin-3-glucoside and d-catechin without acetaldehyde. It was observed that all systems containing acetaldehyde produce far more polymer than does malvidin-3-glucoside alone. These results are in agreement with previous work (Berg and Akiyoshi, 1975; Timberlake and Bridle, 1976).

As expected, storage temperature markedly affected the reactions (Fig. 1–3). At 52°C there was less polymer produced than at the lower temperatures. With or without acetaldehyde, systems at 42°C produced the greatest amount of polymeric material in the least time, but the polymers were most stable to degradation and precipitation at 32°C or less. This is important since thermovinification, commonly done at 50–70°C, is known to produce wines with less color (Amerine et al., 1972). These results show that lower temperatures would be more effective in producing and maintaining wines with good, stable color.

Fig. 4 shows the log plots for malvidin-3-glucoside changes in the reaction mixture containing equimolar malvidin-3-glucoside and d-catechin with excess acetaldehyde. Based upon the decreasing concentration of malvidin-3-glucoside all four temperatures show linearity. These are the most linear of the kinetics curves prepared (concentration, log concentration, inverse of log concentration, or inverse of concentration squared versus time, or zero, first, second, and third order kinetics, respectively). Thus, the condensation reaction appears to be first order with respect to the disappearance of the anthocyanin, but does not agree with any simple reaction order for the appearance of the polymers. This is also true for the reaction mixtures containing equimolar acetaldehyde or no acetaldehyde, though not as pronounced. The lack of linearity for the polymer production curve is probably due to the eventual precipitation of polymers too large to remain in solution.

For the purpose of comparing reaction rates and activation energies, the condensation was assumed to be pseudo first order. Where a decrease in the amount of polymer occurred (Fig. 1–3), the reaction was broken into two parts — the initial phase where polymerization was occur-
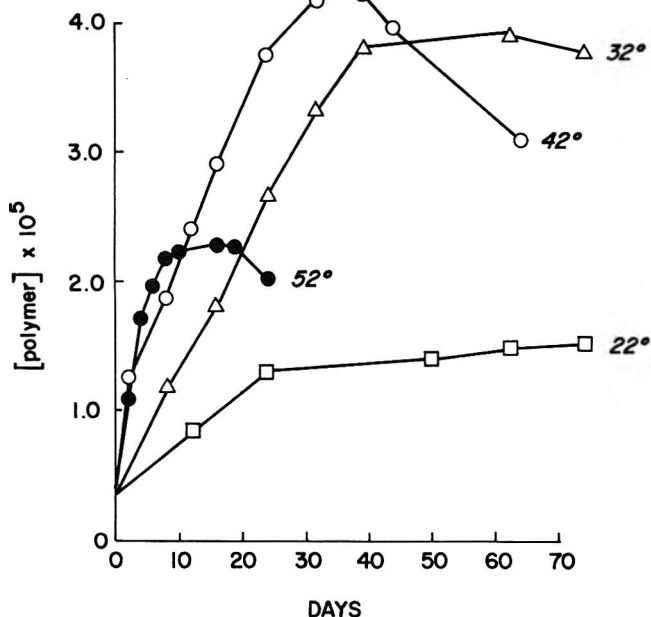
Fig. 1—Effect of temperature on polymer formation with excess acetaldehyde.
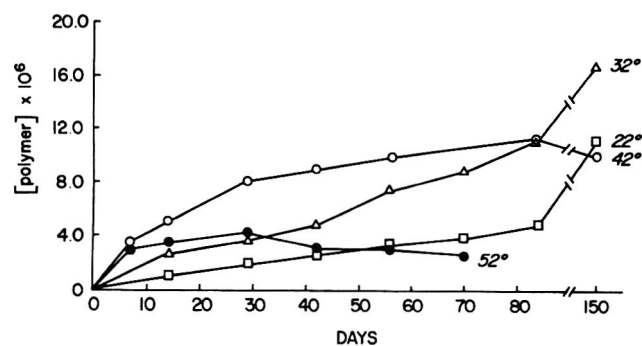
Fig. 2—Effect of temperature on polymer formation with equimolar acetaldehyde.
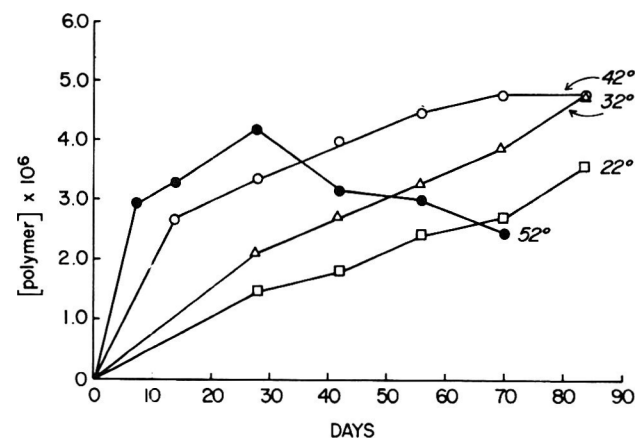
Fig. 3—Effect of temperature on polymer formation without acetaldehyde.

ring, and the final phase where loss of the polymeric color predominated. Precipitation of polymers too large to remain in solution may account for the latter part of this reaction.

The observed reaction rates and activation energies are shown in Tables 1 and 2. As shown in Fig. 5, the activation energies for polymer production and loss of malvidin-3-glucoside were calculated on the basis of the 22–42°C reaction rates, since the reaction rates at 52°C did not increase proportionately with the temperture increase. At a 5% confidence level, there are no significant differences between the activation energies based on the production of polymers (Table 1) and those based on the loss of malvidin-3-glucoside (Table 2) in systems containing d-catechin (least significant differences are: 5.2 for excess acetaldehyde, 9.3 for equimolar acetaldehyde, and 3.7 for no acetaldehyde). Thus, it seems that the majority of the anthocyanin lost is condensing to produce polymeric material.

In the system consisting of malvidin-3-glucoside alone, however, there is a significant difference between the two activation energies (Tables 1 and 2). This indicates that not all of the malvidin-3-glucoside lost is being incorporated into polymeric material. Some malvidin-3-glucoside may be lost to thermal degradations; however, thermal loss of anthocyanins is not considered to be significant until 50 C and above (Adams, 1973). This would, then, be a factor in the 52°C systems, and could also contribute to the 42°C systems. The 32 and 22°C samples should not show significant thermal degradation.

The observed reaction rates (Tables 1 and 2) show an apparent increase as acetaldehyde concentration increases. However, the activation energies do not change significantly. This may be explained by the fact that the direct condensation proposed by Jurd (1965; 1967) requires the flavylium ion form of the anthocyanin. At pH 3.5, however, this ion constitutes only about 10% of the equilibrated anthocyanin solution (Brouillard and Delaporte, 1978). When no acetaldehyde is present, direct condensation of anthocyanin flavylium ions with d-catechin would occur, shifting the malvidin-3-glucoside equilibrium so that more flavylium ion is produced. The reaction would thus continue over a long period of time as seen in Fig. 3. The acetaldehyde-bridged condensation depends only upon the nucleophilic character of carbons 6 and 8 of the anthocy-

anin (Timberlake and Bridle, 1976), and so is only slightly affected by the anthocyanin form. Thus, when excess acetaldehyde is present, the reaction rate is not limited by the state of the anthocyanin equilibrium. If limited acetaldehyde is present, both types of condensation must occur, as shown by the observed reaction rates (Tables 1 and 2). It seems reasonable to propose that if the pH were low enouugh to flavor the flavylium ion, the direct condensation would proceed as rapidly as does the acetaldehyde-bridged condensation.

The kinetic evidence presented points to the occurrence of two types of condensations in wine model systems. At low concentrations of acetaldehyde, both seem to occur simultaneously; when excess acetaldehyde is present, the bridged polymerization predominated. In an actual wine, both condensation reactions would be expected to occur. This work also indicates that the aging of wine could be accelerated by vinification around 42°C, with storage at 32°C, to maximize the production and maintenance of colored polymers.

Table 1—Observed reaction rates and activation energies for production of polymers in model systems

Reactants	Temp. (°C)	Production	
		k (sec^{-1})	Ea (Kcal/mole)
Excess CH$_3$CHO[a]	22	5.0×10^{-7}	
	32	7.0×10^{-7}	
	42	2.2×10^{-6}	11
	52	2.6×10^{-6}	
Equimolar CH$_3$CHO[a]	22	5.4×10^{-8}	
	32	3.1×10^{-7}	
	42	1.8×10^{-7}	12
	52	3.5×10^{-7}	
No CH$_3$CHO[a]	22	4.1×10^{-8}	
	32	2.1×10^{-7}	
	42	1.2×10^{-7}	8.6
	52	1.4×10^{-7}	
M-3-G	22	4.0×10^{-8}	
	32	2.8×10^{-8}	
	42	2.8×10^{-8}	4.2
	52	3.5×10^{-8}	

[a] Systems containing equimolar amounts of malvidin-3-glucoside and d-catechin.

Table 2—Observed reaction rates and activation energies for disappearance of malvidin-3-glucoside in model systems

Reactants	Temp. (°C)	k (sec^{-1})	Ea (Kcal/mole)
Excess CH$_3$CHO[a]	22	2.2×10^{-7}	
	32	4.8×10^{-7}	
	42	9.9×10^{-7}	13
	52	1.6×10^{-6}	
Equimolar CH$_3$CHO[a]	22	5.5×10^{-8}	
	32	2.8×10^{-7}	
	42	3.4×10^{-7}	10
	52	4.9×10^{-7}	
No CH$_3$CHO[a]	22	7.8×10^{-8}	
	32	2.1×10^{-7}	
	42	3.3×10^{-7}	11
	52	5.4×10^{-7}	
M-3-G Alone	22	7.3×10^{-8}	
	32	6.8×10^{-8}	
	42	1.6×10^{-7}	28
	52	1.1×10^{-6}	

[a] Systems containing equimolar amounts of malvidin-3-glucoside and d-catechin.
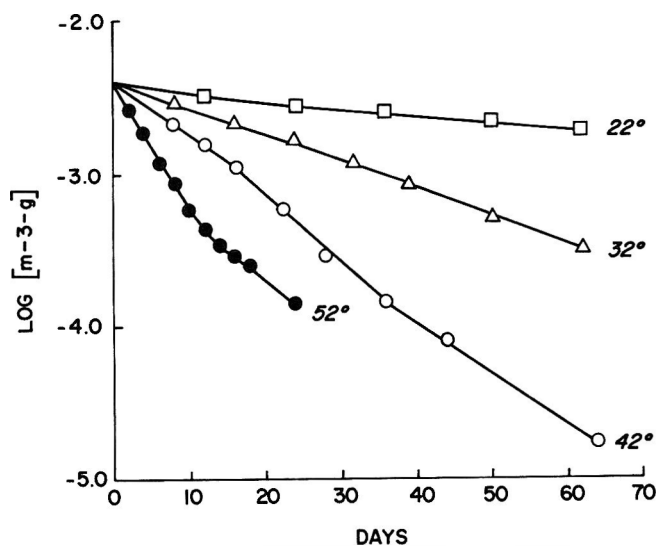
Fig. 4—Malvidin-3-glucoside concentration changes with excess acetaldehyde.

Preconcentration of Apple Juice by Reverse Osmosis

M. J. SHEU and R. C. WILEY

──────── ABSTRACT ────────

Single strength apple juices (10°Brix) were processed by reverse osmosis to 20–25°Brix, primarily at 20°C. A pilot scale plate and frame UF-RO system equipped with cellulose acetate (CA) membranes, CA-865 and/or CA-990, or high resistance (HR) membranes, HR-95 and/or HR-98, was operated at pressures of 35–45 bar. At 45 bar, the larger pore-sized CA-865 possessed the highest processing capacity of 26.9 L/m²/hr (from 10°Brix to 20°Brix) and concentration limits of 35°Brix, but had low recovery of solutes and flavor volatiles. The HR-95 and HR-98 had similar processing capacities of 15–16 L/m²/hr and concentration limits of 20–25°Brix at 45 bar. The recoveries of 97% solutes and 87% apple flavor volatiles were obtained using either the HR-95 or the HR-98.

INTRODUCTION

REVERSE OSMOSIS (RO) or hyperfiltration (HF) is a general process for the separation of substances in a fluid solution by permeation under pressure through an appropriate membrane (Sourirajan, 1978). The potential of developing reverse osmosis as a concentration technique to remove water or undesirable components from liquid food products for commercial use has been of interest to the food industry for about 20 years. Reverse osmosis has several advantages with respect to water removal over traditional evaporation techniques. Because lower heat is used, thermal damage to products is generally eliminated. Other advantages are increases in aroma retention, reduction in energy consumption, and lower capital equipment costs (Merson et al., 1980). Such membrane processing operations will become more and more attractive as membrane technology expands and the cost of energy increases (Deshpande et al., 1982).

The concentration limits of commercial membranes for the reverse osmosis processes on liquid food, at present, range from about 20–35°Brix. This limit restricts the competition of reverse osmosis with evaporation techniques on highly concentrated products; such as commercial frozen concentrated fruit juices, most of these products are about 42°Brix. The reverse osmosis process can either be employed as a preconcentration step to provide a feed juice for evaporation, held in storage, or transported for further processing. The RO-evaporation combination process has the advantages of reducing energy consumption and increasing production capacity (Claussen, 1982).

Another efficient use of the reverse osmosis technique would be to concentrate fruit or vegetable juices two or three-fold, followed by aseptic packaging, which may provide for marketing of shelf-stable consumer-type concentrated juices.

For fruit and vegetable juices low in soluble solids, such as strawberry or carrot juice, or for juices produced by continuous extraction methods (Wiley and Lee, 1978;

Wiley et al., 1979; Binkley and Wiley, 1981; Lee and Wiley, 1981), the low temperature reverse osmosis technique may enable the products to be concentrated to more desirable levels without color degradation (Lee et al., 1982), change of flavor quality, or other quality loss.

Several reports on the physico-chemical properties of membranes during the RO concentration of different types of fruit juices has been studied by Matsuura et al. (1973; 1974; 1975), and Pereira et al. (1976). These studies were theoretical in nature and greatly aided the development of membrane technology. Merson and Morgan (1968) and Merson et al. (1980) used reverse osmosis to concentrate tomato, orange, and apple juices. They concluded that the retention of flavor components is the major factor of concern regarding the quality of the final products. Lee et al. (1982) successfully developed a pilot scale RO technique to concentrate beet juice for use as a natural colorant; however, they were primarily concerned with the removal of beet-like flavor volatiles and concentration of pigment.

Of primary importance to the food industry are the following considerations that still remain unanswered: (1) the extent to which RO techniques may replace evaporation; (2) the quality of the RO concentrated products; and (3) the problems associated with transfering experimental scale technology to industrial applications.

The objectives of these experiments were to concentrate apple juice using pilot scale reverse osmosis equipment, and to evaluate process efficiency and to determine the quality of the concentrated products.

MATERIALS & METHODS

Preparation of feed juices

Aroma stripped frozen apple juice concentrate, 72°Brix, and 150-fold apple essence (same lot) were provided by Knouse Foods Inc., PA. The apple juice concentrate was diluted with distilled water to 10°Brix to simulate average single strength apple juice in the United States. Solutions of 0, 1, 2, and 3X strengths of 150-fold essence were added to 10°Brix apple juice as a feed juice for the RO process. The juice was found to have a pH value of 4.48 and contained 0.106% acids, 5.54% fructose, 2.59% glucose, and 1.77% sucrose.

Thirty liters of feed juice for each pilot scale experiment were prepared one hour before the RO process in order to maintain freshness and integrity.

Apparatus

The De Danske Sukkerfabrikker (DDS) Lab Module 20 plate and frame UF-RO system (Copenhagen, Denmark) as shown in Fig. 1, was used to conduct the RO processes in this study. The membrane section composed of membrane spacers (H), membrane support plates (I), drain papers (J) and membranes (G), were assembled and compressed using a hydraulic pump (C).

Feed juice (D) stored in the reservoir was pumped by suction pressure into the positive pump (B) where it became a high-pressure stream entering the module (A) at a speed of 8 L/min. The juice is concentrated as it flows upward through the 0.3–0.5mm membrane channel then exits from the outlet (E), returning to the reservoir. As juice flows through the membrane section, the permeate passes through the membrane into the thin channels of the membrane support plate (I) and exits via the permeate tubes (F).

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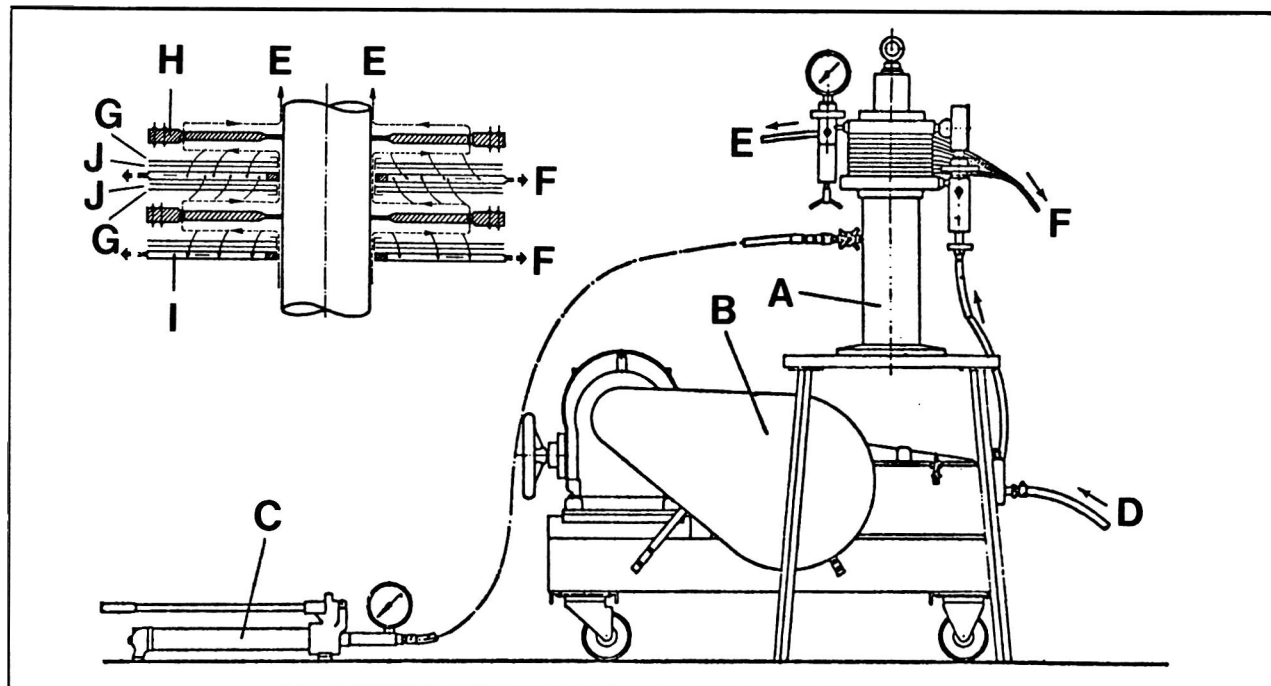
Fig. 1—Construction of DDS Lab Module 20 UF-RO System:

(A) RO Lab Module,
(B) RANNIE pump,
(C) Hydraulic pump,
(D) Feed juice inlet,
(E) Concentrated juice outlet,
(F) Permeate outlet,
(G) Membrane,
(H) Membrane spacer,
(I) Membrane support plate,
(J) Drain paper.

Membranes

Twenty membranes with a total effective membrane area of 0.36m² were installed in the module for each RO process throughout the study. Four types of commercial cellulose acetate (CA)) and high resistence (HR) membranes, CA-865, CA-990, HR-95, and HR-98 (Niro Atomizer Inc., Columbia, MD), were evaluated. The specifications of these four types of membranes and the operating conditions used in this study are listed in Table 1.

Two models of membrane configuration were designed in this study. Model one was equipped with either CA-865 and CA-990 alternately stacked, or HR-95 and HR-98 alternately stacked in the RO module. Model two was equipped with a single type membrane, i.e., CA-865, CA-990, HR-95, or HR-98 in the module. The alternately stacked membrane sets were used to evaluate the characteristic differences between the CA and HR membranes, and the single type membrane sets were used to evaluate the function of each type of membrane.

Reverse osmosis processes

Thirty liters of apple juice were concentrated by RO process on a batch basis for each experiment. Concentrating apple juice using HR membranes or CA membranes was terminated at 20°Brix or 25°Brix, respectively, due to the different concentration limits of membrane types.

Each membrane set was operated at 35, 40, and 45 bar to evaluate the pressure effects on RO concentration. Operating temperature was maintained mainly at 20°C by a heat exchanger,

with the exception of a test run of the alternately stacked HR membranes maintained at 45°C. A total of 84 RO experiments were performed in this study. The processing time of RO processes varied from two to six hours and depended on the type of membrane, operating pressure, operating temperature, and final concentration of product.

The equipment was cleaned in place after each RO process, in order to maintain the flux for the next RO process. Solutions of 1% Terg-a-zyme (DDS, Copenhagen, Denmark) were used to clean CA membranes. Cleanings of HR membranes were accomplished by using 0.1% NaOH solutions.

Analytical

For each RO process, the percent soluble solids contents in apple juice and permeate were monitored by a Bausch & Lomb Abbe 3L (Rochester, NY) refractometer (sensitivity 0.1%) at 20°C. The refractometer was calibrated using distilled water (0°Brix).

The solute compositions of starting feed juice, final concentrate, and composite permeate, collected from each RO process, were analyzed as follows:

Sugar composition analysis. The major sugars which exist in the feed juice, concentrated juice, and permeate, i.e., fructose, glucose, and sucrose, were determined by a carbohydrate column using Waters Associates Model ACC/GPC 244 high performance liquid chromatography (HPLC), equipped with R401 differential refractometer detector (Waters Associates, Milford, MA). The analytical procedures used followed Solomos and Warman (1982), using acetonitrile/water (80/20) as an eluting solvent.

Table 1—Specifications of DDS RO membranes and operating conditions

Membrane type	% NaCl permeability	Approximate cut-off MW value	Recommended operating conditions			Experimental operating conditions		
			pH	°C	bar	pH	°C	bar
CA-865	70	500	2- 8	0-30	0-40	4.5	20	35-45
CA-990	10	350	2- 8	0-30	0-50	4.5	20	35-45
HR-95[a]	5	—	3-11	0-50	0-40	4.5	20, 45	35-45
HR-98[a]	1.5	—	3-11	0-50	0-60	4.5	20, 45	35-45

[a] Thin film composite membrane of proprietary nature

A solution containing 1% fructose, 1% glucose and 1% sucrose was prepared as an analytical standard solution. The sugar content in the sample was calculated from the relative peak heights of the injections of 25 μl standard solution, 4 μl feed juice, 1.5 μl concentrated juice, and 25 μl of permeate.

Titratable acidity. The total acid content in each feed juice, concentrated juice, or permeate sample was analyzed by titratable acidity method. Twenty grams of each sample was titrated to pH 8.3 with 0.1N NaOH using a pH meter. Titratable acidity of a sample is expressed as percent malic acid by weight using following formula:

$$\% \text{ Malic acid} = \frac{\text{ml of NaOH} \times \text{Normality of NaOH} \times 0.067}{\text{Weight of sample}} \times 100$$

Flavor analysis. Forty milliliters of each feed juice, concentrated juice, or permeate sample were placed in a 50 ml volumetric flask, sealed, and incubated in a 55°C water bath before analysis. After allowing 20 min for equilibration of each flask, a 1 ml headspace sample was injected into a 6m x 3.2mm O.D. stainless steel column packed with 15% carbowax 20M on Chromosorb WAW (60/80 mesh) at 100°C isothermally in a Hewlett Packard Model 5830A gas chromatograph (GC) equipped with a flame ionization detector (FID) and a Model 18850A computerized terminal recorder (Hewlett Packard, Palo Alto, CA). Other instrumental conditions were injector temperature; 105°C, detector temperature; 250°C, and flow rate; 30 ml/min using helium as a carrier gas.

Total flavor volatiles of a sample was determined by summation of the integrated peak areas from the GC chromatogram, excluding peak areas of air.

The apple flavor volatiles in the sample were examined by sniffing flavors from the outlet of the gas chromatograph with the detector flame off. The total apple flavor volatiles were determined by summation of those peak areas being identified as possessing fruity or apple-like aroma. These included three major compounds responsible for apple aroma: ethyl-2-methylbutyrate, hexanal, and trans-2-hexenal with retentions of 4.15, 5.05, 9.83 minutes, respectively, and several unidentified compounds which had apple-like nature (Flath et al., 1967; 1969; Jepsen, 1978).

Data calculation

The permeate flux, measured as liters per meter squared per hour (L/m^2/hr) was calculated from the volume of permeate obtained at 30 minute intervals in RO process.

The percent solute recoveries of the final concentrated product after the reverse osmosis process were computed as follows:

$$\% \text{ Solute recovery} = \frac{\% \text{ Solutes} \times \text{Volume of concentrated juice}}{\% \text{ Solutes} \times \text{Volume of feed juice}} \times 100$$

$$\text{or} = \frac{\text{Solutes in the concentrated juice}}{\text{Solutes in the feed juice}} \times 100$$

Statistical analysis

Statistical analyses were performed using the Statistical Analytical System (SAS) (Barr et al., 1979) on an IBM 3033 computer at the Washington Computer Center (WCC). Collected data were analyzed for statistical significance using analysis of variance (ANOVA) and the Duncan multiple range test among the means.

RESULTS & DISCUSSION

Permeate flux and processing capacity

Fig. 2 and 3 show the permeate flux as a function of feed concentration during the RO processes at 35 bar and 45 bar operating pressures, respectively. The osmotic pressure of the feed juice increased as the feed concentration increased during the RO process. This increase in osmotic pressure reduced the difference between operating pressure and osmotic pressure, which caused the reduction of permeate flux. This fact also can be used to explain the low permeate flux at the low operating pressure. Permeate flux about 15 L/m^2/hr is considered to be economically feasible in the RO process.

Recovery of the original flux throughout the study was ensured by cleaning the module after each RO process.

Minimal membrane fouling was found, due to the application of clear apple juices and adequate cleaning processes.

The HR-95, HR-98, and CA-990 membranes had similar characteristics of processing capacity (Table 2) and concentration limits (Fig. 2 and 3). When concentrating apple
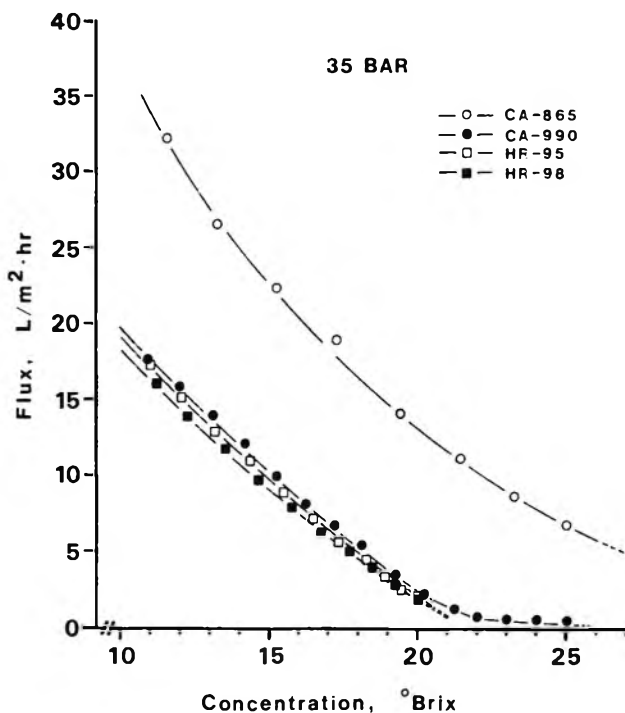
Fig. 2—Flux as a function of feed concentration, using CA-865, CA-990, HR-95, and HR-98 membranes and operated at 35 bar pressure in the RO process; juice flow rate: 8 L/min.
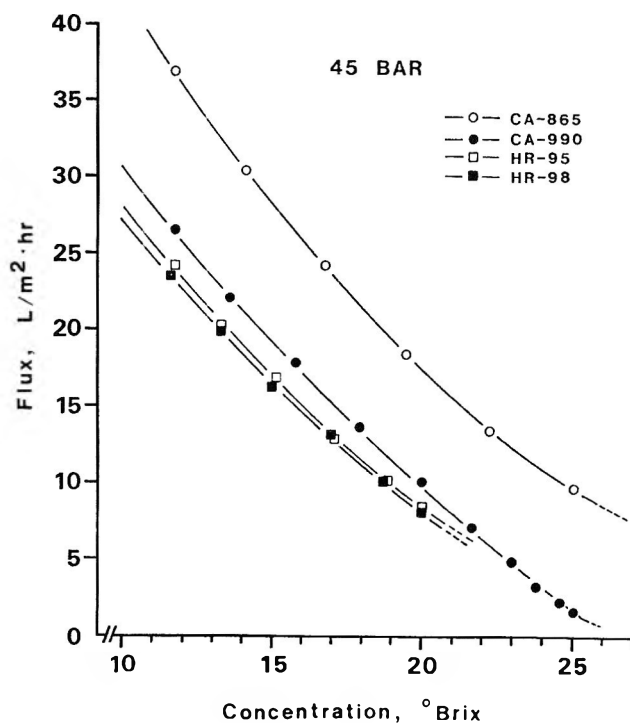
Fig. 3—Flux as a function of feed concentration, using CA-865, CA-990, HR-95, and HR-98 membranes and operated at 45 bar pressure in the RO process; juice flow rate: 8 L/min.

juices at 20°C and 35 bar pressure, these membranes had concentration limits of about 20°Brix, and the average processing capacities of 10–20°Brix ranged from 8.1 L/m²/hr using HR-98 membranes to 8.8L/m²/hr using CA-990 membranes. Using the same membranes operated at 45 bar pressure, the average processing capacities increased to 15.4 L/m²/hr and 17.9 L/m²/hr. Concentration of the apple juice to 25°Brix using these membranes was accomplished at 45 bar pressure. The larger pore-sized CA-865 membrane in RO process had much higher processing capacity of 22.0 L/m²/hr and 26.9 L/m²/hr (from 10–20°Brix), and was able to concentrate apple juice to 25°Brix and 35°Brix, under the 35 bar and 45 bar operating pressures, respectively. Operating CA-865 and HR-95 at 45 bar, which were higher than the recommended operating pressure range (0–40 bar), did not change the functions and the characters of these type of membrane.

Table 2—Average processing capacity of using CA and HR membranes concentrated to 20°Brix and 25°Brix at 20°C

Membrane type	Operating pressure (bar)	Conc to 20°Brix (L/m²·hr)	Conc to 25°Brix (L/m²·hr)
CA-865**	35	22.0	17.1
	40	26.3	21.2
	45	26.9	21.6
CA-990**	35*	8.8*	—
	40	13.5	5.6
	45	17.9	11.1
HR-95*	35	8.2	—
	40	12.3	—
	45	15.9	—
	40 (45°C)	24.4	—
HR-98*	35	8.1	—
	40	11.7	—
	45	15.4	—
	40 (45°C)	23.1	—

* Concentrated to 20°Brix
** Concentrated to 25°Brix

Retention of percent soluble solids and sugars

The 10°Brix apple juices prepared for this study had an average sugar content of 5.54% fructose, 2.59% glucose, and 1.77% sucrose. Retention of sugars, especially fructose because of its sweetness, is considered as the major objective in fruit juice concentration.

Table 3 shows that the CA membranes were unable to retain as much of the soluble solids as the HR membranes, under the same operating conditions. Concentrating apple juice to 25°Brix using CA membranes showed higher loss of soluble solids and sugars, when compared with 20°Brix concentration. All HR membranes were able to retain more fructose than glucose. The low retention of fructose with CA membranes will reduce the sweetness of the concentrated product. In both CA and HR membranes, fructose and glucose appeared to be more permeable than sucrose. This is because sucrose has a lower Taft number. The lower the Taft number for a solute, the higher the retention (Taft, 1956; Matsuura and Sourirajan, 1971).

The HPLC chromatograms of feed juice, concentrated juice, and permeate collected from CA-865 and HR-95 RO processes are shown in Fig. 4. The high sugar content in the CA-865 permeate demonstrates the lower retention of sugar using this membrane, as compared to the HR-95 membrane.

Retention of acid components

While the average acid content (0.106%) in the 10°Brix feed juice was lower than normal single strength apple juice, the lower acid content did not appear to affect the percentage recovery of acid components in the RO processes. Table 3 also indicates that HR membranes had higher retention of acid components than CA membranes. The maintenance of a constant sugar/acid ratio in the processing of apple products is important to assure good flavor quality.

Retention of flavor volatiles

It has been generally recognized that the success of concentrating fruit juice by reverse osmosis depends on the adequate recovery of the flavor components (Matsuura et

Table 3—Percent recovery of percent soluble solids, sugars, and titratable acidity using CA and HR membranes at 20°C

Membrane type	Operating pressure (bar)	Soluble solids at 20°Brix (%)	Soluble solids at 25°Brix (%)	Fructose (%)	Glucose (%)	Sucrose (%)	Titratable acidity (%)
CA-865 and CA-990** alternately stacked	35	80.4[a]	70.2[a]	59.9[a]	66.7[a]	84.9[a]	61.2[a]
	40	85.8[b]	78.0[b]	67.6[b]	74.8[b]	89.0[b]	73.2[b]
	45	85.5[b]	78.4[b]	67.7[b]	75.4[b]	89.1[b]	73.4[b]
CA-865**	35	77.8	65.9	59.6	60.0	72.9	60.6
	40	82.2	70.1	63.0	64.2	76.4	63.9
	45	82.2	73.9	67.2	68.4	76.9	67.7
CA-990**	35*	95.1*	—	90.7*	91.9*	91.3*	95.6*
	40	94.8	93.2	88.7	89.2	88.1	82.6
	45	94.8	93.2	87.4	87.7	86.8	82.6
HR-95 and HR-98* alternately stacked	35	96.4[c]	—	93.5[c]	92.8[c]	96.4[c]	94.0[c]
	40	95.8[c]	—	93.5[c]	92.6[c]	95.7[d]	93.6[c]
	45	95.0[d]	—	93.2[c]	92.4[c]	95.1[e]	92.5[d]
	40 (45°C)	97.7	—	95.3	94.8	98.1	93.7
HR-95*	35	96.7	—	94.7	93.0	96.9	95.2
	40	97.0	—	94.9	93.0	96.7	93.4
	45	96.0	—	93.7	92.0	96.2	92.8
HR-98*	35	96.9	—	93.5	92.6	95.6	98.5
	40	97.0	—	93.3	92.5	95.3	96.6
	45	96.4	—	93.2	92.0	95.1	95.1

*Concentrated to 20°Brix
**Concentrated to 25°Brix
a,b,c,d,eSignificantly different at 5% level

al., 1975). In this study, the use of HR membranes demonstrated excellent retention of flavor volatiles. Recoveries of 79.4% of total flavor volatiles and 81.0% of apple flavor volatiles were obtained under high operating pressure using HR membranes and concentrating to 20°Brix, as shown in Table 4. In addition, operating HR-98 at 45 bar recovered 86.0% of total flavor volatiles and 87.0% of apple flavor volatiles. However, while concentrating with CA membranes to 25°Brix, only 25.2% of total flavor volatiles and 16.9% of apple flavor volatiles could be recovered. In contrast to the HR membranes, the CA-990 membranes showed a lower recovery of flavor volatiles under the same operating conditions to 20°Brix; that is, only 31.3% of total flavor volatiles and 19.1% of apple flavor volatiles were recovered by the CA-990 membrane. The application of CA membranes with their low retention of flavor volatiles resulted in appreciable quality loss, making these types of membranes less suitable for concentrating apple juice. This finding is in agreement with Matsuura and his co-workers (1973; 1974), who reported the lower retention of flavor components using the CA membranes.

Although ethanol has low recoveries in CA membranes, as seen in Table 4, it seems to have better retention than other flavor volatiles. When operating HR membranes at high pressures, the percent recoveries of ethanol were lower than the recoveries of apple flavor volatiles. This indicates that ethanol was more permeable than other flavor volatiles in this type of membrane. Fig. 5 shows the GC chromatograms of feed juice, concentrated juice, and permeate collected from CA-865 and HR-95 membranes. The high content of flavor volatiles in the CA-865 permeate demonstrates the low retention of flavor volatiles of this membrane when compared to the HR-95 membranes. In the same figure, the low retention of ethanol in RO process can be observed from both the CA-865 permeate and the HR-95 permeate.

Effects of pressure on solute and aroma recovery

As seen in Table 3, there is a membrane-pressure interaction when considering these parameters on soluble solids and acid recoveries. Operating with CA membranes at 35 bar pressure resulted in a greater loss of solutes than operating at a higher pressure (45 bar), while the reverse appeared to be true for the HR membranes.

With CA membranes, this may be due to the effects of membrane compaction. The high pressure creating high compaction of the membrane set prevents the loss of solutes during the RO process. For HR membrane, the membrane compaction is minimal, therefore, the fluid containing more solutes was forced through the membrane into the permeate side by the higher operating pressure.

Fig. 4—HPLC chromatograms of feed juice, concentrated juice, and permeate collected from CA-865 and HR-95 membrane processes. (F) Feed juice, 4 µl injection volume; (C) Concentrated juice, 1.5 µl injection volume, (P) Permeate, 25 µl injection volume, (Std) Standard solution, 25 µl injection volume.

Table 4—Percent recovery of flavor volatiles using CA and HR membranes at 20°C

Membrane type	Operating pressure (bar)	Total flavor volatiles (%)	Apple flavor volatiles (%)	Ethanol (%)
CA-865 and CA-990**	35	21.8[a]	14.6[a]	26.4
alternately stacked	40	23.5[ab]	16.7[b]	27.6
	45	25.2[b]	16.9[b]	37.0
CA-865**	35	16.4	11.3	20.1
	40	20.0	13.6	24.8
	45	21.3	13.3	26.7
CA-990**	35*	31.3*	19.1*	47.2*
	40	21.4	13.8	28.8
	45	24.2	15.8	30.3
HR-95 and HR-98*	35	66.7[c]	67.1[c]	57.4
alternately stacked	40	74.5[cd]	75.9[c]	60.3
	45	79.4[d]	81.0[d]	70.1
	40 (45°C)	35.7	40.7	31.0
HR-95*	35	71.4	62.0	73.8
	40	85.2	87.7	75.0
	45	83.8	85.0	77.2
HR-98*	35	68.9	60.9	71.3
	40	86.0	85.6	74.0
	45	86.0	87.0	75.2

*Concentrated to 20°Brix
**Concentrated to 25°Brix
[a,b,c,d]Significantly different at 5% level

The retention of flavor volatiles, using either CA or HR membranes at low operating pressure, resulted in lower recoveries than that at higher operating pressures, as shown in Table 4. This may be due to the increase in osmotic pressure of the feed juice during the concentration process. The increase in osmotic pressure in the feed juice reduced the difference between operating pressure and osmotic pressure, and prolonged the operating time, so that, under the lower operating pressure, the flavor volatiles then have a greater opportunity to diffuse through the membrane into the permeate side. High operating pressure should prevent the loss of apple flavor volatiles during the RO process.

Characteristics of permeates

The examination of the permeate could also verify the functions of each type of membrane. Table 5 describes the permeates which were collected from different membrane processes.

Compared with CA-990, the concentration of apple juice to 25°Brix using the CA-865 membrane removed a large quantity of solvent (72.3% at 45 bar to 75.3% at 35 bar). The high solute content in the CA-865 permeate resulted in the reduction of the final volume of concentrated product. This reduction demonstrated the low recovery of solutes in this type of membrane. The low solute content in the permeates of HR-95 and HR-98 mem-

branes indicated a good retention of solutes.

The permeates collected from 20°C HR-95 and HR-98 membrane operations possessed only a weak apple flavor. These results were confirmed by gas chromatography and verified that the operation of HR membranes under ambient temperature (20°C) showed the best retention of apple flavor volatiles (Fig. 5).

Effects of added essence levels on flavor recovery

Table 6 presents the results of added essence effects on flavor recoveries using CA and HR membranes. With one exception of 1X and 3X essence using the CA membrane on apple aroma recovery, there was not significant difference (P > 0.05) between essence strength and percent flavor recovery. These results suggested that the flavor recoveries of apple essence were not affected by the initial amounts of essence added to the feed juice.

Effects of high temperature RO process on solute recovery

According to manufacturer's specifications, commercial HR and CA membranes can be operated within a temperature range of 0–50°C, and 0–30°C, respectively.

While concentrating apple juice to 20°Brix using HR membranes at 40 bar pressure, the average processing capacities increased from 12.3 L/m²/hr to 24.4 L/m²/hr as the temperature increased from 20 to 45°C (Table 2). Reten-
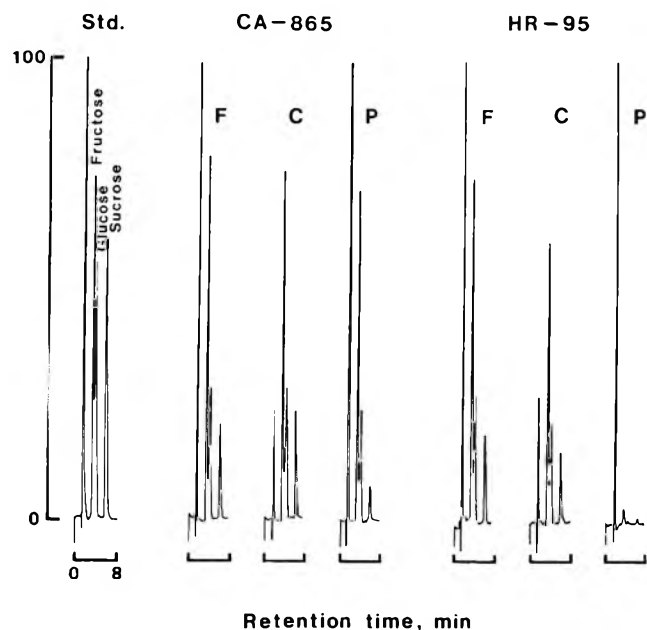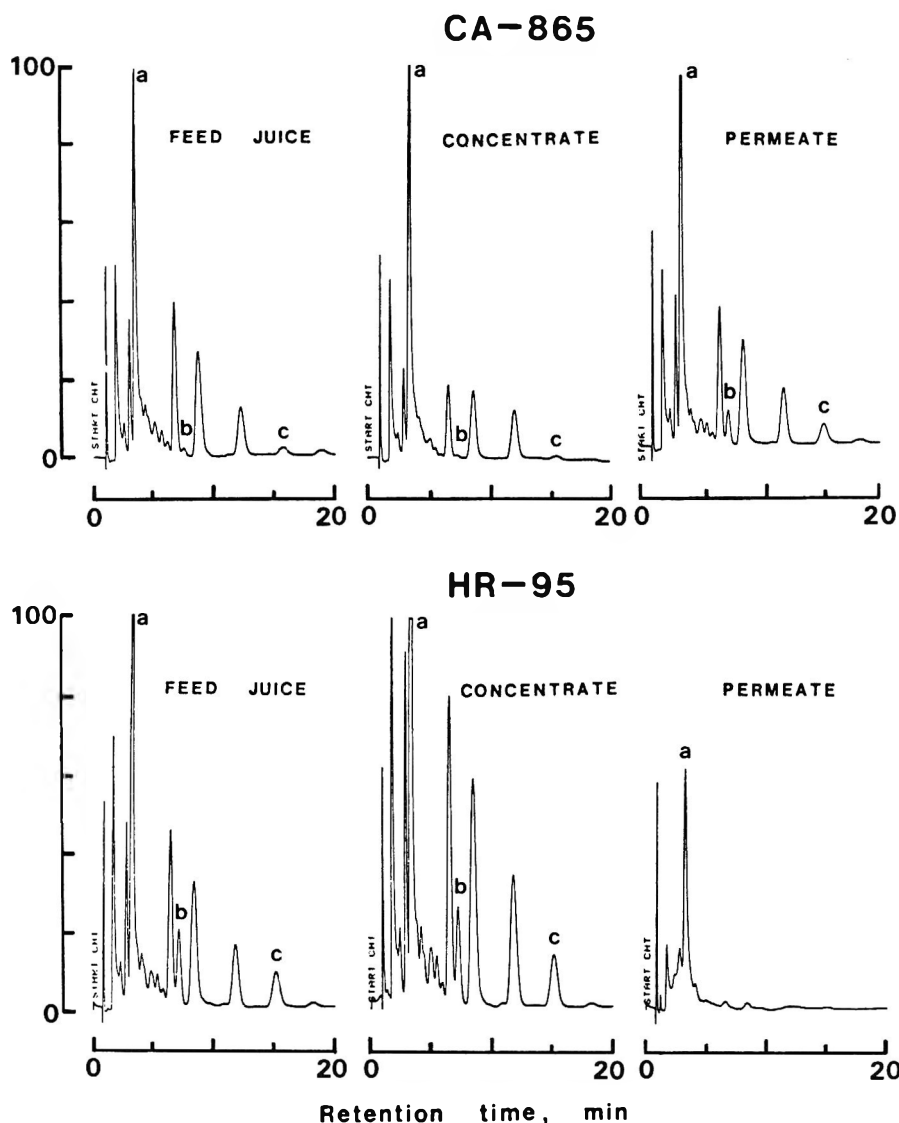

Fig. 5–GC chromatograms of feed juice, concentrated juice, and permeate collected from CA-865 and HR-95 membrane processes. (a) Ethanol; (b) n-Hexanal; (c) trans-2-Hexenal.

Table 5—Characteristics of permeates collected from different membrane processes

Membrane type	Operating pressure (bar)	Water removal (%)	% Soluble solids	pH	Titratable acidity (%)
CA-865**	35	75.3	3.6	4.52	0.051
	40	73.7	3.3	4.53	0.042
	45	72.3	2.7	4.55	0.039
CA-990**	35*	54.4*	0.2*	4.63*	0.012*
	40	65.1	0.4	4.60	0.017
	45	65.1	0.2	4.61	0.015
HR-95*	35	53.7	<0.1	5.91	<0.002
	40	54.0	<0.1	5.92	<0.002
	45	53.5	<0.1	5.89	<0.002
	40 (45°C)	53.2	<0.1	5.82	<0.002
HR-98*	35	53.8	<0.1	5.45	<0.002
	40	53.5	<0.1	5.47	<0.002
	45	53.6	<0.1	5.30	<0.002
	40 (45°C)	53.2	<0.1	5.46	<0.002

* Concentrated to 20°Brix
** Concentrated to 25°Brix

Table 6—Effects of added essence strengths on the percent flavor recovery using CA and HR membranes at 20°C

Membrane type	Essence strength (X)	Total flavor volatiles (%)	Apple flavor volatiles (%)
CA**	1 (normal)	24.5[a]	17.2[a]
	2	22.3[a]	15.7[ab]
	3	23.6[a]	15.2[b]
HR*	1	75.2[b]	73.5[c]
	2	72.4[b]	75.7[c]
	3	73.1[b]	74.8[c]

*Concentrated to 20°Brix
**Concentrated to 25°Brix
[a,b,c]Significantly different at 5% level

tion of soluble solids and sugars was slightly increased at high operating temperatures (Table 3), but retention of flavor volatiles decreased from 74.5% to 35.7% (Table 4). The higher temperature operation seemed to have no effect on total acids recovery (Table 3). The concentration of aroma stripped apple juice by reverse osmosis using HR membrane at higher temperature is feasible with the advantages of high process capacity and high recovery of non-volatile components.

CONCLUSIONS

THE RESULTS of this study have demonstrated that the reverse osmosis using pilot scale equipment is technically feasible for concentrating 10°Brix apple juice to 20–25° Brix at 20°C with good retention of apple flavor volatiles. This amounts to approximately 50–75% reduction in the water content of the juice, which would have potential as a consumer-type product or to be used for further processing.

The processing efficiency improved as the operating pressure increased from 35 to 45 bar. When operating equipment at 45 bar pressure with CA-990, HR-95, and HR-98 membranes, effective concentration limits of about 25°Brix were exhibited, whereas with the larger pore-sized CA-865 membrane, it was possible to concentrate apple juice to 35°Brix. In standardized experiments, i.e., concentrating juice from 10°Brix to 20°Brix, 45 bar pressure at 20°C, the CA-865 yielded a processing capacity of 26.9 $L/m^2/hr$ compared to the HR-95 membrane with a processing capacity of 15.9 $L/m^2/hr$. Permeate flux above 15 $L/m^2/hr$ is considered to be economically feasible.

In order to examine membrane effects, CA-865 and CA-990 membranes were operated concurrently and yielded low solute recoveries with an endpoint juice brix of 25°. Recoveries were 78.4% soluble solids which included 67.7%, 75.4%, and 89.1% retention of fructose, glucose, and sucrose, respectively, 73.4% retention of acids and a poor retention of apple aroma (16.9%). This was contrasted with alternately stacked HR-95 and HR-98 membranes which yielded good quality apple juice at 20°Brix. Recoveries were 95.0% soluble solids, comprising recoveries of 93.2%, 92.4%, and 95.1%, fructose, glucose, and sucrose, respectively, 92.5% retention of acids and 81.0% retention of apple flavor volatiles. The HR membranes generally showed good apple aroma retention and a higher retention of fructose than glucose. The optimal solute recoveries of HR membranes were not greatly affected by the pressures used in these experiments; however, apple flavor volatile recovery of up to almost 88% was found using the HR-95 membranes at 40 bar. This recovery was not affected by 1, 2, and 3X levels of apple essence added to the feed juice.

High temperature operation (45°C) at 40 bar pressure using the alternately stacked HR-95 and HR-98 membranes increased processing capacity from 12.3 $L/m^2/hr$ to 24.4 $L/m^2/hr$, but reduced flavor recovery by more than 50%. One can expect the processing capacity to increase 3–4% for every 1°C increase in temperature. This technique may be used to concentrate previously stripped single strength apple juice because of its high solute recoveries (97.7% soluble solids and 93.7% acids) and high processing capacity.

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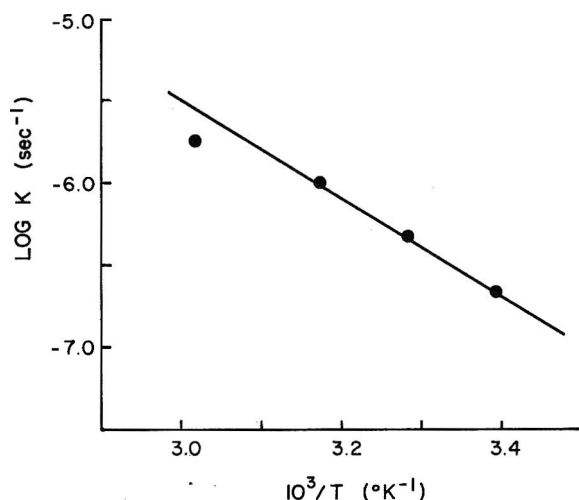
Fig. 5—Arrhenius plot for disappearance of malvidin-3-glucoside with excess acetaldehyde.

Further clarification of these condensation reactions is needed. The isolation and structural analysis of the polymers produced would aid in determining the condensation reaction mechanisms.

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Stability of Color in 'Concord' Grape Juice and Expression of Color

W. A. SISTRUNK and H. L. GASCOIGNE

─────────────ABSTRACT─────────────

The experiment was designed to determine the influence of chemical additives, ascorbic acid and storage time on 'Concord' grape juice color. Adding low concentrations of $CaSO_4$ and $SnCl_2$ increased retention of color as measured by Color Difference Meter (CDM) L, a and b values and by color expression a/L, aL and ab/L. Rapid color changes occurred during storage after 3 months except in juice with added $CaSO_4$ and $SnCl_2$. Ascorbic acid fortification accelerated color loss in some of the treatments. The highest correlations between sensory quality and instrumental color were those between sensory ratings for browning, flavor and hue and CDM L values, anthocyanins and absorbancies at 535/430 nm.

INTRODUCTION

THE LOSS of color during the period between mechanical harvesting and heating of grapes, enzyme treatment, pressing and pasteurization is significant for 'Concord' grapes. During low-temperature, bulk tank storage of the juice, further losses occur as a result of tartration and chemical changes. Greater losses occur in juice with high acid and phenolic content than in juice with low acid and low phenolic content. Certain chemicals such as reducing and chelating agents, antioxidants and acid neutralizers have been shown to stabilize anthocyanins (Acy) in pure juice and grape drinks (Skalski and Sistrunk, 1973). Ascorbic acid (AA) and oxygen contribute significantly to pigment breakdown in grape juice and development of browning (Sistrunk and Cash, 1974; Sistrunk, 1972). AA has been shown to delay browning in the presence of active polyphenoloxidase (PPO) until the AA was degraded to a low level allowing nonenzymatic reactions to accelerate the browning process. Ketose sugars, amino acids and AA increased nonenzymatic browning in grape juice heated at 75°C (Sistrunk, 1972).

A 19 year study of several 'Concord' grape vineyards showed that acidity and color varied considerably between years and vineyards with no predictability in relationship to maturity as measured by soluble solids (Morris et al., 1980). At 16% soluble solids, the juice color ranged from 6.4–15.6 optical density (OD) units between years and from 8.1–15.1 OD units between vineyards during the same period.

Postharvest changes in color of 'Concord' grapes are mainly due to the instability of Acy and occur very rapidly in the fresh grapes following mechanical harvest (Morris et al., 1979). The enzyme PPO was shown to be associated with Acy pigment degradation (Cash et al., 1976), and was also shown to be inhibited by the use of SO_2 (Cash et al., 1976; Morris et al., 1979). Acy are very unstable in fruit juices because of the influence of pH, metal complexes and enzymes (Asen et al., 1969; Skalski and Sistrunk, 1973; Wrolstad et al., 1970). Many of the changes in Acy in grape juice appear to be autodegradative and conditioned by many natural constituents (Skalski and Sistrunk, 1973).

A major cause of color degradation is the loss occurring during the commercial process of heating grapes to a

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temperature of 60°C, adding the pectinase and holding 30–60 min to depectinize the juice. It has been shown that over 50% of the Acy could be lost during this process (Sistrunk, 1972). The phenolic content was higher initially when grapes were extracted at 80–90°C as compared to 60°C, but after 12 months storage there was no difference in phenols between temperatures. The color remained stable after 12 months of storage at 24°C in bottled juice extracted at 80–90°C.

Color of Acy pigmented fruits has been expressed by many color functions when measured by different color instruments. Color Difference Meter (CDM) L and a values have been found to be a useful guide in determining the amount of browning and red color development in grape juice (Sistrunk and Cash, 1974; Morris et al., 1979; Wrolstad et al., 1980). Sastry and Tischer (1952) used OD at 515 nm to measure color degradation of grape juice at various processing temperatures. The absorbancies at 535/420 nm were also used to determine the effects of enzymes, phenolic treatments and pH on 'Concord' grape juice (Skalski and Sistrunk, 1973; Cash et al., 1976; Sistrunk, 1972). Wrolstad et al. (1970) found that CDM L values gave a good correlation with visual scores on color of fresh and frozen strawberries. During storage at 25°C, Acy pigments and ΔE values were used to follow the degradation of pigments in cranberries (Starr and Francis, 1973).

Studies conducted on Zinfandel grapes to determine the effects of heating on grape juice color showed that heating in sealed tubes in a boiling water bath for 1–2 hr caused the juice color to fade. However, after prolonged heating, the juice turned brown (Ponting et al., 1960). The ketose sugar, fructose, in grape juice was found to be more instrumental in producing browning than was sucrose. As AA is degraded rapidly in the presence of air, it produces browning (Sistrunk, 1972).

The objectives of this study were to assess the influences of certain individual additives and combinations of additives on the loss of color in pasteurized 'Concord' grape juice during storage and to determine the color functions that result in the highest correlation with sensory color judgment of grape juice in a wide range of color hues and brown pigment formation.

MATERIALS & METHODS

THE 'CONCORD' GRAPE JUICE was obtained from Welch Foods, Inc. (Springdale, AR) after the juice had been enzyme-hydrolyzed and filtered. The experiment was designed as a factorial with 11 treatments: Control, 0.75% $CaSO_4$, 0.75% $CaSO_4$ + 10 ppm butylated hydroxyanisole (BHA), 0.75% $CaSO_4$ + 25 ppm $SnCl_2$, 25 ppm $SnCl_2$ + 50 ppm $SnCl_2$, 100 ppm $SnCl_2$, 80 ppm SO_2, 500 ppm ethylene-diamine-tetracetic acid (EDTA)-Na salt, 500 ppm EDTA + 80 ppm SO_2, and 500 ppm EDTA + 10 ppm BHA; two ascorbic acid levels: 50 mg/100 ml and no ascorbic acid; and four storage times: 0, 3, 7 and 18 months. The juice was divided into 22 lots of 3,000 ml each, treated with the different additives, and mixed thoroughly. Eleven of the lots were fortified with 50 mg/100 ml of L-ascorbic acid and the other eleven lots contained no ascorbic acid. The juice was heated to 85°C and each lot was filled into 12 "211 x 304" R-enamel cans, sealed and cooled in cold tap water after holding the samples for 5 min. After cooling, the cans were stored at 24°C until the analyses were made.

Duplicate samples of each lot were analyzed the day after processing and the other samples after 3, 7 and 18 months of storage. The vacuum was measured on the cans prior to analysis to follow changes in vacuum during storage. The cans were then opened and the clear juice was decanted from the upper 2/3 of the can to prevent suspended particles from interfering with the color measurement.

Color was measured on 10 ml of juice in a plastic cup with an optical glass bottom on a CDM that was standardized against a white plaque of the following readings: L = 93.2, a = −1.4 and b = 3.2. The depth of the juice was 5 mm, and the white plaque was placed over the top of the cup during the measurement.

One ml of juice was diluted to 50 ml with 1% oxalic acid to determine reduced ascorbic acid by the method of Morell (1941). One ml of juice was diluted with 1% HCl in ethanol to measure total color as anthocyanins (OD units/mg) fresh weight (fw) as previously described (Sistrunk and Morris, 1978). A ratio of the absorbancies at 520/430 nm was used as a measure of color degradation. One ml of juice was diluted to 50 ml with deionized water to measure total phenols by the Folin-Ciocalteau method (Swain and Hillis, 1959). The tartrates and other insoluble solids were measured by filtering the lower 1/3 of the contents of the cans through tared 10 × 10 cm miracloth papers, drying at 70°C for 24 hr and calculating percent dry weight.

Sensory evaluation for color intensity, freedom from browning, color acceptance, and flavor was conducted by a panel of 15 graduate students and faculty on a 9-point hedonic scale: 9 represented intense purple color, free from browning and highly acceptable in color and flavor; whereas 1 represented extremely light color, extremely brown and poorly acceptable in color and flavor. The grape hue (color) was further defined by assigning color values as follows: 1, medium brown; 2, light brown; 3, light red brown; 4, light red; 5, medium red; 6, red purple; 7, medium purple; 8, dark purple; and 9, purple blue. The panel was instructed on the rating scale and shown samples of product that represented the different levels of quality.

The color ratings were made in white 6 cm diameter porcelain dishes with 5 ml of juice in each. Flavor was evaluated by sampling from beakers of juice with a plastic spoon. All ratings were made on an open countertop in a room designed with two solid banks of fluorescent lights so that the room could be lighted with both average and high intensity lighting. The average lighting was used since this was better for good perception by the panelists.

The data were analyzed as factorials by analysis of variance using the Statistical Analysis System (SAS) of the Univ. of Arkansas Computer Center. Wherever F values were significant, the Duncan's Multiple Range Test was used to separate the means of main effects. Significant differences between means of interactive effects were separated by the Least Significant Difference (Snedecor and Cochran, 1967). The means of the main effects and the most important interactive effects are recorded in tables.

RESULTS & DISCUSSION

CONCORD GRAPES grown in Arkansas are usually lower in acidity and more red-purple in color at harvest as compared to the typical purple color and higher acidity of grapes grown in the Northern United States. Improvement of color and color stability by chemical treatment during long-term storage would be an asset to the grape juice industry in the region.

The addition of chemicals at low concentrations did have a significant effect on the color of pasteurized grape juice as indicated by the means of main effects during 18 months of storage at 24°C (Tables 1–4). Initially, the grape juice measured 10.7, 5.7 and −0.8 for L, a and b values, respectively. The Acy were 35.0 and the hue, color, and flavor preference scores were 8.6, 8.1 and 8.5, respectively. When AA was added, the juice was more red in color and slightly less acceptable by the panel. The juice treated with $CaSO_4$ and $SnCl_2$ retained the original color of the juice to a greater extent than the other treatments as shown by the L, a and b values (Table 1). Lee (1976) showed that $CaSO_4$ improved the juice color as demonstrated by higher a values and higher ratings by a sensory panel. The color of the juice of the control, SO_2 and EDTA treatments was redder (higher a and b values). Acy were higher in juice treated with SO_2, EDTA + SO_2 and 50 ppm $SnCl_2$. The browning index was usually higher in juice from these same treatments.

Titratable acidity was higher and pH lower in juice with added $SnCl_2$ (Table 2). Soluble solids were higher on all treatments than the control. All treatments except EDTA had a stabilizing effect on AA. Total phenols were higher in juice treated with 25 ppm $SnCl_2$ and with EDTA + SO_2 than the control. Only $CaSO_4$ + $SnCl_2$ lowered the phenolic content.

The color expression a/L, aL and ab/L generally show the same relationships among treatments as the L, a and b values (Table 3). Lower values in these color expressions indicate better color in grape juice. There was not a wide range in a/L values as compared to aL and ab/L. Some of

Table 1—Main effects of treatment, storage time and ascorbic acid on quality of Concord grape juice[a]

Main effects	L	a	b	Total anthocyanins OD/g fw	Browning index OD 535/430 nm
Treatment					
Control	13.1d	7.07d	0.28c	20.5e	2.39f
CaSO₄(0.75%)	12.3f	5.60e	−1.20e	21.6cde	2.67cd
CaSO₄(0.75%)+BHA (10 ppm)	11.8gh	5.40e	−3.38g	20.9de	2.79bc
CaSO₄(0.75%)+SnCl₂ (100 ppm)	11.7h	5.63e	−4.59h	21.5cde	3.07a
SnCl₂(25 ppm)	13.0d	8.58bc	0.59abc	20.5e	2.50ef
SnCl₂(50 ppm)	12.0fg	5.86e	−2.61f	23.0b	2.97a
SnCl₂(100 ppm)	12.6e	8.47bc	−0.50d	21.7cd	2.74cd
SO₂(80 ppm)	14.1b	9.15b	0.90ab	25.0a	2.91ab
EDTA(500 ppm)	13.5c	8.31c	0.40bc	21.2de	2.62de
EDTA(500 ppm)+SO₂ (80 ppm)	14.5a	10.80a	1.05a	24.8a	2.95ab
EDTA(500 ppm)+BHA (10 ppm)	13.4c	8.56bc	0.48bc	22.3bc	2.48ef
Storage time					
0 months	10.6d	8.50b	−1.46c	38.1a	3.84a
3 months	11.7c	9.25a	−0.72b	24.4b	3.13b
7 months	12.9b	7.12c	−1.22c	15.1c	2.42c
18 months	16.4a	5.47d	0.28a	10.6d	1.55d
Ascorbic acid					
50 mg/100 ml	13.2a	7.98a	−0.65b	21.6a	2.62b
No ascorbic acid	12.6b	7.13b	−0.91a	22.6a	2.84a

[a] Means separated in columns by main effects of Duncan's Multiple Range test. Numbers followed by the same letter are not significantly different.

Table 2—Main effects of treatment, storage time and ascorbic acid on quality of Concord grape juice[a]

Main effects	% Soluble solids	% Titratable acidity as tartaric	Ascorbic acid mg/100g	pH	mg/100g Total phenols
Treatment					
Control	16.9h	0.73e	38.2e	3.78a	138.5cde
CaSO$_4$ (0.75%)	17.2g	0.74e	52.8a	3.68a	199.7abc
CaSO$_4$ (0.75%)+BHA(10 ppm)	17.5def	0.73e	38.1c	3.67a	130.4ef
CaSO$_4$ (0.75%)+SnCl$_2$ (100 ppm)	17.4efg	0.73e	38.5bc	3.64a	171.0f
SnCl$_2$ (25 ppm)	17.7bc	0.93a	40.4bc	3.24b	203.5ab
SnCl$_2$ (50 ppm)	18.0a	0.89b	38.7bc	3.24b	194.9abcd
SnCl$_2$ (100 ppm)	17.9ab	0.85c	42.9b	3.24b	183.6de
SC$_2$ (80 ppm)	17.4fg	0.77d	41.8bc	3.65a	195.9abcd
EDTA(500 ppm)	17.5cdef	0.75de	29.3de	3.64a	191.2bcde
EDTA(500 ppm)+SO$_2$ (80 ppm)	17.7bcd	0.75de	32.7d	3.71a	205.1a
EDTA(500 ppm)+BHA(10 ppm)	17.7bcd	0.73e	28.9de	3.70a	192.3bcde
Storage time					
0 months	18.1a	0.91a	29.6bc	3.63a	211.0a
3 months	17.3bc	0.73c	27.9c	3.63a	189.1b
7 months	14.4b	0.78b	31.7b	3.42c	184.5bc
18 months	17.3c	0.71d	60.7a	3.55b	181.1c
Ascorbic acid					
50 mg/100 ml	17.7a	0.80a	56.4a	3.54a	201.8a
No ascorbic acid	17.4b	0.77b	18.0b	3.57a	180.6b

[a] Means separated in columns by main effects by Duncan's Multiple Range test. Numbers followed by the same letter are not significantly different.

Table 3—Main effects of treatment, storage time and ascorbic acid on quality of 'Concord' grape juice[a]

Main effects	Color expressions a/L	aL	ab/L	% Insoluble tartrates
Treatment				
Control	0.56d	90.5d	0.58e	1.54d
CaSO$_4$ (0.75%)	0.48e	66.9e	0.38f	1.92ab
CaSO$_4$ (0.75%)+BHA(10 ppm)	0.48e	62.8e	0.17h	1.56d
CaSO$_4$ (0.75%)+SnCl$_2$ (100 ppm)	0.50e	64.5e	0.09i	1.33e
SnCl$_2$ (25 ppm)	0.70b	108.2c	0.74bc	2.00a
SnCl$_2$ (50 ppm)	0.50e	71.3e	0.24g	1.79c
SnCl$_2$ (100 ppm)	0.70b	103.8cd	0.66d	1.72c
SO$_2$ (80 ppm)	0.69bc	124.2b	0.75b	1.50d
EDTA(500 ppm)	0.65c	107.9c	0.68cd	1.55d
EDTA(500 ppm)+SO$_2$ (80 ppm)	0.78a	152.5a	0.84a	1.70c
EDTA(500 ppm)+BHA(10 ppm)	0.67bc	111.2bc	0.70bcd	1.81bc
Storage time				
0 months	0.79a	92.4b	0.63b	0.00c
3 months	0.78a	109.8a	0.71a	2.12b
7 months	0.55b	93.3b	0.44c	2.27a
18 months	0.33c	91.4b	0.34d	2.32a
Ascorbic acid				
50 mg/100 ml	0.63a	103.6a	0.56a	1.71a
No ascorbic acid	0.59a	90.0b	0.50a	1.63b

[a] Means separated in columns by main effects of Duncan's Multiple Range test. Numbers followed by the same letter are not significantly different.

Table 4—Main effects of treatment, storage time and ascorbic acid on quality of 'Concord' grape juice[a]

Main effects	Hue	Sensory evaluations Color preference	Flavor preference	Freedom from browning
Treatments				
Control	6.74f	6.41e	6.87b	7.06ef
CaSO$_4$ (0.75%)	7.83bc	7.30b	7.31a	7.56d
CaSO$_4$ (0.75%)+BHA(10 ppm)	8.66a	5.53f	7.19a	9.11ab
CaSO$_4$ (0.75%)+SnCl$_2$ (100 ppm)	8.93a	5.44f	7.41a	9.43a
SnCl$_2$ (25 ppm)	7.54cd	6.87c	5.64e	7.33de
SnCl$_2$ (50 ppm)	8.19b	5.29f	6.33c	8.53c
SnCl$_2$ (100 ppm)	8.56a	7.91a	5.93d	8.90bc
SO$_2$ (80 ppm)	7.02ef	6.68cd	5.78de	6.31f
EDTA(500 ppm)	7.17de	6.46de	6.67b	6.78f
EDTA(500 ppm)+SO$_2$ (80 ppm)	7.24ef	6.56de	5.41f	7.09ef
EDTA(500 ppm)+BHA(10 ppm)	7.26 de	6.39e	6.19c	6.68f
Storage time				
0 months	8.88a	7.74a	7.86a	9.45a
3 months	8.74ab	7.18b	7.64b	8.97b
7 months	8.52b	6.36c	6.74c	7.96c
18 months	4.82c	4.48d	3.48d	4.63d
Ascorbic acid				
50 mg/100 ml	7.45b	6.13b	6.20b	7.45b
No ascorbic acid	8.01a	6.72a	6.63a	8.04a

[a] Means separated in columns by main effects by Duncan's Multiple Range test. Numbers followed by the same letter are not significantly different.

the treatments increased the insoluble tartrates in the juice; however, CaSO$_4$ + SnCl$_2$ decreased tartrates as compared to the control.

Sensory ratings for color indicated that hue and freedom from browning were higher for juice treated with CaSO$_4$ and SnCl$_2$ except the low level of SnCl$_2$ (Table 4). However, color preference was highest in juice treated with CaSO$_4$ (0.75%) and SnCl$_2$ (100 ppm), followed by SnCl$_2$ (25 ppm) and SO$_2$ (80 ppm). Flavor preference was rated highest on juice treated with CaSO$_4$ alone and in combination with BHA and SnCl$_2$.

When stored for 18 months, the color expressions a, −b, Acy and browning index decreased while L values increased as compared to no storage. This indicates that by 18 months the color of most of the juice had badly deteriorated and become brown (Table 1).

Soluble solids, titratable acidity, pH and total phenols decreased with storage time while AA increased, mainly between 7 and 18 month storage periods (Table 2).

Some of the additives had a definite stabilizing effect on AA, resulting in no net change during the first 7 months of storage. The increase in AA at 18 months, especially in some treatments, could have been caused either by the reduction of dehydroascorbic acid to ascorbic acid or by the reaction of the 2,6-dichlorophenolindephenol dye with reductones formed in degraded juice. Earlier studies have shown that reductones formed in an ascorbic acid-anthocyanin-flavanol system can react with the dye (Cornwall and Wrolstad, 1981). The insoluble tartrates increased during storage, indicating that some of the soluble solids, phenols and acids precipitate out as seen in Table 2. The color of the juice as expressed by a/L, aL and ab/L showed that the color degraded during storage although the magnitude of the changes in L, a and b appeared to be greater during 18 months of storage (Table 3).

The sensory ratings for hue, color and flavor preference and freedom from browning decreased during storage indicating a loss of color and an increase in browning (Table 4). There was a drastic change in hue between 7 and 18 months of storage.

With 50 mg/100 ml of AA added to juice the color was lighter red and less purple than in juice with no added AA as indicated by L, a and b values (Table 1). This substantiates work done by Sistrunk and Cash (1974) wherein they found that 25 mg/100 g AA added to grape juice yielded a redder and lighter juice as determined by a and L values. However, AA did not affect the Acy content but made the juice browner as indicated by the browning index.

AA added to juice increased the soluble solids, titratable acidity and total phenols of the juice (Table 2). The addition of AA increased the insoluble tartrates in the juice (Table 3) which could have been partially due to the increase in acidity. Earlier, Sistrunk and Cash (1974) showed that the addition of acids to grape juice increased the amount of insoluble tartrates in the juice. Only the color expression aL was significantly increased by the addition of AA. The higher aL values indicate more redness and a lighter color.

AA decreased the purple color in the juice as indicated by a lower hue value and decreased the color and flavor preferences (Table 4). The addition of AA to the juice promoted browning as compared to juice without added AA although this was not generally true in all the treatments.

There was a significant interaction between treatments and storage time on color and flavor preferences and freedom from browning in stored juice. Very little change in ratings occurred during storage for juice treated with 100 ppm $SnCl_2$, and $CaSO_4$ combined with either BHA or $SnCl_2$ as compared to large changes in ratings for the control, SO_2 and EDTA treatments (data not shown).

The significant interaction between treatments and the addition of AA on color preference indicated that only juice treated with $CaSO_4$, EDTA, and EDTA + BHA was significantly decreased by the addition of AA (Table 6). This same interaction on the color expression a/L showed that the a/L values for the control, $CaSO_4$ and SO_2 treatments were increased by AA, indicating poorer color. These values did not correspond to the color preference scores except in the juice treated with $CaSO_4$.

The color of the samples was measured using both the infinite layer and thin layer technique, but only data on the

Table 5—Interaction of ascorbic acid and storage time on color of 'Concord' grape juice

Storage time	CDM 'a'	Total anthocyanins OD/g fw	Freedom from browning	a/L
Ascorbic acid (50 mg/100 ml)				
0 months	9.16	38.3	9.46	0.84
3 months	9.80	23.0	8.83	0.81
7 months	7.63	15.1	7.49	0.57
18 months	5.40	10.1	4.07	0.32
No ascorbic acid				
0 months	7.82	37.9	9.45	0.73
3 months	8.77	25.9	9.10	0.76
7 months	6.60	15.2	8.42	0.52
18 months	5.53	11.2	5.18	0.34
LSD at 0.05	0.45[a]	1.6	0.44	0.37

[a] Means separated in columns by interactive effects using Least Significant Difference.

Table 6—Interaction of treatments and ascorbic acid on color of 'Concord' grape juice

Treatment	Color preference		a/L	
	50 mg/100 ml Ascorbic acid	No ascorbic acid	50 mg/100 ml Ascorbic acid	No ascorbic acid
Control	6.23	6.60	0.66	0.47
$CaSO_4$(0.75%)	6.14	8.05	0.51	0.43
$CaSO_4$(0.75%)+BHA(10 ppm)	5.55	5.51	0.49	0.47
$CaSO_4$(0.75%)+$SnCl_2$(100 ppm)	5.39	5.49	0.49	0.51
$SnCl_2$(25 ppm)	6.60	7.14	0.71	0.69
$SnCl_2$(50 ppm)	5.21	5.38	0.50	0.51
$SnCl_2$(100 ppm)	7.70	8.11	0.73	0.67
SO_2(80 ppm)	6.54	6.81	0.75	0.63
EDTA(500 ppm)	5.69	7.23	0.64	0.66
EDTA(500 ppm)+SO_2(80 ppm)	6.51	6.61	0.77	0.78
EDTA(500 ppm)BHA(10 ppm)	5.83	6.95	0.68	0.67
LSD at 0.05	0.85[a]		0.06	

[a] Means separated in double columns by measurement by Least Significant Difference.

Table 7—Correlations between sensory evaluation and expression of color by instrumental measurements on 'Concord' grape juice

Color Measurement	Freedom from browning	Sensory evaluations		Visual hue
		Color preference	Flavor preference	
Total anthocyanins OD/g fw	0.661 ***	0.518 ***	0.662 ***	0.596 ***
Browning index OD 535/430 nm	0.785 ***	0.538 ***	0.744 ***	0.761 ***
a/L ratio	0.552 ***	0.644 ***	0.583 ***	0.507 ***
aL	−0.120	0.267 **	−0.099	−0.167
ab/L	0.098	0.547 ***	0.173	0.062
L	−0.859 ***	−0.503 ***	−0.872 ***	−0.879 ***
a	0.277 **	0.536 ***	0.305 ***	0.227 *
b	−0.588 ***	0.058	−0.478 ***	−0.572 ***

*** Significant at 0.0001 probability level.
** Significant at 0.001 probability level.
* Significant at 0.01 probability level.

Some Characteristics of Whole Corn: Whole Soybean (70:30) and Rice: Whole Soybean (70:30) Mixtures Processed by Simple Extrusion Cooking

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———————ABSTRACT———————

Mixtures of corn or rice with dehulled soybeans (70:30) were extruded at three cone openings. Processing temperatures were inversely correlated with cone opening. As cone opening increased fat retention and nitrogen solubility index increased, while dispersibility, protein nutritive value and acceptability (as "atole") by school children decreased. The PER and NPR of the mixtures were significantly (P < 0.05) correlated with processing temperature (r = 0.90 for both) and sensory score (r = 0.83 and 0.82, respectively). No correlation was observed between protein nutritive value and residual antiphysiological factors of the products. After storage of mixtures for 12 wk at 4° and 25°C, fat acidity values were lower than 2%; those stored at 35°C showed nearly 10%. All samples were equally acceptable.

INTRODUCTION

AS STATED in previous studies (Bressani et al., 1978), there is a need for developing simple and relatively inexpensive technological alternatives in order to promote the establishment of successful food industries in small countries such as those of the Central American area. On the other hand, it has been reported that malnutrition in the Central American countries is mostly prevalent in infants and preschool children, and is due not only to a protein deficiency but also to a calorie deficiency (Viteri and Arroyave, 1973). The need exists then to develop alternatives of food products for children, of a high protein quality, high caloric value, high acceptability and relatively low cost, available to the nutritionally most vulnerable groups, which generally are in the low income bracket.

Previous studies (Bressani et al., 1974; Tejerina et al., 1977) have shown that when a corn:soy or rice:soy mixture is prepared in such a proportion that approximately half of the protein comes from the cereal and the remaining 50% from the soybean, the mixture resulted in a higher protein quality food than each individual component. When whole soybeans are used, the above mixture is attained using approximately seven parts by weight of cereal and three parts by weight of whole soybeans. This mixture (using rice or corn as cereal) resulted in a high protein (around 18%) and calorically improved (around 10% fat) vegetable mixture, that when adequately cooked yields a high protein quality (PER between 2.5 and 2.6) and acceptable food product. In the case of the 70:30 whole corn:whole soybean mixture, it has already been reported (Bressani et al., 1978) that a food product of an improved protein quality could be obtained by simple extrusion cooking using the Brady Crop Cooker extruder. Therefore, the possibility exists of preparing an acceptable product for supplementary child feeding that would be nutritionally adequate (protein and calorie-wise) through a simple and relatively inexpensive technology that could, in turn, be the basis for a community or cooperative agroindustry.

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The present work was undertaken to evaluate the protein quality, the acceptability by children and the storage stability of a 70:30 whole corn:whole soybean mixture and a rice:whole soybean one, processed under different conditions using the Brady Crop Cooker extruder.

MATERIALS & METHODS

THE SOYBEANS and yellow corn used in these studies were grown either at INCAP's experimental farm (1,480m above sea level) or in the lowlands of Guatemala. The rice was obtained in a local rice processing plant and was of third grade (broken rice).

Batches of 200 kg of whole yellow corn and whole soybeans were coarsely ground in a hammer mill equipped with a 3 mesh screen. The broken soybeans were then dehulled by air separation using a Ce-Co-Co air blower (Chuo Boeki Goshi Kaisha, Central Commercial Co., Ibaraki, Osaka, Japan) equipped with a 0.3 hp engine. The broken rice was used as such without any further grinding. The particle size of the three different materials was determined using a set of sieves equivalent to 12, 20, 45 and 60 mesh.

With the coarsely milled corn and the broken rice, 450 kg batches of cereal:dehulled, coarsely milled, whole soybean blends were mixed, using a 70:30 weight ratio. A sample of each was taken for chemical analysis. Three portions of 150 kg each were taken from the two cereal:soybean mixtures, and they were processed in a Brady Crop Cooker extruder model 2160 (Brady, Division of Koehring Co., Appleton, WI), using an engine throwing 100 hp to the axis and a cone opening of 0.6, 1.0 and 1.4 mm for each portion, respectively. Before processing the samples, the cone of the extruder was rebuilt through hard-surface welding and refined to the same dimensions of a new cone. The feeding auger speed in the extruder was kept constant at 32 rpm in all cases. From previous observations on similar corn:soy and rice:soy mixtures, such feeding auger speed assures an average feeding rate of 520 kg/hr. No water was added to any sample before processing and all mixtures had an average 12% moisture content. Each mixture was processed in the extruder for approximately 20 min to attain equilibrium conditions before a sample of approximately 50 kg was taken, and the extrusion temperature at the exit cone and the mass flow data were recorded.

Moisture, crude protein (N x 6.25), and oil content were determined according to the AOAC methods (AOAC, 1980). Trypsin inhibitor activity was determined by the method of Kakade et al. (1969), hemagglutinins according to the method described by Jaffé and Brucher (1972), urease activity according to Caskey and Knapp (1944), and fat acidity according to the AOAC (1980). The nitrogen solubility index (NSI) and the dispersibility index (DI) were determined by the methods described by Smith and Circle (1972). In the case of the DI, the supernatant after centrifugation was evaluated for total solids rather than for nitrogen, as indicated in the method to determine protein dispersibility index (PDI) by Smith and Circle (1972). The total solids thus estimated in the supernatant were then compared with the dry matter content in the sample used for the test to estimate the DI. All determinations were done in duplicate.

The sensory trials were carried out with students (boys and girls) from the third and sixth grades of six public schools, three urban and three rural, of Guatemala. In all cases, the samples were presented as an "atole" prepared by suspending one part of the extruded product in ten parts of boiling tap water and adding 0.5 parts of sugar. The mixture was then boiled for 5 min and the "atole" was served hot, without any flavoring agent. In general, this is the way in which the personnel of such schools usually prepare the corn-soy-milk (CSM), "Incaparina," and similar blends used in the school feeding programs in Guatemala. This prepara-

tion assures at least 20g of dry product per glass of "atole" (240g net weight on the average). Acceptability was evaluated using the consumer preference test described by Kramer and Twigg (1966). numerical values were assigned to the levels of the hedonic scale.

The protein efficiency ratio (PER) was determined by the AOAC (1980) method, using rats of the Wistar strain. The net protein ratio (NPR) was estimated according to the method described by Campbell (1963) using rats of the same strain

For the storage trials, the samples were packed in heat-sealed polyethylene bags which were placed inside a triple paper bag. The product was thus packed when it had cooled to room temperature.

Statistical analyses of the data were carried out by the methods described by Snedecor and Cochran (1967).

RESULTS & DISCUSSION

THE PARTICLE SIZE distribution of the ground, dehulled soybeans, ground whole corn and broken rice utilized are summarized in Table 1. Both the ground whole corn and broken rice presented a particle size distribution similar to that defined as coarse in a previous work (Bressani et al., 1978), where it was stated that for extrusion purposes the use of an intermediately or coarsely ground corn is preferable to a finely ground material.

The temperature recorded at the exit cone and the mass flow data obtained at each of the cone openings evaluated for the two cereal:whole soybean mixtures studied are presented in Table 2. The exit cone temperature attained in each of the two mixtures increased as the cone opening of the extruder was reduced, because the Brady Crop Cooker is a thermodynamically autogenous extruder cooker according to the classification of Rossen and Miller (1973). A slight drop in the mass flow rate was observed when the cone opening was reduced.

The protein and fat content of the raw materials and of the extruded products are presented in Table 3. The NSI and DI values obtained for the extruded products are also included. Whole corn flour had a higher oil and protein content than broken rice. Since the whole corn:whole soybean mixture had a higher oil content than the broken rice:whole soybean mixture, this could explain why the temperature attained at the exit cone was relatively lower when processing the former mixture (Table 2), especially considering that, in a thermodynamically autogenous extruder cooker (as the Brady Crop Cooker), the oil of the raw material serves as a lubricant for the operation (Molina et al., 1978). The fact that as the cone opening was reduced, the oil content of any of the mixtures tended to decrease, suggests that as the processing conditions are adjusted to attain a higher extrusion temperaure, some of the oil is expelled out of the material (probably aided by a partial protein coagulation caused by the higher processing temperature) and it is not recovered in the final product. It was noted that the product processed at the higher temperature (narrower cone opening) was quite oily to the touch as compared with that obtained at a lower temperature (wider cone opening). It is considered that the slight decrease in the percent of fat observed in the products processed at the narrower cone opening caused the slight rise in protein content percentage basis of the same products (Table 3).

It is of interest to note that although the NSI of the extruded product tended to decrease as the exit cone opening was reduced, the DI values tended to increase. A lowering in the NSI values in similar mixtures through an increase in the extrusion temperature has been reported by other workers (Harper et al., 1980; Maga, 1976) using the Brady Crop Cooker or similar simple extruders. This decrease in NSI values indicates a higher degree of cooking, suggesting a higher protein digestibility for the mixtures containing whole soybeans extruded at higher temperatures which may be due to more efficient inactivation of the

antiphysiological factors contained in soybeans. Harper et al. (1977) reported a slight protein quality improvement when whole corn:dehulled soybean (70:30) mixture was extruded at 148°C rather than at 132°C. Similar results were reported by Lorenz et al. (1980) with higher PER values for full-fat soybeans evaluated after processing at 143 and 149°C. The increase in the DI values through a decrease in the exit cone opening may be due to an increase in the damaged starch content, causing a decrease in the maximum amylographic viscosity (at 95°C) of the product, as reported by other authors (Anderson et al., 1969, 1970; Molina et al., 1978).

According to the school teachers, each child consumes on the average three glasses (approximately 240 g net weight) of "atole" per day. Each glass, as prepared, contained approximately 209g of water, 10g of sugar and 21g of product, supplying an average of 3.4g of protein. Therefore, the three glasses of "atole" consumed per child per day represented an average protein ingestion of 10 g/child/day, equivalent to 25–30% of the total protein requirement of school boys.

Table 1—Particle size distribution of ground corn and soybeans and broken rice (g/100 g)[a]

Particle size mesh	Ground corn	Broken rice	Ground soybeans
12	85.02	61.08	23.17
20	9.87	19.39	50.28
45	2.83	10.01	13.08
60	1.68	5.18	7.28
80	0.45	3.27	2.81
Pan	0.15	1.07	2.38

[a] Percent retained of each fraction.

Table 2—Processing temperature and mass flow rate attained for the corn:soy and rice:soy (70:30) mixtures when extruded at the cone opening studied

Mixture	Extruding conditions[a]		
	Cone opening (mm)	Exit cone temp (°C)	Mass flow rate (kg/hr)
Corn:soy	0.6	153	509
	1.0	143	511
	1.4	118	516
Rice:soy	0.6	161	506
	1.0	148	509
	1.4	129	513

[a] The feeding auger speed was kept constant at 32 rpm.

Table 3—Some chemical characteristics of the raw materials and of the corn:soy and rice:soy (70:30) mixtures extruded at different cone openings (dry basis)

Material	Extruder cone opening (mm)	Chemical characteristics (%)			
		Protein (N x 6.25)	Ether extract	Nitrogen solubility index	Dispersibility
Corn	—	10.3	3.6	—	—
Rice	—	8.5	0.5	—	—
Dehulled soybeans	—	35.7	26.1	—	—
Corn:soy	0.6	17.8	8.9	32.8	77.1
Corn:soy	1.0	17.1	11.2	40.9	75.0
Corn:soy	1.4	17.0	11.6	48.0	70.6
Rice:soy	0.6	15.1	8.7	25.8	78.1
Rice:soy	1.0	14.9	9.3	29.8	74.8
Rice:soy	1.4	14.8	9.3	35.4	71.9

The nutritional characteristics of the whole corn:whole soybean (70:30) and broken rice:whole soybeans (70:30) extruded products are presented in Table 4. An increase in the extrusion temperature caused a significant (p < 0.05) improvement in the nutritive value of the protein of both mixtures, as indicated by the NPR and PER indices. Although the improvement in NPR and PER values as the extrusion temperature increased agreed with the findings of other authors for corn:soybean mixtures (Harper et al., 1977) or whole soybean alone (Lorenz et al., 1980), in the present case it did not correlate with a decrease in trypsin inhibitors, hemagglutinins or urease activity. Furthermore, the values obtained for these antinutritional factors were low enough in all cases (Table 4) to assure a good protein quality in all samples. The significant (p < 0.05) improvement in protein nutritive value observed as the cone opening was reduced cannot be explained with the available data. However, since animals fed the diets prepared with products processed at higher extrusion temperatures showed a higher intake, it is possible that palatability may be a factor favoring these products. It is also possible that the products processed at the higher temperatures may have a higher damaged starch content which would be more digestible. These points should be investigated further. It should be mentioned, however, that a significant (P < 0.05) positive correlation was found between the NPR and PER values of the products and their corresponding processing temperature (r = 0.90 and 0.91, respectively).

The sensory scores obtained in the different schools is presented in Table 5. An increase in processing temperature exerted a positive effect on the acceptability of the products. Analysis of the data showed that the effect of temperature was statistically significant (P<0.05). Furthermore, a significant (P<0.05) positive correlation was found between the NPR and PER values of the products and their corre-

sponding average (from all schools) sensory score (r = 0.82 and 0.83, respectively). No significant difference was found between the average sensory score of the corn:soybean and that of the rice:soybean mixture extruded at the narrower cone opening (0.6 mm).

The higher degree of preference for products processed at the higher temperatures appears to be due to an improvement in their palatability. Such improvement was identified by the school children as a "better" or "nicer" corn flavor; in some cases even a flavor similar to the highly acceptable "immature corn atole" was reported for the beverages obtained from the products processed at the higher extrusion temperatures. The products processed at lower temperatures were identified as having a "beany-like" or "soybean-like" flavor. The above observations were corroborated by a sensory test carried out at our laboratories with 20 semi-trained panel members. Further, it was reported by our panel members that the sugar (at the concentration used) did not enhance and/or mask the flavor of the mixtures. Therefore, the flavor characteristics reported are thought to be due to the processing temperature.

The fat acidity values of the extruded whole corn:whole soybean and rice:whole soybean mixtures stored for up to 12 wk at different temperatures are presented in Fig. 1 and 2. Both mixtures (whole corn:soybean or rice:soybean) presented the same pattern of behavior. Although the processing temperature or the extruder's cone opening proved not to have any significant effect on the development of fat acidity at any particular storage temperature, it is of interest to note that the lower values were consistently present in mixtures processed at a higher temperature or narrower cone opening.

The storage temperature exerted a significant (P<0.05) effect on the increase in fat acidity values of the extruded mixtures. While the samples stored at 4 or 25°C did not have more than 2% fat acidity during the 12 wk of storage,

Table 4—Some nutritional characteristics of the corn:soy and rice:soy (70:30) mixtures extruded at different cone openings

Mixture	Extruder cone opening (mm)	Net protein ratio	Protein efficiency ratio	Trypsin inhibitors (TUI/mg)[a]	Hemagglutinins[b]	Urease activity[b]
Corn:soybean	0.6	3.22	2.13	5.7	0	0.0
Corn:soybean	1.6	2.87	1.85	4.8	0	0.0
Corn:soybean	1.4	2.75	1.68	3.5	1	0.0
Rice:soybean	0.6	3.31	2.21	4.8	0	0.0
Rice:soybean	1.0	3.05	2.05	4.5	0	0.0
Rice:soybean	1.4	2.63	1.54	4.6	1	0.0
Casein stad. diet	—	3.49	2.38	—	—	—

[a] TUI = Trypsin Units Inhibited.
[b] Hemagglutinin data as highest dilution giving positive test. Urease activity expressed as the change observed in pH units.

Table 5—Sensory score of gruels (atoles) prepared from corn:soy (70:30) and rice:soy (70:30) mixtures extruded at different cone openings and evaluated by grammar school students[a]

Product	Cone opening (mm)	Temp (°C)	Number of students/school[b]						Avg score 348 total students
			54	67	66	64	57	40	
			Sensory score						
Corn:soy	0.6	153	9.0 ± 0.0[c]	7.6 ± 1.9	8.8 ± 0.5	5.8 ± 1.0	7.0 ± 1.8	6.4 ± 2.0	7.4 ± 1.8
	1.0	143	9.0 ± 0.0	6.8 ± 2.5	8.7 ± 0.7	5.8 ± 1.0	6.9 ± 2.2	5.5 ± 1.5	7.2 ± 2.0
	1.4	118	8.8 ± 1.0	7.1 ± 2.2	8.2 ± 0.9	6.6 ± 1.0	6.5 ± 2.0	4.3 ± 2.3	6.9 ± 2.0
Rice:soy	0.6	161	9.0 ± 0.0	7.2 ± 2.3	8.9 ± 0.4	5.9 ± 1.0	8.1 ± 1.5	5.9 ± 1.3	7.5 ± 1.8
	1.0	148	8.8 ± 0.7	6.8 ± 2.3	8.6 ± 0.7	5.6 ± 0.9	6.4 ± 2.4	4.2 ± 2.1	6.8 ± 2.2
	1.4	129	8.9 ± 0.6	6.2 ± 2.5	5.6 ± 3.1	5.2 ± 0.6	6.1 ± 3.3	2.3 ± 1.5	5.8 ± 2.6

[a] Based on a hedonic scale of 1, 3, 5, 7 and 9 according to increased level of acceptance.
[b] Students from the third and sixth grade of the same school.
[c] Figures after ± sign are standard deviation of the mean.
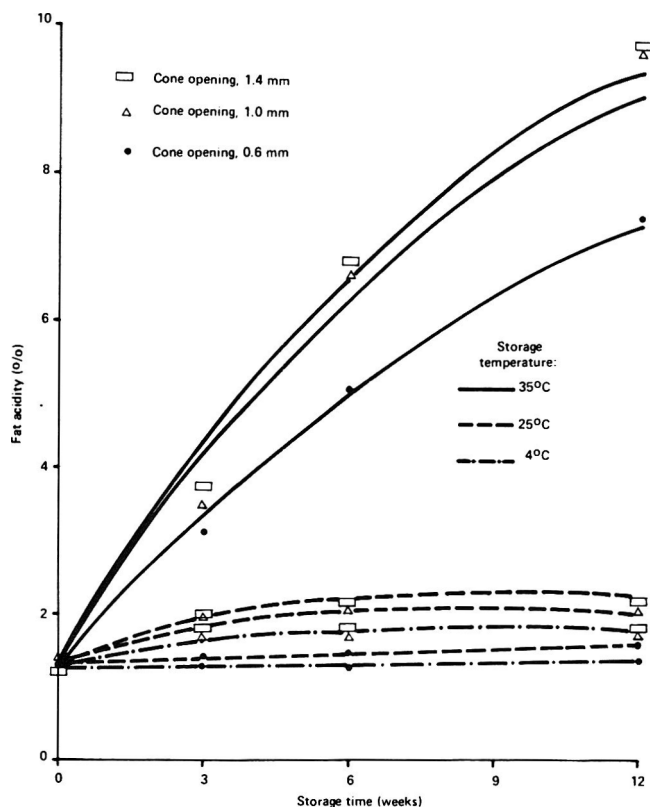
Fig. 1—Fat acidity of the corn:soy (70:30) mixture extruded using different cone openings and stored at three different temperatures.

Fig. 2—Fat acidity of the rice:soy (70:30) mixture extruded using different cone openings and stored at three different temperatures.

those stored at 35°C rose over 2% after the first 3 wk and reached a fat acidity between 7 and 10% after 12 wk of storage. Through another acceptability test run with the same school children it was found, however, that all samples remained acceptable after the 12 wk of storage.

Considering the relatively low processing costs (an average of $0.07 per kg) estimated at a pilot level (500 kg/hr) for the production of an extruded cooked product such as the whole corn:whole soybean or rice:whole soy mixture evaluated here, and considering the high acceptability of the evaluated mixtures, their relatively high caloric density and high protein nutritive value, these products represent viable alternatives as low cost complementary food itmes for infant and child feeding, not only for the Central American area but also on a worldwide basis, as previously stated by other authors (Jansen and Harper, 1980a, b). These extruded products can be consumed in an atole, gruel, or puree form and can be flavored according to taste.

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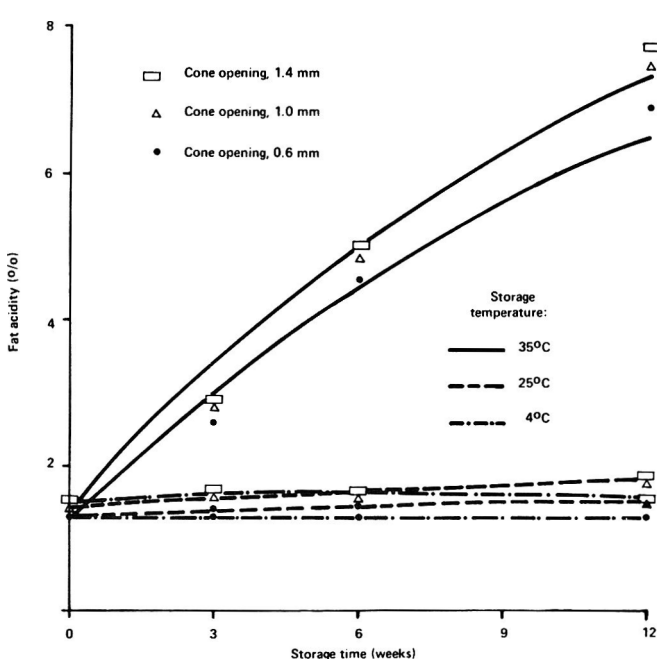
Detection and Control of Soymilk Astringency

J. T. CHIEN and H. E. SNYDER

————————ABSTRACT————————

Soymilk was made less astringent by the addition of skimmed cow's milk (SCM), CaSO4 or citric acid. The additions of CaSO4 and citric acid were not sufficient to cause visible separation of soymilk solids, but there was some difference in mouthfeel due to the additions. Warm temperatures (65°C) of the soymilk resulted in a loss of astringency compared to sensory evaluation at room temperature or 4°C. Analyses for total polyphenols showed statistically significant decreases in apparent polyphenol content per gram of soymilk solids with the first level of addition of SCM, CaSO4, or citric acid but not with subsequent additions.

INTRODUCTION

SOYMILK is a product that would allow human utilization of the lipid, protein, vitamin and mineral nutrients in soybeans. But unfortunately, soymilk is not widely accepted. The objectionable beany flavor can be controlled by heating during disruption of the beans (Wilkens et al., 1967) or before disruption (Nelson et al., 1976). Another flavor defect in soymilk that has received less attention is astringency.

Astringency has long been recognized in fruits and fruit products (Joslyn and Goldstein, 1964) and in wine (Singleton and Esau, 1969). The astringency sensation is generally attributed to polyphenolic substances interacting with mucoproteins in the mouth and throat. Although astringency in soy products has not been well studied, it has been recognized as the throat catching factor (Cowan et al., 1973).

Since astringency is an undesirable flavor sensation, we studied conditions that were calculated to decrease astringency in soymilk. To do this we needed a reliable sensory panel. Herein are reported the procedures used to train the sensory panel, the conditions that decreased astringency, and the associated changes in apparent polyphenol content.

MATERIALS & METHODS

Materials

Davis soybeans of seed quality were used for soymilk preparation. Deionized water was used for soaking, blanching and soymilk preparations.

Preparation of soymilk

Soymilk was made following the procedure of Nelson et al. (1976). Before soaking the beans, all foreign material, hulls, cracked or green beans were discarded. After heating in boiling water for 30 min, the soybeans were disrupted in an Osterizer. The neutralized and diluted (10% solids) slurry was homogenized twice in a Gaulin Model 15M 8TA homogenizer (3,500 psi first stage, 500 psi second stage). The resulting soymilk (4 liter batches from 400g dry beans) was heated to 80°C for pasteurization before storing in the refrigerator.

Before addition of citric acid or CaSO4 the stored soymilk was reheated to 80°C, appropriate additions made, and, for citric acid

additions, neutralized to pH 6.9–7.1. The soymilk was then diluted to 6% solids and homogenized. The SCM additions were made after the reheated, homogenized and diluted soymilk was cooled to approximately 25°C. All soymilk samples were served at approximately 25°C except when temperature was a variable.

A sample of the treated soymilk was lyophilized for subsequent analysis of polyphenols.

Sensory panel training

We attempted to train 12 panel members to sense astringency and report it in standardized terms. This training consisted of approximately 15 sessions over 6 months in which panel members were asked to judge the degree of astringency in soymilk prepared in several different ways. Each session was followed by a discussion. The training was not successful because there was no dependable way to vary astringency, but it did serve to familiarize the panel with astringency.

When we became aware of the utility of SCM additions to soymilk for varying astringency, the same 12 panel members were trained over 6 weeks in five separate sessions. At each session the panel members were presented with six soymilk samples that varied from 0% to 50% added SCM in 10% increments. The panel members were asked to taste each sample starting with 50% SCM: 50% soymilk and to note the first sample in which astringency could be detected. Then the panelists were asked to detect the odd sample in a triangle test in which two samples had the astringency of the adjacent more dilute soymilk sample, and the third sample had the same degree of astringency indicated as detectable.

Panel members unable to detect astringency in soymilk samples with 10% added SCM 60% of the time were excluded from further tasting. Nine panel members qualified.

Sensory panel evaluations

Training and evaluations were done in an open laboratory with fluorescent lighting. A 7-point scoring method (1 being least astringent) was used. As reference samples, panelists were supplied with a 50:50 mixture of SCM and soymilk representing a nonastringent sample and with 100% soymilk as an extremely astringent sample.

The number of samples evaluated by the panel depended on the treatment. For SCM additions five samples were evaluated, for calcium sulfate and citric acid additions four samples for each, and for temperature three samples. The samples were randomized for presentation, and presentation was done by asking panelists to pour a small sample into a paper cup from a larger supply of the treated soymilk.

Panelists were supplied with crackers and water for overcoming the astringency sensation between samples. Each panelist was asked to use his own judgment for the best procedure for overcoming the remaining astringency sensation.

Soymilk treatments were evaluated three times by the nine panelists. At least one day intervened between evaluations.

Polyphenol analysis

Polyphenols in soymilk samples were determined by the method of Hammerschmidt and Pratt (1978). Lyophilized samples (300 mg) were extracted for 2 hr at room temperature with 10 ml of methanol. The extract was filtered, and 0.5 ml of extract was mixed with 10 ml of 2% Na_2CO_3. After 2 min, 0.5 ml of 50% Folin-Ciocalteau reagent was added, and the mixture was allowed to react at room temperature for 30 min. The mixture was centrifuged at 18,000 rpm for 20 min, and absorbancy of the clear supernatant solution was measured at 750 nm (Varian model 6345 spectrophotometer).

Chlorogenic acid was used as a standard, and all polyphenol data are reported as chlorogenic acid equivalents. Each sample treatment was analyzed twice with triplicate replications.

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Table 1—Effect of added SCM on astringency of soymilk

	Percentage of soymilk				
	100	90	80	67	50
Panel score[a]	5.9a[b]	4.4b	3.1c	1.9d	1.3e

[a] Based on a scale of 1 = no astringency to 7 = extremely astringent.
[b] Mean scores with the same letter are not significantly different (p > 0.05) by Least Significant Difference.

Statistical analyses

The sensory panel scores and polyphenol analyses were analyzed further by variance analysis model of fixed effects, completely randomized, one way classification (Yamane, 1967; Snedecor, 1956).

RESULTS & DISCUSSION

WE TRIED SEVERAL PROCEDURES to decrease the astringency of soymilk. Decreasing the astringency would be useful both to improve the sensory properties of soymilk and to train a sensory panel for detection of astringency. The soaking procedure of Rockland (1972) was reported to decrease bitterness and astringency in cooked soybeans, but when we used it to prepare soymilk, we noticed no decrease in astringency. Likewise, $NaHSO_3$ addition, polyvinylpyrolidone addition, and dehulling soybeans had no effect on the astringency sensation of soymilk.

A procedure that was effective in decreasing the astringency sensation was the addition of skimmed cow's milk (SCM). Using 10% gradations between 50:50 soymilk:SCM and 100% soymilk, we were able to train a sensory panel to distinguish degrees of astringency (see Methods). When the trained panel evaluated the astringency of soymilk with SCM added, the results of Table 1 were obtained.

Since tofu is normally nonastringent, we experimented with the effect of added calcium salts and of acidification on the astringency of soymilk. Both treatments decreased astringency.

Table 2 shows the results of the sensory evaluation of varying levels of $CaSO_4$ and citric acid added to soymilk. Each gradation of $CaSO_4$ or citric acid led to a statistically significant (at 5%) decrease in astringency. The largest amounts of $CaSO_4$ (0.2%) and of citric acid (0.4%) were not sufficient to cause visible coagulation of the soymilk, but some of the sensory panel members detected an increased chalkiness or mouthfeel sensation at the higher levels of both additives indicative of protein agglomeration.

The reasons for decreased astringency with addition of SCM, $CaSO_4$, or citric acid are not known. Singleton and Esau (1969) reported that casein was useful in decreasing the astringency of wine. Perhaps casein micelles or soy protein agglomerates sufficiently trap the astringency-causing molecules to prevent them from interacting with proteins of the mouth and throat.

In the Far East, soymilk often is consumed hot. We experimented with the effect of temperature on the astringency sensation. The results in Table 3 show that the trained panel could detect a decrease in astringency when soymilk was tasted at 65–68°C compared to 22–25°C or 4–7°C. There was no difference in astringency sensation between soymilk tasted at 22–25°C and that tasted at 4–7°C. These results show that the common Oriental practice of drinking warm soymilk has advantages in decreasing the astringency sensation.

The polyphenol analyses for the various treatments (SCM, $CaSO_4$, and citric acid additions) are shown in Table 4. Each of the treatments at the lowest level (2) caused a significant decrease in the apparent polyphenol content. This result was unexpected, and we have no good explanation for it. Control experiments showed that the presence of SCM, $CaSO_4$, or citric acid had no influence on the analysis of chlorogenic acid by the Folin-Ciocalteau reagent.

Table 2—Effect of added $CaSO_4$ and citric acid on astringency of soymilk

	Level[a]			
	1	2	3	4
$CaSO_4$ score[b]	61a[c]	4.3b	3.4c	2.6d
Citric acid score	6.3a	4.8b	4.0c	2.6d

[a] Level $CaSO_4$ 1 = 0%, 2 = 0.02%, 3 = 0.06%, 4 = 0.2%
Level citric acid 1 = 0%, 2 = 0.1%, 3 = 0.2%, 4 = 0.4%
[b] Scores are based on a scale of 1 = no astringency to 7 = extremely astringent.
[c] Mean scores with the same letter are not significantly different (p > 0.05) by Least Significant Difference.

Table 3—Effect of temperature on astringency of soymilk

	Temperature of Soymilk		
	4–7°C	22–25°C	65–68°C
Panel score[a]	5.5a[b]	5.4a	4.1b

[a] Scores are based on a scale of 1 = no astringency to 7 = extreme astringency.
[b] Mean scores with the same letter are not significantly different (p > 0.05) by Least Significant Difference.

Table 4—Apparent polyphenol content (mg/g soymilk solids) of soymilk after addition of compounds that decrease the astringency sensation

	Level[a]			
	1	2	3	4
SCM	1.9a[b]	1.4b	1.1c	1.1c
$CaSO_4$	1.8a	1.5b	1.5b	1.5b
Citric acid	1.9a	1.6b	1.6b	1.4c

[a] Level SCM 1 = 0%, 2 = 10%, 3 = 20%, 4 = 50%.
Level $CaSO_4$ 1 = 0%, 2 = 0.02%, 3 = 0.06%, 0.2%.
Level citric acid 1 = 0%, 2 = 0.1%, 3 = 0.2%, 4 = 0.4%.
[b] Means with the same letter are not significantly different (p > 0.05) by Tukey's test (Snedecor, 1956).
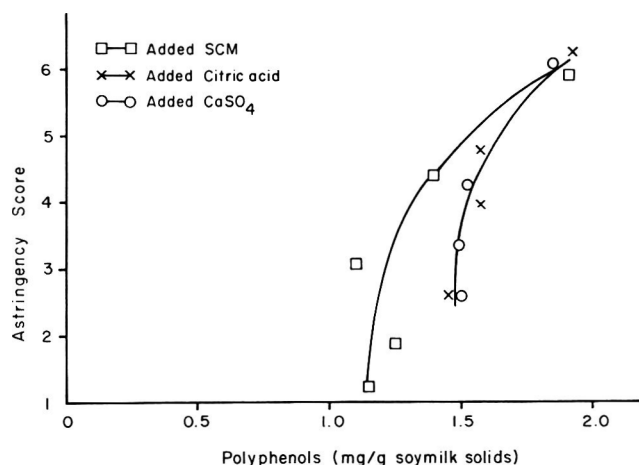
Fig. 1—Relationship between apparent polyphenol content of soymilks and their astringency scores.

Subsequent additions of the three substances had less effect on the polyphenol analysis, but there was a significant difference for SCM at level 3 and for citric acid at level 4. All of these analyses were calculated on the basis of soymilk solids only, so there was no dilution effect due to the added substances.

Astringency is related to apparent polyphenol content in Fig. 1. The loss of astringency and the loss of polyphenol with the first additions of SCM, $CaSO_4$ or citric acid are

evident in Fig. 1. The continued decrease in astringency with further additions of the three substances is obviously not due to decreased amounts of polyphenols. However, as suggested above, the casein micelles or soy protein agglomerates may complete sufficiently with mouth and throat mucoproteins for polyphenols to decrease the astringency sensation.

Astringency is an undesirable taste sensation in soymilk. Further attempts to minimize astringency are justified to widen the appeal and the use of soymilk and its nutrients.

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thin layer (5 mm) method are presented because the correlations with sensory evaluations were higher. Many color expressions were calculated, including the Kubelka-Munk equations, but the data reported in Table 7 were the most meaningful.

The highest correlations were obtained between the color expression L and sensory evaluations freedom from browning, flavor preference and visual hue. Color preference did not correlate as well because of wide variation in color, hue, and development of browning. Browning index, Acy and a/L showed a significant correlation with all sensory evaluations. The b values are negatively correlated with all sensory ratings except color preference. Morris et al. (1980) have shown that b values are a good indicator of color quality in 'Concord' grapes. The a value is more related to color preference than b values.

In conclusion, low concentrations of $SnCl_2$ and $CaSO_4$ had a stabilizing effect on 'Concord' grape juice during 18 months of storage. Some of the stabilizing effect was caused by metal complexing reactions and change in pH. The addition of AA did not accelerate the loss of Acy in the presence of some of the chemical treatments. Certain color expressions correlated well with sensory color judgment. The color expressions that were beneficial in defining color in 'Concord' grape juice during storage were Acy, browning index, a/L ratio and L, a and b values.

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Laboratory Scale Production of Winged Bean Curd

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ABSTRACT

Procedures for the preparation of 100% winged bean or mixed winged bean-soybean curd employing the essential steps of soy curd production have been developed and optimized. Use of magnesium chloride, gluconodelta lactone, acetic acid and calcium sulfate as coagulants was investigated. The results showed that while curd formation was not facilitated by acetic acid, sticky and less cohesive curds could be formed with magnesium chloride and glucono-delta lactone. Calcium sulfate precipitation resulted in a semi-solid and appreciably cohesive curd from winged bean. Winged bean curd had very low hardness value in comparison to soy tofu. Curd products prepared from five ratios of winged bean-soybean mixtures indicate that 50:50 and 25:75 winged bean-soybean combinations by weight resulted in satisfactory products.

INTRODUCTION

THE NUTRITIONAL VALUE of the seeds of winged bean, *Psophocarpus tetragonolobus* L·DC, was brought to the limelight only recently (Philippine Council for Agriculture and Resources, 1978; NAS, 1981). The need for the production of edible food products with high nutritional quality from winged bean seeds has been stressed (NAS, 1981).

Tofu (soybean curd) has found acceptance as a high protein food for human consumption. Tofu production from soybean had been studied extensively by many workers (Loska and Melnick, 1950; Shurtleff and Aoyagi, 1975; Saio, 1979; Tsai et al., 1981) and the effect of calcium concentration on the quality of the soy tofu had also been reported (Skurray et al., 1980; Lu et al., 1980).

No studies had been reported related to the production of curd from winged bean prior to 1977 (International Grain Legume Centre, 1978; Atterado, 1979). Since then, only two reports have appeared. Shurtleff (1978) described a home production method for winged bean curd. Variability of protein content in the tofu samples prepared with HCl coagulation of four selections (TPT-1, TPT-2, TPT-6 and Chimbu) of winged bean was reported recently (Jensen, 1980). Neither of the above reports mentioned the physical or sensory properties of the produced curd.

The objective of the present study was to investigate the possibilities of production of acceptable curd from winged bean. As an initial step an attempt was made to explore laboratory scale production of winged bean curd, and to optimize production conditions. Then combinations of winged bean and soybean were utilized to prepare mixed seed curd products.

The objectives of this work can be categorized into four areas: (1) effect of different coagulants and concentration of coagulants on the winged bean curd product; (2) standardization of conditions for production; (3) preparation of "winged-soy curd" using winged bean-soybean combinations; (4) evaluation of texture of the prepared curds.

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MATERIALS & METHODS

Raw material

SLS-29 and TPT-2 cultivars of winged bean used in this study were obtained from Sri Lanka. Whereas SLS-29 is an indigenous cultivar of Sri Lanka, TPT-2 is of Nigerian origin, but was grown in Sri Lanka. Bonus variety (1979 crop) of soybeans was obtained from the Agricultural Engineering Farm at the Univ. of Illinois.

Laboratory production scale of curd

Thirty grams of winged beans and soybeans were soaked overnight in deionized water at room temperature and at 4°C respectively, and both were dehulled manually. Though soaking at lower temperatures was found to be preferable to avoid bacterial fermentation reactions, room temperature soaking was adopted for winged beans to facilitate more efficient hydration of the beans. Hydrated cotyledons were placed in a Waring Blendor together with boiling deionized water. The ratio of water to beans was 3:1 and the temperature of the water after addition was between 85 and 95°C. The beans and water were blended at full speed for 3 min. The resulting slurry was filtered through eight-layers of cheesecloth to yield two fractions the winged bean milk and a residue consisting mainly of coarse fibrous material (okara). The milk was boiled at 95°C with continuous stirring for 7 min. The milk was then cooled to 70° and divided into 25 ml portions and poured into 50 ml plastic centrifuge tubes. A 2.0 ml portion of coagulant, pre-incubated at 70°C, was added slowly, while the tube was rotated gently to allow the commencement of coagulation. The tubes were incubated at 70°C for 10 min. After incubation, the milk was centrifuged (1500 x g) to aid separation of the supernatant fraction. A flow-sheet for the production of curd is shown in Fig. 1.

For the preparation of winged bean (WB)-soybean (SB) combination curd, 30g of bean cotyledons were used in the following percentage proportions on wt:wt basis. (1) 90:10/WB:SB (2) 75:25/WB:SB (3) 50:50/WB:SB (4) 25:75/WB:SB (5) 10:90/WB:SB.

Coagulants used

Reagent grade calcium sulfate ($CaSO_4 \cdot 2H_2O$), magnesium sulfate ($MgSO_4 \cdot 6H_2O$) and magnesium chloride ($MgCl_2 \cdot 6H_2O$) were obtained from Fisher Scientific Co., NJ and glucono-delta lactone (GDL) were products of Sigma Chemicals, Inc., MO. Acetic acid solutions were prepared by diluting glacial acetic acid.

Yield

The yield of curd was expressed as fresh weight of curd from 100 ml boiled milk.

Chemical analyses

AOAC (1975) procedures were used to determine crude protein (Kjeldahl, N x 6.25); crude fat (ether extract); and moisture contents of samples. Mineral analyses were performed using an Atomic Absorption Spectrophotometer (Perkin-Elmer #306, Norwalk, CT).

Textural evaluation

The Instron Universal Testing Machine (Model TM-M, Canton, MA) was used (Peleg, 1976; Yang and Taranto, 1981). The scale load was calibrated with 1 kg. A plunger with a diameter of 1.0 cm was attached to the moving crosshead. The speed of the crosshead was set at 2 cm/min in both upward and downward direction; the penetration of the plunger into the curd sample was fixed at 60 percent deformation level for 1.2 cm. The recording chart speed was set at 5 cm/min. Two samples of each set of curd, prepared in the form of cylinder (2.0 cm long x 2.5 cm diameter) were equilibrated

at 20 ± 2°C prior to measurements. Three primary textural parameters, hardness, cohesiveness and springiness, as defined by Civille and Szczesniak (1973) were evaluated.

RESULTS & DISCUSSION

THE PROXIMATE ANALYSIS revealed that the winged beans used in this study had protein and lipid composition of 40.1 and 25.3% on a dry weight basis, respectively.

The capacity of various coagulants such as $CaSO_4$, $MgCl_2$, glucono-delta lactone (GDL) and acetic acid, in precipitating the winged bean protein is provided in Table 1. It was observed that of the four coagulants tested, $CaSO_4$ provided a semi-solid, cohesive curd product and the curd prepared with 0.5% concentration seem optimal. Though $MgCl_2$ produced curd of a semi-solid nature, the samples disintegrated into particles when immersed in water. Disintegration was approximately 5% for $MgCl_2$-treated curd and 15% for GDL-treated curd. The fragility of GDL-treated soy tofu in comparison to $CaSO_4$ tofu had been mentioned by Saio (1979). Acetic acid did not provide a satisfactory curd.

Precipitation methods employed by previous workers, for the production of soy tofu differ with respect to the coagulant used. $CaCl_2$ was tested by Miller et al. (1952). Lu et al. (1980) suggested that calcium acetate and $CaCl_2$ as good precipitants, in favor of $CaSO_4$, while Skurray et al. (1980) experimented with $CaSO_4$. In contrast, Holazo et al. (1981) preferred the use of $MgSO_4$ over $CaCl_2$ and vinegar. $CaSO_4$ and bittern (nigari) had been suggested by Tsai et al. (1981) as suitable coagulants. Though Jensen (1980) had cited HCl as the coagulant to precipitate the winged bean protein, the concentration and volume of the acid used was not specified. Furthermore, no descriptions had been made by Jensen of the physical appearance and textural characteristics of winged bean curd obtained from HCl coagulation. For the production of winged bean curd, in the present study, $CaSO_4$ was determined to be the best coagulant; and $CaSO_4$ was utilized at 0.5% level for the remaining studies.

The bean-water ratio used to extract the winged bean milk and the temperature of incubation were two other parameters which were investigated for the production of winged bean curd. It had been reported that the composition of soy milk varied widely according to the amount of water used to make the extraction (Bourne et al., 1976). Three bean-water ratios, 1:5, 1:3 and 1:2, were experimented in this study and of these the ratios of 1:3 and 1:5 resulted in curd of preferable consistency for the winged bean. Of these two, 1:3 bean:water ratio was selected for the preparation of different curd combinations.

Of the four incubation temperatures, 50, 60, 70 and 80°C, tested for winged bean curd production, the curds obtained at 60°C as well as 70°C were of acceptable semi-solid consistency. A temperature of 70°C was chosen since this value falls within the range of 60–80°C, which had been the mid temperature reported by the workers for the production of soy tofu (Saio et al., 1969; Schroder and Jackson, 1972; Holazo et al., 1981; Tsai et al., 1981).

Various mixtures of winged bean and soybean were processed into curd. All curd products containing soybean were firmer and more acceptable. Table 2 shows the yield of curd obtained in different combinations of winged bean-soy mixtures. Data are expressed as weight of fresh curd; and the yield ranged from 52.8–80.0g per 100 ml of boiled milk. These values agree well with the data presented by Tsai et al. (1981) who had used approximately the same volume of milk and coagulant for the production of soy tofu. It can be observed that, while the weight of curd produced from either winged bean or soybean approximates 60–64% of the boiled milk, the curd produced from 50:50/WB:SB and 25:75/WB:SB combinations were in the region of 76–80% of boiled milk. As reduction in the volume of whey is a preferred characteristic in the production of tofu, 50:50/WB:SB and 25:75/WB:SB combinations form suitable choices.

The protein contents of milk, whey and curd samples of the various combinations of winged bean-soy mixture is shown in Table 2. The protein content of the raw milk samples of all the combinations varied only within a narrow range of 5.2–5.5 g/100 ml of milk. The compilation provided by Bourne et al. (1976) reveal that earlier workers extracting the soymilk with a higher bean:water ratio in the range of 1:6–1:15, had soy milk with a protein content of 1.8–4.2%. By using a lower bean:water ratio of 1:3 in this study, the protein concentration of the raw milk samples were elevated. Boiling the milk for 7 min at 95°C, prior to coagulation results in an increase of protein by 4–28% in most of the combinations due to evaporation of water.

The range of protein in the curd samples from various combinations was 6.8–9.3 g/100g on fresh weight basis. Protein content, expressed as percent of solids in the curd samples range from 51.2–67.7. Shurtleff and Aoyagi (1975) cited the range of protein for different three kinds

Fig. 1—Flow sheet for the preparation of curd.

Table 1—Precipitation of winged bean protein by various coagulants[a]

Coagulant	Percent added							
	0.25	0.5	0.8	1.0	1.2	1.4	4.0	8.0
$CaSO_4 \cdot 2H_2O$	+3	+3	+3	+3	+3	+3	+2	+1
$MgCl_2 \cdot 6H_2O$	+3	+3	+3	+3	+3	+3	+2	+1
Glucono-delta lactone	+2	+3	+3	+2	+2	+2	+2	+1
Acetic acid	+1	+1	+1	+1	+1	+1	+1	+1

[a] Mean of four determinations. Rating: +1 coagulation positive, but curd formation absent; +2 coagulation positive, sticky curd formed; +3 coagulation positive, semi-solid curd formed.

Combinations	Raw Milk g/100 ml	Boiled Milk g/100 ml	Whey g/100 ml of boiled milk[b]	Tofu g/100g fresh wt of tofu	Tofu percent of solids	Yield per 100 ml of boiled milk Yield of curd (g)	Yield per 100 ml of boiled milk Yield of Protein in Curd (g)
100/WB	5.0	5.6	0.3	9.3	55.4	60.0	5.6
90:10/WB/SB	5.1	5.4	0.5	8.6	51.2	52.8	4.5
75:25/WB:SB	5.5	5.7	0.6	9.2	56.1	53.6	4.9
50:50/WB:SB	5.2	5.8	0.4	8.8	59.9	76.4	6.7
25:75/WB:SB	5.5	5.4	0.2	6.8	59.1	80.0	5.4
10:90/WB/SB	5.5	6.7	0.3	9.0	62.1	64.0	5.8
100/SB	5.4	6.9	0.4	8.9	67.7	64.0	5.7

[a] Values are averages of at least duplicate determinations.   [b] After coagulation with $CaSO_4 \cdot 2H_2O$

Table 3—solids, ash and mineral contents of winged bean and soybean seeds and curd samples[a]

Seeds and curd samples	Solids percent (Fresh Wt)	Ash percent (Fresh Wt)	Minerals (mg/100g dry wt) Ca	Mg	K	Zn	Fe
NAS (1981) Table Values for WB Seeds[b]	—	3.3—4.9	96—444	132—306	1337—2161	4—6	2—22
Mixed SLS-29 and TPT-2 WB Seeds[c]	—	5.4	420	319	2067	4	7
Bonus 79 SB Seeds[d]	—	3.8	191	322	2919	5	7
100/WB curd	16.8	0.7	1959	169	308	8	9
90:10/WB:SB curd	16.8	0.6	757	190	482	6	10
75:25/WB:SB curd	16.4	0.6	991	185	360	6	10
50:50/WB:SB curd	14.7	0.3	632	151	611	5	8
25:75/WB:SB curd	11.5	0.3	511	128	587	5	6
10:90/WB:SB curd	14.5	0.5	1239	73	301	4	8
100/SB curd	13.2	0.8	1638	151	717	6	7

[a] Values are averages of determinations from two samples.
[b] NAS (1981) values were recalculated on the basis of mg per 100g dry wt using the average 16.7 (of the range 8.7—24.6) for total solids content.
[c] Present study. [The proximate composition in percentage on dry wt basis: protein = 40.1, lipid = 25.3; carbohydrate and fiber = 28.4]
[d] Present study. [The proximate composition in percentage on dry wt basis: protein = 51.2; lipid = 24.8; carbohydrate and fiber = 20.2]

of tofu made from soybean as 5.5—10.6%. No mention had been made by Shurtleff (1980) regarding the protein percentage of winged bean curd.

The range of moisture contents of the different combinations of curd in this study was 83.2—88.5% as evident by the range of solids content 11.5—16.8% (Table 3). The average moisture content of soy tofu, reported by previous workers (Shurtleff and Aoyagi, 1975; Tsai et al., 1981) falls around the range obtained in the present study. Since the amount of water in these curd products are extremely high, these could be included in the category of perishable food product, with a shelf life of only 2 days (Dotson et al., 1977).

Furthermore, the mineral content of the tofu preparations from winged bean and soybean combinations is compared with that of mature winged bean and soybean seeds in Table 3. The mineral content of the winged bean seed used in this study falls within the range reported by NAS (1981). Noticeable differences in the levels of Ca, Mg and K can be seen between the seeds and the curd products; while there is an increase in the Ca levels of the curd samples due to coagulant used. Appreciable decrease in the levels of Mg and K in curd were noticed. The variable content of minerals in the different soy-winged bean curd combinations probably reflects non-specific trapping of minerals during coagulation of the products.

The acceptability of the curd product, depends upon the physical-sensory properties. Hence, rheological parameters of the winged bean curd produced with four coagulants were investigated. Table 4 shows the data obtained with

Table 4—Comparison of rheological parameters of winged bean curd produced with various coagulants[a]

Coagulant	Hardness (g)	Cohesiveness (percent)	Springiness (percent)
$CaSO_4 \cdot 2H_2O$	13	41	67
Glucono-delta lactone	12	37	73
$MgCl_2 \cdot 6H_2O$	18	31	67
$MgSO_4 \cdot 6H_2O$	15	32	60
range	12—18	31—41	60—73
average	14.5	35.3	66.8

[a] Values are averages of duplicate determinations. Coagulant concentrations used is 0.5%. Measurements taken after soaking the curd in water for 24 hr at $4°C$.

regard to the three primary textural parameters, hardness, cohesiveness and springiness. Increased cohesiveness is one of the desirable characteristics. Of the four coagulants tested, $CaSO_4 \cdot 2H_2O$ seems to produce acceptable curd products satisfying this requirement. Lu et al. (1980) also had reported that in the preparation of soy tofu, $CaSO_4$ was preferred to glucono-delta lactone, due to the desirable effect of calcium ion on palatability.

The textural parameters of soy tofu and winged bean curd, prepared with the same coagulant ($CaSO_4$ added at 0.5% of milk volume) reveal noticeable differences (Table 5). Winged bean curd, with a hardness value of 13g is very soft in comparison to the soy tofu which is firm, and having

Table 5—Comparison of rheological parameters of curd[a]

Combinations	Hardness (g)		Cohesiveness (percent)		Springiness (percent)	
	Immediate[d]	After 1-day[e] Soak	Immediate	After 1-day Soak	Immediate	After 1-day Soak
100/WB[b]	—	13	—	41	—	67
90:10/WB:SB	35	44	42	44	60	67
75:25/WB:SB	52	62	41	47	60	80
50:50/WB:SB	33	28	34	38	73	90
25:75/WB:SB	25	25	33	24	84	67
10:90/WB:SB	44	31	28	36	75	80
100/SB[c]	64	35	34	28	87	77

[a] Values are average of duplicate determinations; Coagulant used is $CaSO_4 \cdot 2H_2O$ at 0.5% level.
[b] Winged bean
[c] Soybean
[d] Measurements taken immediately after preparation
[e] Measurements taken after soaking the curd in deionized water for 24 hr at 4°C

a hardness value of 35g. All mixed seed curd products were harder than 100% winged bean curd. Contrasting variation could also be noticed for the values in cohesiveness and springiness as well. Whereas the soy tofu had a light yellow color, the winged bean curd was colorless. The beany flavor was more noticeable in the winged bean curd than in soy or mixed winged bean:soy curd.

The variation in the texture and the nature of winged bean curd, in comparison to that of soy tofu, can be attributed to the differences in the structure of the storage globulin of winged bean. Whereas most legume seeds, including soybean, contain major 7S and 11S storage globulins (Danielsson, 1949), the studies of Gillespie and Blagrove (1980) on the winged bean seed protein show that the major winged bean proteins are of sedimentation coefficients 2S and 6S. Later, Gillespie et al. (1981) reported that the two major components of winged bean have sedimentation coefficients of about 2S and 7S, while no significant 11S fraction was observed.

Saio et al. (1969) investigated the food processing characteristics of tofu made from crude soybean 11S and 7S protein fractions. They observed that the tofu-gel from the 11S fraction was remarkably harder than that from crude 7S fraction, which was described as less adhesive. The palatable hardness of whole soy tofu was attributed by the authors to an adequate proportion of 7S and 11S components present in the soybean protein. It can be inferred that the soft nature of winged bean curd, in comparison to the soy tofu, as revealed by the measurements of hardness (Tables 4 and 5) may be due to the absence of 11S fraction in the winged bean protein.

Among the winged bean-soy mixture curd samples, the values obtained for hardness of 50:50/WB:SB and 25:75/WB:SB combinations lie between those obtained for winged bean curd and soy tofu. These observations indicate that satisfactory curd products with acceptable textural properties can be made with two combinations: (1) 50:50/WB:SB and (II) 25:75/WB:SB. This does not preclude the fact that winged bean alone may not be utilized for the production of a good quality curd, similar to that of soybean. Further processing modification in this direction is currently underway. Some varieties of winged bean may contain quantities of 11S or similar high molecular weight proteins to allow formulation of satisfactory curd gel.

The current studies have brought out some problems with utilization of mature winged bean seed for food use. Efficient and quick removal of the seed coat would be desirable. However, seed coats must be removed by hand as no satisfactory mechanical procedure has been developed to date. The necessity of the removal of seed coat is emphasized by the fact that the seed coat is rich in water-soluble tannin components (deLumen and Salamat, 1980), which if left with the cotyledon imparts an undesirable brownish-

purplish color to the product as well as less acceptable flavor. High tannin concentrations in a diet may also interfere with growth. Another drawback experienced with the winged bean seeds is that approximately 10–20% of the seeds remain unhydrated even after 24 hours of soaking. This observation substantiates the earlier report of Shurtleff (1980) on the hydration behavior of winged bean seeds.

It can be concluded at this stage of research that satisfactory mixed bean curd can be produced using 25–50% winged bean seed. We are continuing to investigate the possibility of producing a good quality curd from winged bean utilizing alternate technology and/or different cultivars of winged bean.

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Application of Enzymes in Soy Milk Production to Improve Yield

SVEND ERIKSEN

─────────── ABSTRACT ───────────

Soy milk was produced from full fat flour by preparing a slurry in water at a water to flour ratio of 10 to 1. The slurry was heated to 50°C, and different enzymes were evaluated in order to optimize protein and solids yields in the milk. The reaction period was 1 hr and after inactivation of the enzymes by boiling for 15 min the mixtures were centrifuged at 1500 x g and the protein and dry matter solubility indices (PSI and DSI) were calculated together with protein and dry matter yields in the obtained milk. The best results were obtained with a neutral proteinase, increasing the protein and solids yields from 33% and 42%, respectively, to 73% and 66%, respectively, at 0.5% dosage level compared to the control sample with no enzyme treatment.

INTRODUCTION

TRADITIONALLY, soy milk is produced by initial soaking of the beans for at least 3 hr depending on the water temperature. The beans are then ground at a water to bean ratio of 10 to 1, and the slurry is filtered and the resulting milk heated to boiling for around 30 min to improve its nutritional value and flavor (Smith and Circle, 1972).

Dry matter and protein yields are around 60% and 70%, respectively, but the traditional process produces a soy milk with a typical beany flavor caused by enzymatic lipid oxidation during the wet grinding process.

The use of toasted seeds (Wilkens et al., 1967) or toasted full-fat soy flour will eliminate the beany flavor but will, however, greatly reduce protein extraction and yield of soy milk. Johnson and Snyder (1978) have investigated the effect of heating soaked beans prior to and during wet grinding. Their results showed that heating before grinding resulted in solids yield as low as 26% due to heat fixing of the protein bodies that are removed by the subsequent centrifugation. Wet grinding in hot water increased the yield to around 43%. Homogenization at high pressure (8000 psi) and high temperatures (75°C) increased the yields in both processes to 49% and 59%, respectively. The authors conclude that homogenization is helpful in redispersing the heat-fixed protein.

Nelson et al. (1976) had previously used this principle in the so-called Illinois process where soaked beans were heat-treated before wet grinding with subsequent homogenization at high pressure and temperature. They reported yields of 89% solids and 95% protein, but there was no separation step in this process. A subsequent centrifugation at low g-values was shown by Johnson and Snyder (1978) to decrease the solids yield to 49%.

Recently Johnson et al. (1981) have reported up to 90% protein yield using a continuous steam-infusion cooking process at 154°C, neutral pH and a holding time of 40 sec. They explain their high yields as a result of optimum heat treatment and extreme shear encountered in steam-infusion and flashing since a certain amount of heat is required to dissociate the protein bodies leading to increased solubility and emulsification.

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The purpose of the present work was an attempt to improve both solids and protein yields in comparison to the traditional process, while maintaining good flavor properties, by means of enzymes that would solubilize the sediment and thus simultaneously decrease the disposal problem of this product.

Preliminary results using a two-step enzyme process have been reported by Olsen and Adler-Nissen (1981). They reported improvements in solids yield of 72% using a proteinase followed by a pectinase.

MATERIALS & METHODS

Preparation of soy milk

Soy milk was produced from toasted full-fat soy flour (Dansk Soyakagefabrik A/S, Copenhagen) by preparing a slurry of flour in water at a water to flour ratio of 10 to 1 (200g water + 20g soy flour). The slurry was heated to 50°C and pH of the slurry was adjusted to the appropriate pH for the given enzyme with either NaOH (4N) or HCl (6N). The pH of the unadjusted slurry was 6.6–6.7. Enzyme was added at two different levels (0.5% and 2% of the flour) and the reaction proceeded for 1 hr.

The reaction was terminated by inactivating the enzyme by boiling for 15 min. The slurry was homogenized in a Sorvall-mixer for 2 min, centrifuged for 5 min at 1500 x g and the milk was obtained as the supernatant. The flow sheet is presented in Fig. 1.

Calculations

The amount of milk was weighed and the yields of solids and protein were determined from the dry matter (105°C, overnight) and protein (N x 6.25) analyses. From mass balance calculations the protein solubility index (PSI) and dry matter solubility index (DSI) were determined as the dry matter and protein centrifugation indices (dci, pci) multiplied by a factor, q, defined as the ratio:

$$q = \frac{\% \text{ water in slurry}}{\% \text{ water in supernatant}}$$

In practice q is determined as:

$$q = \frac{100\% - \% \text{ dry matter in slurry}}{100\% - \% \text{ dry matter in supernatant}}$$

The centrifugation indices are defined as

$$dci = \frac{\% \text{ dry matter in supernatant}}{\% \text{ dry matter in slurry}}$$

$$pci = \frac{\% \text{ protein in supernatant}}{\% \text{ protein in slurry}}$$

The solubility indices are calculated from:

$$PSI = pci \times q$$

$$DSI = dci \times q$$

Those indices represent the maximum obtainable yields, i.e. if all soluble material was recovered by repeated washings of the residue, and thus take into account that centrifugation indices alone slightly overestimate yields due to the presence of insoluble material.

Enzymes

The enzymes used in this study are listed in Table 1 together with their main activities and pH-optima. Except for the experimental enzyme SP-249 (Olsen, 1982) the enzymes are all commercially available (Novo Lab. Inc., Wilton, CT).

RESULTS & DISCUSSION

THE RESULTS of using the different enzymes in the described soy milk process are presented in Table 2.

The results show that no improvements in yields were obtained with Cereflo and Alcalase at pH 6.7, while Neutrase produced yields of almost 60%. The lack of effect of Cereflo indicates that the yield improvement using Neutrase is a proteolytic effect only.

Adjusting the pH of the reaction to the optimum for the given enzyme has only moderate effect on Pectinex, but dramatic effect on both Neutrase and Alcalase. The dry matter and protein solubility indices are the same for the two enzymes, but the relative yield improvement caused by the enzyme in comparison to the control at the optimal pH is higher for Neutrase.

Neutrase is the preferred enzyme, not only because of the above but also due to its neutral pH optimum in comparison to the alkaline pH optimum of Alcalase. Furthermore, the neutralization step can be omitted in the case of Neutrase.

At 0.5% dosage level the protein and solids yield obtained using Neutrase were 73% and 66% respectively, compared to 33% and 42% respectively of the control sample at pH 6.7. This is an improvement of more than 120% on the protein yield compared to no enzyme treatment. At 2% enzyme dosage level the yields were not further improved.

The remaining enzyme tested, SP-249, also produced improvements in yields, but to a lesser extent than both Neutrase and Alcalase.

In the case of SP-249 the yield improvements seem to be dose related, which would produce even higher enzyme dosage levels. This is substantiated in Fig. 2 where the dry matter and protein solubility indices are plotted against dosage level of SP-249. At 8% enzyme the PSI is almost 0.9, while the DSI has stabilized around 0.85.

More effective than increasing the enzyme dosage is prolonging of the reaction time, especially beyond 4 hr, which can be seen by comparison of Fig. 2 and 3. From Fig. 3 it can be seen that a reaction period of 6 hr at 1% enzyme dosage results in a DSI of 0.86 and a PSI of 0.92. This is substantially higher than the results obtained using a reaction period of 1 hr and 6% enzyme (see Fig. 2). Thus, the simple inverse relationship between enzyme dosage and time which is generally valid for enzyme reactions does not hold in the present case, probably due to the insolubility of the substrate.

The results are, however, not particularly impressive considering that the same results were obtained using Neutrase at 0.5% dosage level for only 1 hr! In another experiment the reaction time with Neutrase was increased to 4 hr at 1% enzyme level resulting in a DSI of 0.87 and a PSI of 0.96 which again is no significant improvement to the 1 hr reaction period. The high yields of soy milk obtained using Neutrase at a low dosage level are thus not further improved by increasing the reaction period or the enzyme dosage.

The effect of pH, on the other hand, is very important as can be seen from Fig. 4, where PSI and DSI are plotted against pH. It is essential that the pH is just above 7 to obtain maximum yield and since pH drops a few tenths during the reaction, due to proteolysis, a starting pH of ≈ 7.2 will result in a milk with a pH of 6.9–7.0.

Hackler et al. (1963) pointed out that the protein discarded with the insoluble residue in the traditional soy milk process is superior in its protein efficiency ratio (PER) to the milk itself. This indicates that Neutrase not only improves the yield of soy milk but also the nutritional quality since more than 90% of the protein is solubilized.

Due to lack of laboratory wet milling facilities, full-fat soy flour was used in this study, but the work is continuing in pilot plant scale where hot water grinding of unsoaked soy beans will be applied as a means of obtaining a slurry before addition of enzymes.

CONCLUSIONS

THE PRESENT STUDY has shown that enzymes can improve the yield of soy milk in comparison to traditional

Table 1—Enzymes used in soy milk preparation

Enzyme	pH-optimum	Activities
Neutrase	7.0	Neutral proteinase, β-glucanase
Alcalase	8.0	Alkaline proteinase
Pectinex	5.5	Pectinase
Cereflo	7.0	β-glucanase
SP-249	4.5	Pectinase, cellulase, hemicellulase, proteinase

Table 2—Influence of enzyme on protein and solids yields in soy milk manufacture[a]

Enzyme	% Dosage	pH of reaction	Protein yield (%)	Solids yield (%)	PSI	DSI
Neutrase	0		33	42	0.48	0.61
	0.5	6.7	55	55	0.74	0.74
	1.0		57	56	0.76	0.74
Alcalase	0		32	41	0.45	0.58
	0.5	6.7	28	31	0.44	0.48
	2.0		36	39	0.52	0.57
Cereflo	0		33	42	0.48	0.61
	0.5	6.7	33	41	0.48	0.61
	2.0		29	40	0.42	0.58
Neutrase	0		42	46	0.60	0.66
	0.5	7.0	73	66	0.92	0.84
	2.0		76	68	0.94	0.85
Alcalase	0		56	55	0.78	0.77
	0.5	8.0	70	63	0.95	0.86
	2.0		65	59	0.91	0.81
Pectinex	0		30	38	0.49	0.63
	0.5	5.5	37	44	0.55	0.65
	2.0		41	45	0.63	0.69
SP-249	0		34	44	0.50	0.64
	0.5	4.5	41	46	0.61	0.68
	2.0		60	60	0.79	0.79

[a] Time of reaction: 1 hr.
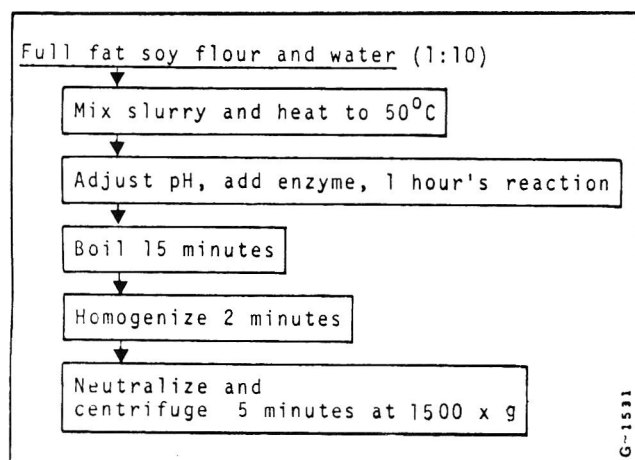
Fig. 1—Flow sheet for preparation of soy milk.

Full fat soy flour and water (1:10)
Mix slurry and heat to 50°C
Adjust pH, add enzyme, 1 hour's reaction
Boil 15 minutes
Homogenize 2 minutes
Neutralize and centrifuge 5 minutes at 1500 × g
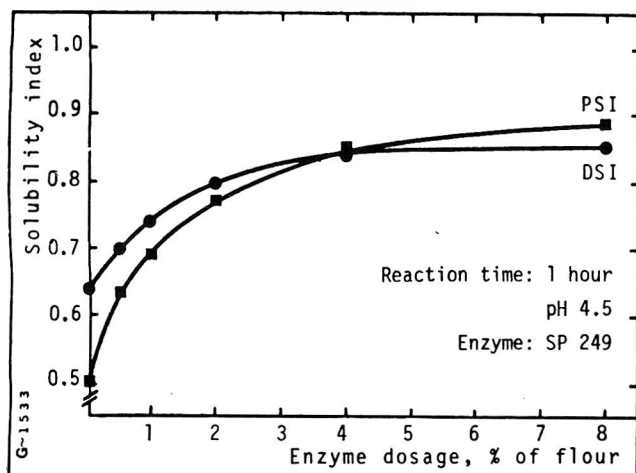
Fig. 2—Effect of enzyme dosage on dry matter and protein solubility indices.

Fig. 3—Effect of time of reaction on dry matter and protein solubility indices.

procedures. Of the different enzymes tested, the neutral proteinase, Neutrase, was found to produce superior results at neutral pH. At 0.5% level the protein and solids yields obtained at 1 hr of reaction were 73% and 66%, respectively, compared to 33% and 42%, respectively, of the control at pH 6.7.

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Fig. 4—Effect of pH on dry matter and protein solubility indices.

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Effects of Packaging Method and Grade Size on Storage Quality of Newly Harvested Peanuts

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——————————ABSTRACT——————————

Newly harvested and cured cv. Florunner jumbo (>8.33 mm dia), split (>6.75 mm dia) and no. 1 (<7.14, >6.35 mm dia) peanuts were packaged as shelled seed in plastic bags, CO_2 atmosphere; in burlap bags, and as farmers stock, bulk. After 1, 3 and 6 mo ambient storage, they were tested for moisture, color of raw seed-coat and color and flavor of peanut butter. Packaging showed no effect on flavor, but seed size did. No. 1's showed a linear loss of flavor with time and generally had the highest moisture level and produced the darkest butter. After 6 mo, burlap-bag storage yielded the darkest raw seed coats, while plastic bag-CO_2 generally maintained the highest moisture level and produced the darkest butter.

INTRODUCTION

THERE IS INCREASING INTEREST in the industry in packaging and storing peanuts in low-oxygen atmospheres at ambient temperatures since quality of shelled seed is maintained and energy costs are reduced (Pearson et al., 1977; Slay et al., 1978; Holaday et al., 1979). Most peanuts are stored inshell (called "farmers' stock") for several months before further processing; however, some peanut shellers desire to shell and package peanuts in CO_2 atmosphere immediately after they are harvested and dried. They have hesitated to do so, because of suggestions that from a few to several months storage in normal atmosphere may be necessary for peanuts to develop their full potential for roasted flavor (Cecil, 1969; Pattee et al., 1971). Also, a reduction in available oxygen has been reported to lower the quality of variable moisture peanuts stored at a "safe" average moisture content of 8% (Cecil, 1976).

Young and Holley (1965) suggested a CO_2 build-up in inshell peanuts as a reason for their having better keeping quality than shelled peanuts, and over 25 years ago the California Agricultural Extension Service recommended a CO_2 atmosphere as a method for home preservation of nut meats and dried fruits and vegetables (Anonymous, 1955).

Newly harvested and dried peanuts were used in this study and evaluated periodically by several quality tests. Because significant quality differences were found between minimum no. 1 and larger seed of cv. Florigiant, a major virginia type variety (Pattee et al., 1982), and with cv. Spantex, a spanish type variety (Sharon, 1965), cv. Florunner, the commercially dominant runner type variety, was selected to add the seed-size parameter to this study. This allowed an examination of relationships among peanut grade sizes, packaging methods and storage times not feasible in previous studies.

MATERIALS & METHODS

THE STUDY was a 3 x 3 x 3 factorial experiment in a completely randomized design with 3 replications; that is, 3 peanut grade sizes (jumbos [>8.33 mm dia], no. 1's [<7.14, >6.35 mm dia] and splits [>6.75 mm dia]) x 3 packaging methods (farmers stock-bulk, shelled —burlap bag and shelled — plastic bag-CO_2 atmosphere) and

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3 storage times (1-mo, 3-mo and 6-mo). The 1980-crop peanuts were obtained from the Dothan Oil Mill Company (Dothan, AL) in early October, soon after harvest, and transported to the National Peanut Research Laboratory in burlap bags.

The shelled grade size x packaging method replications were packaged as individual units. Fifty kg of peanuts for each plastic bag-CO_2 unit were placed in a separate laminated plastic bag and a fiberboard box and flushed with carbon dioxide (13.59 m^{3}h for 15 sec). The bag was then heat-sealed and the box closed. For burlap-bag storage 45.36 kg were sewn up in each bag. For farmers stock-bulk storage a 1.42 m^3 fiberboard box was filled with about 340 kg of inshell peanuts, which were not shelled and sized until after storage. The peanuts were all stored in a metal-clad, warehouse-type structure at ambient temperature and humidity.

After 1-mo, 3-mo and 6-mo, three containers of each of the shelled-peanut treatments were removed from storage, and representative 4.54 kg samples were taken for quality evaluation. Enough farmers stock peanuts were removed from the 1.42 m^3 bulk-storage box to provide 3 representative 4.54 kg samples of each treatment after shelling and screening. Initial (0-mo) quality and population variability were determined on one sample per treatment obtained during packaging. An additional, limited test of another low-oxygen packaging method was introduced outside the factorial design by adding 2 replications of nitrogen gas flush of 50 kg of no. 1's in laminated plastic bag in corrugated box (by the same procedure as with CO_2 flush) for 1-mo and 3-mo storage. These were the storage times and the grade size believed most likely to demonstrate significant influence on the quality of peanuts which might not be completely cured (Schenk, 1961; Baskin and Delouche, 1971; Dickens and Pattee, 1973).

Processing and testing

After storage, moisture, raw seed color (except splits), peanut butter color, and peanut butter flavor were determined for each storage time. Moisture percent was determined by drying subsamples at 130°C for 6 hr (Blankenship, 1977; Young et al., 1982). The remaining peanuts were then dried to 5% moisture before further testing or processing. Hunter L, aL and bL values were recorded for raw seed (skins intact) using a 12 cm-square plastic container having a clear bottom and 4.5 cm-high frosted sides over the 10.16 cm dia viewing port of the inverted optical head of a Hunterlab Color/Difference Meter, Model D25D2L.

Samples for peanut butter color and flavor evaluation for all storage times were prepared by roasting at 177°C for the number of minutes required for a medium roast of the 0-mo sample of the corresponding grade size (Morris and Freeman, 1954; Buckholz et al., 1980; Pattee et al., 1982). The times required were 27.5, 24, and 28 min, respectively, for jumbos, no. 1's and splits. Samples (1 kg) were roasted in a four-compartment, stainless-steel slotted-screen cylinder in a Blue M "Power-O-Matic 60" laboratory oven, modified to rotate the cylinder during roasting. The cooled samples were then blanched in a small NPRL-designed blancher (Barnes et al., 1971), degermed, and hand-picked to remove unblanched pieces and previously hidden damage. The now <1 kg, room-temperature samples were separately ground in a Bauer Bros., horizontal attrition mill with ca. 0.5 mm clearance between grinding plates. The smooth butter was collected in the stainless-steel mixing bowl of a Hobart Model 4-C, KitchenAid, 10-speed food mixer and mixed with the all-purpose beater for 1 min at speed no. 1 and 2 min at speed no. 2. The butter was refrigerated at least over night in oil-retaining, laminated plastic bags and then returned to room temperature for color/flavor evaluation. Color measurement equipment and procedure for the butter samples was similar to that of the raw samples, except that only ca. a 1.5 cm layer of butter was required in the same sample container.

Flavor panel

Usually the same 10 selected and experienced panelists (1 white male, 1 black female and 8 white females; estimated age-range from early 30's through 60's) participated at each storage time. They tasted 9 samples (one replication of the factorial study) at each of 3 sessions for the 6-mo storage time. At 1-mo and 3-mo, 10 samples were presented at 2 of the 3 sessions to allow limited testing of the additional packaging method (N_2 atmosphere in plastic bag). Flavor of the butter was rated on a 5-category ballot (excellent = 1, good = 2, fair = 3, poor = 4 and very poor = 5) in individual, red-masking-lighted booths. Booths are painted blue-purple, a complementary color to the yellow-red region of the products tasted, and chairs and light cabinets are gray. Peanut butter samples were presented in white plastic spoons, coded with random 3-digit numbers and placed on tan colored plastic trays in the order of their listing on the accompanying ballot. The order of listing was rotated among the ballots. Panelists generally tasted at their convenience during mid-morning or mid-afternoon (Kare and Halpern, 1961; Dawson, 1964; Amerine et al., 1965).

Statistical analyses

Factorial analysis of variance (ANOVA) and Duncan's Tests (Helwig and Council, 1979) were run on the grade size x packaging method data for each quality test and for 1-mo, 3-mo and 6-mo separately. ANOVA and Duncan's Tests of the 0-mo data were run, using the grade size x packaging method interaction as the error term. ANOVA and Duncan's Tests were also run on no. 1's packaged 4 ways and stored for 1-mo and 3-mo. Linear regressions with unconverted data and with converted data ($\log_e$, Y; $\log_e$ [X + 1]; $\log_e$ Y, $\log_e$ [X + 1]) and quadratic regressions on 0-mo to 6-mo storage time (as X) were run for means of packaging methods and selected grade sizes for selected color and flavor values (as Y).

RESULTS & DISCUSSION

Comparison of 3 grade sizes and 3 packaging methods

After-storage moisture. No significant moisture differences were detected at the beginning of storage (0-mo), but differences were demonstrated both among grade sizes and packaging methods at each storage period thereafter (Table 1). Shelled seed stored with CO_2 atmosphere in plastic bags generally retained more moisture than bulk, inshell peanuts or shelled seed in burlap bags, as expected. The no. 1's generally retained more moisture than the jumbos or splits, which we attribute to the lesser maturity of the no. 1's (Holley, 1960; Woodroof, 1966; Blankenship and Hutchison, 1970). However, the general results were complicated by significant interaction between storage method and grade size in the 1-mo and 6-mo tests. Why there was significant interaction at 1-mo and 6-mo but not at 3-mo is not clear.

Raw seed color — after storage moisture. Generally, the color of raw no. 1's was lighter than that of jumbos (Table 1). This is in general agreement with a previous study of cv. Florigiant peanut screen sizes (Pearson, 1979) and may be mostly the result of the increase in light reflectance inherent in the decrease in particle size (Clydesdale, 1972).

At 6-mo, raw seeds from burlap bags were darker than from other packages. However, differences among packaging methods and interactions with grade sizes changed from

Table 1—Factorial design (grade size x packaging method)—quality parameters showing significantly (5 to 0.01%) different factor means by ANOVA and separation of different (5%) means by Duncan's tests

Quality parameters	Grade size ANOVA F significance (%)	Grade size means			Packaging method ANOVA F significance (%)	Packaging method means			Grade size x package method significance (%)
		Jumbos	No. 1's	Splits		Farmers stock	Burlap bag	Plastic bag-CO_2	
1-Month storage									
After-storage moisture	0.01	6.78A[a]	6.74B[a]	6.60C[a]	0.01	6.42C[a]	6.56B[a]	7.14A[a]	0.01
Raw color, L	0.01	38.34B	40.27A	—[b]	0.02	39.98A	38.83B	39.10B	1.34
Raw color, aL	2.33	16.02A	14.70B	—	N.S.[c]	—	—	—[d]	N.S.[c]
Butter color, L	0.50	46.92B	47.28B	49.06A	N.S.	—	—	—	N.S.
Butter color, aL	2.25	12.84B	13.89A	12.56B	N.S.	—	—	—	N.S.
Butter color, bL	1.68	24.26B	24.88A	25.13A	N.S.	—	—	—	N.S.
Flavor score	0.07	2.41B	3.02A	2.41B	N.S.	—	—	—	N.S.
3-Month storage									
After-storage moisture	0.02	6.09B	6.73A	6.18B	0.01	5.98B	6.00B	7.01A	N.S.
Raw color, L	0.03	36.54B	38.24A	—	N.S.	—	—	—	N.S.
Raw color, aL	0.01	14.00A	13.34B	—	N.S.	—	—	—	N.S.
Raw color, bL	1.02	12.90B	13.11A	—	N.S.	—	—	—	N.S.
Butter color, L	0.01	45.91B	46.01B	49.08A	0.39	48.24A	47.04AB	45.71B	N.S.
Buttler color, aL	0.01	11.33A	11.27A	9.74B	0.03	10.21C	10.82B	11.31A	N.S.
Butter color, bL	0.19	22.94B	22.78B	23.41A	1.00	23.30A	23.07AB	22.77B	N.S.
Flavor score	0.05	2.62B	3.36A	2.62B	N.S.	—	—	—	N.S.
6-Month storage									
After-storage moisture	0.01	5.80B	6.12A	5.60B	0.01	5.59B	5.51B	6.42A	0.36
Raw color, L	0.02	34.64B	35.74A	—	0.01	35.95A	34.05B	35.58A	0.03
Raw color, aL	0.01	14.37A	13.31B	—	1.07	13.58B	14.05A	13.88A	N.S.
Butter color, L	0.01	47.63A	45.66B	48.26A	0.01	48.57A	46.98B	46.00C	0.22
Butter color, aL	0.01	9.96B	10.83A	9.86B	0.01	9.73C	10.30B	10.61A	0.02
Butter color, bL	0.01	23.29A	22.62B	23.37A	0.02	23.40A	23.09B	22.79C	3.48
Flavor score	0.01	2.53C	3.62A	3.00B	N.S.	—	—	—	N.S.

[a] Means not followed by a capital letter shared with each other are significantly (5%) different.
[b] Splits were excluded from raw color measurement, which was, essentially, skin-color measurement for jumbos and No. 1's.
[c] Not significant at the 5% level.
[d] Results of Duncan's tests not shown unless ANOVA showed significance (5%).

Table 2—Quality parameters showing significantly (10 to 0.04%)[a] different factor means by ANOVA and separation of different (5%) means by Duncan's test for 0-month storage

Quality parameters[b]	Grade size ANOVA F significance (%)	Grade size means			Packaging method ANOVA F significance (%)	Means of sub-lots assigned to packaging methods[c]		
		Jumbos	No. 1's	Splits		Farmers stock	Burlap bag	Plastic bag-CO_2
Raw color, bL	1.88	12.90B[d]	13.53A[d]	—[e]	N.S.	—	—	—
Butter color, aL	2.18	14.20A	13.47B	14.40A	0.04	12.43C	15.27A	14.37B
Flavor score	1.10	2.50B	2.97A	2.50B	9.40	2.50B	2.70AB	2.77A

[a] The ANOVA significance level was dropped to 10% because of the small number of degrees of freedom (no true replication within a factor, and grade size x packaging method interaction supplying the d.f. for error).
[b] Quality parameters not listed unless ANOVA F value significant (10%) for grade size or packaging method.
[c] Samples for testing for 0-month were taken from assigned sub-lots before the sub-lots were packaged and stored.
[d] Means not followed by a capital letter shared with each other are significantly (5%) different.
[e] Splits were excluded from raw color measurement for lack of skin coverage.

Table 3—Peanut butter L-value, grade size x packaging method interaction means at 6-mo storage

	Farmers stock	Burlap bag	Plastic bag-CO_2	Grade size mean
Jumbos	49.3	48.1	45.5	47.6
No. 1's	45.9	45.4	45.7	45.7
Splits	50.6	47.4	46.8	48.3
Packaging method mean	48.6	47.0	46.0	

Table 4—Packaging method evaluation for limited test of nitrogen atmosphere; separation of significantly (5%) different means by Duncan's tests[a]

Quality parameters	Packaging method means			
	Farmers stock	Burlap bag	Plastic bag-CO_2	Plastic bag-N_2
	1-Month storage			
Raw color, L	40.60A[b]	39.80B[b]	40.40AB[b]	41.20A[b]
	3-Month storage			
After-storage moisture, %	5.50C[c]	6.16B[c]	6.77A[c]	6.75A[c]

[a] Results of Duncan's tests not shown unless ANOVA showed significance (5%).
[b] Means not followed by a capital letter shared with each other are significantly (5%) different.
[c] Determined by Motomco Moisture Meter.

one storage time to another without apparent pattern or reason. Further study is needed on these relationships.

Butter color. After storage, splits generally produced the lightest-color butter. By 6-mo, significant interaction had developed between grade size and packaging method, but apparently (Tables 1 and 3), no. 1's produced darker butter than jumbos or splits stored as farmers stock or burlap-bagged peanuts. Plastic bag-CO_2, burlap bag, and farmers stock peanuts produced the darkest to lightest butters, respectively, for jumbos and splits.

Butter flavor. Jumbos and splits shared identical mean flavor values at each storage period until 6-mo, when jumbos rated better than splits (Tables 1 and 2). Butter from no. 1's consistently rated the poorest in flavor from 0-mo to 6-mo. Thus, cv. Florunner, as well as cv. Florigiant (Pattee et al., 1982) and cv. Spantex (Sharon, 1965) peanuts screened to or near the minimum size for no. 1 grade for their market type, can produce butter of significantly inferior flavor compared to that of larger kernels from the same lot.

Only the jumbos and splits showed a hint of flavor improvement, and that was only at 1-mo. Among packaging methods there appeared to be no difference in effect upon flavor development during storage.

Limited test of plastic bag-N_2 atmosphere

In nonfactorial ANOVAs and Duncan's Tests (5% significance), quality values of no. 1's stored in plastic bags with N_2 atmosphere were compared with those of the above 3 packaging methods for 1-mo and 3-mo (Table 4). Significant differences among the packaging-method means were limited to raw-kernel color (L) at 1-mo and to after-storage moisture at 3-mo. In neither case did CO_2 and N_2 atmosphere effects differ significantly.

Effects of storage time on raw seed color (L) for 6 grade size — packaging method combinations

The slopes of the regressions (Table 5) suggest that the seed coats of raw jumbos stored in burlap bags and no. 1's stored in burlap bags or as farmers stock darkened more rapidly than jumbos stored as farmers stock or in plastic bags with CO_2 or no. 1's stored in plastic bags with CO_2.

All best-fit curves had coefficients of determination (r^2) high enough to recommend them for predictive use.

Effects of storage time on flavor of butters from 3 peanut grade sizes

Jumbos did not exhibit significant (5%) change in flavor quality during 6-mo storage by any of the 5 regressions employed (Table 5). The pattern of change for splits best fits a quadratic curve. The no. 1's showed a linear decrease in flavor quality, with an r^2 just high enough to recommend predictive use.

SUMMARY

IN THIS STUDY of peanut grade-size, packaging-method and storage-time relationships, the flavor of butter from newly harvested and cured no. 1 cv. Florunner peanuts was inferior to butter from jumbos or splits. During 6-mo ambient storage, the flavor of jumbos remained essentially unchanged, while the flavor of no. 1's showed a linear decline. Packaging and storing the raw peanuts as farmers stock-bulk, in burlap bags or in plastic bags with CO_2 atmosphere showed no significant differential effect on flavor either from possible additional curing or from 6-mo aging.

After storage, moisture content of raw no. 1's was generally higher than that of jumbos or splits, and storage in plastic bags with CO_2 atmosphere generally maintained moisture at a higher level than storage as farmers stock or in burlap bags.

After 6-mo storage, raw burlap-bagged peanuts were darker colored than those from other packages. Also, no. 1's apparently produced darker butter than jumbos or splits for farmers stock and burlap-bagged peanuts, and both jumbos and splits apparently produced the darkest to lightest butters for plastic bag-CO_2, burlap-bag and farmers stock storage, respectively.

Table 5—Regressions of means of selected color and flavor values of packaging methods and selected grade sizes on storage time (0-6 mo)

Quality parameter/ treatment	Linear with unconverted data					Most practicable other[a]						
	r^{2b}	Significance of F (%)	(A) Intercept	(B) Slope	Standard deviation	Type[a]	r^{2b}	Significance of F (%)	(A)	(B)	(C)	Standard deviation
Raw color, L Grade size— packaging method[c]												
Jumbos- farmers stock	0.73	0.16	40.27	−0.75	1.10	$Log_e (X + 1)^d$	0.87	0.01	41.44	−2.82	—	0.77
Jumbos- burlap bag	0.97	0.01	39.27	−1.07	0.50	—	—	—	—	—	—	—
Jumbos- plastic bag- CO_2	0.89	0.01	38.85	−0.71	0.62	$Log_e (X + 1)$	0.96	0.01	39.81	−2.57	—	0.38
No. 1's- farmers stock	0.94	0.01	41.69	−0.99	0.61	—	—	—	—	—	—	—
No. 1's- burlap bag	0.97	0.01	40.79	−0.94	0.41	—	—	—	—	—	—	—
No. 1's- plastic bag- CO_2	0.93	0.01	40.75	−0.76	0.52	—	—	—	—	—	—	—
Butter flavor score Grade size[e]												
Jumbos	0.06	43.05	2.48	0.01	0.14	—	—	—	—	—	—	—
No. 1's	0.73	0.04	2.96	0.11	0.18	—	—	—	—	—	—	—
Splits	0.59	0.37	2.40	0.09	0.20	Quadratic	0.64	1.07	2.46	−0.01	0.02	0.19

[a] Selected primarily for improvement over r^2 values and significance levels of linear regressions with unconverted data, but also considering ease of computation. All regressions except quadratic were linear, with indicated converstions of data.
[b] r^2 (coefficient of determination)—a value of 0.72 corresponds to an r (correlation coefficient) value of 0.85, commonly regarded as a minimum value recommended for predictive use.
[c] Degrees of freedom for error = 8 for all listed regressions.
[d] Formulas involving log_e of X required addition of 1, i.e., (X + 1) for computation, since log_e of 0 = $-\infty$.
[e] Degrees of freedom for error = 10 for all regressions except quadratic, which have 9.

Significant interactions at 6-mo between grade size and packaging method for after-storage moisture, raw kernel color and peanut butter color suggest the need for further study, including the possibly interacting effects on peanut quality of such other factors as growing season, production region, cultural practices, screen-size-distribution pattern and variations in ambient storage environment.

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High Fructose Corn Syrup: Replacement for Sucrose in Angel Cake

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――――――ABSTRACT――――――

High fructose corn syrup (HFCS) (42% fructose) was used to replace 25, 50, 75 or 100% of the sucrose in angel cakes. Replacement with HFCS of 50, 75 or 100% of sucrose resulted in foams with lower specific gravities; decreased foam beating time; and cakes with lower volume, browner crusts, yellower crumb, firmer texture, and decreased sweetness. Replacement of 25% sucrose with HFCS did not affect greatly the physical measurements or sensory characteristics studied.

INTRODUCTION

COMMERCIAL USE OF corn syrup, dextrose and high fructose corn syrup (HFCS) has increased dramatically since 1970, primarily because corn sweeteners are less expensive than sucrose (Inglett, 1981). The major reason for the increased usage of corn sweeteners was the development in 1967 of first-generation HFCS containing 42% fructose, 52% glucose and 6% higher saccharides (Vuilleumier, 1980). Although other functional properties may differ, HFCS theoretically is equivalent to sucrose in sweetness because it contains glucose and fructose in a proportion similar to that of sucrose. HFCS has found wide application as a less expensive alternative to sucrose in food products such as soft drinks, yeast-leavened baked goods and canned fruits, where the main function of the sugar is to provide sweetness (Inglett, 1981).

One of the most difficult applications of HFCS is in cakes, where the amount and type of sugar present greatly affect the flavor, volume, texture and browning of the product. Thompson et al. (1980) studied the flavor acceptance and taste perception of butter cakes prepared with sucrose or HFCS. The flavor of the crumb of HFCS cakes was as acceptable as that of the sucrose cakes, although some panelists detected a sharp or tart but acceptable flavor in the crusts of HFCS cakes. Volpe and Meres (1976) noted an undesirable sourness in the crumb of white layer cakes in which 60% of the sucrose was replaced with HFCS. They attributed the sour flavor to the high-acid leavening system used and believed that the sourness might be masked by a flavoring system different from vanilla.

Fructose and glucose, as reducing sugars, participate readily in aldoseamine nonenzymatic browning reactions (Hodge and Osman, 1976). Koepsel and Hoseney (1980) found that layer cakes made with HFCS had excessive browning, which Volpe and Meres (1976) minimized in white layer cakes having 60% of the sucrose replaced with HFCS by a high-acid leavening system that acidified the cake batter to below pH 6.0. At the lowered pH, crust browning and crumb yellowing were reduced to an acceptable level. Thompson et al. (1980) reported that although volume of butter cakes with HFCS was lower than that of sucrose cakes, the cakes were equal in tenderness. Koepsel and Hoseney (1980) found that replacement of 100% of the sucrose with HFCS in high-ratio white layer cakes

resulted in greatly decreased volume and open grain. White layer cakes in which 60% of the sucrose was replaced with HFCS had slightly greater volume than 100% sucrose cakes (Volpe and Meres, 1976).

No research studies were found concerning the use of HFCS in angel cakes. This study was undertaken to investigate selected physical and sensory characteristics of angel cakes with 25, 50, 75 or 100% of the sucrose replaced with first-generation HFCS.

MATERIALS & METHODS

Ingredients and formula

The AACC Method 10-95 for testing baking quality of angel cake flour (Kissell and Bean, 1978) was modified by the addition of clear imitation vanilla flavoring; first-generation HFCS (42% fructose) was used to replace 25, 50, 75 or 100% of the sucrose (Table 1). In cakes containing HFCS, the 71% sugar solids content of the HFCS was included in the sugar solids total, and the 29% water content of the HFCS was subtracted from the formula water; thus, all cakes contained 314g sugar solids and 295 ml water.

Mixing procedure

The AACC Method 10-95 for angel cake (Kissell and Bean, 1978) was modified because HFCS was used. Acid salt, sodium chloride, flavoring and all sugar (sucrose and/or HFCS) were whipped with the reconstituted albumen using a Kitchen Aid mixer (model K5-A, Hobart Co., Troy, OH) fitted with whip attachment. The desired specific gravity range of the foam was adjusted to 0.19 ± 0.01 because all formula sugar was added to the foam. The sifted flour then was folded into the foam. Preparation and baking of the cakes proceeded according to AACC Method 10-95 except that the cakes were baked in a household-type electric range (Whirlpool Corp., Benton Harbor, MI).

Foam and batter specific gravities were determined (Kissell and Bean, 1978). Batter pH was read directly from an approximately 10 g sample using a pH meter (Horizon Ecology Co., Chicago, IL). An estimate of volume was determined immediately after the cakes were removed from the pan (Kissell and Bean, 1978). The investigator recorded descriptive statements of symmetry including smoothness of side and bottom crusts, presence of cracks in the top crust and presence of "dips" or other irregularities in the top surfaces of cakes. Percentage moisture in the cake interior was determined from

Table 1—Formulas for angel cakes at five HFCS levels

Ingredient	Replacement level of HFCS for sucrose				
	0%	25%	50%	75%	100%
Bleached cake flour[a], g	110	110	110	110	110
Dried egg albumen[b], g	40	40	40	40	40
Acid salt[c], g	1.5	1.5	1.5	1.5	1.5
Sodium chloride, g	3.0	3.0	3.0	3.0	3.0
Clear imitation vanilla flavoring, ml	7.5	7.5	7.5	7.5	7.5
Extra fine granulated sucrose[d], g	314	236	157	79	–
HFCS[e], g	–	111	222	333	444
Distilled water, ml	295	263	231	299	167

[a] Softasilk, General Mills, Inc.
[b] Type P-20, Henningsen Foods, Inc.; includes approximately 0.1% sodium lauryl sulfate added to dried egg solids by processor.
[c] Monocalcium phosphate Monohydrate, Regent 12XXX, Stauffer Chemical Co.
[d] C & H, California and Hawaiian Sugar Co.
[e] Isomerose 100, Clinton Corn Processing Co.; contains 42% fructose, 52% glucose and 6% higher saccharides.

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10-g samples of crumbs dried in a Brabender Semi-Automatic Moisture Tester (C.W. Brabender Instruments, Inc., South Hackensack, NJ) for 2 hr at 120°C. Crust and crumb color were evaluated using a HunterLab Spectrophotometer (Hunter Associates Laboratory, Inc., Reston, VA). A 4 x 4 x 1.5-cm sample of the crumb or a 4 x 4 x 1.5-cm sample including top crust was evalutated for L (Lightness), a (redness) and b (yellowness). An Instron Universal Testing Machine (IUTM) (Instron Corp., Canton, MA) was used to evaluate firmness and elasticity of the cake crumb. The 50-cm^2 compression anvil was used with the 500 kg load cell. Three 2.5-cm cubes from the midsection of each cake were compressed to 1.0 cm using a 0.2 kg load. Two force curves were recorded using crosshead and chart speeds of 200 mm/min (Fig. 1). The peak height of the first curve (C), converted to kg units, represented the resistance of the crumb to compression, or firmness (Neukom and Rutz, 1980). The distance (in cm) to the peak of the second curve expressed as a ratio of the distance to the peak of the first curve (B/A x 100) was an indication of elasticity of the crumb.

Sensory evaluation

Sensory evaluation of angel cakes was completed within 22 hr of baking by a 10-member experienced panel of faculty and graduate students from the Departments of Foods and Nutrition and Grain Science and Industry. Panelists were seated at partitioned booths and provided water for rinsing during all sessions. During two preliminary sessions, the panel evaluated three samples for tenderness, moistness, sweetness, crust color and crumb color. Five-point category scales were provided for each characteristic, and panelists were instructed to use whole numbers only. After the preliminary sessions, some category scales were modified to reflect better the panelists' perceptions of the products.

Samples from each of the five treatments were evaluated by individual panelists during each of four sessions in the main study. Smples (4 x 4 x 1.5 cm) from the midsection of the cakes were placed on white plates and evaluated for tenderness, moistness and sweetness under red light to eliminate possible color influences. Crumb color was evaluated from 4 x 4 x 1.5-cm samples displayed on white plates under a Super Skylight (Macbeth Daylighting Corp., Newburgh, NY) on daylight setting. Crust color was evaluated similarly using samples including top crusts.

Experimental design and analyses of data

Data for physical measurements from five replications and sensory data from four replications were analyzed by analysis of variance for a randomized complete block design (Snedecor and

Fig. 1—Typical compression curves obtained from the IUTM for determining firmness and elasticity of angel cakes.

Cochran, 1967). Least square means were compared to determine treatment effects. Least significant differences were calculated for sensory data at the 5% level to determine significance of differences between means.

RESULTS & DISCUSSION

Physical measurements

Beating time and foam specific gravity. As egg white is whipped, stiffness and volume of the foam increase, and specific gravity decreases as air is incorporated into the foam (Palmer, 1972). Foams containing 50, 75 or 100% HFCS were beaten a shorter time (P < 0.001) (Table 2) than foams containing 0 or 25% HFCS before reaching the desired specific gravity range (Table 3). Despite less beating time, foams containing 50, 75 or 100% HFCS had lower (P < 0.05) specific gravities than 0 or 25% HFCS foams. Sucrose foams held a stiff, slightly bending peak at a specific gravity of approximately 0.19 (Fig. 2); foams containing HFCS only barely mounded at a specific gravity of approximately 0.18 (Fig. 3). Foams containing 25, 50 or 75% HFCS were less stiff as HFCS increased.

Batter specific gravity. Mean F-values indicated a difference (P < 0.01) in batter specific gravity (Table 2). Batters containing 100% HFCS had higher (P < 0.05) mean specific gravities than any of the other batters (Table 3). Although HFCS egg white foams had lower mean specific gravities than sucrose egg white foams before flour was folded in, batters containing HFCS and no sucrose had higher mean specific gravities than batters containing sucrose (Table 3). These results suggest that foams containing HFCS only are less able to retain air than are sucrose foams at comparable specific gravities as flour is folded in during the last stage of mixing.

Estimate of volume. Mean F-values for estimates of volume were different (P < 0.001) (Table 2). Although there was no difference between the mean volume estimates for 0 and 25% HFCS cakes, the mean volume estimates for 50, 75 and 100% HFCS cakes were lower (P < 0.05) (Table 3). The higher the level of HFCS, the lower the estimated volume. The lower volume of cakes containing HFCS could be influenced by premature starch gelatinization caused by the monosaccharides in HFCS (Bean et al., 1978; Koepsel and Hoseney, 1980) or by decreased stiffness of foams containing HFCS (Palmer, 1972).

Batter pH and percentage moisture. The mean batter pH values for the five treatments were not different (Table 2). The mean range of batter pH was from 5.35—5.42, well within the optimum range of 5.0—6.5 recommended by Pyler (1973) for angel cake. Thompson et al. (1974) reported that layer cakes made with HFCS were moister than sucrose cakes. In this study, mean percentage moisture
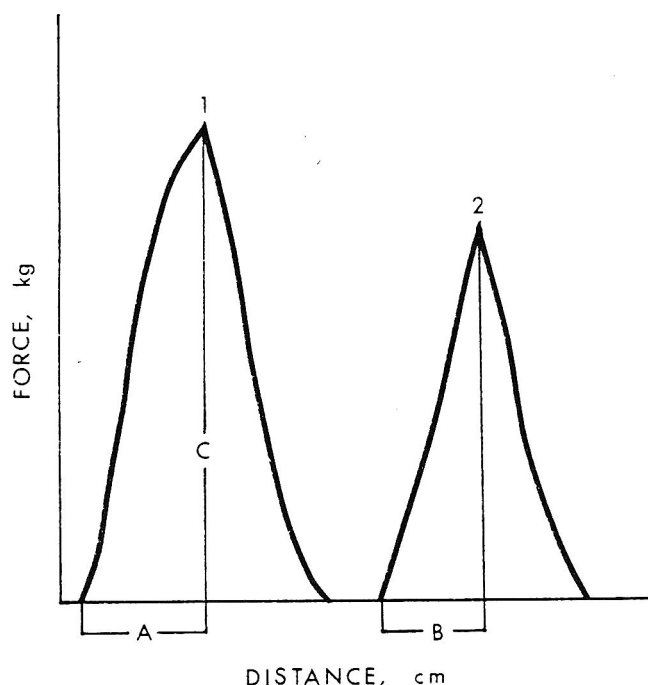
Table 2—F-values from AOV for physical measurements of angel cakes made with five levels of HFCS

Beating time	76.95***
Foam specific gravity	4.74*
Batter specific gravity	5.54**
Estimate of volume	65.13***
Batter pH	1.40
Percentage moisture	1.74
Crust L-value	37.66***
Crust a+-value	24.64***
Crust b+-value	10.94***
Crumb L-value	2.31
Crumb a+-value	2.18
Crumb b+-value	123.55***
Elasticity	1.21
Firmness	7.72**

*, P < 0.05; **, P < 0.01; ***, P < 0.001

readings taken within 24 hours of baking were not different for any treatment (Table 2).

Crust color. Crust color was affected greatly (P < 0.001) by the replacement of sucrose with HFCS (Table 2). In general, as the HFCS increased, the crust color became darker, more red and less yellow, as measured by the HunterLab Spectrophotometer (Table 3). These results agree with previous studies in which the substitution of HFCS for sucrose in layer cakes resulted in much browner exteriors (Koepsel and Hoseney, 1980; Thompson et al., 1980). The b-values (yellowness) for 25, 50 and 75% HFCS cakes were not different (Table 3) whereas b-values for 0 and 100% HFCS cakes were different (P < 0.05) from each other and from all other treatments (P < 0.05). The crusts of the 0% HFCS cakes were yellower (P < 0.05) than the crusts of all other cakes. The crusts of 100% HFCS cakes

were darker (P < 0.05) and less yellow (P < 0.05) than all other cakes. Crust a-values (redness) were not different for 0 and 25% HFCS cakes. Crust a-values for 50, 75 and 100% HFCS cakes were different (P < 0.05) from each other and from those of 0 and 25% HFCS cakes, indicating redder crusts as HFCS increased.

Crumb color. Although crumb mean b-values (yellowness) differed (P < 0.001), crumb L-values (lightness) and a-values (greenness) were not different at any HFCS level (Table 2). Crumb b-values were different (P < 0.05) at each HFCS level (Table 3). As the level of HFCS increased, the b-values increased, indicating a yellower crumb. These results agree with results with shortened cakes in which the use of HFCS caused the development of uncharacteristic yellowish or greenish interior colors attributed to Maillard reactions (Thompson et al., 1980; Volpe and Meres, 1976).
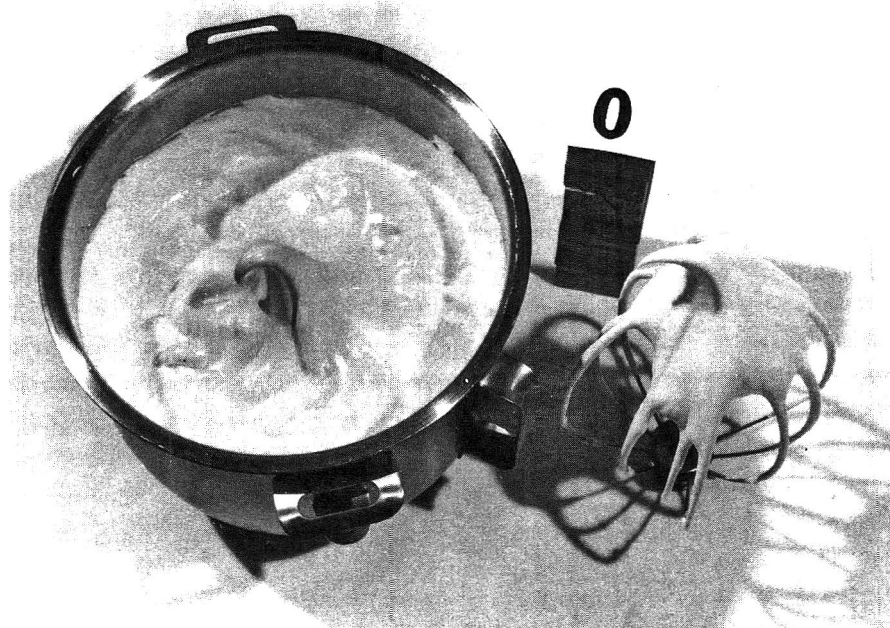
Fig. 2—Typical peak formed by all sucrose egg white foams (specific gravity = 0.193).

Table 3—Least square means[a] for physical measurements of angel cakes made with five levels of HFCS

	Percentage of HFCS				
Physical measurement	0	25	50	75	100
Beating time, seconds	180a	186a	138b	114c	54d
Specific gravity					
Foam	0.191abd	0.193ab	0.182cd	0.183acd	0.180cd
Batter	0.237a	0.238a	0.254a	0.253a	0.272b
Estimate of volume, cc	2933a	2878a	2648b	2566c	2494d
Batter pH	5.42a	5.42a	5.42a	5.36a	5.35a
Percentage moisture	39.06a	39.89a	40.07a	39.70a	40.50a
HunterLab values					
Crust					
L	57.01a	48.57a	47.63a	46.49b	43.50c
a	7.86a	7.83a	8.41b	8.82c	9.23d
b	17.20a	15.24b	15.39b	14.39b	12.88c
Crumb					
L	84.01a	84.23a	84.03a	83.51a	82.46a
a	−3.04a	−2.87a	−2.94a	−2.80a	−2.68a
b	9.72a	10.98b	12.31c	12.85d	14.04e
Instron values					
Elasticity[b]	88.70a	90.53a	91.50a	91.08a	88.72a
Firmness, kg	0.247a	0.234a	0.367b	0.394b	0.466b

[a] Means bearing different letters within the same row differ significantly (P < 0.05).
[b] B/A x 100.

Elasticity and firmness. There were no differences in elasticity among the five treatments; however, mean F-values indicated a difference ($P < 0.01$) in firmness (Table 2). Firmness measurements (kg force) were not different for 0 and 25% HFCS cakes (Table 3). Cakes containing 50, 75 or 100% HFCS were firmer ($P < 0.05$) than 0 or 25% HFCS cakes. The increased firmness measurements of 50, 75 and 100% HFCS cakes could be attributed to either decreased stiffness of the foams or premature starch gelatinization.

Surface symmetry. The bottom crusts of all cakes were smooth. The side crusts of 75 and 100% HFCS cakes were occasionally ragged because those cakes tended to stick to the pan. The top crusts of 0 and 25% HFCS cakes always had pronounced cracks and splits characteristic of angel cake. Cracks were less apparent or not present in the surfaces of 50, 75 and 100% HFCS cakes (Fig. 4). The top surfaces of 0 and 25% HFCS cakes tended to be even, whereas the top surfaces of 50, 75 and 100% HFCS cakes frequently had one or two small, shallow "dips."

Sensory evaluation

Tenderness. Mean tenderness scores were not different for 0 or 25% HFCS cakes (Table 4). Mean tenderness scores indicated cakes became firmer as HFCS increased (Table 4). These results agree with firmness measurements obtained with the IUTM in which 50, 75 and 100% HFCS cakes were firmer than 0 and 25% HFCS cakes (Table 3).

Moistness. There were no differences among moistness scores (Table 4). These results agree with percentage moisture measurements, among which no differences were found (Table 2). Results from this study differ from findings by Thompson et al. (1974), in which frozen and thawed layer cakes made with HFCS were judged moister than sucrose cakes. In this study, results within 24 hr of baking do not agree with the suggestion made by Henry (1976) and Saussele et al. (1976) that HFCS may contribute to moisture retention in baked products to a greater extent than does sucrose.
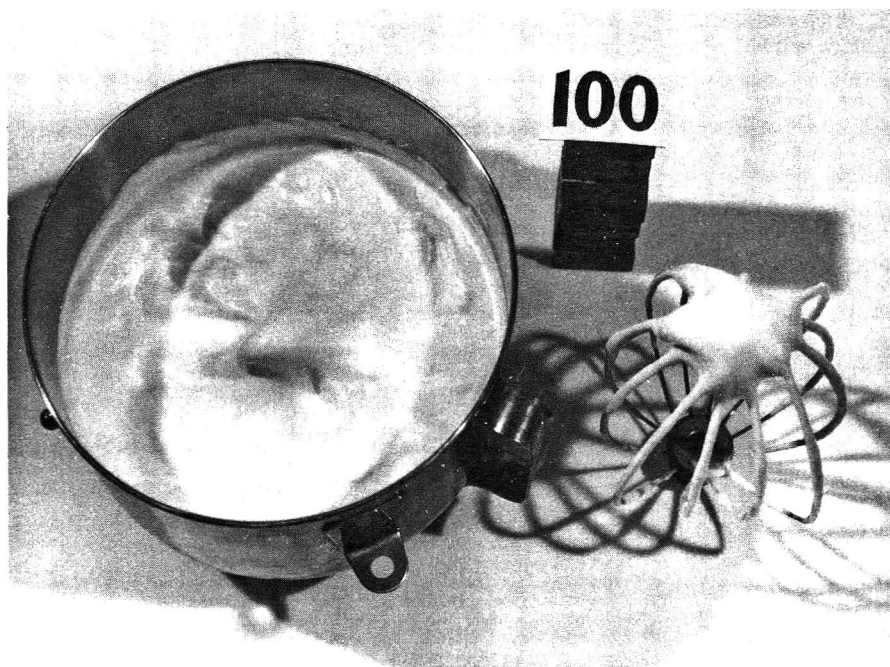
Fig. 3—Typical peak formed by all HFCS egg white foams (specific gravity = 0.183).
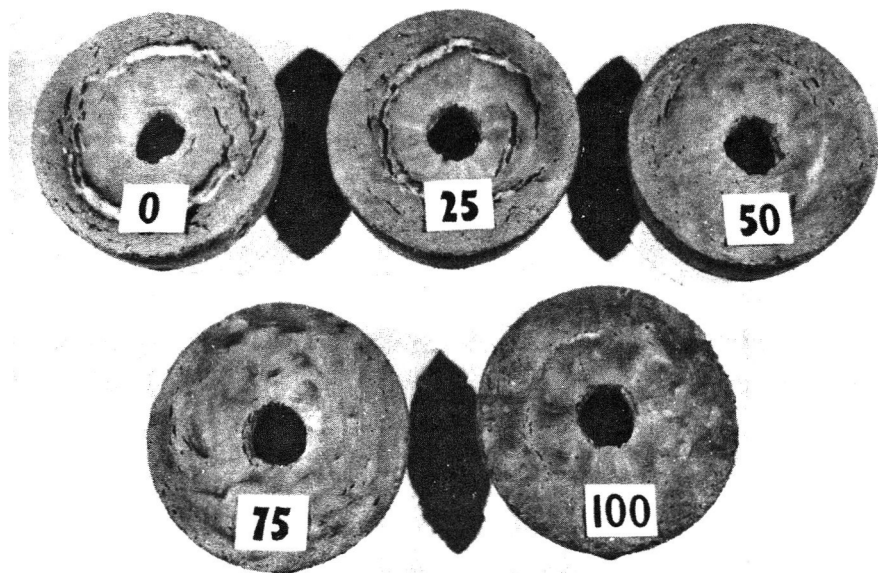
Fig. 4—Top surfaces of angel cakes at five HFCS levels.

Sweetness. Cakes with 25% HFCS had the highest mean sweetness scores, but their scores were not different from those for 0% HFCS cakes (Table 4). The 50 and 100% HFCS cakes were not different from each other in sweetness, but were less sweet than all other cakes. Six of ten panelists detected bitterness in some cakes. The four panelists who did not may reflect the insensitivity to bitter taste occurring in 28 to 40% of Caucasian Americans when tasting solutions of phenylthiocarbamate (Amerine et al., 1965). Consequently, the lower mean sweetness scores for cakes containing 50% or greater levels of HFCS may not reflect the full extent of the bitterness present.

Desirable and undesirable flavor compounds can be produced by caramelization of sugars or by other ingredients of cake as well as by aldoseamine reactions (Hodge and Osman, 1976). Because they are reducing sugars, monosaccharides such as glucose and fructose in HFCS undergo these reactions more readily than does sucrose (Hodge and Osman, 1976). Data for 50, 75 and 100% HFCS cakes suggest that sweetness decreases if HFCS replaces 50% or more of the sucrose in angel cake (Table 4). A different reaction may have occurred in the 25% HFCS cakes, which received the highest mean sweetness scores. Some glucose syrups, when mixed with sucrose syrup, give greater sweetness than predicted from the separate sweetnesses of the component sugars (Hodge and Osman, 1976).

Crumb color. Mean scores for the crumb of 0 and 25% HFCS cakes indicated that they appeared white (Table 4); the crumb of 50% HFCS cakes was judged off white, and that of 75 and 100% HFCS cakes was judged creamy. Panel scores for crumb color agreed with b-values measured by the HunterLab Spectrophotometer, which indicated a yellower ($P < 0.05$) crumb as the level of HFCS increased (Table 3). Volpe and Meres (1976) found that crumb yellowing attributed to Maillard reactions was minimized if the pH of the batters was below 6.0. The mean pH of angel cake batters in this study ranged from 5.35–5.42 (Table 3). At that pH range, Maillard browning reactions may not be as prominent as caramelization (Shallenberger and Birch, 1975). Thus, the off white or creamy color observed in the crumb of 50, 75 and 100% HFCS cakes may be attributable more to caramelization than to Maillard browning reactions.

Crust color. Mean scores for crust color decreased ($P < 0.05$) as the level of HFCS increased (Table 4). Cakes containing only sucrose had light yellowish brown crusts while 100% HFCS cakes had dark golden brown crusts. Panel scores agreed with HunterLab L, a- and b-values, which indicated that the crust of cakes became darker, redder and less yellow as the level of HFCS increased (Table 3). Excessive exterior browning in HFCS containing cakes has been attributed to Maillard browning reactions of monosaccharides (Koepsel and Hoseney, 1980; Thompson et al., 1980). The low pH of cake batters in this study may have favored crust caramelization rather than Maillard browning in cakes containing HFCS.

Cost of ingredients

Using February, 1981, wholesale prices (Inglett, 1981), HFCS is approximately 50% less expensive than sucrose on an equal solids basis. A 25–100% replacement of sucrose with HFCS could result in a 4.5–18% reduction in ingredient cost of angel cakes.

CONCLUSIONS

UNDER THE CONDITIONS of this study, it was concluded that:

(1) Egg white foams containing HFCS are less stiff than egg white foams containing only sucrose at comparable specific gravities.

Table 4—Least square means[a] of sensory scores for selected characteristics of angel cakes made with five levels of HFCS

Sensory characteristic	Percentage of HFCS				
	0	25	50	75	100
Tenderness[b]	4.34a	3.95ab	3.29bc	3.03c	3.18c
Moistness[c]	3.97a	3.74a	3.29a	3.47a	3.47a
Sweetness[d]	3.26ab	3.55a	2.74c	3.16b	2.79c
Crumb color[e]	4.37a	3.66b	2.97c	2.29d	1.84e
Crust color[f]	3.89a	2.79b	2.34c	1.84d	1.21e

[a] Means bearing different letters within the same row differ significantly ($P < 0.05$).
[b] Tenderness: 5 = tender and 1 = tough.
[c] Moistness: 5 = moist, does not require much saliva and 1 = dry, requires much saliva.
[d] Sweetness: 5 = strongly sweet and 1 = moderately sweet, slightly bitter.
[e] Crumb color: 5 = intensely white and 1 = light yellow.
[f] Crust color: 5 = light beige and 1 = dark golden brown.

(2) replacement with HFCS of 50, 75 or 100% of sucrose in angel cake results in foams with lower specific gravities; decreased foam beating time; decreased volume of cakes; browner crusts; yellower crumb; firmer texture; and decreased sweetness.

(3) Replacement with HFCS of 25% of the sucrose in angel cakes does not greatly affect selected physical measurements or sensory characteristics.

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Water Velocity Effect on Heat Penetration Parameters During Institutional Size Retort Pouch Processing

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————————ABSTRACT————————

Institutional size retort pouches (15 X 12 X 1") filled with 10% bentonite were processed in water with overriding air pressure. The heat penetration parameter, f_h, was measured at seven flow rates from 10 gal/min (Re = 3000) to 110 gal/min (Re = 33000). Apparent convection heat transfer coefficients (h-values) were calculated. Significant differences were found for both the h-value and observed f_h value as a function of flow rate. The h-values ranged from 33–48 BTU/hr ft^2 F and the observed f_h values ranged from 23.0–20.1 min for 10 and 110 gal/min, respectively.

INTRODUCTION

MANY QUESTIONS have arisen in regard to the operational parameters involved in the safe thermal processing of retort pouches. Critical processing factors which have been identified include pouch thickness, confinement, effect of residual gas, type of heating media, and overpressure (Beverly et al., 1980, Milleville and Badenhop, 1980). However, research quantifying these critical factors under conditions of practical processing operations is limited in scope.

The circulation rate of heated water has been identified as a critical processing factor which is important for maintenance of uniform retort temperature during processing (Milleville and Badenhop, 1980; Lampi, 1977). Pflug and Borrero (1967) observed an increase in the rate of heat transfer into retail size retort pouches with an increase in circulation rate. This increase was attributed to an increase in the external fluid heat transfer coefficient. However, the experimental equipment used did not lend itself to quantitation of this effect in terms which allow comparison of various systems. No information is available that quantitates the effect of water circulation rate on heat penetration parameters. These parameters are used to calculate process times, and large unknown variations in their value could represent a potential health hazard.

This study was undertaken to determine the effect of flow rate on heat penetration parameters in institutional size retort pouches with a conduction heating model system. Additionally, the determination of dimensionless constants normally associated with heating and cooling processes would provide a means of predicting the performance of retort systems having differing geometries.

MATERIALS & METHODS

Pouch preparation

The experimental procedure utilized a 10% bentonite suspension as a conduction heating model system. The bentonite suspension was prepared by adding 4.5 lb of 325 mesh bentonite (Kennesaw-Wilcox, Atlanta) slowly to 18.4L of continuously stirred distilled water. The suspension was heated to boiling, cooled, and allowed to stand for 24 hr.

The retort pouch material (0.5 mil PET/ADH/0.5 mil Al foil/4 mil PP) utilized was commercially available stock supplied courtesy

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of American Can Company (Greenwich, CT). Three sides of each 12 X 15 in. pouch were sealed with a ¼ in. wide impulse seal (14H/HTV, Vertrod Corp., Brooklyn, NY). A 9-in. stainless steel needle type copper-constantan thermocouple was inserted into each pouch through a stuffing box (O.F. Ecklund Custom Thermocouples, Cape Coral, FL). The thermocouple was maintained in the geometric center of the pouch by two teflon blocks (1.5 X 0.75 X 1.0 in.) which were positioned a minimum of 2 in. from the thermocouple junction (Fig. 1). Each pouch was cold filled with 5 lb of the bentonite suspension to achieve a 1-in. pouch thickness. The fourth seal of the filled pouch was achieved with a vacuum impulse sealer (Multivac M-3-II, Koch, Kansas City, MO). The pouch was subjected to a minimum of 25 in. of vacuum for 1 min prior to sealing and was immediately given an additional cosmetic seal. No measurement of residual gas in the pouches was made; however, after vacuum sealing and multiple processing operations no residual air was apparent. The film appeared to be in intimate contact with the surface of the bentonite solution at room temperature. All thermocouples were grounded to the retort frame during processing (Fig. 1).

The pouched 10% bentonite suspension was stabilized by heating until the center of the pouch was within 1°F of the processing temperature of 250°F (Ball and Olson, 1957). Following stabilization, the pouches were processed and heat penetration data collected.

Pouch processing

Pouches were processed in a retort designed and built at the University of Florida specifically for institutional size retort pouches. The retort has the capability of using both pressurized water or steam-air mixtures for processing a maximum of eight pouches at one time (12 X 18 X 1 in.). Fig. 2 is a schematic diagram of the retort configuration for pressurized water operation. After filling with preheated process water, the water was recirculated by a centrifugal pump capable of giving reproducible flow rates from 10–110 gal/min. Flow rates of below and above 30 gal/min were measured by the pressure differential across an orifice plate in a 1 and 2-in. pipe, respectively. A straight pipe before the orifice meters was included in the retort design to insure a fully developed flow pattern and, consequently, accurate flow-rate measurement. Temperature control was accomplished with a mercury expansion bulb located at the water inlet to the racking system and a pneumatic controller having proportional and integral functions. The racking system (Fig. 3) consisted of four vertical spreader plates made of 16 gauge stainless steel with 40% open area (3/8 in. holes on 9/16 in. staggered centers) in front of the four horizontal racks, each of which contained two 12 X 15 X 1 in. pouches. The racks were made of 16 gauge stainless steel with 40% open area (3/8 in. holes on 9/16 in. staggered centers). A 5/8 in. water channel separated each of the four racks. The racks were designed for a 1-in. pouch profile and provided intimate contact of the pouch and rack on both top and bottom. Temperature of the retort was monitored by five thermocouples in the exit and one in the entrance of the racking system.

The following processing method was used for all experiments. Eight pouches were placed in the retort and preheated to an internal temperature of 180° ± 5°F at a water flow rate of 110 gal/min. Subsequently the water flow rate was adjusted to the appropriate level for a specific experiment. The pre-heating water was
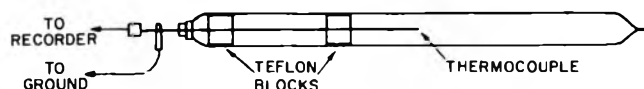
Fig. 1—Thermocouple placement in retort pouches.

drained and the retort was flooded with 265°F water which elevated the entire retort system to 230–240°F. Two to three minutes were required to bring the retort up to the processing temperature of 250°F. The pouches were processed with 10 psi overriding air pressure (total operating pressure of 25 psi) until the slowest heating pouch obtained a center temperature of 245°F. The retort was then drained and flooded with cooling water, which was recirculated while being continuously vented and replenished. Temperature, orifice meter differential pressure, and absolute pressure values were recorded with a data logger (Digistrip II, Kaye Instruments, Bedford, MA).

The heat penetration parameters (f_h, j_h, and j_c; Stumbo, 1973) were calculated by means of a FORTRAN IV computer program using linear regression to estimate the straight line portion of the heating and cooling curves.

Experimental design

The experiment was designed primarily to obtain a relationship between flow rate and heat penetration data. However, part of the experiment was also concerned with determining the effect of rack position on the heating characteristics of the pouches.

To determine if positional effects were significant, an experiment was completed with eight pouches filled with 10% bentonite suspension which were processed at four flow rates (17, 30, 70, and 110 gal/min). Each flow rate was replicated. Individual pouches were evenly distributed between retort positions for different runs to avoid biased results. This procedure included the variation between pouches in the experimental error. Three additional experiments at flow rates of 10, 50 and 90 gal/min were completed in replicate to further define the relationship between flow rate and heat penetration parameters. The trial at a flow rate of 10 gal/min was accomplished on the first set of eight pouches. The 50 and 90 gal/min flow rates were completed in replicate on a second set of eight pouches. Ball and Olson (1957) have stated that insignificant changes in the heat penetration characteristics of prestabilized bentonite suspensions were observed for nine processing runs.

Statistical analysis of the data was accomplished using the Statistical Analysis System (release 79.5, SAS Institute, Cary, NC).

Thermal diffusivity, moisture, and density measurement

Thermal diffusivity of the 10% bentonite suspension was measured by the transient method utilizing the f_h value of the heating curve (Ball and Olson, 1957). A 10% bentonite suspension was prepared and filled into five 303 X 406 cans. After stabilization the five cans were retorted in a still retort (Dixie No. 3) with 100% steam at 250°F. Each can was retorted twice and the average f_h was used in the calculation of thermal diffusivity for a finite cylinder according to Eq. 1 (Ball and Olson, 1957).

$$\alpha = 0.933/(1/a^2 + 0.427/b^2)f_h \qquad (1)$$

where α is the thermal diffusivity, a is the can radius, and b is one-half the can height.

Moisture content was determined by drying approximately 20g of 10% bentonite suspension to constant weight in an oven at 248°F. Density was determined by the weight of a known volume of 10% bentonite suspension.

RESULTS & DISCUSSION

TABLE 1 summarizes the thermal properties of the 10% bentonite suspension. Thermal diffusivity and density were measured, the specific heat was calculated by proportioning a literature value for dry bentonite (Arens, 1951) and the moisture content. Thermal conductivity was calculated from the thermal diffusivity, specific heat, and density.

The apparent convection heat transfer coefficient (h-value) was calculated from the slope of the heating curve. According to Ball and Olson (1957)

$$\lambda_1^2 = 2.303/(\alpha f_h) \qquad (2)$$

for an infinite slab, where α is the thermal diffusivity, f_h is the slope of the heating curve, and λ_1 is the first root of the equation

$$\cot(\lambda a) = k/(h\lambda) \qquad (3)$$

where a is the half thickness of the slab, k is the thermal conductivity of the material being heated, and h is the convection heat transfer coefficient. Substitution of Eq. (1) into Eq. (2) and rearrangement yields the h-value as a function of f_h value at constant k, α and a values; hence

$$h = k \sqrt{2.303/(\alpha f_h)} \ \tan \sqrt{2.303a^2/(\alpha f_h)} \qquad (4)$$

The term, apparent h-value, is used in the context that it included the resistance to heat transfer of the stainless steel rack and pouch material in addition to the external fluid heat transfer coefficient.

The average f_h and apparent h-values found in this experiment appear in Table 2. The j_h and j_c values were not listed since their average values were meaningless, but their values were utilized on an individual basis to calculate process times. The j_h values were not statistically analyzed since retort come-up times varied and a constant temperature distribution could not be guaranteed within each pouch. It should be noted here that all flow rates used were
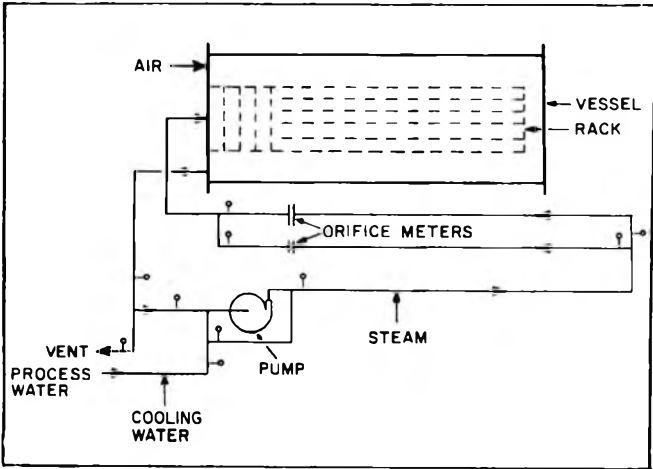
Fig. 2–Experimental pouch retort configuration for processing with heated water.
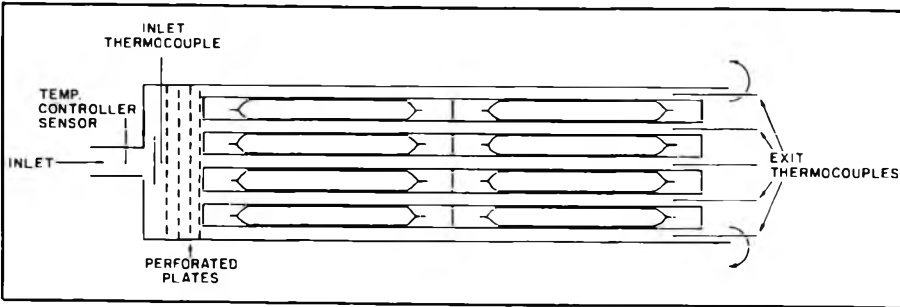
Fig. 3–Experimental pouch retort racking system.

Table 1–Thermal properties of 10% bentonite suspension

Thermal diffusivity	7.26 X 10^{-3} ft^2/hr
Thermal conductivity	0.448 BTU/hr ft °F
Specific heat	0.925 BTU/lb °F
Density	66.8 lb/ft^3

adequate to maintian retort temperature within ±1°F as measured by the entrance and five exit thermocouples in the racking system.

Both f_h and apparent h-values were used as response variables in the statistical analysis of retort position effects. As mentioned earlier, this analysis included only flow rates of 17, 30, 70, and 110 gal/min. No interaction was found between position and flow rate for either f_h or apparent h-values, indicating that it was appropriate to examine the main effects of both position and flow rate. A significant effect of position was not found when positions were considered individually. When positions were split into front and back of the retort, a significant effect was found with f_h value (p = 0.049). The h-value in this case was not significant at the $\alpha = 0.05$ level (p = 0.065). The front of the retort had a lower f_h value (higher apparent h-value) than the rear of the retort. This difference was probably due to entrance effects prior to the establishment of a fully developed flow profile of pressurized water. When retort positions were split into top versus bottom, the apparent h-value and f_h values were not significantly different ($\alpha = 0.05$).

The effect of flow rate was highly significant on both f_h and calculated apparent h-values with p-values of 0.0004 and 0.001, respectively. Table 2 summarizes the effect of flow rate on these two parameters. The f_h value tended to decrease with increasing flow rate. This drop in f_h value may in part be related to the transition from laminar to fully developed turbulent flow. The apparent h-values increased with increasing flow rates as was expected from the previously presented mathematical relationship.

The minimum hypothetical f_h value obtainable was calculated for a 1-in. pouch of 10% bentonite assuming an infinite slab, infinite convection heat transfer coefficient, and taking into account the resistance to heat transfer from a solid rack and pouch material. The minimum f_h value obtainable should be near 14.7 min. During these experiments the lowest f_h value obtained was 19.9 min which does not approach this calculated theoretical value. Initial studies with a 95% steam-5% air mixture as the heating medium at a high flow rate (465 lb steam/hr), indicated that an f_h value of approximately 20 min was the minimum obtainable with the retort system. This discrepancy between actual and calculated minimum f_h values indicated that some undefined resistance existed under these processing conditions. Verification of this resistance is currently under investigation.

The effect of water flow rate on the slope of the heating curve indicated that the assumption of an infinite Biot number (ratio of external convective to internal conductive resistances to heat transfer) is not valid under these conditions. Table 2 summarizes the Biot numbers calculated for each flow rate using the average convection heat transfer coefficient. The range of Biot numbers was from 3.1–4.5.

The low Biot numbers illustrated the importance of the surface heat transfer coefficient when processing with flowing water.

A common empirical method of expressing the relationship between flow rate and convection heat transfer coefficient is the expression

$$Nu = c(Re)^m (Pr)^n \qquad (5)$$

where Nu is the Nusselt number, Re is the Reynolds number, Pr is the Prandtl number, and c, m, and n are experimentally determined constants. A relationship of this type allows heat transfer coefficients from one retort system to be applied to another system, thereby eliminating the system dependence of the data.

Table 2 also summarizes the Reynolds and mean Nusselt numbers obtained for the flow rates used. The Reynolds numbers were calculated using published constants for water at 250°F and the hydraulic diameter of the water channel between racks as the characteristic dimension. Surface roughness was not considered in these calculations. The Reynolds numbers ranged from 3,000–33,000. The lowest Reynolds numbers were in the transition range between laminar to fully developed turbulent flow. The relationship between the Nusselt and Reynolds number found was

$$Nu = 2.31(Re)^{0.15} \qquad (6)$$

Since all experiments were carried out at 250°F the Prandtl number was constant and therefore the term involving the Prandtl number was included in the constant, 2.31. Analysis of this relationship is the subject of current experimentation which address the effects of different rack types and pouch profiles.

Process times (B-value, Stumbo, 1973) were calculated for each of the flow rates using the f_h, j_h, and j_c values. Fig. 4 illustrates the effect of flow rate on the calculated process time. An increase of 9.6% in process time was found from the high to low flow rate.

It has been demonstrated that the circulation rate of heated water is an important critical factor in the processing of institutional size retort pouches. Water circulation rate was shown to be important not only for maintenance of retort temperature, but also for obtaining consistent, reproducible heat penetration parameters and process times. The racking system used in this experiment gave optimal conditions for heat transfer into the pouch be-

Table 2—Mean heat transfer values determined for various water flow rates

Flow rate (gal/min)	Reynolds no.[a]	f_h (min)	h (BTU/hr ft² °F)	Biot no.[b]	Nusselt no.[c]
10	3,000	23.0 ± 0.5[d]	33 ± 2	3.1	7.4
17	5,100	21.2 ± 0.7	37 ± 3	3.4	8.2
29	8,700	20.7 ± 0.6	44 ± 4	4.1	9.8
49	15,000	21.1 ± 0.5	42 ± 2	3.9	9.2
70	21,000	20.4 ± 0.4	45 ± 2	4.2	10.0
90	27,000	19.9 ± 0.5	49 ± 3	4.5	10.8
110	33,000	20.1 ± 0.7	48 ± 5	4.5	10.7

[a] $\rho d_h v/\mu$ where ρ = density, d_h = hydraulic radius, v = water velocity, and μ = viscosity
[b] ha/k
[c] ha/k_f where k_f = fluid thermal conductivity
[d] ± values indicate 95% confidence interval (t test)
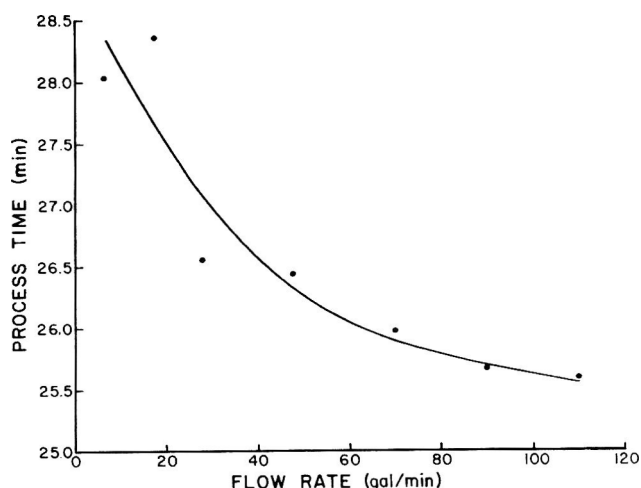

Fig. 4—Effect of flow rate on calculated process time (Stumbo, 1973) z = 18°F, F_o = 6 min, Retort temperature = 250°F, Initial temperature = 180°F.

Kinetics of Protein Quality Loss in Enriched Pasta Stored in a Sine Wave Temperature Condition

J. Y. CHEN, K. BOHNSACK, and T. P. LABUZA

─────────── ABSTRACT ───────────

The rate of loss of protein quality in enriched pasta was studied at constant temperature (30, 37 and 45°C) and under a continuous sine wave temperature fluctuation (25/45°C with a 24-hr period). Both loss of lysine by the fluoro-dinitro benzene (FDNB) method and a bioassay (*Tetrahymena thermophila* growth) for protein quality were employed. Significant loss of protein quality occurs in about 1 yr at temperatures above 30°C. The bioassay method showed that nutrient losses other than lysine could be occurring. Data from the constant temperature studies were used to predict the losses that occurred for the sine wave condition using the Hicks-Schwimmer model as modified with an Arrhenius approach. The prediction model gave about 15% error in comparison to actual losses. In addition, the rate of loss for the sine wave (25/45°C) was greater as predicted than the rate of loss at a constant mean temperature of 35°C.

INTRODUCTION

IN THE COURSE of processing, distribution, and storage of food, temperature fluctuations may occur which can affect the rates of deterioration, especially nutrient loss. In pasta, one of the major losses of nutrients can be the loss of protein quality through the nonenzymatic browning reaction of lysine and reducing sugars. Lysine is usually lost more rapidly than other essential amino acids in the first stages of the Maillard reaction because of the free epsilon amino group. Lea and Hannan (1950), Warren and Labuza (1977), Warmbier et al. (1976), Eichner and Karel (1972), and Labuza and Saltmarch (1982) demonstrated a substantial loss in available lysine before the visual development of brown pigment.

A simplified mathematical method based on first order kinetics has been proposed to predict the effect of temperature and water activity (a_w) on lysine loss for up to about 50% less where $\ln(A/A_0)$ is plotted vs time:

$$\ln(A/A_0) = -k\theta \qquad (1)$$

where A = concentration of lysine at specified time; A_0 = original concentration of lysine at time = 0; k = rate constant in $(\text{time})^{-1}$ = slope of line = $f(a_w$ and temperature); and θ = time.

From studies at several temperatures, the Arrhenius activation energy (E_A) can be obtained which gives a measure of the temperature sensitivity of the reaction. It has been shown that the activation energy for lysine loss in different categories of food products ranges from 10 to 38 Kcal/mole (Saltmarch et al., 1981) indicating that the sensitivity to temperature for lysine loss varies significantly with the food studied. The different methods which are employed to measure lysine loss also give different E_A's. For example, the E_A for dry cod muscle obtained from the work of Miller (1956) was 23.6 Kcal/mole for the FDNB method vs. a value of 37.5 Kcal/mole for available lysine as measured by a chicken growth study (bioassay).

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With respect to nutrient loss in pasta, Fabriani and Frantoni (1972) using the FDNB (fluro-dinitro benzene) procedure showed a 30% loss after storage for 18 wk at 45°C with an activation energy of 13.3 Kcal/mole. Unfortunately, the type of pasta and the a_w used were not presented in their study. Cubadda et al. (1968, 1970) showed about 50–60% loss of lysine in dry pasta (water activity of 0.1) held at 60–80°C for up to 10 hr.

Labuza et al. (1982) have recently studied lysine loss in egg noodles which were subjected to a square wave fluctuating temperature condition as well as to constant temperature. The results showed that for steady state storage, about 30% lysine loss occurred in pasta during storage at 25°C for 1 yr. The data followed first order kinetics. The maximum rate of loss appeared to be at 0.52 a_w, which is close to the range for commercially produced pasta. The calculated activation energy for lysine loss ranged from 13–22 Kcal/mole. There did not seem to be a significant effect of a_w on the E_A. They also presented mathematical models for predicting lysine loss under a fluctuating square wave condition. This model uses the rate constants from constant temperature storage and the Arrhenius plot to make predictions for a variable temperature regime. The prediction showed a range of 2–53% error when compared to actual measured losses for pasta held alternately at 25 and 45°C (1 wk periods) for over a year. This suggested that storage tests at constant temperature, rather than at fluctuating temperatures, are practical for shelf life testing since the Arrhenius plot can be used to make predictions of losses for any temperature distribution. Labuza and Saltmarch (1982) found similar results for lysine loss in stored whey powders also held under a square wave temperature fluctuation. Other studies are needed with a sine wave temperature fluctuation and random variable temperature storage conditions to confirm the applicability of the mathematical model. Thus, the purpose of this study was to determine whether kinetic data from steady state storage conditions could be used to predict protein quality losses in pasta held under a sine wave fluctuating temperature storage condition. In addition, two methods for assaying protein quality loss were compared in this study. A bioassay procedure with the degree of *Tetrahymena thermophila* growth was utilized to analyze the overall relative loss of nutritional value (RNV) in pasta (Warren and Labuza, 1977; Evans and Witty, 1980). This was compared to the FDNB chemical analytical method for available lysine.

MATERIALS & METHODS

Pasta

The pasta used in the present study was prepared at General Mills, Inc. (Minneapolis, MN) by an extrusion and drying process. Enriched semolina (International Multifoods, Minneapolis, MN) with 2% (by weight) of egg solids (Henningson Foods, St. Louis, MO) was used. The pasta was humidified at refrigerated temperature to an a_w of 0.49 over a $Mg(NO_3)_2$ salt solution (a_w of 0.53). The a_w of the pasta was determined by vapor pressure manometry (Lewicki et al., 1978). Replicate 5-g samples were sealed in aluminum laminated pouches and stored at 30°C, 37°C and 45°C for the steady state accelerated shelf life tests. In addition, replicate pack-

age samples were stored in a fluctuating sine wave condition cycling between 25°C and 45°C over a 24-hr period of time. Previous work by Kamman et al. (1981) showed that the come-up/come-down times of the packages were negligible and should not affect the kinetics. When removed for analysis, each sample was ground in a Micro Mill (Chemical Rubber Co., Cleveland, OH) for 90 sec and then ground again as finely as possible in a porcelain mortar just before analysis. Eight samples were analyzed at the onset of the study to determine the initial value and the variability in the measurement. Duplicate samples were analyzed ever 2 or 4 wk during storage over a period of 20–52 wk.

Relative nutritional value (RNV) by means of the tetrahymena thermophila bioassay

The bioassay method, originally developed by Sutton (1978) and modified by Saltmarch (1980), was employed. Two grams of ground pasta were predigested by a buffered enzyme solution containing 5.3 mg/ml trypsin, 10.3 mg/ml α-chymotrypsin and 4.3 mg/ml peptidase at 37°C for 1 hr. The amount of pasta added was determined based on the amount of protein needed to yield a final concentration of about 0.3 mg N/ml bioassay medium. Thereafter, the digested samples were incubated with a 1/100 (v/v) of 3 day old culture of *Tetrahymena thermophila* and incubated at ambient temperature for 72 hr. The organisms were counted by a modification of the Coulter counter method developed by Evancho et al. (1977). Relative nutritional value (RNV) of each sample were obtained by dividing the cell count at a given storage time by the average cell count of samples determined at the beginning of storage.

FDNB available lysine determination

Available lysine (using fluoro-dinitro benzene) was determined according to a modification of Booth's method (1971) which was adapted by Labuza and Saltmarch (1982).

Two grams of powdered pasta were weighed into a 250 ml boiling flask and 10 ml of $NaHCO_3$ (80 g/liter) solution was added to each flask. The flask was then shaken for 1 hr at 37°C and 200 rpm in the New Brunswick environmental shaker incubator model G24 to disperse the sample. Then 15 ml of FDNB solution, which contained 0.4 ml of FDNB in 15 ml of 95% ethyl alcohol, was added. The flasks were shaken again at room temperature and 200 rpm for 3 hr in the same incubator. Thereafter, the samples were treated as in the Booth's method (1971). The results were reported on a solids basis.

RESULTS & DISCUSSION

Protein quality stability-steady state condition

Fig. 1 is the semilog plot of RNV retention for the pasta at an a_w of 0.49; the data show good linearity indicating first order as in the previous studies. At 45°C, about 30% loss occurs at 18 wk, similar to that reported by Fabriani and Frantoni (1972). The rate constants calculated from the slopes of the best regression lines (Freund, 1967) are shown in Table 1. At 30°C about 25% loss of protein quality occurs in around 10 months, while at 37°C it takes about 6 months for 25% loss. The FDNB results were not as good since data were determined at only two constant temperatures and for only several months because of lack of samples. Fig. 2 shows greater scatter than Fig. 1 which is indicative of the lower r^2 in Table 2. In addition, at 37°C about 25% loss of lysine occurs in 8 months while the Tetrahymena assay shows a 25% loss in about 6 months. This indicates that either other changes beside lysine loss in the pasta are occurring which can reduce the growth of the organism or the typical errors of the two methods end up showing a difference in nutritional quality. Since pasta normally has an a_w of between 0.4 and 0.5 and may be stored for up to 12–18 months during distribution, significant loss of protein quality could occur. The Arrhenius plot of log_{10} k vs 1/T is shown in Fig. 3 with the E_A values presented in Table 3. The values for both methods were quite close based on the confidence limits and were similar to that reported by Fabriani and Frantoni (1972) and Labuza et al. (1982).

Protein quality stability-unsteady state conditions

Hicks (1944) and Schwimmer et al. (1955) theorized that the mean or average temperature of periodic temperature fluctuations was inappropriate in predicting the
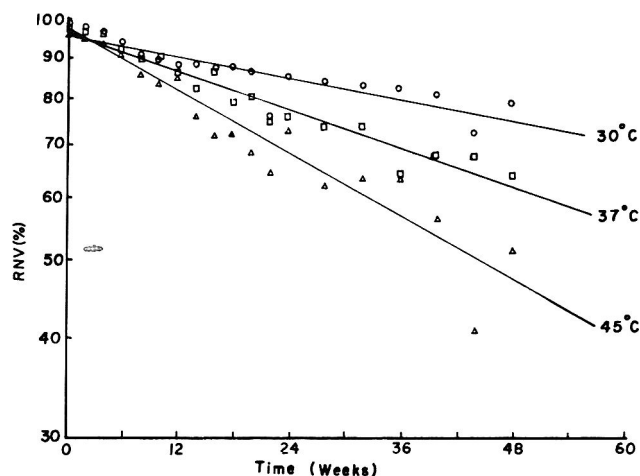
Fig. 1—Fractional retention of relative nutritional value in enriched pasta stored at 30, 37, 45°C and a water activity of 0.49.

Table 1—First order protein quality loss kinetics for enriched pasta (a_w = 0.49) as a function of storage temperature

	Temperature (°C)		
	30	37	45
r^2	0.842	0.953	0.929
k*	61.13 ± 11.89	101.27 ± 10.08	155.77 ± 19.27
θ½ (weeks)**	94.9/140.8	62.25/76.01	39.60/50.78

*Rate constant in (weeks)$^{-1}$ x 10^4 ± 95% confidence limits
**Upper and lower 95% confidence limits for time to reach A = 50% RNV in weeks
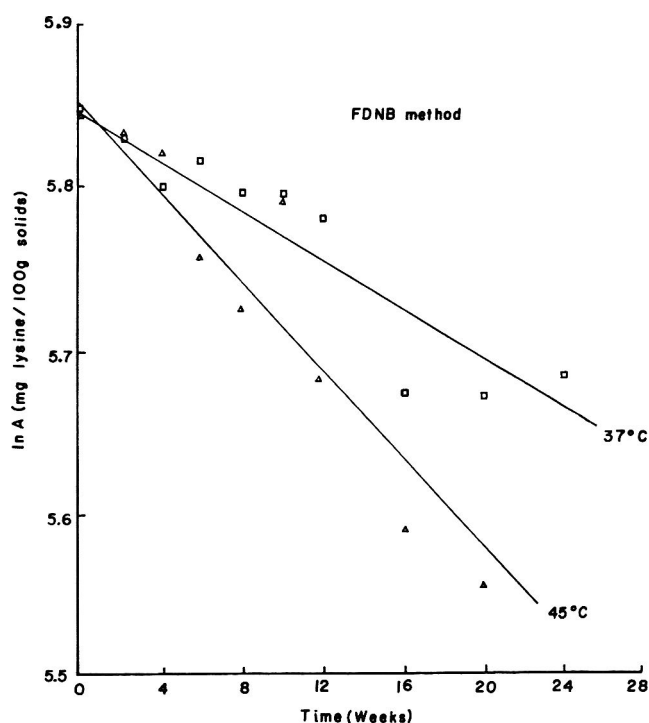
Fig. 2—Available lysine loss (FDNB) in pasta stored at 37°C and 45°C and a water activity of 0.49.

amount of degradation of a food subjected to fluctuating temperatures. Both authors derived equations to predict losses for reactions following zero order kinetics based on their theory.

Labuza (1979) derived a mathematical model to predict the extent of nutrient loss for a first order reaction for product stored under a periodic sine wave fluctuating temperature condition using the kinetic data from steady state temperature conditions. The mathematical model illustrates that the rate of loss during the fluctuation can be equated to the rate of loss at some derivable constant temperature (T_{eff}) which is greater than the mean temperature (T_m) of the periodic fluctuation cycle. The relationship between T_{eff} and T_m is represented in the following equation as shown first by Hicks (1944).

$$T_{eff} = T_m + \frac{1}{b} \ln \Gamma_{sine} \qquad (2)$$
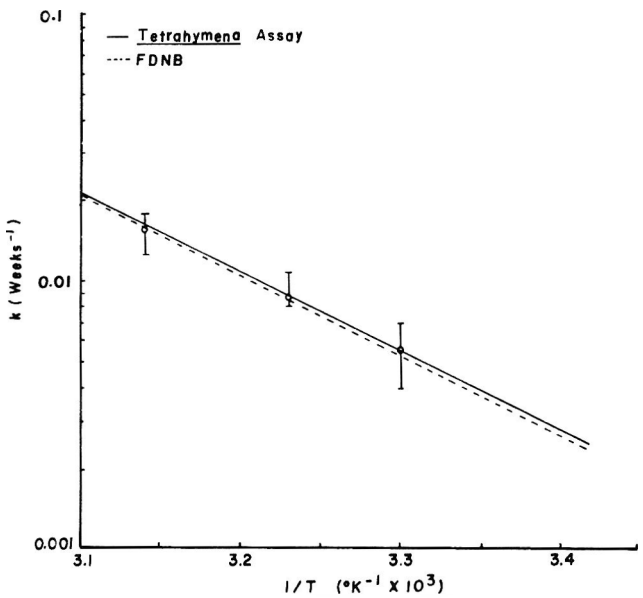
Fig. 3—Arrhenius plot of steady state rate constants versus reciprocal of absolute temperature.

Table 2—First order FDNB available lysine loss kinetics for enriched pasta (a_w = 0.49) as a function of storage temperature

	Temperature (°C)	
	37	45
r^2	0.873	0.928
k*	80.20 ± 32.51	150.09 ± 44.34
$\theta\frac{1}{2}$**	58.7/151.5	34.1/70.4

§k ln (weeks)$^{-1}$ x 10^4 ± 95% confidence limits
**Upper and lower 95% confidence limits for time to reach A = 50% available lysine in weeks

where T_m = fluctuation mean (35°C in this present study) = $(T_{upper} + T_{lower})/2$; b = 0.503 $E_A/(T)$ (T + 10) where T is in °K; and Γ_{sine} = wave function based on steady state data.

For a first order reaction Γ_{sine} can be found from Eq. (3):

$$\Gamma_{sine} = 1 + \frac{(a_o b)^2}{2^2} + \frac{(a_o b)^4}{2^2 4^2} + \frac{(a_o b)^6}{2^2 4^2 6^2} + \cdots \qquad (3)$$

where a_o is the amplitude of the temperature fluctuation (in °C) and b is found from the Arrhenius plot rather than from a shelf life plot as done by Hicks (1944) and Schwimmer et al. (1955). The extent of change for a first order reaction undergoing a sine wave temperature is:

$$A = A_o e^{(-k_{T_m} \Gamma_{sine} \theta)} = A_o e^{-k_{eff} \theta} \qquad (4)$$

where k_{T_m} = rate constant at mean temperature (T_m) interpolated from the Arrhenius plot of the steady state data and $k_{eff} = k_{T_m} \Gamma_{sine}$.

In this study, the rate of loss of protein quality under a sine wave temperature fluctuation was examined and compared to the rates of loss predicted by the above equations using the data generated from the steady state study. The bioassay data from the sine wave storage conditions are shown in Fig. 4 along with the best straight lines from the constant temperature data. Fig. 4 shows that the sine wave data follow a fairly good straight line and proceed at a faster rate than the 37°C constant temperature data. The mean temperature of the sine wave is 35°C. Similar results were found for the FDNB data, as shown in Fig. 5, but with greater scatter. This verifies that the rate of loss in the sine wave temperature condition proceeds faster than the rate at the mean temperature (35°C) of the fluctuation. Table 4 shows that for the bioassay procedure the actual effective temperature T_{eff}, the actual reaction rate constant k_{eff}, and the time for 50% loss indicate a faster reaction than that predicted by the theoretical equations. When the 95% confidence limits are considered for the bioassay, the k_{eff} and $\theta\frac{1}{2}$ values indicate that there is no statistical difference between the actual and predicted values. The actual FDNB data also indicate a faster rate of loss than the predicted values. Since only two temperatures were used, no confidence limits could be calculated. The actual FDNB values do not fit within the 95% limits shown for the bioassay method, which may confirm that the methods may not be measuring the same thing as also occurred with the steady state data.

There is about a 15% error between the predicted and actual lysine values with time, as seen in Table 5, which is somewhat larger than the error found (2–7%) at an a_w of 0.52–0.65 in the square wave study but much less than found at an a_w of 0.44 (53%) where the loss rate was very slow. It should be noted that the predictions are based on a value of 100% RNV at zero time. If the error at zero time is ±5%, at 50 wk the value would be 62.3 ± 9% which makes it overlap the actual values even better. A more detailed analysis of the statistical method for these analyses is found in Labuza and Kamman (1983). These errors might be due to the following factors:

Table 3—Temperature dependency of protein quality loss in unenriched pasta

E_A (± 95% confidence) (Kcal/mole)	r^2	Q_{10} (35/45°C)	Method	Reference
12.06 ± 9.97	0.869	1.84 ± 0.10	Tetrahymena bioassay	This study
15.34*		2.20*	FDNB	This study
12.77 ± 5.16	0.860	1.86 ± 0.41*	FDNB	Labuza et al. (1982)
13.30*		2.12	FDNB	Fabriani et al. (1972)

*Statistics cannot be applied to get ± 95% CL

(1) The prediction of protein quality loss under fluctuating conditions is based on the kinetic data obtained at steady state. A high coefficient of variation was found in this study in assessing protein quality (19% for the bioassay; 12% for FDNB) and, thus, it is impossible to obtain good k values from the steady state tests (Benson, 1960). This magnifies the error in predictions based on the data of such conditions. At the same time, the error in analyzing the protein quality loss under the sine wave fluctuating condition would be generated in the same way, which could cause further inaccuracy in the comparison.

(2) Previous studies on loss of food quality (such as vitamin loss; protein quality loss) showed a possible "history effect" of food deterioration reactions when food products are subjected to fluctuating temperature storage conditions (Kamman et al., 1981; Labuza et al., 1982). A history effect means that an acceleration in the rate of loss at the lower temperature (25°C) is possible due to a nonadditive effect occuring during storage at the higher temperature (45°C). This effect might be attributable to the catalysis effect of degradation products formed at higher temperature. Under such a sine wave fluctuation situation, the accumulated "history effect" due to continuous temperature fluctuation would cause a greater effective T_{eff} and rate constant for protein quality loss than that predicted from steady state data, and thus a shorter shelf life of enriched pasta as was observed in actual fluctuation conditions.

(3) The fact that the sorption isotherm for a food system is strongly affected by temperature has long been noted (Labuza, 1968). Under fluctuating storage conditions such as the sine wave fluctuation in this study, the temperature varies with time periodically which eventually would result in an a_w fluctuation in the food system. A model to predict the response profile of a_w to temperature shifts in porous food products has been mathematically

proposed by Bruin and Liou (1981), although no further experimental confirmation has been made. Many chemical reactions show a dependence of both k and E_A on a_w. It has also been shown that the E_A of protein quality loss kinetics in pasta does not depend on the a_w of the test sample (Labuza et al., 1982); however, the rate constant, k, did show some effect. Therefore, the rate of protein quality loss would be expected to change with temperature cycling due to effects other than temperature itself. However, at this time point it is too complicated to quantitatively determine how much of an effect this would impose on protein quality loss in pasta.
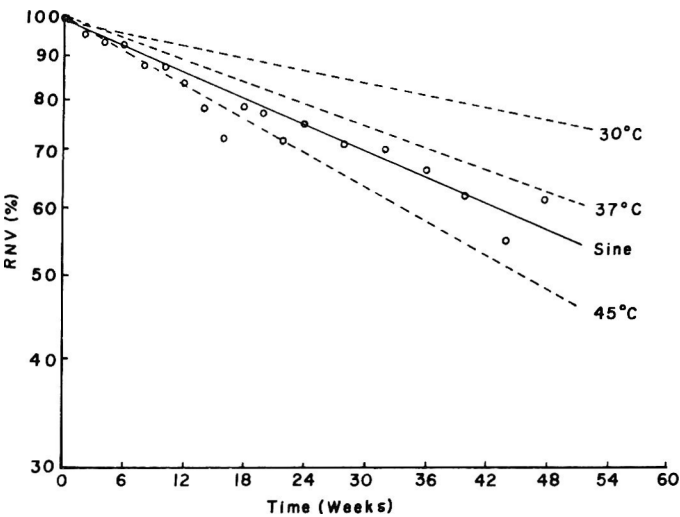
Fig. 4—Fractional retention of relative nutritional value in enriched pasta stored under 25/45°C sine wave condition as compared to steady state storage ($a_w = 0.49$).

Table 4—Comparison of predicted to actual parameters of protein quality change under a sine wave fluctuation condition (± 95% confidence limits)

Method measure	T_{eff} (°C)	k_{eff} (weeks x 10^4)	$\theta_{1/2}$ (weeks)
Tetrahymena			
Predicted	36.53 ± 1.12	93.35 ± 13.23	74.2 ± 10.4
Actual	40.15 ± 2.17	118.32 ± 13.41	57.9 ± 8.2
FDNB			
Predicted	37.00	73.29	94.6
Actual	40.22	103.60	66.9

Table 5—Comparison of predicted to actual extent of protein quality loss under a sine wave fluctuating condition (25/45)

Time (weeks)	RNV (%)*	
	Predicted	Actual
0	100	100
5	95.44 ± 0.63	92.47 ± 2.49
10	91.08 ± 1.19	87.16 ± 2.94
15	86.93 ± 1.70	82.15 ± 3.35
20	82.96 ± 2.16	77.43 ± 3.70
25	79.18 ± 2.57	72.99 ± 3.99
30	75.57 ± 2.94	68.79 ± 4.26
35	72.12 ± 3.26	64.84 ± 4.48
40	68.83 ± 3.54	61.12 ± 4.65
45	65.69 ± 3.79	57.60 ± 4.81
50	62.69 ± 4.00	54.30 ± 4.92

* RNV (%) left is calculated by $\ln (\%RNV) = -k_{eff} \cdot \theta$ with 95% confidence limits

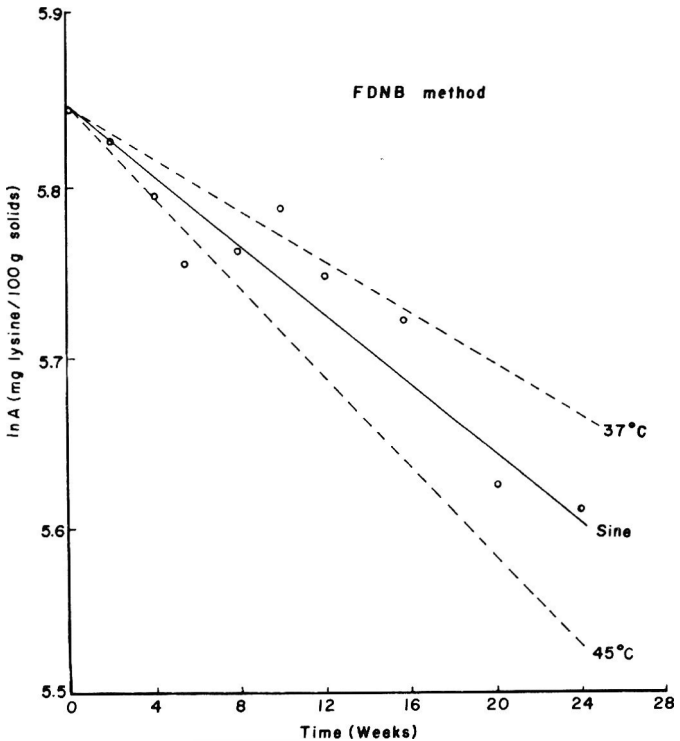
Fig. 5—Extent of available lysine loss (FDNB) in enriched pasta stored under 25/45°C sine wave condition as compared to steady state storage ($a_w = 0.49$).

CONCLUSION

THE PREDICTIVE EQUATIONS that were presented for sine wave fluctuations proved to be within 15% error when compared to the actual rate of loss, giving an underprediction of the extent of change. The mean temperature of the fluctuation was shown to be an inadequate measure of the anticipated loss. The equations are useful in predicting the amount of loss that may occur in food subjected to conditions of variable temperature without the need to gather data on the rate of loss during conditions of variable temperatures. The Hicks-Schwimmer-Labuza model enables reasonable prediction of these losses based on steady state data generated in the laboratory if sufficient data points were supplied with normal experimental variability. A comparison of the two assay methods show that possible nutrient changes other than lysine loss are causing a greater rate of loss for the RNV determination than for the FDNB method under both constant temperature and fluctuating temperature conditions.

From a practical standpoint, significant loss of protein quality in pasta may occur during storage at 25°C or during storage or distribution under fluctuating conditions. Extreme conditions of high temperaure and high relative humidity such as those of southern climate, or unfavorable high temperature such as in the Middle East where desert warehousing of food is anticipated and diurnal temperature fluctuations occur, may adversely affect the protein quality of warehoused pasta and contribute to shortened shelf life.

Since both the response profile of a_w in foods to temperature fluctuation and the effect of a_w of reaction kinetics of food deterioration are not fully understood for most food products, it is strongly suggested that further work in such an area should be done.

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HEAT PENETRATION PARAMETERS – POUCH PROCESSING . . .

cause there was intimate contact between the racks and pouch and the racks had large perforations with a high percent of open area. The relationships presented could change with a different rack design, such as smaller perforations, less open area, or a dead space between the top of the rack and the pouch. These variables are currently under investigation.

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Evaluation of A Modified Gradient Feed Culturing System for Growth of Lactobacillus plantarum Sausage Starter Organism

J. S. GANOUNG, R. H. SCHMIDT, and K. L. SMITH

———————————— ABSTRACT ————————————

An internally controlled gradient feed culturing technique, where a nutrient gradient was initiated and subsequently controlled by the lactic acid synthesis, was compared to conventional batch culturing for growth of a sausage starter organism, Lactobacillus plantarum. Significantly higher cell densities (p < 0.01) were observed with twice the culturing time for gradient feed culturing compared to batch culturing. Cell yields within batch or gradient feed culturing system were not affected by pH control at 5.8 or 6.0. Significantly higher (p < 0.05) cell yields, however, were obtained for gradient feed cultures (pH 6.0) with NH_4OH addition at 50% theoretical lactic acid (TLA) level than were obtained at 10% TLA or without NH_4OH addition.

INTRODUCTION

STARTER CULTURE CONCENTRATES were introduced into the fermented sausage industry about 30 years ago and have been a tremendous aid in processing. The use of starter organisms results in reduced process time, extended shelf life and sausage products of more uniform quality (Everson et al., 1969, 1970; Santa and Mugra, 1969). A commonly used sausage starter organism, Lactobacillus plantarum, is found indigenously in the fresh meat environment. The inhibitory properties of L. plantarum against pathogenic and spoilage organisms found in meat products have been documented (Daly et al., 1973; Raccach and Baker, 1978, 1979a, b; Raccach et al., 1979).

While research has been conducted related to optimizing culturing conditions for the production of dairy product starter concentrates, culturing conditions for sausage starter organisms have not been as extensively investigated. The production of high density cultures with acceptable activity is an essential objective fo successful starter concentrate manufacture. The concept of gradient feed culturing has been used as a technique for production of high density cultures of Cellulomonas (Srinivasan et al., 1977). This approach which involves gradient addition of medium to the culture has not been applied to lactic acid bacteria. The objetive of this investigation was, therefore, to assess the feasibility of the application of this technique to sausage culture manufacture.

MATERIALS & METHODS

Microbial cultures

The organism used in these experiments was a homofermentative L. plantarum 7870 (American Bacteriological & Chemical Research Corp., Gainesville, FL). This strain is characterized by a high degree of growth and acid production in a sausage batter and is used commercially in the production of semi-dry fermented beef and beef/pork sausages. The organism was maintained at 32°C in APT broth (Difco, Detroit, MI) and was transferred daily during experimental

periods. Inocula for each experimental trial were from 15–20 hr APT cultures which yield plate counts of approximately 10^9/ml.

Experimental growth medium

A growth medium was formulated which simulated that used in commercial Lactobacillus starter culture manufacturing. Medium composition was as follows: 11.7g Yeastamin 60 (Amoco Foods, Chicago, IL); 10.0g glucose (Difco); 10.0g O.M. Peptone (Oscar Meyer CO.. Madison, WI); 1.0g sodium tripolyphosphate; 0.8g NaCl and water to 1 liter. The growth medium buffer capacity was calculated from data obtained by titration with lactic acid to pH 6.0 and was determined to be equivalent to 10 mmole NH_4OH/L.

Culturing systems

Gradient feed systems. Each gradient feed trial was begun by inoculating (0.025%) a start-up medium of a composition equivalent to 4.0% of the total nutrients in a volume of 10% of the total fermentor volume. The remaining (96.0% of total nutrients) medium was divided into the gradient reservoirs so as to achieve a 10-fold increase in nutrients from start to finish.

Externally controlled gradient feed systems were essentially as described by Srinivasan et al. (1977) where the nutrient gradient was pumped into the culture as a function of incubation time (14 hr at 32°C). This syystem was modified to an internally controlled gradient feed system in which the culture pH was controlled at pH 5.8 and at pH 6.0. The NH_4OH was added directly to the growth medium. Therefore the rate of medium dispensing was controlled by the growth and acid production rather than by incubation time. The effect of the relative NH_4OH concentration addition on cell yield was evaluated by adding NH_4OH at concentrations sufficient to neutralize 10, 50, and 100% of the theoretical lactic acid (TLA) yield of the growth medium. Calculation of TLA was based on the assumption that 1 mole of glucose yields 2 moles lactic acid. Therefore, 22.0, 111.0, and 222.0 mmoles of NH_4OH were added to the 2L of medium for neutralization at 10, 50 and 100% TLA, respectively. Samples were taken from gradient feed cultures at the end of pumping and near the end of exponential growth phase.

Batch system. In conventional batch culturing systems, growth was allowed in the total medium with pH control at pH 5.8 and at pH 6.0 by addition of concentrated NH_4OH (16.57M) or without pH control. Samples were aseptically taken at 1–2 hr intervals for up to 14 hr incubation at 32°C.

Equipment

A 2-liter fermentor (VirTis Model 40-100, VirTis Co., Inc., Gardiner, NY) and a 7-liter fermentor (New Brunswick Model M-19, New Brunswick Scientific Co., Edison, NJ) were used. In both fermentors, the experimental volume was 2L of medium which was sparged with nitrogen gas in all experiments to reduce oxygen tension. The fermentors were connected to a Versatrol pH module (Assembly Products, Inc., Chesterland, OH) which activated a Cole Parmer peristaltic feed pump (Cole-Parmer, Chicago, IL). A simple gradient mixer with magnetic stirrers was constructed to generate the nutrient concentration gradient (Fig. 1). Two glass reservoirs which were filled with 900 ml each were connected by a stopcock valve. The ability of the gradient mixer apparatus to generate a linear nutrient increase was evaluated by following the increasing glucose concentration in a simulated gradient feed experiment. All equipment in contact with the growth medium was autoclaved (121°C/20 min) except the pH probe which was chemically sterilized in a 200 ppm hypochlorite solution.

Microbial analyses

Serial dilutions from duplicate samples were made in sterile 0.1%

peptone buffer and were plated using Plate Count Agar (Difco). The number of cells per chain was estimated microscopically and was multiplied by the plate count to estimate the total cells per ml. Cell yields obtained toward the end of the exponential growth phase were compared for the two pH levels (5.8 and 6.0) and for the two culturing techniques (batch vs gradient feed). In addition, the number of cells per mole of NH_4OH neutralized was determined at the end of pumping experiments which were controlled at pH 6.0 and comparisons were made between neutralization levels.

Statistical analyses

Analysis of cell yield data was performed using Statistical Analysis System (SAS) programs at the Univ. of Florida computer center. The General Linear Models Procedure was used to compensate for uneven replications within analyses, and variation among days was used as an error term. Mean values of cells/ml and logarithm of cells/ml for culturing techniques, pH levels and levels of added NH_4OH were examined.

RESULTS & MATERIALS

Effect of culturing technique on cell yield

Cell yield data for *L. plantarum* 7870 grown by batch and gradient feed culturing techniques are presented in Table 1. Differences between cell yield data from batch culturing with and without pH control at 5.8 and 6.0 were not significant ($p > 0.05$) due to considerable variability in the data. Similarly, differences between cell yield from gradient feed culturing at pH 5.8 and at pH 6.0 were not significant ($p > 0.05$). However, the general data trend observed for both culturing systems was an apparent increase in cell yield with growth at pH 6.0 compared to growth at pH 5.8. This trend was confirmed when data were analyzed across culturing technique and differences in cell yield between culturing at pH 5.8 and 6.0 were significant ($p < 0.05$). An optimum growth range of pH 6.0–6.5 for lactic acid bacteria has been reported (Cogan et al., 1971; Efstathiou et al., 1975; Lloyd and Pont, 1973). The interaction between culturing system and pH was not significant ($p > 0.05$) indicating that both culture systems were affected similarly by difference in pH control level.

An increase ($p < 0.01$) in cell yield was obtained using pH controlled gradient feed as compared to pH controlled

batch culturing. The geometric mean cell density yield for gradient feed culturing was 2.2×10^9 cells/ml compared to 9.3×10^8 cells/ml for the pH controlled batch system. This approximates a 2.4-fold increase in cell yield by the gradient feed culturing compared to the batch system. This increased yield may result from a shift in the metabolism of the organism from endogenous metabolism in the case of batch culturing to assimilative metabolism as the organisms are poised with increasing nutrient concentrations in the gradient feed system. Increased NH_4OH concentration in the gradient medium resulted in an increase in cell yield (Table 2). Differences between data from trials at 10% and at 50% TLA were significant ($p < 0.01$). NH_4OH at 100% TLA resulted in failure of the system to operate. The culture was not able to produce enough acid from available glucose to neutralize all of the NH_4OH.

The efficiency of the culturing system can be estimated from calculating cell yield per mole of NH_4OH added. Differences in fermentation efficiency relative to level of NH_4OH added in the gradient feed system were not significant ($p > 0.05$). The calculated efficiency for the culturing system controlled at pH 5.8 was 13.38 $\log_{10}$ cells mole^{-1} NH_4OH compared to 13.45 $\log_{10}$ cells mole^{-1} NH_4OH for the pH 6.0 controlled systems.

In conclusion, the internally pH controlled gradient feed culturing system resulted in an increased yield of *L. plantarum* cells compared to batch culturing techniques. However, the process time for the gradient feed system was approximately twice that of the batch system. This increased process time may preclude practical application of

Table 1—Effect of batch culturing[a] and gradient feed culturing techniques[b] on cell yields for Lactobacillus plantarum 7870

Culturing system	N[c]	Cell yield[d] ($\log_{10}$ cells/ml)
Batch		
No pH control	4	8.86
pH control at 5.8	5	8.94
pH control at 6.0	3	9.00
Gradient feed		
pH control at 5.8	4	9.22
pH control at 6.0	5	9.44

[a] In a 2-liter fermentor at $32°C$ agitated at $80 - 100$ rpm, sparged with nitrogen. Samples taken after 14-hr incubation.
[b] As above with gradient addition of nutrients and NH_4OH at 50% theoretical lactic acid (TLA). Samples taken after stopping of nutrient pump.
[c] Number of replicate trials. Duplicate platings done for each replicate.
[d] Mean of the $\log_{10}$ adjusted cell count ml^{-1} on plate count agar.

Fig. 1—Gradient mixer for generating linear gradient concentration of nutrients: (1) Concentrated reservoir; (2) Dilute reservoir; (3) Feed pump; (4) Growth chamber; (5) Sampling port; (6) pH probe; (7) Nutrient inlet; (8) Agitator; (9) pH meter; (10) Controller.

Table 2—Effect of increasing equivalent NH_4OH level in the gradient medium during culturing[a] on cell yield of Lactobacillus plantarum 7870

NH_4OH level[b] (% of TLA)	N[c]	Cell yield[d] ($\log_{10}$ cells/ml)	Cell yield efficiency[e]
0	3	9.03	13.84
10	3	9.09	13.85
50	5	9.44	13.45

[a] In a 2-liter fermentor at $32°C$, agitated at $80 - 100$ rpm, sparged with nitrogen and gradient addition of nutrients and NH_4OH.
[b] Samples taken after stopping of nutrient pump.
[c] Number of replicate trials. Duplicate platings done for each replicate.
[d] Mean of $\log_{10}$ adjusted cell count/ml^{-1} on plate count agar.
[e] Cell yield/mole^{-1} NH_4OH added. Where no NH_4OH added, the buffering capacity of the media was determined by titration with lactic acid.

Effect of Culture pH on D Value, Cell Growth and Sporulation Rates of P.A. 3679 Spores Produced in an Anaerobic Fermentor

KIMBERLY A. PANG, PAUL A. CARROAD, and ALFRED W. WILSON

ABSTRACT

The submerged culture of P.A. 3679 spores was studied in an anaerobic fermentor with pH control. The control of pH was necessary for sporulation in trypticase soy broth medium supplemented with 5.0 g/L or more glucose. D values, growth rates, and sporulation rates showed variation with pH in the range 6.0–8.0. z value appeared to be less sensitive to culture pH.

INTRODUCTION

THE PRODUCTION OF SPORES of Putrefactive anaerobe (P.A.) 3679 for use in thermal processing studies is commonly accomplished in tubes or bottles overlaid with vaspar or other sealing mixture to maintain anaerobic conditions (NCA, 1968; Goldoni et al., 1980). A difficulty with such a method is that the overlaid sealing mixture hinders accessibility to the culture broth. Control of pH, for example, which requires sampling and pH adjustment necessitates breaking the anaerobic seal. The production of spores in an instrumented fermentor which can be maintained under anaerobic conditions offers a useful alternative. In an instrumented fermentor, pH as well as temperature and other environmental factors can be controlled.

Zoha and Sadoff (1958) demonstrated that P.A. 3679 spores could be grown in stirred culture and that sporulation occurred exponentially. Their crude fermentation system consisted of a flask filled with nitrogen-sparged medium, agitated by a magnetic stirrer, with manual pH control. They studied the effect on sporulation of initial glucose concentration in a tryptone medium controlled near pH 7.2, and reported that 2 g/L glucose gave good spore crops. Lower levels of sugar resulted in poor cell growth and higher levels inhibited sporulation.

Anema and Geers (1973) studied sporulation of Clostridium sporogenes in a fermentor with a tryptone medium and added glucose. They concluded that glucose level influences sporulation and thermal resistance through its effect on pH. With initial glucose concentration of 2 g/L and no pH control, sporulation was normal. With 5 g/L and no pH control, sporulation was inhibited and there was lysis of vegetative cells. With 10 g/L, there was no sporulation without pH control. The D_{230} values ranged from 4–80 min, but they did not report z values nor investigate systematic variation with respect to pH.

Alcock et al. (1981) reported flask experiments on P.A. 3679 in which initial pH values in the range 6.0–7.5 influenced sporulation but not heat resistance, and in which controlled pH levels in a fermentor appeared to influence both sporulation and resistance. Differences in resistance with respect to pH were inconclusive since the differences may have been masked by the inocula having been derived from different spore crops.

Fermentor studies have recently been reported for Clostridium botulinum toxin production (Siegel and Metzger, 1979) and spore outgrowth (Smoot and Pierson, 1979). Such reports aided the design of an instrumented fermentation system for this spore study on P.A. 3679.

This study was undertaken to investigate the effect of pH level during fermentation on the production of P.A. 3679 spores in trypticase soy broth medium supplemented with glucose.

MATERIALS & METHODS

Culture medium

The basal medium for all experiments was trypticase soy broth (BBL) containing (in g/L): trypticase peptone, 17.0; phytone peptone, 3.0; sodium chloride, 5.0; dipotassium phosphate, 2.5, and glucose, 2.5 (Rohde, 1968). Cysteine hydrochloride was added to a concentration of 0.05% (Smoot and Pierson, 1979). Glucose (Difco) was added to achieve the desired initial glucose level (5 g/L or 15 g/L) depending upon the experiment. The added glucose was sterilized separately and transferred into the fermentor aseptically through a connecting tube which was in place during sterilization.

Test strain

An initial culture of P.A. 3679 was obtained from the National Food Processors Association (Berkeley, CA). Pure colonies were isolated on anaerobic egg agar (Leininger, 1976). A pure culture was grown in trypticase soy broth (BBL) in overlaid bottles according to the method in NCA, 1968 (i.e. 2 wk incubation at 30°C, 1 wk at 21°C). Spores were harvested by thrice centrifuging in sterile 250 mL bottles (Nalgene) and resuspending and washing spores in sterile M/15 phosphate buffer (pH 7.0). The final pellet of spores was resuspended in skim milk and separated into 80 tubes for lyophilization. The inoculum for each experiment was grown from one such lyophilized tube to maintain uniformity in the inoculum.

Inoculum

To prepare the inoculum for an experiment, one lyophilized pellet of P.A. 3679 spores was transferred to 10 mL of sterile trypticase soy broth (no added glucose) with added cysteine (0.05%). The broth was exhausted of air during autoclaving and inoculated at approximately 80°C. The spores were then heat shocked in the tube at 82–83°C for 13 min (Goldoni et al., 1980). The spores were then incubated 3 days at 30°C in a GasPak anaerobic jar (BBL) according to Gottschalk et al. (1981). Actively growing cells were obtained in 3 days.

The inoculum was standardized to approx. 1 to 5 X 10⁶ cells/mL before introduction into the fermentor. A sample was withdrawn from the tube in the anaerobic jar and a count of cells was made in a Petroff-Hauser counting chamber (Koch, 1981) under phase-contrast using oil immersion (Murray and Robinow, 1981). The cell concentration in the tube always exceeded 5 X 10⁶, and standardization was achieved by diluting the tube with sterile water that was exhausted of air. The inoculum was 10 mL into a fermentor of 1,500 mL liquid volume. This small inoculum was used to extend the exponential growth phase so numerous data could be taken for determination of specific growth rate.

Fermentation

Spores were grown in a 2-L Bioflo fermentor (New Brunswick Scientific Co., Model C3). Temperature was controlled at 30°C by passing water from a large volume water bath controlled by an immersion heater with pump (Braun Thermomix Model 1400)

through heating coils in the fermentor. The pH was controlled and monitored by a dual pump controller (New Brunswick Scientific Co., Model pH22) with addition of 2N HCl and 2N NaOH. The pH controller was equipped with a strip chart recorder for monitoring pH. The pH values studied were 6.0, 6.5, 7.0, and 8.0.
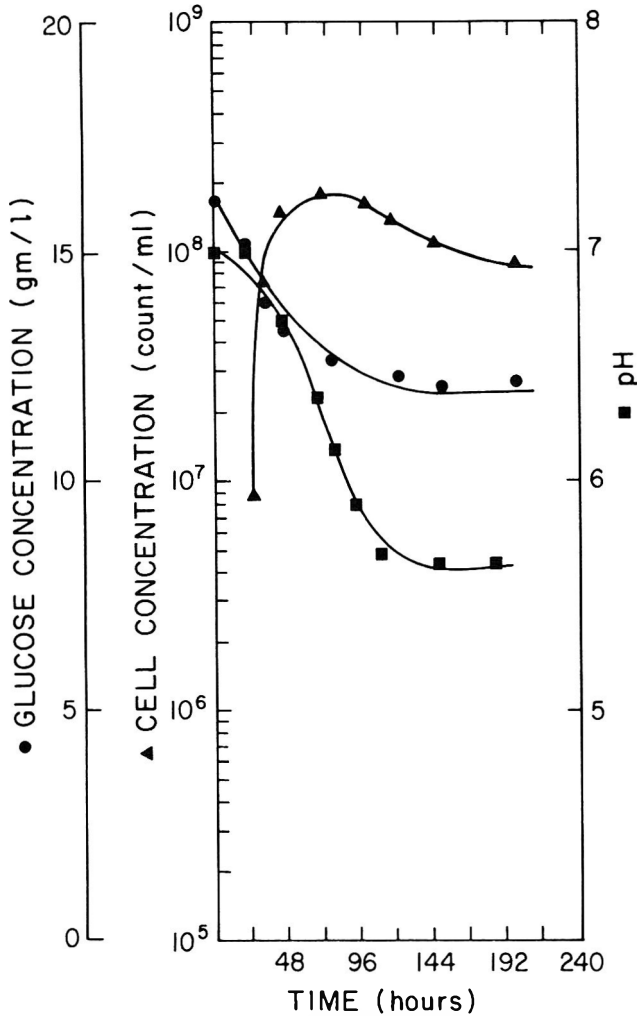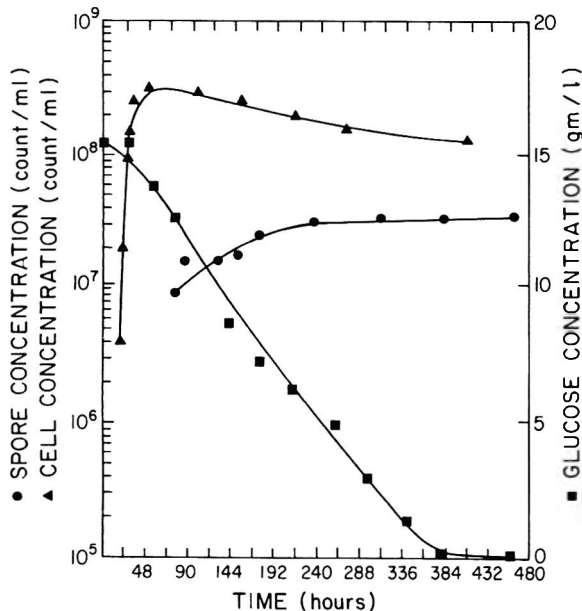
Following autoclaving of medium in the fermentor, nitrogen that was sterilized through a packed bed filter and an absolute filter (0.22 micron) was bubbled through the hot broth at 0.1 VVM (150 mL/min), based on design parameters of Siegel and Metzger (1979) to maintain anaerobic conditions during cooling. After inoculation with spores that were heat shocked as described above, nitrogen was sparged only into the headspace of the fermentor (Siegel and Metzger, 1979). Medium was stirred by a magnetically coupled impeller at 200 rpm. Spores were harvested as described for the inoculum preparation, but after a 20 min aeration of the culture to lyse vegetative cells (Zoha and Sadoff, 1958).

Samples were taken aseptically at no greater than 12 hr intervals over the course of the fermentation for sugar analysis, and cell and spore counts. All assays were made in triplicate and average values are reported [see Pang (1982) for complete data]. All data were used in drawing curves in Fig. 1, 2, and 3, but only representative points are shown for clarity.

Thermal death time (TDT) determination

Heat resistance (D and z values) was determined only on harvested spores, according to the tube technique of NCA (1968) (p. 172–177). The D value was determined in triplicate by count reduction (Stumbo et al., 1950). The initial count was made on spores which had been heat shocked 13 min at 82–83°C (Goldoni et al., 1980). The final count was made on spores surviving the TDT retort, which were not further heat shocked. The spores were suspended in sterile M/15 phosphate buffer (pH 7.0) for heat shocking and for use in the TDT tubes which contained 2.0 mL each. The heat shocked and retorted spores were enumerated by the plate count method. Pour plates of trypticase soy agar were incubated in an anaerobic jar for 72 hr at 30°C and the colonies were counted (Hays and Lynt, 1976). TDT times were selected arbitrarily to achieve at least three D values in the range 230–250°F, for calculation of z values.

In Fig. 3 and 5, as well as other z value determinations, the line through the D value points was fitted by least squares.

Assays

Cell and spore counts during fermentation were made in a Petroff-Hauser counting chamber (Koch, 1981), under phase contrast with oil immersion (Murray and Robinow, 1981). All refractile spores, including those inside cells, were counted.

Fig. 1—Fermentation of P.A. 3679, showing decrease in pH, incomplete sugar utilization, and termination of growth.

Fig. 2—Cell growth and sporulation of P.A. 3679 with complete glucose utilization (pH controlled at 7.0).
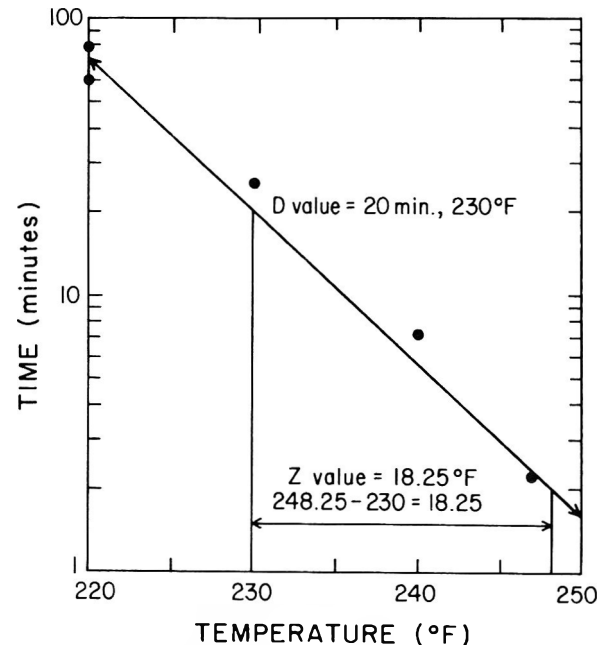
Fig. 3—D and z values for P.A. 3679 spores (initial glucose concentration 15 g/L, pH controlled at 7.0, corresponding to Fig. 2).
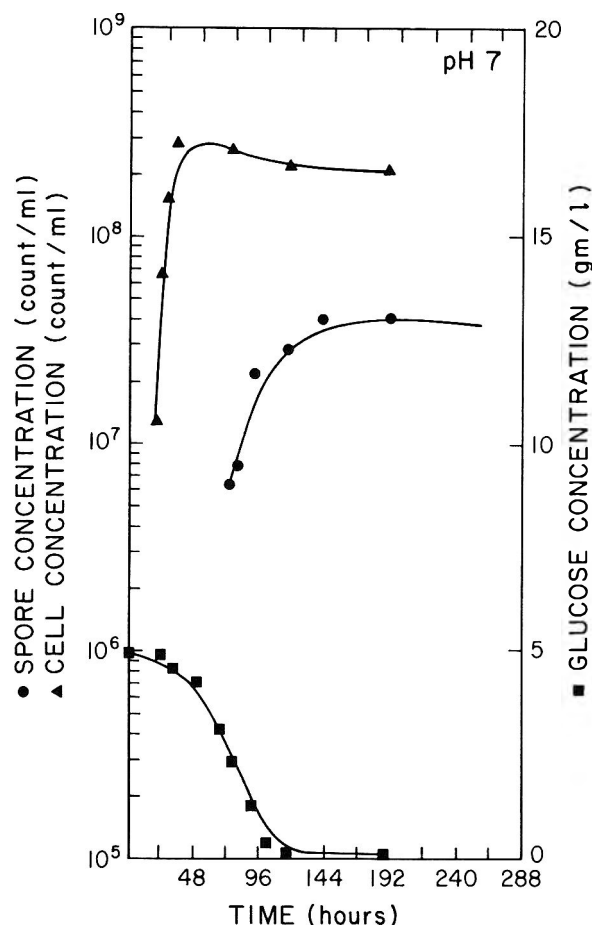
Fig. 4—Cell growth and sporulation of P.A. 3679 with complete glucose utilization (pH controlled at 7.0).

Fig. 5—D and z values for P.A. 3679 spores (initial glucose concentration 5 g/L, pH controlled at 7.0, corresponding to Fig. 4).

Fig. 6—Effect of pH on D_{230} values, z values, and maximum specific growth and sporulation rates of P.A. 3679.

Glucose concentration in the fermentation medium was determined by a reducing sugar assay using 3,5-dinitrosalicylic acid (Sumner and Somers, 1944).

RESULTS & DISCUSSION

AN INITIAL GLUCOSE CONCENTRATION of approximately 15 g/L without pH control was predicted to exceed the fermentable limit for spore production, based on the results of Anema and Geers (1973) and Zoha and Sadoff (1958). Results shown in Fig. 1 confirm this prediction. After a glucose consumption of less than 5 g/L, sufficient acid was produced to lower the pH from 7 to 5.6 and growth ceased. Control of pH at 7.0, as illustrated in Fig. 2, however, permitted cell growth and sporulation to proceed with complete glucose utilization. The D_{230} and z values for the fermentation with an initial glucose concentration of 15.0 g/L and pH control at 7.0 were 20 min and 18.25F°, respectively, as shown in Fig. 3. Subsequent experiments to test the effect of varying pH were conducted with an initial glucose concentration of 5.0 g/L, since this amount exceeded the sugar utilization which was possible without pH control and was consistent with results of previous researchers regarding a glucose level at which sporulation was inhibited without pH control.

Experiments with pH control and initial glucose concentration of 5.0 g/L were conducted at pH levels of 6.0, 6.5, 7.0, and 8.0. Data for the pH 7.0 run, which are typical for all runs, are shown in Fig. 4 and 5. In Fig. 4, the cell, spore, and glucose concentrations are plotted versus time from inoculation. Harvested spores were tested for thermal resistance with results as shown in Fig. 5.

The cells underwent an exponential growth phase consistent with first order growth kinetics. Spore count also increased in exponential fashion, consistent with the results of Zoha and Sadoff (1958), which permitted calculation of an empirically derived maximum specific sporulation rate,

κ, which is defined analogously to maximum specific growth rate, μ (Aiba et al., 1973):

$$\mu = \frac{1}{(\text{cell count})} \left[\frac{d(\text{cell count})}{d(\text{time})} \right]_{\text{maximum}}$$

$$\kappa = \frac{1}{(\text{spore count})} \cdot \left[\frac{d(\text{spore count})}{d(\text{time})} \right]_{\text{maximum}}.$$

Variations of D_{230} values, z values, maximum specific growth rates, and maximum specific sporulation rates with respect to pH are shown in Fig. 6. The D_{230} values show a maximum at pH 6.5. The z values are essentially unchanged among the experiments with an average value of 19.6 ± 1.7°F. The D_{230} value of 10.0 min at pH 7.0 for an initial glucose concentration of 5.0 g/L is consistent with that reported by Anema and Geers (1973) of 13.0 min at similar conditions but in a slightly different medium and with a different strain of *Cl. sporogenes*. They demonstrated that the D_{230} increased as initial glucose concentration increased from zero to 2 g/L. Results of this study showed an increase in D_{230} at pH 7 from 10.0 min to 20.0 min as initial glucose level was raised from 5 g/L to 15 g/L. These trends are consistent, although numerical values are not directly comparable due to differences in strain and medium.

The variations in growth rate and sporulation rate show maxima at approximately pH 7.0. The shapes of the curves are consistent with the report by Russell (1971) that for *B. coagulans* the optimum pH for sporulation is equivalent to that for growth, but that the range is narrower. The maximum cell concentrations for the four runs averaged 3.7 (± 1.8) X 10^8 cells/mL with slightly higher values at pH 7.0 and 8.0 than at pH 6.0 and 6.5. The maximum spore concentration averaged 3.5 (± 1.7) X 10^7 spores/mL, with pH 7.0 and 8.0 levels about twice those for pH 6.0 and 6.5.

Results of this study show that control of pH permits addition of glucose to the 15 g/L level and that variation in pH yields spores whose D values may be varied to suit different thermal processing purposes.

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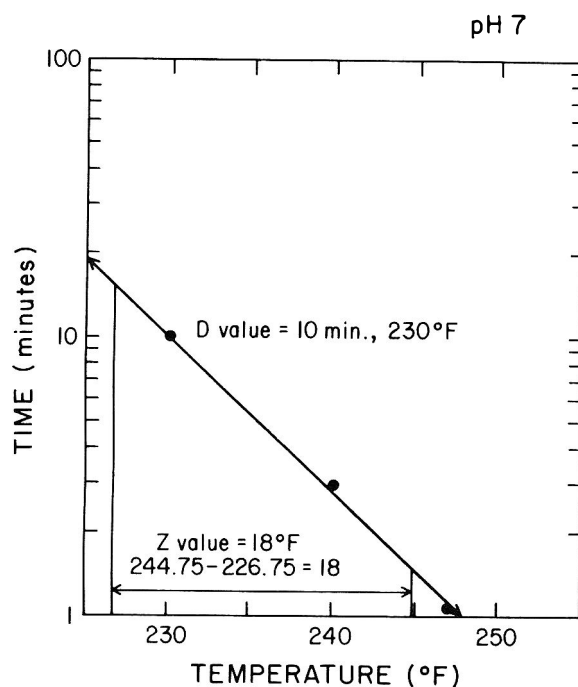this technique in commercial starter culture applications. It is conceivable that with further research the gradient system could be modified to enhance yield and culture activity with decreased process time.

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Determination of Moisture, Protein, Fat, and Calories in Raw Pork and Beef By Near Infrared Spectroscopy

ELAINE LANZA

─────────── ABSTRACT ───────────

The near infrared (NIR) (1100–2500 nanometers) of raw pork and raw beef samples was determined on a Neotec Model 6350 Scanning Spectrocomputer. Spectral curves were correlated with moisture, protein, fat, and calorie data determined by standard methods. A stepwise multiple regression technique was used to determine the optimum wavelengths for predicting each constituent. Correlation coefficients were ≥ 0.987 for moisture, fat, and calories and 0.885 for protein. The coefficients of variation for each of the nutrients were $\leq 3.46\%$.

INTRODUCTION

NEAR-INFRARED (NIR) reflectance spectroscopy is a rapid, effective analytical tool that is used for the determination of moisture and protein in cereal grains (Williams et al., 1978), moisture, protein, and oil contents of oilseeds (Hymowitz et al., 1974; Ben Gera and Norris, 1968a) and major constituents in forages (Norris et al., 1976). This spectroscopic technique was developed to replace laborious and time-consuming conventional methods, i.e. the Kjeldahl method for protein, various solvent extraction methods for fat, and oven-drying methods for moisture, but is not widely used for the analysis of food products. As early as 1968, Ben-Gera and Norris (1968b) used transmission spectroscopy in the NIR range (1500–1850 nm) to determine the fat and moisture contents of meat products. More recently Kruggel et al. (1981) and Martens et al. (1981) determined the moisture, fat and protein content of meats with a fixed-filter NIR reflectance instrument.

This study reports on the use of a NIR scanning instrument (1100–2500 nm) in both the transmission and reflectance modes to determine the moisture, fat, protein and calorie contents of raw pork and beef.

MATERIALS & METHODS

Near infrared spectrocomputer

All NIR measurements were made with a Neotec Model 6350 Spectrocomputer (Neotec, Silver Spring, MD). This instrument has a single-beam scanning monochromator that provides a linear scan over the 1100–2500 nm region. The 6350 takes five scans/ sec. Data were recorded at 2-nm intervals and 50 scans were averaged for every sample. A ceramic disk was used as a reference in the reflectance mode and an empty quartz transmission cell was used in the transmission mode.

In the reflectance mode the light beam is directed down towards the sample. Light reflected off the sample is then picked up by four lead sulfide cells equally spaced at 45° above the sample. In the transmission mode a lead sulfide cell is positioned directly beneath the sample cell. The detected signal is fed into a log amplifier, digitized and sent to a Nova computer (32K words of memory).

The absorbance data were recorded as log 1/R (R = reflectance) which varies approximately linearly with the concentration of the absorber (Norris et al., 1976). The mathematical transformation of the data to first or second derivative is done to reduce multi-

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plicative effects on reflectance spectra, such as particle size, sample temperature, and sample compaction plus breaking intercorrelations between wavelengths. The second derivative transformation, which was used for all data reported in this paper, was approximated by the method of finite differences where:

$$\log \frac{1}{R_{\lambda_1}} - 2 \log \frac{1}{R_{\lambda_2}} + \log \frac{1}{R_{\lambda_3}}$$

A twenty-point scale was used in the derivative calculation.

Before NIR can be used to determine the composition, of a particular sample, the NIR instrument must be calibrated. Pork and beef samples were each divided into two sets, a calibration set and a prediction set; and, the moisture protein and fat values of these samples were determined (Table 1).

A step-forward multiple regression program was then used to find the linear equation which best correlates to the analytical data. Separate equations were found for moisture, protein, fat and calories. The calibration equations used throughout the paper are in the form:

$$Y_i = b_0 + K_1 \frac{\dfrac{d^2 \log (1/R_{\lambda_1})}{d\lambda_1^2}}{\dfrac{d^2 \log (1/R_{\lambda_2})}{d\lambda_2^2}} + K_2 \frac{\dfrac{d^2 \log (1/R_{\lambda_3})}{d\lambda_3^2}}{\dfrac{d^2 \log (1/R_{\lambda_4})}{d\lambda_4^2}}$$

Where, b_0, K_1 and K_2 are the constants of the regression equation and Y_i is the concentration for sample i. The use of a derivative divided by derivative was first suggested by Norris and Barnes (1976) and recently explained in more detail by Norris (1982). The calibration equation should give a high correlation coefficient (r), a low standard error of calibration (SEC) and a low bias. The standard error of calibration measures how well the instrument matches calibration samples and is calculated by

$$SEC = \left(\frac{\sum\limits_{i=1}^{n} (Y_i - \hat{Y}_i)^2}{n - 3} \right)^{\!\!1/2}$$

The bias is calculated as

$$BIAS = \frac{1}{n} \sum\limits_{i=1}^{n} (Y_i - \hat{Y}_i)$$

Once the calibration equation is found, the prediction samples are used to verify the equation. The standard error of prediction (SEP) is the standard deviation of the prediction error.

$$SEP = \sum\limits_{i=1}^{n} \left(\frac{[(Y_i - \hat{Y}_i) - Bias]^2}{n - 1} \right)^{\!\!1/2}$$

where n = number of samples, Y_i = laboratory value of concentration, and $\hat{Y}_i$ = NIR predicted concentration.

Meat samples

Beef and pork samples were obtained from a large meat study conducted at the Beltsville Agricultural Research Center, Beltsville, MD. The beef samples were selected at random from 14 different cuts from 11 carcasses, USDA grades prime through standard; pork samples were selected from 7 retail cuts from 71 carcasses, USDA quality grades 1, 2 and 3. Each sample, representing a retail

cut of meat, was emulsified to a smooth paste in a Robot-Coupe Food Processor for 12 sec at 1500 rpm and for 18 sec at 3000 rpm. Replicate portions of each sample were frozen in 4 oz polyethylene screw top containers and saved for subsequent analyses. A paired t-test showed no significant difference between the moisture content of freshly mixed and frozen samples (n = 20). Moisture and nitrogen (Kjeldahl) were analyzed by AOAC procedures (1975). Total fat was determined by the Folch procedure (Folch et al., 1957) and calories by the procedure of Atwater as described in USDA, Handbook #8 (Watt and Merrill, 1975). Moisture and fat were analyzed in-house, and nitrogen by a commercial laboratory. When the sum of moisture, fat, and protein for any sample was ⩽ 98.5% or ⩾ 101.5%, that sample was not analyzed by NIR. This helped to eliminate errors due to faulty analytical values. All samples were brought to room temperature (20–23°C) mixed thoroughly, and analyzed in the NIR spectrometer using standard Pacific Scientifid quartz sampling cups with quartz covers.

RESULTS & DISCUSSION

INITIALLY PORK SAMPLES were run in the transmission mode, in which cell thickness is critical. When 10 subsamples of pork were run at 0.3 mm thickness the coefficients of variation were 0.7% for H_2O, 2.9% for fat and 1.5% for protein. When sample thickness was increased to 2 mm, the coefficients of variation were 0.3% for H_2O, 1.0% for fat and 1.5% for protein. At that thickness, the amplifier was saturated above 1.85 u due to the absorbance of H_2O. Wavelengths selected by the computer (wavelengths which give the highest correlation coefficient between the chemical analysis and the NIR spectra) for the pork calibration set appear in Table 2. Second derivative math transformation of the spectra was found to give better correlations than first derivative math. The SEC for all four constituents were acceptable, but SEP were excessive for protein and fat. The bias also was high for both protein and fat.

The same pork samples were then scanned in the reflectance mode. The reflectance sample cup contains about 25g of ground meat (10 mm thick). The sample holder by spinning the cup continuously 5/16″ off center increases the exposed surface area of the sample from 3 cm to 10 cm. Coefficeints of variations for 10 reloads of the same sample were 0.5% for H_2O, 0.3% for fat and 0.7% for protein. The wavelengths selected by the computer to give the calibration equations with the lowest standard error of calibrations i.e. the highest r values for the moisture, protein, fat and calories in pork samples are given in Table 3. The standard error of prediction for protein and fat were considerably lower in the reflectance mode, probably due to the increased sample size. Table 4 reports the results for beef samples analyzed by reflectance NIR. The r, SEC, and SEP for all constituents for pork and beef are comparable except for the lower correlation for beef protein.

The similarity of absorbance scans in the reflectance mode between pork and beef (Fig. 1) suggested that data for beef and pork might be combined into one calibration set. The prediction set of pork (Table 3) and the prediction set for beef (Table 4) were combined in a new calibration file of 27 pork and 36 beef samples (N = 63). As in Table 3 and 4, the computer step-forward regression program was used to select the calibration equation with the highest correlation between the absorbance scans and the analytical data. These results are given in Table 5. The SEC and the SEP for all constituents are similar to those reported in Table 3 and 4 except for the SEP for protein. In order to improve these results, a comparison of the wavelengths chosen and the NIR spectra of the individual constituents was made, since the wavelengths chosen by the computer should represent the constituent being quantitated. The fat of both beef and pork were extracted using the Folch procedure. The residues from those extracts after being washed and dried were considered mainly meat protein. The second derivative curves for pork fat, pork protein and water are shown in Fig. 2. Beef fat and protein curves are similar to those of pork. A good predictive wavelength for protein, for example, would be a wavelength which is a minimum or a maximum in the second derivative curve for protein, and where there is either no change or a smaller and opposite change in the second derivative curves for fat and water. The same type of criteria was used to select the best wavelengths for water and fat. In Table 5 only the 1720 nm wavelength selected for fat satisfies the criteria described above. Since it is tedious to examine all 1400 nm of the spectra for each constituent, I reduced the number of wavelengths examined by comparing only those wavelengths with a correlation of ⩾ 0.7. For example, protein had only 11 wavelengths ⩾ 0.7 and only 3(1698 nm, 1744 nm, and 2164 nm) are peaks free of interferences. Each of these wavelengths was then used as a starting point (numerator of the first term) for selecting a stepwise linear multiple regression equation. The equation starting with 2164 nm gave slightly better results. The same procedure was used to select the calibration equations for moisture and fat. For calories a slightly different approach was used, since in meat, it is not the chemical value for calories, but

Table 1—Composition of samples analyzed by chemical methods

Component	Mean	SD	Range
Beef Calibration Set (n = 63)			
Moisture	71.2	1.9	65.7– 74.5
Protein	20.5	1.3	17.8– 23.0
Fat	7.7	2.6	3.1– 14.0
Calories	155.8	19.7	123.5–211.1
Beef Prediction Set (n = 36)			
Moisture	69.0	2.9	65.8– 75.0
Protein	20.0	1.2	18.1– 22.3
Fat	9.3	3.4	4.4– 14.7
Calories	168.9	27.6	125.7–212.8
Pork Calibration Set (n = 64)			
Moisture	71.3	2.2	66.9– 75.9
Protein	20.5	1.2	17.5– 25.6
Fat	7.9	2.9	3.9– 15.5
Calories	159.2	24.6	128.0–226.8
Pork Prediction Set (n = 27)			
Moisture	72.7	2.0	65.2– 75.5
Protein	20.3	1.3	18.4– 22.5
Fat	7.3	2.9	3.9– 11.3
Calories	150.9	33.8	127.3–233.8

Table 2—Wavelengths selected and statistical summary of pork analysis by near-infrared transmission spectroscopy

	λ_1[a] (nm)	λ_2 (nm)	λ_3 (nm)	λ_4 (nm)	r	SEC[b] (%)	SEP[c] (%)	Bias (%)	r
Moisture	1732	1700	1446	1752	0.959	0.54	0.59	0.01	0.979
Protein	1740	1760	1702	1342	0.874	0.56	0.99	0.73	0.739
Fat	1718	1306	1224	1520	0.994	0.26	1.12	1.37	0.828
Calories	1726	1774	1698	1808	0.987	2.89	5.33	2.48	0.973

[a] λ_1, λ_2, λ_3, λ_4 refer to the wavelengths selected for the calibration equation described in Materials & Methods
[b] Standard error of the calibration samples (n = 64)
[c] Standard error of the prediction samples (n = 27)

	λ₁[a] (nm)	λ₂ (nm)	λ₃ (nm)	λ₄ (nm)	r	SEC[b] (%)	SEP[c] (%)	Bias (%)	r
Moisture	1734	1774	2290	1698	0.986	0.52	0.60	−0.033	0.975
Protein	1768	1644	2408	2338	0.940	0.41	0.66	0.04	0.870
Fat	1212	1284	2204	1188	0.998	0.27	0.16	0.06	0.999
Calories	1730	1292	1700	2202	0.996	2.88	4.92	−0.06	0.978

[a] $\lambda_1, \lambda_2, \lambda_3, \lambda_4$ refer to the wavelengths selected for the calibration equation described In Materials & Methods.
[b] Standard error of the calibration (n = 64)
[c] Standard error of the prediction samples (n = 27)

	λ₁[a] (nm)	λ₂ (nm)	λ₃ (nm)	λ₄ (nm)	r	SEC[b] (%)	SEP[c] (%)	Bias (%)	r
Moisture	1178	1640	2438	1344	0.974	0.45	0.52	−0.01	0.982
Protein	1768	1644	1602	1378	0.907	0.56	0.61	−0.01	0.865
Fat	1720	1136	1218	1300	0.997	0.21	0.31	0.18	0.996
Calories	1784	1376	2416		0.985	3.57	4.28	1.00	0.988

[a] $\lambda_1, \lambda_2, \lambda_3, \lambda_4$ refer to the wavelengths selected for the calibration equation described in Materials & Methods
[b] Standard error of the calibration samples (n = 63)
[c] Standard error of the prediction samples (n = 36)

	λ₁ (nm)	λ₂[a] (nm)	λ₃ (nm)	λ₄[a] (nm)	r	SEC[b] (%)	Pork SEP[c] (%)	Pork Bias (%)	r	Beef SEP (%)	Beef Bias (%)	r
Moisture	1732	1700	1990	1218	0.985	0.48	0.66	−0.26	0.93	0.59	0.42	0.96
Protein	1376	1772	1524	1804	0.899	0.53	0.92	−0.19	0.53	1.15	−0.06	0.63
Fat	1720	1308	2388	1890	0.998	0.23	0.28	0.15	0.99	0.27	−0.10	0.995
Calories	1236	1308	1988	1762	0.993	3.38	3.80	1.73	0.97	5.30	−1.02	0.97

[a] $\lambda_1, \lambda_2, \lambda_3, \lambda_4$ refer to the wavelengths selected for the calibration equation described in Materials & Methods
[b] Standard error of the calibration samples (n = 63; 27 pork + 36 beef)
[c] Standard error of the prediction samples (n = 64, pork, n = 63 beef)

the biological value calculated by multiplying specific At-water factors by the protein and fat content of the meat sample which is of interest. Thus, a good wavelength would be one that is either near the minimums and/or maximums for both fat and protein, and where there is a minimum of intereference from water. The numerators in the first term of the regression equations listed in Table 6 all satisfy the criteria given above, as shown in Fig. 2. To test these four multiple regression equations the 63 samples from the pork calibration set (Table 3), and the 64 samples from the beef calibration set (Table 4) were predicted (Table 7). Even though there is a considerable improvement in the SEP for protein in Table 7 compared to Table 5, the correlation coefficient for protein is not as high as the other constituents. One possible explanation for this is that the analytical data for protein contained errors. Since there was not enough sample available to repeat the Kjeldahl analysis, protein content was estimated from the fat and moisture levels (100 minus the fat and moisture). The mean ash content for both beef and pork was so low, approximately 1% ± 0.2, its value was not considered in the calculation. The above data were then used in the combined beef-pork calibration set as an alternate estimate of protein in meat. The r value for protein increased from 0.885 to 0.939, which suggests that there might have been a problem in the protein laboratory data. Another explanation for the lower correlation is that the Kjeldahl method measure nitrogen while NIR measures protein.

Table 8 shows the coefficients of variation for pork and beef calculated from the standard errors of prediction (Table 7) and the means of all samples (Table 1). The coefficients of variation are less than reported by Kruggel
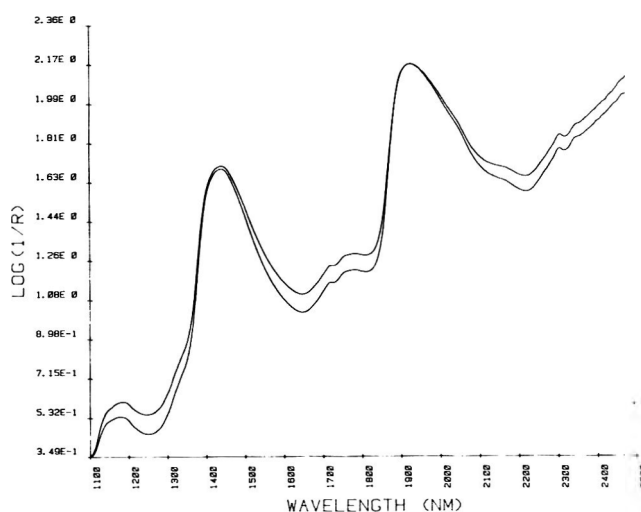
Fig. 1—NIR reflectance scan of pork (upper) and beef (lower curve).

et al. (1981) for beef (moisture, 2.65%, protein, 4.23%, and fat, 11.6%). While the use of NIR as described in this paper is adequate for moisture and fat analysis the SEP for protein needs to be reduced before this technique can be used for the regulatory monitoring of meat.

Table 6—Calibration equations for moisture, protein, fat and calories for pork and beef by near-infrared reflectance analysis using selected wavelengths

	b_0[a]	K_1	K_2	λ_1 (nm)	λ_2 (nm)	λ_3 (nm)	λ_4 (nm)	r	SEC[b] (%)
Moisture	10.03	−19.86	− 17.47	1778	1656	1986	1168	0.987	0.45
Protein	23.79	12.08	2.10	2164	1204	1132	1186	0.885	0.57
Fat	6.84	− 8.41	22.15	1720	1308	2238	1890	0.998	0.23
Calories	100.46	−75.51	−146.04	1730	1320	1756	1136	0.991	3.66

[a] b_0, K_1 and K_2 are constants in the calibration multiple regression equation, $\lambda_1, \lambda_2, \lambda_3, \lambda_4$ are the selected wavelengths, and Y is the concentration for sample i.

$$Y_i = b_0 + K_1 \frac{\dfrac{d^2 \log (1/R_{\lambda_1})}{d\lambda_1^2}}{\dfrac{d^2 \log (1/R_{\lambda_2})}{d\lambda_2^2}} + K_2 \frac{\dfrac{d^2 \log (1/R_{\lambda_3})}{d\lambda_3^2}}{\dfrac{d^2 \log (1/r_{\lambda_4})}{d\lambda_4^2}}$$

[b] Standard error of the calibration samples

Table 7—Prediction of nutrients in beef and pork by near-infrared reflectance using selected wavelengths

	Pork (N = 63)			Beef (N = 64)		
	SEP[a] (%)	Bias (%)	r	SEP (%)	Bias (%)	r
Moisture	0.64	0.08	0.94	0.57	0.19	0.96
Protein	0.55	−0.18	−0.79	0.70	−0.14	0.84
Fat	0.28	0.15	0.99	0.27	−0.10	0.995
Calories	3.29	−1.59	0.98	3.76	−1.47	0.98

[a] Standard error of the prediction samples
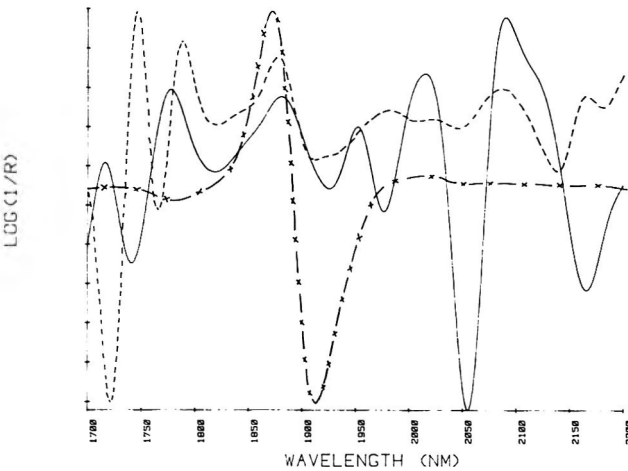
Fig. 2—Second derivative of NIR reflectance scan of protein, fat, and water in pork: -x-x moisture; - - - - fat; and ——— protein.

Table 8—Coefficients of variation for determination of moisture, protein, fat and calories in pork and beef by near-infrared reflectance

	Pork (%)	Beef (%)
Moisture (g/100g)	0.90	0.81
Protein (g/100g)	2.71	3.43
Fat (g/100g)	3.46	3.29
Calories (Kcal/100g)	2.06	2.34

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Electron Microscopic Investigation of *Pseudomonas fragi* ATCC 4973 on Intact and Sarcoplasm-Depleted Bovine Longissimus dorsi Muscle at 21°C

P. LEE WING, R. Y. YADA, and B. J. SKURA

──────────── ABSTRACT ────────────

Scanning and transmission electron microscopy were used to investigate formation and role of glycocalyx material involved in adhesion of *Pseudomonas fragi* to intact and sarcoplasm-depleted beef surfaces. Depletion of sarcoplasm did not decrease attachment of *P. fragi* to bovine muscle. *P. fragi* caused a rapid increase in pH of only intact muscle. Examination of inoculated muscle (washed and intact) by SEM after 1, 2, 3, or 5 day incubation period, revealed a pebbling effect on the bacterium surface as well as a coiling of glycocalyx material. TEM showed two types of polymeric material; one was adherent to the bacterial surface while the other was amorphous. The amorphous type probably corresponded to the coiled glycocalyx revealed by SEM. Close association between glycocalyx and bleb-like evaginations on *P fragi* reinforces previous hypotheses concerning their role and functions in attachment and meat spoilage.

INTRODUCTION

THE INITIAL STAGES of microbial meat spoilage involve some form of contact between the microorganism and the meat surface, with an eventual attachment of the microorganism to the substrate. The attachment process consists of two stages: stage one, or the stage of "reversible sorption," in which microorganisms in contact with the surfaces become subject to short range attraction forces, e.g. hydrophobic or Van der Waals forces (Fletcher and Loeb, 1979); and stage two, or the stage of "permanent adhesion," which involves the formation of a glycocalyx (extracellular polymeric material) between the bacteria and the substrate (Fletcher and Floodgate, 1973). Extensive research by Costerton et al. (1978) demonstrated that microorganisms adhere to substrates by means of a mass of tangled fibers of polysaccharide, with the formation of "feltlike glycocalyx." A better understanding of the spoilage phenomenon would then require a detailed knowledge of the mechanism of attachment and adhesion to meat surfaces by spoilage microorganisms. Notermans and Kampelmacher (1974), showed that the optimum temperature for attachment of Pseudomonas strains was ca 21°C. Gill and Newton (1980), indicated that psychrotrophs continue to compete successfully with mesophilic species at normal ambient temperature, except with meat stored anaerobically at temperatures in excess of 20°C. Storage at 21°C should then favor greater association of pseudomonads with the meat surface and hence may enhance increased glycocalyx formation during the adhesion stage of the attachment process.

At chill temperatures the spoilage flora of meat are composed of psychrotrophic species, with pseudomonads usually predominating under aerobic conditions (Gill and Newton, 1978). At higher temperatures, however, little information is available on the growth of bacteria on meat surfaces (Gill and Newton, 1980).

The object of this study was to investigate: (a) the behavior (growth, pH, and glycocalyx formation) of *Pseu-* *domonas fragi* ATCC 4973 on intact and sarcoplasm-depleted bovine muscle at 21°C; and (b) the formation and role of glycocalyx material in adhesion of bacteria to meat surfaces.

MATERIALS & METHOD

BOVINE longissimus dorsi muscles, 24 hr postmortem, were obtained from a local abattoir and transported on ice to the laboratory. Thin slices (3 mm) of muscle were prepared by the method of Yada and Skura (1981). The slices were randomly organized into two groups. One group did not receive further treatment (intact muscle) while the other was subjected to a washing procedure (Yada and Skura, 1981) to extract water soluble components (washed muscle). Intact and washed muscle slices were sterilized with 10 K Gy of gamma-radiation, inoculated with *P. fragi* (Yada and Skura, 1981) and incubated at 21°C. Sterile controls of intact and washed muscle were also prepared. At time intervals of 0, 1, 2, 3, and 5 day storage, the population of *P. fragi* on intact and washed muscles (inoculated and sterile controls) as well as pH were determined according to the procedure used by Yada and Skura (1981). Samples were concurrently sectioned and treated for examination by means of scanning (SEM) and transmission (TEM) electron microscopy.

Preparation of muscle for SEM

Muscle tissues were sectioned into 2 x 2 cm cubes prior to fixation with 2.5% glutaraldehyde (J.B. EM Services Inc., Quebec, Canada) in 0.05M phosphate buffer (pH 7.0; 24 hr; 4°C). Samples were rinsed twice in 0.05M phosphate buffer (pH 7.0) at 21°C before further fixation with 1% osmium tetroxide (J.B. EM Services Inc.) in 0.05M phosphate buffer (pH 7.0) for 1 hr. This step was followed by dehydration through a graded series of ethanol: 50, 70, 80% for 5-min each, 90% for two 10-min periods and 100% for three 20-min periods. All ethanol dilutions were made with distilled-deionized water. Samples were critical point dried in a Parr-bomb (Parr Instrument Co., Moline, IL) using CO_2 as the transitional fluid. Samples mounted on aluminum stubs with silver paste (J.B. EM Services Inc.) were coated with gold by vacuum evaporation. Samples were viewed on the Cambridge Stereoscan 250, operated at 40 Kv.

Preparation of muscle for TEM

Samples for transmission electron microscopy were prepared by the method of McCowan et al. (1978), with slight modifications. Tissues (5 x 5 mm) were fixed at 4°C for 24 hr with 2.5% glutaraldehyde solution in 0.05M phosphate buffer (pH 7.0) prior to post fixation (2 hr, 21°C) in 0.015% dye [ruthenium red (Aldrich Chemical Co., Milwaukee, WI) or alcian blue (J.B. EM Services, Inc.)] buffered with a 0.05M phosphate solution. Samples, after 2 hr in 0.05M phosphate buffer containing 0.05% dye (DP buffer) at 21°C, were washed in five changes of the same solution for 1 hr each; with one overnight wash included. Samples were postfixed for 2 hr in 2% osmium tetroxide in DP buffer, followed by five 1 hr washes in DP buffer. Dehydration was achieved by immersion of samples in 15, 30, 50, 70, 90 and 100% ethanol. The ethanol was prepared with distilled-deionized water. Samples, following dehydration were washed in two changes of propylene oxide (J.B. EM Services Inc.) for 15 min each, infiltrated with a 1:1 mixture of propylene oxide and Epon 812 (J.B. EM Services Inc.) for 16 hr, then embedded in 100% Epon 812.

Microtomy

Tissues were sectioned on a "Proter-Blum" MT-2 ultramicrotome (Ivan Sorvall Inc., Norwalk, CN), mounted on 3 mm copper grids, then stained with uranyl acetate and lead citrate.

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Electron microscopy of all specimens was performed with a Zeiss EM-10 transmission electron microscope at an accelerating voltage of 80 Kv.

RESULTS

Growth of P. fragi

At day 0 there were between 10^5 and 10^6 P. fragi /cm^2 on intact and washed inoculated muscle (Fig. 1). No bacteria were detected on the intact or washed controls at day 0 or day 5. P. fragi grew rapidly on both intact and washed muscle during the initial 3 days of the storage trial followed by a modest rate of growth during the remainder of the storage trial. The bacterial population at the end of the 5 day storage trial on the washed muscle was not as high as that on intact muscles. A similar phenomenon was observed at $4°C$ (Yada and Skura, 1981).

In order to evaluate statistically the effect of decreased sarcoplasm concentration on growth of P. fragi, data for washed and intact inoculated muscles were subjected to a data transformation for linearization (Fig. 2) using the modified super-simplex optimization method of Fujii and

Nakai (1980) as described previously (Yada and Skura, 1981).

Linearization of the log bacterial count data yielded the following regression equations:

Intact inoculated: $(Y-4.39)^3 = 17.60 X + 2.71$ r=0.99
Washed inoculated: $(V-5.30)^3 = 18.69 U - 3.99$ r=0.97

where: Y = log CFU/cm^2 of intact inoculated muscle; V = log CFU/cm^2 of washed inoculated muscle; X, U = incubation time (days).

The growth rate of P. fragi on washed muscle (slope = 18.69) was similar to that on intact muscle (slope = 17.60). A comparison of the slopes, using the F-test, indicated that there was no significant difference ($P > 0.05$) between intact and washed inoculated muscle in their ability to support growth of P. fragi.

pH

Pseudomonas fragi caused a marked increase in pH of inoculated muscle from pH 6 (day 0) to pH 9 (day 5), but had little effect on the pH of washed inoculated muscles. No pH changes were observed in the control (washed and intact) muscles. Similar observations were noted by Yada and Skura (1981) for P. fragi growing on intact and washed muscle at $4°C$.

Electron microscopy

Scanning electron micrographs of washed and intact muscles as early as day 1 of the incubation period revealed the initial formation of a fiber-like glycocalyx material extending from the surface of the microorganism (Fig. 3a). At higher magnifications a pebble-like extracellular material on the outer surface of the microorganism could be seen (Fig. 3b, 3c). The more detailed sequential stages of fiber-like glycocalyx formation could not be differentiated during examination of samples from day 1, 2 or 3. Examination of both washed and intact muscles after 5-days incubation showed an intensification of the pebbling effect on the bacterial surface, in addition to a coiling of the glycocalyx fiber to form a matted mass of extracellular material (Fig. 4).

No marked differences were observed in samples stained with ruthenium red or alcian blue examined with the transmission electron microscope. Ruthenium red, however, gave a slightly better contrast of structures. Since both ruthenium red and alcian blue are specific for acidic polysaccharides, electron dense areas were not limited to the extracellular polymer but also to acidic polysaccharide present in the meat sample. TEM, combined with ruthenium red and alcian blue staining, demonstrated two types of extracellular material associated with attachment. One type of polymer formed a thin coat on the surface of the bacterial cell, while the other was an amorphous substance that stretched between and around bacteria and muscle surfaces (Fig. 5). A similar pattern of glycocalyx localization and morphology was reported in a study of acidic polysaccharide involved in the adhesion of a marine bacterium to solid surfaces (Fletcher and Floodgate, 1973).

Scanning as well as transmission electron micrographs revealed bleb-like evaginations or protrusions on the surface of P. fragi after a 5 day incubation period (Fig. 6). A close association of these blebs to the site of attachment of glycocalyx fibers to the bacterial surface was also observed (Fig. 6). These phenomena were not evident in samples incubated for 1, 2, or 3 days. The morphology of P. fragi or its extracellular material on washed muscles appeared to be unaffected by diminished quantities of sarcoplasm since similar observations were seen on both washed and intact inoculated muscle types.
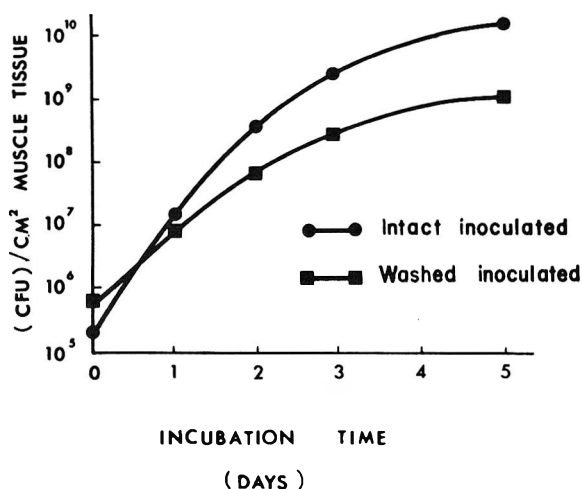
Fig. 1—P. fragi on intact and washed muscle samples, stored at 21°C.
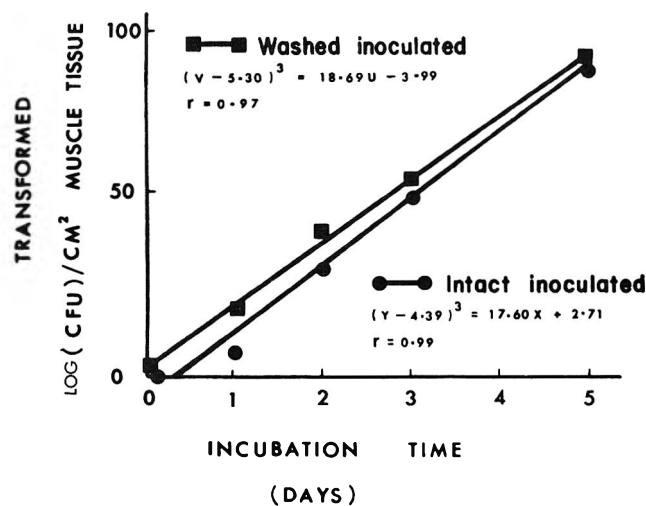
Fig. 2—Linearization of log bacterial population data for intact and washed muscle samples, inoculated with P. fragi, stored at 21°C.

DISCUSSION

INGRAM AND DAINTY (1971) reported that at higher temperatures the respiration of the meat tissue is much greater so that there is likely to be less available oxygen in the tissue near the surface on which the bacteria grow. Very thin samples were used in this study, therefore, an aerobic environment was maintained. Several authors, have suggested a preferential utilization of low molecular weight components by bacteria during meat spoilage (Jay, 1972; Gill and Newton, 1978). The growth rate of *P. fragi*, at 21°C, on sarcoplasm-depleted beef was not significantly

different than that on intact muscle, while at 4°C *P. fragi* grew more rapidly on intact muscle than on washed muscle (Yada and Skura, 1981).

The general trend observed for pH changes in both washed and intact inoculated muscles were in accord with published data (Yada and Skura, 1981). The nature of the biochemical changes occurring, particularly at intermediate temperatures (15–25°C) has not been adequately documented. Contradictory reports, on the correlation between pH and the number of microorganisms present on meat surfaces are found in the literature (Turner, 1960; Rogers and McCleskey, 1961). The increase in pH of intact inocu-
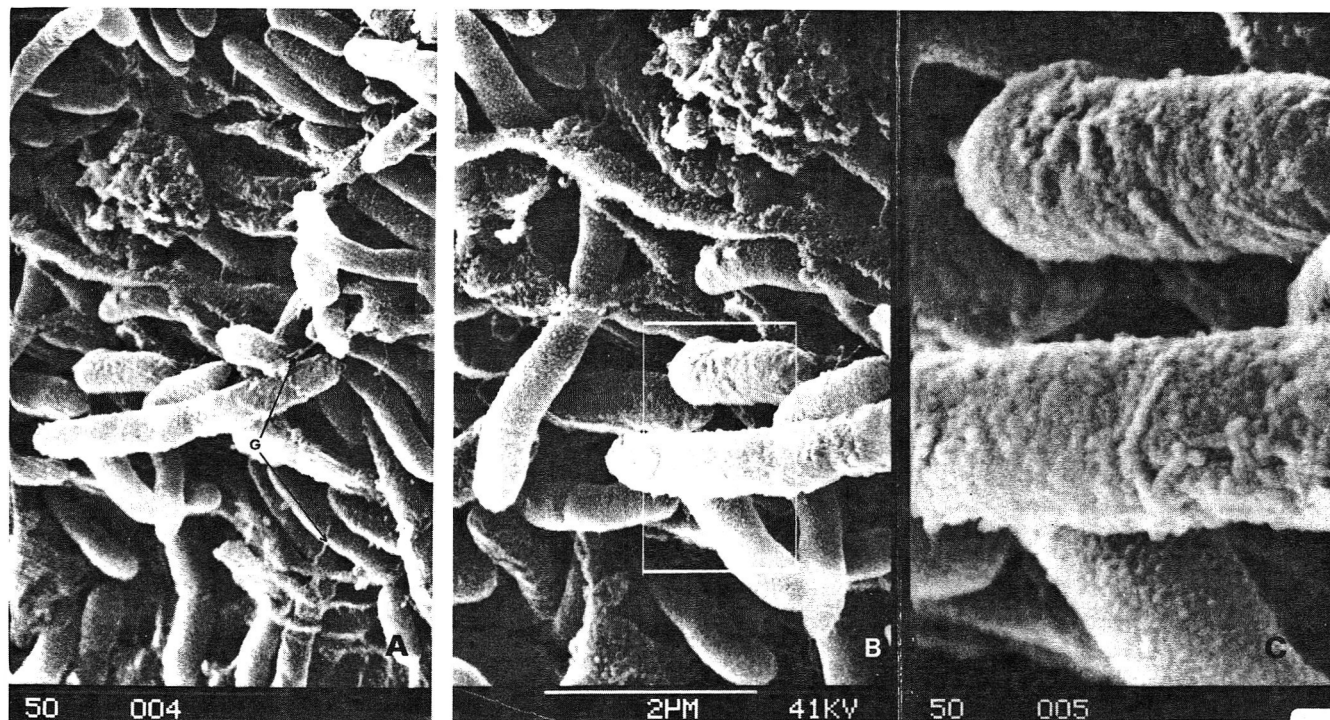
Fig. 3—(a) Scanning micrograph of day 1 intact muscle, inoculated with P. fragi, showing the initial formation of fiber-like glycocalyx material (G). (b) The exterior surface of the bacterium is encased in a mass of pebble-like extracellular polymeric material. (c) This represents a magnification of the area enclosed in the box in micrograph (B).
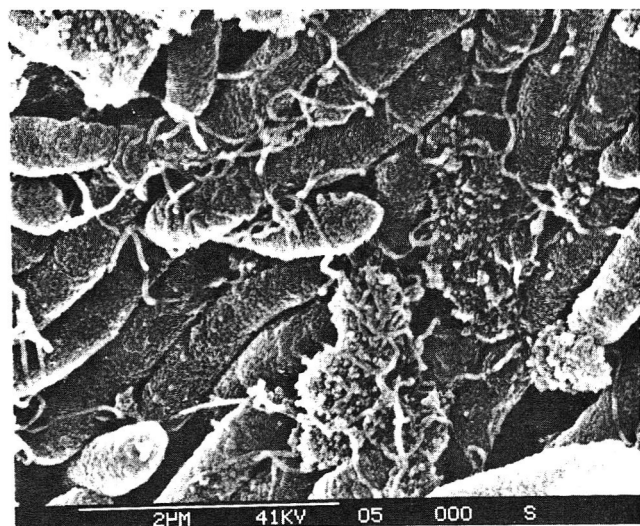
Fig. 4—Scanning micrograph of intact muscle (day 5), inoculated with P. fragi. There is an intensification of the pebbling effect on the bacterium surface, as well as a coiling of glycocalyx fibers.
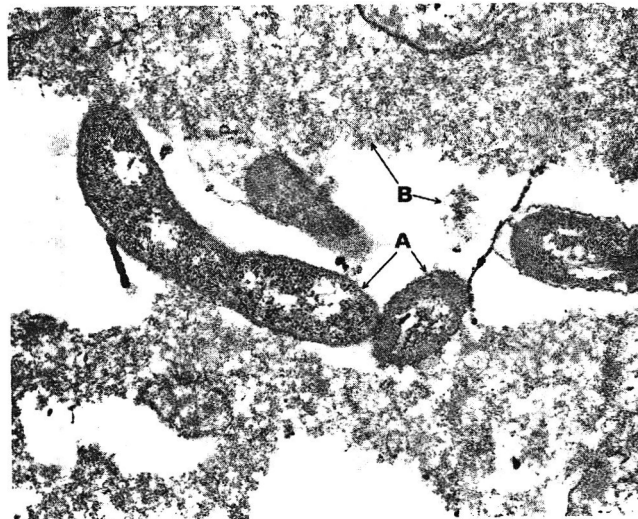
Fig. 5—Electron micrograph of two types of extracellular material associated with attachment. Type one, adherent to the bacterium surface (A) and type two, being amorphous in nature (B).

lated muscle may be due to the production of amines and ammonia by *P. fragi.*

In considering the morphology of the extracellular material as seen under the SEM, it is recognized that all commonly used dehydration and drying methods have their drawbacks and result in shrinkage (Brunk et al., 1981). The structured organization of the fibrous polymer appears to indicate that this material may not be the result of stretching of extracellular polymers when microorganisms are adjacent to each other, as reported by Fletcher and Floodgate (1973). Rather, it suggests an actual outgrowth of long fibrous material which participates in bridging microorganisms to each other or to the substrate. Examination of the bacterium surface for the determination of specific sites for outgrowth of extracellular polymeric material revealed a random pattern of interconnecting glycocalyx material. A close study of various micrographs, however, tend to favor polar sites for outgrowth of the extracellular fibers.

Since polysaccharide material may be involved in adhesion, ruthenium red or alcian blue were used for TEM studies. Although various researchers (Behnke, 1968; Behnke and Zelander, 1970) have used alcian blue as a
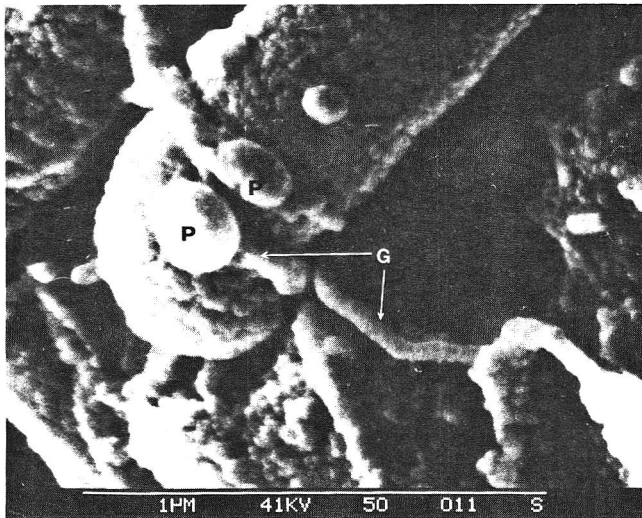
Fig. 6—Scanning micrograph of bleb-like protrusions (P) in association with the glycocalyx fibers (G).
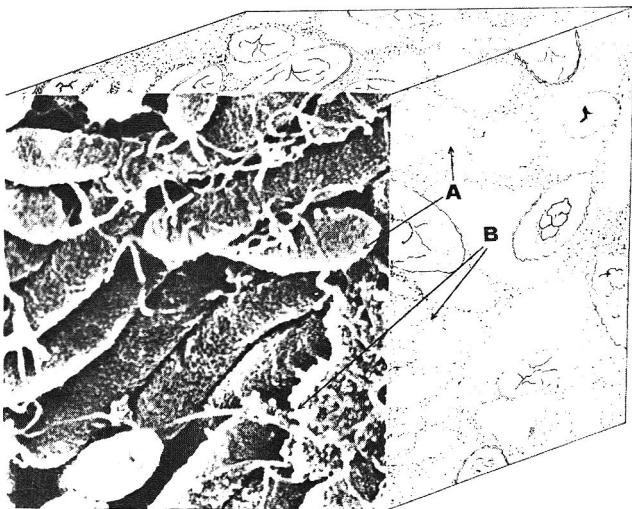
Fig. 7—Schematic illustration of the relationship between the types of extracellular material as seen by means of SEM and TEM. A and B corresponds to the extracellular material types described in Fig. 5.

means of improving fixation or producing electron density in acidic polysaccharides, ruthenium red fixation, in this study, gave better preservation of morphology and increased contrast of structures. This may be due to the homogeneous staining of the acidic polysaccharide by the ruthenium red-osmium tetroxide combination (Dierich, 1979).

TEM verified observations made from scanning electron micrographs, that thin polymeric material was adherent to the bacterial cell. By means of both SEM and TEM, the pebbling effect on the surface of the microorganism may partly be due to the corrugated morphology of the bacterial cell wall as well as dehydration effects during sample preparation. The amorphous substance as seen in transmission electron micrographs, probably corresponds to sections taken through the coiled, matted mass of extracellular material as evident in scanning electron micrographs (Fig. 7). Fig. 7 is a schematic representation of what one would expect to see if the sample, as revealed by SEM, was sectioned and examined by TEM.

Wiebe and Chapman (1968) found that certain nutritional and physiological conditions have been shown to induce the formation of blebs on the cell wall of some pseudomonads. Dutson et al. (1971) stated that since it was possible that bacterial proteolytic activity was responsible for myofibrillar disruption, the enzymes may be secreted into blebs on the bacterial surface which later form globules. The globules then probably release their contents into the muscle tissue surrounding the bacteria. Boethling (1975), reported that proteases and other exoenzymes are usually repressed until the late exponential phase of growth. Other workers (Tarrant et al., 1971; Dainty et al., 1975) have been unable to demonstrate proteolysis until spoilage is well advanced. These findings explain the lack of bleb-like evaginations on *P. fragi* on samples other than those incubated for 5 days.

The close association between the polymeric fibers and blebs reinforces previous studies (Costerton et al., 1978) which suggested that the extracellular polymeric material aids in: (a) positioning the bacteria to the substrate surface; (b) channelling various nutrients towards the bacteria; (c) concentrating and conserving digestive enzymes released by the bacteria.

CONCLUSION

GROWTH OF *P. fragi* on meat surfaces was not suppressed by decreased sarcoplasm concentrations at intermediate temperatures (ca 21°C); however, only intact inoculated muscles showed an increase in pH. Although bacterial numbers were lower in sarcoplasm-depleted muscles, the method of microbial attachment to meat surfaces (both washed and intact) appeared to be similar, since the number of *P. fragi* on freshly inoculated intact and washed muscle were similar. The initial formation of a glycocalyx as well as extracellular fibers in the form of a coiled matted mass of polymeric material was demonstrated by both SEM and TEM.

The formation and localization of blebs in association with the extracellular polymeric fibers, may represent the stages prior to enzymatic meat spoilage. The glycocalyx material appeared to mediate cell-to-cell as well as cell-to-muscle attachment in both washed and intact muscle.

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Electrical Stimulation Effects on Myoglobin Properties of Bovine Longissimus Muscle

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ABSTRACT

Beef longissimus steaks from 40 bull carcasses were used to study the effects of electrical stimulation (ES) on meat color mechanisms and myoglobin properties. Total pigment concentration and ratios of myoglobin forms for ES and control (C) samples were not different. Color panel and several spectrophotometric measurements indicated ES produced a lighter, brighter-red color in displayed steaks. Disappearance of reduced myoglobin during blooming was similar in unaged C and ES steaks, but reduced myoglobin decreased faster during blooming of ES steaks aged 6 days. ES decreased anaerobic metmyoglobin reducing activity and induced more metmyoglobin formation when steaks were in a 1% oxygen atmosphere.

INTRODUCTION

ELECTRICAL STIMULATION improved longissimus (LD) muscle color of beef carcasses at the time of ribbing (Eikelenboom et al., 1981; Davis et al., 1981; Salm et al., 1981) and under retail display conditions (Hall et al., 1980). Others have found, however, no LD muscle color improvement at ribbing (Savell et al., 1979; Grusby et al., 1976) or during display (Claus, 1982). Orcutt et al. (1981) reported that LD muscle from the electrically stimulated (ES) carcasses was lighter and redder than control (C) muscle, but there was no difference in the proportion of oxymyoglobin on steak surfaces. They suggested that color differences may be due to deeper oxygen penetration and/or greater surface light reflection. However, Tang and Henrickson (1980) reported a greater proportion of oxymyoglobin in ES muscle than in C muscle. The objective of this research was to study the effects of carcass ES on color mechanisms and myoglobin properties of beef LD msucle.

MATERIALS & METHODS

Source of materials

Forty Angus bulls, half of which were implanted near birth with 36 mg of Ralgro (a protein anabolic metabolite from *Gibberella zeae*) and re-implanted about every 106 days, were fed a typical feedlot diet for a minimum of 196 days and slaughtered at either 454 or 499 kg. The average USDA carcass quality grade was Good-75. Carcasses were split and alternating left and right sides were ES at 45 min postmortem for 2 min using 420V, 60Hz, AC, 0.68 sec on and 0.32 sec off, with approximately 1 amp delivered through the side. The other side served as a control. A series of 2.5 cm thick LD steaks were removed from the short loin (one at 24 hr and the others at 48 hr postmortem) for use in the color studies. One steak removed at 48 hr postmortem was divided transversely into central, medial, and lateral portions.

Display color stability study

A steak removed at 48 hr postmortem was wrapped in polyvinylchloride (PVC) film and displayed for 7 days at 3°C under 1076 lux of continuous Natural fluorescent lighting. Samples were bloomed in the dark for 1 hr before measuring percentage reflectance

at selected wavelengths and Hunter L*, a*, and b* values (Commission Internationale de l'Eclairage 1976 version and Illuminant C) with a D54 Hunterlab spectrophotometer.

A five member color panel evaluated LD steaks during display for overall muscle color using the scale of 1=very bright red, 2= bright red, 3=slightly dark red or brown, 4=dark red or brown, and 5=extremely dark red or brown (Kropf et al., 1971). Steaks were evaluated visually and spectrophotometrically at 0, 1, 3, 5, and 7 days of display.

Oxygenation studies

One steak per side was removed immediately after ribbing (24 hr postmortem) and transported to the laboratory in an oxygen impermeable bag. The steak was re-faced to expose a new surface and wrapped immediately in PVC film. Percentage reflectance at selected wavelengths and Hunter L*, a* and b* values were measured spectrophotometrically within 15 sec after facing (time 0) and at 1, 2, 3, 4, 5, 8, 15, and 30 min post-facing.

The lateral one-third portion of a steak removed at 48 hr postmortem was vacuum packaged immediately after fabrication in 3 mil Saran-coated surlyn barrier film (<1cc of oxygen/645 cm²/24 hr at 23°C and 0%RH), and stored in the dark at 5°C. After 4 days of storage, the samples were faced, bloomed, and evaluated spectrophotometrically and visually as described for the steaks removed and oxygenated at 24 hr postmortem.

Metmyoglobin reducing activity

The central and medial one-third portions of a steak removed at 48 hr postmortem were vacuum packaged immediately after removal, stored in the dark at 5°C and were used to study anaerobic and aerobic metmyoglobin reducing activity (MRA).

At 10 days postmortem, the central one-third portion was removed from the package, submerged in 1% potassium ferricyanide for 30 sec to induce metmyoglobin (MMb) formation, then the excess solution was blotted. Samples were left unpackaged at 27°C for 30 min and repackaged in oxygen impermeable film. Anaerobic reduction of MMb at 27°C was followed spectrophotometrically at 30, 60, 90, 120, 150, 180, 210, 240, and 300 min after chemical oxidation. Percentage reflectance of samples was read before oxidation to establish the initial percentage of surface MMb.

The medial one-third portion of the steak was stored until 15 days postmortem, removed from the packaging film, and covered with PVC film. Samples were placed in an anaerobic incubator; the incubator was evacuated and flushed with a mixture of 1% oxygen and 99% nitrogen gas (repeated twice) to induce MMb formation (Ledward, 1972). The incubator was stored in the dark at 5°C for 26 hr. Samples were removed (0 time) and immediately scanned spectrophotometrically. During sample removal, the chamber was continuously flushed with the 1% oxygen gas mixture. After initial scanning, samples were stored in the dark at 5°C and aerobic reduction of MMb was measured at 2, 4, 6, 8, 10, 12, and 24 hr after removal from the 1% oxygen atmosphere.

Spectrophotometric and pigment calculations

K/S value ratios of 474/525 nm and 572/525 nm were calculated to determine the percentages of reduced myoglobin (Mb) and MMb, respectively. Oxymyoglobin (MbO) was calculated by difference. Constants used in pigment calculations determined for the spectrophotometer and the two packaging films used in this study were: 0.89 and 0.94 for 100% Mb, 0.40 and 0.51 for 0% Mb, 1.82 and 1.45 for 100% MMb, and 0.62 and 0.51 for 0% MMb for the oxygen permeable (PVC) and impermeable (Saran-coated surlyn) films, respectively. Percentage reflectance differences at 630 nm minus 580 nm (%R630–%R580) were used to indicate differences in redness; the larger the difference, the brighter red the sample.

Total pigment analysis

Total pigment concentration was determined using Hornsey's (1956) acidified acetone procedure and ppm haematin was converted to mg/g wet wt using the 0.026 conversion factor of Franke and Solberg (1971). Pigments were extracted in the dark for 30 min. Preliminary work indicated that the addition of 0.5% DL cysteine hydrochloride for color stability (DeVore and Solberg, 1974) was not necessary.

Statistical analysis

Data were analyzed by analysis of variance procedures and means were separated using Duncan's Multiple Range test (SAS Institute, Inc., 1979). No interactions between cattle management system and electrical stimulation were found; therefore, the data were pooled and analyzed for electrical stimulation effects.

RESULTS

Total pigment concentration

No differences ($P > 0.05$) were found for total pigment concentration of C (5.3 mg/g) and ES (5.6 mg/g) muscle.

Display color stability

Steaks from ES carcasses had brighter red ($P < 0.05$) visual color scores than C steaks initially and at 1, 3, and 5 days of display (Table 1), but color differences were not apparent by day 7. ES samples were ($P < 0.05$) both brighter red (larger %R630–%R580 nm) and lighter in color (larger Hunter L* values) than C samples at most evaluation times. No consistent differences ($P > 0.05$) were found between ES and C samples for Hunter a* and b* values and for percentages of surface Mb, MbO, or MMb.

Oxygenation studies

ES samples bloomed at either 24 hr (Table 2) or 6 days (Table 3) postmortem had brighter red ($P < 0.05$) visual color scores, greater ($P < 0.05$) differences at %R630–%R580 nm, and larger ($P < 0.05$) Hunter L* values than C samples at all evaluation times during blooming. No differences ($P > 0.05$) between ES and C samples bloomed at 24 hr postmortem were observed for Hunter a* and b* values, or for percentages of surface Mb (except at 5 and

Table 1—Means for visual color, spectrophotometric measurements, and myoglobin forms for displayed control (C) and electrically stimulated (ES) bovine longissimus steaks

Display time (days)	Treat-ment	Visual panel score[a]	R630-R580	Hunter (CIE) values			Myoglobin forms[b]		
				L*	a*	b*	%Mb	%MbO	%MMb
0	C	2.5[c]	21.1[d]	38.6[d]	34.3[c]	20.6[c]	8.9[c]	83.3[c]	7.8[c]
	ES	2.0[d]	24.1[c]	41.4[c]	32.8[d]	21.6[c]	5.3[d]	85.0[c]	9.7[c]
1	C	2.7[c]	21.1[d]	40.5[d]	32.6[d]	21.6[c]	1.4[c]	85.5[c]	13.1[c]
	ES	2.2[d]	23.8[c]	43.2[c]	33.8[c]	22.5[c]	0.7[c]	86.6[c]	12.7[c]
3	C	2.8[c]	20.9[d]	40.4[d]	33.4[c]	22.0[c]	1.1[c]	76.8[c]	22.1[c]
	ES	2.5[d]	22.7[c]	42.4[c]	34.0[c]	22.4[c]	2.3[c]	77.4[c]	20.3[c]
5	C	3.1[c]	20.2[d]	39.6[c]	34.2[c]	23.5[c]	6.8[c]	74.1[c]	19.1[c]
	ES	2.8[d]	21.6[c]	41.0[c]	34.2[c]	23.7[c]	6.1[c]	78.4[c]	15.5[c]
7	C	3.2[c]	19.3[c]	40.4[d]	31.5[c]	21.2[c]	4.6[c]	67.6[c]	27.8[c]
	ES	3.0[c]	20.1[c]	42.6[c]	31.2[c]	20.8[c]	3.1[d]	69.8[c]	27.1[c]

[a] 1 = very bright red, 2 = bright red, 3 = slightly dark red or brown, 4 = dark red or brown, 5 = extremely dark red or brown.
[b] Mb = reduced myoglobin, MbO = oxymyoglobin, MMb = metmyoglobin.
[c,d] Means for each time and within each column having different superscripts are different ($P < 0.05$).

Table 2—Means for visual color, spectrophotometric measurements, and myoglobin forms for control (C) and electrically stimulated (ES) bovine longissimus steaks bloomed at 24 hr postmortem

Min in air	Treat-ment	Visual panel score[a]	R630-R580	Hunter (CIE) values			Myoglobin forms[b]		
				L*	a*	b*	%Mb	%MbO	%MMb
0	C	—	15.8[d]	33.8[d]	28.6[c]	10.6[c]	76.2[c]	20.1[c]	3.7[c]
	ES	—	17.1[c]	35.9[c]	25.6[c]	10.6[c]	73.8[c]	17.5[c]	8.7[c]
1	C	—	16.4[d]	34.6[d]	28.7[c]	11.3[c]	68.3[c]	25.7[c]	6.0[c]
	ES	—	17.5[c]	35.9[c]	29.2[c]	11.6[c]	68.1[c]	25.4[c]	6.4[c]
2	C	—	16.5[d]	34.8[d]	28.9[c]	12.0[c]	63.7[c]	31.1[c]	5.2[c]
	ES	—	17.8[c]	36.2[c]	28.6[c]	12.1[c]	62.7[c]	31.1[c]	6.1[c]
3	C	—	16.8[d]	34.7[d]	29.2[c]	12.5[c]	60.8[c]	36.7[c]	2.6[c]
	ES	—	18.0[c]	36.3[c]	29.6[c]	12.7[c]	59.1[c]	36.0[c]	4.9[c]
4	C	—	16.7[d]	34.6[d]	29.2[c]	12.7[c]	58.1[c]	37.7[c]	4.2[c]
	ES	—	18.0[c]	36.5[c]	29.4[c]	12.7[c]	56.5[c]	38.0[c]	5.5[c]
5	C	—	16.9[d]	34.2[d]	29.9[c]	13.2[c]	58.1[c]	37.4[c]	4.5[c]
	ES	—	18.3[c]	36.6[c]	29.5[c]	13.0[c]	54.1[d]	39.3[c]	6.6[c]
8	C	—	17.1[d]	34.9[d]	29.6[c]	13.4[c]	52.8[c]	44.1[c]	3.1[c]
	ES	—	18.4[c]	36.7[c]	29.8[c]	13.6[c]	50.9[c]	44.3[c]	4.8[c]
15	C	—	17.0[d]	35.6[d]	28.8[c]	13.1[c]	47.5[c]	42.6[c]	9.9[c]
	ES	—	18.4[c]	37.4[c]	29.2[c]	13.3[c]	44.5[c]	43.2[c]	12.3[c]
30	C	2.5[c]	17.3[d]	36.5[d]	28.5[c]	13.4[c]	39.4[c]	46.6[c]	14.1[c]
	ES	2.0[d]	19.3[c]	38.5[c]	28.4[c]	13.5[c]	34.9[d]	47.4[c]	17.7[c]

[a] 1 = very bright red, 2 = bright red, 3 = slightly dark red or brown, 4 = dark red or brown, 5 = extremely dark red or brown.
[b] Mb = reduced myoglobin, MbO = oxymyoglobin, MMb = metmyoglobin.
[c,d] Means for each time and within each column having different superscripts are different ($P < 0.05$).

Table 3—Means for visual color, spectrophotometric measurements, and myoglobin forms for control (C) and electrically stimulated (ES) bovine longissimus steaks bloomed at 6 days postmortem

Min in air	Treatment	Visual panel score[a]	R630-R580	Hunter (CIE) values			Myoglobin forms[b]		
				L*	a*	b*	%Mb	%MbO	%MMb
0	C	—	18.1^d	37.7^d	28.4^c	10.8^d	74.0^c	15.7^c	10.3^c
	ES	—	19.8^c	40.0^c	28.3^c	11.3^c	70.2^d	17.7^c	12.1^c
1	C	—	18.5^d	37.4^d	29.1^c	11.8^c	66.7^c	24.3^c	9.0^c
	ES	—	20.0^c	40.7^c	28.3^d	12.1^c	61.2^c	26.9^c	11.9^c
2	C	—	18.6^d	38.1^d	28.8^c	12.3^d	58.5^c	29.3^d	12.1^d
	ES	—	20.4^c	41.1^c	29.3^c	12.8^c	51.9^d	31.9^c	16.2^c
3	C	—	18.9^d	38.3^d	28.9^c	12.8^d	53.1^c	34.0^d	12.9^c
	ES	—	20.6^c	41.0^c	28.8^c	13.3^c	47.5^d	36.5^c	16.0^c
4	C	—	19.1^d	38.3^d	29.2^c	13.3^d	49.3^c	37.9^d	12.8^d
	ES	—	20.8^c	40.8^c	29.0^c	13.8^c	43.4^d	40.8^c	15.8^c
5	C	—	19.2^d	38.4^d	29.2^c	13.4^d	45.7^c	40.5^d	13.8^d
	ES	—	21.0^c	41.5^c	28.9^c	13.9^c	38.5^d	43.5^c	18.0^c
8	C	—	19.2^d	38.3^d	30.0^d	14.7^c	41.6^c	46.8^d	11.3^d
	ES	—	21.5^c	41.4^c	29.7^c	15.2^c	31.8^d	51.7^c	16.5^c
15	C	—	20.4^d	39.0^d	30.5^c	16.0^c	29.1^c	55.9^c	14.9^c
	ES	—	22.4^c	42.4^c	30.0^c	16.2^c	20.0^d	60.8^c	19.2^c
30	C	2.4^c	21.6^d	39.4^d	31.8^c	18.0^c	18.1^c	68.2^c	13.7^c
	ES	2.0^d	23.7^c	42.8^c	31.6^c	18.2^c	10.7^d	72.1^c	17.2^c

[a] 1 = very bright red, 2 = bright red, 3 = slightly dark red or brown, 4 = dark red or brown, 5 = extremely dark red or brown.
[b] Mb = reduced myoglobin, MbO = oxymyoglobin, MMb = metmyoglobin.
[c,d] Means for each time and within each column having different superscripts are different (P < 0.05).

30 min), MbO, and MMb. But, ES samples bloomed at 6 days postmortem had smaller (P < 0.05) Hunter a* values at 1 and 8 min, larger (P < 0.05) Hunter b* values at 0, 2, 3, 4, and 5 min, less (P < 0.05) Mb at 0, 2, 3, 4, 5, 8, 15, and 30 min, more (P < 0.05) MbO at 2, 3, 4, 5, and 8 min, and more (P < 0.05) MMb at 2, 4, 5, and 8 min than C samples.

Table 4 presents a comparison of means from samples bloomed at 1 or 6 days postmortem. Visual color scores were similar for samples bloomed at 1 and 6 days postmortem, but samples bloomed at 6 days postmortem had greater (P < 0.05) differences at %R630–%R580 nm, and had larger (P < 0.05) Hunter L*, a*, and b* values than samples bloomed at 1 day postmortem. Six-day old samples had considerably less (P < 0.05) surface Mb and more (P < 0.05) surface MbO than samples bloomed at 1 day postmortem, but percentages of surface MMb were similar.

Metmyoglobin reducing activity

Percentage surface MMb of ES and C samples was not different (P > 0.05) initially or 30 min after oxidation with potassium ferricyanide (Fig. 1). But, C muscle had less (P < 0.05) surface MMb at 1, 1.5, 2, 2.5, 3, 3.5, and 4 hr after oxidation during anaerobic reduction.

ES samples formed more (P < 0.05) surface MMb (0 time) in 1% oxygen than C samples (Fig. 2) and ES samples had more (P < 0.05) surface MMb at 2, 4, 10, and 12 hr of aerobic reduction.

Fig. 3 presents the percentage of MMb reduced over time under anaerobic and aerobic conditions. C samples in an anaerobic environment reduced a greater (P < 0.05) percentage of MMb at 1, 1.5, 2, 2.5, 3, and 3.5 hr than ES samples. But, the percentage of MMb reduced aerobically was not different (P > 0.05) for ES and C samples at any time.

DISCUSSION

Total pigment concentration

These data, like those of Tang and Henrickson (1980) indicate no differences in total pigment concentration of

Table 4—Means comparing control (C) and electrically stimulated (ES) bovine longissimus samples bloomed at either 1 or 6 days postmortem

Measurement[a]	Treatment	Evaluation time	
		1 day	6 days
Visual color score	C	2.5^b	2.4^b
	ES	2.0^c	2.0^c
R630-R580	C	17.3^e	21.6^c
	ES	19.3^d	23.7^b
Hunter L*	C	36.5^d	39.4^c
	ES	38.5^c	42.8^b
Hunter a*	C	28.5^c	31.8^b
	ES	28.4^c	31.6^b
Hunter b*	C	13.4^c	18.0^b
	ES	13.5^c	18.2^b
Reduced myoglobin, %	C	39.4^b	18.1^d
	ES	34.9^c	10.7^e
Oxymyoglobin, %	C	46.6^d	68.2^c
	ES	47.4^d	72.1^b
Metmyoglobin, %	C	14.1^b	13.7^b
	ES	17.7^b	17.2^b

[a] Samples evaluated after blooming for 30 min.
[b,c,d,e] Means for the same measurement with different superscripts are different (P < 0.05).

ES and C muscle; therefore, ES color differences must be attributed to other factors.

Color display stability

The color display stability results are similar to those reported by Orcutt et al. (1981); steaks from ES carcasses were brighter red and lighter colored than steaks from C carcasses and there were no differences in percentage surface MbO. Hall et al. (1980) reported that adductor steaks from ES carcasses were visually brighter than steaks from C carcasses during display. However, no muscle color differences between ES and C samples from bull, heifer,

and steer carcasses were observed during 4 days of display by Jeremiah and Martin (1980). Claus (1982) reported that no visual color differences were observed in C and ES samples during 5 days of display. Brighter LD muscle color in ES carcasses at a 24 or 48 hr postmortem ribbing time has been reported (Eikelenboom et al., 1981; Davis et al., 1981; Salm et al., 1981).

Oxygenation studies

Although ES muscle was brighter red and lighter than C muscle during blooming at both 24 hr and 6 days postmortem, color differences cannot be attributed solely to different proportions of myoglobin pigments on steak surfaces. More significant differences were observed for percentages of Mb, MbO, and MMb on both C and ES samples bloomed at 6 days postmortem than at 24 hr postmortem, an effect attributed to aging. The extent of change from 0 to 30 min of bloom time in %R630–%R580 and Hunter L* values at both 24 hr and 6 days postmortem was essentially the same for C and ES samples. Apparently, the effects of electrical stimulation on muscle color involves other factors besides pigment concentration and changes in myoglobin forms.

Cross et al. (1979) studied combinations of cloth shrouding and PVC film wrapping of C and ES carcasses. Shrouded ES carcasses had better muscle color than shrouded C carcasses, but no color differences were observed between shrouded ES carcasses, PVC wrapped ES carcasses, and C carcasses that were both shrouded and PVC wrapped. However, C shrouded and PVC wrapped carcasses had better muscle color scores than C shrouded sides. Their results suggest that a possible slower chilling rate of carcasses that were both shrouded and PVC film wrapped resulted in muscle color similar to that of ES carcasses. Conversely, more rapid chilling of muscle than normal can also affect muscle color. Kastner et al. (1980) displayed hot-boned electrically stimulated samples and found no color advantage over displayed C samples, but hot-boned cuts chilled faster than cuts chilled in an intact carcass. Faster chilling of the hot-boned cuts may negate the color lightening effects of ES on muscle color, but the combination of ES and hot-boning does prevent the darkening of muscle color observed in hot-boning alone (Taylor et al., 1981; Claus, 1982).

Savell et al. (1978) observed more structural damage in ES than in nonstimulated beef muscle. This tissue disruption may result in a looser muscle structure that permits deeper oxygen penetration, thus resulting in a thicker MbO

Fig. 1—Mean percentages (±SE) of surface metmyoglobin on control and electrically stimulated bovine longissimus samples initially (I) and during anaerobic reduction. Metmyoglobin was formed by oxidation with potassium ferricyanide.
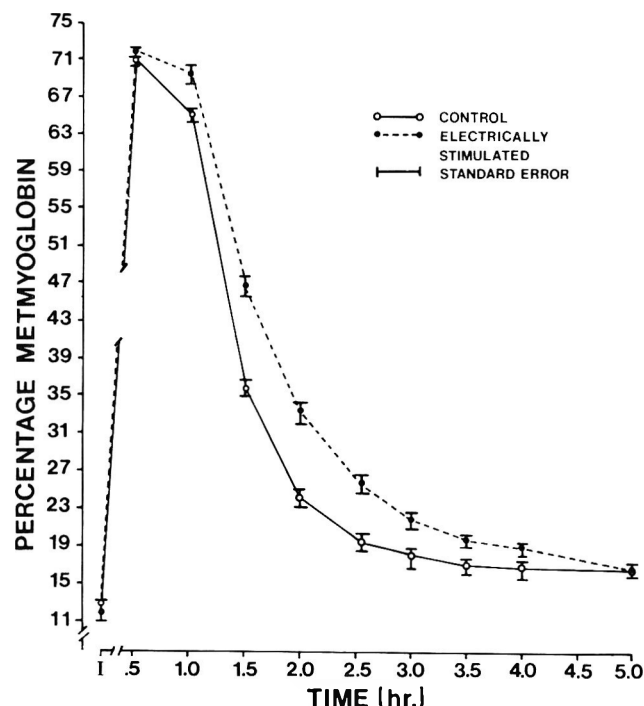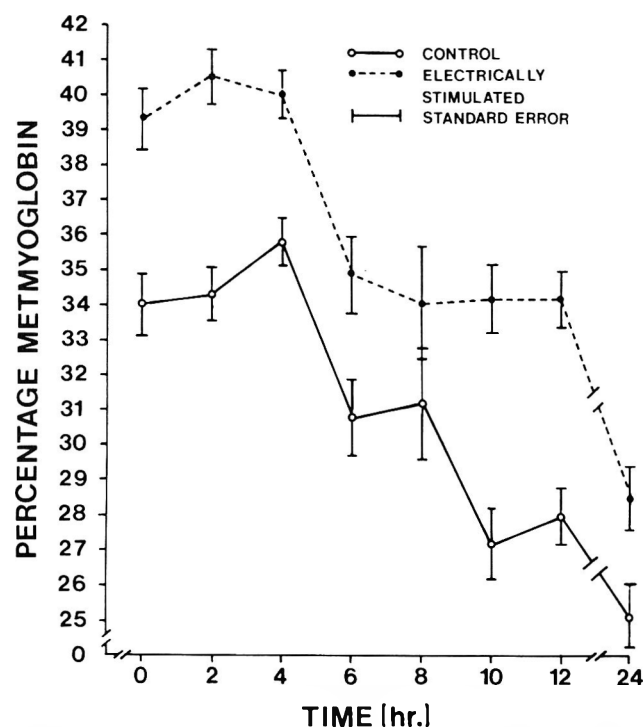

Fig. 2—Mean percentages (±SE) of surface metmyoglobin on control and electrically stimulated bovine longissimus samples during aerobic reduction. Metmyoglobin was formed by oxidation in a 1% oxygen atmosphere.

Fig. 3—Percentage of metmyoglobin reduced by control and electrically stimulated bovine longissimus samples during anaerobic and aerobic reduction.

layer and a deeper MMb layer. The looser structure may also cause more light scatter and consequently a lighter muscle appearance without changing the percentage of surface MbO. George et al. (1980) considered the structural damage in ES bovine muscle similar to that seen in pale, soft and exudative porcine muscle. Dutson et al. (1980) reported that ES ovine muscle had more released lysosomal enzymes; however, a greater percentage of these enzymes were degraded due to a lower pH of ES muscle at a higher muscle temperature. Ashmore et al. (1972) reported that enzymes in dark cutting beef which has a high pH and a tight muscle structure were very competitive for available oxygen. Thus, less oxygen would be available for Mb oxygenation. Although enzyme activity or structural damage were not measured in this study, the spectrophotometric measurements indicate ES muscle was lighter in color and refelcted more light from its surface. If enzymes important to muscle color are also degraded due to temperature-pH conditions in ES carcasses, enzyme activity may be lower in ES muscle which would allow deeper oxygen penetration.

Metmyoglobin reducing activity

The two metmyoglobin reducing activity methods present slightly different results. During anaerobic reduction, C samples reduced MMb faster than ES samples. However, under aerobic conditions, ES samples formed more MMb, but the percentage of MMb reduced over time was similar to C samples. These data supported the suggestion by Giddings (1974) that there are separate enzymatic mechanisms for aerobic and anaerobic MMb reduction. The reduction of MRA by ES probably will not decrease appreciably the shelflife of LD muscle under practical conditions, but, the lower MRA may explain our observations (unpublished data) of decreased myoglobin stability during display of inside round steaks from ES carcasses.

If more lysosomal membranes are ruptured and more enzymes are denatured (Dutson et al., 1980) after ES of carcasses, then possibly other subcellular organelle membranes such as mitochondrial membranes may also be affected. Recent reviews (Giddings, 1974; Livingston and Brown, 1981) discuss the importance of mitochondrial activity to meat color, especially MRA. Watts et al. (1966) reported that the addition of NAD to muscle samples could restore MRA and that oxygen had to be limited for MRA to proceed. They noted that electrons from NADH react preferentially with oxygen rather than with the reducing mechanism. Ledward (1971) reported rapid MRA depletion in the presence of oxygen and Stewart et al. (1965) reported that little MRA occurred below 15°C. Therefore, the temperature conditions (5°C) of the aerobic study may have been rate limiting for both C and ES samples.

Based on this research, it was concluded that: (1) meat color differences between C and ES muscle were not due to total heme pigment concentration; (2) color differences between C and ES muscle were not due to different proportions of surface Mb, MbO, or MMb; (3) ES muscle was lighter and reflected more light from its surface than C muscle, perhaps due to structural changes in ES muscle; and (4) under certain conditions ES muscle was more susceptible to MMb formation and had less MRA.

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Effects of Carcass Maturity on Collagen Solubility and Palatability of Beef from Grain-Finished Steers

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―――――――― ABSTRACT ――――――――

Samples from steers fed a high-energy diet for 185 days prior to slaughter were used to compare collagen solubility and palatability of beef from youthful and mature carcasses. Carcasses representing the two maturity classes were selected specifically to have similar levels of intramuscular and subcutaneous fat, but distinctly different maturity indicators. Under the conditions of this study, maturity class had no effect on sensory properties or shear force values of cooked rib steaks. Total collagen content of samples from the longissimus muscle was higher for mature carcasses, but maturity class means for percentage of soluble collagen were not different. The relationships of these findings to preslaughter feeding regimen and collagen metabolism are discussed.

INTRODUCTION

THE RELATIONSHIP of beef carcass maturity to the palatability traits of beef has been investigated and documented by numerous researchers (Romans et al., 1965; Walter et al., 1965; Breidenstein et al., 1968; Berry et al., 1974). Results of these studies consistently have shown that advancements in physiological maturity of the carcass are detrimental to beef palatability particularly from the standpoint of tenderness. Furthermore, it has been established that the amount of heat-labile collagen in bovine skeletal muscle decreases during maturation and that this decrease is responsible for age-associated toughening of beef (Goll et al., 1964; Hill, 1966; Herring et al., 1967; Bailey, 1972; Shimokomaki et al., 1972; Dutson, 1974).

It is important to recognize, however, that much of the existing information concerning the interrelationships among maturity, collagen properties and beef tenderness has been compiled using samples from animals for which preslaughter nutritional regimen is either unknown or unspecified.

During the past several decades, a wealth of information has accumulated which suggests that intensive preslaughter feeding has a beneficial effect on sensory properties of beef (Black et al., 1931; Wanderstock and Miller, 1948; Meyer et al., 1960; Hawrysh et al., 1975; Kropf et al., 1975; Bowling et al., 1977; Schroeder et al., 1980; Tatum, 1981; Dolezal et al., 1982). Moreover, recent studies have associated increases in collagen solubility with the feeding of high-energy diets to youthful cattle (Aberle et al., 1981; Wu et al., 1981).

In light of these findings, it seems reasonable to speculate that intensive preslaughter feeding may impede age-associated toughening of beef. If that is the case, then beef from youthful and mature animals fed high-energy diets should have similar proportions of soluble collagen and be comparable in tenderness.

This study was conducted to compare collagen solubility and palatability of beef produced by youthful and mature, grain-finished steers.

MATERIALS & METHODS

Source of materials

Approximately 200 crossbred steers exhibiting visual evidence of variation in maturity were identified at the termination of a 185-day finishing period. The ages of the cattle were estimated to range from 2–5 yr. The cattle had been maintained on very low energy pasture prior to finishing. Because of variation in age, length of the backgrounding period also was variable. The steers were finished as a group on a high-energy diet (approximately 2.2 Mcal/kg NEm, 1.4 Mcal/kg NEg expressed on a 100% dry matter basis).

On the day following slaughter, 50 carcasses were chosen for the experiment using USDA descriptions of beef carcass maturity (USDA, 1975). For the purposes of this study, carcasses were categorized into one of two maturity classes – "youthful"or "mature." Carcasses with maturity indicators corresponding to A or B maturity (n=25) were assigned to the youthful maturity class; carcasses representing USDA's C and D maturity groups (n=25) were classified as mature. The distinction between maturity classes was predicated upon carcass maturity constraints for particular USDA quality grades (USDA, 1975). In order to reduce confounding due to between-class variation in marbling and fatness, youthful and mature carcasses were selected in pairs chosen specifically to have similar levels of intramuscular and subcutaneous fat. All carcasses were subjected to identical postmortem chill temperatures ($1° \pm 1°C$).

Carcass data collection and sample preparation

Twenty-four hr postmortem, USDA yield and quality grades were determined (USDA, 1975) and scores were assigned to each carcass for texture and distribution of marbling and lean texture and firmness based on visible characteristics of the longissimus muscle at the 12th – 13th rib interface.

A 10th to 12th rib section of the wholesale rib was removed from the left side of each carcass and transported from the point of slaughter in Grand Island, NE, to the Colorado State Univ. meat laboratory where it was boned, trimmed of subcutaneous fat and epimysial connective tissue, vacuum packaged and stored at 2°C. Ninety-six hr postmortem, three steaks (2.5 cm thick) were removed from the center of each boneless rib section, wrapped and stored at −29°C to be used for palatability determinations. Samples for collagen analysis were obtained from the 12th rib end of each rib section.

Palatability determinations

Sensory evaluation was performed by a six-member, descriptive panel. The six panelists were selected and trained in accordance with the AMSA Guidelines for Cooking and Sensory Evaluation of Meat (AMSA, 1978).

Three steaks from each carcass were thawed (2°C) and broiled on Farberware Open-Hearth broilers to an internal temprature of 70°C (monitored using copper-constantan thermocouples and a recording potentiometer). The 12th rib steak from each 10th to 12th rib section was used for sensory evaluation; the 10th and 11th rib steaks were used for subsequent shear force measurements. The sequence in which samples from each carcass were chosen and prepared for palatability determinations was completely random.

Upon reaching the desired internal temperature, steaks for sensory evaluation were removed from the broiler and portioned

into sections of uniform dimensions (approximately 1.3 cm x 1.3 cm x 1.9 cm). The warm sections were randomly selected and served immediately to the panel. Panelists assigned scores to each sample for juiciness, tenderness, connective tissue amount, and flavor desirability using eight-point, structured rating scales. Samples were evaluated at the rate of six samples per session, one session per day and five sessions per week.

Steaks for shear force determinations were cooled to 20°C and six 2.5 cm cores were removed, parallel to the longitudinal orientation of the muscle fibers, for Warner-Bratzler shear force measurements.

Collagen analysis

Hydroxyproline content was determined for longissimus samples (n=15) chosen randomly from each maturity class. Freeze-powdered subsamples (4g) were heated for 70 min at 77°C in 0.25 strength Ringer's solution (Hill, 1966). Following centrifugation, supernatant and residue fractions were individually hydrolyzed in 6N HCl for 20 hr at 115°C. Following neutralization, hydroxyproline content of each hydrozylate was determined using methods described by Bergman and Loxley (1963). Collagen content was calculated by multiplying the hydroxyproline content of the residue by 7.25 and that of the supernatant by 7.52 (Cross et al., 1973). Percentage of soluble (heat labile) collagen was calculated by dividing the collagen content of the supernatant by the collagen content of the entire sample.

Statistical methods

Means and standard errors were computed for each trait. Effects of maturity class (youthful vs mature) were tested for significance using one-way analysis of variance (Snedecor and Cochran, 1967). Although carcasses originally were selected in pairs, subsampling for hydroxyproline determinations precluded the use of statistical analysis for paired data.

RESULTS

DATA PRESENTED IN TABLE 1 characterize the carcasses used in this study. By design, youthful and mature carcasses had similar degrees of marbling and fat thickness levels, but exhibited distinct differences in carcass maturity and USDA quality grade (P < 0.001). Carcasses received similar scores for lean firmness, marbling texture and marbling distribution regardless of maturity classification. Between class differences in carcass weight and lean texture were relatively small, but were consistent enough for statistical significance (P < 0.05).

Means and standard errors for palatability attributes and collagen characteristics are presented in Table 2. Maturity class had no effect (P > 0.05) on sensory properties or shear force values of cooked steaks. Total intramuscular collagen content was higher (P < 0.05) for mature carcasses; however, maturity class means for percentage of soluble collagen were not different (P > 0.05).

DISCUSSION

PREVIOUS RESEARCH has established a consistent relationship between cooked beef tenderness and age-associated changes in the molecular structure of bovine intramuscular collagen (Goll et al., 1964; Hill, 1966; Carmichael and Lawrie, 1967; Kruggel et al., 1970; Shimokomaki et al., 1972; Cross et al., 1973; Boccard, 1978). During collagen synthesis, aldimine-type bonds form between tropocollagen molecules providing reducible, heat-labile crosslinks which contribute to the organization and structural stability of collagen fibers (Light and Bailey, 1979). The proportion of these reducible intermolecular crosslinks (and heat-soluble collagen) in bovine muscle tissue has been shown to increase from the fetal stage to a maximum at about 12–18 months of age (Shimokomaki et al., 1972; Carmichael and Lawrie, 1967). During subsequent maturation, the crosslinks gradually stabilize to an insoluble, heat-resistant form, causing a reduction in the amount of intramuscular colla-

gen that may be solubilized during subsequent cooking (Hill, 1966; Bailey, 1972). This age-dependent strengthening of bonds and concurrent reduction in collagen solubility provide the basis for the widely accepted maturity-beef tenderness relationship.

In the present study, however, carcass maturity had virtually no effect on collagen solubility or beef tenderness. In attempting to explain these paradoxical findings, it is important to note that changes in collagen crosslink stability have been shown to correspond more closely to growth rate and the physiological maturation process than to the temporal age of the animal (Bailey and Shimokomaki, 1971; Shimokomaki et al., 1972). Thus, factors that influence rates of growth and physiological maturation may have a pronounced effect on collagen crosslink stability.

Aberle et al. (1981) demonstrated relationships among preslaughter feeding regimen, growth rate and collagen solubility and concluded that acceleration of growth via

Table 1—Means and standard errors for carcass traits

Trait	Carcass maturity class[a]		Level of significance
	Youthful	Mature	
Number of observations	25	25	
Carcass maturity[c]	77.5 ± 5.1	262.6 ± 7.3	P < 0.001
Degree of marbling[d]	470.4 ± 19.8	467.6 ± 19.3	NS[b]
USDA quality grade[e]	512.3 ± 10.2	201.6 ± 6.8	P < 0.001
Fat thickness, mm	11.4 ± 0.9	11.0 ± 0.7	NS
Longissimus muscle area, cm[2]	74.9 ± 1.9	76.5 ± 2.4	NS
Carcass weight, kg	316.6 ± 9.5	343.8 ± 8.3	P < 0.05
USDA yield grade[f]	2.7 ± 0.12	2.8 ± 0.12	NS
Lean texture[g]	5.3 ± 0.23	4.6 ± 0.21	P < 0.05
Lean firmness[h]	5.0 ± 0.27	4.7 ± 0.22	NS
Marbling texture[g]	5.7 ± 0.17	5.2 ± 0.27	NS
Marbling distribution[i]	4.6 ± 0.28	4.6 ± 0.20	NS

[a] Youthful = A and B maturity; Mature = C and D maturity (USDA, 1975).
[b] NS = not significant (P > 0.05).
[c] 0 to 99 = A; 100 to 199 = B; 200 to 299 = C; 300 to 399 = D.
[d] 400 to 499 = small.
[e] 200 to 299 = Commercial; 500 to 599 = Choice.
[f] Kidney fat was partially removed at slaughter; estimates of residual kidney, pelvic and heart fat were used to calculate yield grades.
[g] 1 = extremely coarse; 8 = extremely fine.
[h] 1 = extremely soft; 8 = extremely firm.
[i] 1 = extremely uneven; 8 = extremely uniform.

Table 2—Means and standard errors for palatability attributes and collagen characteristics

Trait	Carcass maturity classification[a]		Level of significance
	Youthful	Mature	
Palatability attributes[c]			
Juiciness[d]	4.7 ± 0.21	4.9 ± 0.22	NS[b]
Tenderness[e]	4.8 ± 0.21	5.0 ± 0.17	NS
Connective tissue amount[f]	5.0 ± 0.19	5.3 ± 0.13	NS
Flavor desirability[g]	5.4 ± 0.14	5.5 ± 0.10	NS
Shear force, kg	7.7 ± 0.28	7.6 ± 0.36	NS
Collagen characteristics[h]			
Total collagen, mg/g	11.0 ± 0.57	12.6 ± 0.51	P < 0.05
Soluble collagen, %	33.9 ± 1.32	33.4 ± 1.20	NS

[a] Youthful = A and B maturity; Mature = C and D maturity (USDA, 1975).
[b] NS = not significant (P > 0.05).
[c] Statistics for palatability attributes are based on twenty-five observations.
[d] 1 = extremely dry; 8 = extremely juicy.
[e] 1 = extremely tough; 8 = extremely tender.
[f] 1 = abundant; 8 = none.
[g] 1 = extremely undesirable; 8 = extremely desirable.
[h] 1 = Statistics for collagen characteristics are based on fifteen observations.

intensive preslaughter feeding may exert a direct effect on collagen stability and tenderness of beef. According to Aberle et al. (1981), cattle fed high energy diets experience rapid rates of protein synthesis and, therefore, would be expected to produce beef with a high proportion of newly synthesized, heat-labile collagen. Since collagen turnover normally is relatively slow (Dutson, 1976), any increase in synthesis rate should indeed increase the proportion of immature collagen.

Hall and Hunt (1982) presented an important addendum to the concept of nutritional effects on collagen solubility. The latter study involved steers (280–840 days old) fed varying levels of dietary energy. Among steers fed high-energy (grain) diets for 392 days, collagen solubility (percentage of salt- plus acid-soluble collagen) was highest during the rapid growth phase (0–202 days on feed) and then declined during subsequent growth (202–392 days on feed). The reduction in collagen solubility was even more pronounced when animals fed high-energy diets from 0–202 days on feed were placed on maintenance rations from 202–392 days on feed. These data support the rationale that nutritional acceleration of growth during the rapid growth phase is accompanied by concomitant increases in collagen synthesis and the proportion of reducible crosslinks which are characteristic of newly synthesized collagen. However, after a certain degree of maturity is attained, declining growth rate—occurring either as a natural consequence of maturation or in response to restricted energy intake—apparently is associated with slower rates of collagen synthesis and increased stability of existing collagen crosslinks.

A particularly interesting aspect of the data presented by Hall and Hunt (1982) is the fact that steers fed low-energy (forage) diets continuously from approximately 280–840 days of age had among the highest proportions of soluble intramuscular collagen. The authors postulated that cattle fed low-energy diets grow and mature at slower rates than cattle fed high-energy diets, so that at a given chronological age, forage-fed cattle would be physiologically less mature than their grain-fed contemporaries. These findings suggest that if cattle are subjected to a restricted supply of energy during the phase of growth which normally is characterized by rapid growth and development, collagen is synthesized at a relatively slow rate and maturation of reducible collagen crosslinks is delayed.

The foregoing relationships among growth rate, collagen synthesis and the proportion of soluble intramuscular collagen provide a plausible explanation for the results of the present study as well. The fact that maturity had no apparent effect on collagen solubility or tenderness suggests that the intermolecular collagen crosslinks in the musculature of the mature animals had not stabilized to their heat-resistant form, even though there was skeletal evidence of advanced physiological maturity. Though these findings seem contradictory, it may be possible for animals to exhibit relatively mature characteristics in some tissues of the body, and yet appear relatively immature with respect to other tissues. In that connection, previous research has demonstrated that various tissues in the animal's body grow and develop in a specific sequence of priority and that under conditions of restricted energy intake, available nutrients will be utilized for growth and development of tissues of highest priority (Hammond et al., 1972). For example, during periods of restricted energy intake, skeletal development precedes development of the musculature; prolonged dietary energy restriction could cause substantial divergence in the degrees of maturity of these two tissues. Therefore, it is conceivable that youthful and mature animals in the present study could have been extremely diverse with respect to skeletal maturity, but comparatively

similar in stage of muscular development at the onset of this finishing period.

Although elucidation of the exact cause and effect relationships is beyond the scope of this study, it may be theorized that restricted dietary energy intake during the rapid growth phase, followed by intensive preslaughter feeding (a) delayed maturation and stabilization of intermolecular collagen crosslinks and (b) accelerated the rate of collagen synthesis uniformly for both youthful and mature cattle, resulting in similar proportions of soluble collagen and comparable tenderness for both maturity classes.

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Assay Precision and Accuracy of Calcium-Dependent Proteinase Activity in Rat Skeletal Muscle

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————————————ABSTRACT————————————

The accuracy and precision with which Ca^{++}-dependent proteinase (CAF) activity can be assayed in skeletal muscle tissue was determined by using two experimental approaches: (1) repeated sampling of a homogeneous batch of minced rat skeletal muscle to estimate variation among CAF assays done on fractions made from the same muscle tissue and to ascertain the effect of sample size on assays of CAF activity; and (2) comparison of CAF assays done on muscle samples of similar weights obtained from different animals that had been treated alike. Muscle CAF activity can easily be detected in 0.5g muscle samples, but the measured activity is not accurate and increases with increasing sample size. The decreased CAF activity assayed in small muscle samples seemed to originate from failure to extract all the CAF in these samples, possibly because of the different homogenizer that must be used to homogenize small samples. If a Waring Blendor is used at 8000 rpm, muscle samples must be 19g or larger to obtain accurate assays of CAF activity. The coefficient of variation for duplicate assays of CAF activity on the same P_{0-45} crude CAF fraction was 5.85% (assay variation); for assays of CAF activity on different samples of the same muscle tissue, 7.18% (sampling variation and variation in procedure for preparing crude CAF fractions); and for assays of CAF activity on muscle tissue obtained from the different groups of animals that had been treated alike, 10.30% (animal variation). Hence, CAF activity can be measured with acceptable precision in skeletal muscle tissue, but treatments designed to alter muscle CAF activity must cause changes of at least 20% to be detectable against the natural variation of muscle CAF activity in different animals.

INTRODUCTION

SEVERAL YEARS AGO, a Ca^{++}-activated factor (CAF) that very quickly removed Z-disks from myofibrils was discovered in minced skeletal muscle tissue (Busch et al., 1972). This factor was subsequently purified and found to be a Ca^{++}-activated proteinase that was not located in lysosomes (Dayton et al., 1976a; Reville et al., 1976). This proteinase specifically degraded those components that seem at least partly responsible for keeping the contractile proteins assembled in the proper three-dimensional array. Because the effects of this proteinase were limited largely to these components and because of other properties of the proteinase, it was suggested that CAF may be responsible for initiating metabolic turnover of myofibrils in skeletal muscle cells (Dayton et al., 1975).

Shortly after the discovery of CAF, it was found that the structural and biochemical alterations occurring in myofibrils during postmortem storage were remarkably similar to the changes that CAF causes in myofibrils (Goll et al., 1975; Olson et al., 1976; 1977; Stromer et al., 1974). For example, both CAF treatment and postmortem aging result in: (1) degradation of the Z-disk that initially is heralded by increasing myofibril fragmentation during homogeniza-

tion (Dayton et al., 1976b; Goll et al., 1974; MacNaughtan, 1978a, b; Olson et al., 1976, 1977); (2) degradation of the M-line at a slower rate than Z-disk degradation (Dayton et al., 1976b; Henderson et al., 1970); (3) a very specific and limited degradation of the myofibrillar proteins that results in disappearance of troponin-T and appearance of a group of polypeptides with mass near 30,000 daltons but no ostensible degradation of actin or myosin (Dayton et al., 1975; Olson et al., 1977; Penny, 1980; Samejima and Wolfe, 1976); (4) degradation of the intermediate filament protein, desmin, that makes up part of the cell cytoskeleton (O'Shea et al., 1979; Robson et al., 1981; Young et al., 1981); and (5) an increase in the Mg^{++}-modified ATPase activity of myofibrils (Arakawa et al., 1970; Goll and Robson, 1967; Suzuki and Goll, 1974). It was also shown that bovine semitendinosus and longissimus dorsi muscles had more CAF activity and increased more in tenderness during postmortem aging than psoas muscles (Goll et al., 1974; Olson et al., 1976; 1977). The amount of 30,000-dalton polypeptide, which evidently is produced by CAF (Cheng and Parrish, 1977; Olson et al., 1977; Yamamoto et al., 1979), and degree of myofibril fragmentation, which can be related to level of CAF activity (Goll et al., 1974; Olson et al., 1976). are both directly related to tenderness of bovine longissimus dorsi (MacBride and Parrish, 1977; Olson and Parrish, 1977). Finally, Penny and coworkers (Penny, 1976; Penny et al., 1974) have shown that tenderness of freeze-dried bovine semitendinosus is greatly increased when such muscle is reconstituted with saline solutions containing CAF and that postmortem biochemical changes in porcine muscle can be mimicked by CAF treatment. Clearly, considerable evidence exists to indicate that CAF is one of the principal causes of postmortem changes in the myofibrillar proteins.

Because of CAF's suspected role in muscle growth, certain muscle pathologies, and meat tenderness, it is important to have a convenient assay that provides accurate and precise measurements of CAF activity. Very large quantities of muscle, up to 5000–6000g fresh weight, are required to obtain mg quantities of purified CAF (Dayton et al., 1976a). Hence, it is impractical, and in many instances, impossible to quantitate CAF in muscle tissue by determining how much purified CAF can be isolated from a specified amount of tissue. Although assays of CAF activity in crude muscle homogenates have been described (Goll et al., 1974; Olson et al., 1977), no studies have been done to determine whether these assays were accurate estimates of CAF activity, what the major sources of variation are in assays of muscle CAF activity, and whether CAF activity can be assayed accurately in small samples of muscle tissue such as might be obtained by biopsy. The purposes of this study, therefore, were: (1) to determine the major sources of variation in assays of muscle CAF activity and to quantitate the precision with which muscle CAF activity can be estimated; this goal includes estimating the variation in muscle CAF activity among animals that have been treated alike; and (2) to ascertain whether CAF activity can be measured accurately in small samples of skeletal muscle tissue weighing 2g or less.

MATERIALS & METHODS

Experimental design

Because the purpose of this study was to determine the accuracy and precision with which muscle CAF activity can be assayed rather than attempt to catalog muscle CAF activities in domestic animals under various conditions, rat skeletal muscle was used. Our experience indicates that the procedures and results described here for rat skeletal muscle also apply to beef skeletal and cardiac muscle, and probably to skeletal muscle from other species as well. Rats, however, were a more convenient and cheaper source of muscle tissue than domestic animals. Also, it was possible by using rats as a source of tissue to assay muscle CAF activity in a number of different animals that had similar genetic background and that had been treated alike.

Two experimental approaches were used in this study. The first approach used minced rat skeletal muscle that had been obtained by pooling muscle tissue from six different animals, and mixing and grinding it to produce as homogeneous a sample as possible. This homogeneous pool of rat skeletal muscle was sampled repeatedly to determine the precision with which muscle CAF activity can be assayed, and the effect of muscle sample size on CAF activity. Three separate experiments were done using muscle tissue samples ranging from 0.5–29.0g. All experiments used an experimental design identical to that shown in Fig. 1 for the experiment assaying samples ranging from 9–19g. Two experiments were done with quintuplicate samples at each of three weights, 0.5, 1.0, and 2.0g (15 samples total), and one experiment was done using quadruplicate samples at each of three larger weights, 9.0, 19.0, and 29.0g (12 samples total; Fig. 1).

The second experimental approach used muscle from rats that had been subjected to alloxan-injection (Brooks et al., 1983a), to different regimens of fasting and refeeding (Brooks et al., 1983b) or to no treatment at all (control groups of rats). The purpose of this second approach was to determine variation in muscle CAF activity among animals that were of similar genetic background and that had been treated alike rather than determine whether differences in muscle CAF activity existed among different treatment groups. Therefore, each treatment group was taken as a group of animals that had been treated alike, although some groups had been starved, some had been injected with alloxan, others had received no treatment, etc. Each treatment group contained between four and seven animals, and one $P_{0.45}$ crude CAF fraction was prepared from each animal. Duplicate assays were done on all $P_{0.45}$ crude CAF fractions, so eight to fourteen assays were done for each group. Thirty-one such groups of rats comprising 159 animals in four different experiments were included in this second experimental approach. Initial analyses indicated that the different treatments had no effect on variation in muscle CAF activity among animals within a treatment group, so all groups in each experiment were pooled to calculate a coefficient of variation for CAF activity in muscle from animals treated alike. Similarly, coefficients of variation for duplicate assays of each $P_{0.45}$ crude CAF fraction were calculated over all groups in each of the six experiments included in this study (Table 4). Repeated sampling to produce different $P_{0.45}$ crude CAF fractions from the same muscle tissue was done in only the three experiments from the first experimental approach; separate coefficients of variation were also calculated for each of these three experiments.

Protein preparations and assays

All protein preparations were done at 2°C using precooled solutions. Rats were sacrificed by decapitation, and all available skeletal muscle was removed immediately. In the first experimental approach, muscle from six different animals was pooled, diced finely by using a scalpel, mixed, and then passed through a meat grinder to ensure complete mixing. This entire batch of ground muscle was frozen, and portions were then removed as needed for the three experiments testing precision of muscle CAF assays and the effect of sample size on assay of muscle CAF activity. In the second experimental approach, skeletal muscle tissue from each animal was diced finely and was then subjected directly to homogenization. The minced muscle was suspended in 10 vol (w/v) of 4 mM EDTA, pH 7.2, by homogenizing in a 1L Waring Blendor for samples weighing 9g or more, or in a micro Eberbach attachment to a Waring Blendor for samples weighing 2g or less. Homogenization was done in three bursts of 30 sec each with a 30-sec cooling period interspersed between the bursts. The pH of the homogenized samples was be-

tween 6.5 and 7.0, so it was unnecessary to adjust pH or add additional buffer to maintain homogenate pH above 6.5. Because CAF precipitates between pH 4.9 and 6.2 (Dayton et al., 1976a), homogenate pH should be kept above 6.2–6.5 during preparation of $P_{0.45}$ crude CAF fractions to prevent loss of CAF.

Although this homogenization procedure confounds type of blender with sample size when comparing results from samples smaller than 9g with results from samples larger than 9g, it is impossible to homogenize small samples of 2g or less in a 1-L blender and very difficult to homogenize large samples of 19g or more in a 15 ml Eberbach attachment. Therefore, this experimental design is appropriate for the situation that would be encountered when actually doing CAF assays on muscle samples between 0.5 and 29g. Moreover, the experimental results indicated that the two types of blenders used in the present study had no effect on amount of muscle CAF activity measured when muscle sample sizes were the same. To prevent any implication of confounding homogenizer type with sample size, however, statistical comparisons of the effects of muscle sample size on assays of CAF activity were done only among samples homogenized with one type of blender.

A $P_{0.45}$ crude CAF fraction was prepared from each of the homogenized samples by using the procedure described by Dayton et al., (1976a), except that samples were salted out between 0 and 45% ammonium sulfate saturation instead of between 0 and 40% ammonium sulfate saturation. The higher ammonium sulfate saturation was used to ensure total precipitation of CAF even from the smallest muscle samples used in this study. The higher ammonium sulfate saturation precipitated very little additional nonCAF protein above that precipitated at 40% ammonium sulfate saturation. In those experiments involving samples of less than 5g muscle tissue, fresh weight, precipitation at pH 6.2 was omitted during preparation of the $P_{0.45}$ crude CAF fractions because no detectable protein was precipitated from these samples at pH 6.2. Omitting precipitation at pH 6.2 has no effect on the amount of CAF activity measured in a $P_{0.45}$ crude CAF fraction, although it increases slightly the amount of total nonCAF protein in these fractions. It was crucial, however, to precipitate CAF from these extracts at pH 4.9 before the final ammonium sulfate precipitation at 45% saturation to remove a CAF inhibitor protein (Fagan and Goll, 1983). The presence of this inhibitor prevents accurate assay of CAF activity.

Casein, Hammersten quality, was obtained from the United States Biochemical Corporation and ultrapure ammonium sulfate from R-Plus Research Laboratories, Inc. Unless otherwise indicated, all solutions were made with double-distilled, deionized water that had been redistilled in glass and stored in polyethylene containers. Protein concentrations were determined by using the biuret method (Gornall et al., 1949) as modified by Robson et al. (1968) or the Folin-Lowry procedure (Goll et al., 1964; Lowry et al., 1951).

Measurement of CAF activity

CAF activity was measured in duplicate on each $P_{0.45}$ crude CAF fraction by using casein as a substrate and the ionic conditions and controls described by Dayton et al. (1976a). This procedure determines the extent of proteolysis by measuring the amount of peptide material that is released from casein by CAF and that is soluble in 2.5% trichloroacetic acid. The solubilized peptides are measured by absorbance at 278 nm. Activity of a CAF-EDTA control (Dayton et al., 1976a) was measured for each $P_{0.45}$ crude CAF
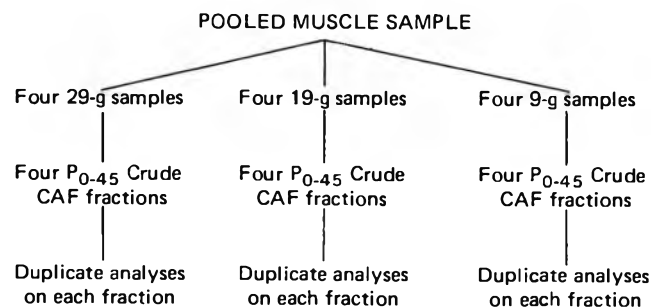
Fig. 1—Experimental design used to test the accuracy and precision with which CAF activity can be measured in small samples of muscle tissue. The design shown is for an experiment using 9, 19, and 29-g samples but similar experiments were done with 0.5, 1.0, and 2.0-g samples.

fraction; this activity was subtracted from proteolytic activity measured for the same fraction in the presence of 5 mM Ca^{++} to give Ca^{++}-specific proteolytic activity for each P_{0-45} crude CAF fraction. Total CAF activity in a P_{0-45} crude CAF fraction was calculated by multiplying Ca^{++}-specific proteolytic activity of a given P_{0-45} crude CAF fraction, measured as absorbance units at 278 nm released from casein at 25°C per 30 min per mg P_{0-45} crude CAF protein times mg of protein in the P_{0-45} crude CAF fraction. This total CAF activity was then divided by g of skeletal muscle, fresh weight, used to prepare the P_{0-45} crude CAF fraction to obtain OD_{278} units of CAF activity per g skeletal muscle, fresh weight. Our laboratory has shown (Brooks et al., 1983a; b) that the P_{0-45} crude CAF fraction prepared as described in this paper contains only one Ca^{++}-activated proteolytic activity assayed by using the procedure described in this section, and that this proteolytic activity is CAF.

RESULTS

Effect of sample weight on accuracy of CAF assay

Several preliminary experiments showed that CAF activity assayed in muscle samples weighing 0.5–2.0g was less than 50% as high as when it was assayed in the same batch of muscle but using 20–30g samples. It was noticed during these preliminary experiments that the Eberbach homogenizer used for the 0.5–2.0g samples did not disperse these samples as thoroughly as the 1-L Waring Blendor dispersed the larger 5–50g samples. Thorough homogenization seems essential for extraction of CAF activity from its intracellular attachments (Dayton et al., 1976a; Dayton and Goll, unpublished observations). It seemed likely that the low CAF activity assayed in 0.5–2.0g samples was due to incomplete extraction of CAF because of less efficient homogenization. Ultrastructural observations have shown that CAF is associated with the Z-disk and the plasma membrane in skeletal muscle cells (Dayton and Schollmeyer, 1981). Therefore, two experiments were done in which 1%, final concentration, v/v, Triton X-100, a nonionic detergent, was added to the homogenization medium. It was anticipated that Triton X-100 would dissolve the plasma membrane and release CAF completely into the extract. These experiments were difficult technically because Triton X-100 interferes with ammonium sulfate precipitation (protein complexes with Trition X-100 micelles which float rather than sediment) and very extended dialysis periods, up to 5–7 days, are required to dialyze out enough Triton X-100 to permit unimpeded ammonium sulfate fractionation. Assays of the Triton X-100 extracts showed that specific CAF activity in 0.5–2.0g muscle samples homogenized in the presence of 1% Triton X-100 was five to six times higher than in similar size samples homogenized without Triton X-100 and was approximately 1.8 times higher than in 19–29g samples homogenized in the absence of Triton X-100 (cf. Table 1 and Table 2). As shown subsequently in this paper, the amount of CAF activity extracted from rat skeletal muscle under conventional conditions reaches a maximum at sample sizes of 19g and remains constant thereafter. Consequently, Triton X-100 either solubilizes a fraction of CAF that is never extracted under the usual homogenization conditions used to prepare CAF, or Triton X-100 has a direct effect on CAF itself. We have recently found that addition of 1% Triton X-100 to assays of purified CAF increases CAF activity 1.6–2.0-fold (Tan and Goll, unpublished observations), which is almost exactly the amount that specific activities of 1.0 and 2.0g samples homogenized in Triton X-100 were elevated over those of 19 and 29-g samples homogenized without Triton (cf. Tables 1 and 2). Therefore, it seems very likely that the unusually high specific activity of the 1.0 and 2.0-g samples homogenized in 1% Triton X-100 is due to a direct effect of Triton X-100 on CAF rather than solubilization of some heretofore unextractable fraction of CAF.

Even in the presence of 1% Triton X-100, the assayed specific acitvity of CAF decreased as sample size decreased from 2.0 to 1.0 to 0.5g muscle, fresh weight (Table 1). Because it was difficult to homogenize and to prepare P_{0-45} crude CAF fractions from samples containing Triton X-100, it was decided to omit Triton X-100 from the homogenization medium and to attempt to learn how large muscle samples had to be before a reproducible amount of CAF activity could be extracted with the homogenization and sample preparation procedures used in this study. Preliminary experiments suggested that this sample weight was between 10 and 20g muscle, fresh weight, so a third experiment was done using muscle tissue samples of 9, 19, and 29g. Results of this experiment indicated that CAF activity could not be assayed accurately in samples weighing 9g, but that assays of 19 and 29g samples gave identical activities, both higher than the activities assayed in the 9g samples (Table 2). Therefore, muscle sample sizes of 19g or larger, fresh weight, were used in all subsequent studies.

Sources of variation in assays of CAF activity

The experiments described in the preceding section can also be analyzed to compare variation among duplicate chemical assays of the same P_{0-45} crude CAF fraction with variation among CAF assays done on quadruplicate or quintuplicate samples of the same muscle tissue. Such an analysis is shown in Table 3 for the experiment using 9, 19, and 29g samples. Variation among assays of CAF activity in different samples from the same muscle tissue is significantly ($P < 0.005$) greater than variation among duplicate assays of the same P_{0-45} crude CAF fraction (Table 3). Hence, CAF activity assays are similar to most other chemi-

Table 1—Effect of sample weight on CAF activity when assayed in very small samples of rat skeletal muscle extracted with 1% Triton X-100

Sample wt (g)	CAF activity[a] (OD_{278} Units/30 min/g skeletal muscle)
0.5	0.526 ± 0.063[b]
1.0	0.601 ± 0.053[b]
2.0	0.688 ± 0.070[b]

[a] Figures are means plus or minus standard errors of 20 determinations on 10 different samples.
[b] All means are significantly different at $P < 0.05$.

Table 2—Effect of sample weight on assay of CAF activity in rat skeletal muscle

Sample wt (g)	CAF activity[a] (OD_{278} Units/30 min/g skeletal muscle)
9	0.233[b]
19	0.354
29	0.372

[a] Figures are means of eight determinations on four different samples.
[b] Significantly different from the means for 19 and 29g samples at $P < 0.01$.

Table 3—Analysis of sources of variation associated with repetitive CAF assays of skeletal muscle samples weighing, 9, 19, or 29g

Source of variation	d.f.	Mean square	"F" Ratio	
Weight of tissue assayed	2	0.0460	8.08	$P < 0.01$
Different samples of the same wt and from the same muscle tissue	9	0.00569	7.40	$P < 0.005$
Separate assays of the same P_{0-45} crude CAF fraction	12	0.000770		

Table 4—Comparison of the sources of variation associated with assay of CAF activity in rat skeletal muscle

Experiment	No. of animals involved	Source of variation[a]		
		Duplicate assays of the same P_{0-45} crude CAF fraction	Assays of quadruplicate or quintuplicate samples of the same weight from the same muscle samples	Assays of different animals in the same treatment group
0.5, 1.0, and 2.0g samples homogenized in 1% Triton X-100-I	N/A[b]	7.69 (30)	8.70 (30)	—
0.5, 1.0, and 2.0g samples homogenized in 1% Triton X-100-II	N/A[b]	3.65 (30)	4.48 (30)	—
9, 19, and 29g samples homogenized without Triton X-100	N/A[b]	6.14 (24)	8.35 (24)	—
Starvation and refeeding - I	41	—	—	9.90 (41)
Starvation and refeeding - II	33	6.41 (66)	—	12.01 (66)
Starvation and refeeding (Brooks et al., 1983b)	43	6.47 (86)	—	9.32 (86)
Alloxan-injection (Brooks et al., 1983a)	42	4.75 (84)	—	9.99 (84)
Average for all acceptable	—	5.85±0.58[c]	7.18±1.35[c]	10.30±0.49[c]

[a] Figures are coefficients of variation calculated from the number of determinations of CAF activity indicated in the parenthesis
[b] N/A = not applicable
[c] Means plus or minus standard error for the number of experiments shown

cal assays in that variation among duplicate chemical analyses done on the same sample is much less than variation among assays done on different samples of the same material.

It is important when designing experiments to determine the effect of different treatments on muscle CAF activity or when attempting to relate muscle CAF activity to meat tenderness, etc. to have an estimate of the variation in CAF activity that exists among animals that have been treated alike. Therefore, muscle CAF activities were determined on samples larger than 19g from groups of rats that had been treated alike. The results of these analyses expressed as coefficients of variation are shown for four different experiments in Table 4. These results averaged over several different experiments confirm that variation among duplicate assays of the same P_{0-45} crude CAF fraction (C.V. = 5.85%) is less than variation among CAF assays done on quadruplicate or quintuplicate samples of the same muscle tissue (C.V. = 7.18%). The coefficient of variation of 10.30% (Table 4) for muscle CAF assays from animals treated alike indicates that differences in muscle CAF activity would have to be at least 20% before a significant effect of any treatment on muscle CAF activity could be detected.

DISCUSSION

ALTHOUGH ACCURATE and precise measurement of muscle CAF activity is essential when attempting to relate differences in rate of postmortem tenderization to muscle CAF levels or when determining whether rate of muscle loss in various muscular dystrophies or extent of muscle damage in infarcted myocardium is related to CAF activity, this is the first reported attempt to determine the precision with which CAF activity can be measured and the potential errors in assays of CAF. Up to now, almost all assays of muscle CAF activity have used a Waring Blendor to homogenize minced muscle tissue (Dayton et al., 1976a; 1979; Hattori and Takahashi, 1979; Locker et al., 1977; Olson et al., 1977; Ouali and Valin, 1980–81; Parrish et al., 1981; Penny, 1976). The present study shows that, if this homog-

enization procedure is used, muscle sample sizes must be 19g or larger to obtain accurate measurements of CAF activity. The currently used procedures can easily detect CAF activity in muscle samples as small as 0.5g, fresh weight, such as might be obtained by biopsy, but activities measured on extracts prepared in the usual way from these small muscle samples are too low and are not accurate estimates of true muscle CAF activity. It seems that improvements in homogenization procedures or solutions will be required before CAF activity can be accurately measured in skeletal muscle samples less than 19g. Until such improved procedures are devised, muscle sample sizes should be 19g or larger when assaying CAF activity. The currently used procedures do produce reasonably precise measurements of muscle CAF activity, with a coefficient of variation of only 7.18% for assays done on different samples of the same tissue. That variation in assays of CAF activity in different samples (or P_{0-45} crude fractions) from the same muscle tissue is significantly larger than variation among duplicate assays of the same P_{0-45} crude CAF fraction, however, indicates that better estimates of muscle CAF activity would be obtained by preparing duplicate P_{0-45} crude CAF fractions from one muscle tissue sample than by doing duplicate assays of one P_{0-45} crude CAF fraction. This procedure does not seem to have been followed in most assays of muscle CAF activity done thus far (Dayton et al., 1979; Olson et al., 1977; Parrish et al., 1981).

Very few studies have reported specific activities of CAF per unit of muscle tissue. Two groups (Gerard and Schneider, 1980; Kar and Pearson, 1976) have used a glass homogenizer to disperse muscle tissue. Because striated muscle is notoriously difficult to homogenize, and glass homogenizers are generally inefficient in dispersing striated muscle tissue from postnatal mammalian organisms, the muscle CAF activities reported by these two laboratories are likely too low and must be regarded skeptically. Unfortunately, neither of these laboratories have expressed their specific activities in a form that would permit comparison with the specific activities obtained in the present study. Dayton et al. (1979) do not indicate the size of muscle sample used

but report a specific CAF activity of 0.29 OD_{278} units/30 min/g muscle, fresh weight, for normal rabbit skeletal muscle, in good agreement with the specific activity of 0.37 OD_{278} units/30 min/g muscle, fresh weight, obtained in the present study for rat skeletal muscle. Bovine skeletal muscle, assayed in 100g samples, has been reported to have specific CAF activities ranging from $0.08-0.09$ OD_{278} units/30 min/g muscle, fresh weight, immediately after death (Olson et al., 1977) to $0.108-0.121$ OD_{278} units/30 min/g muscle, fresh weight, after $10-14$ days of postmortem aging (Parrish et al., 1981). These three studies together with the present study provide some indication of specific CAF activity in mammalian skeletal muscle and suggest that CAF activity may be slightly lower in bovine skeletal muscle than in either rat or rabbit skeletal muscle.

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Ames Test for Mutagenicity on Pacific Whiting Treated with Hydrogen Peroxide

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─────────── ABSTRACT ───────────

Raw Pacific whiting (*Merluccius productus*) treated with hydrogen peroxide or potassium bromate was tested for mutagenicity by the Ames *Salmonella*/microsome assay with strains TA 98 and TA 100 without and with S-9 activation. To examine the effects of long-term exposure, cooked whiting treated with hydrogen peroxide and stored up to 6 months at −26°C was also tested. In contrast to the raw-treated fish, the cooked sample contained 78% catalase-reactive peroxide up to 6 months later. For testing, acidic, neutral and basic fractions were obtained by modifying the procedure of Felton et al. (Mutat. Res., 1981) to reduce emulsion formation. No extract from either potassium bromate or hydrogen peroxide treatment produced mutagens.

INTRODUCTION

PACIFIC WHITING (*Merluccius productus*) represents a vast resource of high quality protein. In 1979, fishing fleets mainly from the USSR and Poland caught 141,622 mt of Pacific whiting (FAO, 1980). At present, this species is rarely marketed in the United States because the flesh often becomes mushy on slow cooking. This soft-textured fish is not intrinsically mushy. The problem develops in association with parasitization by myxosporidian protozoa of the species *Kudoa* (Patashnik et al., 1982; Kabata and Whitaker, 1981; Tsuyuki and Williscroft, 1982). During slow cooking, proteolytic enzymes formed by the protozoa rapidly hydrolyze the muscle. *Kudoa paniformis* probably causes the proteolysis. Kabata and Whitaker (1981) found that whiting from the Strait of Georgia contained only *K. thyrsitis*, while whiting taken from the Pacific Ocean southwest of Vancouver Island contained both *K. thyrsitis* and *K. paniformis*. Patashnik et al. (1970) noted that whiting from Puget Sound, like the Strait of Georgia somewhat isolated from the open ocean, generally did not suffer from the textural problem. Tsuyuki and Williscroft (1982) found that the enzyme from *K. paniformis* possessed a much higher temperature optimum (above 60°C) than that from *K. thyrsitis* (below 40°C). Thus the enzyme from *K. paniformis* would be active nearly throughout cooking by a slow method, whereas that from *K. thyrsitis* would be inactivated more readily during heating.

Investigations at this laboratory have developed a number of methods for improving the texture of Pacific whiting. Patashnik et al. (1982) showed that rapid heating, such as deep frying, eliminates the problem, as long as the slices are limited to 1 cm in thickness or less. To expand the uses of whiting, Miller and Spinelli (1982) studied chemical means of inactivating the proteolytic enzyme. They found that several oxidants, including hydrogen peroxide and potassium bromate, were effective.

Hydrogen peroxide has been used in food processing since the early 1950's. In cheese-making for instance, it is used to destroy the normal microorganisms in milk, prior to fermentation with pure cultures. In the preparation of marinated herring fillets, hydrogen peroxide not only lightens the tissue, but also extends the shelf life of the product (Sims, 1975). To expand the utilization of minced flesh recovered from small fish or filleting waste, Young et al. (1980) have studied the bleaching effect of hydrogen peroxide on cod (*Gadus morhua*), saithe (pollock) (*Pollachius virens*), and mackerel (*Scomber scrombrus*).

Use of hydrogen peroxide in food processing is regulated by individual countries. The United States Food & Drug Administration considers hydrogen peroxide to be generally recognized as safe (GRAS) in the concentrations used for disinfection and bleaching (Hile, 1981). In Japan hydrogen peroxide was used to improve the texture of surimi (minced fish flesh) and to sanitize the surface of kamaboko (steamed fish cake made from surimi) until scientists at Hiroshima University reported it to be weakly carcinogenic in mice. At dosages of 0.1 and 0.4% in drinking water it produced tumors of the duodenum (Ito et al., 1981). Japanese authorities now require the decomposition or removal of hydrogen peroxide from food products (Ministry of Health and Welfare of Japan, 1980).

Potassium bromate is used by the baking industry to improve the texture of bread dough. It is reduced rapidly during baking (Tsen, 1968).

The Japanese (Sato, 1907), motivated by the high incidence of gastric cancer in their country, have been studying potential carcinogens in food for some time. Sugimura and his associates have published extensively on carcinogens and mutagens in broiled meat and fish (Sugimura et al., 1977; Matsukura et al., 1981). Krone and Iwaoka (1981) have studied the production of mutagens during cooking of fish.

This paper presents the results of Ames tests on extracts from raw and cooked Pacific whiting treated with hydrogen peroxide and raw whiting treated with potassium bromate. The study was designed to learn whether the interaction of hydrogen peroxide or potassium bromate with fish would produce mutagenic substances.

MATERIALS & METHODS

REAGENTS were analytical grade. Organic solvents were distilled-in-glass grade from Burdick & Jackson (Muskegon, MI).

Raw whiting

Fillets of parasitized Pacific whiting were thawed, ground in a Hobart Silent Cutter, mixed thoroughly with hydrogen peroxide (30%, diluted 1:6 with distilled water), potassium bromate (0.5%), or, as a control, sodium chloride (0.1M, approximately the ionic strength of muscle) in the ratio of 10 parts fish to 1 part solution (Miller and Spinelli, 1982) and frozen at −20°C. Nonparasitized fish were also treated with 0.1M NaCl.

Cooked whiting

Frozen fillets were thawed, and a single layer was wrapped in aluminum foil and steamed 20 min. After cooling, the fish were

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ground and treated with hydrogen peroxide or, for the control, distilled water and frozen at $-26°C$.

Parasitization assay

To measure hydrolysis caused by the proteolytic enzyme, ground fish (3g) was incubated for 30 min at 45°C in a 50-ml polycarbonate centrifuge tube covered with parafilm (At 37°C the enzyme was not sufficiently active to monitor accurately.) To stop the reaction, 3 ml of trichloroacetic acid were added and allowed to react for 30 min at room temperature. After centrifuging for 10 min at 9750 x g, 1 ml of the supernatant was assayed for protein by the Lowry method (Lowry et al., 1951). To correct for soluble protein present initially in the sample, a portion incubated at 0°C was assayed in parallel and the protein content subtracted from the content of the sample incubated at 45°C.

Hydrogen peroxide and potassium bromate determination

The method of Miller and Spinelli (1982) adapted from Price and Lee (1980) and the AOAC (1975) handbook was used. Thus, 4 ml water, 1 ml oxidant standard or 1g fish, 1 ml saturated potassium iodide, and 1 ml 0.001M ammonium molybdate in 0.1N sulfuric acid were shaken 1 min, titrated to a light yellow with 0.1N sodium thiosulfate, a few drops of 1% starch solution added and the titration continued to the end point. When catalase was included, a suspension of 20 mg bovine liver catalase, 3600 units/mg (Sigma Chemical Co., No. C-10) in 5 ml water replaced the 4 ml water normally used.

Extraction and fractionation of samples

The extraction method of Felton et al. (1981), chosen to avoid ammonium ions, which produce artifacts (Iwaoka et al., 1981), was modified to reduce emulsion formation, so typical in extraction of fishery products. Samples of 200g fish were homogenized with a Tissumizer (Tekmar Co.) with 200 ml acetone and filtered through glass-fiber paper under suction. The process was repeated twice. The combined filtrate was rotary evaporated under reduced pressure below 32°C until the acetone had been removed.

The initial extract was separated into acidic, neutral and basic fractions as follows (Fig. 1): the residue from the acetone extract was treated with 250 ml of a diluting solution containing 100g sodium sulfate and 4.6 ml concentrated HCl in 600 ml distilled water. The pH was adjusted to 3 with concentrated HCl. The mixture was extracted three times with 100 ml methylene chloride with gentle rocking to avoid emulsification. The organic layer contained the acidic and neutral components. The aqueous phase was made basic to pH 11 with 20% NaOH and extracted three times with methylene chloride. The combined organic phase was dried over anhydrous sodium sulfate and evaporated below 32°C to give the basic fraction. The solution containing the acidic and neutral components was treated with 100 ml of an extracting solution con-

taining 100g sodium sulfate and 0.2g NaOH in 600 ml distilled water. The pH was adjusted to 12 with 20% NaOH. The mixture was rocked and the aqueous layer removed. The organic layer was extracted twice more with 100 ml portions of the extracting solution. The residual organic phase contained the neutral fraction. The combined aqueous phase was acidified with concentrated HCl to pH 3 and extracted three times with 100 ml methylene chloride to obtain the acidic fraction. After rotary evaporation, the three fractions were stored at $-20°C$ in sterile borosilicate vials with Teflon-lined caps. Before mutagen testing, the samples were diluted with dimethyl sulfoxide (DMSO), to concentrations representing 0.01, 0.10, 0.25, 0.5, 1, 5, and 10g fish/plate.

Mutagenicity testing

We used the *Salmonella*/mammalian-microsome mutagenicity test of Ames et al., (1975). We obtained bacterial cultures from Professor B. N. Ames, Aroclor 1254 induced rat liver S-9 from Litton Bionetics, Inc., and stored them at $-75°C$. For the assay, 2 ml of molten top agar containing 0.05 mM L-histidine, 0.05 mM biotin, and 0.09 M sodium chloride were inoculated at 44°C with 0.1 ml overnight culture of *S. typhimurium* strain TA 98 or TA 100 in Oxoid nutrient broth No. 2 (K.C. Biological), and dosed with 50 μl DMSO or sample in DMSO, and, when desired 0.5 ml S-9 mix. The mixture was poured onto a glucose minimal plate and incubated 72 hr at 37°C. The hisitidine-independent colonies were counted with a Biotran II (New Brunswick Scientific Co., Inc.) colony counter. Known carcinogens were tested in each experiment. Assays were repeated to confirm the findings. The genetic markers of the bacterial strains were verified routinely.

Each glucose minimal plate contained 25 ml Difco Bacto-agar in 1% glucose/VB salts in a Falcon Muta-assay plate or well-aged standard plastic Petri dish. For the S-9 mix, 2 ml S-9 fraction were added to 0.4 ml of a solution of 0.4M magnesium chloride and 1.65M KCl, 0.1 ml 1M glucose-6-phosphate, 0.8 ml 0.1M nicotinamide adenine dinucleotide phosphate (NADP), 10 ml 0.2M sodium phosphate buffer (pH 7.4) and 6.7 ml demineralized distilled water.

To test the efficacy of the system in detecting mutagens in food products, we also tested the basic fraction of an extract of over-cooked fried hamburger, cooked 10 min at 190°C (Pariza et al., 1979). Basic fraction equivalent to 20g hamburger produced 998 ± 133 mutant colonies/plate with *Salmonella* strain TA 98 in the presence of S-9.

RESULTS & DISCUSSION

WE CHOSE THE AMES TEST to assay for mutagenic activity because of its widespread application for the screening of not only pure compounds, but also complex mixtures such as coal fly ash (Fisher et al., 1979), hair dye (Ames et al., 1975), and mussels (*Mytilus edulis*) from polluted waters (Parry et al., 1976). We were looking for
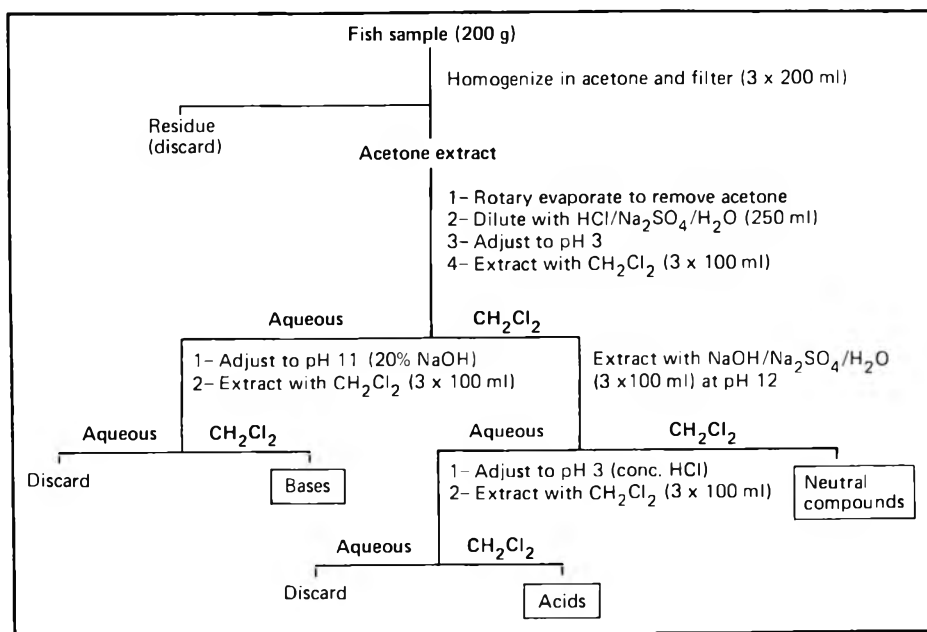
Fig. 1—Procedure for extraction and fractionation of Pacific whiting for mutagen testing.

a preliminary indication of potentially hazardous substances resulting from treatment of Pacific whiting with hydrogen peroxide or potassium bromate.

We used two bacterial strains in our testing. One strain, TA 98, is sensitive to mutagens which cause frame-shift alterations is DNA. The second strain, TA 100, detects base-pair as well as some frame-shift defects. In addition, the Ames test employs S-9 fraction from mammalian livers to approximate the complex xenobiotic metabolizing capacity of the human liver. Many substances require biotransformation in order to produce a mutagenic response. The S-9 fraction from the livers of rats induced with Aroclor 1254 contains a combination of mixed function oxidases and cytochromes capable of converting many promutagens into ultimate mutagens, that is, direct-acting compounds which cause mutation of the test strains of bacteria.

The Ames test is a reverse mutation assay that measures the formation of histidine-independent prototrophic colonies from the original auxotrophs which require histidine for growth. For a mutagenic response to be significant, the number of revertants must be at least double the normal spontaneous number of revertants of the strain. To avoid effects produced by changing the concentration of DMSO itself, all plates including spontaneous controls contained 50 μl DMSO (Rosin and Stich, 1979).

Raw whiting

Four samples of raw Pacific whiting were assayed in the Ames test:
(1) parasitized whiting containing 0.45% hydrogen peroxide
(2) parasitized whiting containing 0.045% potassium bromate
(3) parasitized whiting (control)
(4) nonparasitized whiting (control)

The nonparasitized whiting was included in order to eliminate the possibility that any mutagenicity observed in parasitized samples might derive from the parasitization itself rather than the chemical treatment. The extent of parasitization of these samples was measured by assaying for proteolysis. Parasitization increased proteolysis nearly 13-fold (Table 1).

No raw samples elicited any mutagenic response. Acidic, neutral, and basic fractions from acetone extracts were tested with strain TA 98 or TA 100 both without and with metabolic activation. Hydrogen peroxide decomposes rapidly, however, in contact with raw fish, oxygen being evolved even during mixing. One function of catalase, a normal constituent of muscle, is to destroy the hydrogen peroxide formed during aerobic metabolism. In the case of raw whiting, little time was available for peroxide interactions to occur. Although potassium bromate remained intact in raw whiting after storage for 1 month, nonetheless no mutagenic response was observed in any fraction of the extract from this treatment.

Cooked whiting

To determine the long-term effect of hydrogen peroxide in tissue lacking catalase activity, cooked whiting was treated with hydrogen peroxide. The whiting was steamed for 20 min to inactivate the enzyme without formation of heat-induced mutagenic substances. Although hydrogen peroxide disappeared rapidly in the raw samples, in cooked whiting it was surprisingly stable. Sixty-nine percent of the original peroxide remained after 3 days in a refrigerator at 3°C. After 6 months of frozen storage at −26°C, 78% remained (Table 2). The difference in concentration of hydrogen peroxide between samples from the two storage temperatures may result from bacterial action at 3°C or inhibition of some oxidative reaction at the lower temperature.

The residual activity that we attribute to hydrogen peroxide, could relate to some other oxidant. The ammonium molybdate/thiosulfate reagent may not distinguish between hydrogen peroxide and reactive substances formed from it. To shed some light on the nature of the oxidative activity, cooked whiting treated with peroxide and stored for 7 months was treated with catalase. The rapid loss of peroxide-like activity when diluted 1:10, favors hydrogen peroxide itself (Table 3). Aebi (1974) says that catalase decomposes hydrogen peroxide much more rapidly than it oxidizes H donors with the consumption of peroxide. The slower rate of reaction in the more concentrated solution is attributable to inactivation of the catalase at the higher concentration of hydrogen peroxide in the original sample (Aebi, 1974).

We tested cooked whiting immediately after treatment and 1, 3, and 6 months later. We included extracts representing 0.01, 0.10, 0.25, 0.5, 1, 5, and 10g fish/plate, in an effort to detect any mutagenic effect that might be obscured by a cytotoxic response at higher concentrations of fish extracts. Mutagenic substances can be cytotoxic, i.e., reduce growth or kill, and nonmutagenic compounds in extracts can also be cytotoxic, especially at higher concentrations. The media for the Ames test contains a small amount of histidine to allow limited growth of the histi-

Table 1—*Proteolysis in raw Pacific whiting*

	Soluble protein content[a] (μg/ml)	Increase in proteolysis
Nonparasitized	311	1
Parasitized	4011	12.9
(Highly parasitized)[b]	(8000)	(22.9)

[a] Sample incubated 30 min at 45°C, quenched with trichloroacetic acid, and centrifuged 10 min at 9750 x g. The protein content of the supernatant was determined by the Lowry method (Lowry et al., 1951).
[b] Included to give an indication of possible high levels of proteolysis

Table 2—*Fate of hydrogen peroxide in cooked Pacific whiting during storage*

Sample	Temperature (°C)	Hydrogen peroxide (%)[a]
Control[b]		0
1 hr	3	91
1 day	3	69
3 days	3	69
2 months	−26	75
4 months	−26	78
6 months	−26	78

[a] Percent of the initial 0.45% remaining
[b] With water in place of hydrogen peroxide

Table 3—*Effect of catalase on residual hydrogen peroxide in cooked Pacific whiting treated with hydrogen peroxide*

Sample	Calc. conc (%)	Peroxide content (milliequiv)	
		In water	In catalase solution[a]
Hydrogen peroxide	0.45	0.236	0
Cooked whiting	0	0	0
Treated[b] cooked whiting			
Immediately	0.45	0.151	0.019
After 30 sec[c]	0.45	N.A.[d]	0.007
After 60 sec	0.45	N.A.[d]	0.006
Diluted 1:10[e]	0.045	0.014	0

[a] 5 ml of catalase (4 mg/ml)
[b] Cooked whiting was treated with 0.45% hydrogen peroxide and stored 7 months at −26°C.
[c] To allow for reaction of catalase
[d] Not analyzed
[e] To avoid inactivation of catalase at elevated hydrogen peroxide concentrations

dine-dependent bacteria. Reproduction for a few generations is required to elicit mutation; therefore the presence of an even confluent bacterial lawn indicates normal growth (van Kooij et al., 1977). In the presence of cytotoxic substances, widely spaced larger individual colonies sometimes visible to the naked eye replace the confluent microscopic growth of the normal lawn. The few cells which are resistant to the toxic substances in the test extract grow larger than usually possible because of the reduced number of living cells competing for the limited amount of histidine in the test medium. These large colonies, resistant to toxic substances, are nonetheless histidine-dependent. Without microscopic examination of the bacterial lawn they can be confused with the somewhat larger colonies of histidine-independent organisms. True mutagenic activity is confirmed by regrowth in the total absence of histidine. In the present study, the bacterial lawns appeared normal except occasionally at concentrations of 5 and 10g fish/plate.

Data for peroxide-treated cooked whiting, stored 6 months at $-26°C$, are shown in Table 4. Means for all concentrations were combined to emphasize the limited effect of the fish extracts on the normal number of revertants from spontaneous mutation. Generally both the mean and the standard deviation for extracts from the hydrogen peroxide treated fish were similar to those without the treatment. Apparently hydrogen peroxide treatment had less effect than constituents extracted from the fish regardless of treatment. It is possible that mutagens were produced, but either were not extracted or decomposed during the extraction process.

Prolonged exposure of frozen cooked whiting to hydrogen peroxide did not produce mutagenic substances which could be detected by the Ames test. Minced flesh can be treated with hydrogen peroxide to reduce the bacterial load, inactivate proteolytic enzymes, modify the texture or lighten the color without the formation of mutagenic byproducts. Following reaction, excess hydrogen peroxide can be destroyed rapidly with catalase to leave a peroxide-free product.

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Table 4—Mutation rates of fractions from hydrogen-peroxide treated, cooked Pacific whiting after storage for 6 months at $-26°C$

Fraction	H_2O_2 Treatment	Without S-9 mix TA 98	Without S-9 mix TA 100	With S-9 mix TA 98	With S-9 mix TA 100
None[b]	—	18±3	197± 8	26± 8	190±22
Basic	—	24±2	175±27	38±12	188±18
Basic	+	24±7	160±22	30± 6	185±24
None[b]	—	25±2	110± 4	37± 6	104± 4
Neutral	—	25±3	111± 6	37± 6	117±10
Neutral	+	31±3[c]	d	28± 5	d
Acidic[e]	+	27± 3	117±10	36± 3	122±11
2-Nitrofluorene (10 µg/plate)		1098			
Sodium azide (1 µg/plate)			528		
2-Aminoanthracene (2.5 µg/plate)				466	975

[a] Mean and standard deviation of 7 concentrations from 0.01—10g fish/plate; mean of 2 plates at each concentration
[b] Mean and standard deviation for 3 plates
[c] Two highest concentrations contained particles of lipid which increased the count irrelevantly
[d] Not tested
[e] Acidic fraction of fish without hydrogen peroxide treatment lost during isolation

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Determination of Hypoxanthine in Fish Meat with an Enzyme Sensor

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ABSTRACT

An enzyme sensor specific for hypoxanthine (Hx) was developed using immobilized xanthine oxidase-membrane and an oxygen probe. Xanthine oxidase (E.C. 1.2.3.2.) was covalently immobilized on a membrane prepared from cellulose triacetate, 1,8-diamino-4-amino-methyloctane, and glutaraldehyde. Hx is oxidized to uric acid by the immobilized enzyme, the output current of the oxygen probe decreasing due to oxygen consumption. A linear relationship was obtained between current decrease and Hx concentration in the range 0.06–1.5 mM. The enzyme sensor could be used for more than 100 assays without decrease of output current. After 30-day-storage at 5°C, no remarkable decrease of output current was observed. The enzyme sensor system was applicable to the simple, rapid, and economical determination of Hx in several fish meats including sea bass, saurel, mackerel, yellowfish, and flounder.

INTRODUCTION

ESTIMATION of fish meat freshness is important for the food industries for the manufacture of high quality products. After the death of a fish, the decomposition of ATP in the fish meat sets in and ADP, AMP, and other related compounds are subsequently generated according to the following sequence (Saito et al., 1959).

$$ATP \xrightarrow{(a)} ADP \xrightarrow{(b)} AMP \xrightarrow{(c)} IMP \xrightarrow{(d)} HxR \xrightarrow{(e)} Hx \xrightarrow{(f)} X \xrightarrow{(g)} U$$

where ATP is adenosine-5'-triphosphate, ADP is adenosine-5'-diphosphate, AMP is adenosine-5'-monophosphate, IMP is inosine-5'-monophosphate, HxR is inosine, Hx is hypoxanthine, X is xanthine, and U is uric acid. In most cases, the rate determining step is (e) or (f) depending upon the species of fish, and consequently HxR or Hx is accumulated with increase of storage time. HxR and Hx may be used as an indicator of the freshness. Therefore, simple and rapid methods for the determination of Hx are required in food industries.

Various methods have been described for the HxR and Hx determination (Jones et al., 1964; Burt et al., 1968; Ehira and Uchiyama, 1969; Kobayashi and Uchiyama, 1970; Tanaka et al., 1970; Jahns et al., 1976). However, each requires complicated and time consuming procedures. An enzymatic assay method was introduced for the determination of HxR and Hx (Ehira and Uchiyama, 1969). The reactions and enzymes involved are as follows:

$$HxR + Pi \xrightarrow{\text{Nucleoside phosphorylase}} Hx + Ribose-P \quad (3)$$

$$Hx + O_2 \xrightarrow{\text{Xanthine oxidase}} X + H_2O_2 \quad (4)$$

$$X + O_2 \xrightarrow{\text{Xanthine oxidase}} Uric\ acid + H_2O_2 \quad (5)$$
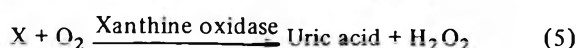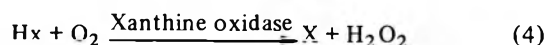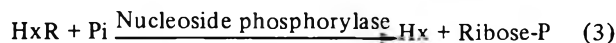
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Uric acid formed was measured spectrophotometrically at 290 nm. However, this enzymatic method generally requires a long time for the determination and expensive enzymes.

Since the principle of an enzyme electrode was proposed, many enzyme electrodes have been developed and proven to be useful. Some of them were applied to clinical and food manufacturing process analyses (Updike and Hicks, 1967; Aizawa et al., 1974; Burns, 1976; Leon et al., 1976; Satoh et al., 1976; Huntington, 1978; Nikolelis and Mottola, 1978; Kayama et al., 1980; Karube et al., 1980, 1982). In the present study, a flow-type enzyme sensor system has been developed and applied to a rapid and simple determination of hypoxanthine according to reactions (4) and (5). In this paper, xanthine oxidase is immobilized on a cellulose triacetate membrane and attached to an oxygen electrode. Oxygen consumption due to the oxidation of hypoxanthine was monitored by the oxygen electrode. The enzyme sensor was applied to the determination of Hx in several fish meats.

MATERIALS & METHODS

Materials

Xanthine oxidase (E.C. 1.2.3.2, from bovine spleen) was obtained from Boehringer Co. Histamine was obtained from Sigma Co. Hypoxanthine, inosine and 50% glutaraldehyde were purchased from Tokyo Kasei Co. Dichloromethane, 1,8-diamino-4-aminomethyloctane and cellulose triacetate were obtained from Kokusan Kagaku Co., Asahi Kasei Co. and Eastman Kodak Co., respectively. Other reagents were analytical grade or laboratory grade materials. Distilled water was used throughout the experiments.

Sea bass, *Lateolobrax japonicus*, saurel, *Trachurus japonicus*, mackerel, *Scomber japonicus*, yellowfish, *Seriola quinqueradiata*, and flounder, *Lepidopsetta bilineata*, were purchased from a fish market and stored in ice.

Determination of hypoxanthine (Hx) concentration

Hx was extracted from 2g of fish muscle with 10% perchloric acid (PCA) according to Ehira's method (Ehira and Uchiyama, 1969). After the Hx solution was neutralized with 10N KOH, 0.65 ml of 0.5M phosphate buffer (pH 7.8) containing 1.54 mM cysteine was added to the solution, and adjusted to 10 ml with a 10% neutralized PCA solution (final pH 7.7). The final concentration of cysteine was 0.1 mM.

Thin-layer chromatography for aldehydes

Thin-layer chromatography was carried out to determine the presence of aldehydes in sample solutions, because aldehydes are also decomposed by xanthine oxidase. A 6N HCl solution (0.5 ml) was saturated with 2,4-dinitrophenyl hydrazine and added to 1 ml of the sample solution, and then it was boiled for 10 min. The solution was cooled to room temperature, a small amount of chloroform added and the mixture vigorously shaken. Hydrazone derivatives of aldehydes in the chloroform layer were separated and identified by silica gel thin-layer chromatography using a developing solvent of petroleum ether and ethyl ether (4:1).

Preparation of the immobilized enzyme membrane

Cellulose triacetate (250 mg) was dissolved in 5 ml of dichloromethane and, then 200 μl of 50% glutaraldehyde was added and mixed. After stirring the mixed solution to make it homogeneous,

1 ml of 1,8-diamino-4-aminomethyloctane was added gradually and fully mixed. The mixture was spread on a glass plate and allowed to stand for 2 days at room temperature to complete intermolecular cross-linking of 1,8-diamino-4-aminomethyloctane and glutaraldehyde. The thin layer formed on the glass plate was cut into small membrane pieces (0.7×0.7 cm^2) and peeled off in distilled water, then washed with a 0.05M phosphate buffer (pH 7.5). These membranes were soaked in a 0.1% (w/v) glutaraldehyde (0.05 Tris-HCl buffer, pH 8.4) solution at 30°C for 2 hr. After washing with a sufficient amount of distilled water, 10 sheets of the membrane were immersed in 3 ml of 0.05M phosphate buffer (pH 7.8) containing 300 μl (120 units) of xanthine oxidase at 5°C for 48 hr. In order to improve the stability of the enzyme membranes, they were further incubated at 5°C for 24 hr in 5 ml of Tris-HCl buffer (0.05M, pH 8.4) containing 0.1% (w/v) glutaraldehyde and a stabilizing reagent such as cysteine (0.1 mM) of histamine (0.1 mM). The enzyme bound membranes were washed with the buffer and stored in a 0.05M phosphate buffer solution (pH 7.8) containing 0.1 mM cysteine. The enzyme membranes were stored at 5°C to examine their storage stability. The relative activity of stored membranes were determined every 24 hr.

Apparatus

Fig. 1 shows the schematic diagram of the sensor system for the hypoxanthine determination. The oxygen probe used in this system was a Clark type electrode consisting of a platinum cathode, a lead anode, alkaline electrolyte (KOH) and an oxygen-permeable Teflon membrane. The enzyme membrane prepared was tightly fixed on the Teflon membrane of the probe and covered with a dialysis membrane (cellulose acetate). The temperature was controlled at 32°C during the enzyme reaction.

Assay procedure

A phosphate buffer (0.05M, pH 7.8) solution containing 0.1 mM
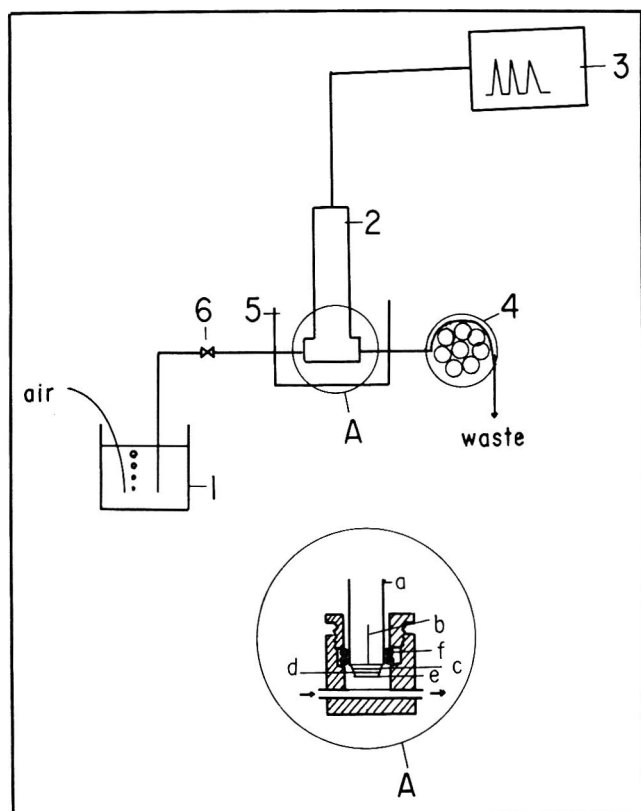
Fig. 1—Schematic diagram of the enzyme sensor system for hypoxanthine determination: 1. Buffer tank, 2. Oxygen electrode, 3. Recorder, 4. Peristaltic pump, 5. Thermostated bath, 6. Injection port, A: a. Oxygen electrode, b. Platinum cathode, c. Teflon membrane, d. Enzyme membrane, e. Cellulose acetate membrane, f. Rubber ring.

cysteine was transferred continuously to the sensor by a peristaltic pump. After the output current became steady, a 20–50 μl aliquot of the hypoxanthine solution or a fish extract solution was injected into the flow line and the current change was recorded. The maximum current decrease was used as a measure of Hx concentration. A conventional enzymatic measurement according to Ehira and Uchiyama (1969) was also employed.

RESULTS & DISCUSSION

Effects of immobilizing conditions on stability of the immobilized enzyme

The stability of immobilized enzymes is very important for the performance of biosensors. Therefore, the stability of the immobilized xanthine oxidase (XOD) was examined. It has been reported that free XOD was stable at 5°C for 5 months (Ehira and Uchiyama, 1969). The stability of the enzyme generally increases with immobilization. The ability of XOD immobilized at 5°C for 48 hr was higher than that immobilized at 25°C for 30 min as shown in Fig. 2, (a), (b). On the other hand, the stability of XOD was markedly improved when XOD was treated with a Tris-HCl buffer solution (0.05M, pH 8.4) containing glutaraldehyde (0.1%) and cysteine (0.1 mM) or histamine (0.1 mM) [Fig. 2 (c), (d)]. The presence of free cysteine was also effective for the stabilization of the immobilized XOD as shown in Fig. 2 (e). Therefore, cysteine and histamine might act as stabilizers for XOD. No decrease of XOD activity was observed for 7 hr (e).

Response of the enzyme sensor

After a current output was stabilized, a 20 μl aliquot of Hx solution (Hx 30 n mol) was injected into the flow line (Fig. 3, a↓). The output current began to decrease within 30 sec, and then a minimum current was obtained within 1 min. One assay could be completed within 100 sec. Another aliquot (Hx 1.25 n mol) was then injected and a small response was obtained (Fig. 3, b↓). The current decrease between the initial and the minimum currents was used as the measure of Hx concentration.
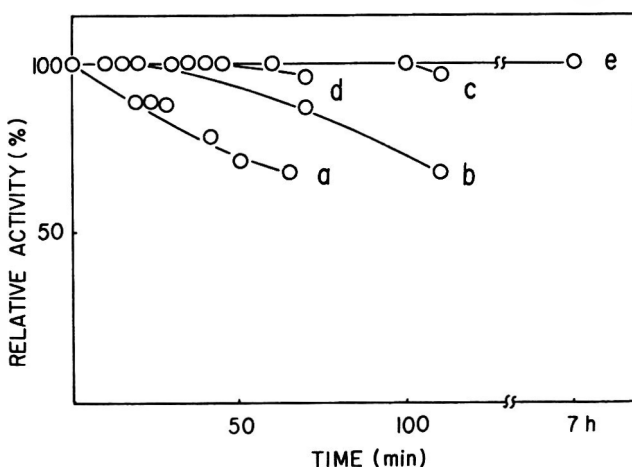
Fig. 2—Effect of immobilization and operational conditions on the activity of the immobilized enzyme. The activity of the membranes were assayed at pH 7.8, 32°C. The same amount of substrate (20 n mol in 20 μl) was used for the assay. (a) The membrane was immersed in xanthine oxidase solution (0.05M phosphate buffer, pH 7.8) at 25°C for 30 min. (b) The membrane was immersed in a xanthine oxidase solution at 5°C for 48 hr. (c) The membrane (b) was treated with a Tris-HCl buffer containing 0.1% glutaraldehyde and 0.1 mM cysteine. (d) The membrane (b) was treated with a Tris-HCl buffer containing 0.1% glutaraldehyde and 0.1 mM histamine. (e) The membrane (c) was used in the presence of cysteine (0.1 mM).

Effects of assay conditions on the response
of the enzyme sensor

Fig. 4 shows the effects of the flow rate on the response of the enzyme sensor. Below the flow rate of 1.2 ml·min^{-1}, the response was almost constant. However, it decreased considerably above 1.2 ml·min^{-1} possibly due to insufficient time for reaction of the substrate with the immobilized enzyme. Fig. 5 indicates the effects of the sample volume on the response of the sensor. When a volume of sample solutions was between 20 and 50 μl, the current decrease obtained was almost constant. The pH and temperature also affected the activity of the immobilized enzymes. The effects of pH and temperature on the current output of the sensor are shown in Fig. 6 and 7. The maximum response was obtained when the temperature and pH were approximately at 41.5°C and 7.7, respectively. However the long term stability of the immobilized enzyme depended on operational temperature. Therefore, the stability of the sensor was examined at 32°C and 37°C respectively (Fig. 8). No decrease of current output was observed when the sensor was used for 15 times at 32°C, while it gradually decreased to 70% of the initial response when it was used at 37°C. From these results, the optimum conditions for the assay by the sensor were determined as follows: flow rate 1.0 ml·min^{-1}, sample volume 20μl, temperature 32°C, pH 7.8. The sensor was able to withstand more than 100 assays at this conditions. Furthermore the storage stability of the sensor was examined. The sensor was stable for more than 30 days at 5°C.
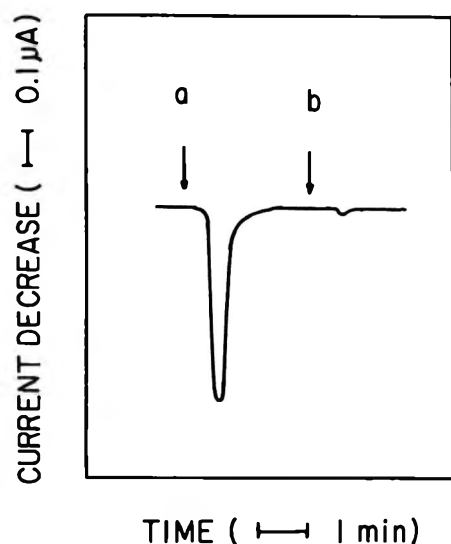
Fig. 3—Response of the enzyme sensor to Hx. A 20 μl aliquot of Hx (a:30 nmol, b:1.25 nmol) was injected and the output current was recorded. The flow rate, temperature, and pH were 1.0 ml·min^{-1}, 32°C, and 7.8 respectively.

Fig. 4—Effects of the flow rate on the current decrease. A sample volume was 20 μl containing 20 nmol Hx. Other conditions were the same as Fig. 3.

Fig. 5—Effects of the sample volume on the current decrease. Other conditions were the same as Fig. 4.
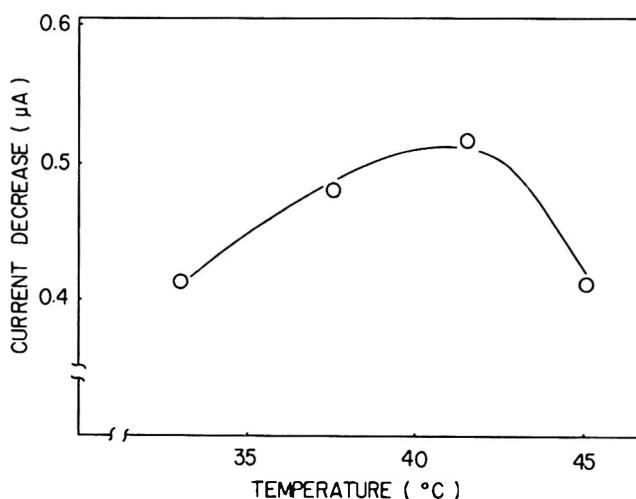
Fig. 6—Effect of temperature on the current decrease of the sensor. The sample volume was 20 μl. Other conditions were the same as Fig. 4.

Calibration curve

Standard sample solutions of Hx were applied to the enzyme sensor system. When a sample solution containing 20 nmol of Hx was injected, the current decrease obtained was 0.4 μA. A linear relationship was obtained in the range from 62.5 μM to 1.5 mM (1.25–30 nmol in 20 μl as shown in Fig. 9. The minimum concentration for determination was 8.5 mg of Hx per liter. The reproducibility of the current decrease was examined using the same sample. The current decrease was reproducible within +8% for a sample containing 136 mg·l^{-1} of Hx. The standard deviation was ±0.2 mg·l^{-1} in 100 experiments.

Comparison of the enzyme sensor system and the conventional enzymatic assay method

Sample solutions containing Hx were prepared from sea bass, saurel, mackerel, yellowfish, and flounder, Hx concentration was determined by both the enzyme sensor method and the conventional enzymatic assay method.

Fig. 7—pH profile of the enzyme sensor. The sample volume was 20 μl. Other conditions were the same as Fig. 4.
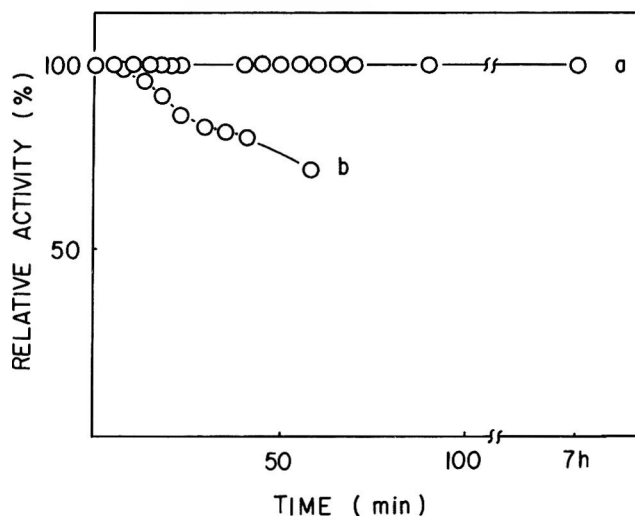
Fig. 8—Effects of operational temperature on the stability of the enzyme sensor. Measurements were performed at 32°C (a) or at 37°C (b). Other conditions were the same as Fig. 4.

Fig. 9—Calibration curve of the enzyme sensor for Hx. Measurements were done at the conditions described in Fig. 4.
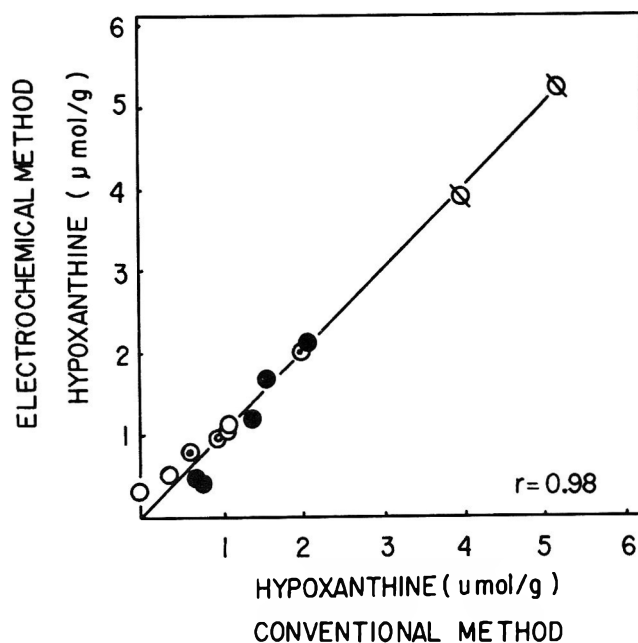
Fig. 10—A correlation between the Hx concentrations determined by the enzyme sensor method and by the conventional enzymatic assay method. (ʘ) sea bass, (●) saurel, (ο) mackerel, (◉) yellowfish, (ↄ) flounder.

Since xanthine oxidase has been shown to react with adenine and aldehydes (Greenlee and Handler, 1964), the contents of both compounds in the sample solution were assayed. However, the content of adenine in a sample solution was negligibly low (Jones and Murray, 1960; Saito, 1961). Furthermore, formaldehyde and acetaldehyde were not detected by a silica gel thin-layer chromatography.

As shown in Fig. 10, a linear correlation was observed between the values determined by the both methods. The correlation coefficient was 0.98. These results suggest that the sensor proposed is an economical and reliable method for analysis for Hx in fish meats. However, the Hx content in fish meats depends on species. For example, the Hx content is quite high in flatfish, *Hippoglossoides dubius (Schmidt)*, white croaker, *Argyrosomus argentatus (Houttuyn)*, big-eye, *Scombrops boops (Houttuyn)*, relatively high in pacific halibut, *Hippoglossus stenolepis Schmidt*, hairtail, *Trichiurus lepturus Linnaeus*, shark, *Prionace glauca* (Linnaeus) and low in skipjack, *Katsuwanus pelamis* (Linnaeus), yellow fin tuna, *Neothunnus albacore (lowe)*. (Ehira and Uchiyama, 1973). Therefore the Hx content alone cannot be used as an indicator of fish freshness. Further studies in our laboratory are directed towards developing a biosensor which can simultaneously analyze Hx and HxR.

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Electrophoresis and Chromatography of Heat-Treated Plain, Sugared and Salted Whole Egg

S. A. WOODWARD and O. J. COTTERILL

──────ABSTRACT──────

The effects of heat treatments on the proteins in plain, 10% sugared and 10% salted whole egg were studied by polyacrylamide disc gel electrophoresis and diethylaminoethyl-Sephacel ion exchange chromatography. Electrophoretograms of products heated from 57–87°C for 3.5 min showed a wide range of protein stability. Livetins and some globulins were most heat sensitive, while conalbumin and oval-bumin were most stable. Sugar and salt increased heat stability of proteins by 1.6 and 7.9°C, respectively. Heat sensitive proteins were most stabilized by sugar and salt. Heating whole egg and sugared whole egg changed the chromatograms substantially, while heating salted whole egg caused fewer changes.

INTRODUCTION

THE SENSITIVITY of egg components to heat denaturation has been reported by a number of researchers. Polyacrylamide gel electrophoresis (PAGE), ion-exchange chromatography (IEC), and differential scanning calorimetry (DSC) are important techniques used to determine the heat stability of specific egg proteins, both in native egg systems and as isolated proteins in buffered solutions. Chang et al. (1970) used PAGE to separate the proteins of albumen, yolk and centrifuged whole egg into 12, 19 and 16 bands, repectively. They compared electrophoretic patterns from raw products to those heated at 61.7–65.6°C for 3 min. Donovan et al. (1975) observed three peaks in DSC thermograms of egg white at 65, 74 and 84°C. They indicated these to be denaturation temperatures of conalbumin, lysozyme and ovalbumin, respecitvely. McKinney (1977) developed a diethylaminoethyl (DEAE)-cellulose IEC procedure for separating whole egg proteins. This was used in conjunction with PAGE to determine the effects of processing on whole egg, including pasteurization (60°C for 3.5 min). Dixon and Cotterill (1981) used PAGE and IEC to determine the response of yolk proteins to heat (54–84°C, 3.5 min). Matsuda et al. (1981) conducted a similar study of egg white (54–90°C, 3 min) using vertical flat-sheet PAGE. The information from these studies on the thermal response of proteins in native egg systems compares very well with that from studies of isolated egg proteins.

The main objective of this study was to determine the sensitivity of whole egg proteins to heat treatments ranging from pasteurization to coagulation. Past studies of whole egg have focused on pasteurization temperatures, at which little damage was reported. The effects of higher temperature treatments have not previously been reported for whole egg. An additional objective was to determine the effect of added sugar and salt on heated whole egg.

MATERIALS & METHODS

Egg samples

Day-old eggs were obtained from a genetically controlled flock of Single Comb White Leghorns. Whites and yolks from six or more eggs were blended in a Waring Blendor without incorporation of air,

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sufficient to provide a homogeneous mixture, then filtered through four layers of cheesecloth. The pH of the mixture was 7.2.

Salt (NaCl) and sugar (sucrose) were added to the egg mixture at a concentration of 10% on a weight:weight basis. This concentration is typical of commercial use, and is equivalent to 1.89M NaCl and 0.31M sucrose in whole egg. The egg samples will be hereafter designated WE, SuWE and SaWE for plain, 10% sugared and 10% salted whole egg, respectively.

Heat treatments

Samples for electrophoresis were prepared by placing 2 ml of WE, SuWE, or SaWE in thermal death time tubes (13.0 cm × 0.7 cm i.d.) with a hypodermic syringe. Tubes were immersed in a water bath at 48°C for 1 min, then transferred immediately to another bath at the treatment temperature (57–87°C in increments of 3°C) for 3.5 min, followed by 1 min in an ice bath. Treated samples were refrigerated overnight prior to electrophoresis. Upon removal from refrigeration, samples were diluted with 2 ml deionized water and mixed on a Vortex mixer. Coagulated samples were broken with a stainless steel rod and mixed. All tubes were then centrifuged at 1000 rpm for 10 min to remove insoluble precipitates or clumps.

The laboratory tubular pasteurization unit described by Cotterill and Glauert (1969) was used in heat treatment of WE, SuWE and SaWE samples for chromatography. Approximately 500 ml of product were pumped through the pasteurizer for 1 min at 48°C, 3.5 min at the treatment temperature, and 1 min at 0°C. An aliquot collected from the middle portion of the run was stirred to homogeneity on a magnetic stirrer and diluted 1:1 (wt:wt) with the starting chromatographic buffer (0.05M tris-HCl, pH 6.8) for application to the column. All SaWE samples were dialyzed 48 hours against this buffer prior to dilution to remove salt.

Electrophoresis

PAGE was performed with a Model 1200 PAGE Canalco unit (Miles Laboratories, Inc., Elkhart, IN) as described by Dixon and Cotterill (1981). The 7% polyacrylamide separating gel resolves proteins ranging from 10^4–10^6 molecular weight. Low pH gels (loading and stacking gels, pH 5.0, separating gel, pH 4.3) and buffers for reversed polarity electrophoresis (RPAGE) were prepared according to instructions from Canalco. RPAGE was used in the separation of lysozyme. A 5-μl sample was placed on each gel, containing ca 300 μg protein.

Chromatography

DEAE-Sephacel (Pharmacia Fine Chemicals, Uppsala, Sweden) supplied in a pre-swollen form was equilibrated with the starting buffer, 0.05M tris-HCl at pH 6.8. Excess buffer was decanted and the slurry was packed into a 2.0 × 30 cm column at a flow rate of 60 ml/hr. The column was layered with 2 ml of diluted sample containing ca 120 mg protein.

A continuous salt gradient of concave shape (See Table 3) was formed by a 9-chamber MRA Gradient Maker (MRA Corp., Clearwater, FL) using five buffers of 200 ml each at pH 6.8 (0.05M tris-HCl) with NaCl concentrations of 0, 0, 0.10, 0.30 and 0.60M, respectively. Peaks were eluted at a constant flow rate of 50 ml/hr with a Model 312 Metering Pump (ISCO, Lincoln, NE). Absorbance of eluant was monitored at 280 nm by a Model UA-2 Ultraviolet Analyzer (ISCO, Lincoln, NE). Fluorescence was detected by exciting the eluant stream at 254 nm and measuring emission at 350 nm in a Model 111 Fluorometer (Sequoia-Turner Corp., Mountainview, CA). Fractions of 20 ml were collected, dialyzed, freeze-dried and rehydrated for identification by PAGE and RPAGE.

RESULTS & DISCUSSION

Physical appearance

The appearance of heated WE began to change at 60°C, becoming slightly opaque. Tubes of WE became increasingly opaque from 63–72°C. A soft coagulum formed at 75°C, with harder curds forming at higher temperatures. The appearance of heated SuWE was similar to WE, with the same changes occurring at 3°C higher temperatures. SaWE was drastically different. Upon addition of salt, the raw liquid became clear yellow with a much lower viscosity, indicative of increased protein solubilization. At 75°C slight cloudiness developed. Opaqueness increased through 81°C, with a soft gel forming at 84 and a firm curd at 87°C.

Electrophoresis

Electrophoretic separation of WE gave 20 distinguishable protein bands, 11 attributed to egg white, 6 to yolk and 3 with mobilities not characteristic of either (see Fig. 1). These are probably albumen-yolk protein interactions. Bands were identified according to previous studies (Chang et al., 1970; McKinney, 1977; Galyean and Cotterill, 1979; Dixon and Cotterill, 1981; Matsuda et al., 1981), and by comparison to electrophoretograms of albumen, yolk and isolated proteins. Not all proteins in WE can be separated by PAGE. The gel pore size (7% polyacrylamide gel) excludes proteins larger than 1×10^6 MW. These include ovomucin of egg white and some lipoproteins. Several known yolk protein bands were not discernible due to overlap with the more pronounced egg white proteins. Conalbumin masked γ-livetins and ovoinhibitor, while ovalbumins masked α-livetins and phosvitins. Egg white globulins and yolk β-livetins appeared in the same zone and were difficult to distinguish. The albumen proteins were more prominent due to their greater concentration.

Table 1 summarizes the major protein bands in order of their electrophoretic appearance. The temperatures listed are the result of direct visual examination of electrophoretic gels for band intensity and location. Major changes in electrophoretograms appeared at temperatures above 63, 66, and 75°C for WE, SuWE and SaWE, respectively.

The stability of lysozyme (63°C in WE, 60°C in SuWE, 72°C in SaWE, pH 7.2, see Fig. 2) is generally lower than that reported by others. Donovan et al. (1975) reported a denaturation temperature (T_d) of 74.0°C for lysozyme in egg white at pH 7.0. Addition of 10% sucrose increased the T_d by 2°C. However, in our study lysozyme was 3°C more sensitive in SuWE than in WE. Johnson and Zabik (1981) found that an isolated lysozyme preparation coagulated at 81.5°C at pH 8. Donovan et al. (1975) obtained T_d's of 75.0 and 72.5 for isolated lysozyme at pH 7 and 9, respectively. Privalov and Khechinashvili (1974) showed that the heat stability of lysozyme is strongly dependent on pH. They reported T_d's ranging from 48°C at pH 1.5 to 78°C at pH 4.5. The disappearance of lysozyme from RPAGE electrophoretograms at low temperatures in this study may be due to interactions with yolk components (Cunningham and Cotterill, 1971). Galyean et al. (1972) stated that lysozyme was inactivated by yolk components and partially reactivated by salt. They suggested that electrostatic interactions were responsible for loss of activity. These interactions could cause a loss of heat stability similar to the effect of pH. The increased heat stability with addition of 10% salt supports this hypothesis.

The immobile band (top of gels, Fig. 1) is comprised of the high molecular size lipoproteins. The disappearance of protein from this region coincided with the point of coagulation for all three products, when the aggregates became insoluble and were removed by centrifuging.

Ovomacroglobulin (OMG) is a large egg white protein which barely migrates into the gel due to its size (650,000 MW). It was the most heat labile protein in WE (60°C), but its stability improved to 63 and 78°C in SuWE and SaWE, respectively. Matsuda et al. (1981) reported the presence of OMG in egg white up to 66°C, with a heating time of 3 min. Donovan et al. (1969) reported a transition temperature of 60°C for OMG at pH 7. They determined that it breaks into subunits by heating or by exposure to denaturants. We recovered OMG from PAGE and subjected it to the action of β-mercaptoethanol (37°C for 30 min), which breaks disulfide bonds. The resultant subunits when applied to PAGE migrated to the ovalbumin region, which is consistent with the reported isoelectric point (pI) of OMG. Thus, in the WE samples, the ovalbumin bands prevented the subunits from being seen in electrophoretograms at temperatures above the initial disappearance temperature.

The heat stability of lipovitellins coincides with that of

Table 1–Highest temperature of electrophoretic band stability

Band no.	Identity	Relative mobility	WE Plain (°C)	WE + 10% Sucrose (°C)	ΔT[a]	WE + 10% NaCl (°C)	ΔT[a]
	RPAGE						
	Lysozyme	0.79–0.84	63	60	(−3)	72	(9)
	PAGE						
1	Immobile	0	72	72	(0)	87	(15)
2	Ovomacroglobulin	0.02	60	63	(3)	78	(18)
3	Lipovitellins	0.08–0.19	72	72	(0)	87	(15)
4	Unidentified	0.26	69	72	(3)	75	(6)
5	Conalbumin	0.27–0.32	78	81	(3)	78	(0)
6 & 7	Ovoinhibitors	0.29, 0.32	78	78	(0)	78	(0)
8 & 9	G_2 globulins	0.35, 0.39	81	81	(0)	78	(−3)
10	G_{3A} globulin	0.45–0.50	63	66	(3)	75	(12)
11 & 12	G_{3S} globulins	0.52, 0.55	63	66	(3)	72	(9)
13–16	β-livetins	0.58, 0.60, 0.62, 0.64	63	69	(6)	72	(9)
17	G_{3B} globulin	0.66–0.68	72	72	(0)	78	(6)
18	Ovalbumin A_2	0.73–0.80	75	78	(3)	81	(6)
19	Ovalbumin A_1	0.81–0.90	75	78	(3)	81	(6)
20	Tracking Dye	1.00	—	—	—	—	—
Avg. Temperature			69.9	71.5		77.8	
Avg. Protective Effect					(1.6)		(7.9)

[a] Protective effect of sugar and salt shown in parentheses.

WHOLE EGG

omg

lipovitellins

conalbumin

γ - livetins

G₂

G₃ₐ

β - livetins

globulins

G₃ₛ

G₃ᵦ

ovalbumins

α - livetins

phosvitins

dye

WHOLE EGG + 10% SUCROSE

omg

lipovitellins

conalbumin

γ - livetins

G₂

G₃ₐ

β - livetins

globulins

G₃ₛ

G₃ᵦ

ovalbumins

α - livetins

phosvitins

dye

WHOLE EGG + 10% NaCl

omg

lipovitellins

conalbumin

γ - livetins

G₂

G₃ₐ

β - livetins

globulins

G₃ₛ

G₃ᵦ

ovalbumins

α - livetins

phosvitins

dye

Cᵤ 57 60 63 66 69 72 75 78 81 84 87

Temperature, °C

Fig. 1—Polyacrylamide gel electrophoretograms of plain, 10% sugared and 10% salted whole egg heated at temperatures from 57–87° C for 3.5 min. C_U = unheated control.
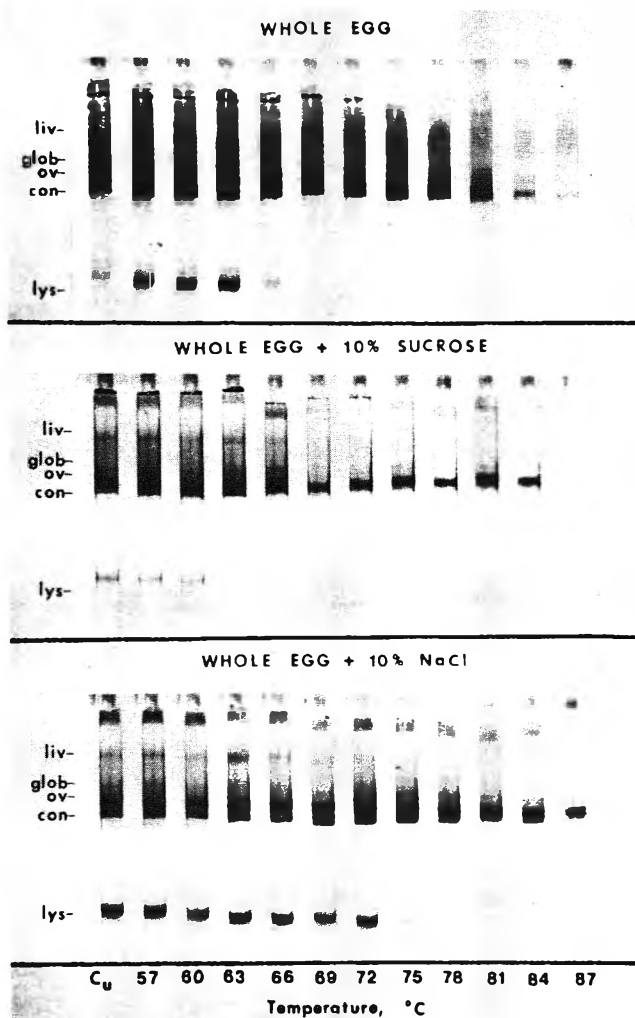
Fig. 2—Reversed polarity electrophoretograms of plain, 10% sugared and 10% salted whole egg heated at temperatures from 57—87°C for 3.5 min. Liv = livetins, glob = globulins, ov = ovalbumin, con = conalbumin, lys = lysozyme, C_u = unheated control.

Table 2—Identification of chromatographic peaks

A.	Lipoproteins (high MW)
B.	Lysozyme
C.	Unidentified EY protein (γ-livetin)
D.	Unidentified EY protein
E.	Conalbumin + ovoinhibitors + G_2 globulins
F.	Unidentified globulin
G.	G_{3A} globulin + β-livetins
H.	$G_{3A,S}$ globulins + β-livetins
I.	$G_{3S,B}$ globulins + β-livetins
J.	Ovalbumin A_3
K.	Ovalbumin A_2
L.	Ovomacroglobulin
M.	Ovalbumin A_1
N.	Phosvitin + Lipovitellin
O.	Lipovitellin + phosvitin
P.	α-livetin
Q.	Unidentified EY protein
R.	Flavoprotein

Table 3—Calculated NaCl concentration in eluant

Volume (ml)	[NaCl] (M)
0	0
100	0
200	0.02
300	0.05
400	0.10
500	0.15
600	0.22
700	0.29
800	0.38
900	0.49

the immobile proteins. This suggests that these proteins could have the same T_d as the larger yolk lipoproteins, and could participate in the coagulation mechanism. The temperatures of stability are consistent with the results of Dixon and Cotterill (1981), in which salt stabilized lipovitellin in heated egg yolk.

Conalbumin had a markedly increased heat stability (78°C in WE) compared to that reported in egg white by many researchers (60–65°C, Chang et al., 1970; Donovan et al., 1975; Hegg et al., 1978; Nakamura et al., 1979). This is due to the iron-conalbumin complex, with iron coming from the yolk (Cunningham and Lineweaver, 1965). Donovan and Ross (1975) reported the presence of four forms of conalbumin with different heat stabilities: conalbumin, T_d=63°C, two iron-conalbumin intermediates with one iron atom each, with T_d's of 67.5 and 76.5°C, and Fe_2-conalbumin, T_d=83.5°C. The two sharp bands directly below conalbumin appear to be ovoinhibitors. Matsuda et al. (1981) detected three similar bands in egg white as the conalbumin band disappeared due to heat denaturation. These were stable to about 72°C in egg white at pH 7.

The two bands in the G_2 globulin zone had different mobilities than the single G_2 band encountered in egg white. This may be the result of interaction with yolk proteins. The stability of G_2 (81°C) and the relative instability of G_{3A} globulin (63°C) are in agreement with the

results of Matsuda et al. (1981) for these proteins in egg white, and Chang et al. (1970) for whole egg. The β-livetins showed less stability in whole egg than in yolk (Dixon and Cotterill, 1981).

Ovalbumin is one of the more heat stable proteins in whole egg. Donovan et al. (1975) reported its stability in egg white at 84°C, with no change due to pH. Hegg et al. (1979) found that an ovalbumin preparation coagulated at 78°C. The change in band intensity for ovalbumin follows a similar pattern in the paper of Matsuda et al. (1981). Thus, it appears that ovalbumin has the same heat stability in whole egg as in egg white.

Sucrose and salt (Table 1) increased the heat stability of the protein in whole egg by 1.6 and 7.9°C, respectively, based on the average increase in electrophoretic band stability. Donovan et al. (1975) reported that 10% sucrose in egg white increased protein heat stability by 2°C. They suggested that the effect was due to reduced water activity. Salt increases the ionic strength of the liquid surrounding the proteins and its stabilizing effect is due to this ionic change. In the case of OMG, the protein was stabilized against breakdown into subunits by heat. Other proteins were stabilized against denaturation. Nakamura et al. (1979) found that anions stabilized conalbumin to heat, with nitrates, chlorides and sulfates being capable of protecting against heat damage. The protection afforded by salt to proteins was highly variable. Those with greatest heat sensitivity were protected to a higher degree, while the more stable proteins showed little or no protection.

Chromatography — Whole egg

DEAE-Sephacel chromatography resolved WE into 18 distinguishable peaks (Fig. 3 — unheated), compared to 11 peaks by the DEAE-cellulose procedure of McKinney (1977). Peaks identified by PAGE and RPAGE generally showed multiple protein bands (see Table 2), indicating incomplete resolution. Protein resolution is based on net charge, molecular size and homogeneity of proteins. DEAE-
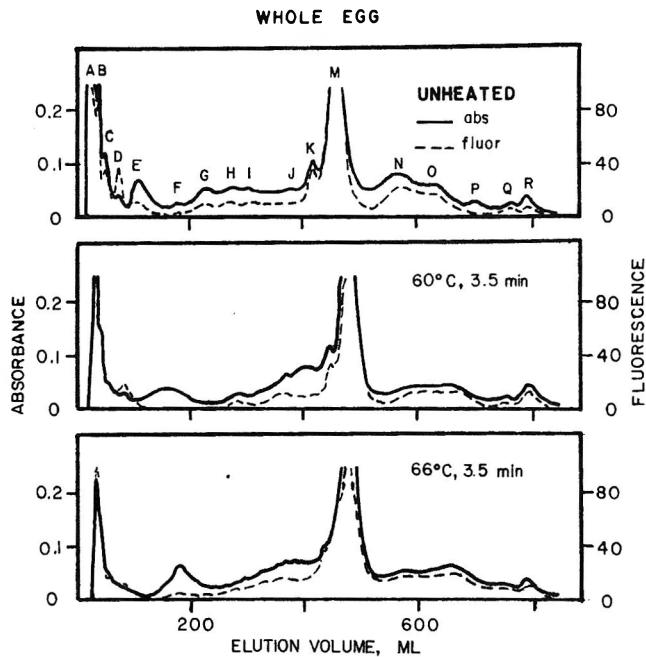
Fig. 3—DEAE-Sephacel chromatograms of unheated and heated (60 and 66°C for 3.5 min) whole egg.

Fig. 4—DEAE-Sephacel chromatograms of unheated and heated (61.1 and 67.1°C for 3.5 min) whole egg containing 10% sugar.

Fig. 5—DEAE-Sephacel chromatograms of unheated and heated (63.3 and 69.3°C for 3.5 min) whole egg containing 10% salt.

Sephacel is a beaded cellulose with an exclusion limit for globular proteins of 1×10^6 Daltons. Proteins of larger size do not exchange with the DEAE sites. All smaller proteins are separated on a basis of net charge. Peak broadening or loss of resolution occurs with increasing molecular size below the exclusion limit. In the chromatogram, ovalbumins (peaks J, K and M, MW 45,000) were better resolved than conalbumin (peak E, MW 80,000) and lipovitellin (peaks N and O, MW 400,000).

Many of the proteins of whole egg have been well characterized with respect to molecular size and pI (Powrie, 1977). Proteins were generally eluted in order of decreasing pI. Peaks A and B, eluted at the end of the void volume, contained all proteins which were not exchanged. A large lipoprotein fraction was present as evidenced by the yellow floating lipid layer in the eluate. Analysis of this fraction by PAGE showed about 20 weak bands, typical of the pattern of native egg yolk (Chang et al., 1970). Apparently traces of all yolk proteins were eluted as an aggregate consisting mainly of a high molecular weight lipoprotein. One lipoprotein fraction was occasionally eluted in the region of peaks F through J along with lysozyme. The broadness of the eluted lipoprotein suggests a protein in the molecular size range of 1×10^6 Daltons, the exclusion limit. It was too large to penetrate the separating gel in PAGE, but formed a floating yellow layer in the eluate. Its pI is estimated to be between 5.5 and 6.5 OMG appeared either in peak A or peak L as confirmed by PAGE. Because OMG is near the exclusion limit of Sephacel, its ability to exchange with the DEAE sites is limited. The reported pI of 4.5–4.7 suggests its elution with the ovalbumins (pI-4.6), but it was most often present with peak A.

Lysozyme was also present in peaks A and B, as expected from its pI of 10.7. It was also occasionally eluted in peaks F through J as carried there by a lipoprotein as mentioned above. This is further evidence for a lysozyme-yolk protein interaction as discussed earlier. Parkinson (1967) reported the loss of lysozyme from soluble proteins of WE and its presence among insoluble proteins. In a DEAE-cellulose separation of whole egg, his initial peak contained mostly insoluble proteins. He suggested these were the result of yolk-albumen protein interactions, since the peak did not appear in chromatograms of egg white or egg yolk.
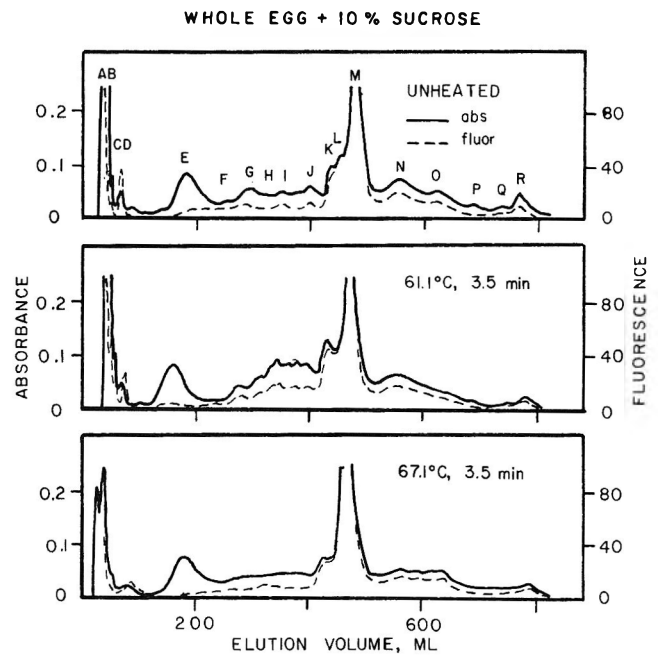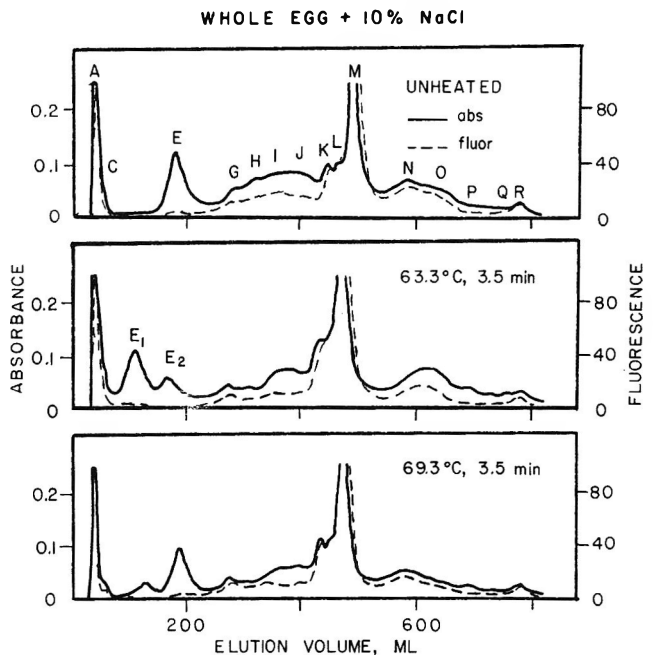
Peaks C and D were unidentified egg yolk proteins, distinct because of their high fluorescence relative to absorbance. Peak E was eluted as a brown band, the major constituent of which was conalbumin. Analysis by PAGE revealed six bands, including conalbumin, globulins and ovoinhibitors. Conalbumin binds one or two ferrous ions per molecule, giving rise to three possible forms of conalbumin with zero, one or two ions complexed and corresponding pI's of 6.73, 6.25 and 5.78, respectively (Wenn and Williams, 1968). Peak E often separated into two peaks of varying size (see Fig. 5). The probable cause for this peak separation was the charge difference based on the degree of iron saturation.

The region of peaks F through I contained a mixture of egg white globulins and egg yolk β-livetins. The most promi-

nent electrophoretic band was G_{3A} globulin, which appeared most strongly in peak G and also in F, H and I. The poor resolution and high baseline in this region are indicative of the heterogeneity of these proteins.

Peaks J, K and M correspond to ovalbumins A_3, A_2 and A_1, the most abundant proteins in egg white. Separation into three peaks is based on the charge effect of zero, one, or two phosphates bound per protein molecule. The reported proportion of $A_3:A_2:A_1$ as 3:12:85 is confirmed by the relative size of these three peaks (Perlmann, 1952). OMG was the constituent of peak L as discussed earlier. It was seldom resolved in the WE chromatogram, but appeared regularly in the chromatograms of SuWE and SaWE.

Peaks N and O contained both phosvitin (three electrophoretic bands) and lipovitellin, which are complexed in native yolk (Radomski and Cook, 1964). Peak P was identified as an α-livetin while Q was an unidentified yolk protein. Flavoprotein with a pI of 4.1 was the main component of peak R.

The chromatograms in Fig. 3–5 were not entirely reproducible. Some of the reasons for variations have been discussed. The WE pattern (Fig. 3, unheated) was refined from a series of experiments until reproducibility was good. The other chromatograms (Fig. 3–5) represent three to six replications (average 4) and those shown were the most typical.

Heat treatments

The chromatograms give a semi-quantitative measure of the response of WE proteins to heat treatment (Fig. 3). At the pasteurization temperature of 60°C, many peaks were reduced in size, while no changes were detected in PAGE electrophoretograms. The reduction of peak A was related to precipitation of high MW components induced by heat, with probable interactions of yolk and albumen proteins. The reduction of peak E could indicate the denaturation of iron-free conalbumin. Major changes in the globulin and livetin peaks also occurred, as well as the lipovitellin-phosvitin peak N. At 66°C, more reductions of the same proteins occurred, along with some loss of ovalbumin peak resolution. The increase in the peak at the region of E and F is not well understood. The peak contained a high concentration of conalbumin and globulin G_2. It is possible that at 66°C iron-binding by conalbumin was enhanced. The iron-binding protein of yolk, phosvitin, began to lose electrophoretic stability at 69°C, according to Dixon and Cotterill (1981). Note also the reduction of phosvitin peak N in the chromatogram. The destabilization of phosvitin could promote enhancement of iron transfer to conalbumin, resulting in its greater heat stability.

The chromatogram for unheated SuWE was very similar to that of WE, with the addition of OMG at peak L (Fig. 4). Heat treatments at 61.1 and 67.1°C modified peak heights, but with somewhat less effect than for WE. Conalbumin was much better protected at both temperatures. This may be due to enhanced iron binding. At 61.1°C, the region of peaks G through I reflected the late elution of a lipoprotein-lysozyme complex as discussed earlier. Much more protein damage was evident at 67.1°C, with a flattening of peaks F through J (globulins and livetins) and a reduction of OMG and peaks N and R.

The chromatograms for SaWE were obtained after dialysis of both heated and unheated samples to remove NaCl (Fig. 5). The differences in the chromatograms for unheated SaWE and WE can be partially attributed to precipitation of proteins during dialysis. The main differences were the narrowing of peak A, loss of B and D, poorer resolution of G through J, and narrowing of M. Heating to

63.3°C caused little actual damage. The appearance of an additional peak at E was not due to heat, but appeared as an extra conalbumin fraction as discussed earlier. The conalbumin peaks E_1 and E_2 were not predictable in terms of which would be larger or whether they would appear as one or two peaks. The stability to heat is due to the complexing of iron by the protein. The chromatogram of SaWE at 69.3°C showed decreases in peak A and the N and O peaks. Note the presence of OMG (peak L). Salt stabilized OMG to 78°C according to PAGE results.

The chromatograms gave a more quantitative measure of protein stability to heat than visual appraisal of electrophoretograms. The extent of heat damage to unprotected proteins in WE is contrasted to the protection offered by 10% sugar and salt. The development of a chromatogram for WE has enabled an evaluation of stability of specific proteins in a native system to processing conditions.

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On Sodium Chloride Action in the Gelation Process of Low Density Lipoprotein (LDL) from Hen Egg Yolk

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──────────ABSTRACT──────────

Gelation of 40% LDL solution with 1~10% NaCl was inhibited during frozen storage at −20°C and −25°C. Frozen storage of LDL solutions with more than 4% NaCl at −30°C, −40°C and −60°C induced the gelation, whereas gelation was inhibited by addition of 1 and 2% NaCl at temperatures lower than −30°C. Differential scanning calorimetry analyses revealed that when NaCl acts as an inhibitor of gelation, it increased the unfrozen water in the LDL solutions through formation of LDL-water-NaCl complex where the water is hardly frozen; and when it acts as an accelerator of gelation, it promoted removal of water from the complex.

INTRODUCTION

IRREVERSIBLE INCREASE in viscosity and decrease in solubility of egg yolk induced by freezing and thawing has been termed gelation. Many investigators have shown that low density lipoprotein (LDL) participates in yolk gelation (Powrie et al., 1963; Saari et al., 1964; Mahadevan et al., 1969; Sato and Takagaki, 1976; Wakamatu et al., 1982a). Several factors, such as frozen storage time and temperature (Powrie et al., 1963; Wakamatu et al., 1981a), freezing and thawing rate (Lopez et al., 1954; Powrie, 1968) and addition of salt and sugars (Powrie et al., 1963; Palmer et al., 1970; Sato and Aoki, 1975; Wakamatu et al., 1980) are known to affect the extent of gelation. However, the mechanism or process of gelation has hardly been elucidated.

Since Moran (1925) reported that yolk gelation occurred on frozen storage below −6°C and supercooling of shell egg at −11°C for 7 days did not cause the alteration of yolk fluidity, it has been generally recognized that transformation of a definite amount of water from liquid to ice state is a prerequisite for yolk gelation.

Moran (1925) suggested that concentrated salts in frozen yolk due to transformation of water to ice might cause the irreversible precipitation of lipoprotein. Soliman and van den Berg (1971) assumed that LDL aggregation might be attributed to not only salt concentration but also pH change in the unfrozen phase. Chang et al. (1977) suggested that granules of yolk are disrupted during freezing by a high concentration of soluble salts in the unfrozen phase. They also found that sodium chloride, added to yolk plasma (containing only low-density lipoproteins and globulins), inhibited gelation. On the other hand, Sato and Aoki (1975) demonstrated that LDL gelation was inhibited by addition of salts when it was frozen at higher than the eutectic temperature of coexisting salt. From this finding, they suggested that the salt concentration itself was not a key factor in gelation, and that LDL aggregation might be caused by progressive removal of water from LDL induced by ice formation. Kamat et al. (1976) have suggested that the inhibitory effect of salt on LDL gelation, which was exhibited on freezing at above the eutectic temperature of

the salt, might be attributed to solvating the adsorbed layers stabilizing LDL particles during freezing. Although Sato and Aoki (1975) showed that the extent of LDL gelation with sodium chloride depended remarkably on the sodium chloride concentration and frozen-storage temperature, no experimental evidence has been advanced to provide a reasonable explanation for this sodium chloride effect.

The purpose of this study is to verify the action of sodium chloride on the LDL gelation by tracing the process of water transformation during freezing and thawing with a differential scanning calorimeter.

MATERIALS & METHODS

Materials

Infertile eggs obtained from Zex hens were used within 2 days after laying. LDL and granule fractions were prepared as described in the previous study (Wakamatu et al., 1982a), and then exhaustively dialyzed against distilled water for 3 days at 5°C. Bovine serum albumin (BSA) was obtained from Wako Pure Chemical Industries Co. of Japan.

Freezing treatment

Sodium chloride was added to 40% LDL and 40% granule fraction in its concentration range of 1~10% (w/w) and stored overnight at 5°C. Fifteen grams of the sample containing a given amount of sodium chlordie were frozen and stored in the range from −20°C to −60°C for 72 hr after sealing in a stainless steel vessel (3 x 3 x 3 cm), and then thawed at 25°C for 30 min.

Apparent viscosity measurement

The apparent viscosity was measured on a model RVT, Brookfield synchro-lectric viscometer at 25°C.

Turbidity measurement

The absorbance at 660 nm of a diluted sample (0.5% or 1% solid) with 1M sodium chloride was measured by electrophotometer (model 200-20, Hitachi Seisakusho).

Determination of unfrozen water

Differential scanning calorimetry (DSC) analyses were performed on a Model SSC-540 Dainiseikosha differential scanning calorimeter. Transition heats of a 40~60 mg of sample in an aluminum vessel (diameter 4 mm, height 8 mm) during freezing and thawing were measured by two methods described below.

Method I. A sample was scanned from room temperature to −55°C at the rate of 0.6°C/min, and then from −55°C to room temperature at the same rate.

Method II. A sample was scanned from room temperature to −21°C at the rate of 0.6°C/min and held at −21°C for an hour after the appearance of an exothermic peak. It was then scanned from −21°C to room temperature at the same rate.

The areas under the recorded peaks of the thermal transition were found by weighing the paper; the weights were compared with the weight of a standard area of known enthalpy (18.5 mV, 600 sec). The amounts of frozen water were calculated by dividing the measured exothermic heat and endothermic heat by transition heat of pure water. In the previous study they were found to be 78.0 cal/g water in cooling and 81.1 cal/g water in heating (Wakamatu and Sato, 1979). The unfrozen water was calculated by subtracting the frozen water from the whole water in a sample. The tempera-

ture measured by differential scanning calorimeter was corrected by setting the melting point of pure water and mercury as 0°C and −38.9°C, respectively.

Determination of protein

After digestion of a sample by heating in sulfuric acid, nitrogen was determined by the Nessler method. Protein was given by multiplying the nitrogen value by 6.25.
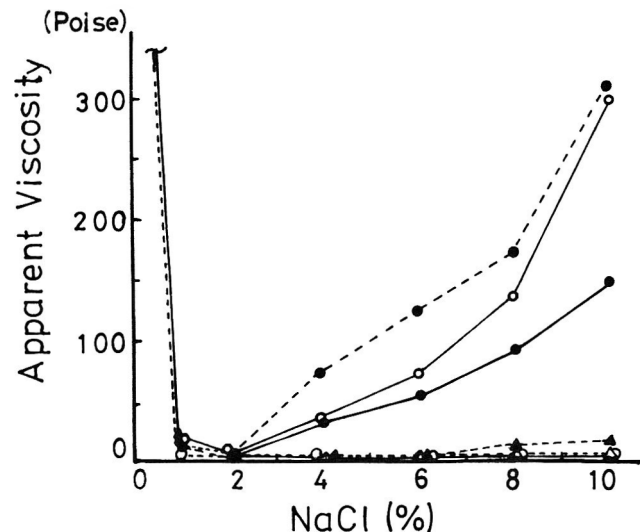
Fig. 1—Sodium chloride effect on apparent viscosity of frozen-thawed LDL. Freezing temperature: o····o, unfrozen; △——△, −20°C; ▲···▲, −25°C; •——•, −30°C; •····•, −40°C; o——o, −60°C. Fifteen grams of LDL solution with NaCl was frozen and stored at each temperature for 72 hr. Each point is the mean value for duplicate measurement.
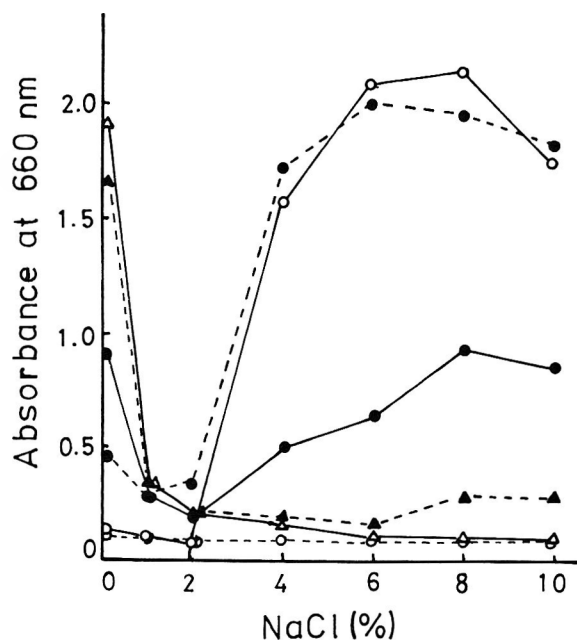
Fig. 2—Sodium chloride effect on turbidity of frozen-thawed LDL. Freezing temperature: o····o, unfrozen; △——△, −20°C; ▲···▲, −25°C; •——•, −30°C; •···•, −40°C; o——o, −60°C. Fifteen grams of LDL solution with NaCl was frozen and stored at each temperature for 72 hr. Turbidity was measured after dilution of the LDL solution to 1% with 1M NaCl. Each point is the mean value for duplicate measurements.

RESULTS

Effect of sodium chloride on viscosity and turbidity of frozen-thawed LDL

The effect of sodium chloride on the viscosity and the turbidity change of LDL solution during frozen storage at −20°C, −25°C, −30°C, −40°C, and −60°C is shown in Fig. 1 and Fig. 2. The frozen storage at −20°C and −25°C had almost no effect on the viscosity of LDL solution with 1~10% sodium chloride. In the range of more than 4% sodium chloride, the viscosity of frozen-thawed LDL solution increased with the increase in the soidum chloride amount during frozen storage below −30°C, whereas the viscosity change of LDL solution with 1% and 2% sodium chloride almost never occurred during frozen storage below −30°C. The turbidity of frozen-thawed LDL without salts decreased as frozen storage temperature fell, and the turbidity increment almost never was observed during frozen storage at −60°C (Fig. 2). The effect of frozen storage temperature on the viscosity of LDL without salts could not be exhibited clearly, because LDL without salts showed a high viscosity even before freezing (Fig. 1). These effects of sodium chloride on the viscosity and turbidity of LDL solution at each temperature were almost the same as those shown earlier in the case of yolk (Wakamatu et al., 1980), except that the range of sodium chloride concentration where yolk gelation occurred on frozen storage below −30°C, was more than 6% against over 4% in the case of LDL.

As shown in Fig. 3, the viscosity change of the granule fraction with 1~10% sodium chloride during frozen storage in the range from −20°C to −60°C was within 50 poises. These findings indicate that LDL is the main component responsible for yolk gelation even in the presence of sodium chloride.

Effect of sodium chloride on unfrozen water of LDL solution at −21°C

During DSC scanning of LDL without salts and LDL solution with 1~10% sodium chloride from room temperature to −21°C and then from −21°C to room temperature, one exothermic transition on cooling and one endothermic transition on heating occurred in all samples. Table 1 shows the composition of the samples, the exothermic heat (ΔH exo), the endothermic heat (ΔH endo) and the unfrozen water calculated from these transition heats and water content of each sample. The ΔH exo and ΔH endo decreased
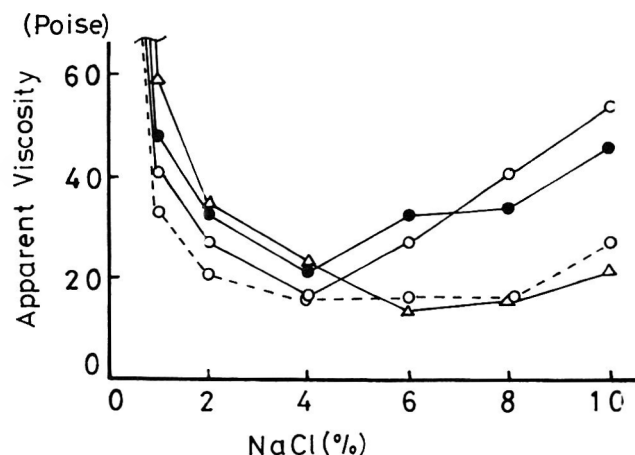
Fig. 3—Sodium chloride effect on apparent viscosity of frozen-thawed granule fraction. Freezing temperature: o····o, unfrozen; △——△, −20°C; •——•, −30°C; o——o, −60°C. Fifteen grams of granule fraction was frozen and stored at each temperature for 72 hr. Each point is the mean value for duplicate measurements.

Table 1—Transition heat during freezing and thawing, and unfrozen water of LDL solutions with sodium chloride[a]

Composition			ΔH(cal/g sample)[b]		Unfrozen water (g/g solid)		
NaCl	H$_2$O	LDL	ΔH exo	ΔH endo	Exo	Endo	Average
0	0.580	0.420	41.9 ± 0.4	42.4 ± 0.8	0.10	0.14	0.12
0.01	0.574	0.416	41.0 ± 1.6	40.1 ± 0.5	0.11	0.19	0.15
0.02	0.568	0.412	37.3 ± 0.1	39.1 ± 0.4	0.21	0.20	0.21
0.04	0.557	0.403	29.3 ± 0.1	30.4 ± 1.2	0.41	0.41	0.41
0.06	0.545	0.395	23.5 ± 0.2	25.5 ± 0.4	0.54	0.51	0.53
0.08	0.534	0.386	19.5 ± 0.3	21.5 ± 0.2	0.61	0.58	0.60
0.10	0.522	0.378	14.0 ± 1.5	15.0 ± 1.5	0.72	0.71	0.72

[a] DSC analyses were carried out at room temperature to −21°C. [b] Average ± standard deviation for duplicate analyses.

Table 2—Turbidity change of frozen-thawed LDL solutions with sodium chloride during differential scanning calorimetry[a]

	Absorbance at 660 nm[b]	
NaCl (%)	Turbidity I[c]	Turbidity II[d]
0	1.20 ± 0.35	1.20 ± 0.22
1	0.16 ± 0.06	0.26 ± 0.01
2	0.09 ± 0.01	0.10 ± 0.01
4	0.06 ± 0.01	2.23 ± 0.04
6	0.07 ± 0.02	2.41 ± 0.04
8	0.06 ± 0.01	2.47 ± 0.02
10	0.07 ± 0.01	2.50 ± 0.13

[a] Frozen-thawed LDL was diluted to 0.5% with 1M NaCl solution.
[b] Average ± standard deviation for duplicate measurements.
[c] DSC analyses were carried out at room temperature to −21°C.
[d] DSC analyses were carried out at room temperature to −55°C.

Table 3—Freezing and melting points of LDL solutions with sodium chloride during freezing and thawing[a]

	Temperature (°C)		
NaCl (%)	F.P. 1[b]	F.P. 2[c]	M.P.[d]
0	−16.2 ± 0.8	−	−0.6 ± 0.2
1	−14.4 ± 3.0	−	−3.7 ± 0.2
2	−14.0 ± 1.5	−	−5.8 ± 0.6
4	−19.3 ± 2.6	−	−22.3 ± 0.2
6	−20.3 ± 1.3	−40.9 ± 1.1	−21.7 ± 0.2
8	−25.4 ± 3.4	−39.5 ± 0.6	−21.9 ± 0.3
10	−27.6 ± 2.0	−38.6 ± 0.5	−21.9 ± 0.2

[a] Average ± standard deviation for three measurements.
[b] Onset temperature of the first exothermic peak.
[c] Onset temperature of the second exothermic peak.
[d] Melting point.

with sodium chloride concentration, and consequently the unfrozen water increased with increase of the sodium chloride amount. Because of a small difference in the amount of unfrozen water calculated from ΔH exo and ΔH endo, the average is regarded as the unfrozen water of the sample.

The turbidity of the samples after DSC analyses was shown in Table 2 as turbidity I. The turbidity I decreased with increase of sodium chloride amount in the range from 0−2%, whereas no turbidity increment was found in the range of more than 4% sodium chloride. This turbidity change coincided with that at −20°C shown in Fig. 2, so it was considered that the data obtained by DSC analyses well reflected the process of water transformation during freezing at −20°C and thawing.

Effect of sodium chloride on unfrozen water of LDL solution at −55°C

Fig. 4 shows the representative DSC thermograms of LDL without salts and with 1∼10% sodium chloride obtained by scanning the samples from room temperature to −55°C and then from −55°C to room temperature. An exothermic transition on cooling and an endothermic one on heating occurred during DSC scanning of LDL without salts [Fig. 4(A)], LDL with 1% (figure was omitted) and 2% sodium chloride [Fig. 4(B)], whereas two exothermic transitions on cooling and two endothermic transitions occurred on scanning of LDL with more than 6% sodium chloride [Fig. 4(D), (E), (F)]. In the case of LDL with 4% sodium chloride, one exothermic transition on cooling and two endothermic transitions on heating were detected [Fig. 4(C)]. Although this kind of discrepancy in the number of transition peaks between cooling and heating has been shown also in the case of yolk with 6% sodium chloride (Wakamatu et al., 1980), the reason is not clear.

The onset temperatures of the exothermic peak appearing at a higher temperature (peak 1) and a lower one (peak 2) were represented respectively as freezing point 1 (F.P. 1) and 2 (F.P. 2) and shown in Table 3. Although two

endothermic peaks appeared on heating LDL solution with more than 4% sodium chloride, the onset temperature of the endothermic peak at a higher temperature could not be determined precisely because of the overlap of the two peaks. In this case the onset temperature of the endothermic peak at a lower temperature was taken as the melting point (M.P.) (Table 3). The F.P. 1 was virtually unaffected by the sodium chloride concentration and was almost the same as that of the LDL without salts in the range of less than 2% sodium chloride, whereas F.P. 1 decreased with increase of sodium chloride concentration in the range from 4−10%. On the other hand, F.P. 2 was almost constant (about −40°C). The M.P. shifted toward a lower temperature with an increase of sodium chloride concentration in the range of less than 2%, and they were higher than the F.P. 1 of the same sample. The M.P. of LDL solution was about −22°C in the range over 4% sodium chloride and corresponded well with the eutectic temperature of the sodium chloride-water system (−21.13°C).

The exothermic heat of peak 1 (ΔH exo 1) and peak 2 (ΔH exo 2) and the endothermic heat (ΔH endo) were shown in Table 4. Although two endothermic peaks were found in the thermograms of LDL solution with more than 4% of sodium chloride (Fig. 4), they could not be separated correctly because of the overlap of the two peaks, so the two endothermic heats were combined and represented as ΔH endo in this case. The ΔH exo 1 of all samples decreased with increase of the sodium chloride amount. The relationship between the ΔH exo 2 (cal/g sample) and sodium chloride content (x:g/g sample) calculated by the least squares method was expressed by the following equation:

$$\Delta H \text{ exo } 2 = (219.8 \pm 20.6)x - (6.5 \pm 1.7)$$

The amount of water transformed from liquid to ice per g of sodium chloride was calculated to be 2.8g by dividing the slope of the equation by the transition heat of pure water (78.0 cal/g H$_2$O). This value corresponds approximately to that obtained in the case of a sodium chloride solution (2.5∼2.7 g/g NaCl) (Wakamatu and Sato, 1979).

In addition, the M.P. of LDL solutions with more than 4% sodium chloride were about −22°C. Their temperature coincided with the eutectic temperature of sodium chloride
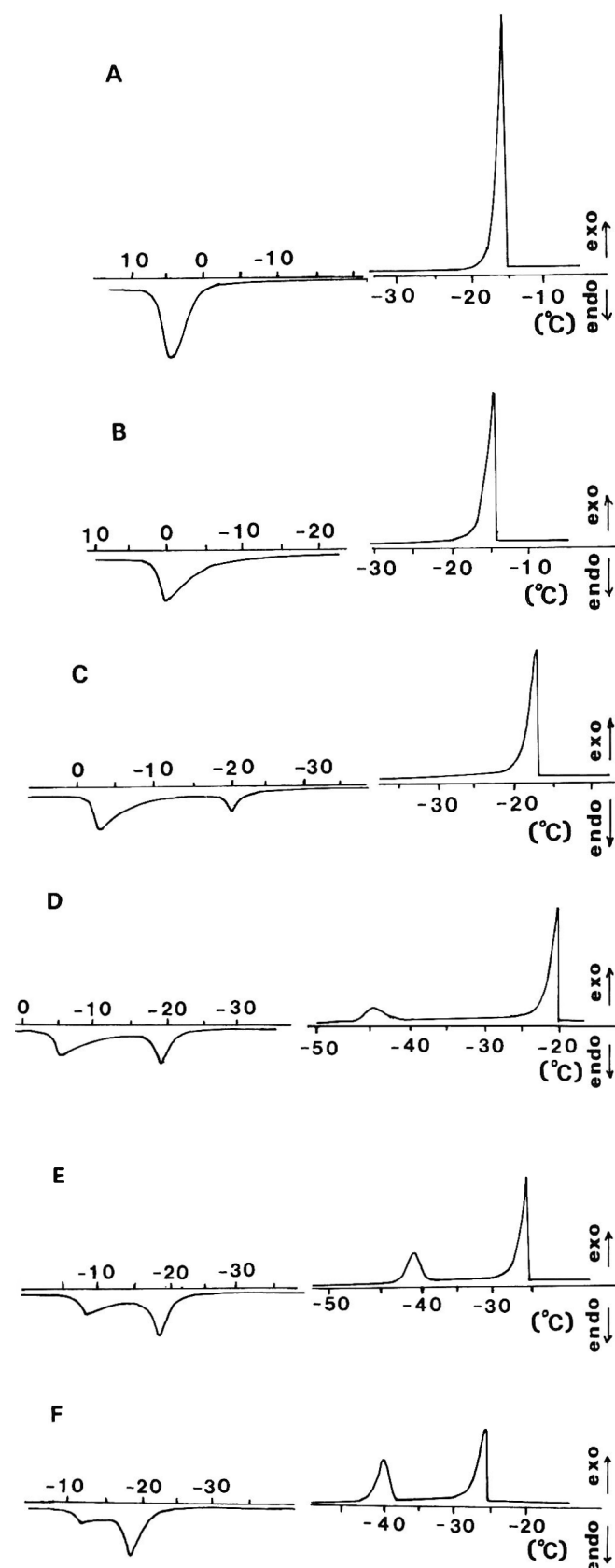
Fig. 4—Differential scanning calorimetry thermograms of LDL solutions with sodium chloride at room temperature to −55°C. NaCl conc.: (A), 0%; (B), 2%; (C), 4%; (D), 6%; (E), 8%; (F), 10%.

(−21.13°C). Based on these results and the phase diagram of sodium chloride-water system shown by Landolt (1962), it is considered that the ΔH exo 1 is caused by transformation of relatively free water into ice, and that the ΔH exo 2 is caused by formation of an eutectic mixture of water and sodium chloride, namely, by transformation of bound water of sodium or chloride ion into ice. The term bound water of ions is used, in the present study, to denote the water which participate in the formation of the eutectic mixture.

The unfrozen water of each sample at −55°C calculated from the total exothermic heat (ΔH exo: sum of ΔH exo 1 and ΔH exo 2) and the total endothermic heat (ΔH endo) were shown in Table 4. A small difference was found in the unfrozen water calculated from the ΔH exo and the ΔH endo, so their average was represented as the unfrozen water of the sample. In the case of LDL with 4% sodium chloride, the unfrozen water calculated from the ΔH exo was much smaller than that calculated from the ΔH endo. This difference is explained by the fact that two endothermic transitions occurred in heating, whereas only one transition took place in cooling. Thus, the unfrozen water calculated from ΔH endo was regarded as the unfrozen water of the sample. The unfrozen water of LDL with less than 2% sodium chloride increased with increase of sodium chloride amount like the case of the DSC scanning to −21°C. On the other hand, the unfrozen water of LDL with more than 4% sodium chloride decreased with an increase of sodium chloride amount, in spite of the ΔH exo 1 decrease. As shown in Table 2, the increment in the turbidity measured after DSC analyses (turbidity II) of the LDL solution with 1% and 2% sodium chloride was small, and it tended to decrease with sodium chloride concentration in the range from 0−2%. On the other hand, LDL solution with 4~10% sodium chloride exhibited a remarkable increase in turbidity. These turbidity changes agreed well with the changes in the unfrozen water shown in Table 4. Although the turbidity of the LDL solution without salts increased during DSC scanning from room temperature to −55°C (Table 2, turbidity II), the frozen storage at −60°C and thawing at 25°C did not induce a turbidity increase of LDL solution without salts (Fig. 2). This disagreement is probably caused by the difference in thawing rate between the two experiments. On the other hand, the turbidity change of LDL solution with sodium chloride (turbidity II) corresponded well with that induced by frozen storage at −40°C and −60°C (Fig. 2). These findings suggest that the effect of thawing rate on the turbidity of LDL solution without sodium chloride is more conspicuous than with sodium chloride at such a low freezing temperature.

DSC analyses of BSA-sodium chloride-water system below freezing point

During DSC analyses between room temperature and −55°C of BSA solutions with less than 4% sodium chloride, one exothermic transition in cooling and one endothermic transition in heating was found [Fig. 5(A)]. In the case of BSA solutions with 6% [Fig. 5(B)] and 8% sodium chloride (figure was omitted), one exothermic transition in cooling and two endothermic transitions in heating occurred. During scanning of BSA solution with 10% sodium chloride, two exothermic and endothermic transitions were found [Fig. 5(C)].

Table 5 shows the composition of the BSA solutions, the exothermic heats (ΔH exo 1 and ΔH exo 2), the total endothermic heat (ΔH endo) and the amount of unfrozen water of each sample. The sodium chloride concentration (g/g BSA, g/g protein and mole/mole protein) in BSA solution with 4% sodium chloride where an eutectic mixture of sodium chloride and water was not formed and that of BSA

Composition			ΔH (cal/g solid)[b]			Unfrozen water (g/g solid)		
NaCl	H₂O	LDL	ΔH exo 1	ΔH exo 2	H endo	Exo	Endo	Average
0	0.596	0.404	43.3 ± 1.9	0	45.2 ± 1.0	0.10	0.10	0.10
0.01	0.590	0.400	39.9 ± 1.1	0	40.5 ± 2.2	0.19	0.22	0.21
0.02	0.584	0.396	36.9 ± 0.8	0	36.4 ± 0.1	0.27	0.32	0.30
0.04	0.572	0.388	33.2 ± 0.4	0	37.8 ± 1.3	—	0.25	0.25
0.06	0.560	0.380	30.9 ± 0.6	6.9 ± 0.1	38.6 ± 1.0	0.17	0.19	0.18
0.08	0.548	0.372	26.0 ± 0.9	10.6 ± 0.5	39.0 ± 1.2	0.17	0.15	0.16
0.10	0.536	0.364	23.5 ± 0.5	15.7 ± 0.3	39.3 ± 1.5	0.07	0.11	0.09

[a] DSC analyses were carried out at room temperature to −55°C.　　[b] Average ± standard deviation for three measurements.

solution with 6% sodium chloride where the eutectic mixture was formed, were shown in Table 6. From these data, the lowest sodium chloride concentration for the formation of the eutectic mixture (critical sodium chloride concentration) is regarded to be about 160~250 mole/mole BSA. Similarly, the critical sodium chloride concentration for LDL is regarded to be about 2,800~5,600 mole/mole protein (Table 6). Yolk is composed of many proteins with different molecular weights, so the critical sodium chloride concentration could not be represented as mole per mole protein (Table 6).

DISCUSSION

WHEN SODIUM CHLORIDE solutions with less than 24% (w/w) solute were scanned from room temperature to −55°C with the differential scanning calorimeter, two exothermic transitions due to transformation of free water into ice and the formation of a eutectic mixture of water and sodium chloride occurred (Wakamatu and Sato, 1979). On the other hand, only one transition (peak 1) was detected during cooling of LDL solution with 1% and 2% sodium chloride to −55°C [Fig. 4(B)]. These results suggest that free sodium or chloride ions are absent in them. Thus, virtually all of the sodium chloride is involved in a complex composed of LDL, water and sodium chloride. The gelation of the LDL solutions with 1% and 2% sodium chloride was inhibited during frozen storage at temperatures between −20°C and −60°C (Fig. 1 and Fig. 2). This finding indicates that the water in the complex is hardly frozen, and this water may protect LDL from freezing damage and prevent the mutual contact of LDL particles. Such a complex was also observed in BSA solutions with sodium chloride (Fig. 5), so it was assumed that the protein moiety of LDL mainly participates in the formation of an LDL-water-sodium chloride complex.

The basic and acidic amino acids in a BSA molecule are each about 100 mole/mole BSA, respectively, whereas the critical sodium chloride concentration for BSA solution was about 160~250 mole/mole BSA (Table 6). These data mean that sodium or chloride ions of about 60~150 mole cannot pair with polar amino acids, even if all of the polar amino acids might be present on the surface of the BSA molecule. From these findings, it is assumed that these ions are not bound tightly by proteins, in contrast to divalent metal ions. Rather, they are weakly adsorbed on the proteins, sharing with a part of each bound water. This idea accords well with the model on the binding of small ions to proteins, as proposed by Klotz and Luborsky (1959). According to their statement, the stability of such a complex is largely attributed to the cooperative formation of ice-like hydration lattice between the complexing species. In the case of LDL, the critical sodium chloride concentration was about 2,800~5,600 mole/mole LDL and the basic and acidic amino acids in a LDL molecule are about 460 mole and 680 mole, respectively. These estimates were based on the amino acid composition of apo-VLDL shown
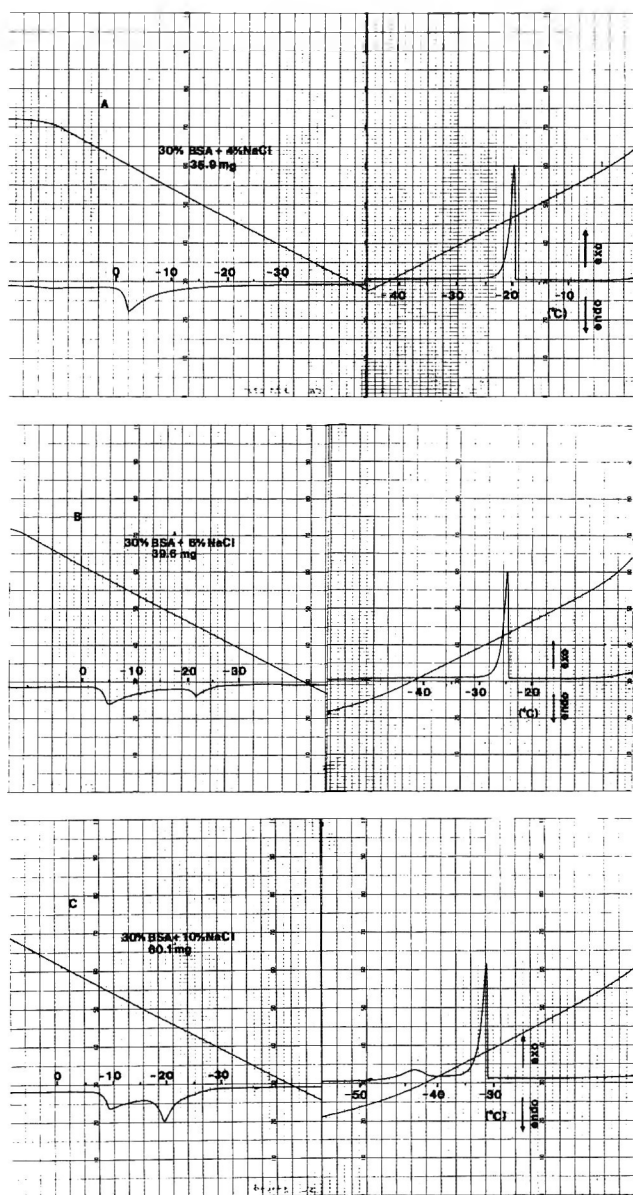
Fig. 5—Differential scanning calorimetry thermograms of BSA solutions with sodium chloride at room temperature to −55°C. NaCl conc.: (A), 4%; (B), 6%; (C), 10%.

by Kurisaki and Yamauchi (1977) and the apparent molecular weight of protein moiety of LDL (4.1 × 10⁵) which was calculated from the molecular weight of LDL (3.3 × 10⁶) and the protein content (12.5%). Thus, the critical sodium chloride concentration is much higher than for the polar amino acids of LDL, and the ratio of the sodium chloride to the polar amino acids for LDL is higher than for

Table 5—Transition heat during freezing and thawing and unfrozen water of BSA solutions with sodium chloride[a]

| Composition | | | ΔH (cal/g sample)[b] | | | Unfrozen water (g/g solid) | |
NaCl	H$_2$O	BSA	ΔH exo 1	ΔH exo 2	ΔH endo	Exo	Endo
0.02	0.686	0.294	41.2 ± 0.9	0	43.4 ± 1.7	0.50	0.48
0.04	0.672	0.288	36.8 ± 1.4	0	37.1 ± 3.1	0.61	0.65
0.06	0.658	0.282	32.7 ± 0.4	0	37.7 ± 0.6	0.70	0.56
0.08	0.644	0.276	29.5 ± 0.2	0	37.6 ± 0.1	0.75	0.51
0.10	0.630	0.270	26.8 ± 0.4	9.7 ± 0.6	36.8 ± 0.8	0.44	0.48

[a] DSC analyses were carried out at room temperature to −55°C. [b] Average ± standard deviation for three measurements.

Table 6—Critical sodium chloride concentration of BSA, LDL and yolk for the formation of the eutectic mixture of sodium chloride and ice

| | BSA | | | LDL | | | Yolk | |
NaCl (%)	NaCl g[a] /g BSA	NaCl g /g protein	NaCl mole[b] /mole protein	NaCl g[c] /g LDL	NaCl g /g protein	NaCl mole[d] /mole protein	NaCl g[e] /g yolk	NaCl g /g protein
2	—	—	—	0.05	0.40	2800	—	—
4	0.14	0.14	160	0.10	0.80	5600	0.08	0.28
6	0.21	0.22	250	—	—	—	0.12	0.42
Protein (%)	96.6			12.5[f]			28.4[f]	

[a] Calculated from the data shown in Table 5.
[b] Molecular weight of BSA is 66,000.
[c] Calculated from the data shown in Table 4.
[d] Calculated as molecular weight of LDL is 3.3 x 10^6 and protein content is 12.5%.
[e] Calculated from the data shown by Wakamatu et al. (1980).
[f] Cited from the data shown by Wakamatu et al. (1982a).

BSA. In addition, it was found that nearly all of the sodium in plasma fraction of yolk is dialyzable (Wakamatu et al., 1982b). These findings support the model for the adsorption of small ions to proteins described above and suggest that the phospholipids as well as protein in LDL may partially participate in the formation of the LDL-water-sodium chloride complex.

When LDL solution with more than 4% sodium chloride was scanned from −55°C to room temperature, two endothermic transitions occurred, and the water involved in the eutectic mixture increased with increase of sodium chloride amount. In addition, gelation of these solutions occurred during frozen storage at below −30°C, so the findings suggest that the sodium or chloride ions in them are adsorbed on LDL almost to the limit. Moreover, a part of sodium chloride may exist as free ions. Thus, the water in them may be classified into bound water of LDL (W b), bound water of sodium or chloride ions which are adsorbed by LDL (W ia), bound water of free ions (W if) and free water (W f). When these solutions are cooled to −60°C, W f is frozen at first and this will induce a gradual increase of sodium chloride concentration in the unfrozen phase. An extremely concentrated sodium chloride may compete with the ions adsorbed by LDL for W ia. In addition, onset of the formation of the eutectic mixture of sodium chloride and water (W if) may accelerate the migration of W ia from the complex to the eutectic mixture; finally, W ia may decrease to the level of the unfreezable water of LDL, where the protective effect of W ia on freezing damage is lost. It is also assumed that the formation of a eutectic mixture, or transformation of W if into ice may induce the disruption of the LDL-water-sodium chloride complex; thus, there is the resultant release of sodium or chloride ion with W ia from the complex.

The LDL gelation was inhibited by addition of sodium chloride in the range from 1−10% when these samples were frozen at −20°C and −25°C (Fig. 1, Fig. 2). DSC analyses of these samples from room temperature to −21°C revealed that only one transition (peak 1) had occurred, and that the unfrozen water increased with increase of the sodium chloride amount (Table 2). These findings indicate that only W f is frozen and W if is not, under these conditions, even if free sodium or chloride ions exist. Consequently, the disruption of the LDL-water-sodium chloride complex may

not be induced.

In the present study, it was shown that when sodium chloride acts as an inhibitor of LDL gelation, it increases the unfrozen water in the sample; and when it acts as an accelerator of gelation, it promotes removal of water from the LDL-water-sodium chloride complex.

Based on the results shown in the previous studies (Wakamatu et al., 1980, 1982c), it is assumed that LDL or yolk gelation is induced by at least two steps: (1) the unfrozen water is reduced during freezing to less than the critical moisture of LDL (0.11~0.16 g/g LDL), which is the indispensable amount of water for stabilizing LDL, and (2) the micro-ice crystals, which may be formed between LDL particles or the freezing period, are removed by merging with macro-ice crystals during frozen storage and thawing. In addition, it was found that frozen storage at −20°C for 72 hr did not cause the change in the unfreezable water of LDL. Furthermore, the unfreezable water consisted of monolayer and multilayer water of LDL molecule on the desorption isotherm (Wakamatu, 1981). These findings suggest that a part of free or capillary-condensed water which corresponds to the difference between the critical moisture and the unfreezable water may play an important role in preventing LDL aggregation. The present results confirm this reasonable assumption and support the view that at least in the case of sodium chloride, the salt concentration itself is harmless to yolk protein and that progressive removal of water is a key factor in gelation.

Although the gelation mechanism, especially in respect to the structural change of LDL, is not fully elucidated, a certain change may be induced in the hydrated parts of LDL, namely protein and phospholipid moiety, through dehydration due to ice formation. This in turn may cause the irreversible structural change of LDL molecules. In this respect, further study on the conformational change in phospholipid moiety of LDL during frozen storage is necessary, because phospholipids in water exhibit a thermotropic mesomorphism depending on water content (Chapman, 1973).

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Refractive Index of the Dispersed Phase in Oil-in-Water Emulsions: Its Dependence on Droplet Size and Aging

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———————— ABSTRACT ————————

A simple method of determining the refractive index of the dispersed phase was applied on different gum-stabilized oil-in-water emulsions, in which this refractive index is taken to be equal to that of the continuous phase at (extrapolated) zero optical density point. Refractive indices of dispersed phases were usually lower than those of the oil part alone. In dilute emulsions, the refractive index of the dispersed phase increased with time, while optical density of emulsion decreased. In aged concentrated emulsions, both refractive index and optical density decreased with time. At a given composition, the refractive index and optical density varied directly with droplet size. The results are interpreted in terms of reasonable physical and chemicl phenomena.

INTRODUCTION

INCREASE in the refractive index difference between the oil phase and the aqueous phase will increase the turbidity of an emulsion, and thus a smaller amount of oil would be required to obtain a given level of cloud. Use of a smaller amount of oil is, in general, favorable for oil-in-water emulsion stability.

The refractive index of the dispersed phase of an oil-in-water emulsion containing surface-active materials may be different from that of the oil in its bulk, or nondispersed state. The difference can reflect the exchange of components between the water and oil phases; the accumulation of ingredients at the interface; and physical effects.

The optical density (O.D.) of a layer of nonabsorbing dilute monodisperse emulsion can be obtained by applying Lambert-Beer's Law (Lothian and Chappel, 1951)

$$O.D. = \log_{10}(\frac{Io}{I}) = \tfrac{1}{4}\,\pi\,D^2\,(\log_{10} e)\,KNl \qquad (1)$$

where Io and I are the intensities of the incident and transmitted light, respectively; D the diameter of the droplet; K the light scattering coefficient; N the number of droplets per unit volume; and l the optical path length.

Furthermore,

$$K = 2 - \frac{4\sin\rho}{\rho} + \frac{4(1 - \cos\rho)}{\rho^2} \qquad (2)$$

where the particle size parameter ρ, expressed in radians, is defined by

$$\rho = \frac{2\,\pi\,D\,(n_1 - n_2)}{\lambda} \qquad (3)$$

λ being the wavelength of light, and n_1 and n_2 the refractive indices of dispersed and continuous phases, respectively (Goulden, 1958).

The outcome is an oscillatory relationship between O.D. and ρ. A given change in droplet size, or in refractive index difference between phases, or in wavelength of light, can either increase or decrease the optical density of the emul-

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sion depending on the initial values of ρ and O.D. The limiting value of O.D. is zero (clarity) as D approaches zero (homogeneity), as λ approaches infinity, or as $n_1 - n_2$ approaches zero. This last relationship (O.D. = 0 when $n_1 = n_2$) has been used to determine the refractive index of the dispersed phase of emulsions (Walstra, 1965) and latex suspensions (Smart and Willis, 1967). We have applied it here to study the effects of composition, dispersity, and age upon the refractive indices of the oil phases in certain oil-in-water emulsions.

MATERIALS & METHODS

Materials

Orange oil and orange flavor samples were obtained from The Coca-Cola Company, Foods Division (Plymouth, FL). Brominated vegetable (soy) oil was purchased from Estech Specialty Chem. Corp. (Chicago, IL). Ester gum (glycerol ester of wood rosin) was purchased from Hercules Inc. (Wilmington, DE). Spray-dried gum acacia and Purity Gum® (a modified food starch) were from Meer Corp. (North Bergen, NJ) and National Starch and Chemical Corp. (Bridgewater, NJ), respectively. Food-grade sodium benzoate was from Pfizer, Inc. (New York, NY).

Tap water was treated by filtration through an Everpure cartridge, #359-PM U-Fil (Everpure Inc., Westmont, IL), to eliminate particulate matter and chlorine. Sucrose (A.C.S. Certified) was from Fisher Scientific Co.; a stock solution was prepared and diluted as necessary.

Emulsions

Three different types of emulsions, hereinafter referred to as "original" emulsions, were prepared:

#1 Oil part. 271.4g orange oil (typical analysis $\sim$ 95% limonene by GLC) and 152g ester gum. Water solution: 768g gum acacia, 3.6g sodium benzoate, and 2604g water.

#2 Oil part. Same as #1. Water solution: 200g Purity Gum®, 3.6g sodium benzoate, and 3172g water.

#3a, 3b, 3c Oil part. 240g orange flavor (>99.5% w/w orange oil; typical analysis $\sim$ 94% limonene by GLC), 30g brominated vegetable oil, and 100g ester gum. Water solution: 912g gum acacia and 2718g water.

The oil parts were added to the water solutions with continuous stirring, then the mixture was homogenized in a Gaulin M3 homogenizer (Gaulin Corp., Everett, MA). The homogenization conditions used for preparing these emulsions are: Emulsion #1—2000 psi (first stage)/500 psi (second stage), 2 passes; #2—500/500, 2 passes; #3a—3600/400, 1 pass; #3b—4000/500, 1 pass; #3c—1350/150, 2 passes.

Measurements

The original emulsions were diluted to different extents for different measurements.

For droplet size measurements (Coulter Counter® Model TAII with a 15-micron aperture tube and data converter, from Coulter Electronics, Inc., Hialeah, FL), original emulsions were diluted 1000-fold with treated tap water, then 0.4 ml of this diluted emulsion was mixed with 90 ml 5% NaCl solution.

For spectrophotometric measurements, the original emulsions were first diluted with distilled water in two steps. The emulsions were diluted 15-fold in the first step and 20-fold in the second step. In each step the newly diluted emulsion was stirred slowly for 1 hr. The stock emulsions thus prepared were therefore 300-fold dilute, relative to the original emulsions. To these 300-fold diluted emul-

sions, the required amounts of stock sucrose solution and distilled water were added to attain different refractive indices of continuous phase. The highest concentration of sucrose in the continuous phase was 60% w/v. The optical densities of these diluted emulsions (1500-fold diluted, relative to the original emulsions, unless otherwise noted) were determined by Beckman Model 25 spectrophotometer with recorder, vs reference solutions (water solutions of the corresponding concentrations of gum and sucrose). One-cm path length, self-masking cells (Precision Cells, Inc., Hicksville, NY) were used to prevent backscattered light from contributing to the transmitted beam. At the emulsion concentrations used, the absorbance of light due to the oil components was found to be insignificant.

Refractive indices were measured on solutions corresponding to the continuous phases of these 1500-fold diluted emulsions. The refractive index at 589 nm was determined by Abbe refractometer; for measurement at 435 nm, a Precision® refractometer with mercury vapor lamp and a combination filter of Corning #3389 and #5113 was used. Both instruments are from Bausch and Lomb (Rochester, NY).

RESULTS

FIG. 1 SHOWS EXAMPLES of optical density of 1500-fold diluted emulsions as a function of wavelength. Optical density values at 425 and 589 nm have been plotted against refractive indices of the continuous phases. The data were amenable to straight line extrapolation. A few such plots are shown in Fig. 2A and 2B. The refractive index of the continuous phase, extrapolated to zero optical density, was taken as the refractive index of the dispersed phase of the diluted emulsion. Optical density values at lowest refractive index, corresponding to water being the continuous phase, were always above the line when measured on the freshly diluted emulsions (Fig. 2A). These points were ignored in making the least square plots.

Table 1 shows refractive indices of dispersed phases and optical densities, at two wavelengths, for 1500-fold diluted emulsions, obtained by extrapolation. Both refractive index of dispersed phase and optical density of emulsion were higher for fresh original emulsions, than for the same emulsions after 3 months storage, all measurements being made at the same time after dilution. As the time interval between dilution and measurement increased, so did the refractive index of the oil phases for all the emulsions; the optical densities of the 1500-fold diluted emulsions decreased with age. When some of the original emulsions were diluted 500-fold instead, the results were almost identical.

Emulsions #3a, #3b, and #3c all had the same composition, but were prepared under varying homogenization pressures; the ratios of their interfacial areas were 1:1.5:2.2; geometric mean diameters were 1.30μ, 0.87μ, and 0.59μ, respectively. The refractive indices of the oil phases and the optical densities of the emulsions decreased with decreasing mean diameter of the dispersed phase droplets, when measured under the same conditions.

DISCUSSION

AS MENTIONED EARLIER, we have used two hydrocolloids, gum acacia (also known as gum arabic) and Purity Gum®, to stabilize our emulsions. Gum acacia exists in nature as a neutral or slightly acidic salt of a complex polysaccharide containing calcium, magnesium, and potassium. The accepted molecular weight (MW) is 600,000 daltons, with a broadly skewed distribution (Anderson et al., 1968). Hydrolysis of gum acacia yields L-arabinose, L-rhamnose, D-galactose, and D-glucuronic acid. Gum acacia is insoluble in oils; its addition to water results in a lowering of the surface tension (Banerji, 1952). It has been
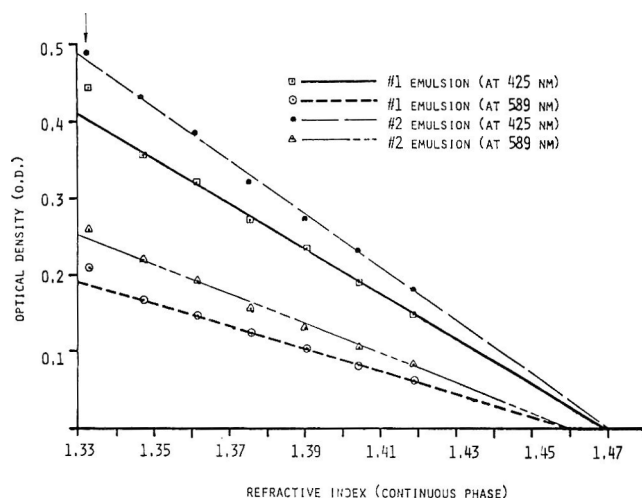
Fig. 2A—*Examples of extrapolative determination of refractive indices of diluted emulsions from turbidity spectra. The refractive index values on the abscissa are at 589 nm. A dispersion value of 0.007, average for water and sugar solutions (Krüss, 1920; Rosenhauer, 1969) should be added to the plotted refractive indices in order to obtain the correct values at 425 nm. Measured 2 hr after 1500-fold dilution from original emulsions.*

Fig. 1—*Optical density of 1500-fold diluted emulsions as a function of wavelength. In each case, sugar concentration of continuous phase was zero. Measurements were made 2 hr after dilution.*
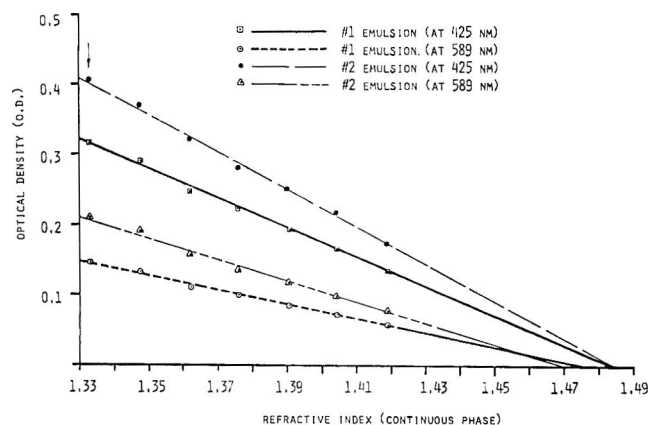
Fig. 2B—*Same as 2A, except measured 24 hr after 1500-fold dilution from original emulsions.*

shown (Veis and Eggenberger, 1954; Warburton, 1966; Anderson and Rahman, 1967) that the acacia molecule in solution exists as a stiff coil with numerous side chains; the end-to-end dimension varies from 0.06μ at zero charge to 0.24μ (root mean square) at maximum charge.

Purity Gum® (a modified food starch) is primarily amylopectin, a polymer of glucose, that has been chemically modified to contain some lipophilic groups and is mildly anionic in aqueous solution. Amylopectin is highly branched; in solution it shows a rather compact body with a dense core and a roughly spherical shape when not distorted (Rutenberg, 1980). The molecular weight (Mw) of Purity Gum® is about 400,000 with a wide distribution range (Trubiano, 1981). We found that its surface activity is almost as high as that of gum acacia.

It should be noted that, in general, the primary layer of polymers adsorbed on an oil droplet are extremely difficult to desorb; statistically, many segments would have to be simultaneously lifted off and replaced with bound water (Healy, 1978). Studies on the interfacial films formed by hydrocolloids, such as gum acacia, have shown that the first layer of gum deposited at the oil-water interface was irreversibly adsorbed, while the subsequent layers were reversibly adsorbed (Shotton and White, 1963; Shotton et al., 1964).

The refractive index arrived at for the dispersed phase is an average of the refractive indices of the individual droplets. The assumption is made that the thickness of the interface is small enough, relative to the wavelength of the inci-

Table 1—Refractive indices of dispersed phases and optical densities of emulsions

Original emulsion	Interval between 1500-fold dilution and measurement (hr)	Refractive Index 589 nm	425 nm	O.D. (at 0 sugar in continuous phase) 589 nm	425 nm
#1 Emulsion (Fresh)	2	1.4610	1.4761	0.213	0.445
	24	1.4744	1.4905	0.146	0.318
#1 Emulsion (3-month old)	2	1.4519	1.4655	0.170	0.361
	24	1.4582	1.4722	0.132	0.282
	48	1.4666	1.4812	0.114	0.249
#2 Emulsion (Fresh)	2	1.4588	1.4770	0.260	0.492
	24	1.4695	1.4912	0.210	0.405
#2 Emulsion (3-month old)	2	1.4572	1.4738	0.256	0.485
	24	1.4648	1.4842	0.207	0.398
	48	1.4701	1.4879	0.182	0.354
#3a Emulsion (Fresh)	2	1.4732	1.5005	0.470	0.712
	24	1.4810	1.5091	0.383	0.593
	48	1.4905	1.5220	0.318	0.498
#3b Emulsion (Fresh)	2	1.4581	1.4774	0.401	0.687
	24	1.4731	1.4953	0.306	0.534
#3c Emulsion (Fresh)	2	1.4495	1.4643	0.320	0.638
	24	1.4636	1.4793	0.243	0.496
Oil Part of Emulsion #1, 2		1.4963	1.5120[a]		
Oil Part of Emulsion #3a, 3b, 3c		1.4930	1.5081[a]		
d-Limonene		1.4723[b]	1.4889[c]		

[a] Extrapolated from experimentally determined values at 589 nm and 435 nm.
[b] Internatonal Critical Tables (1930), p. 52, McGraw-Hill Book Co., NY.
[c] Extrapolated from values at 589 nm and 435 nm, Intl. Critical Tables (1930).

dent light, that the droplets (interface plus oil phase) behave optically as a single dispersed phase.

The refractive index of the dispersed phase in the emulsions was usually found to be lower than that of the oil blend per se. (This was always true for freshly diluted emulsions.) This observation suggests that the refractive index of the hydrated gum at the interface is lower than that of the oil part. We are not aware of any refractive index data of hydrated gum at such an interface. However, our measured values of $n_D = 1.401$ and 1.405 for very viscous aqueous solutions of gum acacia and Purity Gum®, respectively (each ca. 57% w/w), lend support to the above interpretation.

The observed decrease in refractive index of dispersed phase during three months storage of original emulsions (as measured after dilution), might reflect gradual solubilization of oil components, or of the products of hydration (Askar et al., 1973) and oxidation (Schenck et al., 1965) of d-limonene and oxidation (Moore and Lawrence, 1958) of ester gum, into the aqueous phase. It is also possible that interfacial equilibrium had not yet been established in these concentrated emulsions when the first (fresh) measurements were made, and that hydrocolloid continued to accumulate at the interface during storage. These two phenomena (solubilization of oil part components, and accumulation of additional hydrocolloid at the interface) are not incompatible; they might occur simultaneously. Both increase the ratio of interfacial hydrocolloid-to-oil.

The observed decrease in optical density during three months storage of the original emulsions (as measured after dilution) might similarly be attributed to solubilization of oil components or their reaction products. A less likely, but possible explanation is coalescence or flocccculation of droplets during storage. Calculations based on Eq (1), (2), and (3) suggest that substantial increases in droplet size, via combination of droplets, could decrease the optical densities of our emulsions #1 and #2 (initial geometric mean diameters ∿0.7μ). In these calculations, the emulsions were treated as though they were monodisperse.

The observed increases in refractive index of the dispersed phase, during 24–48 hr storage of 1500-fold diluted emulsions, can be interpreted to reflect loss, upon dilution, of some of the outer layer hydrocolloid from the interface into the suddenly more dilute aqueous phase. Loss of hydrocolloid from the interface may also be invoked to account for the observed decreases in optical densities during storage of the 1500-fold diluted emulsions. While such loss is expected to increase refractive index, as noted, it would also decrease droplet size. Unlike the situation in which changes in droplet size result from combination or fragmentation of droplets, in this case, there would be no counter-directional change in the number of droplets, N. Under these circumstances, calculations similar to those noted above indicate that any decrease in droplet diameter would tend to decrease optical density for our emulsions, and that this effect would predominate over the opposite effect of the increase in refractive index.

The direct relationship between refractive index of dispersed phase and mean droplet diameter may be attributed to the expected direct relationship between available interfacial area and quantity of hydrocolloid adsorbed. A similar argument was invoked (Dudo, 1973) to explain analogous results with $0.18\mu-0.74\mu$ latex particles dispersed in aqueous media with emulsifier. Gold sol titration (to be reported elsewhere) of our emulsions #3 indicated that the quantity of free gum (i.e., not adsorbed at the interface) is greatest in #3a and least in #3c.

The direct relationship between optical density of emulsion and mean droplet diameter is consistent with the results of calculations based on equations (1), (2), and (3) over this range of diameters ($1.30\mu-0.59\mu$).

A finding of particular interest is that decreases in optical density of emulsion and in refractive index of dispersed phase, upon storage of original emulsions, is much more pronounced in the case of the emulsion stabilized by gum aracia, than in that stabilized by Purity Gum®.

Another interesting result is the much higher measured optical density for emulsions #3 than for #1 or #2.

These apparently dramatic effects of composition of interface and oil phase upon the optical properties of the emulsions may warrant further investigation.

Sufficiently long storage of dilute emulsions can, at least in some cases, result in refractive index of dispersed phase exceeding that of the separate oil part (e.g., emulsion #3a, Table 1). A possible explanation is that the first irreversibly adsorbed layer of gum at the interface contains virtually no water and therefore has a refractive index higher than that of the oil; whereas subsequent layers of gum, which are desorbed during storage of dilute emulsions, have a lower refractive index than oil, by virtue of incorporation of water.

Since the method of extrapolating the data (least squares straight line plot) was arrived at empirically, it might not be the method of choice for all emulsions. Also, because of the different environment, the refractive index of the dispersed phase in dilute emulsion may not exactly correspond to that in more concentrated emulsion — as strongly suggested by the observed rapid changes in refractive indices of dispersed phases of newly diluted emulsions.

The above findings could be useful where the turbidity of emulsions is a matter of importance, e.g. cloudy beverages. Beverages prepared from old concentrated emulsions may show lower level of cloud than those prepared from fresh emulsions. Manipulation of the emulsion composition, particularly that of the oil part, seems to be a promising way to effect large increase in beverage cloud.

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Characterization of Polymer and Solute Bound Water by Pulsed NMR

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——————————ABSTRACT——————————

Pulsed NMR signals from water in combination with two polymers and two solutes, individually and in mixtures, was measured over a_w range 0.75–0.95. Both spin-lattice (T_1) and spin-spin (T_2) relaxation curves showed negligible slopes for water with starch and casein (polymer water) and large for water with sucrose and salt (solute water). Mixtures of polymer and solute waters showed intermediate slopes. Dilution of a sucrose solution gave T_1 and T_2 responses approaching that of pure water. T_2 from NaCl indicated less and from sucrose more water structure than pure water. NMR data coincided with sorption isotherms. It was concluded that polymer and solute waters show different NMR responses and can coexist in a food.

INTRODUCTION

A TYPICAL WATER sorption isotherm for foods is sigmoidal in shape and has been classified as a type II isotherm by Brunauer (1943). Kuprianoff (1958), Labuza (1968), Kuntz and Kauzmann (1974) have analyzed the sigmoidal characteristics of this sorption isotherm to show that different types of bound water are present, depending on water activity (a_w). However, boundaries between the different regions representing a given type could not be quantitatively determined (Kuprianoff, 1958; Labuza, 1968). Rockland (1969), using the Henderson equation was able to demarcate the isotherm into three distinct regions called "local isotherms." Each of these local isotherms showed different Henderson constants considered to represent the three different types of water.

Both Rockland (1957) and Kuprianoff (1958) considered three types of water to be bound within a food. Rockland recognized the first two types to be true bound water while Kurpianoff felt that only the first type should be considered. Both researchers agreed that the third type was condensed water trapped within microcapillaries. Gur-Arieh et al. (1967) found three greatly different densities of water bound by a low protein fraction of wheat flour; the first showed 1.48, the second 1.11 and the third 0.97 g/cc.

Lang and Steinberg (1981), using the Smith (1947) linear sorption isotherm, were able to show marked differences in sorption behavior between macromolecules and solutes. They reported that each of these sorbed a different type of water over a broad a_w range. The boundaries of this range as well as the quantity of water sorbed at any a_w in this range could be characterized from the linear isotherm parameters. The water sorbed by the macromolecules was termed "polymer" water while that sorbed by solutes was termed "solute" water.

Pulsed NMR has become an important technique for studying water in foods. It provides information about the physical characteristics of the bound water molecule such as nuclear relaxation times. These instrumental signals can be used to categorize the mobility of the different

types of bound water (Leung et al., 1976). This mobility may be related to the physical properties of the given type of bound water. Walter and Hope (1971), Kuntz and Kauzmann (1974) and Fennema (1977) have reviewed kinetic methods such as pulsed NMR for measuring bound water.

Hazelwood et al. (1969), using a high resolution NMR spectrometer, found that water in muscle existed in at least two ordered phases. This has been supported by Cope (1969) with work on water in muscle and brain tissues using pulsed NMR. Based upon proton relaxation time, many researchers have used pulsed NMR to characterize the water phases (Brey et al., 1968; Woessner and Snowden, 1970; Leung et al., 1976; Hansen, 1976).

Duckworth (1972) studied a starch-sucrose mixture and Duckworth and Kelly (1973) studied an agar-sucrose mixture. Both mixtures showed a noticeable increase in pulsed NMR signal when moisture content was increased to the point that "the sugars had become mobilized." However, they did not relate this signal increase to type of bound water. Although the workers referenced here as well as others (Shanbhag et al., 1970; Mousseri et al., 1974; Leung et al., 1976) have differentiated between free and bound water by means of NMR, none have differentiated between different *states* of bound water.

Prehistoric man knew that moisture content determines storage stability because he dried food for preservation. He also knew that a_w was important because he added salt to fish and meat to reduce a_w. Now we seek to add a *third dimension* by demonstrating that a food simultaneously contains different *states* of water; the different characteristics of these waters may be of crucial importance to stability and other important attributes of the food. Therefore, the objective of this work was to learn how to differentiate between "polymer" and "solute" waters by means of pulsed NMR signals and to be able to use these signals as an analytical tool to determine the presence of each in a food.

MATERIALS & METHODS

Materials

Corn starch, Argo brand (Best Foods, Inc., Englewood Cliffs, NJ), casein, purified (Fisher Scientific Co., Fairlawn, NJ), and sucrose, Domino brand (Amstar Corp., New York, NY) were used.

Moisture determination

This was determined by the vacuum oven method (AOAC, 1970) using 60°C and 29.8 in Hg vacuum for 36 hr. Determinations were made in triplicate.

Sample equilibration

Equilibration of starch, casein, starch-sucrose (90:10 wet basis) and casein-salt (95:5, wet basis) was done against various saturated salt solutions as described by Lang et al. (1981). In each case, the a_w value for the saturated salt was obtained either directly from those accepted by the National Bureau of Standards (K_2SO_4-0.976; KNO_3-0.946; $(NH_4)_2SO_4$-0.813; NaCl-0.755; NaBr-0.591; $MgCl_2$-0.331) (Greenspan, 1977) or were determined in this laboratory by calibration against these accepted values using cellulose as a secondary standard ($BaCl_2$-0.910; $ZnSO_4$-0.890; K_2CrO_4-0.859; Li_2SO_4-0.842) (McCune et al., 1981). —*Continued on next page*

Solute preparation

Solutions of sucrose and sodium chloride were prepared individually. The solution moisture contents and their corresponding water activities were determined from isotherms reported by Lang ar.d Steinberg (1981).

Pulsed NMR

Pulsed NMR measurement was carried out using a Praxis PR-102 Pulsed NMR Spectrometer (The Praxis Corp., San Antonio, TX) at 20°C. T_1 was determined from the 180°-VTD-90° pulse sequence by plotting the difference in signal from the two pulses against the VTD. This was repeated for samples equilibrated to several a_w; in order to relate these data to a_w, the difference in signal was taken at a single VTD, 6×10^4 m sec, for each a_w. T_2 was determined with the spin echo technique using the 90°-VTD-180° pulse sequence.

Approximately 2g of the solid material or 3 ml of the solutions were placed into a 10 × 75 mm sample tube and the tube was inserted into the magnet probe. For each measurement, the NMR signal recorded was the average of two separate determinations. This was done by placing the sample into the probe two times. After sufficient time was allowed for signal equilibration, the signal was recorded.

RESULTS & DISCUSSION

BOTH SPIN-LATTICE (T_1) and spin-spin (T_2) relaxation time measurements were made with the Pulsed NMR instrument. Both polymer water sorbed by macromolecules such as starch and casein and solute water sorbed by solutes such as sucrose and sodium chloride as well as mixtures of these, were measured.

Spin lattice measurements

Constant a_w. Fig. 1 shows the first pulse minus second pulse signals with increasing Variable Time Delay (VTD) for three samples equilibrated to 0.785 a_w. Under the range of VTD used here, the casein gave a flat line. Thus, water bound to a macromolecule, i.e., polymer water, showed a constant difference between first and second pulse signals with increasing VTD, under these conditions. Since T_1 is the negative reciprocal of the slope of this line (Farrar and Becker, 1971), it is infinitely large here. In contrast, the sodium chloride showed a decreasing signal with increasing VTD. This means that the slope, and thus T_1, are finite.
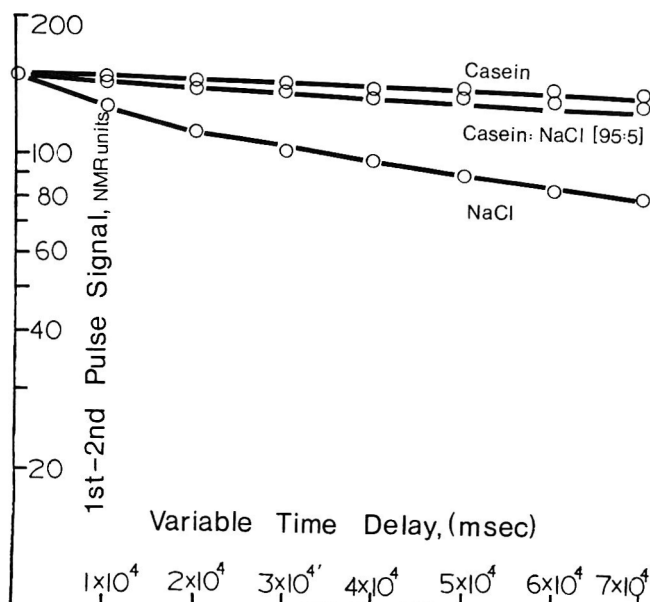
The mixture of casein and salt gave, as expected, a line intermediate between the two individuals, close to that for casein.

A similar experiment was performed wi⁺h starch and sucrose at 0.9C a_w (Fig. 2). The response for starch was the same as that for casein, leading to the same conclusion. The response for sucrose was similar to that of NaCl, showing again that solute water gave an NMR spin-lattice signal under these conditions. The starch:sucrose (90:10) mixture gave an intermediate line close to that for starch, as expected.

Solute concentration. In order to further investigate the response from solute water, sucrose solutions of four concentrations were analyzed. The data in Fig. 3 show that the slope was steepest for the saturated solution and became more flat with increasing dilution. The line was horizontal for distilled water, as expected.

Effect of a_w. It was considered important to relate NMR signal from polymer and solute waters to a_w. For this, data such as that in Fig. 2 at one a_w were obtained for a series of a_w. In each case, the lines were entered at a VTD of 6×10^4 msec and the first pulse-second pulse signals thus obtained were plotted against the respective a_w in Fig. 4. At each a_w from 0.30—0.83, both the starch and the starch:sucrose mixture gave the same response so that all the points fell on the same horizontal line. At a_w above 0.83, the starch response continued on the same horizontal line to 0.95, but the mixture showed a separate line with a steep slope.

The sucrose response (Fig. 4) was interesting. At this VTD, the crystals in contact with the saturated solution gave essentially no second pulse response, so that the observed signal is due to the sucrose protons which are in fast exchange with water. As long as the sucrose solution is supersaturated, the a_w will remain at 0.86 and the NMR

Fig. 1—Spin-lattice relaxation times (T_1) plots for water on casein, salt and a casein:salt (95:5) mixture, each equilibrated to 0.785 a_w.
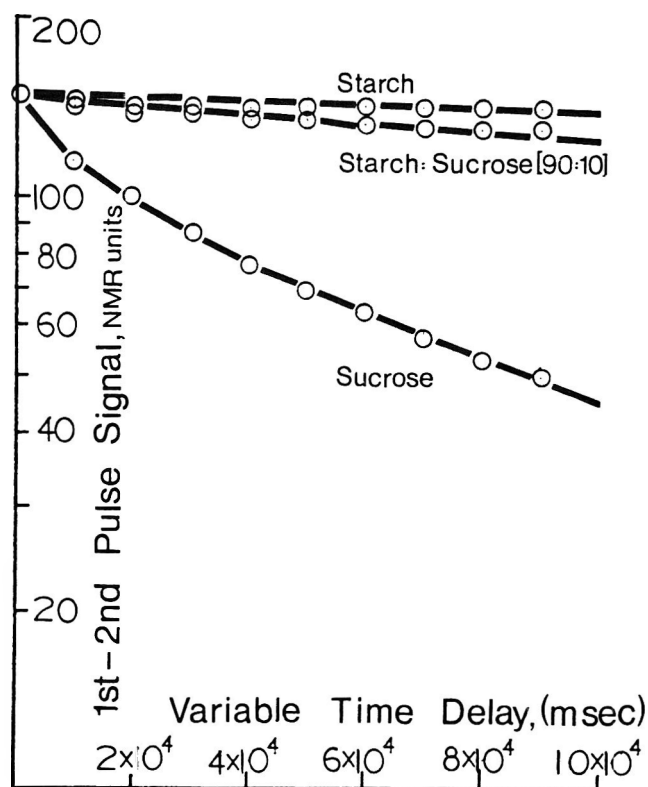
Fig. 2—Spin-lattice relaxation times (T_1) plots for water on starch, sucrose and a starch:sucrose (90:10) mixture, each equilibrated to 0.91 a_w.

signal will increase because the ratio of crystals to saturated solution is decreasing; the magnetic susceptibility change caused by crystals is decreasing and this will result in an increasing NMR signal as observed. From Fig. 3 we see that the NMR signal at 60 sec VTD increased with increasing a_w above 0.86. Increasing a_w corresponds to a dilution of the sucrose solution. The faster decay of the NMR signal of sucrose solution in comparison to the decay of free water in Fig. 3 may be attributed to a fast exchange of protons between sucrose with its water of hydration and bulk water. As the fraction of these exchanging protons decreases with increasing a_w, its contribution to the observed decay decreases. This also explains the ascending slope for the sucrose line in Fig. 4, above 0.86 a_w.

We see in Fig. 4 that the starch-sucrose mixture above 0.86 a_w behaves markedly different from either hydrated starch itself or hydrated sucrose alone. Therefore, this distinct behavior of the starch-sucrose mixture is caused by the interaction between the starch and hydrated sucrose. A possible model of this interaction involves hydrogen bonding of water molecules between hydrated sucrose and starch. This would somewhat immobilize the hydrated sucrose molecules, thereby decreasing the NMR signal. Thus, with increasing a_w above 0.86, we expect the fraction of hydrogen-bonded sucrose which is linked to starch to increase and this will decrease the NMR signal as observed.
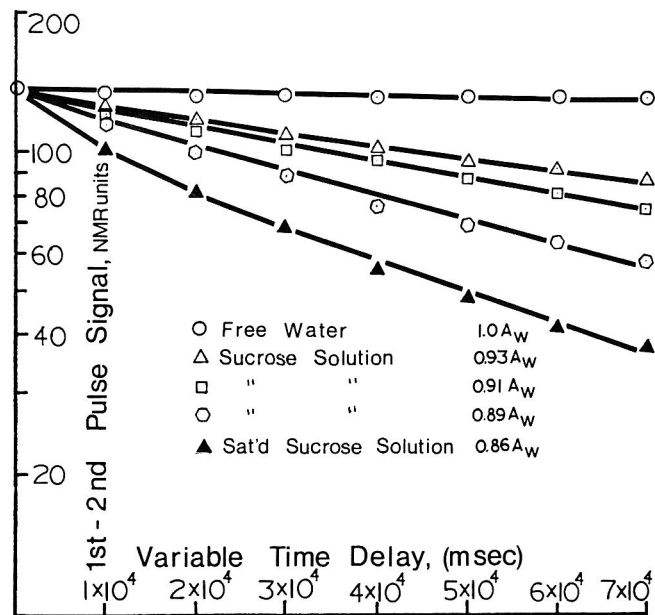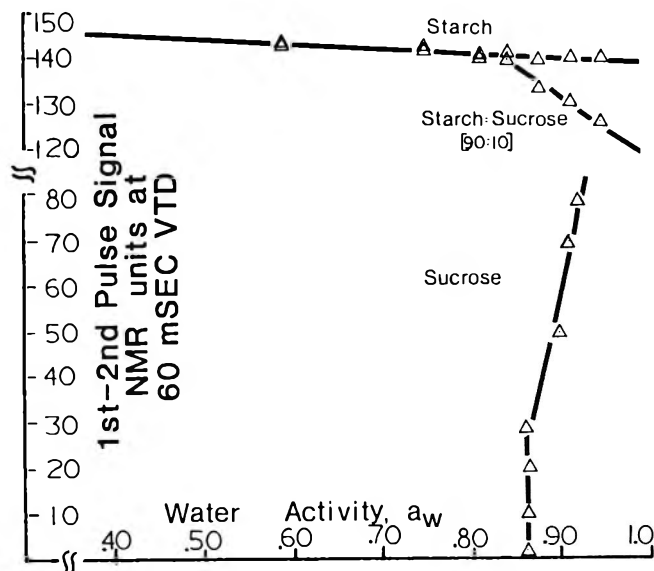
Conclusions. It was concluded from the data in Fig. 1−4 that polymer water under the instrument parameters used here, showed an infinite T_1, while solute water showed a finite T_1. Furthermore, the single line for starch and the broken lines for the sucrose and the starch:sucrose mixture in Fig. 4 closely resemble in graph and in meaning the corresponding linear sorption isotherms presented by Lang and Steinberg (1981). This coincidence strengthens the validity of the concept that at least two different types of water can coexist in a mixture at the same a_w in the intermediate moisture range.

Spin-spin relaxation measurements

Solute water in saturated solutions. Fig. 5 shows the echo signal from saturated NaCl was linear with VTD. Since the slope of the spin-spin line is equal to $-2/T_2$ (Farrrar and Becker, 1971), T_2 here was calculated to be 1750 msec. The saturated sucrose solution showed an early curved portion attributed to the sucrose (Leung et al., 1976) followed by a long linear portion. T_2 calculated from this line was 1110 msec. Thus, the water in the two saturated solute solutions showed comparable T_2 values indicating similar but not identical characteristics.

Free water was measured for comparison to solute water. When the instrument was tuned for saturated sucrose, the free water curve was above the sucrose line and when the instrument was tuned for saturated NaCl, the free water curve was below the NaCl line. This may be explained on the basis of structure. Since a lower curve on an echo plot such as Fig. 5 indicates a higher degree of water structure, the higher free water curve related to saturated suc-

Fig. 3—Spin-lattice relaxation times (T₁) plots for water on saturated sucrose and three unsaturated sucrose solutions.

Fig. 4—Variation of pulsed NMR signal with water activity for starch, sucrose and a starch:sucrose (90:10) mixture.
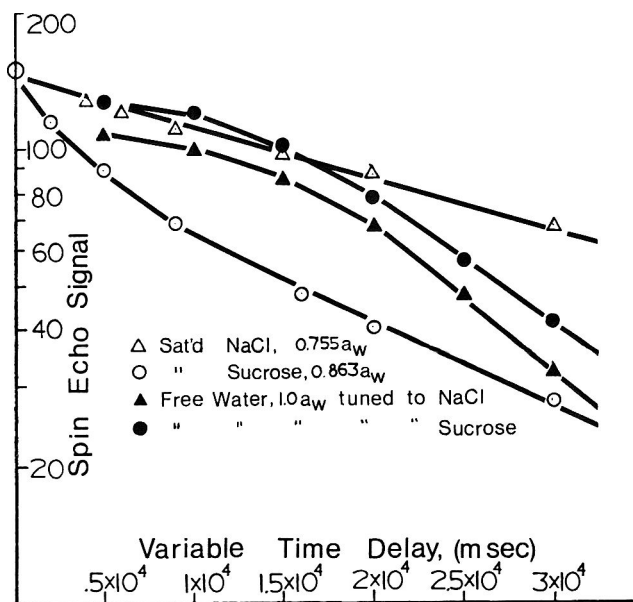
Fig. 5—Pulsed NMR echo signal from free water and water bound to sucrose and sodium chloride.

rose indicates free water has less structure than the water associated with sucrose. Conversely, the lower free water curve related to saturated sucrose indicates free water has more structure than the water associated with NaCl. This explanation is corroborated by the statement that sucrose is a "structure-former" while NaCl is a "structure-breaker" (Fennema, 1976).

Solute water in unsaturated solutions. Fig. 6 shows the NMR echo signal from four sucrose solutions ranging from saturated to 0.875g H_2O/g sucrose. The slopes show an orderly progression from −0.0010 to −0.0019. This follows from the discussion just above that dilution of a sucrose solution decreases structuring.

Polymer water. Corn starch samples equilibrated to eight a_w were read in the instrument tuned to starch. They all gave the *same* echo curve. This means that increasing moisture content had no effect on the signal and leads to the conclusion that polymer water does not give a spin-spin NMR signal under these conditions.

This negative finding supports a significant implication. It is well known that oil will give a signal but upon hydrogenation to a solid the signal will disappear; this is the basis for NMR determination of the solids/fat ratio. By analogy, since polymer water did not give a signal it must also be "solid" or, to use the Ling (1972) expression, "ice-like." This implication is confirmed by thermodynamic studies (Lang et al., 1982); polymer water showed a high negative enthalpy while solute water showed a less negative enthalpy, indicating that polymer water has lost its latent heat of fusion so it must be solid while solute water is still liquid.

Solute-polymer water mixture. To prepare this sample, starch was moistened to 0.946 a_w and sucrose was moistened to the same a_w. These were mixed in the proportion starch:sucrose (60:40) on an original wet basis. The echo signal, Fig. 7, gave regression line parameters of a = 1.8115 and b = −0.0027, with r = 0.9955. The saturated sucrose solution curve from Fig. 6 is included for comparison. It was concluded that solute water in a mixture with polymer water shows a strong T_2 signal indicating that pulsed NMR can be used to detect solute water in a formulated food

containing macromolecules as well as solutes. Quantification of the solute water in such a food will require further research.

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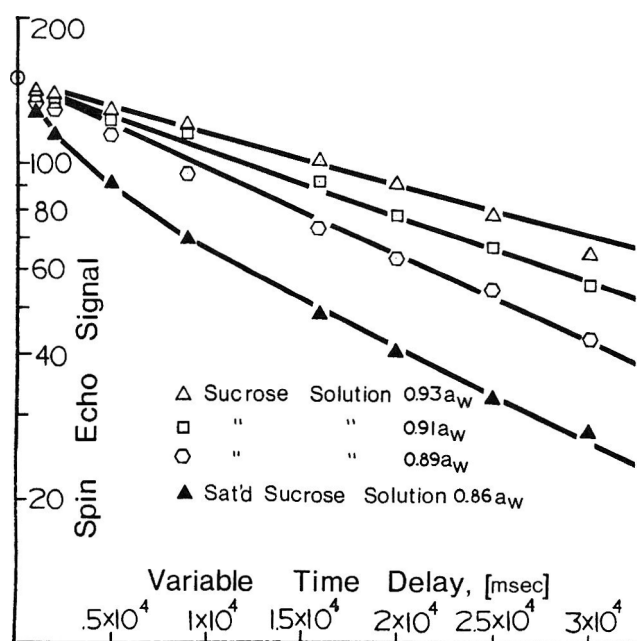
Fig. 6—Pulsed NMR echo signal for a saturated and three unsaturated sucrose solutions.
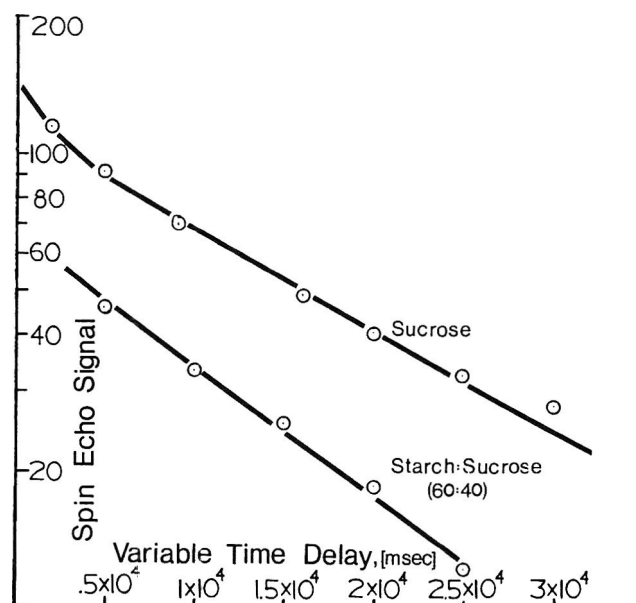
Fig. 7—Pulsed NMR echo signal from a saturated sucrose solution and a starch:sucrose (60:40) mixture equilibrated to 0.946 a_w.

Qualitative and Quantitative Determination of Some Yellow, Orange and Red Food Dyes by Resonance Raman Spectroscopy

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─────ABSTRACT─────

The use of resonance Raman spectroscopy in the determination of food dyes was studied with the yellow, orange and red dyes registrated by the European Economical Community. The natural dyes studied did not exhibit a resonance Raman spectrum, but most of the artificial dyes give spectra that can be used for analytical determinations. The detection and identification limits are sufficiently low for practical use. The identification capability and its advantage over absorption spectrometric measurements are demonstrated on a commercial bubble gum sample. Quantitative measurements indicated a good accuracy for this method.

INTRODUCTION

THE WIDESPREAD USE of artificial colorants in food has given rise to certain questions about the toxicity of these compounds to man. Although few exclusive results have been obtained on this matter, it has however urged not only national but also international authorities to put forward legal regulations and restrictions for the use of these compounds.

The supervision of the observation of these rules requires adequate analytical methodology. Commonly, this is being done by using colorimetric instrumentation but mainly by paper, thin-layer and high performance chromatography (Dixon and Remyk, 1982; Graichen, 1975; McKone and Ivie, 1980; Lepri et al., 1978; Masalia-Tsobo, 1980; Sasaki, 1979). Both methods have some disadvantages. Visible absorption techniques are usually not very much specific. Chromatographic techniques have the disadvantage to rely on retention time, which may vary with differences in the medium. Moreover, sample preparation procedures are very cumbersome, Even the combination of the two techniques (HPLC analysis and UV/VIS scanning) may not entirely resolve the problem.

In this study, the use of resonance Raman spectroscopy for the detection, identification and quantitation of food dyes is investigated. The Raman spectrum of a sample is obtained by irradiating it with an intense monochromatic laser beam and analysing the fraction of the scattered light which has a lower frequency. From the polarizability theory of Placzek (1934), it follows that if the frequency of the incident beam is as close as possible to the absorption maximum of the compound being studied, the intensities of the spectral Raman bands are increased by several orders of magnitude (up to 6). This permitted the development of a new technique, called Resonance Raman Spectroscopy, which has been used in this study. Few studies concerning the use of resonance Raman spectroscopy in food dye investigations have been published. Brown and Lynch (1976) showed that red food colorants could be detected and identified in commercial food stuffs. Higuchi et al. (1978) did a more profound study on three dyes by this technique. Both studies, however, were based on a very limited number of

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dyes and did not treat the qualitative and quantitative aspect simultaneously.

In the present study, the whole range of yellow, orange and red water-soluble dyes used for food coloring, registrated by the European Economical Community, was investigated qualitatively and quantitatively by resonance Raman spectroscopy and compared with the traditional colorimetric method.

MATERIAL & METHODS

THE SPECTRA of the food colorants were recorded on a Raman spectrometer Coderg PHO using its standard illumination chamber and the 0.3 ml liquid cell. A slit width of approximately 13 cm^{-1} in combination with a time constant of 2.5 sec were used. The spectral range extended from 800–1900 cm^{-1}. To excite the samples, a Spectra Physics Model 164 Ar+ laser was used.

Table 1—Names and classifications of the dyes investigated

Names	ECC	Color Index	FD&C
Yellow dyes:			
Tartrazin Tartrazin RS Tartar Yellow Hydrazin Yellow CI Acid Yellow	E102	19140	Yellow 5
Chrysoine S Yellow T Tropaeoline 0 CI Acid Orange 6	E103	14270	
Quinolidine Yellow Quinidine Yellow KT	E104	47005	Yellow 10
Orange dyes:			
Sunset Yellow Sunset Yellow FCF	E110	15985	
Orange GGN	E111	15980	
Red dyes:			
Carmoisine A Azorubine Azorubine S	E122	14720	
Amaranth Amaranth R CI Acid Red 27	E123	16185	Red 2
Cochineal Red A New Coccine Ponceau 4R Brilliant Ponceau 3R Brilliant Ponceau 4R Brilliant Ponceau 5R Xylidine Ponceau Naphtalene Scarlet 4R	E124	16255	
Scarlet GN	E125	14815	
Ponceau 6R Crystal Ponceau 6R	E126	16250	
Erythrosine Erythrosine B Erythrosine extra bluish S 887	E127	45430	Red 3

Fig. 1—Raman spectra of some food dyes: (A) E102, 10 ppm; (B) E103, 10 ppm; (C) E104, 10 ppm; (D) E110, 10 ppm; (E) E111, 10 ppm.

After amplification of the primary signals provided by a S20 photomultiplier tube, the data were stored on a Facit 4070 paper-tape punch. The tapes were read by a Facit 4030 photoreader and stored in a HP 1000 minicomputer, based on a 21 MX-E processor with 128 k words of semiconductor memory. At this point data manipulation, such as smoothing and baseline correction, were carried out by appropriate programs (Van Haverbeke and Janssens, 1981). Plotting of the spectra was done on a HP 9872B digital plotter.

For UV measurements, a Cary 17D, coupled on-line with the HP 1000 minicomputer was used. All UV spectra were recorded in the range 825–200 nm. Switching of the tungsten-lamp (VIS) to the deuterium-lamp (UV) was made at 360 nm. No absorption nor T suppression was required and the overall used pen-period was 5 sec, with the slit control setting being 2.3.

All the food dyes used were purchased from Gurr (Hopkins & Williams, Essex, UK) or were obtained in powdered form through the courtesy of N.V. Sluys (Boechout, Belgium). Stock solutions were made up in a concentration of 500 ppm in distilled water and were used after appropriate dilution. Commonly available bubble gum was used as commercial food samples.

RESULTS & DISCUSSION

THE DIFFERENT DYES used in the food industry can be referenced by their commonly used names or by one of the classification numbers that are available today. In Table 1, the yellow, orange and red food dyes, both natural and artificial, that are classified by the EEC are listed, together with the most frequently used names and synonyms, their color index number (CI), and the FD&C classification where possible.

The absorption maxima of the different dyes vary considerably (around 420 nm for yellow dyes, 470 nm for orange dyes, and 500 nm for red dyes). For the red dyes, both the 488 and 514.5 nm laser lines of the Argon ion laser are suitable. For the yellow and orange dyes, one has to choose between the 488 nm line that is very high in intensity, but slightly off the absorption maximum, and one of the lower wavelength lines that are much closer to the absorption maxima but are less intense. Except for E104, for which a better result was obtained with the 458 nm line, the best resonance Raman spectra were recorded using the blue-green 488 nm laser line. The same excitation frequency can thus be used for all but one dye.

Resonance Raman spectra were recorded from the dyes cited in Table 1 at concentrations of 10 ppm. Since no considerable photochemical decomposition was found, the standard 0.3 ml liquid cell was used.

None of the natural food dyes studied here showed a noticeable resonance Raman spectrum. The reason for this phenomenon must be found in the chemical composition of these natural food colorants. We were not able to record resonance Raman spectra of compounds E105 and E127. These compounds exhibit a huge fluorescence on which the Raman spectrum is superimposed. Because the intensity of the fluorescence band is very much higher and causes much
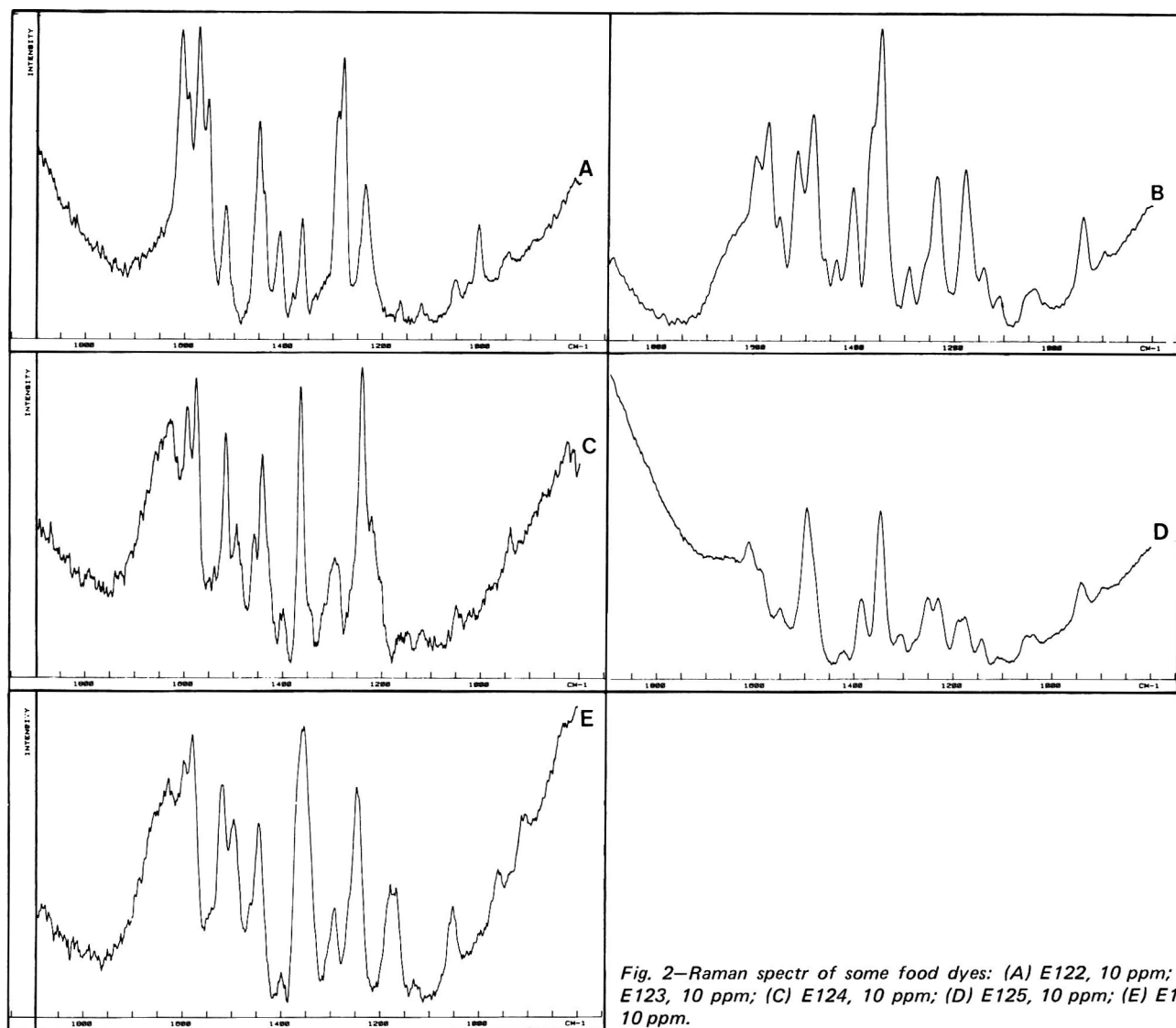
Fig. 2—Raman spectr of some food dyes: (A) E122, 10 ppm; (B) E123, 10 ppm; (C) E124, 10 ppm; (D) E125, 10 ppm; (E) E126, 10 ppm.

more spectral noise, the information from the resonance Raman spectrum is almost lost.

The resonance Raman spectra of the remaining dyes are shown in Fig. 1 and 2. The most important bands are listed in Table 2. As can be seen, the differences in the spectra are very large, to the extent that these spectra can be used as a fingerprint to identify the dye present in the sample.

The advantage of this technique over the traditional visible absorption technique is displayed in Fig. 3 and 4. The outer layer of a commercial yellow bubble gum ball is dissolved in 50 ml distilled water and filtered through a Millipore 0.3 μm filter. Fig. 3B and 4B show respectively the resonance Raman and visible spectra of this solution. For comparison purposes, the resonance Raman and visible spectra of E102 and E104 are shown in the Fig. 3C and 4C and Fig. 3D and 4D, respectively. Using the visible absorption spectra, it is difficult to conclude whether E102 or E104 are present in the bubble gum ball. The resonance Raman spectra however show a difference between the spectra of the two yellow dyes, and very good agreement between the dye in the bubble gum ball and E102.

This feature of the technique becomes even more interesting for a mixture of two food dyes with totally different colors. In this case, by appropriate selection of the exciting wavelength, it is possible to have one dye respond to the resonance conditions while the other does not. In such a situation, the resonance Raman spectrum of the former dye will be seen very clearly, while the ordinary

Raman spectrum of the latter will be very weak and will not be noticed. An example of this is also included in Fig. 3. Spectrum 3A is recorded from the filtered solution of the outer layer of a commercial green bubble gum ball in 50 ml distilled water. To obtain a green color, either a green dye can be used or a mixture of a yellow and a blue dye can be applied. Using the 488 nm laser line puts the yellow dye in resonance conditions, but the blue dye, having an absorption maximum around 600 nm, is well off reso-nance. The spectrum of this solution shows a very good agreement with the yellow dye E102, from which we can conclude that the green color is formed by mixing E102 with a blue dye. For comparison, Fig. 4A shows the visible spectrum of the same solution. From this plot, we can only assume that a mixture of a blue and a yellow dye is present, but we can not say with certainty whether the yellow dye is E102 or E104.

The detection limits of the different dyes were deter-mined by making solutions of lower concentrations by con-secutive dilutions and recording the spectra. We considered a compound detectable when at least one band has a signal-to-noise ratio of 3. All the dyes studied had detection limits somewhat below the 100 ppb level. This value is higher than the detection limit mentioned by Higushi et al. (1978). However, the detection limit is of little or no value because it only indicates the presence of any dye of the series, since the most intense band in the spectra will usually occur in the $1300-1500 \text{ cm}^{-1}$ region where the

Table 2—Tabulation of the most important Raman bands of the dyes[a]

Dye	Frequency[b] (relative intensity)						
E102	812(36)	839(23)	878(21)	916(17)	1050(9)	1100(12)	1129(25)
	1158(20)	1179(18)	1219(12)	1281(23)	1352(64)	1415(27)	1481(67)
	1502(78)	1600(100)	1639(78)				
E103	875(23)	1009(7)	1131(21)	1194(26)	1278(41)	1316(43)	1326(42)
	1338(40)	1392(83)	1424(49)	1473(30)	1502(50)	1615(100)	
E104	1121(9)	1165(11)	1229(36)	1239(36)	1282(23)	1292(24)	1309(21)
	1372(100)	1419(40)	1441(56)	1470(33)	1478(33)	1485(31)	1492(31)
E110	990(30)	1041(14)	1099(18)	1133(6)	1189(41)	1236(76)	1263(19)
	1343(60)	1394(69)	1428(31)	1487(61)	1507(87)	1555(44)	1609(100)
E111	991(20)	1035(6)	1100(14)	1179(18)	1229(98)	1341(66)	1387(72)
	1440(36)	1487(100)	1553(53)	1600(92)			
E122	942(25)	1001(33)	1053(11)	1121(6)	1162(7)	1234(47)	1278(89)
	1290(71)	1336(10)	1365(34)	1382(10)	1410(31)	1451(68)	1516(40)
	1555(75)	1575(100)	1594(78)	1609(99)			
E123	942(37)	1039(13)	1109(11)	1141(20)	1177(53)	1258(51)	1292(20)
	1348(100)	1366(67)	1407(43)	1439(18)	1487(67)	1516(59)	1555(37)
	1575(69)	1600(57)					
E124	942(20)	1053(6)	1100(14)	1179(18)	1229(98)	1341(66)	1387(72)
	1440(36)	1487(100)	1553(53)	1600(92)			
E125	893(46)	939(51)	1034(19)	1050(19)	1107(6)	1141(18)	1175(27)
	1228(40)	1250(39)	1304(18)	1346(100)	1384(43)	1421(10)	1492(97)
	1550(31)	1589(51)	1613(64)				
E126	908(68)	961(46)	1052(32)	1131(6)	1167(40)	1182(40)	1246(77)
	1292(32)	1355(100)	1400(9)	1444(64)	1497(66)	1521(78)	1573(96)
	1598(87)	1627(89)					

[a] Bands with a relative intensity smaller than 5 were omitted.
[b] Expressed in cm^{-1} displacement versus the excitation line.
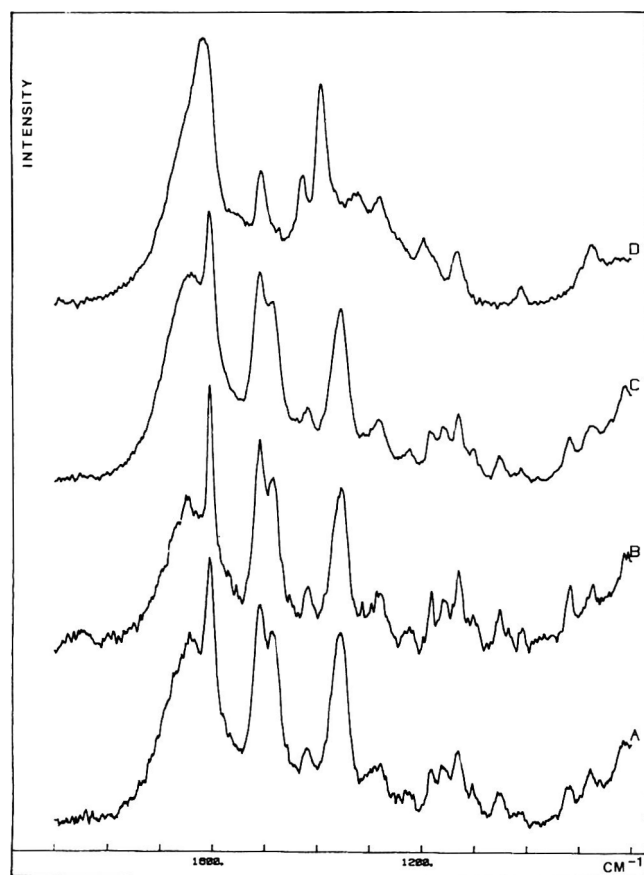
Fig. 3—Raman spectra of food dyes and candy: (A) Colored layer of a green bubble gum (see text); (B) Colored layer of a yellow bubble gum (see text); (C) E102; (D) E104.
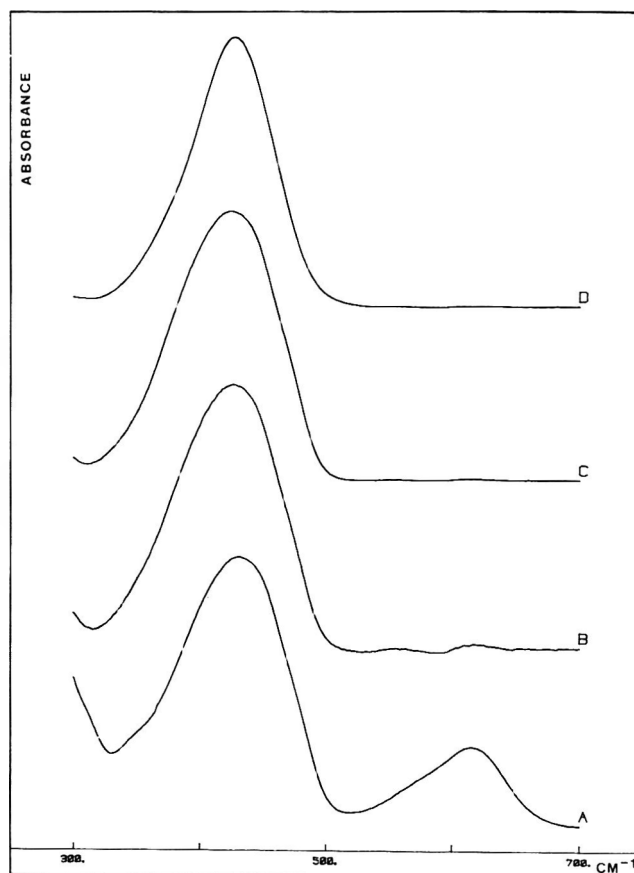
Fig. 4—UV/VIS spectra of food dyes and candy: (A) Colored layer of a green bubble gum (see text); (B) Colored layer of a yellow bubble gum (see text); (C) E102; (D) E104.

stretching frequency of the N=N bond, present in most dyes, will occur. Much more important is the identification limit, which is the concentration for which the resonance Raman spectrum shows at least five bands with a signal-to-noise ratio of at least 3. Usually, these five bands are enough to reveal the identity of the dye. Values between 200 and 300 ppb were obtained for the dyes which gave resonance Raman spectra. An upper detection limit of 30 ppm is found when, due to absorption, the intensity of the resonance Raman spectrum becomes too small to perform adequate measurements. Although absolute intensity measurements for quantitative analysis can be performed, their accuracy is highly questionable because the intensity of a Raman spectrum depends on a number of parameters which can not be controlled entirely by the operator of the instrument. Therefore, it has become common practice to do relative intensity measurements in all types of Raman spectroscopy: the ratio of the intensity of the band to be measured versus a band from an internal standard of known concentration is determined. For this purpose, the 1352 cm^{-1} band of E102 was measured and the ratio to the 1640 cm^{-1} band of water determined. A calibration plot was made with solutions of E102 varying from 1–20 ppm. On these measurement points, several least squares regression methods were performed. The best results were obtained with a single power regression of the type $Y = aX^b$ where b is different from 1. For values of 15.1 and 0.92 for a and b respectively, we obtained a correlation coefficient of 0.99.

Using this calibration plot, the concentration of E102 in the above mentioned solutions of a yellow and a green commercial bubble gum ball in 50 ml distilled water was determined. Since the concentrations of these solutions did not fall in the range of the calibration plot, appropriate dilutions were made. For the original solutions, concentrations of 27 and 47 ppm, respectively, were found for the yellow and green bubble gum ball, which results in 1.35 and 2.40 mg E102, respectively, per bubble gum ball, with a standard deviation of 0.10 mg. A comparative study with visible absorption spectroscopy revealed amounts of 1.35 and 2.45 mg each with a standard deviation of 0.05 mg.

CONCLUSION

FROM THE FOREGOING, we may conclude that resonance Raman spectroscopy can be used for the determination of

yellow, orange and red dyes in some kinds of food. The different dyes can be identified at concentrations that are low enough for practical purposes. Furthermore, since water has only a very weak Raman spectrum, measurements in water are easily done, and application to water-soluble foodstuffs is therefore relatively straight-forward. A very interesting feature is the fact that artificial dyes can be determined in the presence of the natural dyes studied here, which makes the method capable of detecting the addition of small amounts of aritifical coloring in naturally colored foodstuffs.

Presently, this study is being extended over the whole range of food dyes, as well as the ones that are officially regulated and those that are forbidden. Library search methods for identification of the dye from the recorded spectrum using computer search methods are also being developed. The quantitative aspect will be studied extensively, and the problems associated with dye mixtures will be evaluated from a number of different approaches.

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Inter- and Intra-Laboratory Variation in Amino Acid Analysis of Food Proteins

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————————————ABSTRACT————————————

Amino acid compositions of casein, egg white, beef, soy isolate, rapeseed concentrate, pea flour and wheat flour were studied by seven collaborating laboratories. Samples were hydrolyzed with 6N HCl, performic acid + 6N HCl, and (with one exception) 4.2N NaOH. Amino acids were then determined by ion-exchange chromatography using automatic analyzers. One laboratory, however, determined tryptophan by treatment with p-dimethylaminobenzaldehyde. The colorimetric determination of tryptophan was comparable to the tryptophan values determined by ion-exchange chromatography. Interlaboratory variation of tryptophan (coefficients of variation, CV, up to 24%), cystine and methionine (CV up to 17%), was greater than that of most other amino acids (CV up to 10%). Intralaboratory variation for all amino acids was less than 5%.

INTRODUCTION

RELIABLE AND RAPID MEANS of assessing protein quality of foods need further development for scientific and regulatory purposes. Theoretically, a logical way to assess protein quality is by comparing the amino acid content of a food with human amino acid requirements, for example by calculating an amino acid or chemical score (FAO/WHO, 1973). This method also provides information about the most limiting amino acid and supplementary or complementary value of proteins. Nevertheless, a serious problem associated with this and other *in vitro* methods which use amino acid profiles, is that of obtaining reliable amino acid composition. Canada and the United States have no official methods for amino acid analysis of food proteins. The use of different amino acid procedures in various laboratories may result in very different estimates of protein quality. Therefore, determining and controlling the variation in the commonly used methods of amino acid analysis become important for improving *in vitro* indices of protein quality.

Most amino acids in proteins can be satisfactorily determined with an automatic amino acid analyzer after properly controlled acid hydrolysis (Blackburn, 1978; Moore and Stein, 1963). Hydrolysis with 6N HCl at 110°C for 18–24 hr is commonly used for the determination of all amino acids except tryptophan. The hydrolysis time, which is selected by some analysts arbitrarily, has been repeatedly found to influence release of amino acids from proteins

(Cherry, 1981; Hackler, 1981; Moore and Stein, 1963). Although cystine and methionine are determined in 6N HCl hydrolysates by some analysts, separate hydrolyses are preferred for analysis of sulphur amino acids and tryptophan since they are destroyed to varying degrees during acid hydrolysis. Cyst(e)ine and methionine are more accurately determined after oxidation by performic acid to their stable forms, i.e. cysteic acid and methionine sulfone, respectively by the method of Moore (1963). Methods of tryptophan analysis have been critically reviewed by Friedman and Finley (1971). Alkaline hydrolysis is the most feasible way of quantitative determination of tryptophan in proteins when using an automatic analyser (Blackburn, 1978). In this respect, Hugli and Moore (1972) developed a simple procedure (in which proteins are hydrolyzed with NaOH containing starch as an antioxidant) permitting accurate determination of tryptophan.

Although suitable methods for determining all amino acids have been developed, interlaboratory comparisons of these methods for routine use are either incomplete or limited in scope (Happich et al., 1981). Knipfel et al. (1971) reported between-laboratories mean coefficients of variation (CV) of up to 7% for amino acids in casein, soybean meal and fish flour. Much higher variability has been reported for methionine (relative standard deviation, RSD, 16.3%) and cystine (RSD, 27.7%) in soybean meal (Cavins et al., 1972) and for cystine (CV values of up to 38%) in seven protein sources (Williams et al., 1979) as well as for tryptophan (CV values of up to 53%) in soybean, peanut and cottonseed protein concentrates (Westgarth and Williams, 1974). The lower variability reported by Knipfel et al. (1971) may have resulted because the collaborators analyzed samples of the same hydrolysates, prepared in one laboratory. In routine work, however, laboratories would prepare their own hydrolysates. Therefore the results of Knipfel et al. (1971) do not provide a realistic estimate of the variation. Moreover, cystine and tryptophan were not studied by these workers. The objectives of the present investigation were twofold: (a) to obtain more information on inter- and intra-laboratory variability in the currently used methods of amino acid analysis of protein products; (b) to re-investigate the effect of time of hydrolysis on the release of amino acids.

MATERIALS & METHODS

SAMPLES of seven proteins were prepared and distributed by the organizing laboratory (laboratory 1) as described previously in a preliminary report (Sarwar et al., 1981). The casein (ANRC Reference Protein) was purchased from Humko Sheffield Chemical, Kraftco Corporation (Madison, WI). Egg white solids and soy assay protein were bought from Teklad Mills, ARS (Sprague-Dawley, Division of the Mogul Corp., Madison, WI). The samples of pea flour and rapeseed protein concentrate were kindly provided by Griffith Laboratories (Toronto) and Dr. J.D. Jones, Food Research Institute, Agriculture Canada (Ottawa), respectively. Minced beef (lean grade) and whole wheat flour (Five Roses brand) were purchased from a local supermarket. The meat sample was freeze-dried and defatted before analysis.

All the samples were finely ground (40 mesh) and mixed for 30 min before subsampling and shipping to the collaborating laboratories. A vial (5.5 ml) of Beckman Calibration Standard for amino

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Lab. no.	Sample weight (mg protein)	6N HCl used (ml)	Method of hydrolysis[a]	Method of removal of 6N HCl after hydrolysis	Column system(s)	Amino acid analyzer
1	50	175	Sealed flask under N_2	Evaporated under N_2 at 45°C in analytical evaporator	Two	Beckman 121MB
2	30-180	80	Sealed jar under N_2	Evaporated at 45°C under vacuum	One	Technicon TSM-IR
3	90	50	Sealed flask under N_2	Evaporated at 40°C under vacuum	One	Beckman 119BL
4	5	5	Evacuated and sealed tube	Evaporated at 40°C under vacuum	One	Durrum D500
5	30-35	10	Evacuated and sealed flask	Evaporated in vacuum desiccator over NaOH	Two	Beckman Spinco 120
6	75	300	Refluxed under N_2	Evaporated in rotary evaporator	Two	Beckman 120C
7	5	2	Evacuated and sealed tube	Evaporated in vacuum desiccator over NaOH	Two	Beckman 121

[a] Temperature = 110°C; Time = 11, 22, 44 or 66 hr

acid analyzers (No. 31220) was also sent to each of the participating laboratories.

Each laboratory analyzed the samples for dry matter and nitrogen contents. Laboratory 1 also analyzed the samples for ether extractables, insoluble dietary fiber (Mongeau and Brassard, 1982), and carbohydrates (other than insoluble dietary fiber). Moisture, nitrogen, ether extractables and ash were determined by AOAC (1975) procedures.

For each of the seven proteins, three hydrolyses (6N HCl, performic acid + 6N HCl and 4.2N NaOH) were carried out in duplicate by all collaborating laboratories, except laboratory 5, where the basic hydrolysis was replaced by p-dimethylaminobenzaldehyde treatment of intact proteins (Spies and Chambers, 1949). In addition, two protein sources (casein and rapeseed protein concentrate) were hydrolyzed in duplicate with 6N HCl at 110°C for 11, 44 or 66 hr.

Samples were hydrolyzed with 6N HCl to obtain hydrolysates suitable for analysis of all amino acids except cystine + cysteine and tryptophan (in all laboratories) and methionine (in some laboratories). The 6N HCl hydrolysates were prepared by each laboratory's normal procedure (Table 1). All collaborators hydrolyzed the samples at 110°C for 11, 22, 44 or 66 hr, but widely different sample weights and volumes of acid were used. The sample to acid ratios (mg protein/ml acid) varied from 0.4–4.0. Three different methods of hydrolysis, i.e. in evacuated and sealed tubes or flasks (three laboratories), sealed flasks or jars (three laboratories) or under reflux (one laboratory) were used. Four laboratories used two column analyzers while the other three used one column analyzers. The collaborators used their own buffer systems and calibration standards in addition to the common amino acid calibration standard.

Samples oxidized with performic acid were also hydrolyzed with 6N HCl to obtain hydrolysates suitable for the determination of cystine + cysteine as cysteic acid (all laboratories) and of methionine as methionine sulfone (laboratories 1, 2, 4, 5, and 6) by the method of Moore (1963).

Samples were hydrolyzed with 4.2N NaOH for the determination of tryptophan using the procedure of Hugli and Moore (1972) in all laboratories except laboratory 5, in which unhydrolyzed samples were treated with p-dimethylaminobenzaldehyde by a method of Spies and Chambers (1949).

All amino acids, except tryptophan in laboratory 5, were determined by ion-exchange chromatography in an automatic amino acid analyzer (Table 1). In laboratory 5 tryptophan was determined colorimetrically with a Bausch and Lomb Spectronic 20 spectrophotometer.

Laboratories were instructed to record results as mg amino acid/g nitrogen. All results were based on the nitrogen and moisture analyses carried out individually in each collaborating laboratory.

All results were statistically evaluated on an "as reported" basis and after adjustment to the same nitrogen values (as reported by

Table 2—Means, standard errors (SE), and coefficients of variation (CV) for recovery of nitrogen in seven protein sources

Protein source	Mean[a]%	Between laboratories SE[a]	CV%[a]	Within laboratories CV%
Casein	99.4	2.9	2.9	0.8
Egg white solids	94.6	7.9	8.4	1.3
Minced beef	93.7	4.2	4.5	0.8
Soy assay protein	98.5	3.7	3.7	1.1
Rapeseed protein conc	96.7	3.6	3.7	0.6
Pea flour	94.9	5.5	5.8	1.1
Whole wheat flour	93.6	4.0	4.3	0.9

[a] These data were previously reported as preliminary results (Sarwar et al., 1981).

Laboratory 1). Before statistical analyses, the units for amino acids were changed from mg/g N to g/16g N by the multiplication factor 16/1,000.

The arithmetic means for each protein were computed for 18 amino acids (arginine, histidine, isoleucine, leucine, lysine, methionine, cystine, phenylalanine, tyrosine, threonine, tryptophan, valine, alanine, aspartic acid, glutamic acid, glycine, proline and serine), ammonia, and nitrogen recovery values. Analysis of variance techniques were used to obtain measures of between-laboratories and within-laboratories variability (Steiner, 1975) for each amino acid within each protein source hydrolyzed for 22 hr. The between-laboratories standard error is the estimated standard error of the mean of the two duplicate determinations in this investigation. It is defined as $\sqrt{\bar{\sigma}_A^2}$, where $\bar{\sigma}_A^2$ is the between-laboratories mean square from the analysis of variance. The between-laboratories coefficient of variation (CV) is defined as $\frac{\bar{\sigma}_A}{\sqrt{2} \text{(mean)}} \times 100$ and is relative measure of variation expressed as a percentage.

Within-laboratories variances were obtained by using the within-laboratories mean squares, $\bar{\sigma}_W^2$, as a pooled estimate of variance. From these variances, within-laboratories coefficients of variation were calculated by the following formula using the same overall mean as was used above.

$$CV \text{ (within laboratories)} = \frac{\bar{\sigma}_W}{\sqrt{2} \text{ (mean)}} \times 100$$

RESULTS & DISCUSSION

MEANS, SE, AND CV VALUES for recovery of nitrogen (calculated from quantitative analysis of 18 amino acids plus ammonia) in seven protein sources are given in Table 2. The mean recovery values were 93.6–99.4%. Casein, soy

Table 3—Proximate composition (% dry basis) of seven protein sources

Protein source	Protein[a,d] (N × 6.25)	Ether extractables[b,d]	Insoluble dietary fibre[b]	Ash[b,d]	Carbohydrates[c] (other than insoluble dietary fibre)
Casein	94.7 ± 0.8	0.2	0.1	0.4	4.6
Egg white solids	87.7 ± 0.5	0.2	0.1	5.7	6.3
Minced beef	95.2 ± 1.1	0.4	0.1	3.9	0.4
Soy assay protein	95.6 ± 0.8	0.2	0.2	1.8	2.2
Rapeseed protein concentrate	69.1 ± 0.8	0.9	9.8	8.0	12.2
Pea flour	30.8 ± 0.4	1.1	1.8	3.8	62.5
Whole wheat flour	16.4 ± 0.1	2.1	9.2	1.5	70.8

[a] Mean ± SEM of seven laboratories.
[b] Determined in laboratory 1.
[c] Calculated by difference.
[d] These data were previously reported as preliminary results (Sarwar et al., 1981).

Table 4—Means, standard errors (SE), and coefficients of variation (CV) of amino acids in casein[a]

Amino acid (g/16 gN)	Mean	Between laboratories SE	Between laboratories CV%	Within laboratories CV%
Arginine	3.71	0.34	9.1	1.8
Histidine	2.97	0.18	6.0	1.6
Isoleucine	5.36	0.26	4.8	1.3
Leucine	10.16	0.44	4.3	0.7
Lysine	8.44	0.42	5.0	0.6
Methionine	3.02	0.13	4.2	0.8
Cystine	0.47	0.08	17.6	2.6
Phenylalanine	5.47	0.39	7.1	1.5
Tyrosine	6.04	0.42	6.9	1.2
Threonine	4.64	0.32	7.0	1.5
Tryptophan	1.31	0.19	14.3	1.1
Valine	6.85	0.30	4.4	1.4
Alanine	3.30	0.19	5.8	1.8
Aspartic acid	7.71	0.31	4.0	0.9
Glutamic acid	24.00	2.53	10.5	1.3
Glycine	2.00	0.13	6.4	1.3
Proline	11.72	0.78	6.6	1.9
Serine	6.10	0.22	3.6	1.6
Ammonia	1.98	0.19	9.6	5.9

[a] Parts of the data contained in this Table were reported previously as preliminary results (Sarwar et al., 1981).

Table 5—Means, standard errors (SE), and coefficients of variation (CV) of amino acids in egg white solids[a]

Amino acid (g/16 gN)	Mean	Between laboratories SE	Between laboratories CV%	Within laboratories CV%
Arginine	5.69	0.50	8.9	1.2
Histidine	2.25	0.25	10.9	1.4
Isoleucine	5.28	0.59	11.2	2.2
Leucine	8.76	0.74	8.4	1.7
Lysine	6.98	0.62	8.8	2.5
Methionine	3.83	0.32	8.4	1.8
Cystine	2.81	0.31	11.0	1.7
Phenylalanine	6.21	0.57	9.3	1.0
Tyrosine	4.40	0.34	7.7	2.5
Threonine	4.68	0.46	9.9	2.7
Tryptophan	1.46	0.29	19.7	1.5
Valine	6.78	0.64	9.5	2.0
Alanine	5.99	0.60	10.0	4.2
Aspartic acid	10.46	1.06	10.1	1.7
Glutamic acid	14.00	1.94	13.9	2.2
Glycine	3.59	0.27	7.4	1.6
Proline	3.76	0.40	10.5	3.8
Serine	6.83	0.64	9.4	2.3
Ammonia	1.64	0.45	27.5	4.6

[a] Parts of the data contained in this Table were reported previously as preliminary results (Sarwar et al., 1981).

isolate, and rapeseed concentrate seemed to give higher nitrogen recovery values than other protein sources. Interlaboratory variation in nitrogen recovery appeared higher for egg white and pea flour than for other samples. Part of the variation may have been due to differences in the determination of nitrogen in the samples which were analyzed independently in each laboratory (Table 3). The use of different nitrogen values for a given sample will also influence estimates of interlaboratory variation for amino acids in that sample. In order to investigate this point, amino acid data were adjusted by using the same nitrogen values as determined by Laboratory 1. The adjusted means, SE and CV values (not reported here) for 18 amino acids plus ammonia and recovery of nitrogen in most cases changed only by a very small amount. Therefore, only the original data, which give more reliable estimate of the error, are reported.

Means, SE and CV values for 18 amino acids and ammonia in casein, egg white, beef, soy isolate, rapeseed concentrate, pea flour, and wheat flour (all hydrolyzed for 22 hr) are given in Tables 4, 5, 6, 7, 8, 9 and 10, respectively. The estimates of inter- and intra-laboratory variation differed for each amino acid within a protein source (Tables 4—10). In all cases, intralaboratory variation was smaller than the variation between laboratories.

In casein, the interlaboratory variation (estimated as CV) for all amino acids except cystine (17.6%), tryptophan

(14.3%) and glutamic acid (10.5%) was less than 10% (3.6—9.1%, Table 4), whereas the intralaboratory variation for all amino acids was lower than 3% (0.6—2.6%).

In egg white, the between-laboratories CV values for all amino acids except tryptophan (19.7%) and glutamic acid (13.9%) were below 12% (7.4—11.2%, Table 5). The within-laboratories CV values for most amino acids were below 5% (1.0—4.2%).

In beef, four amino acids (tryptophan, cystine, methionine and glutamic acid) had between-laboratories CV values of more than 10% while the within-laboratories CV values for most amino acids were less than 3% (Table 6).

Tryptophan exhibited the highest between-laboratories CV of 23.7% in soy isolate whereas the values for all other amino acids except histidine (12.3%), cystine (11.4%) and methionine (11.3%) were below 10% (3.3—9.4%, Table 7). Methionine had the highest within-laboratories CV of 4.5%, while the values for all other amino acids were below 3%.

In rapeseed concentrate, the interlaboratory variation for five amino acids (cystein, tryptophan, tyrosine, methionine, and proline) was higher than 10% (Table 8). This variation was particularly high for cystine (CV = 17.5%), and tryptophan (CV = 15.2%). The estimates of intralaboratory variability for all amino acids except cystine and isoleucine were below 4%.

The between-laboratories CV values for cystine, methio-

Table 6—Means, standard errors (SE), and coeffients of variation (CV) of amino acids in minced beef[a]

Amino acid (g/16 gN)	Mean	Between laboratories SE	Between laboratories CV%	Within laboratories CV%
Arginine	6.55	0.57	8.7	1.1
Histidine	3.20	0.24	7.6	3.7
Isoleucine	4.18	0.14	3.4	0.8
Leucine	7.75	0.46	5.9	1.4
Lysine	7.94	0.75	9.5	1.2
Methionine	2.25	0.24	10.5	1.4
Cystine	1.02	0.13	12.8	2.7
Phenylalanine	3.88	0.33	8.6	2.0
Tyrosine	3.14	0.22	7.1	2.1
Threonine	4.21	0.17	4.0	2.7
Tryptophan	0.99	0.17	16.9	2.6
Valine	4.54	0.25	5.5	2.3
Alanine	6.57	0.47	7.2	2.3
Aspartic acid	8.99	0.54	6.0	2.2
Glutamic acid	15.28	1.58	10.3	1.4
Glycine	7.44	0.50	6.8	1.9
Proline	5.47	0.48	8.8	2.6
Serine	3.90	0.26	6.6	1.9
Ammonia	1.40	0.16	11.4	7.1

[a] Parts of the data contained in this Table were reported previously as preliminary results (Sarwar et al., 1981).

Table 7—Means, standard errors (SE), and coefficients of variation (CV) of amino acids in soya assay protein[a]

Amino acid (g/16 gN)	Mean	Between laboratories SE	Between laboratories CV%	Within laboratories CV%
Arginine	7.87	0.74	9.4	1.4
Histidine	2.54	0.31	12.3	1.5
Isoleucine	4.72	0.16	3.3	2.5
Leucine	8.51	0.40	4.7	2.0
Lysine	6.34	0.37	5.8	1.9
Methionine	1.24	0.14	11.3	4.5
Cystine	1.19	0.14	11.4	2.4
Phenylalanine	5.62	0.39	6.9	1.4
Tyrosine	4.04	0.18	4.4	2.0
Threonine	3.84	0.34	8.8	1.1
Tryptophan	1.14	0.27	23.7	3.7
Valine	4.91	0.33	6.8	1.4
Alanine	4.37	0.26	6.0	2.3
Aspartic acid	11.80	0.57	4.8	2.2
Glutamic acid	20.70	1.71	8.3	2.0
Glycine	4.22	0.26	6.2	2.0
Proline	5.42	0.36	6.6	2.3
Serine	5.30	0.38	7.2	1.4
Ammonia	1.98	0.22	11.4	2.2

[a] Parts of the data contained in this Table were reported previously as preliminary results (Sarwar et al., 1981).

Table 8—Means, standard errors (SE), and coefficients of variation (CV) of amino acids in rapeseed protein concentrate[a]

Amino acid (g/16 gN)	Mean	Between laboratories SE	Between laboratories CV%	Within laboratories CV%
Arginine	6.90	0.57	8.2	1.2
Histidine	2.91	0.19	6.5	3.5
Isoleucine	4.16	0.38	9.1	4.1
Leucine	7.84	0.41	5.2	0.9
Lysine	5.70	0.33	5.8	2.9
Methionine	1.97	0.25	12.8	1.7
Cystine	2.46	0.43	17.5	4.3
Phenylalanine	4.24	0.40	9.5	1.8
Tyrosine	2.82	0.38	13.5	3.9
Threonine	4.19	0.35	8.3	3.6
Tryptophan	1.50	0.23	15.2	2.9
Valine	5.24	0.35	6.7	2.5
Alanine	4.71	0.19	4.1	2.1
Aspartic acid	7.54	0.24	3.1	1.9
Glutamic acid	20.32	1.84	9.0	1.1
Glycine	5.20	0.22	4.2	1.8
Proline	6.28	0.79	12.6	1.2
Serine	4.41	0.21	4.8	2.1
Ammonia	2.35	0.48	20.3	2.8

[a] Parts of the data contained in this Table were reported previously as preliminary results (Sarwar et al., 1981).

Table 9—Means, standard errors (SE), and coefficients of variation (CV) of amino acids in pea flour[a]

Amino acid (g/16 gN)	Mean	Between laboratories SE	Between laboratories CV%	Within laboratories CV%
Arginine	8.07	0.71	8.8	1.9
Histidine	2.44	0.22	9.1	2.7
Isoleucine	4.27	0.34	7.9	4.7
Leucine	7.67	0.55	7.1	3.1
Lysine	7.68	0.52	6.8	2.8
Methionine	1.11	0.18	16.1	4.7
Cystine	1.51	0.21	14.1	4.3
Phenylalanine	4.99	0.49	9.8	2.0
Tyrosine	3.50	0.57	16.4	3.1
Threonine	4.17	0.22	5.4	1.7
Tryptophan	0.87	0.16	18.5	3.0
Valine	4.96	0.50	10.0	2.3
Alanine	4.73	0.28	6.0	0.7
Aspartic acid	11.60	0.69	6.0	1.2
Glutamic acid	16.36	1.47	9.0	1.1
Glycine	4.62	0.27	5.9	0.9
Proline	4.31	0.28	6.5	2.7
Serine	4.94	0.38	7.7	3.3
Ammonia	1.73	0.29	16.9	0.9

[a] Parts of the data contained in this Table were reported previously as preliminary results (Sarwar et al., 1981).

nine, tyrosine, and tryptophan (14.1−18.5%) in pea flour were higher than 10% (Table 9). The within-laboratories CV values for all amino acids except methionine, isoleucine and cystine were lower than 4%.

In wheat flour, the interlaboratory variation for six amino acids (tryptophan, tyrosine, cystine, arginine, methionine and glutamic acid) was more than 10% with tryptophan (CV = 22.4%) having the highest value (Table 10). The within-laboratories CV values for all amino acids except tyrosine and methionine were below 4%.

The effects of different times of 6N HCl hydrolysis on the release of amino acids from casein and rapeseed concentrate are shown in Tables 11 and 12, respectively.

The release of five amino acids (isoleucine, lysine, phenylalanine, valine and serine) from casein was significantly affected by the length of hydrolysis (Table 11). Eleven hours of hydrolysis produced the lowest values for

isoleucine and valine. In addition, the 11 hydrolysis gave lower lysine and phenylalanine values than those obtained with 22 or 44 hr of hydrolysis. Sixty-six hr of hydrolysis produced a lower serine value than those obtained with 11 or 22 hr of hydrolysis. In general, the 22 hr hydrolysis gave optimum results.

In the case of rapeseed concentrate, seven amino acids (arginine, isoleucine, leucine, lysine, valine, alanine and serine) were significantly influenced by the hydrolysis time (Table 12). Eleven hours of hydrolysis produced the lowest values for arginine, isoleucine, leucine, lysine and valine. Sixty-six or forty-four hours of hydrolysis gave a lower serine value than the twenty-two hour hydrolysis period.

In general, there seemed to be no advantage in increasing the length of hydrolysis from 22 hr to 44 or 66 hr except for the highest release of valine and isoleucine after 44

and/or 66 hr of hydrolysis (Tables 11 and 12). Similar observations about the differences in recovery of isoleucine and valine with variation in hydrolysis time have previously been made (Hackler, 1981; Happich et al., 1981).

The estimates of inter- and intra-laboratory variation in amino acid analysis obtained in the present investigation (Tables 4–10) compare favorably with those reported in the literature (Cavins et al., 1972; Happich et al., 1981; Kreienbring, 1981; Westgarth and Williams, 1974; Williams et al., 1979). For example, the interlaboratory variability for most amino acids in soy isolate (Table 7) was lower than that reported for amino acids in soybean meal (relative standard deviations of up to 25.7%) by Cavins et al. (1982). Our between- and within-laboratories CV values for lysine (5.0–9.5%; 0.6–2.7%) in casein, beef, soy isolate and wheat flour were in good agreement with the CV values reported for lysine (4.2–8.4%; 3.0–4.9%) in these protein sources by Happich et al. (1981). But the interlaboratory variation for valine (CV = 4.4–10.0%) obtained in this

study was lower than that (CV = 8.5–14.4%) reported by Happich et al. (1981). When Happich et al. (1981) corrected their data by removing outliers, the interlaboratory variation for valine was lowered by about 50% and their corrected CV values (5.2–8.5%) were similar to our results.

The between-laboratories CV values for cystine in casein (17.6%), soy isolate (11.4%), and wheat flour (14.1%) obtained in this study were lower than those reported for these protein sources (casein = up to 38%, soybean = up to 18%, wheat = up to 23%) by Williams et al. (1979). Similarly, interlaboratory variation for tryptophan observed in this investigation (CV values of up to 24%) was lower than noted in Trial I (CV values of up to 53%) of Westgarth and Williams (1974). However, these workers were able to reduce the interlaboratory variation by repeating the collaborative study, and CV values for tryptophan in the second trial were below 10%. In our study, one laboratory (No. 5) determined tryptophan by the methods of Spies and Chambers (1949) while the other six laboratories used the Hugli and Moore (1972) method. Two of the laboratories, however, did not have experience with this method. Therefore, repeating the study for tryptophan determination in the two laboratories that did not have experience with the ion-exchange procedure may reduce variation between laboratories for this amino acid. It should be noted that in this study, the determination of tryptophan by the colorimetric method of Spies and Chambers (casein = 1.6, egg white = 1.7, beef = 1.1, soy isolate = 1.4, rapeseed concentrate = 1.7, pea flour = 0.9, wheat flour = 1.2 g/16g N) was comparable to the values obtained by ion-exchange chromatography (casein = 1.3, egg white = 1.4, beef = 1.0, soy isolate = 1.1, rapeseed concentrate = 1.5, pea flour = 0.9, wheat flour = 1.1 g/16g N, average of six laboratories).

To compare our results with those of Knipfel et al. (1971), amino acid data were transformed (T = $\log_e$) to achieve normality of residuals and the corrected data were also subjected to analysis of variance. The within- and between-laboratories CV values for transformed data of ten amino acids were previously reported (Sarwar et al., 1981, Tables 13.6 and 13.9). According to the transformed data, interlaboratory variation (expressed as CV values) for all 18 amino acids in the seven protein sources was not more than 5%. The CV values for tryptophan (3.3–5.0%), cystine (2.1–5.0%), and methionine (0.8–3.8%) were higher than the values for other amino acids, which were in most cases below 2%. The CV values calculated from the

Table 10—Means, standard errors (SE), and coefficients of variation (CV) of amino acids in whole wheat flour[a]

Amino acid (g/16 gN)	Mean	Between laboratories		Within laboratories
		SE	CV%	CV%
Arginine	4.31	0.56	13.0	2.8
Histidine	2.19	0.20	9.3	1.7
Isoleucine	3.34	0.24	7.2	2.2
Leucine	6.85	0.31	4.6	1.7
Lysine	2.66	0.19	7.3	2.7
Methionine	1.65	0.20	12.2	4.0
Cystine	2.22	0.31	14.1	2.0
Phenylalanine	4.87	0.42	8.6	1.0
Tyrosine	2.91	0.42	14.5	4.5
Threonine	2.93	0.28	9.7	1.5
Tryptophan	1.12	0.25	22.4	2.7
Valine	4.27	0.35	8.2	1.7
Alanine	3.48	0.17	4.8	1.5
Aspartic acid	5.04	0.25	5.0	3.3
Glutamic acid	30.95	3.96	12.8	1.7
Glycine	3.95	0.19	4.8	1.5
Proline	10.44	0.78	7.5	1.7
Serine	4.39	0.39	9.0	3.0
Ammonia	3.33	0.59	17.7	3.3

[a] Parts of the data contained in this Table were reported previously as preliminary results (Sarwar et al., 1981).

Table 11—Effect of time of 6N HCl hydrolysis on amino acid composition of casein

Amino acid (g/16 gN)	Hydrolysis time (hr)[a]			
	11	22[b]	44	6
Arginine	3.5	3.7	3.7	3.8
Histidine	2.8	3.0	3.2	3.0
Isoleucine	4.6a	5.3b	5.6b	5.6b
Leucine	9.5	10.2	10.0	9.8
Lysine	7.9ab	8.4c	8.5c	8.3bc
Phenylalanine	5.1ab	5.5c	5.3c	5.2bc
Tyrosine	5.7	6.0	5.8	5.6
Threonine	4.4	4.6	4.6	4.3
Valine	5.9a	6.8b	7.1b	7.1b
Alanine	3.2	3.3	3.2	3.2
Aspartic acid	7.7	7.7	7.7	7.5
Glutamic acid	22.6	24.0	23.4	22.8
Glycine	2.0	2.0	2.0	2.0
Proline	11.4	11.7	11.2	11.2
Serine	5.9c	6.1c	5.5bc	5.0ab

[a] Values for a given amino acid followed by different letters differ significantly (P < 0.01).
[b] The 22hr data were taken from Table 4.

Table 12—Effect of time of 6N HCl hydrolysis on amino acid composition of rapeseed protein concentrate

Amino acid (g/16 gN)	Hydrolysis time (hr)[a]			
	11	22[b]	44	6
Arginine	6.2a	6.9b	6.9b	6.9b
Histidine	2.7	2.9	2.9	2.9
Isoleucine	3.5a	4.2b	4.4b	4.5b
Leucine	7.2a	7.8b	7.7b	7.7b
Lysine	5.3a	5.7b	5.6b	5.8b
Phenylalanine	3.9	4.2	4.2	4.2
Tyrosine	2.6	2.8	2.7	2.7
Threonine	4.0	4.2	4.0	4.0
Valine	4.1a	5.2bc	5.5cd	5.6d
Alanine	4.3a	4.7b	4.5ab	4.5a
Aspartic acid	7.4	7.5	7.4	7.5
Glutamic acid	19.4	20.3	19.9	19.7
Glycine	4.8	5.2	5.1	5.2
Proline	6.0	6.3	6.2	6.4
Serine	4.2cd	4.4d	4.0bc	3.7ab

[a] Values for a given amino acid followed by different letters differ significantly (P < 0.01).
[b] The 22hr data were taken from Table 8.

transformed data compare favorably with those reported by Knipfel et al. (1971). These workers employed a similar transformation in analyzing their data and reported average CV values of 2.1–7.4%, for casein, soybean flour, and fish flour. This favorable comparison was quite encouraging but unexpected, because, in the study by Knipfel et al. (1971), all laboratories analyzed the same hydrolysates which were prepared in one laboratory, as compared to independently prepared hydrolysates in this investigation. The lower variation obtained in this study may be attributed to the use of improved amino acid analyzers.

Adjustment of amino acid data to constant total recovery values has been reported to improve the comparability between laboratories (Knipfel et al., 1971). Application of a similar correction (amino acid data adjusted to a total nitrogen recovery of 100% in each laboratory) to our data also resulted in reduced interlaboratory variability (Table 13) for most amino acids in six protein sources (casein, egg white, minced beef, soy protein, rapeseed concentrate and pea flour). In the case of wheat flour, the adjustment decreased the CV values for six amino acids (histidine, cystine, valine, alanine, glutamic acid and serine) whereas the CV values for other 11 amino acids were increased (Tables 10 and 13).

The high between-laboratories CV values for tryptophan (up to 24%) and sulphur amino acids (up to 17%) obtained in the present investigation would suggest that there is a need for further standardization of methods for the determination of these amino acids. Although the interlaboratory variation for most other amino acids (released by 6N HCl hydrolysis) was less than 10%, this variability could probably be further reduced by following a more uniform experimental protocol than the one used in the present study (Table 1). Similar conclusions about the need for further standardization and collaborative testing of methods of amino acid analysis were reached at the 1981 AOAC Workshop on "Amino Acid Analysis." To develop an official method for amino acid analysis, researchers need to study and optimize hydrolysis conditions and instrument operation. This could be done by conducting separate collaborative studies on the two variables (i.e. hydrolysis and analysis) involved in the determination of amino acids, as recommended by the participants of the 1981 AOAC Workshop on "Amino Acid Analysis."

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A New Calculation Method for Distinguishing Endo- from Exo-Polygalacturonases

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ABSTRACT

A method which uses a comparison ratio (CR) of fluidity $(1/\eta sp)/$ μmoles reducing groups is proposed for comparing and distinguishing endo- and exo-polygalacturonases (PGases). This method is faster, easier and as accurate a method as the percent hydrolysis method. The CR method can also be used as a measure of the purity of endo-PGase with respect to exo-PGase.

INTRODUCTION

EXO- AND ENDO-POLYGALACTURONASES (EC 3.2.1.-15 and EC 3.2.1.40, respectively) (PGases) hydrolyze polygalacturonic acid (PGA) chains to yield smaller units, each unit containing a reducible carbon. Exo-PGases hydrolyze mono- and di-galacturonic acid from the terminal ends of the polymer. This reaction requires hydrolysis of 10–20% of the glycosidic bonds to reduce initial substrate viscosity by half (Whitaker, 1972). Endo-PGases hydrolyze PGA randomly along the polymer, and thus rapidly reduce substrate size and viscosity. This reaction requires only a small percentage of the glycosidic bonds to halve the initial substrate viscosity (Whitaker, 1972).

The two modes of PGase attack, terminal and random, can be differentiated by comparing the rate of substrate-size reduction with the rate of hydrolysis, as measured by viscosity (Whitaker, 1972) and by a reducing sugar assay (Nelson, 1944), respectively.

Many researchers (Liu and Luh, 1978; Whitaker, 1972; Pressey and Avants, 1973) have compared the rate of viscosity loss with the rate at which glycosidic bonds are hydrolyzed by a hydrolysis-viscosity (HV) method. The HV value is determined from the μmoles of bonds that are hydrolyzed at 50% viscosity. This method is most accurate in determining the mode of PGase attack when endo- and exo-PGases are clearly separated. On impure extracts the presence of endo-PGase is easy to see viscometrically even with large amounts of exo-PGase present but the small amount of endo-PGase also obscures the presence of the exo-PGase. When the HV method is applied to mixtures of both PGases, the intermediate results may be used as a crude indication of the purity of exo-PGase with respect to endo-PGase.

This paper presents a fast, easy method for comparing viscometric and reductometric assay results.

MATERIALS & METHODS

ENDO- AND EXO-PGASE extracts were semipurified from placental tissue of fresh ripe papayas (*Carica papaya*) Solo cultivar according to the method of Chan and Tam (1982). The reaction mixture and the viscometric and reductometric assays were taken from Chan and Tam (1982). The reaction mixture consisted of a ratio of 1.0 ml enzyme solution to 5.0 ml 1.2% polygalacturonic acid (PGA) (Sigma Chemical Co.) in 0.03M acetate buffer. Six ml of the reaction mixture were used for the viscometric assay and 18 ml of the same reaction mixture were used for determining the amount of reducing groups formed. Substrate degradation was carried out at 37°C to 18

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and 23% hydrolysis for endo- and exo-PGase, respectively. Viscosity outflow times were recorded at various intervals. Reducing groups were measured at various intervals by removing 1 ml samples from the reaction mix, acidifying the sample with 0.17 ml 2N HCl and making slightly alkaline with 2.3 ml 0.167N NaOH. Concentrated solutions of reducing groups were appropriately diluted before assaying according to Chan and Tam (1982). The data from the viscometric assay and the assay for reducing sugars were analysed by the hydrolysis-viscosity (HV) and the comparison ratio (CR) methods described below. The analysed data appear in Table 1.

Hydrolysis-viscosity method (HV) – % hydrolysis at 50% viscosity

In determining the percent hydrolysis at 50% viscosity, the Cannon-100 (Cannon Instrument Co.) viscometer outflow times were converted to percent viscosity by the equation of Roboz et al. (1952).

$$A = \left(\frac{Vo - Vt}{Vo - Vs}\right) 100 = \% \text{ viscosity loss} \tag{1}$$

where Vo = outflow time at start of reaction; Vt = outflow time at time (t); and Vs = outflow time of solvent with no PGA.

By rearrangement and assuming the desnity of the reaction mix and solvent to be similar:

$$A = \left[\frac{\left(\frac{Vo}{Vs} - 1\right)\left(\frac{Vt}{Vs} - 1\right)}{\left(\frac{Vo}{Vs} - 1\right)}\right] 100 = \left(\frac{\eta spI - \eta spX}{\eta spI}\right) 100 \tag{2}$$

where ηspI = specific viscosity of reaction mixture at the start of reaction; and ηspX = specific viscosity of reaction mixture at time (t).

$$\eta spX = \left(1 - \frac{A}{100}\right) \eta spI \tag{2a}$$

One ml samples were removed from the duplicate reaction mixture and assayed for reducing sugar (μmoles/ml). The percent viscosity and the μmoles/ml reducing groups were plotted versus time. From the curve for percent viscosity, the time (T_{50}) at which the substrate reaches 50% visiocisyt loss was determined. Applying time (T_{50}) to the reductometric curve, the number of μmoles/ml of reducing groups was determined. To compute the percent hydrolysis the following equation was used:

$$\% \text{ hydrolysis} = \frac{(\mu\text{moles/ml}) \times 100}{(\mu g \text{ PGA/ml})/\text{Avg M.W. of PGA subunit}} \tag{3}$$

Comparison ratio (CR) method

In the comparison ratio method, the viscosity outflow times were converted to fluidity units $(1/\eta sp)$ by the equation:

$$\left(\frac{1}{\eta sp}\right) = \left(\frac{Vt}{V_s} - 1\right)^{-1} \tag{4}$$

where Vt = outflow time at time (t); and Vs = outflow time of solvent with no PGA. Both fluidity and the μmoles of reducing groups produced were linear with time during the initial phase of PGA degradation. To compute the CR, the rate of increase in fluidity was divided by the rate of increase in reducing groups:

$$CR = \frac{\Delta \text{ fluidity } (1/\eta sp)/\text{time}}{\Delta \mu\text{moles reducing groups/time}} \tag{5}$$

$$CR = \frac{(\eta spI - \eta spX)}{(\eta spI)(\eta spX)(\mu\text{moles})} \tag{5a}$$

Table 1—Comparison of the CR method with the HV method

| Enzyme | Time period | Rates of reaction | | % Visc. | | CR | Calculated HV |
		Fluidity min	μmoles/ml min	loss (eq. 1)	HVa	(eq. 5)	(eq. 3 & 6)
Exo-PGase	0.4 - 8 hr	5.18×10^{-4}	5.72×10^{-3}	17	6.5	0.091	23.7
	8 - 19 hr	4.13×10^{-4}	3.40×10^{-3}	31	10.8	0.122	17.7
	19 - 28 hr	3.95×10^{-4}	3.03×10^{-3}	38	13.7	0.130	16.6
	28 - 47 hr	3.03×10^{-4}	2.30×10^{-3}	48	18.0	0.132	16.4
	47 - 69 hr	2.98×10^{-4}	2.05×10^{-3}	55	22.8	0.145	14.8
	HV method (% hydrolysis at 50% viscosity) = 21.5						
Endo-PGase	4.5 - 28 min	1.74×10^{-1}	8.54×10^{-2}	79	4.0	2.04	1.06
	18 - 48 min	2.02×10^{-1}	8.94×10^{-2}	86	6.0	2.26	0.96
	48 - 75 min	2.05×10^{-1}	8.36×10^{-2}	92	11.4	2.45	0.88
	75 - 150 min	1.40×10^{-1}	4.68×10^{-2}	95	17.5	2.99	0.72
	HV method (% hydrolysis at 50% viscosity) = 1.07						

a Total bonds available in 1% PGA = 56.7 μmoles/ml.

The fluidity and reductometric slopes became nonlinear during the later phases of PGase degradation. The CR was then computed by taking the fluidity and reducing groups slopes from a short time interval. The slopes of the short time intervals approximate the instantaneous slope at any one time.

Since fluidity and the number of reducing groups were measured in duplicate enzyme reaction mixtures, the time and the enzyme concentration units cancel out in the CR equation. The CR therefore is a measure of the amount of fluidity increase for a given number of μmoles reducing sugar produced under the stated conditions. Furthermore, CR values measured at different time intervals can be compared.

An HV value (% hydrolysis at 50% viscosity loss) can be calculated from a CR value. The μmoles of reducing groups is first calculated from the CR value using Eq. (6) which was obtained by substituting Eq. (2) and (2a) into Eq. (5a) and rearranging.

$$\mu moles/ml = \frac{\% \text{ viscosity loss}}{(100\text{-}\% \text{ viscosity loss}) (\eta spI) (CR)} \qquad (6)$$

where ηspI = Initial specific viscosity; and % visc. loss obtained from Eq. (2). For 50% viscosity loss: μmoles = $(CR \times \eta spI)^{-1}$. To compute the percent hydrolysis (HV value), Eq. (3) was used.

The ability to convert the CR to HV data makes it possible to compare CR data with other published research.

RESULTS & DISCUSSION

THE PLOT of fluidity vs time for endo-PGase (Fig. 1) was linear up to 11% hydrolysis after which the slope decreased rapidly. In a similar plot for exo-PGase (Fig. 1), the rate of increase in fluidity appeared linear initially up to 14% hydrolysis but when extended to 23%, hydrolysis was seen to gradually decline. Chan and Tam (1982) show the complete curves for specific viscosity and reducing groups.

An accurate CR can be measured at any point from zero to 18% hydrolysis for semipure extracts of both exo-PGase and endo-Pgase (Table 1). The small increase in the CR value within a single extract as the percent hydrolysis increases was insignificant compared to the large variation between the CRs of endo- and exo-PGase extracts (Table 1). The CRs for a single extract increased by a factor of 1.45 for exo-PGase and 1.12 for endo-PGase as the percent hydrolysis increased to 18%. Conversely, the CR for the semipurified endo-PGase extract (Table 1) (CR = 3.0) was 20 times larger than that for a similarly purified exo-PGase (CR = 0.15). Moreover, enzyme extracts purified one step further through gel permeation (CR = 11.5) (Chan and Tam, 1982) was 200 times larger than the CR for a similarly purified exo-PGase (CR = 0.056) (Chan and Tam, 1982). Since the CR was relatively constant during the reaction course, a CR measured for one extract at its initial phase may be compared to a CR measured for another extract at a latter phase of PGA degradation up to 18% degradation.
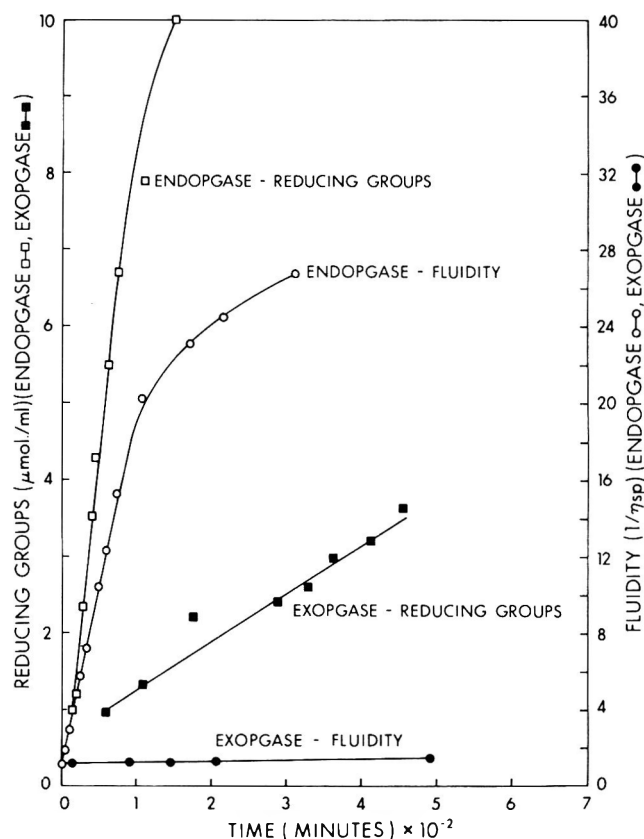
Fig. 1—Initial reaction rates for endo- and exo-polygalacturonase. Assay by Chan and Tam (1982).

Moreover, exo-PGases or dilute enzyme extracts may be measured much quicker during a short initial period of reaction.

Our CR values range from 0.06 to 11.5 depending on the proportion of endo- and exo-PGase present. The CR value thus may possibly be used as a rough first measure of the proportion of endo-PGase with respect to exo-PGase by extrapolation from the CR values for both pure enzymes.

A comparison of the CR and HV methods shows that the calculated percent hydrolysis at 50% viscosity from initial CRs, closely approximates the actual percent cleavage at 50% viscosity obtained by the HV method. For exo-PGase, the calculated percent hydrolysis at 50% viscosity from the initial CR number was 23.7 vs the actual 21.5%

Statistical Evaluation of Water Activity Measurements Obtained with the Vaisala Humicap Humidity Meter

GUILLERMO FAVETTO, SILVIA RESNIK, JORGE CHIRIFE, and CONSTANTINO FERRO FONTÁN

──────────── ABSTRACT ────────────

The objective of this study was to determine the suitability, precision and accuracy of the Vaisala Humicap electric hygrometer for a_w measurements in the range 0.752–0.974 (at 25°C). Results of the statistical evaluation indicated that this hygrometer, when calibrated and operated as described here, provides a convenient and fast means of a_w measurement having adequate accuracy and precision for most food applications.

INTRODUCTION

IT IS GENERALLY ACCEPTED that water activity (a_w) is an important factor affecting the shelf life of intermediate moisture foods (IMF). In their recent book, Troller and Christian (1978) adequately reviewed the effect of a_w on the microbial and physicochemical stability of foods. For this reason there is a demand in research and quality control in the food industry for accurate, fast and convenient methods of measuring a_w. Electric hygrometers (or other types) have been used to a large extent in the food area, (Labuza et al., 1976; Gál, 1975, 1981) but as noted by Troller (1977) there have been few attempts to evaluate their precision and accuracy. Stoloff (1978) reported the results of a collaborative study for calibration or check of suitable equipment for measurement of a_w.

Although quite a number of commercially available hygrometers were used, various problems have been noted; mainly, the instability of various instruments (i.e. need for frequent calibration), loss of accuracy with sensory aging, and inaccuracy at certain levels of a_w (Troller, 1977).

The objective of this study was to determine the suitability, precision and accuracy of the Vaisala Humicap humidity meter for a_w measurements in the range 0.752–0.974 (at 25°C). This range was selected because it represents the range of a_w wherein microbial growth is most likely to occur (Troller. 1977). In addition, evaluation of the time necessary to reach equilibrium between food samples and sensing element was investigated. The Vaisala Humicap instrument was also statistically compared with a dial type polyamide thread hygrometer of current use in many food laboratories.

MATERIALS & METHODS

Instruments

A Vaisala Humicap humidity meter manufactured by Vaisala Oy (PL 26 SF-00421 Helsinki 42, Finland) was used. It consists of a relative humidity and temperature indicator HMI 14 A and a humidity and temperature probe HMP 14 A. This probe employs the Vaisala Humicap humidity sensor which is based on capacitance

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changes in a polymer thin film capacitor. The same sensor covers the whole range of relative humidity, 0–100%. The sensor, according to a modification developed by Driesen and Kern (Postfach 1126, 2000 Tangstedt, West Germany), is mounted together with a very small fan within a Teflon head which is used to cap the sample holder. The fan serves to decrease the time of equilibration between the sample and sensing element. Fig. 1 pictures the sensor arrangement and sample holder.

Measurements were made by placing the sample in the holder which consists of a plastic cup of about 190 ml and the sensor was then used to cap the sample holder. Readings may be taken directly from a 0–100% equilibrium relative humidity scale and converted to a_w by dividing by 100. However, this was not used since dial readings can be made only to about 0.01 a_w; instead the output terminals of the indicator were connected to a Digital Multimeter Model 248, Data Precision, U.S.A. which allowed a_w readings to the third decimal place.

It is known that adequate calibration is a key factor in obtaining accurate a_w measurements with electric hygrometers, such as the one used here. The calibration procedure recommended by the equipment manufacturer for measurement in high humidity (above 75% RH) is as follows. The probe is kept over a saturated solution of K_2SO_4 overnight and then the sensor is set to the proper a_w level by adjustment of a screw near the sensor. This procedure, however, was not used and calibration was performed as described in the Calibration section of this article.

Some determinations of a_w were done using a dial-type polyamide thread hygrometer, the "a_w-Wert Messer" manufactured by Firma LUFFT (Stuttgart, West Germany). According to the manufacturer's recommendations calibration is performed using a saturated solution of $BaCl_2$ (a_w = 0.902 at 25°C) and adjusting the indicator to this value with a set screw. However, and in order to improve the accuracy of a_w measurements, calibration was performed as described by Chirife and Ferro Fontán (1980). After adjusting the indicator to a_w = 0.902 the instrument was checked against other standard saturated salt solutions and a calibration curve was obtained in the a_w range of interest.

Methods

The holder and sensor were always maintained in an air-circulating constant temperature cabinet at 25°C ± 0.1°C. All a_w measurements in the range studied (0.752–0.974) were approached from a lower level which corresponded to near equilibrium with ambient relative humidity.

The samples, either saturated salt solutions or foods were always stored at 25°C for long periods in order to insure thermal equilibrium. In all measurements the volume of sample used corresponded to about 20 ml.

a_w Standards

Saturated salt solutions were used as standards for measurement of a_w. These saturated salt solutions were arbitrarily chosen to provide a range of a_w levels most representative of those in the microbiological growth range. The standard a_w values (SV) listed in Table 1 are those recently recommended by Chirife et al. (1982).

RESULTS & DISCUSSION

Calibration

In order to improve the accuracy of a_w measurements the following procedure was used. The sensor was first set to the proper a_w level after equilibration over a saturated solution of $BaCl_2$ (a_w = 0.902 at 25°C). The sensor was then equilibrated over other standard saturated salt

solutions to cover the range of interest, and a calibration curve was obtained. Due to the short time needed to reach equilibrium (as described below) a complete calibration curve (six a_w values in the range 0.752–0.974) was obtained in about 3 hr. It was found that in the range studied the sensor exhibits an almost linear response, that is the calibration curve is fitted by a straight line. However, for better accuracy the calibration data are best expressed by an equation of the type

$$y = a \cdot e^{bx} \qquad (1)$$

where x is the meter reading and y is the "true" water activity. Fig. 2 shows a calibration curve and the linear and exponential fits. In present work equation (1) was used for all calculations although it is obvious that the linear approximation will also work well for most uses.

The stability of the calibration curve was also studied as follows. The sensor was equilibrated and adjusted to the proper a_w level over a saturated solution of $BaCl_2$ ($a_w = 0.902$) and then a calibration curve was obtained and parameters a and b [Eq. (1)] were computed. Following this calibration the a_w of saturated $(NH_4)_2SO_4$ ($a_w = 0.802$) and saturated KNO_3 ($a_w = 0.926$) was periodically measured over 120 days. For each measurement the sensor was adjusted only with saturated $BaCl_2$ ($a_w = 0.902$) and readings were transformed to "true" a_w using the original calibration parameters (a,b). The results are shown in Table 2.; it can be seen that over the period studied (120 days) the calibration remains quite stable since the accuracy at 0.802 and 0.926 a_w is within ± 0.003 and ± 0.005, values which are adequate for most research and quality control needs (Troller and Christian, 1978).

Equilibration time

The time required to attain equilibrium within the sensor chamber is a function of various parameters, among them physical nature of the sample, a_w level, specific surface area, and transport conditions in the air space above the sample.

Fig. 3 and 4 show the rate of approach to equilibrium during a_w measurements of various standard saturated salt solutions and IMF such as sucrose solution, milk jam and salted beef (ground or cut in thin slices). The curves are not exactly equivalent because the initial value (sensory reading at time zero) depended on the ambient relative humidity which was not always the same. Nevertheless, they all show that the rate of approach to equilibrium is very fast; it can be seen that an equilibration time of 30 min is enough for a_w measurement of saturated salt solutions or foods of varied physical nature and a_w. Consequently, all measurements were performed following a 30 min equilibration period.

Table 1—Water activities at 25°C of six standard saturated salt solutions[a]

Saturated salt	a_w
NaCl	0.752
$(NH_4)_2SO_4$	0.802
KCl	0.843
$BaCl_2$	0.902
KNO_3	0.926
K_2SO_4	0.974

[a] Source: Chirife et al. (1982)
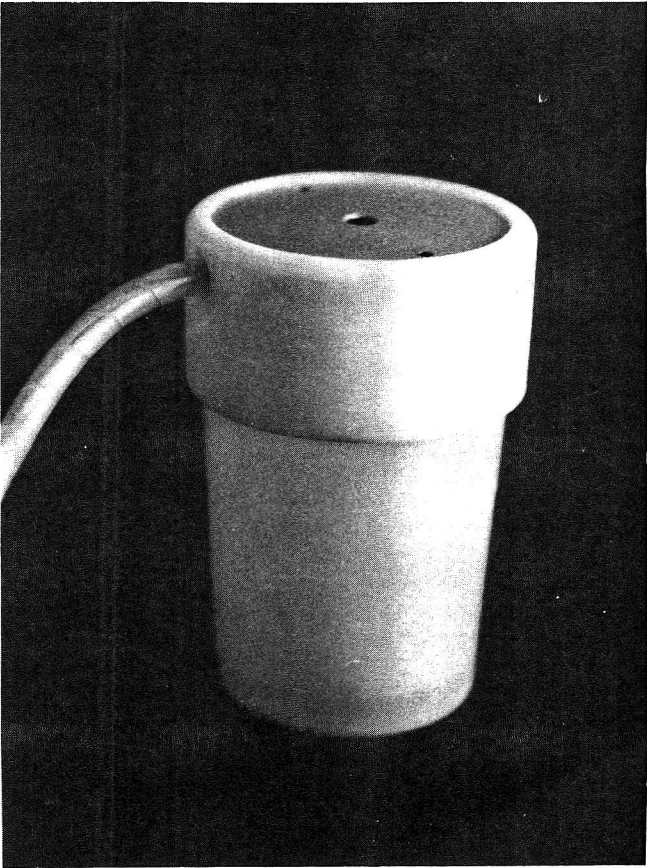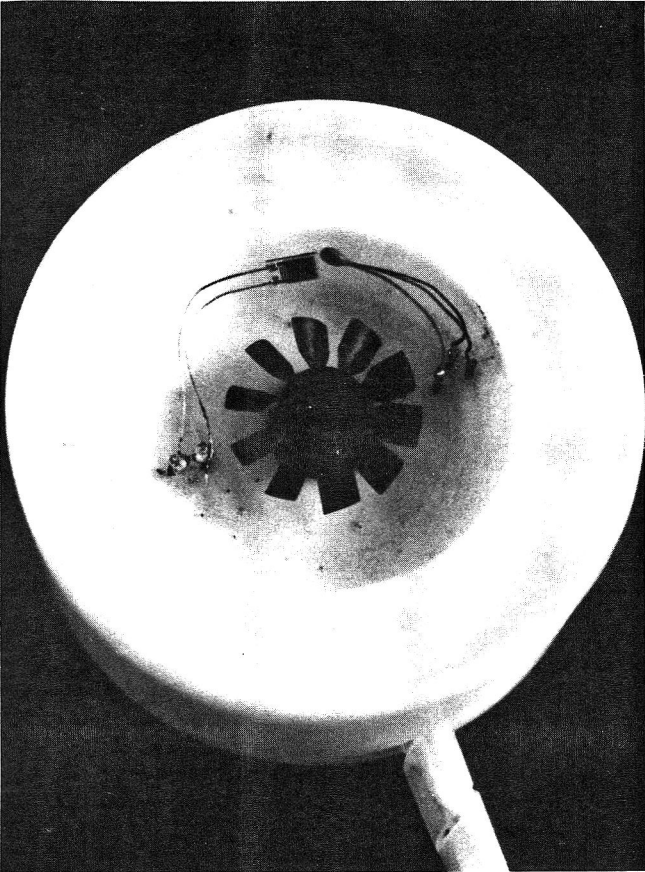
Fig. 1—View of sensory arrangement and fan (left), and sample holder (right).

Precision

Precision represents repeatability or the difference between replicates in a series of identical tests. Table 3 shows mean values and standard deviations for measurements of five different standard saturated salt solutions and three different foods having a_w in the range of present concern. The results for saturated salt solutions compare favorably with those obtained by Troller (1977) using the Sina Scope electric hygrometer for a_w measurement in similar saturated salt solutions. Troller (1977) found that standard deviations decreased with increase in a_w levels. Present data do not show such correlation and standard deviations varied randomly with a_w level. The excellent precision observed for saturated solutions was also obtained with the three foods studied. The data show virtually no food-related differences in precision. The precision obtained in these experiments seems to be slightly better than that obtained by Troller (1977) with the Sina Scope electric hygrometer and two foods (fruit jelly and chocolate syrup) having related values of a_w.

Replication

The confidence interval for μ, the mean of a normal distribution is expressed through the statistical variable t,

$$t = \frac{\overline{X} - \mu}{S/\sqrt{n}} \qquad (2)$$

where n is the number of replicates and S is the standard deviation. The number of replicates required to produce a given level of data confidence can be calculated from

$$|\overline{X} - \mu|_{5\%} \leqslant \frac{t_{.05}\, S}{\sqrt{n}} \qquad (3)$$

It was found that, depending on the particular a_w level, only three or four replications one enough to achieve a confidence interval of $\pm 0.005\ a_w$ which is adequate for most research needs in the food area (Troller and Christian, 1978). The results also indicate that the number of replicates needed to obtain a given confidence level do not correlate with a_w level, but varied randomly.

Fig. 2—Calibration curve at 25°C for the Vaisala Humicap meter.

Table 2—Stability of the calibration curve as a function of time

Time (days)	$a_w{}^a$	$a_w{}^b$
0	0.803	0.925
30	0.801	0.927
32	0.804	0.925
36	0.804	0.931
46	0.799	0.924
50	0.803	0.928
119	0.800	0.922
122	0.799	0.925

[a] Standard a_w value 0.802.
[b] Standard a_w value 0.926.

Fig. 3—Equilibration time for a_w measurements of various saturated salt solutions.
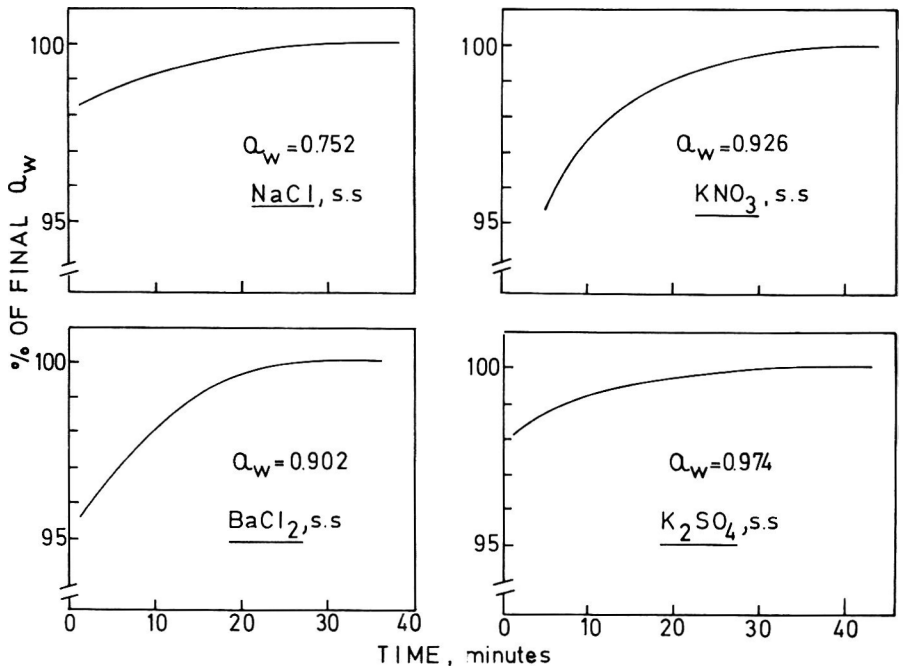
Comparison of a_w measurement with the Vaisala and LUFFT hygrometers

Table 4 shows the results of the a_w found for three food samples measured with the Vaisala and the LUFFT hygrometers. Analysis of variance of data is shown in Table 5 which indicates that there is no significant interaction between products and methods used to measure a_w. This means that the measured a_w of a given product does not depend on the method used, as it is to be expected when reliable (properly calibrated) instruments are used.

CONCLUSIONS

THE RESULTS of this work indicated that the Vaisala electric hygrometer, when calibrated and operated as described in this work, will produce accurate and precise a_w measurements for most food research needs. It is important to stress that the accuracy obtained with this hygrometer depends on obtaining a calibration curve in the a_w range of interest; poorer results are to be expected when the meter is calibrated according to manufacturer's instructions (adjusting the sensor with one standard saturated solution).

Perhaps, one of the most relevant characteristics of the hygrometer here studied is the short time needed for measurement. As shown here, 30 min is enough for measurement of a_w in a diversity of materials including saturated salt solutions and liquid, semisolid or solid foods of varied a_w level.

The results obtained with various IM foods showed that the high level of precision prevailing with saturated salt solutions is reproduced in real foods. However, we believe

Table 3—Statistical estimates of precision[a] of a_w measurement of saturated salt solutions and three different foods

Sample	Standard values	Mean value
NaCl	0.752	0.751 ± 0.0025
$(NH_4)_2SO_4$	0.802	0.799 ± 0.0008
KCl	0.843	0.840 ± 0.0027
$BaCl_2$	0.902	0.900 ± 0.0019
K_2SO_4	0.974[b]	0.976 ± 0.0016
Condensed milk (sweetened)		0.833 ± 0.0017
Milk jam		0.842 ± 0.0015
Tomato paste (triple concentrated)		0.934 ± 0.0013

[a] Twelve replicates at each a_w level, 30 min.
[b] Eight replicates.

Table 4—Results of multiple analysis for a_w measurement in foods by the Vaisala and LUFFT hygrometers

Condensed milk (Sweetened)		Tomato paste (Triple conc.)		Milk jam	
LUFFT	VAISALA	LUFFT	VAISALA	LUFFT	VAISALA
0.836	0.834	0.935	0.931	0.845	0.845
0.836	0.834	0.935	0.933	0.842	0.844
0.831	0.833	0.929	0.933	0.838	0.842
0.835	0.832	0.931	0.934	0.842	0.841
0.833	0.836	0.931	0.934	0.841	0.841
0.831	0.834	0.925	0.934	0.837	0.842
0.832	0.832	0.931	0.934	0.841	0.841
0.835	0.831	0.926	0.934	0.840	0.841

Table 5—Analysis of variance: measurement of a_w values for food items in comparative study (ref. Table 5)

Source of variation	Degrees of freedom	Sum of squares	Mean square	F	$F_{.05}(DF, 40)$[a]	$F_{.01}(DF, 40)$[a]
Between foods	2	0.095642	0.047821	9496.37	3.23	5.18
Between methods	1	0.000021	0.000021	4.24	4.08	7.31
Interaction	2	0.000023	0.000011	2.26	3.23	5.18
Error	47	0.00021				
TOTAL	52	0.095897				

[a] DF: Degrees of freedom.
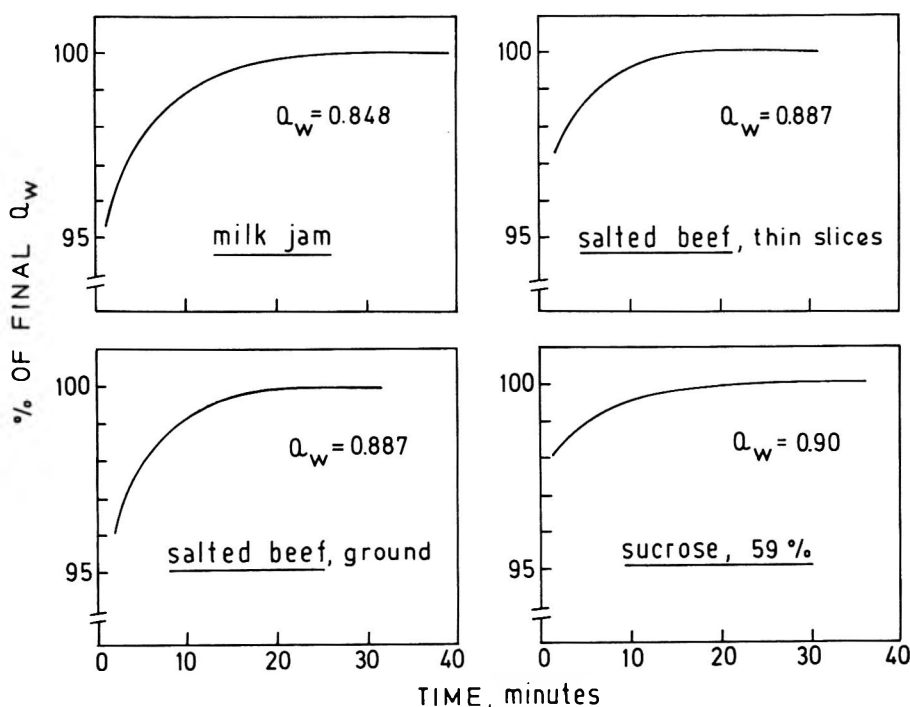
Fig. 4—Equilibration time for a_w measurement of various foods.

that additional studies should be made using other foods of varying composition in order to check if the same levels of precision are obtained. It is reasonable to assume that some foods which contain organic volatiles may contaminate the sensor, as reported for glycols (Sloan and Labuza 1975; Troller 1977). This will be the subject of future studies.

The comparison of the Vaisala hygrometer with the LUFFT (fiber-demensional hygrometer) lead to very satisfactory results.

It may be concluded that the Vaisala hygrometer when operated as described here, provides a convenient and fast means of a_W measurements having adequate accuracy and precision for most food applications.

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CALCULATING ENDO- FROM EXO-PGases . . . From page 533 ■■■■■■

(Table 1). For endo-PGase the calculated percent hydrolysis at 50% viscosity was 1.06 vs the actual 1.07%.

Other researchers have reported the use of a ratio of viscometric change over reductometric increase to compare and distinguish endo-PGase from exo-PGase. Endo (1964), using the unit 10 min/T_{50} for visocsity, obtained a ratio by dividing viscosity units by the μmoles of reducing sugar per minute. T_{50} was the time to reach 50% viscosity loss. Measuring T_{50} was very slow for exo-PGases and for dilute PGase extracts. Hatanka and Imamura (1974) used a ratio of the sugars produced to the viscometric activity.

The CR method differs from the above methods by using the rate of increase in fluidity as the measure of viscosity change. Fluidity, unlike ηsp and percentage loss in viscosity increased linearly with time. The slope of fluidity over time was also found to be linear with enzyme concentration. Fluidity was directly proportional to the average degree of polymerization of PGA for endo-PGases (Rombouts and Pilnik, 1972).

In conclusion the CR method was faster, easier and as accurate to compute as the HV method for comparing

endo- and exo-PGases.

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Nonenzymatic Browning of Freeze-Dried Sucrose

JAMES M. FLINK

————————————ABSTRACT————————————

The appearance of nonenzymatic browning products in sucrose-based systems (5–20%; pH 1–5) during freeze drying and during subsequent room temperature storage in the dry state was investigated. Results showed that following the completion of the primary sublimation stage of drying, sucrose hydrolysis to glucose and fructose was initiated. Glucose and fructose undergo further breakdown, and at the same time products which absorb in the UV (280 nm) were formed. A short time later observable brown colors are noted (absorbance at 400 nm). The extent of UV absorbance and brown color formation increased with decreasing sucrose concentration and decreasing pH in the initial solution.

INTRODUCTION

SMYRL AND LE MAGUER (1978) reported results of an investigation on the effect of pH on volatile retention in freeze-dried model solutions in which they measured the retention of carvone, piperitone, and eugenol in sucrose or gum arabic solutions over the pH range 3–11. They showed retention to increase as pH deviates from the neutral range. Retention was measured from the ratio of UV absorption for the rehydrated, freeze-dried sample and the initial solution. Absorption should arise solely from the model volatile, and it was reported that no interfering UV absorbing products were formed when sucrose was freeze-dried in the absence of the model volatiles.

While conducting a similar investigation with benzaldehyde and sucrose over an extended pH range (2–12), samples at the low pH values showed a shifted absorption maxima and eventually brown color formation.

While Maillard type, nonenzymatic browning reactions in dry products have been widely reported in the literature (recently reviewed by Troller and Christian, 1978), there is much less information on acid-catalyzed browning of pure sugar systems in the dry state. Karel and Labuza (1978) reported that a freeze-dried model system containing sucrose, avicel and citric acid could undergo nonenzymatic browning at 55°C at a moisture content of 0.22g water/100g solids. Resnik and Chirife (1979) noted that air-dried apple slices underwent nonenzymatic browning at temperatures from 55–83°C, even when in the dry state. The possible presence of nitrogen-containing compounds in the apple was not noted. Kapelman et al. (1977) noted that freeze-dried lemon crystals stored in the dry state underwent nonenzymatic browning at temperatures as low as 25°C. Storage at 4°C was required to prevent browning. Their notation regarding carbon dioxide formation could indicate that nitrogen compounds were taking part in the reactions. Similar results had been demonstrated earlier (Karel and Nickerson, 1964) for dehydrated orange juice.

The chemistry of nonenzymatic browning reactions has been described by Hodge and Osman (1976). The first step in the acid-catalyzed browning of sucrose is the hydrolysis of the sucrose molecule. Schoebel et al. (1969) and Karel

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and Labuza (1968) have described that acid hydrolysis can occur in acid containing, freeze-dried sucrose systems at temperatures of 37°C. They noted that it appears that monolayer adsorbed water is available for the hydrolysis reaction. Lund et al. (1969) investigated the acid-catalyzed hydrolysis of sucrose in frozen solution. The reaction rate deviated from Arrhenius behavior (e.g. remained constant with falling temperature) over the temperature range -8.5 to $-16.5°C$. This is presumably due to the increase in reactant concentration, especially H^+, during the freezing process. Williams-Smith et al. (1977) and Van Den Berg (1966) have indicated that the pH of a solution can change by up to 3 pH units during freezing.

As conditions which could be conducive for nonenzymatic browning could occur in the freeze-drying process or during subsequent storage, this study investigated the appearance of nonenzymatic browning products in freeze-dried sucrose based systems, both during freeze-drying and during subsequent storage in the dry state.

The study reported here was conducted to investigate the causes of the brown color formation in freeze-dried sucrose samples of low pH and determine when in the freeze-drying process it begins.

MATERIALS & METHODS

THE TEST SYSTEM consisted of aqueous solutions which have been adjusted to desired pH values with hydrochloric acid (HCl) prior to freeze-drying. Sucrose concentrations of 5–20% (w/w) and pH values of 1–5 (initial solution basis) were investigated. Samples were evaluated at various stages of the freeze-drying process (initial solution, frozen solution at various times, during freeze drying at various times, and during storage). Sampling during freeze-drying could be accomplished in under 10 seconds. Prior to analysis of partially dry or dry samples, deionized water was added to bring the sample back to its initial weight. The fractional weight remaining (weight at a given time/initial weight) was used as a measure of the extent of drying.

In most tests, samples were analyzed for absorbance at 280 nm (presumably HMF). In one series of studies, samples were analyzed (by enzymatic methods) for glucose, fructose and sucrose and for spectrophotometric absorbance at 280 and 400 nm. Moisture contents of the freeze-dried samples were evaluated by drying at 70°C. Since the nonenzymatic browning reaction which occurs with the 70°C drying contributes to the weight loss, the gravimetric method will normally tend to overstate the actual moisture contents. However, by measuring the weight loss at half-hourly intervals for 4 hr, it was possible to extrapolate the time dependence of weight loss (water), after the rapid initial weight loss in the first hour, to t = 0 to obtain a good approximation of the correct moisture content value.

RESULTS & DISCUSSION

FIG. 1 shows a weight loss curve for freeze-drying of a 10% sucrose solution at pH = 2, together with the UV absorbance (280 nm) of the same samples. It can be seen that the appearance of UV absorbance occurs simultaneously with the approximate end of primary freeze-drying, and that it increases rapidly thereafter. The samples eventually take on a brown color. Fig. 2 shows that similar behavior is noted when dry samples are removed from the freeze-dryer and stored at $a_w = 0$. The fact that browning can occur in the
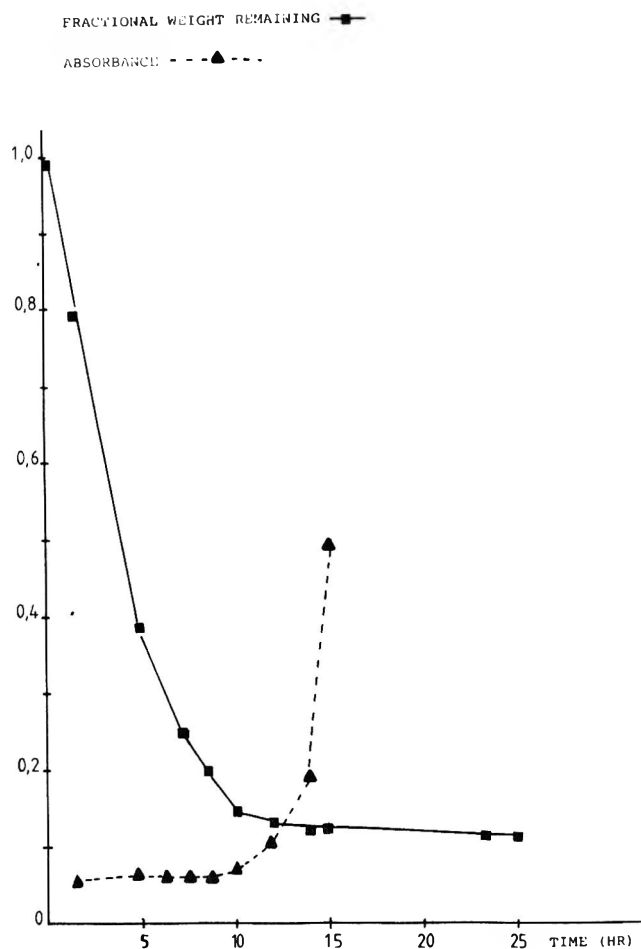
Fig. 1—Fractional weight remaining and spectrophotometric absorbance (280 nm) of 10% sucrose solution at pH = 2 sampled during freeze-drying.

Fig. 2—Effect of a_W of storage on formation of compounds absorbing at 280 nm (10% sucrose at pH = 2).

freeze-dryer during an extended secondary drying period (desorption step) could give the impression that the nonenzymatic browning had occurred during the sublimation step of the freeze-drying process, while in fact it appears to be related to the attainment of the ice-free state in the sample. Fig. 2 also shows the effect of the storage a_w on formation of compounds absorbing at 280 nm for freeze-dried 10% sucrose at pH = 2. It can be seen that the UV absorbance is lower as the water activity of storage increases, indicating that the presence of water slows the reaction. Such an effect could result for a reaction in which water is a product. It was observed that samples held at a_w = 0 tended to lose weight during storage, which further indicates that production of water can be occurring in the browning reaction.

Comparative studies have also been conducted with frozen samples and concentrated sucrose solutions to note if similar UV absorbance occurs. Increasing UV absorbance was noted for room temperature storage of a 60% sucrose solution at pH = 1, but it was much slower than for freeze-dried sucrose. There was little UV absorption observed for 60% solutions at pH = 2 or 3. Frozen samples held at −20°C for 7 days showed essentially no reaction. Fig. 3 shows the relationship of weight loss in freeze drying, concentrations of sucrose, glucose and fructose, and absorbance at 280 and 400 nm for a freeze-dried 10%, pH = 2 sucrose solution stored at a_w = 0. The moisture content after 6 hr of freeze-drying is 2.8g water/100g sucrose, which is well below the BET-monolayer value for freeze-dried sucrose. Hydrolysis of the sucrose apparently begins

during the final stages of freeze-drying, as the disappearance of sucrose and formation of glucose and fructose are noted to begin at this time. Absorbance at 280 nm appears soon after this time. The appearance of visual browning (400 nm) follows somewhat later, and appears to be especially related to the decrease in fructose concentration. Hodge and Osman (1976) noted that in the absence of buffers, fructose is the more reactive of the products from sucrose hydrolysis, and that a major product of the subsequent dehydration reaction is hydroxymethylfurfural (HMF), which absorbs at 280 nm.

In Fig. 4, the effect of sucrose concentration and pH on absorbance at 280 nm following a 2-day freeze-drying process is shown. The product has been dry for much of the time that it has been held in the freeze-dryer in this process. The formation of compounds which absorb at 280 nm increases for decreasing pH and decreasing sucrose concentration. As it is presumed that an acid-catalyzed sucrose hydrolysis, followed by dehydration of the glucose and fructose is responsible for the UV-absorbing compounds, the effective hydrogen ion concentration is important in determining the reaction rate. In freezing and freeze-drying, H^+ concentration is increased relative to that present in the original solution, first due to the formation of the concentrated solute phase and then later a second, larger increase associated with the removal of the sorbed water (this occurring simultaneously with a rise in product temperature). For samples at a given initial pH (i.e. a fixed initial amount of hydrogen ions per unit of solution) the final H^+ concentration following freeze-drying will be highest for the samples with the lowest sucrose concentra-

tion. This can also arise because the more dilute solution will originally contain more water and thus more H$^+$. Similarly, in freezing, the lower solids concentration gives more ice formation and thus the degree of concentration of the concentrated solute phase is larger for dilute solution. Dilute samples will thus have lower effective pH values for both freezing and after freeze-drying, though the extent of the pH decrease will depend on a number of factors, including final sample moisture. At a given sucrose concentration, the lower the initial solution pH, the higher H$^+$ will be in the freeze-dried sample. The UV absorption follows expected behavior for acid-catalyzed browning reactions if effective pH instead of initial solution pH is considered.

Fig. 5 shows that initial sucrose concentration and solution pH influence UV absorbance (280 nm) during storage at a$_w$ = 0 in a manner similar to that observed after freeze-drying (Fig. 4).

SUMMARY

THE BROWNING REACTION observed apparently results from the large increase in hydrogen ion concentration (due to reduction of water concentration) which occurs with the passage of the ice interface in freeze-drying. This increase in H$^+$, together with the rise in temperature in the dry layer leads to a rapid acid-catalyzed hydrolysis of sucrose to glucose and fructose, which then undergo acid-catalyzed dehydration reactions (primarily the fructose). Storage at a$_w$ = 0 appears to accelerate this reaction, probably due to
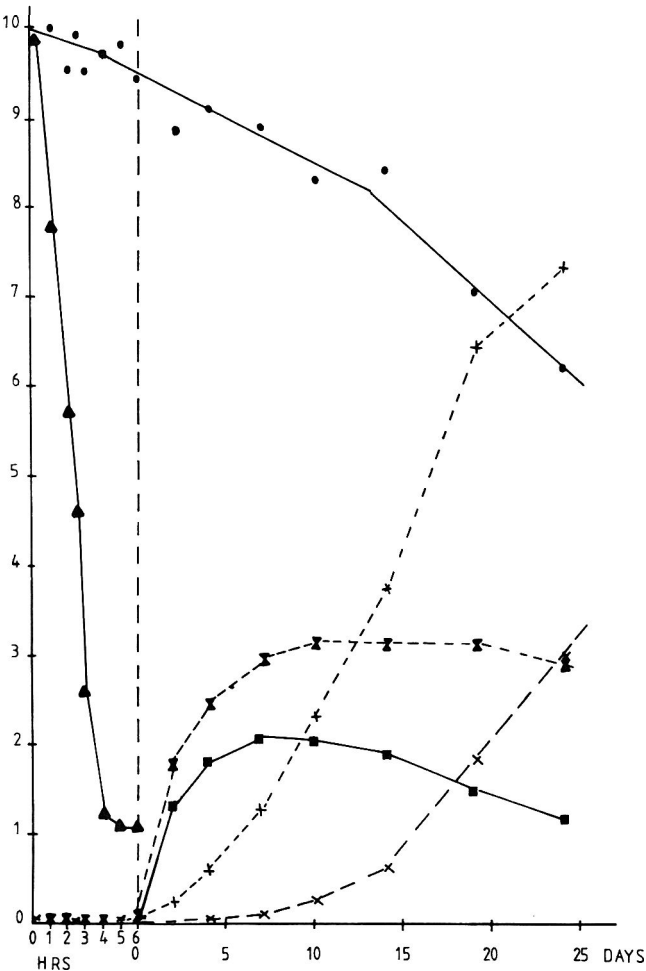
Fig. 3—Fractional weight remaining in freeze-drying, concentration of sucrose, glucose and fructose, and absorbance at 280 and 400 nm for a freeze-dried 10%, pH = 2 sucrose solution stored at a$_w$ = 0: ▲ Fractional Weight Remaining, X 10; ● Sucrose (g/L), X 0.1; ⲭ Glucose (g/L), X 0.1; ■ Fructose (g/L), X 0.1; + Absorbance at 280 nm, X 0.02; and X Absorbance at 400 nm, X 1.
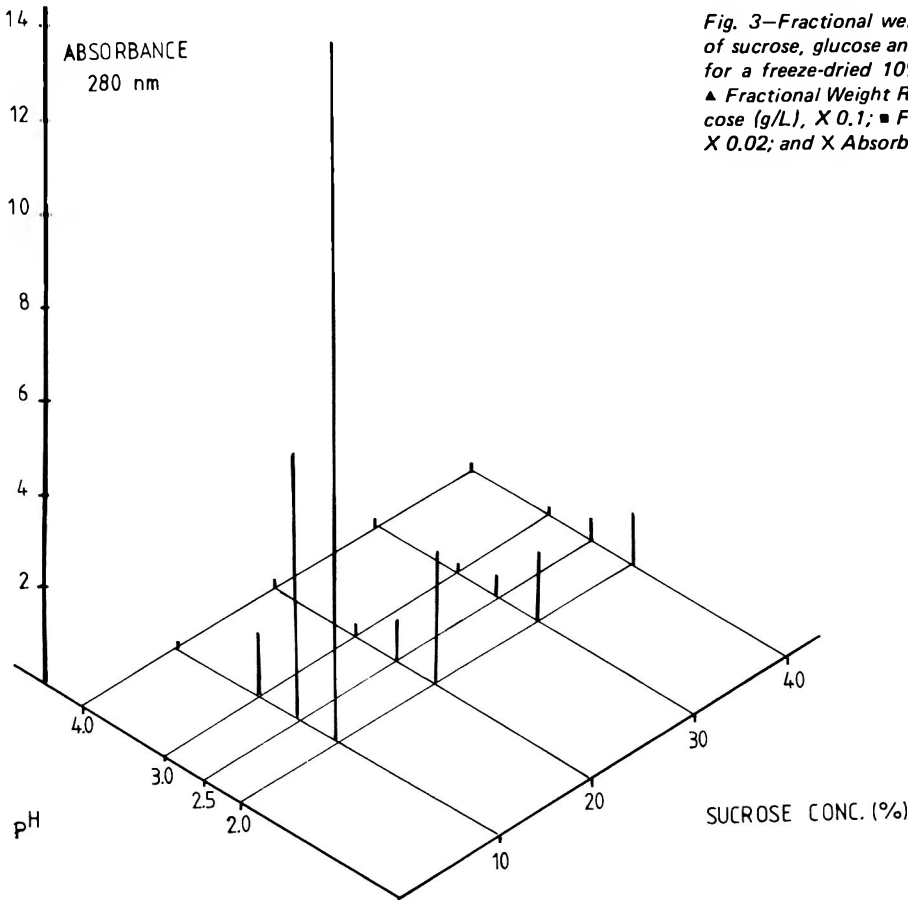
Fig. 4—Effect of sucrose concentration and pH on absorbance at 280 nm following freeze-drying.
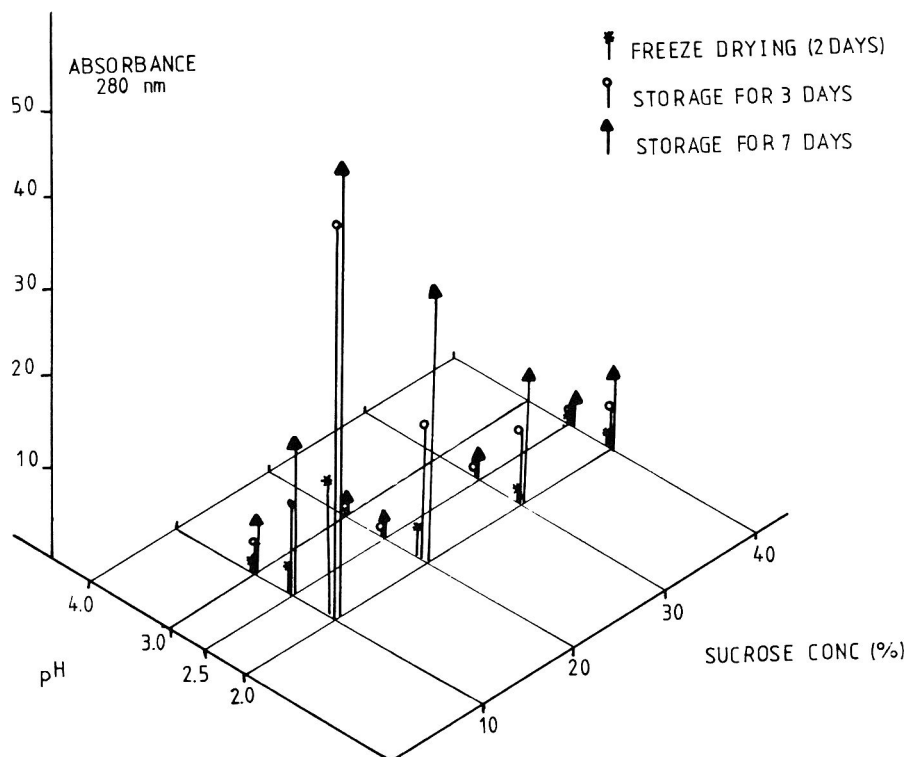
Fig. 5—Effect of sucrose concentration and soution pH on absorbance at 280 nm following freeze-drying and storage at $a_w = 0$.

the removal of water (a reaction product) from the sample environment. Further steps in the reaction pathway give products which absorb at 280 nm (presumably HMF), this eventually followed by brown color formation. For acid-catalyzed nonenzymatic browning of liquids to occur at rates similar to those observed in freeze-dried materials, higher temperatures will generally be required as the H^+ concentration normally present in liquid systems is much lower than that present during freeze-drying, or in subsequent storage of freeze-dried samples.

The conclusions of this study will be of importance for interpretation of evaluations of product stability during freeze-drying and storage stability for high acid, sugar-containing materials, such as food products (for example, freeze-dried fruit juices, especially citrus) or other blended aritifical compositions. In this respect, it is important to note that the observance of brown color in the freeze-dried product may result from other causes than the traditional Maillard browning reaction, or the action of too high sample temperatures during the drying process. In addition, for the situation where samples are being freeze-dried as a preparation step for subsequent chemical analysis, it is important to realize that changes may be occurring to the sample during the freeze-drying step, even though the drying is occurring at low temperatures.

In addition, from the results of this study, it was possible to explain the cause of the high absorption values that had

been noted in the flavor retention study.

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Kinetics of the Maillard Reaction Between Aspartame and Glucose in Solution at High Temperatures

J. A. STAMP and T. P. LABUZA

―――――――――――― ABSTRACT ――――――――――――

Comparison was made of the extent of browning during accelerated storage tests of glucose/aspartame and glucose/glycine model systems under steady state conditions of 70, 80, 90, and 100°C and an a_w of 0.80. Browning of aspartame followed zero order kinetics with a time to 0.1 absorbance units at 420 nm of 11.40, 5.3, 2.15 and 1.0 hr for each respective temperature. The activation energy (E_A) was 22.0 Kcal/mole for the glucose/aspartame system and 15.5 Kcal/mole for the glucose/glycine system. The temperature sensitivities (Q_{10}) for the model systems were 2.4 and 1.9, respectively. The predicted shelf life to reach 0.1 absorbance units in an aqueous system at 45°C is 62 days compared to the actual value of 60 days.

INTRODUCTION

ASPARTAME (1-methyl-N-L-aspartyl-L-phenylalanine) is a dipeptide composed of two amino acids, the methyl ester of phenylalanine and aspartic acid. These, along with other amino acids, are normal constituents of protein foods consumed as part of a normal diet. Aspartame has been approved for use as a sweetening agent and caloric reduction agent (21 CFR 172.804) in foods such as cold breakfast cereals, chewing gum, and as a dry base for puddings, gelatins and beverages.

Nonenzymatic browning via the Maillard reaction is one important mode of deterioration of aspartame in foods. Since aspartame is a dipeptide, it can react with reducing sugars in the presence of water via the Maillard reaction. This interaction may result in off-flavors, the loss of sweetening power, or undesirable color changes (Hodge, 1953).

Both temperature and water content during storage affect the Maillard reaction. The Maillard reaction increases with increasing temperature and can be considered to be a zero order reaction when reactant concentrations are not limiting (Labuza, 1970). It is well established that the nonenzymatic browning reaction rate increases above the BET monolayer moisture content as a_w increases. An intermediate a_w from about 0.6–0.8 results in a rate maximum which then decreases again as a_w increases due to dilution effects on the reacting species (Eichner and Karel, 1972; Labuza, 1980).

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In this study the participation of aspartame in the browning reaction in a glucose solution under steady state conditions of temperature and water activity was studied. With this knowledge, the stability of aspartame in liquid food systems, such as syrup, can be predicted.

MATERIALS & METHODS

For the collection of browning data, a model system was designed composed of either (1) glucose (Mallinckrodt Chemical Co.) and aspartame (L-aspartyl-L-phenylalnine methyl ester, G.D. Searle & Co.) or (2) glucose and glycine (Sigma Chemical Co.).

Aqueous solutions were prepared with glycerol (Spectrum Chemical Mfg. Co.) added as a humectant at a ratio of 108g water to 100g glycerol achieving an a_w of 0.80. Water activity measurements were done on an electric hygrometer (Kaymont Instruments). The model systems were designed so that the solutions had an initial glucose to aspartame or glycine molar ratio of 3:1 as shown by Warmbier et al. (1976) to give the greatest rate of brown pigment formation. The amount of glucose added was 3.0g per 100 ml of glycerol water solution with either glycine at 0.5g or aspartame at 1.5g. All solutions were adjusted to pH 4 with 1 molar HCl to maximize solubility of the aspartame. Sample solutions were sealed in 2.0 ml hydrolysis vials (Supelco Inc.) to minimize heat transfer limitations in come-up time and placed into a circulating, controlled temperature, oil bath at each temperature (45, 70, 80, 90, and 100°C). At each sample time, samples were quickly placed into an ice bath to inhibit further brown pigment formation. The development of brown pigment was measured at absorbance at 420 nm on a Beckman DB-G spectrophotometer (Beckman Instruments Inc.), and a time (θ_s) to 0.1 absorbance units was used as the time for initial visual browning as was found by Warren and Labuza (1977) and Warmbier et al. (1976) to be the point where a significant amount of amine (>40%) has been reacted. Duplicate measurements of three replicates were analyzed for single samples at each temperature during storage.

Rate constants were obtained for brown pigment formation assuming a zero order rate relationship based on the work by Labuza and Saltmarch (1981). This relationship is defined as follows;

$$B = B_0 + k_z t$$

where B is the brown pigment concentration at t; B_0 is the brown pigment concentration at t = 0; k_z is the zero order rate constant; t is the time. The temperature dependence of browning was determined by use of the typical Arrhenius relationship of $\ln k$ vs $1/T$ to obtain activation energies (E_A) and Q_{10} values (Labuza, 1979).

RESULTS & DISCUSSION

RATE CONSTANTS (OD/hr) for brown pigment formation listed in Table 1 were obtained from the slopes of linear plots of measured brown pigment concentration versus time

Table 1—Brown pigment formation rate constants (OD/hour) x 10^2*

System	a_w	70°C	80°C	90°C	100°C	E_A Kcal mole	Q_{10}
Glucose/Aspartame	0.80	0.89 ± 0.001	2.04 ± 0.002	5.22 ± 0.004	12.6 ± 0.460	22.0	2.38
$\theta_{s_{0.1}}$ (hours)		11.40	5.30	2.15	1.0		
Glucose/Glycine	0.80	—	17.3 ± 0.015	32.9 ± 0.051	59.7 ± 0.12	15.5	1.89
$\theta_{s_{0.1}}$ (hours)			0.580	0.250	0.120		

* ± 95% confidence limits; for all cases $r^2 > 0.97$
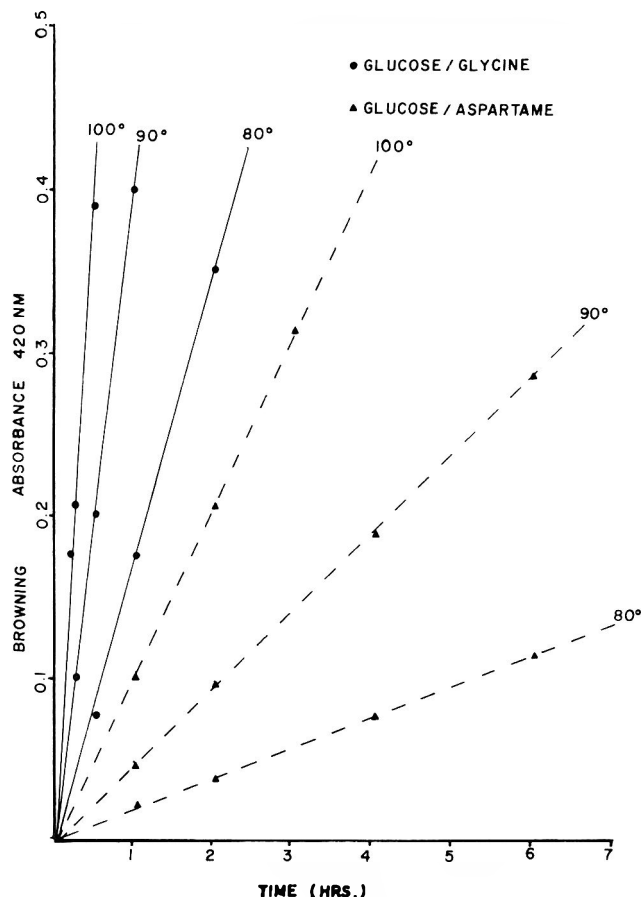
Fig. 1—Brown pigment formation in aqueous model systems at various temperatures.
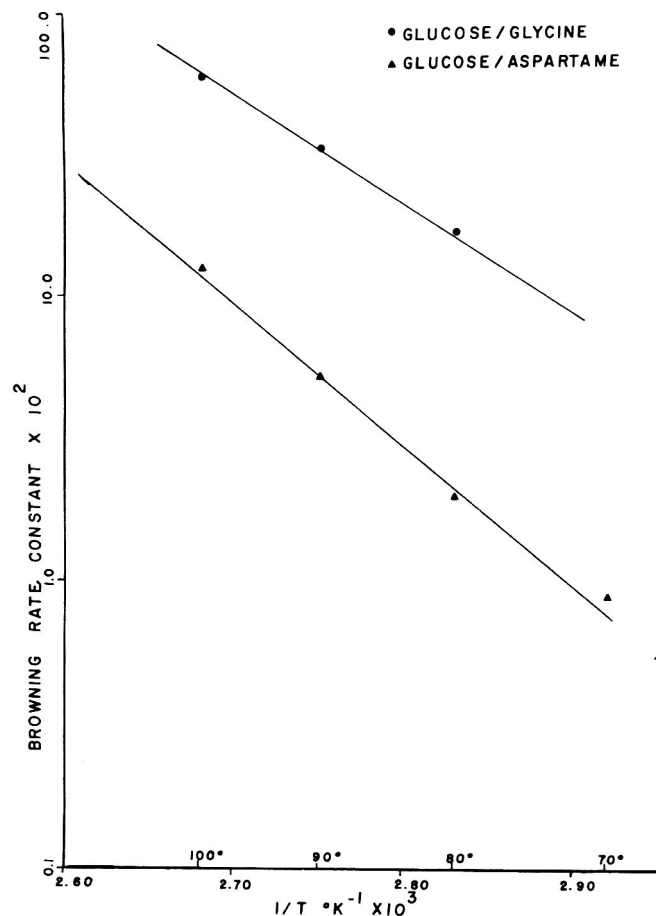
for each temperature condition tested. Fig. 1 shows the plot of the extent of browning as a function of time for both systems held at 80, 90, and 100°C. As can be seen, the data exhibit zero order kinetics. It is also obvious from Fig. 1 that the aspartame/glucose system degrades at a slower rate at all three temperatures than does the glucose/glycine systems. As seen in Table 1, the 95% confidence limits of the constants are small, indicating little analytical error. In addition, the average time to a browning value of 0.1 is also presented in Table 1. At high process temperatures, the browning rate of aspartame in the presence of glucose is significant. At 100°C it only takes 1 hr for the level of browning to reach the end of shelf life in the model system. Thus for a boiling water acid canned food process (such as a carbonated beverage) at 100°C, significant loss of aspartame, causing sweetening loss as well as undesirable color production, can occur if the product also contains reducing sugars. With the high cost of aspartame it is possible that not all the sugar will be replaced and that high fructose corn syrup (high in fructose and glucose) will be used. It should be noted that the rate of loss of the free amino acid, glycine, is much faster, probably because the amino group is not sterically hindered as is the case in aspartame.

Fig. 2 is the Arrhenius plot relationship using constants determined by linear regression of the extremes of the 95% confidence limits for each test temperature. Because of the high degree of confidence for the browning data, good correlations exist for the activation energies. Table 1 lists the values calculated for the activation energies with the glucose/aspartame system giving a value of 22.0 Kcal/mole while the glucose/glycine model system gave an E_A of 15.5 Kcal/mole for the browning reaction. These values are in the range expected for the browning reaction as reviewed

Fig. 2—Arrhenius plot (rate constant vs 1/T) for browning of aqueous model systems at three temperatures.
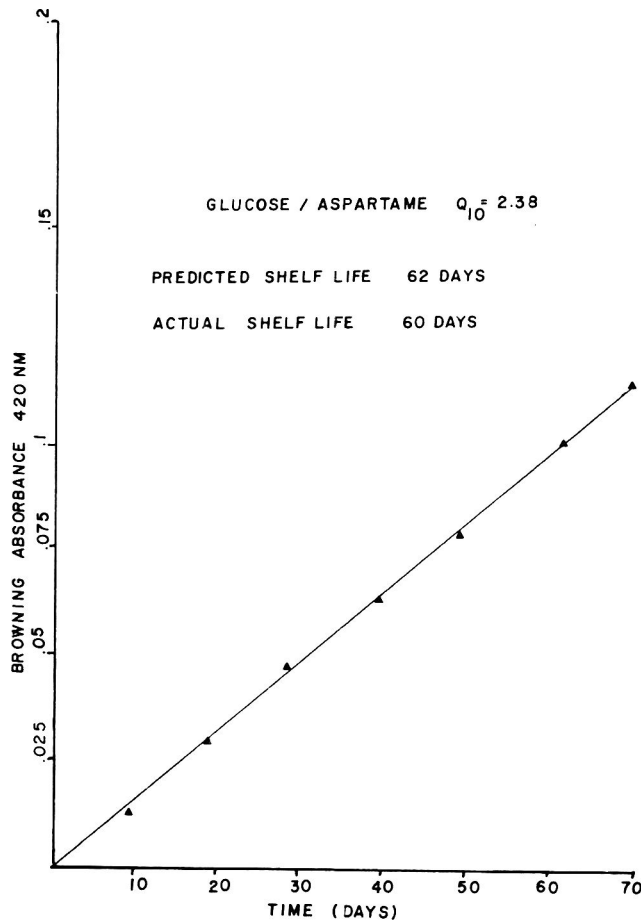
Fig. 3—Experimental values during storage of aqueous model systems at 45°C (Browning (OD/day)).

Tyramine in Cocoa and Derivatives

M. JALON, C. SANTOS-BUELGA, J. C. RIVAS-GONZALO, and A. MARINE-FONT

ABSTRACT

A method is described for the analysis of tyramine in cocoa and derivatives by sand-column extraction with alkaline ethyl acetate as eluant and later transference to a 0.2N HCl acid phase. Tyramine is determined quantitatively by fluorometry after reaction with α-nitrous-β-naphthol. Qualitative confirmation is carried out by spectrofluorometry and thin layer chromatography. Average recovery of the method was 88.7% and relative standard deviation was 4.61%. The method was applied to samples of derivatives of cocoa sold in Spain and to intermediate substances in the industrial processes used in the production of sweetened cocoa powder. The amounts of tyramine found ranged between 0.1 and 2.8 μg/g.

INTRODUCTION

OF THE MANY INTERACTIONS between ingested foods and the health of individuals, the one which occurs between the intake of certain foods and migraine is well known. Among such foods, the derivatives of cocoa, especially chocolate, are outstanding and the related migraine has been attributed to the presence of tyramine in them (Smith et al., 1970; Rivas-Gonzalo et al., 1978). This has not been entirely confirmed, however, and other vasoactive amines have also been implicated, principally serotonin (Ghose et al., 1978; Hardebo et al., 1978) and phenylethylamine (Sandler et al., 1974; Gonsalves and Stewart, 1977).

Tyramine in foods is also of importance because it is able to interact with mono-amine-oxidase inhibitor drugs (IMAO), giving rise to hypertensive crises of variable consequence (Marine-Font, 1978).

Other authors have described the relationship between the fermentation process undergone by certain foods and their tyramine content (Rice et al., 1976; Rice and Koehler, 1976). In the particular case of cocoa, fermentation takes place during the manipulations which the seeds are subjected to in exporter countries (Minifie, 1980). Kenyhercz and Kissinger (1977) reported greater amounts of tyramine in fermented seeds (11.5 ± 0.1 μg/g), than in unfermented seeds (3.9 ± 0.1 μg/g), which seems to support this relationship between tyramine and fermentation in cocoa, too.

The few studies appearing in the literature (Kenyhercz and Kissinger, 1977; Ingles et al., 1978; Hurst and Toomey, 1981) report amounts of tyramine in cocoa derivatives ranging from undetectable to 14.6 μg/g; relatively low when compared with the amounts detected in cheese (undetectable to 2170 μg/g) (Rivas-Gonzalo et al., 1978) or meat derivatives (undetectable to 1500 μg/g) (Santos-Buelga et al., 1981). These latter have been related to the appearance of migraine to a much lesser extent, though they have frequently been implicated in cases of interaction with IMAO drugs (Blackweel and Mabbit, 1965; Rice et al., 1976).

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This work includes data on the tyramine content in cocoa derivatives commercialized in Spain as well as data on intermediate products of the industrial production of cocoa powder.

Even though moisture is very low in this kind of product, the water content was analyzed in all samples.

MATERIALS & METHODS

THE METHOD used was an adaptation of that proposed by Santos-Buelga et al. (1981) for the analysis of tyramine in meat products.

Preparation of sample

Two grams of cocoa or derivative were weighed into a 250 ml beaker (when the products are compact it is necessary to freeze and then grate until a granular texture is achieved). Ten ml borate buffer pH = 10.4 (50 ml of 0.1 N solution in boric acid and potassium chloride + 46.5 ml of 0.1N sodium hydroxide) and 1−1.2g of sodium carbonate were added to obtain a pH value of 10.4 ± 0.1. The alkalinized substance was then mixed with 70−80g fine sand and 50−60g anhydrous sodium sulphate to obtain a dry mass.

Extraction and separation

One hundred milliliters of ethyl acetate were added to the dry mass and left to stand for 30 min, stirring occasionally to avoid aggregation of the sand. The mass was placed in a 3 cm diameter column provided with a stopcock. The beaker was washed with 100 ml ethyl acetate, which was added to the column. The 200 ml ethyl acetate were eluted over a period of approximately 2 hr. The eluate was extracted three times with 8 ml 0.2N HCl in a separatory funnel. The acid extracts were combined and adjusted to 25 ml.

Quantitative determination

Two milliliters of the final extract were transferred into a test tube. One milliliter of the solution of α-nitrous-β-naphthol (0.1% [w/v] in 95% ethyl alcohol) and 1 ml of the nitric solution (1M nitric acid containing 2% [v/v] sodium nitrite at 2.5% [w/v], prepared fresh) were added. This was mixed well and heated at 60°C for 1 hr, cooled to room temperature and 10 ml 1,2-dichloroethane added. The mixture was shaken vigorously for 1 min to extract the excess α-nitrous-β-naphthol. The top layer was recovered and used for fluorometric determination of tyramine at 545 nm resulting from activation at 450 nm.

Tyramine concentrations were calculated from a comparison of the fluorescence intensities with respect to a calibration curve, based on varying concentrations of free tyramine subject to the α-nitrous-β-naphthol reaction. A blank was used to correct native fluorescence of the reagents.

Qualitative identification

Spectrofluorometry. Activation and emission spectra

were determined to ensure that they coincided with those of the fluorophore formed from standard tyramine.

Thin-layer chromatography. The method used was that of Rivas-Gonzalo et al. (1979). Specifications were: The final acid phase was brought to dryness in a rotovapor at 40°C. The residue was redissolved in 1 ml 0.2N HCl, and 20 µl were spotted onto TLC plates.

— Support: Cellulose MN-300.
— Eluent: Butanol/Acetic acid/Water (12:5:3).
— Color development: 0.1% α-nitrous-β-naphthol in 95% ethyl alcohol + 3M nitric acid containing 0.05% sodium nitrite.

Determination of moisture

The extract was dried at 100–105°C (Casares, 1967).

RESULTS & DISCUSSION

THE METHOD EMPLOYED was subjected to recovery and accuracy assays before being applied to the sample. Recovery assays were carried out by adding different amounts of standard tyramine (1, 2, 5, 10, and 15 µg/g) to a sample of sweetened cocoa powder. The method was applied five times for each of the amounts shown, simultaneously carrying out an assay with a sample of the same sweetened cocoa powder to which no tyramine had been added in order to

Table 1—Tyramine content (referring to overall product) and moisture of Spanish products derived from cocoa

Sample	Tyramine (µg/g)	Moisture (%)
Dark chocolate		
a	0.5	2.4
b	0.5	1.2
c	0.7	1.4
d	0.7	1.6
e	0.9	4.1
f	1.1	2.3
Drinking chocolate[a]		
a	0.2	2.7
b	0.4	2.5
c	0.5	2.8
d	0.5	1.0
e	0.6	2.8
f	0.7	2.2
g	0.8	1.3
h	0.8	1.3
Milk chocolate		
a	0.1	1.7
b	0.2	1.6
c	0.3	2.0
d	0.3	1.6
e	0.4	1.2
f	0.5	1.5
Substitute chocolate		
a	0.1	2.6
b	0.1	2.4
c	0.5	2.0
d	0.6	2.0
Sweetened cocoa powder		
a	0.3	3.6
b	0.4	2.3
c	0.4	7.8
d	0.7	1.4
e	0.8	4.4
f	0.8	1.8
Chocolate granules		
a	0.4	1.6

[a] "Drinking chocolate" is plain or milk chocolate, in bar form, to which cereal flour has been added to a maximum of 15%, for grating and heating before comsumption.

correct, in each case, the amount of the amine present in the sample. Average recovery values ranged between 83.7% and 93.8%, overall recovery being 88.7%.

To test accuracy, nine succesive analyses for tyramine content were carried out on a sample of sweetened cocoa powder. Relative standard deviation was 4.61%.

The method employed was then used to analyze two main groups of samples:

(a) Samples of different cocoa derivatives purchased from food stores, including dark chocolate, drinking chocolate, milk chocolate, chocolate substitute, sweetened cocoa powder and chocolate granules (see Table 1). The highest tyramine contents were found in dark chocolate (average content 0.7 µg/g) and the lowest in milk chocolate and chocolate substitute (average content 0.3 µg/g), which is logical since these latter have a lower proportion of cocoa. Sweetened cocoa powder and drinking chocolate have intermediate values (average content 0.6 µg/g). Though references to the tyramine content in this kind of product is scarce, our findings are in general lower than those reported by other workers. Hurst and Toomey (1981) found that for milk chocolate levels ranged between 3.76 and 12.02 µg/g and Kenyhercz and Kissinger (1977) found 8.3 µg/g for sweetened cocoa powder. It should be noted that we found tyramine in all the samples studied, in disagreement with the findings of Ingles et al. (1978), who found none in dark chocolate, milk chocolate and white milk chocolate. They did, however, report levels of 2 µg/g in drinking chocolate.

(b) Samples obtained during the industrial manufacturing processes of sweetened cocoa powder. Figure 1 outlines the principal technological processes and intermediate products together with the tyramine content at each stage.

Tyramine content does not seem to be markedly affected by the techniques involved and the small alterations it does undergo could be explained by the separation of fractions and the addition of other products. It may be seen that tyramine is already present in the original product
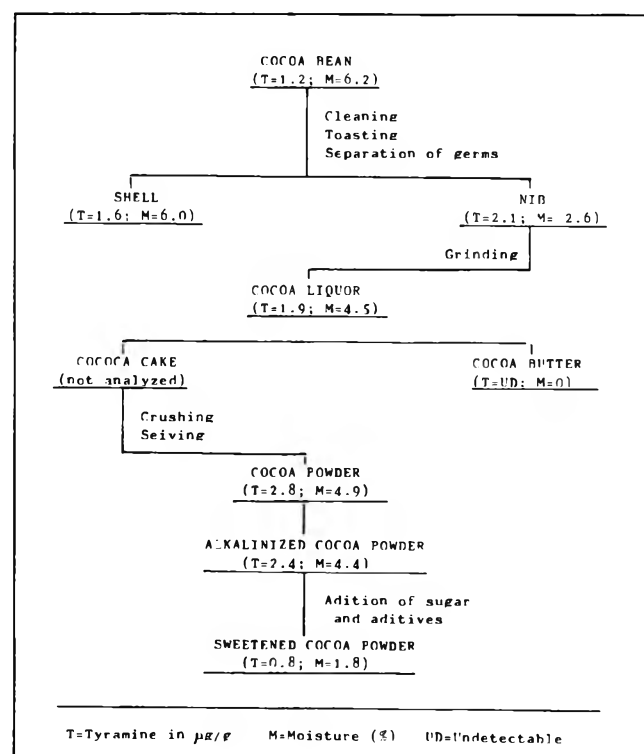
Fig. 1—Tyramine content (referring to overall product) and moisture of intermediate products in the process of making cocoa powder.

(the fermented and roasted cocoa bean) and it must there-fore have first appeared earlier, possibly during fermenta-tion. This is plausible since the formation of tyramine is always associated with a fermentative or maturation proc-ess; furthermore, this is in agreement with the findings of Kenyhercz and Kissinger (1977), who reported that fer-mented beans had a greater tyramine content (11.05 ± 0.1 µg/g) than unfermented beans (3.9 ± 0.1 µg/g). Hurst and Toomey (1981), reported great variations in the tyra-mine content of cocoa liquor of different origins (0.73–14.6 µg/g). Our own results (1.9 µg/g) are of the same order as the lowest values reported by other workers.

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by Warmbier et al. (1976). In addition, the Q_{10}'s are listed with glucose/aspartame having the higher Q_{10} of 2.38 as compared to the glucose/glycine system which gave a Q_{10} of 1.89. Thus the aspartame system is more sensitive to temperature change at higher temperature levels.

From data at accelerated temperature, one can predict what the shelf life of a particular product will be at a lower temperature (Labuza, 1982). The predicted shelf life at 45°C for the aspartame/glucose system gives a value of 62 days. Fig. 3 shows that the actual time to an O.D. of 0.1 for the aspartame/glucose system held at 45°C was 60 days, indicating that the Arrhenius relationship holds very well in this model system. Whether this would be true in a real food is not known. The accelerated storage data presented here can also be applied to processed food products such as reduced calorie syrups and soft drinks which are sterilized in boiling water, in predicting the browning that would occur with aspartame as part of the formulation. We are currently investigating the kinetics of the loss of sweeten-ing power during the Maillard reaction. This work will be published at a later date.

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Method of Analysis for (-)-Epicatechin in Cocoa Beans by High Performance Liquid Chromatography

H. KIM and P. G. KEENEY

─────────ABSTRACT─────────

A sensitive high performance liquid chromatographic method for analysis of (-)-epicatechin (3,3',4',5,7-pentahydroxyflavan) in cocoa beans is described. Bean samples were extracted in 80% acetone with subsequent sample clean-up on a Waters Associates C_{18} SEP-PAK. Separation of (-)-epicatechin was accomplished on a μBonda-pak C_{18} column using a mobile phase of water:methanol:acetic acid (87:8:5). (-)-Epicatechin was detected at 280 nm and quantified by comparing peak height of sample to those of standards. The method demonstrated excellent reproducibility and recoveries of added (-)-epicatechin averaged over 90%. UV scans and mass spectrometric anlaysis of collected eluate from the chromatograph provided positive identification of the compound in cocoa bean extracts.

INTRODUCTION

CHOCOLATE is one of the most complicated natural flavors. Over 300 volatile compounds have been identified in roasted cocoa beans (Keeney, 1972). Many of these flavor compounds, generated during roasting, arise from precursors developed during fermentation and drying of freshly harvested cocoa beans. Studies (Rohan, 1963, 1964; Rohan and Connell, 1964) indicate that flavanoids (polyphenols), along with sugars and amino acids may be the flavor precursors in fermented cocoa beans.

During fermentation and drying, complex chemical changes in polyphenols occur which affect the flavor and color of chocolate. Anthocyanins are hydrolyzed enzymatically to anthocyanidins which polymerize along with simple catechins to form high molecular weight leucocyanidins or complex tannins (Roelofsen, 1958; Forsyth and Quesnel, 1963). These complex tannins in turn interact with proteins by the tanning process. Thus, astringency and bitterness associated with polyphenols are reduced while unpleasant flavors and odors of roasted proteins are depressed. During the drying stage, (-)-epicatechin and leuco-cyanidins are oxidized enzymatically resulting in the brown color characteristic of chocolate (Griffiths, 1957; Quesnel, 1966). Further changes during roasting and conching may occur to affect chocolate flavor, but information is lacking.

Although most polyphenols are reduced during fermentation and drying, they are still present in chocolate and impart astringent and bitter tastes. Moreover, tanning probably contributes to the low digestibility and poor biological value of cocoa proteins (Chatt, 1953). The polyphenols and their reactants may be the anti-cariogenic factors observed in chocolate, but they are also suspect relative to their toxicity and carcinogenicity (Singleton and Kratzer, 1973; Singleton, 1981). Although cocoa polyphenols received considerable attention in the past, very little has been published in recent years. With the availability of modern analytical techniques, it is appropriate to take a new look at this important class of compounds in cocoa.

Most investigations of cocoa polyphenols utilized paper chromatography and spectrophotometric measurements.

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The disparity of such analytical methods requiring large samples and lengthy analysis time leads to difficulty in assessing the reliability of experimental results. However, recent developments in high performance liquid chromatography (HPLC) suggest this approach as being promising in overcoming past analytical difficulties. Methods of analysis for sugars, alkaloids, emulsifiers, aflatoxin, artificial colors, and artificial flavors by HPLC have been developed for products of the chocolate confectionery industries (Hurst et al., 1980). In cocoa beans, (-)-epicatechin is the major catechin which polymerizes to form complex tannins during fermentation and is the major substrate for enzymatic browning during drying. The purpose of this investigation was to develop a suitable method of extraction and HPLC analysis of (-)-epicatechin in cocoa beans and to evaluate the accuracy and precision of the entire procedure. The effects of bean variations due to varietal types, fermentations, and roasting on the concentration of (-)-epicatechin will be the source of another study.

MATERIALS & METHODS

HPLC apparatus and operating conditions

Analytical column: μBondapak C_{18}, 30 × 0.4 cm i.d. (Waters Associates, Milford, MA); guard column: C_{18}/Corasil (37-50μ), 40 × 20 mm i.d. (Waters); pump: Model 6000-A (Waters); injector: Model U6K (Waters); detector: Model 450 variable wavelength (Waters) set at 280 nm; recorder: Beckman 10" recorder set at 0.2"/min; mobile phase: water:methanol:acetic acid (87:8:5), 2.0 ml/min.

Standards and solvents

(-)-Epicatechin and (+)-catechin: Sigma Chemical Company, St. Louis, MO; chlorogenic acid: ICN Pharmaceuticals, Inc., Cleveland, OH; methanol: HPLC grade, Fisher Scientific Company, Pittsburgh, PA; acetic acid: glacial, Fisher Scientific Company.

Sample preparation

Whole, oven-dried unfermented Forastero cocoa beans (supplied by Centro de Pesquisas de Cacao, Itabuna, Bahia, Brazil) were de-shelled and ground in a Tekmar model A-10 analytical mill to particle sizes passing through a 20-mesh screen. Pieces of dry ice were added with the beans to prevent melting of cocoa lipids due to frictional heat of grinding. The ground samples were defatted with hexane for 16–18 hr in a soxhlet apparatus and the residual hexane was removed in a vacuum oven at 65°C. The resulting defatted samples were stored in the dark in desiccators over $CaSO_4$ prior to extraction procedures.

For the extraction of (-)-epicatechin, 0.5g of defatted cocoa powder and 80 ml of 80% aqueous acetone in a 125-ml Erlenmeyer flask were sonicated 30 min in a sonic cleaning device filled with ice water. Sonication as an aid in solubilizing (-)-epicatechin was preferred over shearing in a Waring Blendor since the latter promoted oxidation of the polyphenols and browning of the extract.

After filtering the extract under vacuum through Whatman #1 paper on a Buchner funnel and washing the glassware and residue with 80% aqueous acetone, the total filtrate was brought to 100 ml in a volumetric flask. A measured portion, 5 or 10 ml from a volumetric pipette, was dried under vacuum at 45°C in a 125-ml rotary evaporator flask. This residue was resuspended into two 5.0 ml aliquots of distilled water by swirling 2 min in a 45°C water bath. The solutions were pooled and injected through a Waters Associates C_{18} reverse-phase SEP-PAK which had been pre-condi-

tioned with 2 ml of methanol followed by 5 ml of water. (-)-Epicatechin, which was retained in the SEP-PAK, was eluted with 40% aqueous methanol into a 10-ml volumetric flask. Ten microliters of this final solution was injected into the liquid chromatograph, (-)-epicatechin being monitored at 280 nm.

Identification of (-)-epicatechin

To obtain enough of the (-)-epicatechin for UV and mass spectral analysis, eluates from several HPLC injections of cocoa extract were pooled. Detector sensitivity had been lowered (i.e., 2 AUFS) to adjust for the increase in sample injection load. After evaporation to dryness on a rotary evaporator, the residue from pooled eluates was resuspended with 5.0 ml of methanol. This solution was scanned (260–300 nm), using a model 25 UV-VIS recording spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) equipped with quartz cuvettes. The resulting spectrum was compared to the spectrum of standard (-)-epicatechin in methanol.

Mass spectrometric analysis was conducted with an LKB 9000A Gas Chromatograph-Mass Spectrometer (LBK-Produkter ab Stokholm-Bromma 1 Sweden) equipped with a Direct Inlet System (LKB 9042). Five microliters of the methanol solution containing approximaterly 0.25 μg of (-)-epicatechin was placed in a direct inlet system sample vial, dried, and injected into the analyzer. (-)-Epicatechin was fragmented by electron bombardment at 70 eV and the ion fragments were recorded with a Datagraph Recording Oscillograph (Consolidated Electrodynamic Corp., Pasedena, CA). The resulting spectrum was compared to the spectrum of standard (-)-epicatechin. High resolution mass spectrometry of standard (-)-epicatechin was conducted with a Kratos MS9/50 (Kratos Ltd. Barton Dock Road, Urmston Manchester M31 2LD) double focusing high resolution instrument equipped with Kratos D55 computer system (Data General NOVA/4). Approximately 1.0 mg of the sample was analyzed with a direct probe at 250°C and fragmented by electron bombardment at 50 eV.

Quantitative analysis of (-)-epicatechin

Preparation of standard curves. Standard (-)-epicatechin samples at six concentrations (0.29, 0.64, 1.19, 2.07, 3.24, and 4.37 μg) were prepared in duplicate. Average peak height (mm) response from two HPLC injections of each sample at 0.04 AUFS was recorded. Linear regression was determined by the least squares method and the correlation coefficient (r) of peak heigh versus concentration was calculated.

Reproducibility and recovery studies. Reproducibility of the entire method was determined by conducting extractions of six separate lots of a homogeneous defatted cocoa powder. Duplicate injections of each extract were analyzed on the liquid chromatograph, results being expressed as milligrams of (-)-epicatechin per 0.5g of defatted sample. Relative standard deviation and a 95% confidence interval were calculated to assess the reliability of the procedure. For recovery tests, 0.5g samples of "spiked" cocoa containing 0.0, 5.6, 11.2, 15.2, and 20.0 mg of standard (-)-epicatechin were placed in 125-ml Erlenmeyer flasks and analyzed as previously described.

RESULTS & DISCUSSION

(-)-Epicatechin identification

At the outset it was recognized that cocoa beans contain several polyphenols, including chlorogenic acid and (+)-catechin (Forsyth and Quesnel, 1963) which exhibit strong UV absorbance and might have chromatography properties similar to (-)-epicatechin. However, since separation by paper chromatography has been demonstrated (Roberts and Wood, 1953), it was a reasonable assumption that an even better separation would be realized through HPLC. This is evident in Fig. 1 which shows effective separation of these phenolic compounds on the reverse-phase μBondapak C_{18} column.

In following the isolation of (-)-epicatechin and its identification in cocoa extracts, several qualitative tests were performed. Typical chromatograms of a cocoa extract and an extract "spiked" with standard (-)-epicatechin are presented in Fig. 2. Co-elution of peak "a" and the standard is clearly evident. Peak "b", eluting subsequent to (-)-epicatechin, was tentatively identified as caffeine through

similar spiking trials with authentic caffeine. Obviously, caffeine and other compounds were extracted with 80% aqueous acetone. Fortunately, the highly efficient HPLC
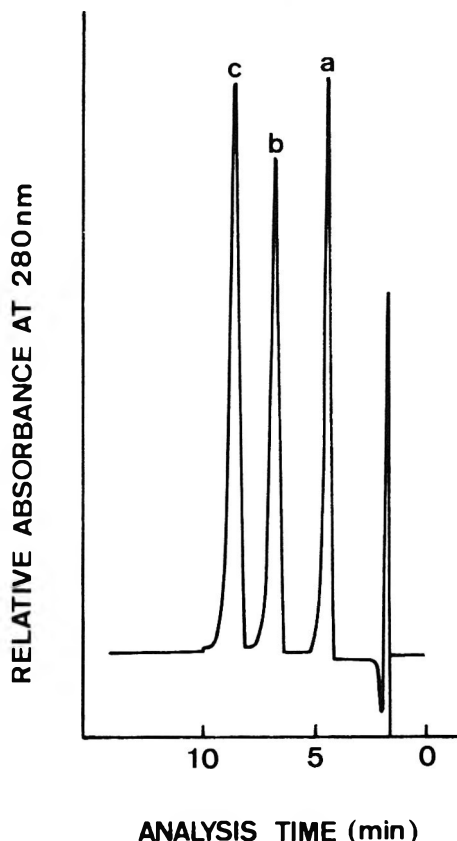
Fig. 1—HPLC separation of (-)-epicatechin from suspected polyphenols occurring in cocoa: (a) (+)-catechin; (b) chlorogenic acid; (c) (-)-epicatechin.
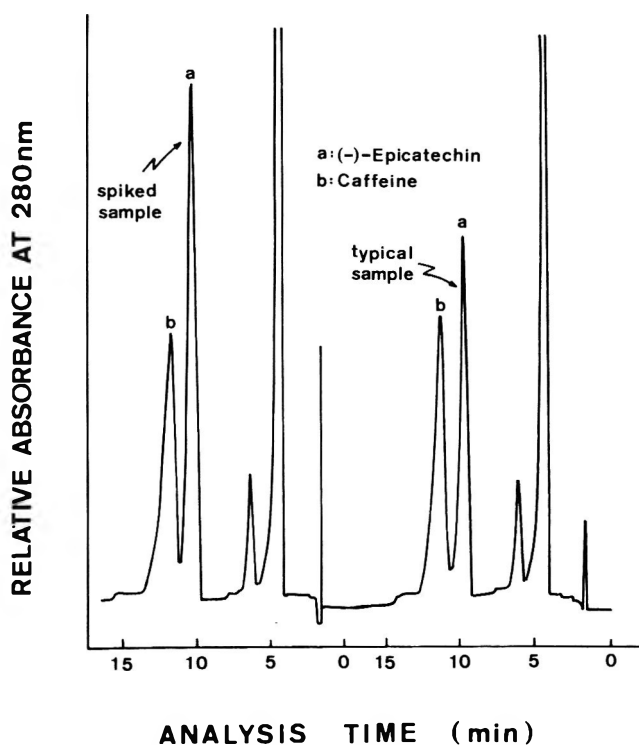
Fig. 2—HPLC chromatograms of: right, cocoa extract; left, cocoa extract spiked with standard (-)-epicatechin.
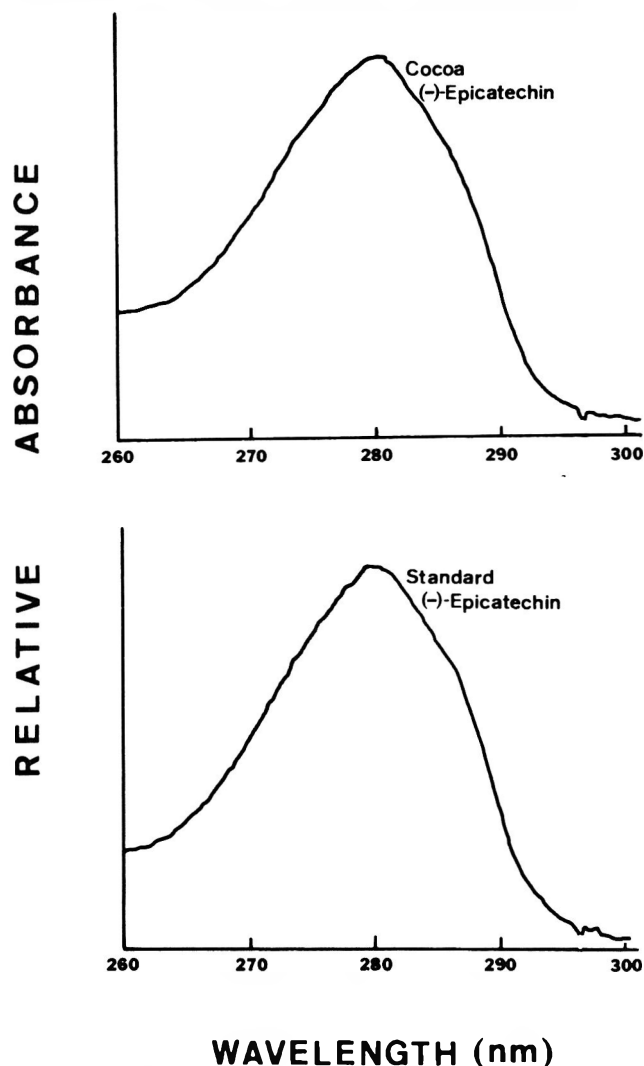
Fig. 3—Absorption spectra of (-)-epicatechin determined by Beckman spectrophotometer: top, cocoa (-)-epicatechin; bottom, standard (-)-epicatechin.
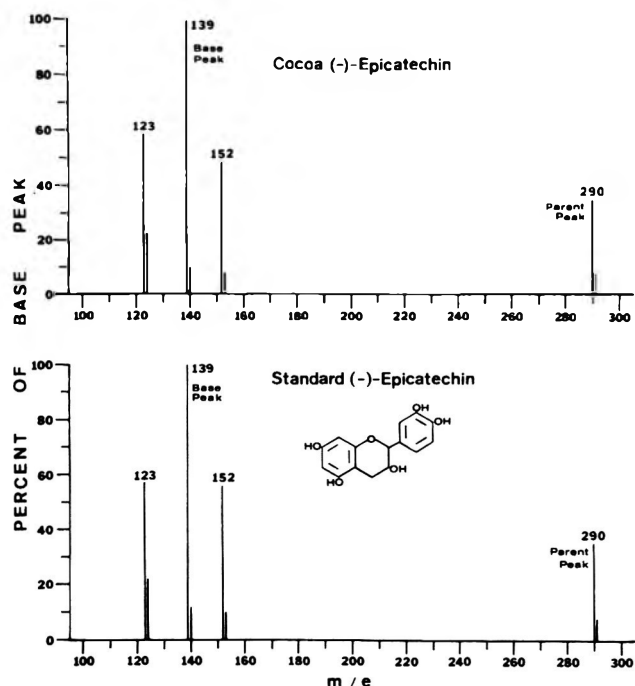
column and the mobile phase provided excellent separation of caffeine and (-)-epicatechin.

The absorption spectrum of the collected cocoa (-)-epicatechin peak, scanned between 260 and 300 nm, was identical to the standard (Fig. 3). Mass spectra of cocoa (-)-epicatechin were identical to the authentic standard with major ions at m/e of 123, 139, 152, and 290 (Fig. 4). The parent peak at m/e 290 corresponds to the molecular weight of (-)-epicatechin. The base peak was at m/e 139 with no major ions below m/e 100.

To elucidate the possible structures of the major ion fragments, high resolution mass spectrometry of standard (-)-epicatechin was performed. Results confirmed the major ions to be: 123.0424 ($C_7H_7O_2$), 139.0434 ($C_7H_7O_3$), 152.0459 ($C_8H_8O_3$), and 290.0793 ($C_{15}H_{14}O_6$). The proposed structures of these ion fragments are presented in Fig. 5.

Quantitative analysis of (-)-epicatechin

Peak height (mm) response for (-)-epicatechin in cocoa extracts was compared to standard curves for quantification purposes. Curves for standard (-)-epicatechin were obtained at six concentrations ranging from 0.29 μg to 4.37 μg with detector sensitivity at 0.04 AUFS (Table 1). Excellent linearity for detector response was shown by the correlation coefficient of 0.99.

Reproducibility and recovery studies

A relative standard deviation of 2.57% (Table 2) based on extractions of six separate lots of the same cocoa powder provides an estimation of the error involved. Injection errors and extraction variations contribute to the final error of the procedure. The narrow range (21.72 ± 0.59) for a 95% confidence interval demonstrates the precision of the entire method.

Reliability of the entire method was enhanced by utilizing a guard column and a C_{18} reverse-phase SEP-PAK for sample clean-up. Extracted (-)-epicatechin, when resuspended in water, was effectively retained by the SEP-PAK while more water-soluble contaminating materials passed through. Subsequent elution with 40% aqueous methanol released (-)-epicatechin, caffeine and other compounds,

Fig. 4—Mass spectra of (-)-epicatechin: top, cocoa (-)-epicatechin; bottom, standard (-)-epicatechin.
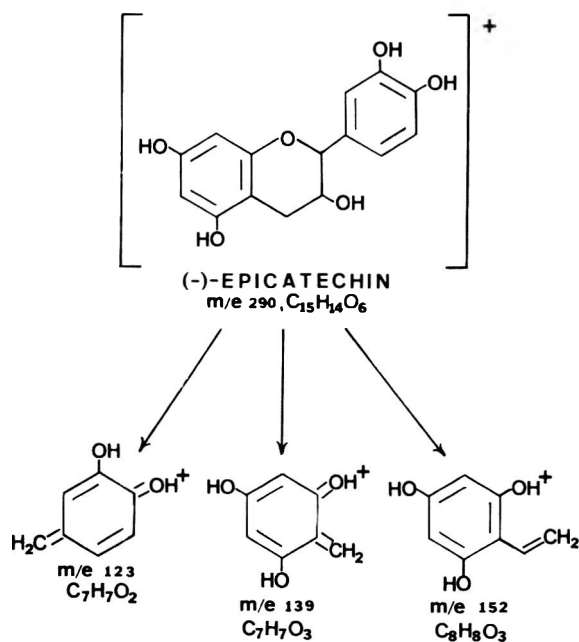
Fig. 5—Proposed structures of major ion fragments obtained by mass spectrometric analysis of (-)-epicatechin.

Table 1—Linearity of standard (-)-epicatechin injected vs peak height

μg (-)-Epicatechin standard injected (Y)	Peak height (mm) at 0.04 AUFS[a] (X)
0.29	10.0
0.64	30.2
1.19	59.3
2.07	103.4
3.24	156.1
4.37	210.0
$Y = 0.026X + 0.017$[b], $r = 0.99$[c]	

[a] AUFS = Absorbance Units Full Scale.
[b] Linear regression equation derived by least squares method.
[c] Correlation coefficient.

Table 2—Reproducibility of (-)-epicatechin determination

Lots of same cocoa sample extracted	mg/0.5g Dry, defatted sample[a]
1	20.80
2	21.40
3	22.35
4	22.14
5	21.90
6	21.73
Average ± 95% Confidence interval	21.72 ± 0.59
Relative standard deviation	2.57%

[a] Values represent mean of duplicate injections.

while substances having higher affinities for the C_{18} stationary phase were visibly retained as purple pigments. Use of a reverse-phase (C_{18}/Corasil) guard column, as recommended by the column manufacturer, helped remove possible contaminants in the mobile phase. Furthermore, the recommended practice of washing the analytical column with methanol after 10–15 injections favors removal of accumulated contaminants. These precautions help to maintain column efficiency and performance life. Throughout this investigation, column performance was monitored by frequent calibrations with standard (-)-epicatechin.

Extraction efficiency of any compound from a complex food matrix, in this case (-)-epicatechin from defatted cocoa powder, is always difficult to assess. For these studies, efficiency was evaluated by determining recovery of known amounts of authentic (-)-epicatechin added to defatted cocoa. Samples analyzed were from the same source utilized for reproducibility studies. High rates of recovery for the procedure, averaging 91.7%, were realized (Table 3). The data show a trend towards decreased recovery with increasing concentration of added (-)-epicatechin. This can be attributed at least in part to the limited sample load capacity of the SEP-PAK (low milligrams) in retaining (-)-epicatechin during removal of more water soluble contaminants. Nevertheless, high accuracy in recovery of (-)-epicatechin can still be claimed.

Table 3—Recovery of standard (-)-epicatechin added to defatted cocoa powder

(-)-Epicatechin naturally present in cocoa powder (mg/0.5g sample)	(-)-Epicatechin added (mg)	(-)-Epicatechin recovered (mg)	Recovery[a] (%)
21.72	5.6	25.82	94.5
21.72	11.2	31.14	94.6
21.72	15.2	33.26	90.1
21.72	20.7	36.59	87.7
		Average	91.7

[a]
$$\% \text{ Recovery} = \frac{(-)\text{-Epicatechin recovered}}{\text{Sample }(-)\text{-epicatechin} + \text{Added }(-)\text{-epicatechin}} \times 100$$

SUMMARY

THIS INVESTIGATION was conducted primarily to develop a rapid HPLC method for determination of (-)-epicatechin in cocoa beans. The cocoa (-)-epicatechin peak on HPLC chromatograms was identified by comparing retention times, UV scans, and mass spectra with those of the authentic standards. Excellent reproducibility of the entire method was demonstrated by the observed relative standard deviation of 2.57% and a 95% confidence interval at 21.72 ± 0.59. Recoveries of standard (-)-epicatechin added prior to the extraction procedure averaged 91.7%.

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Influence of Temperature on the Measurement of Water Activity of Food and Salt Systems

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—————————ABSTRACT—————————

The water activity of four salt slurries (barium chloride, potassium bromide, cobalt chloride and sodium bromide) and four foods (cheese spread, fruit preserves, chocolate frosting and fudge sauce) was determined 10 times at approximately 20°C, 25°C and 30°C using an electric hygrometer. In general, an increase in temperature resulted in a decrease in water activity. The magnitude of the decrease was typically greater between 25°C and 30°C than between 20°C and 25°C. This decrease was also greater when the substrate under test was in the lower a_w range. Thus temperature control is very important in inter-laboratory comparisons and when measuring water activity levels near critical values.

INTRODUCTION

WATER EXISTS in foods in free and bound forms; only the unbound water is available for microbial growth and biochemical reactions (Prior, 1979; Troller, 1980). Measurement of the unbound water is termed water activity (a_w) and is defined as the ratio of the partial vapor pressure of water in air to the vapor pressure of water vapor in saturated air at the same temperature (Scott, 1957). This is numerically equal to the relative humidity at equilibrium expressed as a fraction. Techniques for measurement of a_w have been reviewed by Prior (1979) and Troller and Christian (1978) and two collaborative studies have compared the various methods (Labuza et al., 1976; Stoloff, 1978).

Since the proportion of free and bound water varies with temperature, it has been noted that a_w is temperature dependent (Labuza et al., 1979; Prior, 1979; Stoloff, 1978). Based on theoretical considerations, Stoloff (1978) stated that temperature control is a critical parameter, particularly the temperature differential between sample and vapor phases. Additional work by Wexler and Hasegawa (1954) and Young (1967) indicated a direct relationship between temperature and relative humidity above saturated salt solutions. Nevertheless, water acitvity is measured at a variety of temperatures in the food industry, and often only minimal attempts are made to control the temperature. This could explain in part the variation in a_w readings between laboratories. The purpose of this study was to develop specific data on the influence of temperature on the measurement of water activity as it relates to such analyses in the food industry.

MATERIALS & METHODS

THE EQUIPMENT used for water activity measurement was a Beckman/Sina electric hygrometer. The sample dish was modified by inserting a thermocouple through the wall of the sample holder in order to measure the temperature of the sample directly. The thermocouple was attached to a Leeds and Northrup potentiometer from which the temperature was obtained. Water acitvity was recorded on a Beckman Hygroline recorder. Temperature was maintained by a Precision Scientific low-temperature incubator. A hole was drilled through the side to accommodate the sensor lead wire and allow positioning of the sample dish and sensor inside the incubator.

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Preliminary temperature distribution studies were performed in order to determine the temperature variation inside the incubator. Eleven thermocouples were placed in various locations throughout the incubator. The temperature was monitored over time and recorded at 1-min intervals. After changing the temperature setting and rearranging the thermocouples the procedure was repeated. For each area of the incubator, the mean temperature and standard deviation were calculated. For both repetitions the coldest and warmest spots in the incubator were the same and differed in mean temperature by 1°C. For any particular area the temperature varied by no more than ±0.5°C. The center of the top shelf was chosen as the working area because the mean temperature was in the mid-range of the temperature means and the standard deviation was relatively low (±0.07°C).

Salt slurries were prepared using reagent grade salts and distilled water demineralized and filtered using a Barnstead NANOpure system. Four salts (potassium nitrate, potassium chloride, sodium chloride and magnesium nitrate) were used for standardization of the equipment. Four additional salts (sodium bromide, cobalt chloride, potassium bromide and barium chloride) were used as test salts. Whenever possible, the a_w values used for each salt and temperature were those of Greenspan (1977). Other literature values were used when a value was not given by Greenspan. The a_w of those salts chosen for this study covers the range of water activities commonly encountered in many food systems.

Salts were equilibrated at a given temperature for at least 12 hr. The three target temperatures used were 20°C, 25°C and 30°C. These temperatures encompass the range commonly used in the food industry. The equipment was calibrated with the salt standards according to the manufacturer's instructions. Salts were run until equilibrium was indicated by a plateau on the recorder chart. The temperature was recorded at this time. This was repeated 10 times for each salt at each temperature. Data for each salt were subjected to linear and second order polynomial regression analyses. Goodness of fit tests were conducted to determine the best equation model for the data. The methods used are described by Kleinbaum and Kupper (1978).

Precision was defined as the reproducibility of the method and is represented by the standard deviation of the water activity for each salt at each temperature. Accuracy refers to the agreement between the mean a_w value and the literature value. This was calculated as the percent error by subtracting the literature value from the measured mean, dividing by the literature value and multiplying by 100. The a_w of food samples (cheese spread, raspberry preserves, chocolate frosting and fudge sauce) was measured as for the salts and the data analyzed by regression as above.

RESULTS & DISCUSSION

SUMMARIES of the regression analyses and the equations which were derived to define the lines are contained in Tables 1 and 2. Results indicate that in all but one instance (cheese spread) the first order equation adequately describes the data (p < 0.001). It should be noted from Table 2, however, that with two exceptions ($BaCl_2$ and preserves) the addition of the second order term, x^2, provides a marked improvement in the fit of the line generated from these equations to the data.

Fig. 1 and 2 are graphic representations of the lines defined by the appropriate first or second order equations for the salt slurries and foods, respectively. The two figures indicate that, in general, water activity decreased as temperature increased. This observation is supported by the fact that the regression coefficients listed in Table 1 which define the slope of the line are negative. It is of note that

the regression coefficients become even more negative as substrates of progressively lower a_w are tested. A comparison of the coefficients using a large sample z test indicates that with one exception (NaBr), this conclusion is supported (p $<$ 0.001). Additionally, the fact that the data generally are better described by a second order equation indicates that the magnitude of decrease in a_w per unit increase in temperature is greater as the temperature approaches 30°C.

A decrease in relative humidity of salt slurries as temperature increases has been reported previously (Greenspan, 1977; Wexler and Hasegawa, 1954; Young, 1967). Wexler and Hasegawa (1954), using the dewpoint method, observed that the relative humidity decreased with increasing temperature for seven salts. NaCl showed an increase in relative humidity up to 25°, followed by a decrease. Greenspan (1977) recorded decreasing relative humidities between 20 and 30°C for most saturated salts. Young (1967) graphically depicted the change in relative humidity of saturated salt solutions with temperature; several salts showed little effect of temperature on a_w, however, for most salt slurries a_w decreased as temperature increased. In only one case did a_w increase as temperature increased.

Decreases in a_w of foods as temperature increased were somewhat unexpected since sorption isotherms (Wolf et al., 1973) had indicated that at a constant moisture level a_w increased as temperature increased. The physico-chemical reasons for a decrease in a_w with an increase in temperature are not fully understood at this time. It is possible that more salt or food components are solubilized with the temperature increase, thereby decreasing a_w. Another hypothesis is that the vapor pressure of the solutions is increased more slowly than the vapor pressure of pure water, causing a decrease in a_w. In foods a_w may decrease because of component interactions with water as dissociation occurs in amino acid side chains of proteins.

To study the potential for error in a_w readings produced by variations in temperature from the calibration point, the a_w of the test salts and foods was measured 2°C above and below the standardization temperature of 25°C. Table 3 shows the average of two values obtained at 23°C and 27°C

compared with the values obtained at 25°C for both salts and foods. The change in temperature resulted in expected a_w values based on the graph of water activity versus temperature (Fig. 1 and 2). These data demonstrate that temperature drift in the incubator can result in erroneous a_w values. Temperature distribution data for the incubator (data not shown) showed variations of up to 1°C between various points in the incubator. The results indicate that it is important to measure and report the temperature of the sample itself rather than the incubator temperature.

The precision of the method (standard deviation) is shown in Table 4. Troller (1977) considers ±0.005 a_w adequate precision for most applications. The precision for most salt slurries was good (±0.004 or less). Cobalt chloride and sodium bromide at 25°C had slightly higher standard deviations. Precision for food samples based on ten determinations (data not shown) was somewhat less (higher standard deviations). This was attributed to slight dehydration of the samples in the incubator, since at each temperature a downward trend of a_w was noted with time. For this reason, food data analyses in this paper are based on four determinations rather than ten. Estimates of standard
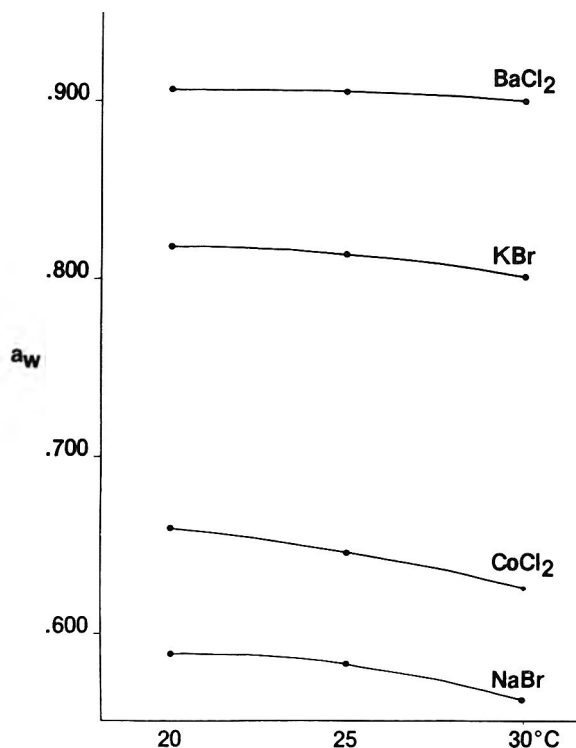
Fig. 1—Effect of temperature on the a_w of salt slurries. Each point represents the mean of ten determinations.

Table 1—Summary of first order regression statistics

Sample	Predicted a_w (30°C)	Regression equation	P[a]
BaCl$_2$	0.901	y=0.9257 − 0.00028x	p $<$ 0.001
KBr	0.802	y=0.8818 − 0.00092x	p $<$ 0.001
CoCl$_2$	0.627	y=0.7833 − 0.00181x	p $<$ 0.001
NaBr	0.562	y=0.6887 − 0.00144x	p $<$ 0.001
Cheese spread	0.952	y=0.9853 − 0.00035x	p $>$ 0.10
Preserves	0.826	y=0.8949 − 0.00080x	p $<$ 0.001
Frosting	0.793	y=0.9241 − 0.00149x	p $<$ 0.001
Fudge sauce	0.765	y=0.9663 − 0.00231x	p $<$ 0.001

[a] Probability that the equation does not fit the data

Table 2—Summary of second order regression statistics

Sample	Predicted a_w (30°C)	Regression equation	R^2[a]	S[b]	P_1[c]	P_2[d]
BaCl$_2$	0.901	y=0.7793 + 0.0036x − 0.000025x^2	42.6	0.0028	p $<$ 0.001	p $<$ 0.10
KBr	0.802	y=0.5799 + 0.0070x − 0.000051x^2	88.2	0.0027	p $<$ 0.001	p $<$ 0.001
CoCl$_2$	0.627	y=0.5461 + 0.0044x − 0.00040x^2	93.1	0.0039	p $<$ 0.001	p $<$ 0.05
NaBr	0.562	y=0.1515 + 0.0182x − 0.000091x^2	88.0	0.0044	p $<$ 0.001	p $<$ 0.001
Cheese spread	0.952	y=0.2752 + 0.0182x − 0.000120x^2	87.4	0.0023	p $<$ 0.001	p $<$ 0.001
Preserves	0.826	y=0.6914 + 0.0045x − 0.000034x^2	75.0	0.0040	p $<$ 0.005	p $>$ 0.10
Frosting	0.793	y=0.2131 + 0.0171x − 0.000121x^2	90.8	0.0044	p $<$ 0.001	p $<$ 0.01
Fudge sauce	0.765	y=0.2549 + 0.0163x − 0.000121x^2	94.3	0.0050	p $<$ 0.001	p $<$ 0.025

[a] Amount of variation in a_w accounted for by including temperature (x) in the equation
[b] Standard deviation of a_w
[c] Probability that the overall equation does not fit the data
[d] Probability that the addition of x^2 does not significantly improve the fit of the equation to the data

Table 3—Water activity determinations at 2°C above and below the calibration temperature of 25°C

Sample	23° C[a]	25° C[b]	27° C[a]
BaCl$_2$	0.907	0.906	0.897
KBr	0.818	0.814	0.805
CoCl$_2$	0.655	0.646	0.635
NaBr	0.582	0.582	0.562
Cheese spread	0.973	0.965	0.953
Preserves	0.843	0.835	0.830
Frosting	0.813	0.816	0.797
Fudge sauce	0.800	0.795	0.778

[a] Mean of two determinations
[b] Mean of ten determinations (salts) or four determinations (foods)

Table 4—Precision of water activity measurements[a]

Sample	20° C	25° C	30° C
BaCl$_2$	±0.002	±0.003	±0.003
KBr	±0.002	±0.003	±0.004
CoCl$_2$	±0.003	±0.006	±0.002
NaBr	±0.004	±0.007	±0.001
Cheese spread	±0.003	±0.001	±0.002
Preserves	±0.005	±0.002	±0.003
Frosting	±0.003	±0.005	±0.005
Fudge sauce	±0.006	±0.002	±0.005

[a] Standard deviations based on ten determinations for salts and four determinations for foods

Table 5—Accuracy of water activity determinations (% variation from literature values)

Salt	20° C	25° C	30° C
BaCl$_2$	−0.44	+0.67	+1.24
KBr	+0.12	+0.62	−0.12
CoCl$_2$	−1.64	−0.46	+1.29
NaBr	−0.51	+1.04	+0.36

deviation for each salt and food product as determined from data about the second order regression equations can be found in Table 2. These estimates of standard deviation range from approximately 0.002–0.005 and provide a more precise estimate of this parameter when a substantial portion of the variation due to temperature is accounted for.

The accuracy of the a_w determinations on salt slurries given as % deviation from literature values is shown in Table 5. These were less than 2% in all cases and usually less than 1%. The values obtained for precision and accuracy support the contention that a_w values should be reported to two rather than three decimal places (Labuza et al., 1976) for routine measurements based on low numbers of replicates.

In conclusion, the data indicate that temperature variation within the range of 20–30°C has an inverse effect on water activity for most salts and foods tested. The magnitude of the effect was greater as temperature increased. The greatest effect was observed with substrates of relatively lower a_w. Conversely, the effect was least noted on substrates of higher a_w, indicating that strict temperature control may not be as critical at this level.

The data presented indicate that the temperature range used for a_w measurement in the food industry could potentially result in inter-laboratory differences of approximately 0.01–0.05 a_w units if one laboratory uses 20°C and another uses 30°C. The a_w of fudge sauce measured at 25°C and 30°C differed by 0.04 a_w units and frosting a_w values differed by 0.03 a_w units. The importance of temperature control is further substantiated by inspection of
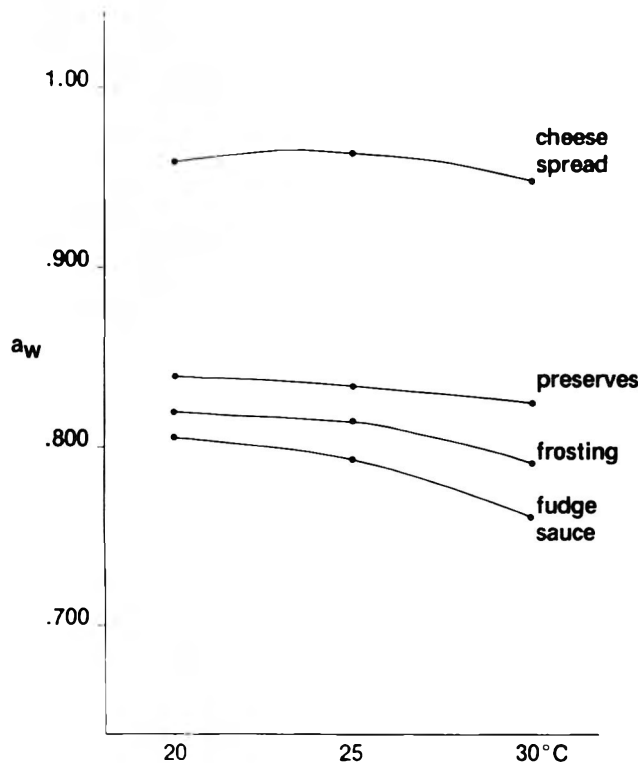
Fig. 2—Effect of temperature on the a_w of foods. Each point represents the mean of four determinations.

the coefficients of determination (R^2) listed in Table 2, which indicate that up to 94% of total variation in a_w readings on some products may be accounted for by including temperature in the regression equation. Thus temperature control is very important for inter-laboratory comparisons and when measuring a_w levels near critical values such as those set by federal regulations. The authors suggest 30°C as the standard temperature for a_w measurement because this temperature is easier to control and usually would not require an incubator with cooling capacity.

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Freezing Time Prediction for Slab Shape Foodstuffs by an Improved Analytical Method

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—————————————ABSTRACT—————————————

An improved analytical method for predicting the freezing time with one dimensional heat transfer for slabs was developed. Tylose-MH-1000 was used as a model test material. The new model is similar to Plank's equation, but has a more theoretical basis. Total enthalpy difference instead of latent heat and weighted average temperature difference instead of the temperature difference between initial freezing point and freezer temperature were used in the improved prediction method. Linear regression was used to estimate shape parameters. Four different foods were used to test the model. Predicted times for foods were within 6% of the measured times.

INTRODUCTION

REFRIGERATION has been an accepted method of food preservation since the turn of the century. For quality, processing and economic reasons, it is important to predict the freezing time for foods.

A number of models have been proposed to predict freezing time. However, when heat transfer involves phase change, most of the standard unsteady state solutions can not be used. Most analytical freezing time prediction techniques apply only to specific freezing conditions. Latent heat is removed over a wide temperature range, but many methods assume a unique freezing point. Thermal properties of the product vary significantly with temperature but few methods account for this variation. All of these factors make most models less reliable. Numerical techniques can easily overcome the problems mentioned above. However, finite difference methods have limitations for irregular shaped or nonhomogeneous products. Finite element methods are effective for irregular shapes and nonhomogeneous products and predictions are accurate, but the numerical methods require substantial computer facilities. The computations involved in numerical methods are too numerous for hand calculations even when solving very small problems. This paper focuses on analytical methods because of application ease.

The term "freezing time" has been defined several ways in the literature. The most adopted term is effective freezing time, the time required to lower the product temperature from its initial temperature to a given temperature at its thermal center (International Institute of Refrigeration, 1972). This time is related to the total process time for food in the freezer.

Many formulas available for predicting freezing time were reviewed by Hung and Thompson (1983), Kinder and Lamb (1974), and Bakal and Hayakawa (1973). Newmann (1860) obtained a solution for freezing or melting in the semi-infinite region bounded by a plane. This method assumes no thermal property changes with temperature except for phase change, the system has a distinctive freezing point and constant initial temperature and boundary condi-

tions. Charm (1971) suggested an extra thickness term to modify the constant temperature boundary condition. Plank (1913, 1941) presented a method for infinite slabs, cylinders and spheres. The initial sensible heat above the freezing temperature was not included so the predicted times are less than experimental results. In order to overcome this deficiency, some workers have proposed modifications or new emperical methods (Ede, 1949; Nagaoka et al., 1955; Eddie and Pearson, 1958; Lorentzen and Rosvik, 1959; Fleming, 1967; Cowell, 1967; Matsuda, 1971; Mellor, 1976). All of the above modified methods assume that the heat removal rate is uniform over the product surface and that the product has a regular shape. Teider (1963) presented a method for brick-shaped objects with varying heat transfer coefficients on different sides. Tanaka and Nishimota (1959, 1960, 1964) presented a method that can be used for conical, double conical or rectangular shaped foods. Schwartzberg (1977) presented a solution for freezing that occurs over a range of temperatures by using the first term of a Fourier Series solution and effective heat capacity as calculated by the Schwartzberg (1976) and Schwartzberg et al. (1977) methods. Dimensionless equations have been extensively used where theoretical mathematical analysis is difficult (Khatchaturov, 1958; Tchigeov, 1958; Mott, 1964; Tao, 1967; Riley et al., 1974). Cleland and Earle (1977; 1979a, b) used dimensionless variables to predict the shape factors in Plank's equation. Loeffen et al. (1981) used the mean bulk initial temperature to simulate nonuniform initial temperature.

The objective of this paper was to develop an improved analytical method for predicting freezing with one dimensional heat transfer in slab shaped foodstuffs. The method is applicable to most freezing processes, many food materials and the prediction accuracy competes with other methods. The input information for this method can be obtained from the literature or through calculations.

MATERIALS & METHODS

THE EXPERIMENTAL DATA were collected in a walk-in freezer (6.7m x 1.8m x 2.4m) at Michigan State University. Two evaporators maintained the room temperature at the design temperature (> -34°C) to ± 1.8°C (Chavarria, 1978). Convective boundary conditions were provided by a low speed wind tunnel in the freezer room. The wind tunnel had a length of 3.8m, a round cross-section diameter of 0.46m and a test section located near the center of the length. The blower provided mean air velocities between 2 and 18 m/sec. The air speed was monitored by pitot tube and micromanometer. The accuracy of the micromanometer was to ± 2.45 Pa. The pitot tube was positioned near the test sample. Test sample was supported by a flat plate with a sharp leading edge in the test section of the wind tunnel. The plate was made from Urethane board (k = 0.0187 w/mK) to insure one-dimensional heat transfer perpendicular to the air flow. Heat transfer through the sample support was evaluated experimentally and was less than 0.3% of the total heat transfer. The plate dimensions were 0.38m x 0.46m with a thickness dependent on sample thickness. The sample holder located at the center of the flat plate was 0.20m x 0.20m with the sample thickness specified in the experimental design. The walls and bottom of the sample holder were constructed of 1.6 mm plexiglass sheets to ease the food sample preparation.

Temperatures were measured with 30 gauge copper-constantan thermocouples located at the bottom of the sample. The lead wires

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were run parallel to the air flow direction. Three thermocouples were located at 5 cm intervals with the middle one located at the center of sample space. Two other thermocouple junctions monitored temperature at the surface of the test sample. A Kaye Instruments Digistrip II digital multipoint recorder was used to print temperature data. The recorder was sensitive to 0.1°C.

The unfrozen water content (Heldman, 1974) for any final center temperature was calculated by the freezing point depression equation (Moore, 1962).

Thermal activities are estimated by the Kopelman (1966) method for product temperature above freezing. Frozen product thermal conductivity values were calculated as suggested by the Heldman and Gorby (1975) modification of the Kopelman equation. This method first considers water the continuous phase for the water-ice system, then the water-ice system is considered as the continuous phase and products solids as the discontinuous phase.

The procedure suggested by Charm (1971) was used to calculate the specific heat based on product composition. The density was calculated from the mass to volume ratio. In this experiment, density was assumed to be independent of temperature.

The total heat removed during freezing was calculated by the enthalpy difference between the initial temperature and the final center temperature of the product. A single freezing temperature was used to simplify the calculations.

$$\Delta H = Cp_l (T_i - T_f) + Cp_s (T_f - T_t) + (M_a - M_n) L \qquad (1)$$

The temperature difference between product and freezing medium was calculated by the weighted average temperature difference, ΔT, for the cooling above and below freezing, and freezing sections of the process. The equation was derived from

$$\Delta T = \frac{(T_i - T_f)(Cp_l)(\frac{T_i + T_f}{2} - T_a) + (T_f - T_t) Cp_s (\frac{T_f + T_t}{2} - T_a)}{\Delta H}$$

$$+ \frac{(M_a - M_n) L (T_f - T_a)}{\Delta H} \qquad (2)$$

This reduces to

$$\Delta T = (T_f - T_a) + \frac{(T_i - T_f)^2 \frac{Cp_l}{2} - (T_f - T_t)^2 \frac{Cp_s}{2}}{\Delta H} \qquad (3)$$

An aluminum slab for surface convection coefficient measurement was located at the same place as the food sample. The size and shape of the aluminum slab was identical to the food samples so the coefficients were expected to be similar. The mean convective heat transfer coefficient at each particular mean air speed and specific ambient temperature was obtained by plotting a dimensionless temperature $(T - T_a)/(T_i - T_a)$ versus time on semi-log paper. The slope of this line was found by regression analysis, and the convective heat transfer coefficient was calculated as

$$h = - \frac{\rho \cdot V \cdot C_p \cdot slope}{A} \qquad (4)$$

The shape parameters, P and R, (Plank, 1941) written in dimensionless form are:

$$Fo = \frac{P}{U \cdot Ste \cdot Bi} + \frac{R}{U \cdot Ste} \qquad (5)$$

The dimensionless freezing time, Fo, is also dependent on external conditions and thermal properties of the freezing material, the relationship can be written as:

$$Fo = f (Bi, PK, Ste, U) \qquad (6)$$

Each set of test conditions (T_i, T_a, h, a) generates one group of dimensionless variables. An empirical relationship between these variables for all tests and the Fourier number, Fo, was found by linear regression, "Multreg Statistics Package" (Weisberg, 1979). Comparison of the regression equation and Eq (5) allowed the empirical terms to be partitioned into P and R equations by trial and error. The prediction model was then written similar to Plank's equation.

$$t_f = \frac{\rho \cdot \Delta H}{\Delta T} (P \frac{a}{h} + R \frac{a^2}{k_s}) \qquad (7)$$

"Karlsruhe test substance" is a test material for freezing studies and its enthalpy versus temperature data are very similar to lean beef (74% water) (Riedel, 1960). This test substance is a 23% gel made of methyl hydroxyethyl cellulose (Tylose-MH-1000) The American Hoeschst Corporation). The thermal properties of the Karlsruhe test substance were obtained from Cleland (1977). This test material was reused. It was stored in a closed environment between uses, and the water content measured before each use. If the water content varied more than ± 1% from the original 77%, a new sample was prepared.

Four foods were selected to test the model. The thermal property data were directly taken from the literature when available. Other properties were predicted by the methods mentioned before. Fresh top round lean beef from the Michigan State University meat laboratory was used, and all visible fat and connective tissues were removed. This meat was stored in a cooler at 4°C. Enthalpy/temperature data were obtained from Riedel (1960) and thermal conductivity data from Morely (1972). Ground beef was also obtained from Michigan State University meat laboratory and stored at 4°C. The heat removed and thermophysical properties were calculated by the previously mentioned prediction methods. One day old fresh mechanically deboned minced carp (Bibum, Type SDX13, meat separator) was used for the fish sample. It was taken from Saginaw Bay of Lake Huron. After deboning the carp was stored at 4°C. The enthalpy difference and thermophysical properties were calculated by the prediction methods. Commercial instant mashed potatoes were from the Pillsbury Company. They were mixed with water to form a 82% water content test sample. The thermal properties published by Cleland (1977) were used.

The water content was measured by using the air oven method of the AOAC (1980, 24.003). The fat content was determined by using the Goldfisch extraction method of the AOAC (1980, 24.005). All thermophysical property values used in this study are in Table 1. Further experimental information is in Hung (1982). Freezing time in this experiment was calculated from when the product was put into the freezer until the product center temperature attained −18°C.

Four variables were examined, according to a Hoke D₆ experimental design (Hoke, 1974) plus the center point was replicated four times. Twenty-three freezing experiments were selected to cover the range of conditions that are commonly used in food freezers. The initial temperature of the material to be frozen was varied from 2.9–30.9°C, the freezer temperature was varied from −19.9 to −32.4°C, the thickness of the test objective was varied

Table 1—Thermo-physical data for freezing test materials

	Karlsruhe test material	Lean beef	Mashed potato	Ground beef	Carp
Thermal conductivity above freezing (W/mK)	0.55	0.51	0.53	0.40[a]	0.70[b]
Thermal conductivity below freezing (W/mK)	1.65	1.55	1.90	1.62	1.72
Specific heat above freezing (J/m³K)	3.71 × 10⁶	3.65 × 10⁶	3.66 × 10⁶	3.38 × 10⁶	3.58 × 10⁶
Specific heat below freezing (J/m³K)	1.90 × 10⁶	1.90 × 10⁶	1.95 × 10⁶	2.20 × 10⁶	2.22 × 10⁶
Latent heat (J/m³)	209 × 10⁶	209 × 10⁶	235 × 10⁶	188 × 10⁶	218 × 10⁶
Initial freezing point (°C)	−0.6	−1.0	−0.6	−1.2[c]	−0.8[c]
Water content (%)[d]	77	62.67	71.85	74.80	82.84
Fat content (%)[d]	—	18.36	11.86	1.70	—
Density (kg/m³)[e]	—	1041.7	1035.5	1098.7	1089.3[f]

[a] Referenced from Sörenfors (1974).
[b] Referenced from Khatchaturov (1958).
[c] Experimental initial freezing point.
[d] These results are the average of 9 experimental data.
[e] These results are the average of 10 random sampling results.
[f] Referenced from Mott (1964).

from 1.0–4.8 cm, and the surface heat transfer coefficient from 9–110 W/m²K. Table 2 lists the measured test conditions, calculated dimensionless variables and freezing times.

RESULTS & DISCUSSION

AN EMPIRICAL EQUATION for predicting the Fourier number, Fo, as a function of the other dimensionless variables was determined by multiple linear regression (Weisberg, 1979). The following equation was chosen by the R^2 statistic and included a minimum number of terms.

$$Fo = -0.2656 - 15.43 \frac{1}{Bi \cdot U} + 0.2079 \frac{1}{Ste \cdot U} + 0.7306 \frac{1}{U \cdot Bi \cdot Ste}$$

$$- 1.083 \frac{PK}{U \cdot Bi \cdot Ste} + 0.01329 \frac{1}{U \cdot Bi^2} + 15.40 \frac{1}{Bi} \qquad (8)$$

$R^2 = 1.000$ degrees of freedom = 16

The partial relationship of P and R with the dimensionless terms in Eq. (7) is:

$$P = 0.7306 - 1.083 PK + Ste \left(15.40 U - 15.43 + 0.01329 \frac{Ste}{Bi}\right) \qquad (9)$$

$$R = 0.2079 - 0.2656 U \cdot Ste \qquad (10)$$

For verification, freezing times predicted by the improved model were compared with real food experimental data (Table 3). The lean beef experimental freezing times varied from 32 to 846 min. The percent difference between experimental and predicted freezing times were from −2.8% to +4.66%. The average absolute difference was 1.24%. For mashed potato the experimental freezing times varied from 18 to 811 min. The differences between experimental and predicted times were between −4.54% to +6.14%. The average absolute difference was 2.64%. For carp the experimental freezing times varied from 18 to 869 min. The freezing time differences were between −3.70% to +3.30%. Average absolute difference was 2.02%. For ground beef the experimental freezing times varied from 22 to 885 min. The freezing time differences were between −3.54% to +2.81%. The average absolute difference was 1.18%.

The Plank (1913), the Nagaoka et al. (1955), the Mott (1964) and the Cleland (1977) methods were selected to compare with the improved method at randomly selected conditions. The thermal properties in Table 1 were used unless other sources are mentioned. The test conditions and results are shown in Table 4. The Plank (1913) method does not include sensible heat. It under-predicted the experimental freezing times by 4.54 to 48.25 percent. The Nagaoka et al. (1955) method accounts for sensible heat but assumes all water is freezable. This assumption may be the reason that it predicted freezing times greater than the experimental data by 5.82–77.50%. The Mott (1964) dimensionless variable method predicted freezing times too low by 12.73–59.38%. Cleland (1977) provided an empirical method using total enthalpy difference, but the sensible heat above freezing was not included. The driving temperature gradient is assumed to be the difference between the initial freezing temperature and the freezer temperature. Although he claimed the accuracy of his method for an infinite slab is ± 5%, the test gave +8.35 to −24.79% variation with experimental data. This is not as accurate as the improved method (−3.54 to +6.14%).

The model was used to predict the sensitivity of freezing time to product property variation and measurement errors. The conditions selected for this were ground beef at an initial temperature of 17°C in a freezer at −25°C with convective heat transfer coefficient of 68 W/m²K. The sample was 3.0 cm thick.

Water volume increases when the phase changes from liquid to solid. This is the main reason for the food changing density and volume expansion during the freezing process. In ground beef the measured density changed from 1041.7 kg/m³ above freezing to 991.2 kg/m³ below freezing. The sample holder confined the sample to expansion in thickness only. The 3.0 cm thick sample increased 0.15 cm in thickness. This volume expansion increased the predicted freezing time by 6.3% (Fig. 1).

Thermal conductivity depends on product composition and temperature. Accurate values are difficult to obtain even by direct measurement. Also thermal conductivity changes during the process cannot be ignored by using the average thermal conductivity. Fig. 1 illustrates the predicted

Table 2—Experimental test conditions and results of Karlsruhe sample

Run #	Int. temp (°C)	Medium temp (°C)	Surface heat transfer coefficient (w/m² K)	Thickness (m)	Water content (%)	Exp. freezing time (min)	Fo	Bi	Pk	Ste	U
1	5.1	−19.9	9	0.013	76.0	184	56.73	0.0709	0.085	0.147	0.951
2	18.3	−25.0	11.5	0.028	76.5	302	20.07	0.195	0.234	0.154	1.051
3	2.9	−30.1	9	0.013	77.1	122	37.61	0.0709	0.0531	0.229	0.963
4	3.7	−30.1	11	0.048	77.3	374	8.458	0.32	0.064	0.225	0.965
5	29.6	−30.1	11	0.012	75.1	138	49.93	0.08	0.338	0.169	1.143
6	30.2	−20.4	11	0.047	76.1	791	18.66	0.313	0.340	0.112	1.222
7	3.5	−29.3	108	0.029	76.4	33	2.044	1.898	0.0595	0.213	0.965
8	26.3	−32.4	110	0.011	76.0	12.5	5.383	0.733	0.294	0.178	1.098
9	29.6	−29.3	108	0.045	76.3	87.5	2.251	2.945	0.318	0.155	1.139
10	16.8	−30.9	108	0.012	76.9	13.3	4.824	0.785	0.211	0.188	1.029
11	4.2	−29.4	108	0.048	76.9	73.5	1.662	3.142	0.0686	0.210	0.967
12	4.4	−25.7	106	0.011	77.3	12.7	5.454	0.707	0.0709	0.182	0.963
13	30.2	−20.3	104	0.010	76.7	20	10.42	0.63	0.319	0.105	1.210
14	4.5	−20.0	104	0.048	77.1	111	2.51	3.025	0.0724	0.141	0.952
15	4.4	−20.1	104	0.011	76.3	16	6.89	0.693	0.0717	0.144	0.952
16	3.8	−30.6	69	0.011	77.0	14.8	6.388	0.46	0.0632	0.221	0.967
17	18.0	−25.6	68	0.044	76.7	116	3.122	1.813	0.222	0.153	1.046
18	30.9	−24.9	68	0.027	76.4	74	5.289	1.113	0.327	0.129	1.179
19	17.4	−20.1	67	0.030	76.6	93	5.384	1.218	0.217	0.121	1.052
20	17.3	−25.0	68	0.027	76.6	61.8	4.420	1.113	0.215	0.151	1.041
21	17.3	−24.7	68	0.027	76.4	62	4.431	1.113	0.216	0.149	1.040
22	17.4	−25.2	68	0.027	76.5	60	4.288	1.113	0.217	0.152	1.041
23	17.9	−25.1	68	0.028	77.4	67	4.453	1.154	0.220	0.149	1.045

Table 3—Difference between experimental freezing times and predicted results for testing food materials

Run #	Int. temp (°C)	Freezer temp (°C)	Surface heat transfer coefficient (w/m²k)	Thickness (m)	Water content (%)	Fat content (%)	Exp. freezing time (min)	Pred. freezing time (min)	Time difference (%)
1A	30.8	−28.0	107	0.030	76.1	0.82	58.5	58.0	−0.85
2A	17.4	−25.2	106	0.048	77.0	0.52	108.3	108.5	+0.18
3A	4.8	−20.3	104	0.019	74.8	2.06	32.2	33.7	+4.66
4A	4.3	−27.6	69	0.047	76.2	0.66	104.8	104	−0.76
5A	29.8	−25.8	68	0.014	74.0	1.63	34.8	34.6	−0.57
6A	17.9	−20.8	67	0.036	73.5	2.90	118	114.7	−2.80
7A	17.2	−30.3	8.5	0.014	74.2	1.60	163.5	162.6	−0.55
8A	4.8	−25.2	8.5	0.033	73.7	2.57	390	391.5	+0.38
9A	30.8	−20.7	10.5	0.047	73.7	2.57	946	842.9	−0.37
1B	30.4	−30.9	108	0.028	82.2	−	44	46.7	+6.14
2B	16.8	−25.5	106	0.044	82.5	−	88	90.1	+2.39
3B	4.6	−20.0	104	0.010	83.4	−	17.6	16.8	−4.54
4B	4.0	−30.6	69	0.046	82.2	−	89.8	90.3	+0.55
5B	29.2	−26.0	68	0.010	82.5	−	23.0	24.2	+5.22
6B	17.5	−20.2	67	0.028	83.4	−	88.5	88.8	+0.34
7B	17.8	−29.6	8.5	0.010	82.2	−	130.8	128.4	−1.83
8B	4.0	−25.4	10.5	0.032	83.8	−	356.3	352.5	−1.07
9B	30.2	−20.5	10.5	0.045	83.4	−	811	822.6	+1.43
1C	31.3	−30.2	108	0.029	71.8	11.6	49	50.5	+3.06
2C	18.2	−23.9	105.5	0.046	69.6	15.3	103	104.3	+1.26
3C	5.3	−20.2	104	0.012	73.1	10.3	18.2	18.8	+3.30
4C	3.9	−28.7	68.5	0.048	71.8	11.6	99	96.3	−2.73
5C	29.9	−25.1	68	0.010	69.6	15.3	27	26	−3.70
6C	18.1	−20.1	67	0.030	72.0	11.6	96.5	95.5	−1.04
7C	17.4	−29.6	8.5	0.010	72.7	10.6	124.8	124.0	−0.64
8C	6.1	−25.0	8.5	0.030	73.1	10.3	362	364.8	+0.77
9C	30.4	−20.0	10.5	0.045	73.1	10.3	869	883.5	+1.67
1D	30.6	−27.9	107	0.027	63.1	17.7	48.5	48.5	+0.62
2D	17.6	−25.0	106	0.045	62.2	19.1	85.5	87.9	+2.81
3D	5.0	−20.1	104	0.015	63.6	17.8	21.8	21.6	−0.92
4D	3.9	−30.9	69	0.048	62.5	18.3	79	76.2	−3.54
5D	30.2	−25.4	68	0.012	62.2	19.1	30.4	30.3	−0.33
6D	17.8	−20.3	67	0.031	62.3	19.2	91.3	91.2	−0.11
7D	17.3	−30.1	8.5	0.011	63.6	16.1	124.5	122.6	−1.53
8D	4.3	−25.0	8.5	0.031	62.3	18.7	317	317.7	+0.22
9D	30.3	−19.0	10.5	0.045	62.3	19.2	885	890.2	+0.59

A. For lean beef
B. For mashed potato
C. For carp
D. For ground beef

influence of thermal conductivity (10% difference) on the freezing curve. A longer freezing time is obtained when using lower thermal conductivity values, but the effect on freezing time is small (2.71%).

Specific heat is also influenced by the product composition and temperature. The predicted influence of specific heat is shown in Figure 1. As expected, higher specific heat (10% difference) will increase the total enthalpy difference and that will lead to a longer freezing process (2.56%).

Not all the water in a product is frozen in the freezing process. The amount of unfrozen water not only affects the latent heat removed, but also affects the thermal properties. Fig. 1 compares 8.66% unfreezable water with no unfreezable water. The result is a 12.45% freezing time difference.

The initial freezing point is also an important parameter affecting the freezing curve. Fig. 1 shows the estimated influence of initial freezing points of −1.2% and −1.7°C. The product with a higher freezing point will have faster ice formation rate and a shorter freezing time (3.73%).

Some water (usually less than 2%) evaporates from the product during freezing (Long, 1955). The evaporation heat is contributed by the product, so the total enthalpy removed by the freezer is decreased. Most of this change takes place at the beginning of the process and should influence the cooling curves. No mass transfer is assumed by the model. With mass transfer, the cooling curve will move to the left (decreased time). The mass transfer rate is a function of air velocity, initial product temperature, product moisture and air humidity.

The surface heat transfer coefficient is a function of location and temperature (Chavarria, 1978). The experimental data were obtained from a smooth aluminum plate. The food sample surface is not this smooth. Thus even the measured experimental data are not truly representative.

From the above example results, the volume expansion and the unfreezable water content are the most significant parameters contributing to error in freezing time predictions.

CONCLUSIONS

ANALYTICAL METHODS to solve the freezing problem have advantages in some situations. Existing approximate analytical solutions or empirical relationships predicted freezing time with varying accuracy, and each had some specific limitations. The improved method reported here not only gives predictions that agree very closely with experimental data but also require approximate composition measurement for thermophysical properties prediction when literature data are unavailable.

Table 4—Predicted freezing time comparison

Product	Int. temp (°C)	Medium temp (°C)	Surface heat transfer coefficient (W/m²K)	Thickness (m)	Water content (%)	Freezing time (min)					
						Experimental	Developed method	Plank	Nagaoka	Mott	Cleland
Lean	17.2	−30.3	8.5	0.014	74.2	163.5	162.6	130.4	203.3	114.4	143.4
	4.3	−27.6	69	0.047	76.2	104.8	104.0	91.2	112.5	62.0	92.9
Beef	30.8	−28.0	107	0.030	76.1	58.5	58.0	36.8	70.6	28.3	44.0
Mashed	17.8	−29.6	8.5	0.010	82.2	130.8	128.4	102.5	157.3	93.2	117.8
	4.0	−30.6	69	0.046	82.2	89.8	90.3	78.7	95.5	53.9	82.1
Potato	30.4	−30.9	108	0.028	82.2	44	46.7	42.0	78.1	38.4	36.2
Carp	17.4	−29.6	8.5	0.010	72.7	124.8	124.0	86.9	140.4	79.0	130.9*
	3.9	−28.7	68.5	0.048	71.8	99	96.3	77.0	129.0	52.7	100.0*
	31.3	−30.2	108	0.029	71.8	49	50.5	27.6	56	21.6	47.2*
Ground	17.3	−30.1	8.5	0.011	63.6	124.5	122.6	84.0	139.7	75.7	129.8*
	3.9	−30.9	69	0.048	62.5	79	76.2	64.3	83.6	43.5	85.6*
Beef	30.6	−27.9	107	0.027	63.1	48.5	48.8	25.1	52.1	19.7	43.8*
Freezing time difference range (%)							−3.54 to +6.14	−48.25 to −4.54	+5.82 to +77.50	−59.38 to −12.73	−24.79 to +8.35
Average ǀdifferenceǀ							1.9	25.48	19.92	40.57	9.71

*Total enthalpy difference is calculated by Meller's (1976) method.
$$H = L + \tfrac{1}{2} Cp_l (T_i - T_f) + \tfrac{1}{2} Cp_s (T_f - T_a)$$

LINE NO. | LINE FORM | CONDITIONS

1 ——— STANDARD =
$T_i = 17°C$ $T_a = -25°C$ $T_f = -1.2°C$
$a = 3.0$ cm $k_s = 1.62$ W/mK $k_l = 0.4$ W/mK
$h = 68$ W/m²K $Cp_s = 2.2 \times 10^6$ J/m³K $Cp_l = 3.38 \times 10^6$ J/m³K
8.66 % WATER UNFROZEN

2 ——— STANDARD EXCEPT 0 % WATER UNFROZEN
3 ------- STANDARD EXCEPT $T_f = 1.7°C$
4 —·— STANDARD EXCEPT $a = 3.15$ cm
5 —··— STANDARD EXCEPT $k_s = 1.46$ W/mK $k_l = 0.36$ W/mK
6 ——— STANDARD EXCEPT $Cp_s = 2.42 \times 10^6$ J/m³K $Cp_l = 3.72 \times 10^6$ J/m³K
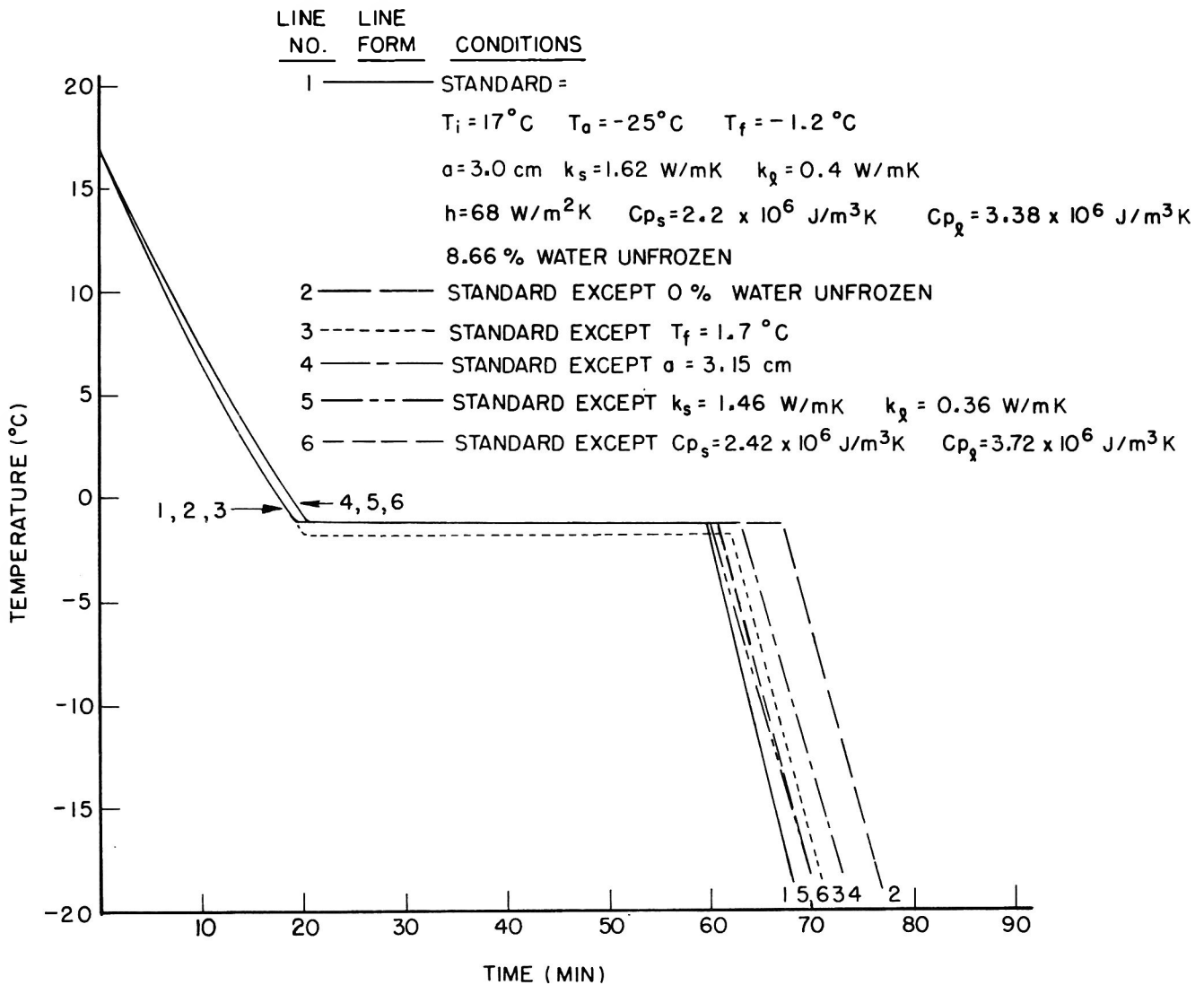
Fig. 1—Influence of property changes on the predicted freezing curve.

SYMBOLS

A   Surface area, (m^2)

a   Thickness, (m)

Bi   Biot number, ha/k, (dimensionless)

Cp   Specific heat, (J/kgK)

Cp_l Specific heat above freezing, (J/kgK)

Cp_s Specific heat below freezing (J/kgK)

Fo   Fourier number, $\alpha t/a^2$, (dimensionless)

ΔH Total enthalpy difference between initial and final temperature, (J/kg)

h   Surface heat transfer coefficient, $(W/m^2 K)$

k_s Thermal conductivity below freezing, (W/mK)

L   Latent heat, (J/kg)

M_a Mass fraction of water, (dimensionless)

M_n Mass fraction of unfrozen water, (dimensionless)

P   Constant for equations 4 and 8 (dimensionless)

PK   Plank number, $Cp_l(T_i - T_f)/\Delta H$, (dimensionless)

R   Constant for equations 4 and 9 (dimensionless)

Ste   Stefan number, $Cp_s(T_f - T_a)/\Delta H$, (dimensionless)

T_a Freezer temperature, $(^\circ C)$

T_f Initial freezing point, $(^\circ C)$

T_i Initial temperature, $(^\circ C)$

T_t Final center temperature, $(^\circ C)$

ΔT Weighted average temperature difference, $(^\circ C)$

t_f Freezing time, (s)

U   Dimensionless variable, $\Delta T/T_f - T_a$, (dimensionless)

V   Volume, (m^3)

α Thermal diffusivity, $K/\rho c_p$, (m^2/s)

ρ Density, (kg/m^3)

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Ms received 7/14/82; revised 11/12/82; accepted 11/15/82.

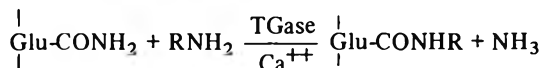# Crosslinking Between Different Food Proteins by Transglutaminase

MASAO MOTOKI and NORIKI NIO

ABSTRACT

Guinea pig liver transglutaminase has been found to catalyze the formation of inter- and intramolecular ϵ-(γ-glutamyl)lysyl crosslinks in proteins. In this paper we study the formation of polymers between different proteins by reacting them with transglutaminase. Analysis of reaction products between acetylated α_{s1}-casein and several proteins (α_{s1}-, κ-casein, β-lactoglobulin, soybean 11S and 7S globulin) by sodium dodecyl sulfate polyacrylamide gel electrophoresis and high-performance liquid chromatography indicated that the different proteins were polymerized through the formation of intermolecular crosslinks. This reaction may represent a procedure to improve the properties of food proteins.

INTRODUCTION

TRANSGLUTAMINASE (TGase) has been found to catalyze the incorporation of primary amines into certain proteins and polypeptides through a replacement reaction to yield modified protein and ammonia (Mycek et al., 1959; Folk and Chung, 1973). It has also been shown that the reaction proceeds via calcium-dependent acyltransfer between the γ-carboxyamide group of protein-bound glutaminyl residues and various primary amines (Folk and Finlayson, 1977). When protein-bound lysyl residues act as acyl receptors, inter- or intramolecular ϵ-(γ-glutamyl)lysyl crosslinks can be formed.

$$\underset{|}{\overset{|}{Glu}}\text{-}CONH_2 + RNH_2 \xrightarrow[Ca^{++}]{TGase} \underset{|}{\overset{|}{Glu}}\text{-}CONHR + NH_3$$

As requirements for food proteins of high quality increase, it will become more important to be able to modify their properties. One possibility is to use enzymatic modifications to improve functional properties and nutritional values. Whitaker (1977) suggested that it would be interesting to consider the possibility of crosslinks in proteins used to fabricate texturized products. Ikura et al. (1980) reported that transglutaminase could be used to polymerize bovine casein components through the formation of intermolecular crosslinks that do not affect their functional properties. It was further shown that soybean globulins could also be used as substrate for this reaction. Recently, Ikura et al. (1981) and Iwami et al. (1981) succeeded in introducing methionine into soybean globulins and lysine into wheat gluten. It is considered, therefore, that transglutaminase can also be utilized to improve the nutritional value of food proteins.

It is interesting to note that polymers created by the action of transglutaminase on protein mixtures could have different rheological properties and nutritional values. Thus, transglutaminase could be used to produce new food materials with unique properties. The present study examines the formation of products between acetylated α_{s1}-casein and various food proteins to prove the formation of polymers by crosslinks between different proteins.

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MATERIALS & METHODS

Transglutaminase

Transglutaminase was prepared from fresh guinea pig livers by the method of Connellan et al. (1971). The purified enzyme gave a single band on polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) and a single peak on high-performance liquid chromatography (HPLC). Eight hundred grams of guinea pig livers yielded a 12.3 ml solution with a specific activity of 12.7 units/mg and a protein concentration of 9.6 mg/mL. Enzyme activity was determined by the colorimetric hydroxamate procedure using N-carbobenzoxy-L-glutaminylglycine (Folk and Cole, 1966). Protein concentration was determined by the method of Lowry et al. (1951). The enzyme solution was stored at $-30°C$.

Protein substrates

α_{s1}- and κ-casein were prepared from fresh whole milk by the method of Zittle and Custer (1963). β-lactoglobulin was obtained from ICN Pharmaceutical, Inc. (Cleveland, OH). Crude 11S and 7S proteins were prepared from defatted soybean flour (Ajinomoto Co., Inc., Tokyo) by the method of Thanh et al. (1975). These were further purified on Sepharose 6B (Pharmacia Fine Chemicals) at 5°C.

Acetylation of α_{s1}-casein

Acetylation of α_{s1}-casein was performed by modification of the method of Franzen and Kinsella (1976). α_{s1}-Casein (500 mg) was dissolved in 6.6M urea (20 ml) and added to 2N Tris-HCl buffer (pH 7.6, 20 ml). Acetic anhydride was added in 80 μl increments up to a total of 1.5 ml over a 2-hr period while keeping the mixture stirred and at 0°C. During acetylation pH was maintained at 8.0 with 2N NaOH. After pH stabilization the solution was dialyzed against water (3°C, 24 hr) to remove impurities and excess of reagents. Acetylated α_{s1}-casein (480 mg) was recovered by lyophilization. It was found that by this procedure approximately 100% of the amino groups had been acetylated. The degree of acetylation was determined by the ninhydrin assay (Paik and Kim, 1972).

Crosslinking of Proteins

The protein crosslinking reaction was performed by a slight modification of the method of Ikura et al. (1980). Typically, a protein solution (5 mg/ml) was prepared in a reaction buffer containing 0.1M Tris-HCl, pH 7.6, 5mM $CaCl_2$ and 10mM dithiothreitol. Transglutaminase (90 μg/ml) was added to this solution. Experiments were performed at 37°C and 0.5 ml aliquots were taken at adequate intervals and added to 0.4M EDTA (pH 8.0, 50 μl) at 0°C. Reaction products were analyzed by SDS-PAGE and HPLC.

SDS-polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis in the presence of SDS was performed on slab gels. Gels containing 14% acrylamide were prepared from a stock solution of 30% by weight of acrylamide and 1.5% by weight N,N-bis-methyleneacrylamide. The final concentrations in the gel were as follows: 0.37M Tris-HCl (pH 8.8) and 0.1% SDS. The gels were polymerized chemically by the addition of 0.025% by volume of tetramethylenediamine and 200 mg ammonium persulfate to 119.8 ml of gel solution. Protein solutions were prepared for electrophoresis by mixing samples with an equal volume of 50% glycerol, 2% SDS, 5% 2-mercaptoethanol in 0.02M phosphate buffer (pH 7.2). Following 2 min incubation at 100°C, 0.4 ml of denatured sample were mixed with 0.1 ml of 0.05% bromphenol blue. The gel electrophoresis was done at a constant voltage (125 V for 2 hr). The gels were electrophoretically stained with 0.1% Coomassie Brilliant Blue R-250 in methanol:acetic acid:

H$_2$O (5 : 2 : 13, V/V/V). They were electrophoretically destained in methanol:acetic acid:H$_2$O (3 : 1 : 6, V/V/V).

High performance liquid chromatography (HPLC) of crosslinked proteins

The analysis by HPLC was performed on an Hitachi Model 635A Liquid Chromatograph. The reaction mixture (10 μl) was injected onto a TSK-GEL G3000SW Column (600mm × 7.5mm i.d., Toyo Soda Manufacturing Co., Ltd., Tokyo). Solvent flow was adjusted to 1 ml per min. The mobile phase was 0.1M phosphate buffer (pH 7.0). The detector monitored absorbance at 280nm.

Chemicals

N-Carbobenzoxy-L-glutaminyglycine was obtained from Kokusan Chemical Works (Tokyo). Coomassie Brilliant Blue was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo). Other reagents were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo).

RESULTS & DISCUSSION

Crosslinking of various proteins

Reaction products for various proteins were analyzed by SDS polyacrylamide gel electrophoresis and HPLC to detect the presence of polymers formed by intermolecular crosslinks catalyzed by transglutaminase. Since the reaction mixture included a reducing reagent (20 mM dithiothreitol) and the electrophoresis procedure included 2-mercaptoethanol, polymers detected could not have been formed by disulfide bonds. Fig. 1 shows that as the reaction proceeds the monomer fractions diminished or disappeared while the polymer fraction increased. This was the case for α_{s1}-casein (Fig. 1A), κ-casein (Fig. 1B) and β-lactoglobulin (Fig. 1C). The acidic subunits of 11S globulin almost disappeared while polymer content increased, although the basic subunits could still be detected after 120 min (Fig. 1D). The three subunits of 7S globulin also diminished while polymer content increased (Fig. 1E). In addition, the band of transglutaminase also diminshed in all cases. Therefore, the polymer bands may contain the polymerized transglutaminase (Jeleńska et al., 1980). In general, these are identical to those of Ikura et al. (1980), except for β-lactoglobulin which was not studied by them.

HPLC analysis confirmed the presence for polymer fraction as shown by the marked increase for the peak at void volume with increasing time of incubation (Fig. 2). κ-Casein apparently is polymerized by intermolecular disulfide linkage under the reaction condition (Brunner, 1977). This is shown by the peak at 0 min on HPLC (Fig. 2B) and the band having the high mobility on SDS-PAGE which was analyzed under the high reductive condition.

Reaction with acetylated α_{s1}-casein

Treatment of α_{s1}-casein with acetic anhydride acetylated approximately 100% of available amino groups. When acetylated α_{s1}-casein was incubated with transglutaminase,

Fig. 1—Confirmation of the polymerization reaction catalyzed by transglutaminase. Reaction products analyzed by SDS-polyacrylamide gel electrophoresis. See "Materials & Methods" for experimental details. Samples were taken at 0, 60, 120, and 180 min and corresponding gels were labeled a, b, c and d. Proteins studied were: (A) α_{s1}-casein (α_{s1}); (B) κ-casein (κ); (C) β-lactoglobulin (β); (D) 11S globulin (11S; AS and BS indicate the acidic and basic subunits, respectively); (E) 7S globulin (7S; α,α' and β indicate the three subunits). TG=transglutaminase; P=polymers.
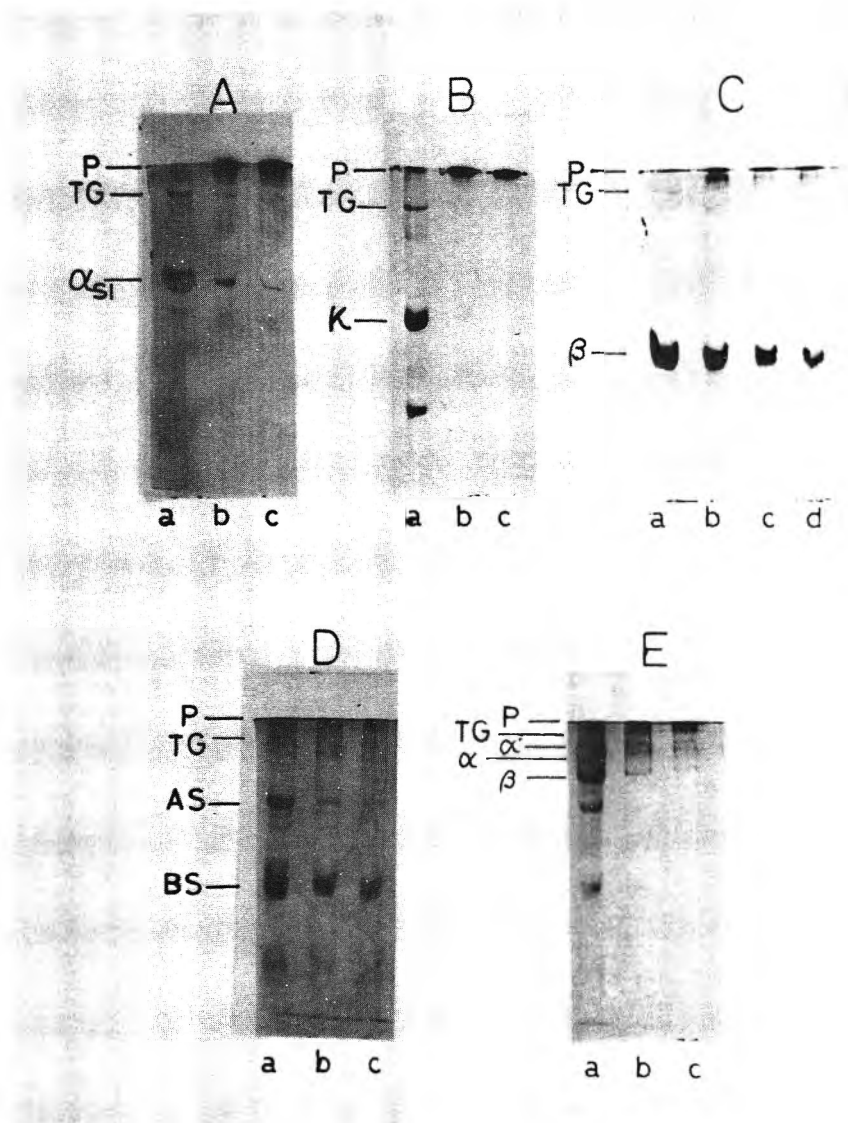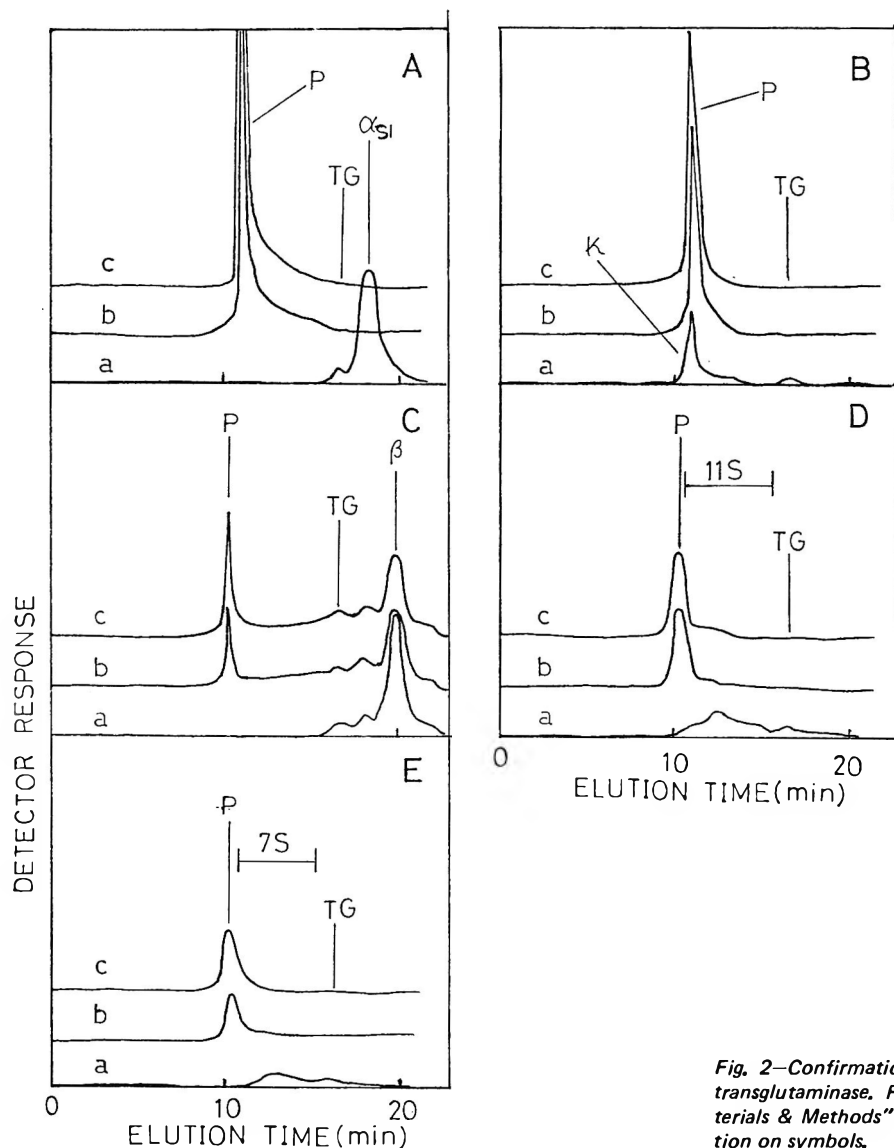
Fig. 2—Confirmation of the polymerization reaction catalyzed by transglutaminase. Reaction products analyzed by HPLC. See "Materials & Methods" for experimental details. See Fig. 1 for explanation on symbols.
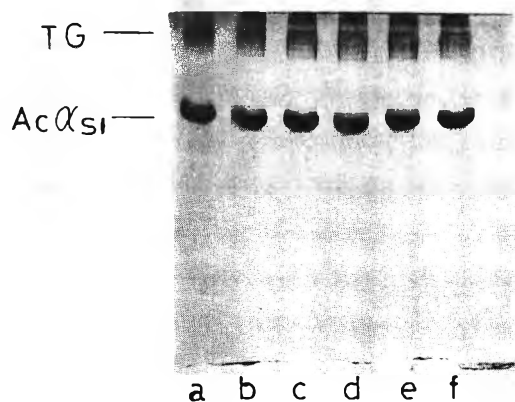
Fig. 3—SDS-polyacrylamide gel electrophoresis for the reaction with acetylated α_{s1}-casein (Acα_{s1}). See "Materials & Methods" for experimental details. Results on gels a thru f are for samples incubated ro 0, 10, 20, 30, 60 and 120 min, respectively. TG=transglutaminase.
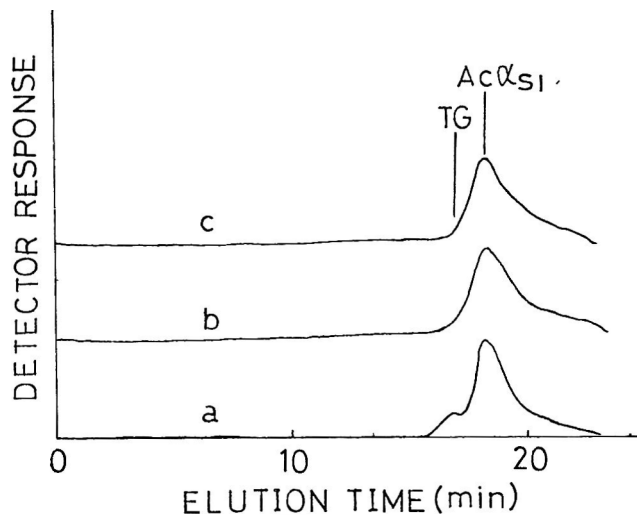
Fig. 4—HPLC analysis for the reaction with acetylated α_{s1}-casein (Acα_{s1}). See "Materials & Methods" for experimental details. Results shown on curves a, b and c are for samples incubated for 0, 60 and 120 min, respectively. TG=transglutaminase.

the monomer fraction did not decrease and no polymer formation was observed as shown by electrophoresis and HPLC (Fig. 3 and 4). This shows that 100% acetylated α_{s1}-casein is not a substrate for transglutaminase. The reaction sites, the ϵ-amino groups of lysine residues, have been blocked by their acetylation.
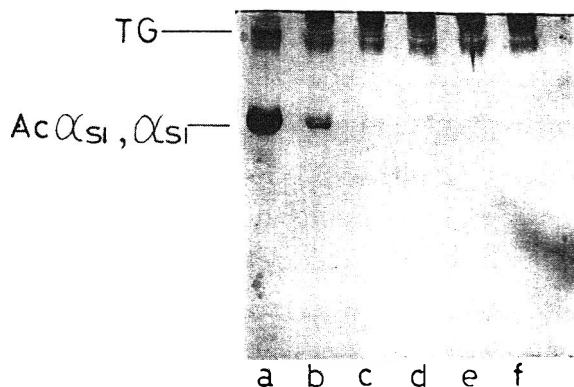
Fig. 5—SDS-polyacrylamide electrophoresis for the reaction with a mixture of acetylated (Acα_{s1}) and native α_{s1}-casein (α_{s1}). See "Materials & Methods" for experimental details. See Fig. 3 for explanation on symbols.
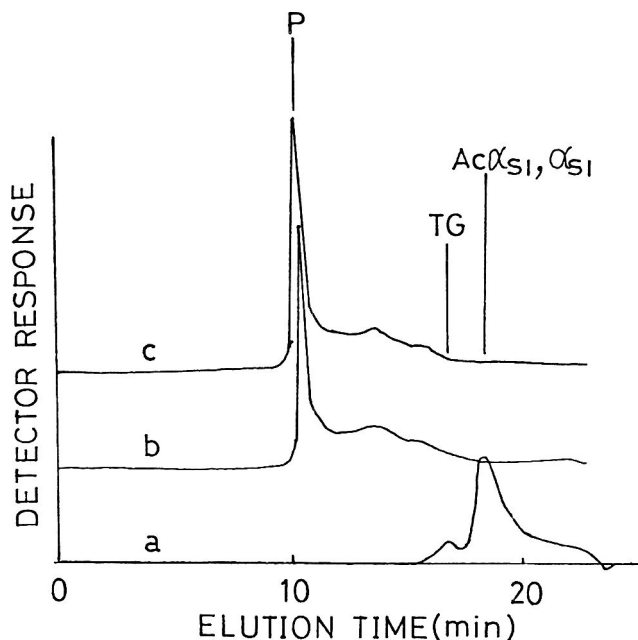
Fig. 6—HPLC analysis for the reaction with a mixture of acetylated (Acα_{s1}) and native α_{s1}-casein (α_{s1}). See "Materials & Methods" for experimental details. See Fig. 4 for explanation on symbols.
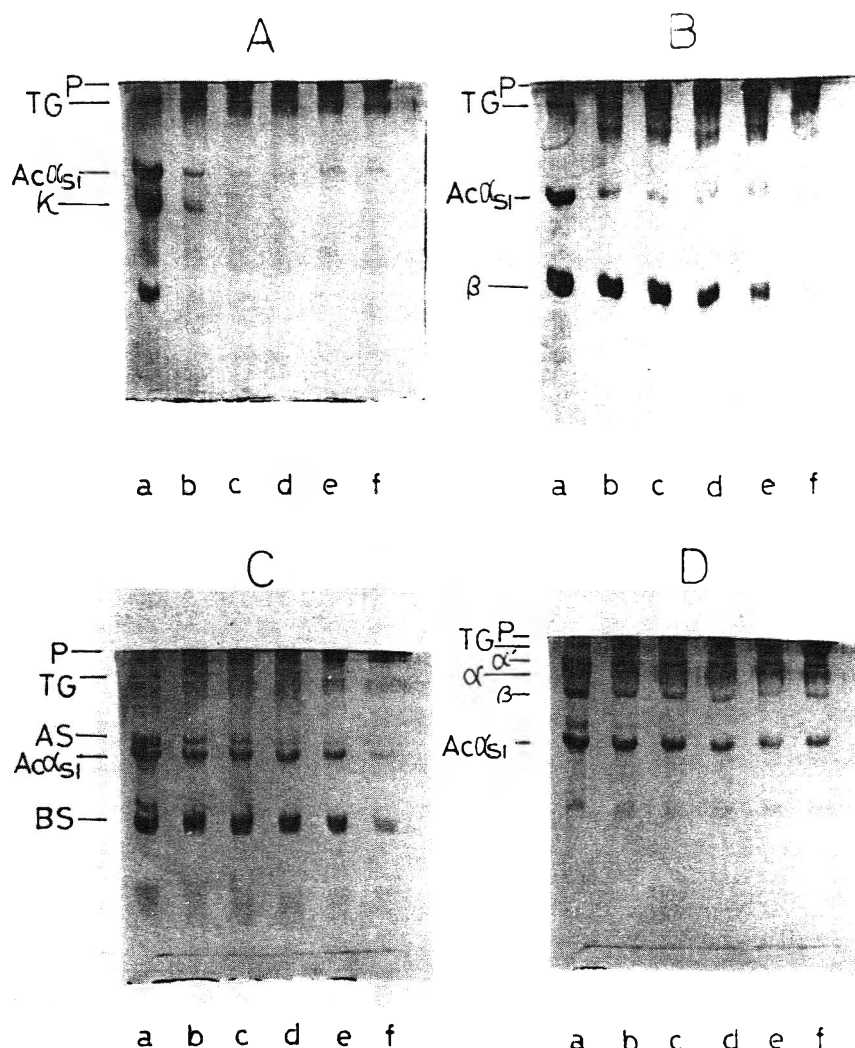
Fig. 7—SDS-PAGE analysis for the crosslinking reaction between acetylated α_{s1}-casein (Acα_{s1}) and various food proteins. 20 mg of each protein mixture (Acα_{s1}:food protein = 1:1) were dissolved in 4 ml reaction buffer and incubated with 80 μl transglutaminase solution. Results on gels a thru f are for samples incubated for 0, 10, 30, 60, 120 and 180 min, respectively. Food proteins studied were: (A) κ-casein (κ); (B) β-lactoglobulin (β); (C) 11S globulin (11S; AS and BS indicate the acidic and basic subunits, respectively); (D) 7S globulin (7S; α, α' and β indicate the three subunits). TG=transglutaminase; P=polymers.

Reaction with a mixture of acetylated and native α_{s1}-Casein

When the reaction buffer included an equivalent weight of acetylated and native α_{s1}-casein there was a progressive disappearance of the monomer fraction while a polymer fraction was formed. This was shown by SDS-PAGE and HPLC (Fig. 5 and 6). This indicates that acetylated α_{s1}-casein can participate in the polymerization reaction with native α_{s1}-casein. It should be noted that both acetylated and native α_{s1}-casein had the same R_f and retention time values.

Crosslinking between different food proteins

The transglutaminase reaction between acetylated α_{s1}-casein and various food proteins was examined next. After incubation of κ-casein, β-lactoglobulin, soybean 11S and 7S globulin with acetylated α_{s1}-casein in a 1:1 ratio, electrophoresis and HPLC showed that the monomer fractions disappeared and polymers were produced (Fig. 7 and 8). When a 1:3 ratio was used, reduction in acetylated α_{s1}-casein fraction and polymer formation were still detected by HPLC (Fig. 9). We had already shown (see Fig. 5 and 6) that similar results were obtained when acetylated and native α_{s1}-casein were incubated with transglutaminase. On the contrary no polymers were detected (see Fig. 3 and 4) when only acetylated α_{s1}-casein was included in the reaction mixture.

These findings show that transglutaminase catalyzes the formation of homologous and heterologous polymers by ϵ-(γ-glutamyl)lysyl crosslinks between the glutamyl residues of acetylated α_{s1}-casein and the lysyl residues of the various native proteins considered in this study.

CONCLUSION

THIS STUDY PROVES that crosslinks between acetylated α_{s1}-casein and various food proteins were catalyzed by transglutaminase. We have also confirmed the formation of polymers when a single, native protein was incubated with transglutaminase. Therefore, this enzyme catalyzes the formation of homologous and heterologous polymers between different food proteins.

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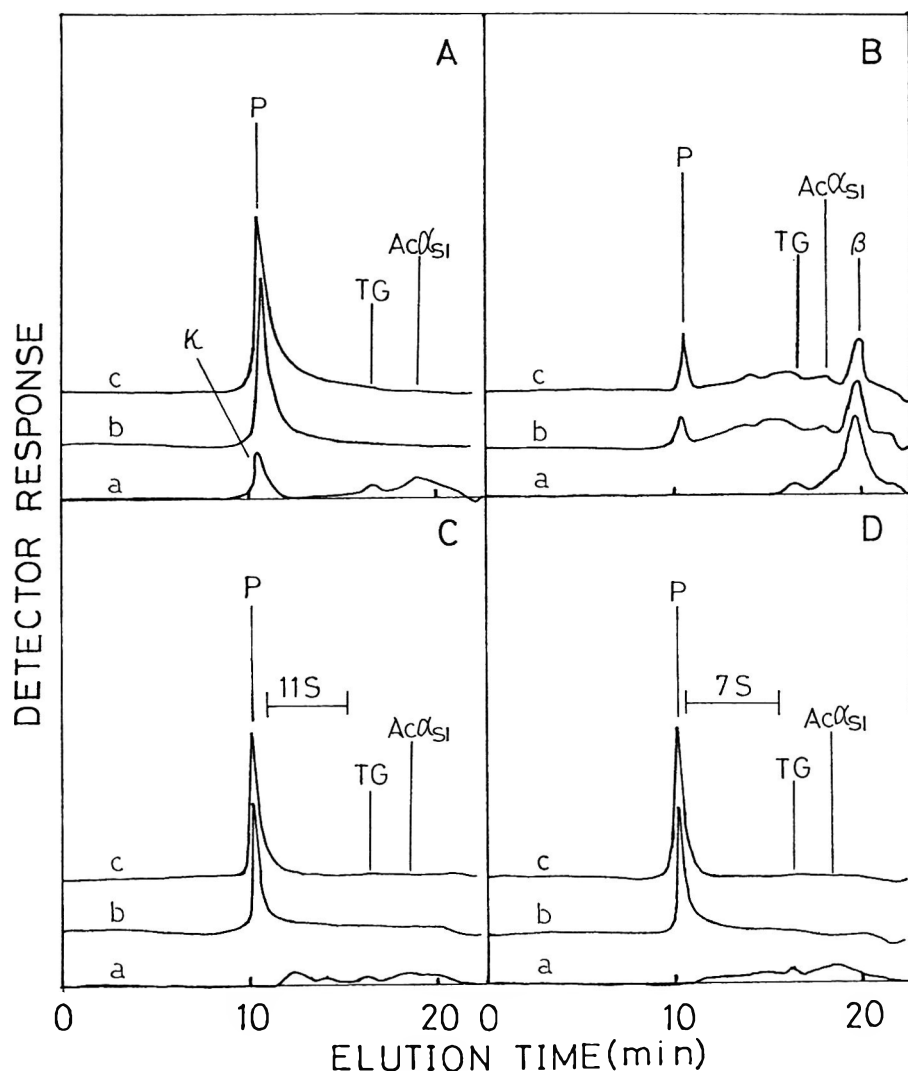
Fig. 8—HPLC analysis of the crosslinking reaction between acetylated α_{s1}-casein (Acα_{s1}) and various food proteins. See Fig. 7 for experimental details and explanation on symbols. Results shown on curves a, b and c are for samples incubated for 0, 60 and 120 min, respectively.
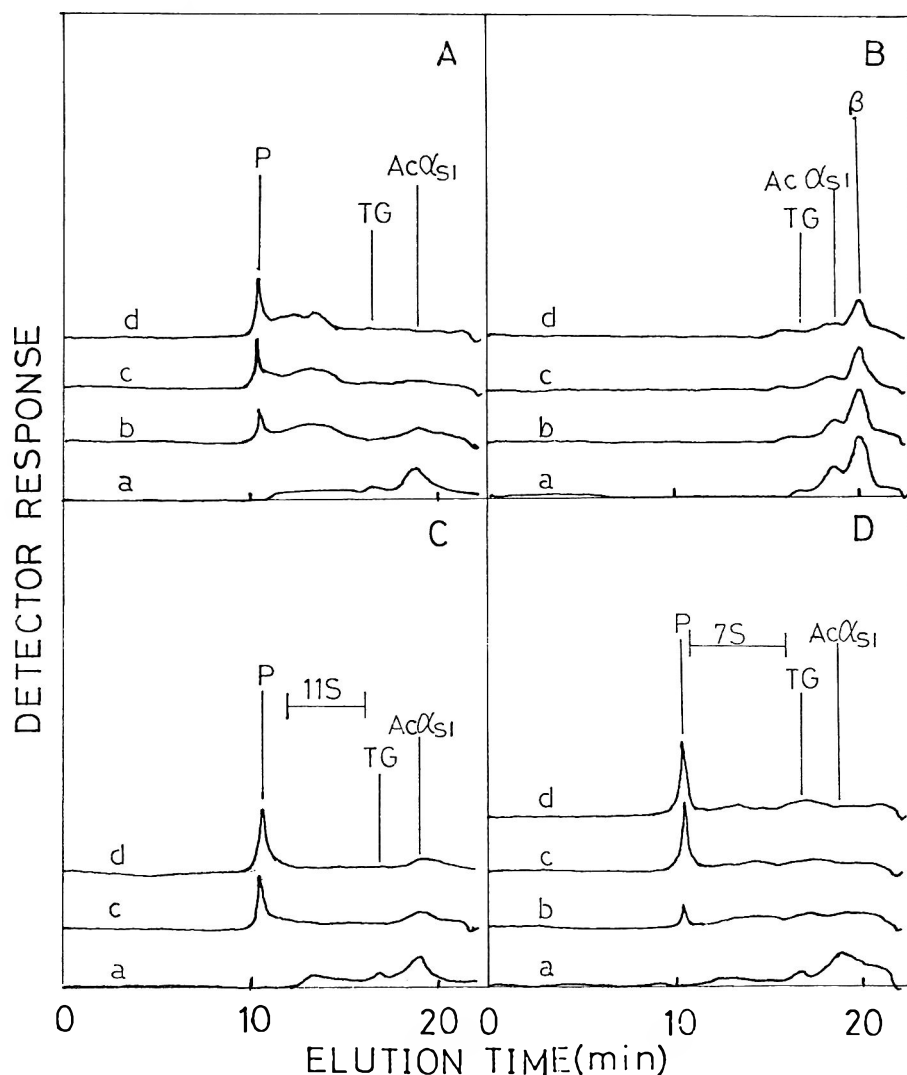
Fig. 9—HPLC analysis for the crosslinking reaction at a higher acetylated α_{s1}-casein (Acα_{s1}): food protein ratio (=1:3). 40 mg protein mixture were dissolved in the reaction buffer (5 ml) and indicated with transglutaminase (100 μl). Results shown on curves a thru d are for samples incubated for 0, 10, 60 and 120 min, respectively. See Fig. 7 for explanation of symbols.

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Zinc, Iron and Copper Availability as Affected by Orthophosphates, Polyphosphates and Calcium

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―――――――――ABSTRACT―――――――――

Male weanling rats were fed an adequate diet supplemented with 0.42% P from either hexametaphosphate or orthophosphate, each at two levels of Ca intake (0.53% or 1.06%). Polyphosphates represented 13% of the total fecal phosphorus at the 0.53% Ca intake; this fraction was increased to 27% at the 1.06% Ca intake. Hexametaphosphate caused a 15% increase in fecal iron, a 12% decrease in liver iron and a 15% decrease in fecal zinc losses. These effects were markedly enhanced by supplemental Ca. Orthophosphate was without effect at the 0.53% Ca intake, but caused an 11% increase in fecal zinc losses at the 1.06% Ca intake.

INTRODUCTION

SIMPLE (ortho) and condensed (poly) phosphates have a broad range of functionalities (e.g. buffer, acidulant, sequestrant, dispersant, and emulsifier) in food systems (Ellinger, 1972). As a consequence, calculations indicate that their use increases the phosphorus content of ordinary American diets by 250−1000 mg per day (i.e. 25−100%) or more. Greger and Krystofiak (1982) have estimated that 20−30% of the phosphorus in the average adult's diet is derived from phosphorus-containing additives, although some individuals were shown to ingest a substantially greater quantity of these additives.

Polyphosphates accounted for 17% of the phosphorus contained in food-grade additives produced in 1973 (Alexander Grant & Co., 1975 as cited by Greger and Krystofiak, 1982). Polyphosphates are highly charged anions which act as polyelectrolytes; this characteristic is responsible for many of their food applications, including their ability to sequester metals.

Orthophosphates have been reported to decrease zinc (Cabell and Earle, 1965) and iron (Hegsted et al., 1949; Mahoney and Hendricks, 1978) absorption and utilization in animals. Furthermore, this effect is exacerbated by a high level of calcium intake (Cabell and Earle, 1965). Pond et al. (1978) found that simultaneously increasing the levels of calcium and phosphorus in the diets of growing pigs caused a decrease in the iron concentration of the radius-ulna; however, phosphorus alone was without effect. Similarly, data from human studies indicate that orthophosphates depress iron abosrption only when calcium intake is concommitantly elevated (Monsen and Cook, 1976; Snedeker et al., 1982).

It is likely that polyphosphates, if left unhydrolyzed in the gastrointestinal tract, will interfere with the absorption of several metals to a greater extent than orthophosphates, since polyphosphates have a much greater affinity for most metals than do orthophosphates. Mahoney and Hendricks (1978) compared the effects of orthophosphate, metaphosphate, pyrophosphate and tripolyphosphate on calcium and iron metabolism in growing and mature rats. All four phosphate salts depressed calcium absorption to about the same degree in growing animals, but only tripolyphosphate

caused consistent decreases in bone calcium content. In addition, all four phosphate salts caused decreases in iron absorption, hemoglobin concentration and total liver iron content; pyrophosphate and tripolyphosphate caused the largest decreases in iron utilization.

Ivey and Shaver (1977) demonstrated enzymatic hydrolysis of polyphosphates, apparently mediated by a non-specific alkaline phosphatase, in in vitro intestinal preparations from rats and pigs; however, hydrolysis of polyphosphates with a chain length of five or greater was slow. Zemel and Linkswiler (1981) reported that hexametaphosphate was substantially (80.5 ± 5%) hydrolyzed to orthophosphate when calcium intake was low (399 mg/day). However, polyphosphate hydrolysis might be expected to be reduced at higher levels of calcium intake due to the formation of a stabilized calcium-polyphosphate complex resistant to phosphatase attack. Analagous effects of calcium on the hydrolysis of phytic acid by yeast (Zemel and Shelef, 1982) and by rat intestinal microflora (Wise and Gilburt, 1982) have recently been demonstrated.

The objectives of the present study were to (1) compare the effects of ortho- and polyphosphates on zinc, iron and copper absorption in growing rats; and (b) to determine whether a two-fold increase in calcium intake will results in a decrease in polyphosphate hydrolysis and thereby enhance the effects of polyphosphates on zinc, iron and copper absorption.

MATERIALS & METHODS

THIRTY MALE, weanling Sprague-Dawley derived (Spartan) rats were divided into five groups of six each and assigned to one of the following dietary treatments for four weeks: (1) a basal diet containing 0.53% Ca and 0.42% P (AIN-76 diet, ICN Nutritional Biochemicals, Inc. Cleveland, OH); (2) high polyphosphate (0.53% Ca, 0.84% P; basal supplemented with 0.42% P from hexametaphosphate); (3) high orthophosphate (0.53% Ca, 0.84% P; basal supplemented with 0.42% P from orthophosphate); (4) high calcium, high polyphosphate (1.06% Ca, 0.84% P; basal supplemented with 0.53% Ca from calcium gluconate and 0.42% P from hexametaphosphate); (5) high calcium-high orthophosphate (1.06% Ca, 0.84% P; basal supplemented with 0.53% Ca from calcium gluconate and 0.42% P from orthophosphate).

The polyphosphate supplement was Graham's salt (sodium hexametaphosphate), and the orthophosphate supplement was provided as an equimolar mix of sodium mono- and dibasic orthophosphates.

All animals were kept in stainless steel metabolic cages designed for quantitative separation of urine and feces. Diet and deionized glass distilled water were available ad libitum throughout the study. Complete fecal collections were made throughout the study and separated into weekly composites. Upon completion of the study, all animals were sacrificed in a CO_2 chamber and their livers were immediately removed.

Aliquots of all weekly fecal composites, liver samples and the five diets were ashed in a muffle furnace at 450°C for approximately 12 hr; the ashed samples were then dissolved in concentrated nitric acid, heated at 100°C for 1 hr, re-dissolved in concentrated hydrochloric acid and diluted for analysis. All samples were analyzed for zinc, copper and iron using atomic absorption spectrophotometry; diet samples were analyzed for calcium and phosphorus as well, using atomic absorption spectrophotometry and the technique of Fiske and Subbarow (1925), respectively.

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The distribution of the various phosphate species in the feces was determined using thin-layer chromatography (Rossel, 1963). Dried fecal samples were diluted 1:1 (w/w) with distilled water and homogenized with a vortex mixer; 20 mg aliquots of the homogenates were then applied to the plates. Following development, the spots were scraped from the plates and then eluted, hydrolyzed and analyzed for phosphorus as described by Karl-Kroupa (1956). Complete resolution of all polyphosphate spots was not readily obtained by this method; consequently, all of the polyphosphate spots (i.e. Graham's salt and its polyphosphate hydrolysis products) were combined to yield a single polyphosphate value.

All data were analyzed using analysis of variance and the least significant difference (LSD) test to separate means at the 5% alpha level.

RESULTS

POLYPHOSPHATES represented 13% of the total fecal phosphorus from the animals consuming the 0.53% Ca, high polyphosphate diet. However, this fraction was significantly (p < 0.01) increased to 27% in the feces from the animals consuming the high (1.06%) Ca, high polyphosphate diet.

The addition of hexametaphosphate to the basal diet caused a 15% increase in fecal iron losses which resulted in a decrease in the apparent absorption of iron, from 64.2% from the basal diet to 45.3% from the low calcium-high polyphosphate diet (Table 1). In addition, total liver iron was reduced by 12% in animals fed the low calcium-high polyphosphate diet when compared to those fed the basal diet (Table 2). Supplementing the basal diet with both calcium and hexametaphosphate (i.e. the high calcium-high polyphosphate diet) caused a 54% increase in fecal iron losses; this increase in fecal iron was not reflected in a further decrease in apparent absorption of iron when compared to the low calcium-high polyphosphate group;

the reason for this discrepancy was a slight, but not statistically significant, increase in food intake in the high calcium-high polyphosphate group (Table 3). In contrast to the effects of hexametaphosphate, the orthophosphate supplement had little effect on fecal or liver iron at either level of calcium intake.

Supplementing the basal diet with hexametaphosphate alone did not affect fecal or liver copper. However, the addition of both calcium and hexametaphosphate caused a 17% increase in fecal copper, thereby reducing copper absorption from 49.2% from the basal diet to 31.0% from the high calcium-high polyphosphate diet (Table 4). As with iron, the orthophosphate supplement had no effect on copper absorption or liver copper at either level of calcium intake.

The addition of hexametaphosphate alone to the basal diet caused a significant decrease in fecal zinc (Table 5). Apparent absorption of zinc from the basal diet was 26.8% and was increased to 30.1% from the low calcium-high polyphosphate diet; this increase was not statistically significant. Simultaneously supplementing the diet with both calcium and hexametaphosphate had a much more pronounced effect, causing apparent absorption of zinc to increase to 42.2%. In contrast, the orthophosphate supplement had no effect on zinc absorption, and the simultaneous addition of calcium and orthophosphate to the basal diet caused an 11% increase in fecal zinc losses, thereby reducing apparent absorption of zinc to 23.6%.

There were no statistically significant differences in growth rate or total food consumption among the groups (Table 3). However, the low calcium-high polyphosphate group had the lowest growth rate and total food consumption; both decreases were proportional to one-another and,

Table 1—Effects of level and form of phsophate and level of calcium intake on iron absorption[a,b]

Diet	Intake (μg/day)	Fecal excretion (μg/day)	Apparent absorption (μg/day)	Apparent absorption (%)
Basal	1196.4 ± 88.2a	427.9 ± 28.6a	768.5 ± 60.7a	64.2
High polyphosphate	1071.0 ± 58.6a	585.7 ± 34.3b	485.4 ± 50.0b	45.3
High orthophosphate	1196.1 ± 98.2a	418.9 ± 18.6a	777.2 ± 83.2a	64.6
High Ca-high polyphosphate	1232.1 ± 85.7a	658.2 ± 17.5c	573.9 ± 72.1c	46.6
High Ca-high orthophosphate	1276.8 ± 87.9a	553.2 ± 22.5a	723.6 ± 84.2a	56.7

[a] In each column, nonmatching superscripts denote significant (p < 0.05) differences.
[b] Data are expressed as mean ± standard deviation.

Table 2—Liver iron, zinc and copper as affected by level and form of phosphate and level of calcium intake[a,b]

Diet	Liver iron (μg)	Liver copper (μg)	Liver zinc (μg)
Basal	1427.4 ± 65.3a	76.6 ± 4.3a	275.0 ± 13.6a
High polyphosphate	1215.7 ± 52.5b	75.4 ± 5.1a	268.7 ± 12.5a
High orthophosphate	1338.4 ± 53.0a	102.2 ± 12.2a	270.3 ± 14.9a
High Ca-high polyphosphate	1169.2 ± 61.8b	109.3 ± 12.9a	263.2 ± 13.8a
High Ca-high orthophosphate	1285.1 ± 139.0a	82.7 ± 5.2a	244.1 ± 18.2a

[a] In each column, nonmatching superscripts denote significant differences.
[b] Data are expressed as mean ± standard deviation.

Table 3—Body weight gain, total food consumption and gain:feed ratio for the 28-day period[a,b]

Diet	Body weight gain (g)	Total food consumption (g)	Gain: feed ratio
Basal	215.7 ± 25.3a	447.2 ± 32.9a	0.482a
High polyphosphate	191.0 ± 17.4a	400.0 ± 21.8a	0.477a
High orthophosphate	211.3 ± 21.5a	446.5 ± 21.8a	0.473a
High Ca-high polyphosphate	215.8 ± 21.1a	460.0 ± 31.7a	0.469a
High Ca-high orthophosphate	215.4 ± 21.2a	476.7 ± 70.1a	0.451a

[a] In each column, nonmatching superscripts denote significant differences.
[b] Data are expressed as mean ± standard deviation.

Table 4—Copper Intake, fecal excretion and apparent absorption as affected by level and form of phosphate and level of calcium intake[a,b]

Diet	Intake (μg/day)	Fecal excretion (μg/day)	Apparent absorption (μg/day)	Apparent absorption (%)
Basal	103.8 ± 7.6a	52.7 ± 3.4a	51.1 ± 4.6a	49.2
High polyphosphate	92.9 ± 5.1a	47.5 ± 3.7a	45.4 ± 4.7a	48.9
High orthophosphate	103.7 ± 5.1a	48.2 ± 4.4a	55.5 ± 5.1a	53.5
High Ca-high polyphosphate	106.8 ± 7.4a	73.7 ± 14.2b	33.1 ± 3.8b	31.0
High Ca-high orthophosphate	110.7 ± 7.0a	61.7 ± 8.1a	49.0 ± 5.0a	44.3

[a] In each column, nonmatching superscripts denote significant differences ($p < 0.05$).
[b] Data are expressed as mean ± standard deviation.

Table 5—Effect of level and form of phosphate and level of calcium intake on zinc intake, fecal excretion and absorption[a,b]

Diet	Intake (μg/day)	Fecal excretion (μg/day)	Apparent absorption (μg/day)	Apparent abosrption (%)
Basal	535.7 ± 40.0a	397.5 ± 35.7a	138.2 ± 30.0a	25.8
High polyphosphate	485.7 ± 26.4a	339.3 ± 16.4b	146.4 ± 27.9a	30.1
High orthophosphate	540.4 ± 47.1a	403.6 ± 28.6a	136.8 ± 28.2a	25.3
High Ca-high polyphosphate	558.6 ± 38.6a	323.6 ± 27.1b	235.0 ± 47.1b	42.1
High Ca-high orthophosphate	578.9 ± 85.7a	462.1 ± 26.8c	116.8 ± 31.8c	20.2

[a] In each column, nonmatching superscripts denote significant differences ($p < 0.05$).
[b] Data are expressed as mean ± standard deviation.

as a consequence, the gain:feed ratio was unaffected. Both high calcium groups had somewhat higher levels of food consumption then the other three groups, but body weight gain was unaffected; as a result, the high calcium-high polyphosphate and high calcium-high orthophosphate groups had the lowest gain:feed ratios.

DISCUSSION

THE RESULTS of this study demonstrate that the gastro-intestinal hydrolysis of dietary polyphosphate is significantly reduced by increasing the level of calcium intake in the rat. This effect is presumed to be due to the formation of stable calcium-polyphosphate complexes resistant to attack by intestinal phosphatases; polyphosphates have a high affinity for calcium, and moderately stable calcium-polyphosphate complexes are readily formed (Van Wazer, 1971). Similarly, phytate (which may be considered to be a substituted cyclic polyphosphate) has a high affinity for calcium, and supplemental calcium has been shown to reduce phytic acid hydrolysis by yeast (Zemel and Shelef, 1982) and by rat intestinal microflora (Wise and Gilbert, 1982).

The hexametaphosphate supplement caused a significant decrease in the bioavailability of iron and an increase in that of zinc. These effects may be due to the sequestration of iron and zinc by hexametaphosphate, forming an unabsorbable complex in the case of iron. The reason for the improvement in zinc absorption by the hexametaphosphate is not yet clear. However, it appears likely that complexing zinc with hexametaphosphate may protect the zinc from other dietary factors which inhibit its' absorption. Hexametaphosphate is effective in reducing the precipitation of zinc, but not calcium, iron or copper, by phytate under simulated gastric and intestinal conditions (Zemel and Petoskey, 1982). In addition, it is possible that the zinc-hexametaphosphate complex is readily absorbed. Other chelating agents, such as EDTA, citrate and diiodoquin, have been shown to enhance zinc abosrption (Oberleas et al., 1966; Jackson et al., 1981).

The effects of hexametaphosphate on zinc and iron absorption were exacerbated by the supplemental calcium, presumably due to the effect of calcium on polyphosphate hydrolysis. Furthermore, calcium and hexametaphosphate

exhibited an interaction that depressed copper absorption. This effect may be secondary to the observed increase in zinc absorption, since copper and zinc exhibit an antagonism (Magee and Matrone, 1960; Van Campen, 1966). Orthophosphates, on the other hand, had little or no effect on zinc, iron or copper availability at the lower level of calcium intake, and depressed zinc absorption at the higher level of calcium intake. Mahoney and Hendricks (1978) have reported that tripolyphosphate depressed hemoglobin concentration and liver iron while orthophosphate had only slight effects on iron metabolism in the rat. Orthophosphates have been reported by some investigators to decrease zinc (Cabell and Earle, 1964) and iron (Hegsted et al., 1949; Mahoney and Hendricks, 1978) utilization in animals although others have found no effect (Schryver et al., 1974; Pond et al., 1975, 1978). Pond et al. (1978) reported that simultaneously increasing the levels of calcium and phosphorus in the diets of growing pigs caused a decrease in iron utilization, while phosphorus alone was without effect. Cabell and Earle (1965) found a similar potentiating effect of calcium on the phosphate-zinc interaction. Data from human studies also indicate that orthophosphates depress iron utilization only when the level of calcium intake is elevated simultaneously with phosphorus (Monsen and Cook, 1976; Snedeker et al., 1982). However, in the study of Snedeker et al. (1982) this effect was not statistically significant, and phosphorus was found to be without effect on zinc absorption or retention at either level of calcium intake. In the present study, orthophosphate had no effect on zinc or iron absorption at the lower level of calcium intake and caused a significant depression in zinc absorption at the higher level of calcium intake. The high calcium-high orthophosphate diet also caused a slight decrease in iron absorption when compared to the basal diet, but this effect was not statistically significant.

The results of this investigation indicate that polyphosphates have a much more pronounced effect on zinc and iron utilization than do orthophosphates. These effects should be considered when using phosphates in food systems. This is a significant consideration especially in the case of substitute and imitation products such as textured

Production of Glycosidases by Psychrotrophic Bacteria

A. MARIN and R. T. MARSHALL

————————ABSTRACT————————

Six of 19 psychrotrophic bacteria common in milk and meat hydro-lyzed large amounts of p-nitrophenyl-glycosides during 48 hr in minimal growth broth at 20°C. Four of the 19 were inactive against the four glycosides used in the screening tests, and nine were weakly active. In tests of the six active cultures against 11 p-nitrophenyl-glycosides, strains of *Enterobacter* had more glycosidase activity than strains of *Pseudomonas*, β-anomers were hydrolyzed more rapidly than α-anomers, more p-nitrophenol was released from the β-D-galactoside than from other substrates, there was activity against the D form but not the L form of fucoside, and p-nitrophenyl-β-D-glucoside was hydrolyzed at about the same rate by each culture.

INTRODUCTION

PSYCHROTROPHIC BACTERIA produced large amounts of extracellular hydrolases including phospholipases (Fox et al., 1976), proteases, and lipases. Reports regarding proteases and lipases abound in current literature. Guffanti and Corpe (1976) reported the partial purification and characterization of α-glucosidase from *Pseudomonas fluorescens* W. However, we found no other references to production of glycosidases by psychrotrophic bacteria in an extensive search of the literature. In fact, when Ingraham and Stokes (1959), Witter (1961) and Morita (1975) reviewed psychrophilic bacteria, and when Kraft and Rey (1979) updated the literature on psychrotrophic bacteria in foods, production of glycosidases was not mentioned.

Glycosidases in foods containing glycocomplexes, such as glycolipids and glycoproteins, could provide carbohydrates for metabolism by the bacteria. Hydrolysis of glycocomplexes may also render the noncarbohydrate portion of a molecule accessible to hydrolases specific for it. Thus, glycosidases may be important determinants of microbial growth and activity in some foods.

Because we suspected that psychrotrophs would be found to produce several glycosidases we selected representative isolates from the many previously studied in our laboratory and determined their abilities to produce extracellular glycosidases. Synthetic substrates for glycosidases were chosen based on information found in the literature about glycoconjugate linkages in natural substrates such as milk. The β linkage is the most common one found in natural substrates.

MATERIALS & METHODS

Selection of glycosidase-producing bacteria

Nineteen cultures of psychrotrophic bacteria of the Univ. of Missouri-Columbia Food Microbiology collection were inoculated into Trypticase soy broth (TSB) and incubated for 2 days at 20°C. Stock cultures were streaked on Trypticase soy agar (TSA) slants, incubated for 2 days at 20°C and stored at 5°C until used. Transfers to fresh TSA were made monthly and to TSB as needed.

Glycosidase activity of these cultures was assayed following a modification of the procedure described by Zahler and Doak (1975). Bacteria were inoculated into a glucose-fortified "minimal

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growth broth" (MGB) in duplicate. This broth contained 7% (v/v) of each of dihydrated sodium citrate (2.0 mM), ammonium sulfate (9.2 mM), glucose (2.0 mM), and salt mixture solution containing heptahydrated magnesium sulfate (2.1 mM), dihydrated calcium chloride (2.3 mM) and sodium chloride (0.086M); 4% (v/v) citric acid (0.1M)-dibasic sodium phosphate (0.2M) buffer at pH 6.0; and 68% (v/v) distilled water. To 5 ml MGB were added the following para-nitrophenyl-glycosides (Sigma Chemical Co.) as synthetic substrates: β-D-fucoside (2.8 mg), α-D-mannoside (3.0 mg), β-D-glucoside (3.0 mg) or β-D-galactoside (3.0 mg). The final substrate concentration was 2 mM. This substrate-broth mixture was sterilized by membrane filtration (0.22 mm) then inoculated with one loopful of 48 hr culture in TSB and incubated. After incubation for 2 days at 20°C, 5 ml glycine (0.1M), adjusted to pH 10.5 with sodium hydroxide (0.1M), was added to stop the enzymatic activity and to stabilize the yellow color of the p-nitrophenol (PNP) freed from the synthetic substrates during incubation. Bacteria were removed by centrifuging the broth at 30,000 x g for 15 min. The absorbance of the yellowish solution was determined at 400 nm, the wavelength for maximal absorbance of PNP, with a Beckman dual-beam spectrophotometer, model 25. Uninoculated samples were tested concurrently as controls. When absorbance values approached 2, samples were diluted with MGB. These absorbance values were converted to concentration (M) of PNP by means of a standard curve.

A standard curve for absorbance vs. concentration of PNP was prepared using MGB with added glycine (pH 10.5). Concentrations of PNP in the MGB ranged from $10^{-9}M$ to $10^{-7}M$. Samples were made in duplicate. Absorbance was determined with a Bausch and Lomb Spectronic-20 at 400 nm. The regression equation and the correlation coefficient of the curve were calculated using the mean of the two absorbance readings.

Production of bacterial pigments

Each of the six microorganisms that produced significant amounts of glycosidases in experiments described above was incubated for 2 days at 20°C in glucose-fortified MGB without synthetic substrate. Absorbance of samples was determined at 400 nm using uninoculated samples as controls.

Growth of nonglycosidic bacteria

Extent of growth of nonglycosidic microorganisms in the MGB containing synthetic substrate was determined in duplicate by determining transmittance at 520 nm after incubation at 20°C for 48 hr. Transmittance was set at 100% with uninoculated MGB, and bacteria were added to the medium until transmittance decreased to 95%.

A standard curve, transmittance versus aerobic plate count (APC), was prepared using *Alcaligenes sp.* A, the bacterium with the poorest growth in the glucose-enriched MGB. Aliquots of broth were inoculated in duplicate with *Alcaligenes sp.* A to produce transmittance values of 40–95%. Numbers of the bacterium in the inoculated broth samples were then determined by the aerobic plate count method according to the *Compendium of Methods for the Microbiological Examination of Foods* (Speck, 1976), except that the incubation temperature was 20°C. Averages of two counts were used to plot the standard curve.

Linkage specificity of glycosidases

Tubes of MGB containing respectively, 2 mM concentrations of the following p-nitrophenyl-glycosides (Sigma Chemical Co.,) were inoculated from fresh slants with selected psychrotrophic bacteria: α-L-fucoside, β-L-fucoside, β-D-fucoside, α-D-mannoside, β-D-mannoside, α-D-glucoside, β-D-glucoside, α-D-galactoside, β-D-galactoside, N-acetyl-β-D-glucosaminide and N-acetyl-β-D-galactosaminide. Since preliminary tests showed that β-fucosidase and α- and β-D-manno-

sidases were weakly active, extent of the hydrolysis of the fucosides and mannosides was measured after incubation at 20°C for 24 hr. Samples containing each of the other substrates were incubated for 5 hr at 20°C. Mean molar concentrations of PNP released per hour of incubation were reported for each glycosidase.

Statistical analyses

Statistical analyses of the tests of linkage specificity of glycosidases were done after modifying the data by the square root transformation, $(x + 1)^{1/2}$. This transformation was used to stabilize the variance which was statistically proportional to the mean (Snedecor and Cochran, 1967). The experimental design was a two-way replicated 6 x 11 factorial.

Analysis of variance (Snedecor and Cochran, 1967) was used to study the effects of independent variables (replicates, treatments and bacteria) and to test for their possible interaction with the dependent variable (concentration of free PNP). Duncan's Multiple Range and Least Significant Difference (LSD) tests (Steel and Torrie, 1960) were used to determine differences among means when F values were significant (P < 0.05).

RESULTS & DISCUSSION

Tests for glycosidase production

Of the nineteen psychrotrophs tested, six hydrolyzed large amounts of PNP from β-D-fucoside, β-D-glucoside and β-D-galactoside (Table 1). The same bacteria hydrolyzed small quantities of PNP-α-D-mannoside. (The alpha form was used in this experiment because the beta form was not available at that time.) Overall, 15 of the bacteria showed some activity against PNP-β-D-fucoside.

Data for the standard curve (Fig. 1), used to convert absorbance values of the broth solution to concentration of PNP, produced a linear response, and the data fit the regression curve well (r=0.999).

Citrobacter intermedius 16, *Enterobacter spp.* A1, 13, 22 and 44 and *Pseudomonas fluorescens* 7 produced considerable glycosidic activity with the four synthetic substrates (Table 1). *Pseudomonas fluorescens* J also showed some glycosidic properties against all four substrates, but, since this response was weak, this microorganism was not used in further studies. *Enterobacter sp.* A1 was the most active β-D-fucosidase producer. Each bacterium produced only a small amount of β-D-mannosidase. On the contrary, the six glycosidase-producing microorganisms were excellent producers of β-D-glucosidase and β-D-galactosidase.

The six glycosidase-producing cultures were tested in a confirmatory experiment under the conditions of incubation and analysis of the previous experiment. As before, quantities of PNP released from p-nitrophenyl-β-D-glucoside and -β-D-galactoside were markedly higher than quantities released from p-nitrophenyl-β-D-fucoside. The small amounts of PNP released from p-nitrophenyl-β-D-mannoside approximated those released in the first experiment from the α-D-anomer. The hydrolysis of PNP-β-D-glucoside was remarkably similar among the six bacteria. *Enterobacter sp.* A1 was the most active producer of β-D-fucosidase and β-D-galactosidase.

Interestingly, all four strains of *Enterobacter* produced large amounts of glycosidases whereas only one of 11 pseudomonads showed high activity. Although these cultures do not represent the entire population of psychrotrophs in foods, the data suggest that species of *Enterobacter* are more likely to produce glycosidases than are species of *Pseudomonas*.

Production of bacterial pigments

Some species of *Pseudomonas* produce pigments, such as pyoverdin, pyocyanin and fluorescein (Banwart, 1979; Frazier, 1967). Therefore, the extent of production of bac-

$$y = 19.2\ X - 0.009$$
$$r = 0.999$$
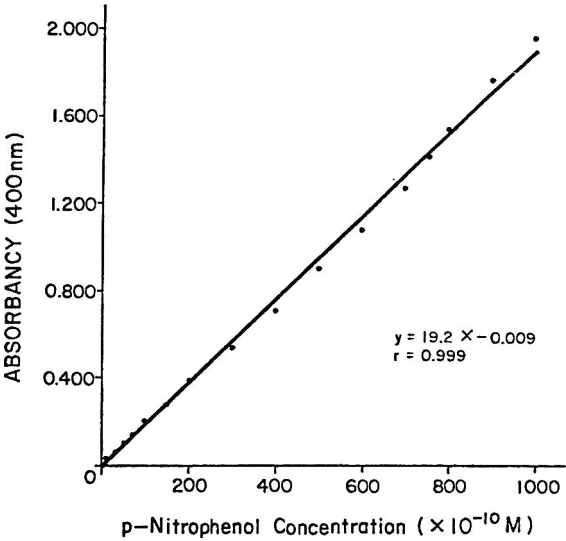
Fig. 1—Absorbancies of p-nitrophenol at different concentrations.

Table 1—Concentrations[a] of p-nitrophenol released from the p-nitrophenyl-glycoside substrates during incubation (2 days at 20° C)

Bacterium	p-nitrophenol concentration $(x10^{-10}M)$			
	β-D-fucoside	α-D-mannoside	β-D-glucoside	β-D-galactoside
Acinetobacter calcoaceticus 24	8	0	0	0
Acinetobacter calcoaceticus 46	7	0	0	0
Alcaligenes sp. A	0	0	0	0
Citrobacter intermedius 16	329	7	3589	1245
Enterobacter sp. Al	1943	17	3526	4828
Enterobacter sp. 13	438	15	3651	3234
Enterobacter sp. 22	202	11	3812	4912
Enterobacter sp. 44	630	29	3516	5078
Pseudomonas fluorescens 1	6	0	0	7
Pseudomonas fluorescens 7	229	28	3729	4781
Pseudomonas fluorescens 14	9	0	0	7
Pseudomonas fluorescens 23	0	0	0	0
Pseudomonas fluorescens 26	18	0	0	0
Pseudomonas fluorescens 27	0	0	0	0
Pseudomonas fluorescens 37	5	0	5	0
Pseudomonas fluorescens J	12	8	50	12
Pseudomonas fragi Co 1	6	0	0	0
Pseudomonas fragi K₁	6	5	0	8
Pseudomonas fragi 27	0	0	0	0

[a] Averages of two determinations

terial pigments, which might absorb at 400 nm, was investigated. None of the inoculated and incubated MGB absorbed at 400 nm when compared to sterile controls of MGB. Thus, bacteria with glycosidic properties did not produce pigments that absorbed at the wavelength at which the absorbance of PNP was determined.

Growth of nonglycosidic bacteria

Bacteria with no glycosidic activity grew well in the synthetic substrate-containing MGB in which they were inoculated. This was evidenced by the generally large decreases in transmittance (at 520 nm) of inoculated samples after incubation compared to 95% transmittance before incubation.

Alcaligenes sp. A grew poorly in all the broths containing synthetic substrates. Transmittance values of the growth medium ranged from 80–86%. However, the population of this microorganism approximated $9 \pm 2 \times 10^6$ cells per milliliter of broth. This bacterial population was considered large enough to have produced detectable enzymatic activities had there been significnat glycosidase(s) produced. Thus, *Alcaligenes sp.* A was a nonglycosidase producer which grew poorly in MGB.

Linkage specificity of glycosidases

Since the literature on the structure of glycocomplexes in foods has reported the presence of only β-D-hexosamines, synthetic substrates such as p-nitrophenyl-α-D-hexosaminides were not used in this test. Other p-nitrophenyl-glycosides were chosen with α- and β-glycosidic linkages as were the L and D families of fucose.

The release of PNP into solution was directly dependent on the glycosidic capabilities of each culture and was not a function of only their populations. Since the stationary phase of growth was reached in a few hours due to the heavy inoculum, populations were similar among the six cultures.

Mean molar concentrations of PNP released per hour of incubation at $2C^{\circ}C$ by glycosidases along with the statistical analysis are presented in Table 2. After statistically analyzing ($P < 0.05$) the square root-transformed data of linkage specificity, the glycosidase activity of *Pseudomonas fluorescens* 7, averaged over all substrates, was found significantly higher than that of the other glycosidase producers. Among all glycosidases, β-D-galactosidase (lactase) was significantly the most active enzyme of *Pseudomonas fluorescens* 7. This enzyme freed almost 1.5 times the amount of PNP released by N-acetyl-β-D-galactosaminidase, the second most active glycosidase of this bacterium.

On the other hand, *Citrobacter intermedius* 16 liberated significantly lower amounts of PNP than did the other four bacteria (averaged over all substrates). This microorganism had poor hexosaminidase activities and its β-D-galactosidase released almost ten times less PNP into solution than did that of *P. fluroescens* 7.

Averaged over all microorganisms, p-nitrophenyl-β-D-galactoside was the most hydrolyzed synthetic substrate. The amounts of PNP released by β-D-fucosidase, both α- and β-D-mannosidases and α-D-glucosidase were significantly much less than amounts released by β-D-glucosidase, α- and β-D-galactosidases and both β-D-hexosaminidases. The latter five glycosidases only needed a 5 hr incubation to free significant amounts of PNP into solution compared to the 24 hr incubation needed by β-D-fucosidase and both α- and β-D-mannosidases. Samples containing PNP-α-D-glucoside were incubated only for 5 hr, based on an asumed rapid rate of hydrolysis, but 24 hr incubation is recommended.

Also, β-anomers of glucoside and galactoside were hydrolyzed more rapidly by glycosidases than were their respective α-anomers. No significant differences was found between mannoside anomers in rates of hydrolysis.

The L series of p-nitrophenyl-fucosides was not hydrolyzed by any bacterial fucosidase. This result was a surprise in that L-fucose is more commonly found in nature than is D-fucose.

Table 2—*Mean molar concentrations* * *of p-nitrophenol released per hour of incubation at $20^{\circ}C$ by glycosidase-producing bacteria from p-nitrophenyl-glycoside substrates with different glycosidic bonds*

Bacterium	α-L-fucoside	β-L-fucoside	β-D-fucoside	α-D-mannoside	β-D-mannoside	α-D-glucoside	β-D-glucoside	α-D-galactoside	β-D-galactoside	N-acetyl β-D-glucosaminide	N-acetyl-β-D-galactosaminide
						p-nitrophenol concentration ($x^{\cdot}0^{-10}M$)					
Citrobacter intermedius 16	0^a_1	0^a_1	5.9^b_1	$0.2^{a,b}_1$	1.8^b_1	5.8^b_1	166^d_1	23.7^c_1	123^d_1	132^d_1	36.9^c_1
Enterobacter sp. A1	0^a_1	0^a_1	23.4^c_2	0.6^b_1	3.5^b_1	19.7^c_2	185^e_1	59.3^d_2	353^f_4	202^e_2	562^g_3
Enterobacter sp. 13	0^a_1	0^a_1	6.1^b_1	1.0^b_1	3.6^b_1	84.5^c_3	166^d_1	105^c_3	284^e_3	246^e_2	505^f_3
Enterobacter sp. 22	0^a_1	0^a_1	6.6^b_1	$0.5^{a,b}_1$	1.3^b_1	30.0^c_2	$174^{e,f}_1$	90.4^d_3	373^g_4	150^e_1	210^f_2
Enterobacter sp. 44	0^a_1	0^a_1	$9.1^{b,c}_{1,2}$	1.4^b_1	1.1^b_1	$21.4^{c,d}_2$	172^g_1	30.6^d_1	177^g_2	120^f_1	60.8^e_1
Pseudomonas fluorescens 7	0^a_1	0^a_1	$9.2^c_{1,2}$	1.1^b_1	$5.2^{b,c}_1$	24.9^d_2	211^f_1	92.7^e_3	946^i_5	330^g_5	734^h_4

*Averages of four determintions. The statistical analysis was done on the square root-transformed data, i.e.

$$\sum_{i=1}^{4} (C_i + 1)^{1/2} \div 4.$$

[a-i] Means on the same line followed by different superscipts differ significantly ($p < 0.05$).
[1-5] Means in the same column followed by different subscripts differ significantly ($p < 0.05$).

Enterobacter spp. Al and 44 and *P. fluorescens* 7 had the most active β-D-fucosidases. *Enterobacter sp.* Al had a fucosidic activity almost twice higher than that of the other bacteria. The two *Enterobacter* species and *Pseudomonas* did not differ in β-D-fucosidase activity.

There were no significant differences in α- and β-D-mannosidase and β-D-glucosidase activities among bacteria.

Enterobacter sp. 13 had the highest α-D-glucosidase and α-D-galactosidase activities. The activities of these two glycosidases did not differ significantly as in the case of those produced by *Enterobacter sp.* 44.

As with β-D-galactoside, N-acetyl-β-D-galactosaminide was hydrolyzed much faster by *P. fluorescens* 7 than it was by the other microorganisms. *P. fluorescens* 7 also hydrolyzed more rapidly N-acetyl-β-D-glucosaminide than did the other bacteria.

In summary, we found that six out of nineteen psychrotrophic bacteria produced significant amount of various glycosidases. The genus *Enterobacter* was an active producer of glycosidases, but *P. fluorescens* 7 had the highest glycosidic activity among all psychrotrophs, averaged over all substrates. More in-depth biochemical studies of the glycosidases of *P. fluorescens* 7 will soon be reported.

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vegetable protein products and imitation cheeses in which mineral bioavailabilities approximately equivalent to those found in the original products are desirable.

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Influence of Potassium Sorbate and Reduced pH on the Growth of Vegetative Cells of Four Strains of Type A and B *Clostridium botulinum*

J. C. BLOCHER and F. F. BUSTA

──────── ABSTRACT ────────

Growth of vegetative cells of four strains of *Clostridium botulinum* was measured as the average A_{630nm} of five replicate peptone-yeast extract broth cultures at 37°C. Media pH values ranged from 7.1 to 5.5 and potassium sorbate concentrations of 0 or 0.26%. Growth ratios (GR = treatment/control) based on the time to reach an A = 0.35 were calculated. Predicted GR's from the linear regression of log (GR) vs log (undissociated sorbic acid concentration) were used to compare strains. Reduced pH (>5.5) had minimal effects on GR. Sorbate inhibited the growth of all strains at 250 mg/L undissociated sorbic acid. This was indicated by predicted GR's of 2.3–3.4. Responses of cells derived from different spore suspensions of the same strain were similar.

INTRODUCTION

POTASSIUM SORBATE has been studied extensively as a potential antibotulinal agent for use in cured meats. Most investigators have studied the effects of sorbate on spores of *Clostridium botulinum*. Sofos et al. (1979) and Smoot and Pierson (1981) reported that sorbate inhibits spore germination. Essentially no quantitative information is available on the effects of sorbate on vegetative cells of *C. botulinum*. Similarly there is little information available on the effects of reduced pH on the growth of *C. botulinum* vegetative cells at reduced nonlimiting pH. The effects of sorbate are known to be pH dependent. Separation of the effects of reduced pH from those of sorbate is necessary to understand the mechanism of action of either of these agents.

Blocher et al. (1982) reported that the degree of resistance of *C. botulinum* spores to both reduced pH and sorbate was strain dependent. Relative resistance to these agents also varied among different spore suspensions of the same strain. The origin of this variation could not be directly determined from the spore inocula studied.

The objectives of this study were to determine if vegetative cells of *C. botulinum* were inhibited by reduced pH or sorbate or both and if the degree of resistance of the cells was strain or spore suspension dependent.

MATERIALS & METHODS

PEPTONE-YEAST EXTRACT BROTH (PYEG) was prepared as previously described (Blocher et al., 1982). Potassium sorbate (Monsanto, St. Louis, MO) was added at 0.26% to appropriate treatments prior to pH adjustment with 1N HCl or NaOH. The pH range for all trials was 7.1–5.5. The medium was dispensed into 12.7 mm glass colorimeter tubes (5 ml/tube), autoclaved for 15 min at 121°C, cooled and stored 16 hr or less under anaerobic conditions (85% N_2, 10% CO_2, 5% H_2) until inoculated.

Spores of four strains of *C. botulinum* (36A, 62A, 12885A, and 53B) were used to prepare vegetative cell inocula. Spores were received from Swift and Co. (Oakbrook, IL) and two spore suspensions of each strain were prepared by the method of Christiansen et al. (1973). Cell inocula were prepared by inoculating PYEG at pH 7.0 with spores of the appropriate strain and spore suspension. The

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inoculated PYEG was heat shocked at 80°C for 15 min and incubated at 37°C for 12–16 hr. Concentration of the inocula was adjusted by diluting the cultures in PYEG to an absorbance at 630 nm (A_{630}) of 0.10; and 0.1 ml aliquots were used to inoculate five replicate tubes of experimental medium per treatment. Inoculation was done under anaerobic gas conditions (85% N_2, 10% CO_2, 5% H_2) in a Coy anaerobe chamber. After inoculation the tubes were overlaid with sterile molten vaspar and incubated at 37°C. Inoculum concentration was determined for each strain in each trial using PYEG plus agar (30 g/L) in Lee tubes (Ogg et al., 1979). The average inoculum was approximately 5 x 10^4 CFU/ml.

The A_{630} of each tube was measured at approximately 4-hr intervals and the time for each treatment to reach an average A_{630} of 0.35 calculated as previously described (Blocher et al., 1982). Growth ratios (GR) for each experimental treatment were calculated. In treatments with reduced pH and 0% sorbate the GR for each strain was calculated as: (hr to A_{630} of 0.35 at reduced pH)/(hr to A_{630} of 0.35 at pH 7.0). In treatments containing sorbate the GR for each treatment was calculated as: (hr to A_{630} of 0.35 with sorbate)/(hr to A_{630} of 0.35 at the same pH and 0% sorbate). The undissociated sorbic acid concentration was calculated as previously described (Blocher et al., 1982).

For treatments containing sorbate a linear regression of log (GR) vs log (undissociated sorbic acid in mg/L) was used to calculate the GR at 250 mg undissociated sorbic acid/L and the predicted values of GR with associated 95% confidence intervals (based on the t distribution) were compared (Blocher et al., 1982). Correlation coefficients for the regression lines ranged from 0.77–0.90. For treatments at reduced pH and 0% sorbate the mean GR and associated 95% confidence intervals for pH values between 5.75 and 5.55 were used to compare strains. All statistical analyses used Multreg (Weisberg, 1979), a statistical computer program from the Univ. of Minnesota, Dept. of Applied Statistics.

RESULTS & DISCUSSION

DUE TO THE APPARENT insensitivity of most strains to reduced pH between 5.75 and 5.55, the average of the 13–18 values for GR obtained for each strain in this range was used to compare resistance to reduce pH. Fig. 1 presents the mean GR and associated 95% confidence interval for cells originating from two spore suspensions of each strain (designated as spore suspensions 1 and 2). Strain 36A, 62A, and 12885A had mean GR's ranging from 1.31–1.54. These strains, and the two spore suspensions from which the cells were derived, were not significantly different from one another at the p = 0.05 level indicated by the overlap of their 95% confidence intervals. Strain 53B grown from either spore suspension had larger mean GR's of 2.05 and 2.46 with overlapping confidence intervals indicating no significant differences between spores suspensions. Based on the overlap of 95% confidence intervals cells grown from spore suspension 1 of the strain 53B were significantly different only from cells derived from spore suspension 2 of strain 36A, while cells derived from spore suspension 2 of strain 53B were significantly different from all strains except cells derived from spores suspension 1 of strain 53B. The variances of the mean GR's of cells derived from spore suspensions of strain 53B were significantly greater than the variances of the mean GR's of the other three strains at the p = 0.05 level of significance.

The larger mean GR's of strain 53B indicated that this strain was the most sensitive to reduced pH in the range
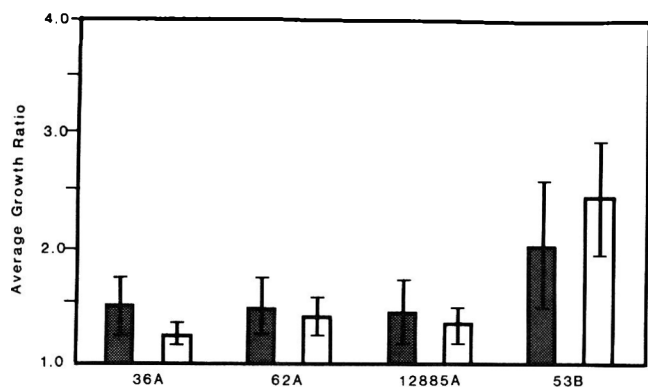
Fig. 1—Average growth ratios and 95% confidence intervals for growth in PYEG between pH 5.75 and 5.55 (0% sorbate) for vegetative cells of four strains and two spore suspensions of Clostridium botulinum. Dark bars are cells from spore suspension 1 and light bars are cells from spore suspension 2.
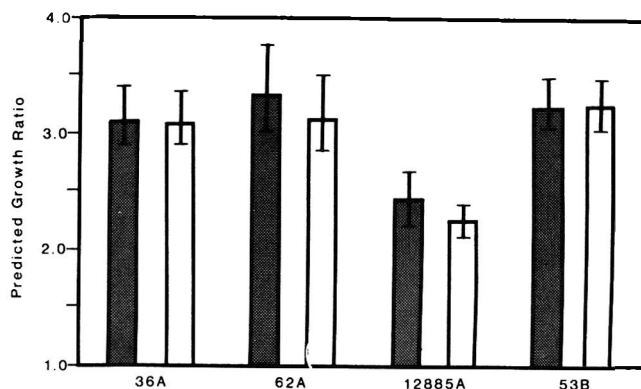
Fig. 2—Predicted growth ratios and 95% confidence intervals for growth in PYEG with 250 mg/L undissociated sorbic acid (pH 5.65 and 0.26% sorbate) for vegetative cells of four strains and two spore suspensions of Clostridium botulinum. Dark bars are cells from spore suspension 1 and light bars are cells from spore suspension 2.

5.75–5.55 as well as the most variable in its response to reduced pH in this range.

Previous work testing spore inocula of these strains of *C. botulinum* in this system reported predicted GR's at pH 5.65 ranging from 2.2–3.8 for strains 36A, 62A, and 12885A (Blocher et al., 1982). The smaller values for the mean GR's with vegetative cells in this pH range indicate that the delays demonstrated with spores are probably due to inhibition of spore germination for strains 36A, 62A, and 12885A. This is in agreement with observations dating back to Dozier (1924), that vegetative cells were less affected by reduced pH than were spores.

The results for strain 53B are less clear in that the vegetative cells exhibited larger GR's (2.05–2.46) and consequently appear to be more inhibited by reduced pH than are the spores (GR 1.5–1.6, Blocher et al., 1982). Measurements with spores involved germination and outgrowth which may have influenced the calculated GR's. The average time to A_{630} = 0.35 at pH 7 for strain 53B was 19 hr with a spore inoculum (unpublished data) and 7 hr with a vegetative cell inoculum. This difference in the denominator of the GR equation for growth at reduced pH makes the GR with a cell inoculum more sensitive to short delays in growth than the GR calculated with a spore inoculum. The larger GR for strain 53B with vegetative cells as compared to spores may be a reflection of the increased sensitivity of GR calculated for the cell inoculum.

Linear regressions of 15–25 values for log (GR) vs log (undissociated sorbic acid in mg/L) were used to calculate the GR's of each strain at 250 mg/L undissociated sorbic acid (pH 5.65 and 0.26% sorbate). When these calculated values and their respective 95% confidence intervals (Fig. 2) were compared, strains 36A, 62A, and 53B all had predicted GR's in the range 3.1–3.4. Confidence intervals of GR's for these three strains overlapped for cells derived from both spore suspensions, indicating no significant differences among the three strains or between the two spore suspensions. Cells from either spore suspension of strain 12885A had smaller GR's of 2.25 and 2.43. The confidence intervals for the GR'S of cells derived from the two different spore suspensions of strain 12885A overlapped one another but did not overlap those of the other three strains. This indicated that the cells derived from the different spore suspensions of strain 12885A were not significantly different from one another, but that cells of strain 12885A were significantly less inhibited by sorbate than cells of the other three strains.

To our knowledge this is the first report that vegetative cells of *C. botulinum* are inhibited by potassium sorbate at concentrations proposed for use in cured meats. Sorbate has been previously reported as inhibitory to *C. botulinum* because it delayed or prevented spore germination (Sofos et al., 1979; Smoot and Pierson, 1981). This study indicates that while spore germination is one point of inhibition, growth of vegetative cells of *C. botulinum* is also inhibited by sorbate. Ability to affect germination and cell growth suggests that sorbate may have more than one site against which it is active.

Lack of variation among cells of the same strain derived from different spore suspensions is also a significant observation. Blocher et al. (1982) reported significant differences in sorbate and pH resistance among spores of different strains of *C. botulinum* and among different spore suspensions of the same strain. The site of this variation could not be determined from the data, but it was hypothesized that variation among different spore suspensions of the same strain was due to minor structural differences in the spores which might affect germination properties of the spores. The current study indicates that variation noted with a spore inoculum was the result of differences in the properties of the spores and not due to changes in the metabolic capabilities of the resulting vegetative cells.

This study has shown that vegetative cells of *C. botulinum* are inhibited by potassium sorbate, but reduced pH has minimal effect on the growth of vegetative cells at pH values above 5.5. Relative resistance to sorbate or reduced pH is strain dependent and no significant differences were found among cells derived from different spore suspensions of the same strain.

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Effect of BHA, BHT, TBHQ and PG on Growth and Toxigenesis of Selected Aspergilli

C. C. S. LIN and D. Y. C. FUNG

————————————ABSTRACT————————————

The antifungal effect of butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ) and propyl gallate (PG) alone or in combination on three toxigenic strains of aspergilli (NRRL 2999, NRRL 4123, NRRL 5835) and three nontoxigenic strains of aspergilli (NRRL 5521, NRRL 5917, NRRL 5918) was examined in a solid medium and in salami. BHT and PG (0.001, 0.005, 0.01, 0.02g per plate) did not inhibit growth, sporulation, and toxigenesis of all six cultures. Aflatoxin production by toxigenic aspergilli (B_1, B_2, G_1 and G_2) in the presence of BHA, TBHQ, and a combination of BHA and TBHQ was reduced significantly ($P < 0.05$). In salami BHA, TBHQ alone or in combination at 100 ppm significantly ($P < 0.05$) decreased the aflatoxin production by aspergilli when compared to control samples. A combination of BHA and TBHQ showed synergistic inhibition in both studies (solid medium and salami studies).

INTRODUCTION

PRODUCTION of carcinogenic aflatoxins by certain strains of aspergilli has been studied extensively since the discovery of this class of toxins in the early 1960's. Aflatoxins continue to be a public health hazard due to their high toxicity and the ubiquitous nature of aspergilli in the environment and in the food chain.

In the United States, regulated tolerances for foods or feeds are 20 ppb totals (Food and Drug Administration, 1974).

Molds often grow on meats, especially cured meats during storage or aging. Some of these molds have a toxigenic potential. The production of aflatoxins in beef, bacon, ham and salami was observed by Bullerman and colleagues (1968, 1969a, 1969b) who reported that the highest level of toxin was produced at 20°C compared with 30°C. A temperature below 15°C and a relative humidity of less than 75% was found to prevent aflatoxin development during the aging of salami.

Strzelecki et al. (1969) isolated 10 fungal species from a moldy country cured ham. Four fungi were identified as toxigenic strains of *A. flavus*. The amount of aflatoxin B_1 and B_2 production by these four strains in YES medium are from 49–85 μg/50 ml of medium. Aflatoxin G_1 and G_2 were not detected. Sutic et al. (1972) also investigated mold contamination in country cured hams. Of 562 mold isolates from 356 country cured hams, 403 were *Penicillium*, 123 were *Aspergillus*, and 36 were other mold isolates. The number of aspergilli isolated depended on the age of the hams and on the amount of moisture in the storage room. Of 121 aspergilli isolates, only two strains were able to produce aflatoxin. Only aflatoxin B_1 was produced in detectable amounts by either strain.

Oldham et al. (1971) studied the production of aflatoxin in salami, New England loaf, and bologna inoculated with *A. flavus* ATCC 15517 and incubated for 12 days at 4.4, 7.2 or 25°C. They found that all samples were negative for aflatoxin production except those incubated at 25°C.

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Antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ), and propyl gallate (PG) commonly used to prevent rancidity in lipids and lipid-containing products were found to have antimicrobial properties.

A relatively large body of information is now available concerning the effects of antioxidants on bacteria and their metabolites (Change and Branen, 1975; Shih and Harris, 1977; Erickson and Tompkin, 1977; Ayaz et al., 1978; Pierson et al., 1980; Robach and Stateler, 1980; Gailani and Fung, 1980; Lahellec et al., 1981; Davidson et al., 1981). Relatively less information is available concerning the effect of antioxidants on growth and toxin production of *Aspergillus parasiticus* and *A. flavus*.

Fung et al. (1977) tested BHA and BHT against 6 toxigenic and 6 nontoxigenic strains of *A. flavus* by using a method similar to an antibiotic susceptibility test. BHT was found not to be inhibitory to *A. flavus* growth, sporulation, pigmentation, or toxigenesis at any of the levels tested. BHA was found to inhibit *A. flavus* growth at 0.005–0.02g per plate of solid media (60 or 100 mm in diameter). The production of aflatoxins B_2, G_1, and G_2 was completely inhibited by 0.01g BHA in a 60-mm-diameter plate of solid medium.

The objectives of this study were: (1) To determine the effects of BHA, BHT, TBHQ, and PG alone or in combination on growth, sporulation, and aflatoxin production by selected aspergilli on solid medium; (2) To evaluate the efficiency of these antioxidants in preventing the production of aflatoxins in salami.

MATERIALS & METHODS

Effect of antioxidants on growth and aflatoxin production on solid medium

Organisms tested. Three toxigenic strains of aspergilli (*Aspergillus parasiticus* NRRL 2999, NRRL 4123, NRRL 5835) and three nontoxigenic strains of aspergilli (*A. flavus* NRRL 5521, NRRL 5917, NRRL 5918) obtained from the Northern Regional Research Laboratory (Peoria, IL) were used in this study. Stock cultures were maintained on Potato Dextrose Agar (Difco). Spore suspensions (10^5–10^6 spores/ml) were made by washing the slants of 7 day-old culture with 3 ml of sterile distilled water with 2 drops of Tween 80 added.

Solid medium used

The agar medium of Hara et al. (1974) was used. In this study, $HgCl_2$ was excluded from the medium because it has an inhibitory effect on growth of aspergilli. The solution was adjusted to pH 5.5 before the addition of the agar. The sterile medium was poured into 100 mm diameter glass petri dishes and incubated overnight before use. Each plate contained 15 ml of medium.

Antioxidants tested

Antioxidants. Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ), and propyl gallate (PG) were obtained from Eastman Chemical Products Inc. (Tennessee). A stock solution of each antioxidant was prepared by dissolving 10g or 1g of the antioxidant in 10 ml of 95% ethyl alcohol. Appropriate amounts were then transferred from the stock solution onto sterile blank disks by use of a micropipett.

Growth inhibition studies on solid medium

Spore suspensions were swabbed on the agar medium (Hara et al., 1974) and then the disk, with desired amount of antioxidant, was placed in the center of the plate. Small amounts (0.0002–0.02g per plate) of BHA, BHT, TBHQ, and PG alone or in combination were used to challenge the test organisms. The plates were incubated at room temperature (23 ± 1°C) for 5 days. The diameters of inhibition zones, when present, were measured in cm. The plates were then placed under ultraviolet light (365 nm) to detect fluorescence in the agar.

Quantification of aflatoxins

The thin-layer chromatography procedure of Pons et al. (1968) was used to identify and quantify the four primary aflatoxins (B_1, B_2, G_1, G_2).

Effect of antioxidants on salami

Salami was chosen as a meat model system to study the efficacy of antioxidants in preventing the production of aflatoxins.

Preparation of cooked beef salami. The processing of cooked beef salami was done at the Dept. of Animal Sciences and Industry, Kansas State Univ., Manhattan. The ingredients of salami are: 10 lb 25/75 beef trim (25% fat); 3 lb water; 100g salt; 40g sugar; 23g Heller salami seasoning; 11g Prague powder (contains 6.25% sodium nitrite) dissolved in ½ cup warm water; 2g sodium erythorbate; 100 mg/L antioxidant (BHA or BHT or TBHQ or PG or combination of BHA and TBHQ).

The following procedures were used for manufacturing salami: (1) grind beef trim through ½ inch plate and then through 1/8 inch plate; (2) add salt to ground beef and mix in a mixer; (3) add water and mix for 1 min; (4) add sugar and Heller seasoning and mix for 30 sec; (5) add Prague powder and mix for 30 sec; (6) add sodium erythorbate and mix for 15 sec; (7) add antioxidant and mix for 20 sec; (8) stuff in shirred fibrous casing that has had one end tied with cord; (9) tie stuffed casing with cord; (10) hang on smoke stick and put into smokehouse to cook until internal temperature of salami reaches 66–68°C (150–155°F); (11) remove from smokehouse and shower; (12) chill to 3.5°C (38°F).

Six batches of salami were made, the control batch contained no antioxidants; the others contained different antioxidants; BHT, PG, BHA, and TBHQ all singly or BHA plus TBHQ, respectively.

Growth inhibition studies in salami. Salami samples were sliced with an alcohol-flame spatula, weighed in a sterile petri dish. One milliliter of Aspergillus spore suspension was applied to the sliced salami. Plates were incubated at room temperature (23 ± 1°C) for 5 days or refrigerator temperature (7°C) for 8 wk and then incubated at room temperature for an additional 5 days. The stored salami samples were palced in a 400 ml sterilized Stomacher Bag (Dynatech Lab., Inc., Alexandria, VA) with 125 ml of extraction solution and mixed in a Stomacher Lab-Blender 400 (Model No. BA6021, A.J. Seward, London, Eng.) for 1 min. Meat slurries were transferred into 500 ml Erlenmeyer flasks. The quantitative analysis of aflatoxin in the meat slurry was made according to the thin-layer chromatography procedure of Pons et al. (1968).

Statistical analysis of the data was performed using two-way analysis of variance and Duncan's multiple range test at 0.05 significance level (Snedecor and Cochran, 1980) with the aid of SAS program.

RESULTS & DISCUSSION

Effect of antioxidants of growth, sporulation, and aflatoxin production on solid medium

Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and tertiary butylhydroquinone (TBHQ) were applied to three toxigenic strains of aspergilli (Aspergillus parasiticus NRRL 2999, NRRL 4123, NRRL 5835) and three nontoxigenic strains of aspergilli (A. flavus NRRL 5521, NRRL 5917, NRRL 5918). At all levels tested (0.001, 0.005, 0.01, 0.02, g per plte), BHT and PG did not inhibit growth, sporulation, and toxigenesis of all six cultures. However, in the presence of BHA and TBHQ (alone or in combination) all cultures were affected. Three distinct zones appeared on the plate containing BHA or TBHQ or in a combination of BHA and

TBHQ in equal amounts: (1) an inner zone of no growth, (2) a middle zone with growth and no sporulation, (3) an outer zone with growth and sporulation. Fig. 1 shows the inner inhibition zone increases in size as the amount of BHA applied increases. Good growth, sporulation, and toxigenesis were observed for all six tested cultures in the absence of antioxidants.

Effect of antioxidants on toxigenic aspergilli

The effects of BHA, TBHQ, and combination of BHA and TBHQ (BHA/TBHQ) on growth, sporulation, and toxigenesis of toxigenic aspergilli (NRRL 2999, NRRL 4123, and NRRL 5835) were studied. Since the effects were similar for all three strains, only data for A. parasiticus NRRL 2999 are presented in detail (Table 1 and Table 2). The inner and middle zones increased in size as the amount of BHA, TBHQ and BHA/TBHQ applied increased. The outer zone showed growth and sporulation of the organism indicating that antioxidants had little effect at that region. The minimum inhibitory concentration (MIC) of BHA and TBHQ was 0.0005g and 0.001g per plate, respectively, while 0.0003g per plate of BHA/TBHQ was inhibitory to NRRL 2999. At 0.005g application of BHA or TBHQ, plates showed brighter fluorescence at the outer zone than the middle and inner zones indicating that more aflatoxins were produced by the organism at a distance from the antioxidants. Further increase of BHA to 0.01 and 0.02g, and TBHQ to 0.02g resulted in no fluorescence in the inner zone of the plates. Combination of BHA and TBHQ was the most efficient group among three treatments. At 0.005g per plate level, no fluorescence could be detected in the inner zone of the plates.

To confirm the presence of aflatoxin in the plates, the agar of control plates without antioxidants, and plates with 0.0005, 0.001, 0.005, and 0.01g of BHA or TBHQ or BHA/TBHQ were analyzed for all four primary aflatoxins. Data in Table 2 indicate that in the presence of BHA, TBHQ, and BHA/TBHQ, aflatoxin production by A. parasiticus NRRL 2999 was inhibited. At 0.0005g per plate level, BHA showed selective effect on aflatoxin G_2 only, while TBHQ and BHA/TBHQ resulted in a selective inhibition on the production of aflatoxin G_1 and G_2. The effect of these antioxidants on aflatoxin production was greater in the G Group (G_1 and G_2) than in the B group (B_1 and B_2). Aflatoxin G_1 was almost totally inhibited by 0.005g per plate of BHA and BHA/TBHQ. Combination of BHA and TBHQ showed a slightly synergistic effect on aflatoxin production by A. parasiticus NRRL 2999.
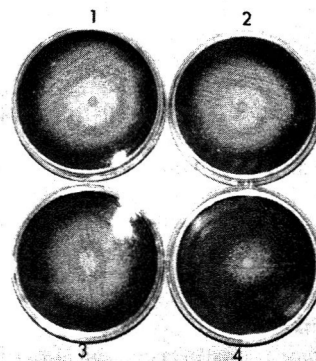
Fig. 1—Growth of A. parasiticus NRRL 2999 on solid medium in the presence of different amounts of BHA disk at the center area: (1) 0.01g, (2) 0.005g, (3) 0.001g, (4) 0.0005g.

Table 1—Effect of BHA, TBHQ, and combination of BHA and TBHQ on growth, sporulation, and toxigenesis of A. parasiticus NRRL 2999 on solid medium[a]

Amount of antioxidants per plate (g)	BHA					TBHQ					BHA and TBHQ[b]				
	I[c]	M[d]	Fluorescence			I	M	Fluorescence			I	M	Fluorescence		
			I	M	O[e]			I	M	O			I	M	O
0.0200	2.60	5.20	−[f]	+[g]	+	1.97	3.80	−	+	+	3.18	5.50	−	+	+
0.0100	2.58	5.20	−	+	+	2.03	3.91	+	+	+	3.13	5.30	−	+	+
0.0050	2.16	4.80	+	+	+	1.77	3.92	+	+	+	3.02	5.30	−	+	+
0.0020	1.70	4.30	+	+	+	0.95	3.15	+	+	+	2.30	4.70	+	+	+
0.0010	0.93	2.70	+	+	+	0.11	1.09	+	+	+	1.88	3.93	+	+	+
0.0005	0.10	1.38	+	+	+	0	0.62	+	+	+	1.84	2.90	+	+	+
0.0004	0	1.33	+	+	+	0	0.11	+	+	+	0.18	0.57	+	+	+
0.0003	0	0	+	+	+	0	0	+	+	+	0.13	0.37	+	+	+
0.0002	0	0	+	+	+	0	0	+	+	+	0	0	+	+	+

[a] Values indicate the mean of 8 samples.
[b] BHA and TBHQ were combined in equal amounts.
[c] I = "inhibition zone" without growth. A 100 mm diameter petri dish was used in this study. The diameter of the zone is expressed in cm.
[d] M = "middle zone" with growth but no sporulation.
[e] O = "outer zone" with growth and sporulation.
[f] − = no aflatoxin diffusion was observed under UV light.
[g] + = UV detection of aflatoxin diffused into agar medium. It is presumptive toxigenesis.

Table 2—Production of aflatoxins of A. parasiticus NRRL 2999 in the presence of BHA, TBHQ, and combination of BHA and TBHQ on solid medium[a]

Amount of antioxidant per plate (g)	Aflatoxin (µg/ml of agar)			
	B_1	B_2	G_1	G_2
Control	71.43	3.75	12.50	2.14
BHA				
0.0005	71.43	3.75	12.50	0.36
0.0010	65.00	2.60	8.30	0.05
0.0050	45.00	1.20	0.50	0.39
0.0100	45.00	2.55	4.00	0.45
TBHQ				
0.0005	71.43	3.75	9.40	0.45
0.0010	65.00	1.65	4.00	0.15
0.0050	55.00	2.70	2.40	0.33
0.0100	52.50	2.25	1.90	0.33
BHA and TBHQ[b]				
0.0005	71.43	3.75	6.25	0.27
0.0010	62.50	2.50	1.60	0.21
0.0050	40.00	1.80	0.40	0.06
0.0100	40.00	1.65	0.40	0.06

[a] values indicate the means of 6 samples.
[b] BHA and TBHQ were combined in equal amounts.

Effect of antioxidants on nontoxigenic aspergilli

The effects of BHA, TBHQ, and BHA/TBHQ against nontoxigenic strains of aspergilli (A. flavus NRRL 5521, NRRL 5917, and NRRL 5918) were studied. Since the effects were similar for all three strains, only data for A. flavus NRRL 5521 are presented in detail (Table 3). All three treatments were found to be effective in inhibiting growth and sporulation. The inner and middle zones increased in size as the amount of BHA, TBHQ, and BHA/TBHQ increased. Antioxidants have no effect on outer area of plates. No fluorescence was detected. The MIC of BHA and BHA/TBHQ were 0.0005g while TBHQ was 0.001g per plate. A good deal of synergistic inhibition was shown for the BHA/TBHQ group; the greatest inhibition zone (3.75 cm) was observed in 0.02g per plate level.

The effect of BHA, TBHQ, and BHA/TBHQ at all treatment levels against three toxigenic strains of aspergilli and three nontoxigenic strains of aspergilli, as compared to the control, was analyzed using two-way analysis of variance and Duncan's multiple range test. All three treatments (BHA, TBHQ, and BHA/TBHQ) were found to inhibit growth, sporulation, and aflatoxin production of the test cultures significantly ($P < 0.05$). Kinds of antioxidants and cultures, levels of antioxidants used, and types of aflatoxins resulted in different ($P < 0.05$) inhibitory effects. The higher the amounts of antioxidants applied, the greater the inhibitory effect observed. Antioxidants are known to have synergistic antioxidant effects when used in combination with other antioxidants (Dugan, 1976). For inner zone development and aflatoxin production, the combination of BHA and TBHQ showed significantly ($\alpha = 0.05$) better inhibition than the two antioxidants applied alone. The inhibitory effect of BHA on growth, sporulation, and aflatoxin production was significantly better than that of TBHQ at all levels applied. Antioxidants had no significantly different effect on inner inhibition zone among three nontoxigenic strains of aspergilli and antioxidants were more effective against nontoxigenic strains than toxigenic strains. Statistical correlation indicates that cultures producing greater amounts of toxin were more resistant to antioxidants ($\alpha = 0.05$). Antioxidants had selective inhibitory effects on the production of the four primary aflatoxins, with B_1 (35%) least inhibited, while B_2 (41%), G_1 (66%) and G_2 (82%) were more inhibited.

This study indicated that BHA and TBHQ alone or in combination are effective in inhibiting growth, sporulation, and aflatoxin production of aspergilli on laboratory medium. In general, the combination of BHA and TBHQ had synergistic effectiveness.

Growth inhibition studies of antioxidants in cooked beef salami

Three toxigenic strains of apsergilli (A. parasiticus NRRL 2999, NRRL 4123, NRRL 5835) and one nontoxigenic strain of aspergillus (A. flavus NRRL 5521) were applied to salami for this study. The salami with cultures and 100 mg/L antioxidants were stored for varying lengths of time at room temperature (23 ± 1°C) and refrigerator temperature (7°C). Mold growth was estimated visually.

There was abundant growth and sporulation of aspergilli on salami after incubation at room temperature for 5 days (RT). Bacteria and yeasts present on salami may have reduced aflatoxin production of Aspergillus at room temperature.

No growth of any test cultures in the salami was observed at refrigeration temperature for 8 wk; likewise, no aflatoxins were found at this temperature. After 8 wk in the refrigerator, salami with cultures were then incubated at room temperature for 5 days (RR) to study the effect of temperature shift on growth and toxigenesis of test cultures on salami. All cultures began to grow at the third day. Profuse

Amount of antioxidants per plate (g)	BHA			TBHQ			BHA and TBHQ[b]		
	I[c]	M[d]	Fluorescence[e]	I	M	Fluorescence	I	M	Fluorescence
0.0200	3.10	5.00	—	2.50	5.20	—	3.75	6.70	—
0.0100	2.95	5.10	—	2.55	5.56	—	3.65	6.40	—
0.0050	2.80	4.25	—	2.00	4.60	—	3.40	5.80	—
0.0020	2.15	3.20	—	1.10	3.20	—	1.45	2.50	—
0.0010	0	0.90	—	0.40	2.60	—	0.60	2.00	—
0.0005	0.30	0.80	—	0	1.00	—	1.00	1.70	—
0.0002	0	0	—	0	0	—	0	0	—

[a] Values indicate the mean of 8 samples.
[b] BHA and TBHQ were combined in equal amounts.
[c] I = "inhibition zone" without growth. A 100 mm diameter petri dish was used in this study. The diameter of the zone is expressed in cm.
[d] M = "middle zone" with growth but no sporulation.
[e] = no fluorescence detected since this is a non-toxigenic strain.

growth and sporulation were obtained at the end of 5 days incubation.

All salami samples from two different storage periods were analyzed by thin-layer chromatography. Salami inoculated with nontoxigenic aspergillus (*A. flavus* NRRL 5521) showed no aflatoxin in a comparable study.

The effects of antioxidants against aflatoxin production of aspergilli (*A. parasiticus* NRRL 2999, NRRL 4123 and NRRL 5835) on salami were studied. Since the effects were similar for all three strains, only data for aspergillus (*A. parasiticus* NRRL 2999) are presented in detail (Table 4). The 100 mg/L of BHA, TBHQ, and combination of BHA and TBHQ (BHA/TBHQ) were found to suppress aflatoxin production of NRRL 2999 at both storage periods. An inhibitory effect of BHT and PG against aflatoxin production of NRRL 2999 was not noted at RR storage period, as compared to the controls. Although reaction of BHT and PG at two storage periods are not consistent, no significant ($\alpha = 0.05$) difference between the control and the antioxidants (BHT or PG) was observed. Salami with culture (NRRL 2999) produced larger amounts of aflatoxins with RR incubation period than at room temperature for 5 days. Effects of BHA, TBHQ, and BHA/TBHQ on the production of four aflatoxins (B_1, B_2, G_1, and G_2) by the test organism were greater after RT storage than RR storage. Butylated hydroxyanisole was the most effective in suppressing aflatoxin production by NRRL 2999 than the other two treatments (TBHQ and BHA/TBHQ). Due to the small amounts of aflatoxin B_2 and G_2 produced by NRRL 2999 at room temperature for 5 days incubation period, productions of aflatoxin B_2 and G_2 by NRRL 2999 were almost totally inhibited by BHA, TBHQ, and BHA/TBHQ, None of the antioxidants tested were highly effective against aflatoxin B_1 and G_1 production by *A. parasiticus* NRRL 2999. The antimicrobial effectiveness of the antioxidants tested was markedly reduced in a food system containing lipid.

Antioxidants are known to have less antimicrobial activity when applied to foods than when used in laboratory media (Robach et al., 1977; Klindworth et al., 1979). The presence of as little as 0.25% lipid decreased inhibition of 200 mg/L BHA by approximately 30% (Ahmed, 1977). The same author also found that BHA was more effective in preventing mold growth in low fat product (applesauce) than in high fat products (cheese). The fat content of this salami was 10.25%; this was assumed to be high enough to cause a decrease in the antifungal activity of BHA or TBHQ or BHA/TBHQ against aspergilli. Unsaturated lipids are extremely reactive compounds which undergo autoxidation with the subsequent formation of free radicals and hydroperoxides (Dugan, 1976). Antioxidants such as those tested are active in preventing autoxidation by donating a hydrogen atom to the unstable lipid free radical. The antimicrobial activity of the antioxidants may be destroyed by the

Table 4—Production of aflatoxins by A. parasiticus NRRL 2999 in the presence of antioxidants on salami[a]

Treatment	Storage period	Aflatoxin (µg/g of salami)			
		B_1	B_2	G_1	G_2
Control	RT[b]	7.0671	0.0050	11.7786	0.0050
	RR[c]	21.1268	0.0391	21.1268	0.0391
BHT	RT	7.2674	0.0045	7.2674	0.0045
	RR	21.1370	0.0395	21.1370	0.0395
PG	RT	6.1475	0.0050	6.1475	0.0050
	RR	21.3068	0.0393	21.3068	0.0393
BHA	RT	5.3097	Tr[d]	7.9646	Tr
	RR	18.3150	0.0332	18.3150	0.0332
TBHQ	RT	6.1224	Tr	9.1837	Tr
	RR	20.2156	0.0387	20.2156	0.0387
BHA and TBHQ	RT	5.6818	Tr	11.3636	Tr
	RR	19.1327	0.0335	19.1327	0.0335

[a] Values indicate the means of 6 samples.
[b] RT = incubation at room temperature (23 ± 1°C) for 5 days.
[c] RR = incubation at refrigeration temperature (7°C) for 8 wk and then room temperature (23 ± 1°C) for 5 days.
[d] Tr = trace amounts.
[e] BHA and TBHQ were combined in equal amounts.

reaction between the antioxidant and a lipid molecule. The hydrophobic nature of antioxidants and their solubility in lipid compounds might cause the antioxidants to be localized within the lipid portion of foods, thus becoming unavailable to act against microorganisms.

It is unlikely that BHA, TBHQ, BHT, or PG could be used alone to control the growth of microorganisms in foods, since the required amounts appear to exceed by far the legally allowed amounts (0.02% based on weight of fat). However, the antioxidants could be useful in combination with other antimicrobials, especially those such as sorbic acid and monoglycerides which show synergistic action with the antioxidants (Branen et al., 1980).

The effect of antioxidants against the test microorganisms on salami, as compared to the control, was analyzed using two-way analysis of variance and Duncan's multiple range test. No difference occurred ($P > 0.05$) between control and antioxidants BHT and PG at the concentration tested, while BHA, TBHQ, and BHA/THBQ inhibited ($P < 0.05$) growth of all test strains. The combination of BHA and TBHQ was the most effective treatment ($\alpha = 0.05$) against aflatoxin production by aspergilli. No difference was noted between BHA and TBHQ alone. Statistical analysis indicated that cultures producing higher amount of toxins were more resistant to antioxidants ($\alpha = 0.05$).

Little work has been done on elucidation of mechanisms of microbial inhibition by the phenolic antioxidants. It is most likely that they act on the cytoplasmic membrane of

the microorganisms. Surak et al. (1976) reported that BHT caused leakage of intracellular contents from *Tetrahymena pyriformis*. Davidson and Branen (1980) found that BHA caused leakage of intracellular protein from *Pseudomonas fluorescens*. The mechanism of microbial inhibition might also be related to the destruction or inactivation of essential enzyme and/or genetic material (Branen et al., 1980).

Butylated hydroxyanisole, an analogue of one of the five rings of aflatoxin, when present in the growth medium, may be incorporated by the organism into precusors of aflatoxins instead of the correct component in the biosynthetic pathway and thus inhibit toxin formation. This activity probably accounted for the antifungal effect of BHA against aflatoxin production by *A. flavus* (Fung et al., 1977).

CONCLUSION

THE RESULTS of growth inhibition studies on agar demonstrated that BHA and TBHQ alone or in combination were effective in inhibiting growth, sporulation, and aflatoxin production by three toxigenic and three nontoxigenic strains of aspergilli. Combination of BHA and TBHQ was effective as a synergistic inhibitor. Propyl gallate and BHT did not show an inhibitory effect on the growth and aflatoxin production of aspergilli. The higher the amounts of antioxidants used, the greater the inhibition zone obtained on solid medium.

The antioxidants added to salami were less inhibitory to the growth of the test microorganism in salami than they were in laboratory medium. All three treatments (BHA, TBHQ, and BHA/TBHQ), at 100 mg/L tested level, in salami were found to inhibit significantly ($P < 0.05$) growth of the test organisms when compared to control samples. Combination of BHA and TBHQ showed synergistic effect.

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Thermal Stability of Folic Acid and 5-Methyltetrahydrofolic Acid in Liquid Model Food Systems

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ABSTRACT

The effects of temperature, iron and ascorbate fortification, and oxygen concentration on the stabilities of folic acid (FA) and 5-methyltetrahydrofolic acid (5-CH₃-THF) were examined in liquid model food systems. Small retort pouches were used as reaction vessels and the model systems were processed at 100°C, 120°C, and 140°C. A cation-exchange procedure was devised to provide sample extract purification before quantitation using high-performance liquid chromatography (HPLC). FA and 5-CH₃-THF were found to be very stable under these conditions. Lactose, protein, iron and ascorbate were all found to be capable of reducing the oxygen partial pressure within the model systems, thereby enhancing the stability of FA and 5-CH₃-THF. FA and 5-CH₃-THF degradation was not zero or first order but possibly second order as a result of limiting oxygen concentrations.

INTRODUCTION

SEVERAL NUTRITION SURVEYS have documented the widespread existence of marginal folacin status among certain American population groups, such as infants, adolescents, pregnant women, and the elderly (Chung et al., 1961; Ten-State Nutrition Survey, 1968–1970; Bailey et al., 1980). Inadequate folacin nutriture may result from insufficient content, instability of dietary folacin or inadequate bioavailability of certain forms of the vitamin. Limitations in analytical methodology for the determination of total folacin complicate the problem of assessing dietary adequacy and folacin nutritional status.

Several studies have shown that extensive losses of folacin may occur during the thermal processing of foods (e.g. Ford et al. 1969; Burton et al., 1970; Rolls and Porter, 1973; Miller et al., 1973; Taguchi et al., 1973; Keagy et al., 1975; Malin, 1977; Leichter et al., 1978). Herbert (1968) estimated that as much as 95% of the initial folacin in food could be lost by oxidative processes during heating. Ramasastri (1965) determined that the folacin requirement for infants can be met fully by a milk diet; however, boiling cow's milk destroys heat-labile folacin (Ghitis, 1966; Ford, 1967; Burton et al., 1967; Ek and Magnus, 1980).

The rates of the reactions responsible for folacin degradation are dependent upon several factors, such as temperature and length of heating time, source of heat, oxygen, light, chemical environment, pH, and leaching loss (Malin, 1975); however, the effects of these factors on the stability of the individual folacin derivatives in realistic model food systems have not been previously investigated. Also, the specific effects of individual food components, such as proteins, reducing sugars, and metal ions, on folacin stability, have not been studied. Previous research involving folacin stability in distilled water or aqueous buffers (Dick et al., 1948; Garrett, 1956; Colman et al., 1975; O'Broin et al., 1975; Cooper et al., 1978; Chen and Cooper, 1979; Paine-Wilson and Chen, 1979; Ruddick et al., 1980) has little practical value in the food industry since food components and other environmental factors can have a dramatic effect

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on nutrient stability. Furthermore, the use of impractical or economically infeasible experimental conditions (e.g. 0.1% ascorbic acid, extreme pH's and nitrogen storage) have little applied value except for perhaps indicating trends that might be observed under more realistic food conditions.

MATERIALS & METHODS

Preparation of model food systems

Model food systems were based on a typical commercially available liquid infant formula ("Enfamil," Mead Johnson & Co.) and were prepared by dissolving 7% w/v D(+)lactose (Mallinckrodt Inc.) and 1.5% w/v potassium caseinate (prepared from isoelectric casein obtained from United States Biochemical Corp.) in 0.1M potassium phosphate (pH 7.0). The model systems were supplemented with 10 μg/g levels of folic acid (FA) and 5-methyltetrahydrofolic acid (5-CH₃-THF) (Sigma Chemical Co.). In studies concerned with the identification of folacin degradation products, the model systems were individually fortified with various levels of FA or 5-CH₃-THF. Nutritional levels of iron [added as ferrous sulfate heptahydrate at 25% NAS/NRC RDA for infants per 10-oz serving (1.34 mg Fe/100 ml)] and/or ascorbic acid [added as sodium ascorbate at 50% NAS/NRC RDA for infants per 10 oz. serving (5.64 mg ascorbic acid/100 ml)] were systematically added to selected model systems so as to provide unfortified, iron, iron and ascorbate, and ascorbate fortified systems. All of the model systems were prepared in duplicate and individually pouched for each processing temperature and time combination. Control model systems received no thermal processing. During constitution, the systems were protected from light so as to minimize the degradation of ascorbate, FA, and 5-CH₃-THF prior to thermal processing. FA and 5-CH₃-THF supplementation solutions were prepared as outlined by Day and Gregory (1981), except that ascorbate was not used in the diluting solutions. These folacin solutions were prepared immediately prior to use and added to the fully constituted model systems.

Thermal pouch processing

Aliquots of the fully constituted model systems (ca 10 ml) were siphoned into small (7.5 x 7.3 cm) retort pouches (American Can Co.). The pouch material consisted of a lamination of polyester, aluminum foil and polypropylene copolymer films with the polypropylene exposed to the model system so that the possibility of metal contamination was eliminated. Undisclosed levels of antioxidant(s) are routinely incorporated into food-grade polypropylene. The filled pouches were sealed through the liquid, using a Vertrod Thermal Impulse Heat Sealer, thereby providing effectively no headspace gas above the pouched model systems. All of the model systems were pouched within 30 min of folacin supplementation.

The pouched model systems were placed in a rack (9 x 9 cm) which could hold up to 10 pouches at any one time and prevented undue movement of the pouches during thermal processing. The separation of approximately 3mm between the pouches has been found to be sufficient to provide equal thermal treatments to all the individual pouches in the rack (Roop and Nelson, 1981; Hiner, 1982). The model systems were then processed for various times at 100°C (in a boiling water bath), 120°C, and 140°C in a thermal death time retort similar to that used by Schmidt et al. (1955). A 60 psi steam line and high pressure water line allowed for rapid (<30 sec) come up and cool down periods respectively. Temperature control was effected by throttling down the 60 psi steam line with a globe valve while a bleeder valve was utilized for fine control. Processing temperatures were stabilized to ±1°C within 1 min.

Retort and pouch temperatures were monitored using thermo-couples and a Speedomax recorder (Leeds and Northrup Co.). The retort pouches had a 10-sec lag time behind the retort temperature during the come up and cool down periods. The short and compensating heating and cooling phases, as well as the rapid heat transfer through the small retort pouches, enabled a steady-state procedure (Lenz and Lund, 1980) to be assumed in kinetic modeling. Unprocessed control and thermally processed model systems were analyzed immediately or frozen at $-20°C$ before subsequent analysis.

Extraction and preparative chromatography

A 5-ml aliquot of each unprocessed control and processed model system was diluted with 4.87 ml of 0.1M potassium phosphate containing 1.0% w/v ascorbate (pH 7.0). The pH was adjusted to 4.5 with 6N HCl (0.13 ml) and the resulting suspension was centrifuged at 25000 x g for 5 min. A 5-ml aliquot of the supernatant was applied to a column (0.7 x 20 cm, ca 12 cm bed height) containing 100–200 mesh Bio-Rad AG 50W-X8, K^+ form (Bio-Rad Laboratories), which had been previously equilibrated with 0.1M potassium acetate containing 1.0% w/v ascorbate (pH 4.0). The cation-exchange resin was then washed with 10–20 ml of the pH 4.0 acetate-ascorbate buffer. Under these conditions, FA and 5-CH₃-THF were selectively retained while many other components were effectively eluted from the column. FA and 5-CH₃-THF were then eluted with 20 ml of 0.1M potassium phosphate containing 1.0% w/v ascorbate and 9.0% v/v acetonitrile (pH 7.0) into a 25 ml volumetric flask which was diluted to volume with the phosphate-ascorbate-acetonitrile buffer. The column eluate was filtered (0.45 μm) before HPLC analysis. One analysis was performed for each pouched model system. Recoveries for FA and 5-CH₃-THF were found to range from 70–90% and depended primarily on the volume of the acetate-ascorbate wash used. Those model systems which received a severe heat treatment required a greater volume wash (up to 20 ml) to elute the higher concentrations of contaminants (thought to be predominantly Maillard browning components). The greater volume wash caused a reduction in the FA and 5-CH₃-THF recoveries obtained.

HPLC analysis of folacin

FA and 5-CH₃-THF concentrations in the control and processed model systems were determined using the HPLC procedure previously developed (Day and Gregory, 1981). The experimental conditions were identical to those previously reported except that the post-column derivatization was not utilized and the acetonitrile concentration in the isocratic mobile phase and the mobile phase flow rate were increased to 11% v/v and 1.0 ml/min, respectively, so as to reduce the FA retention time.

FA and 5-CH₃-THF standards (0.1–10 μg/ml) were prepared by dilution of the model system supplementation solutions with 0.1M potassium phosphate (pH 7.0). HPLC injections were made at ambient temperature using a 100 μl sample loop. Both FA and 5-CH₃-THF were monitored by ultraviolet absorbance at 280 nm, and the quantitation of eluting peaks was achieved by peak height analysis.

HPLC and thin-layer chromatographic analysis
of folacin degradation products

The identification of postulated folacin degradation products, such as pterin-6-carboxylic acid (pt-6-COOH), p-aminobenzoyl glutamic acid (pABG), and 5-methyldihydrofolic acid (5-CH₃-DHF), was achieved using HPLC and thin-layer chromatography (TLC). Standards of pt-6-COOH and pABG (Sigma Chemical Co.) were initially dissolved in 5% w/v dibasic potassium phosphate (pH 9.1) before dilution in 0.1M potassium phosphate containing 0.25% w/v ascorbate (pH 7.0). 5-CH₃-DHF was prepared by the method of Maruyama et al. (1978) which involved the oxidation of 5-CH₃-THF by 2% KMnO₄. The pt-6-COOH, pABG, and 5-CH₃-DHF standards were prepared immediately prior to use, protected from light, and stored at 4°C.

Pt-6-COOH and pABG were detected using the HPLC procedure of Day and Gregory (1981), with an acetonitrile concentration in the isocratic mobile phase of 8.0% (v/v). 5-CH₃-DHF was found to coelute with 5-CH₃-THF using several different HPLC conditions. Therefore, a modification of the TLC procedure of Scott (1980) was used to adequately separate 5-CH₃-DHF from 5-CH₃-THF.

Unfortified model systems (containing no iron or ascorbate) were prepared as previously outlined. These model systems were supplemented with 100 μg/g of 5-CH₃-THF, pouched, and processed for 30 min at 140°C. Control model systems, which received no thermal processing, were also prepared. The absence of iron and ascorbate fortification in the model systems and the extensive thermal processing were selected to enhance the possible formation of 5-CH₃-DHF. The TLC mobile phase used was 0.1M sodium phosphate containing 0.5% w/v ascorbate (pH 7.0). The 5-CH₃-THF and 5-CH₃-DHF standards were applied to precoated plastic plates (20 x 20 cm) of nonionic 13254 cellulose powder containing fluorescent indicator No. 6065 (Eastman Kodak Co.). The folacin standards were applied in the minimum possible volume (usually less than 10 μl) so that approximately 5 μg of each derivative was applied. The control and processed model systems were also directly applied in the minimum volume to achieve a concentration greater than approximately 150ng of folacin activity per sample. The TLC plates were dried with nitrogen gas, before and after development, to minimize oxidation.

Oxygen partial pressure measurements

The oxygen partial pressures of selected model systems were measured utilizing a YSI Biological Oxygen Monitor (Yellow Springs Instrument Co.). The oxygen probe used was a Clark-type polarographic electrode and all measurements were made at 25°C. Unfortified, iron, iron and ascorbate, and ascorbate foritified model systems, as well as an iron fortified model system containing no folacin supplementation, were prepared. Also, lactose, potassium caseinate, ascorbate, iron, and the folacin standards were individually constituted in 0.1M potassium phosphate (pH 7.0). All the systems were pouched and processed for 2 hr at 100°C. Distilled water and 0.1M potassium phosphate (pH 7.0) were similarly pouched and thermally processed. Control model systems received no thermal processing. After processing the model systems were cooled to 25°C. The retort pouches were opened and immediately analyzed to minimize the diffusion of atmospheric oxygen into the individual systems.

RESULTS

Thermal stability of FA and 5-CH₃-THF

Typical HPLC chromatograms of an unfortified model system which had received various thermal treatments are shown in Fig. 1. Similar HPLC chromatograms were obtained from the various model systems. FA was adequately resolved in all the model systems analyzed after processing at 100°C, 120°C and 140°C whereas 5-CH₃-THF was only resolved adequately after processing at 100°C and 120°C. Model systems which did not receive FA or 5-CH₃-THF supplementation exhibited no components which could interfere with the quantitation of FA or 5-CH₃-THF when processed as described above.

Fig. 2, 3, and 4 illustrate the thermal stability of FA in unfortified, iron and ascorbate, iron, and ascorbate fortified model systems processed at 100°C, 120°C, and 140°C, respectively, for various times. Each point on these and subsequent graphs, represents the mean of duplicate processing runs, with agreement between duplicates generally within 5%. FA was found to be relatively stable in all the model systems, with 75–92% retention after processing at 100°C for 2 hr, 68–85% retention after processing at 120°C for 20 min (a typical commercial thermal treatment for infant formula), and 40–55% retention after processing at 140°C for 15 min. A general trend observed after all thermal treatments was that FA had the highest stability in the iron fortified model systems; had intermediate stability in the ascorbate, and iron and ascorbate fortified model systems; and was the least stable in the unfortified model systems. Fig. 5 illustrates the semi-logarithmic plot of FA degradation at 100°C, 120°C, and 140°C in an ascorbate fortified model system. The curved degradation lines observed at 120°C and 140°C were also obtained from the other model systems, which gives strong evidence that the FA degradation in these model systems was not zero, first, or pseudo-

first order, but higher than first order and possibly second order.

Fig. 6 and 7 illustrate the retention of 5-CH$_3$-THF in unfortified, iron and ascorbate, iron, and ascorbate forti-fied model systems processed at 100°C and 120°C for various times. 5-CH$_3$-THF was found to be very stable in all the model systems with 86–91% retention after processing at 100°C for 2 hr, and 88–94% retention after processing at 120°C for 20 min. Due to this high stability, very little variability in 5-CH$_3$-THF stability was observed between the different model systems. However, composition effects
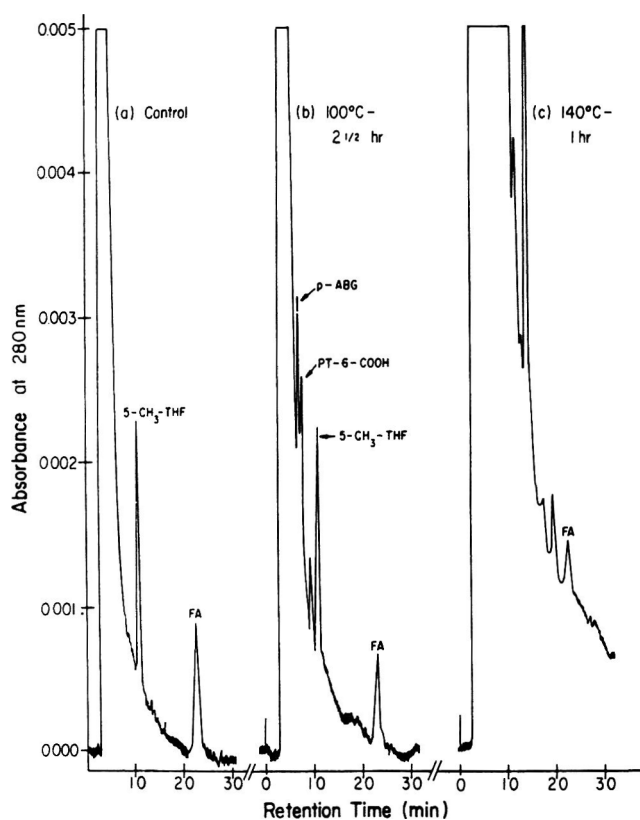
Fig. 1—Typical HPLC chromtograms of an unfortified model food system which had received various thermal treatments.
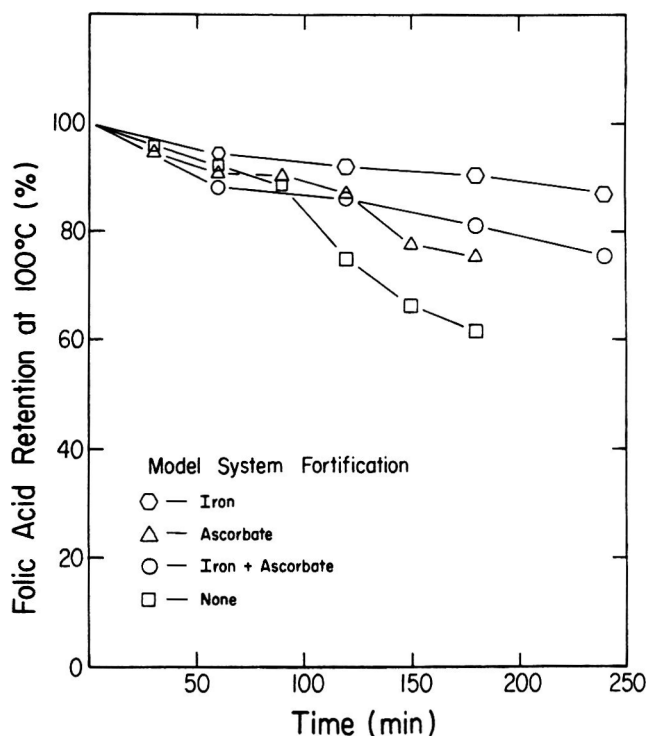
Fig. 2—Thermal stability of folic acid in different model food systems processed at 100°C for various times.
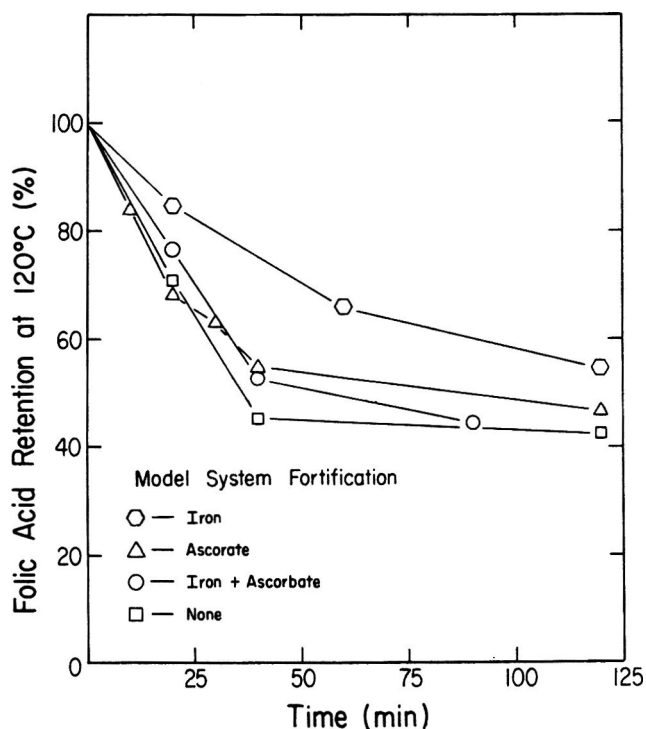
Fig. 3—Thermal stability of folic acid in different model food systems processed at 120°C for various times.
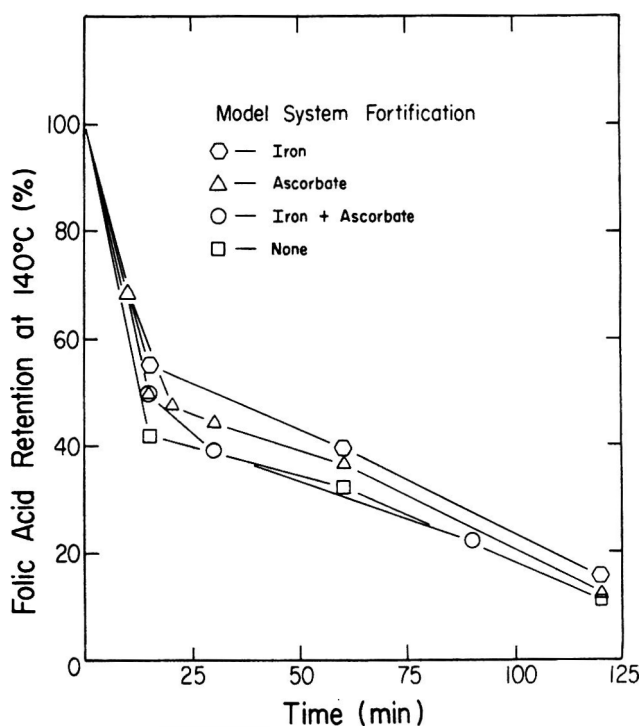
Fig. 4—Thermal stability of folic acid in different model food systems processed at 140°C for various times.

similar to those obtained for FA at 100°C were observed after processing at 120°C for longer than 30 min. A similar curved degradation line at 120°C to that obtained for FA in Fig. 5 was obtained for 5-CH₃-THF degradation from all the model systems when the 5-CH₃-THF thermal stability data were plotted semilogarithmically. These results give strong evidence that the 5-CH₃-THF degradation in these systems, like that of FA, was not zero, first, or pseudo-first order but higher than first order and possibly second order.

Data for the thermal stability of 5-CH₃-THF in the different model systems processed at 140°C was not obtained because of coeluting contaminants which precluded HPLC quantitation. These contaminants were thought to be predominantly Maillard browning components which were not selectively eluted from the Bio-Rad AG 50W-X8 column during the wash phase. The fact that both FA and 5-CH₃-THF were found to be very stable at 100°C and 120°C suggests that 5-CH₃-THF would have probably followed a similar stability trend to that of FA at 140°C.

Identification of folacin degradation products

Pt-6-COOH and pABG were tentatively identified as degradation products of FA on the basis of HPLC retention. These degradation products were found to be formed solely from FA supplemented thermally-processed model systems, and not from similar 5-CH₃-THF supplemented model systems. The rapidly elution pt-6-COOH and pAGB peaks (Fig. 1) were fully resolved from the ascorbate peak by decreasing in the acetonitrile concentration from the normal 9.5 to 8.0% in the isocratic HPLC mobile phase. The peak heights of these FA degradation products were found to increase while the corresponding FA peak height decreased with the increasing severity of thermal processing employed, although no attempts were made to quantitate the degradation products.

Fig. 8 illustrates the TLC separation of 5-CH₃-THF and 5-CH₃-DHF. The observed R$_f$ values for 5-CH₃-THF and 5-CH₃-DHF were 0.86 and 0.58 respectively, which compared very closely to the R$_f$ values of 0.86 and 0.56 quoted

by Scott (1980). 5-CH₃-THF and 5-CH₃-DHF were detected as faint blue fluorescent spots at 254 and 366 nm, in contrast to the quenched spots observed by Scott. This anomaly was probably caused by the different fluorescent
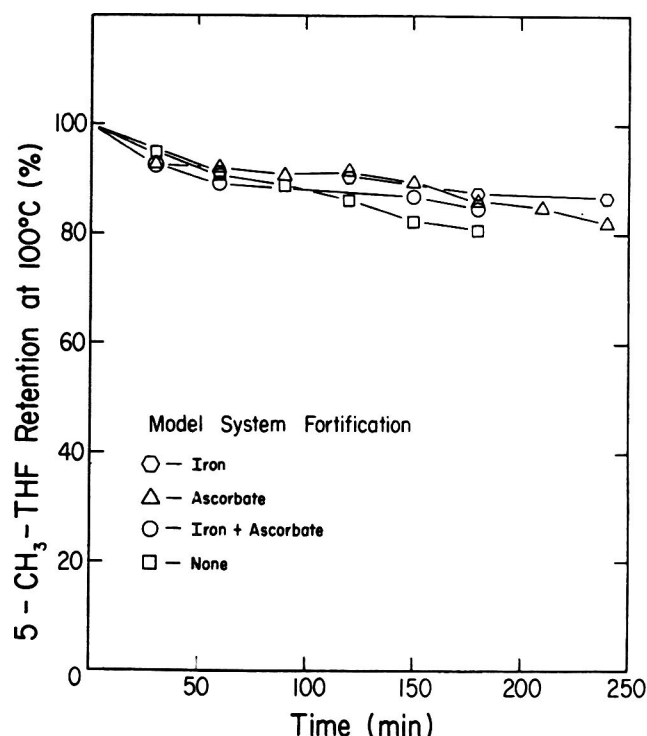
Fig. 6—Thermal stability of 5-methyltetrahydrofolic acid (5-CH₃-THF) in different model food systems processed at 100°C for various times.

Fig. 5—Semi-logarithmic plot of folic acid degradation at 100°C, 120°C, and 140°C for various times in ascorbate fortified model food systems.
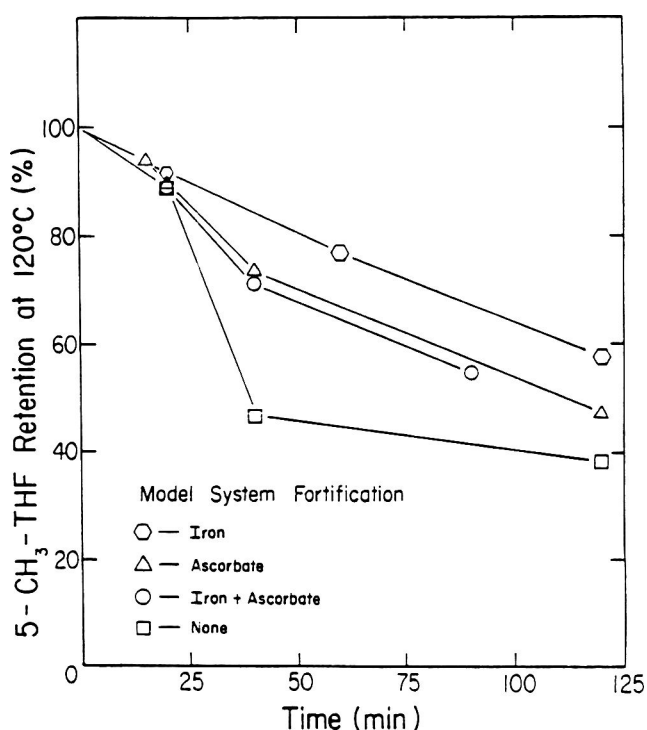
Fig. 7—Thermal stability of 5-methyltetrahydrofolic acid (5-CH₃-THF) in different model food systems processed at 120°C for various times.

indicator used in this study. The TLC separations achieved from control and processed model systems, fortified with 5-CH₃-THF, are also shown in Fig. 8. The model system components were not found to influence the mobility of 5-CH₃-THF and 5-CH₃-DHF. Fig. 8 shows that 5-CH₃-DHF was not detected after processing at 140°C for 30 min. On the basis of the similar stability of FA and 5-CH₃-THF, at least two-thirds of 5-CH₃-THF would be expected to degrade after such a severe thermal treatment. If 5-CH₃-THF oxidized completely to 5-CH₃-DHF, then approximately 3.3 μg of 5-CH₃-DHF would be expected since 5.0 μg of 5-CH₃-THF was initially present in the spotted control model system. The minimum detectable level of 5-

CH₃-DHF was determined to be approximately 130 ng. Therefore, it can be concluded that 5-CH₃-DHF was not a degradation product or was a negligible degradation product of 5-CH₃-THF (less than 4% conversion), under the conditions employed in this study. This TLC procedure additionally gave evidence that pABG was not formed as a product of 5-CH₃-THF degradation under the conditions employed in this study. Scott (1980) found that pABG had an R_f value of 0.94 but Fig. 8 shows that no response was observed in either the control or processed model systems, at this R_f value. The unidentified blue fluorescent spot with an R_f value of 0.80 which was observed in both control and processed model systems was due to some model system component(s) since it was observed consistently in both unsupplemented control and processed model systems.

Oxygen partial pressure measurements

The oxygen partial pressure measurements on selected model systems are shown in Table 1. The values quoted represent the mean of duplicate analyses. Oxygen concentration and molar oxygen values were calculated directly from these partial pressure values. The results obtained indicate that the oxygen partial pressures of the individual systems were dependent on the system composition and the thermal processing conditions employed. The separate model system components, i.e. the folacin standards, ascorbate, iron, potassium caseinate, and lactose, all had the ability to reduce the oxygen partial pressure in their respective systems. These reductions seemed to be additive since the oxygen partial pressure of all the model food systems assumed very low values even at ambient temperature. Lactose and potassium caseinate were the most effective model system components at reducing the oxygen partial pressure, however on a molar basis, iron and secondly ascorbate were considerably more effective in this respect. Processing at 100°C for 2 hr caused a reduction in the oxygen partial pressure in all model systems examined. This reduction was most marked in the iron, lactose, potassium caseinate, and ascorbate systems. A very small reduction in the oxygen partial pressure was observed after processing the model food systems, while a slight reduction was observed after processing the distilled water, phosphate buffer, and folacin standards in buffer, systems.

—Continued on next page

Fig. 8—Thin-layer chromatographic separation of 5-methyltetrahydrofolic acid (5-CH₃-THF) and 5-methyldihydrofolic acid (5-CH₃-DHF). Samples: 5-CH₃-THF (5 g); 5-CH₃-DHF (5.1 µg); model food system (M.F.S.) containing 5 g 5-CH₃-THF; M.F.S. containing 5 g 5-CH₃-THF and processed at 140°C for 30 min. Circled areas on drawing denote blue fluorescing components under excitation at either 254 or 366 nm.

Table 1—Oxygen measurements on selected systems

System	Control pO₂ (mm Hg)	Control O₂[a] (ppm)	Control O₂[b] (µmol/g)	100°C for 2 hr pO₂ (mm Hg)	100°C for 2 hr O₂[a] (ppm)	100°C for 2 hr O₂[b] (µmol/g)
Distilled water	149	7.8	0.24	134	7.0	0.22
0.1M potassium phosphate buffer	146	7.6	0.24	130	6.8	0.21
5-CH₃-THF and FA in buffer	133	7.0	0.22	118	6.2	0.19
Ascorbate in buffer	118	6.2	0.19	99	5.2	0.16
Iron in buffer	109	5.7	0.18	61	3.2	0.10[c]
Potassium caseinate in buffer	99	5.2[c]	0.16[c]	61	3.2[c]	0.10[c]
Lactose in buffer	86	4.5[c]	0.14[c]	43	2.3[c]	0.07[c]
Unfortified M.F.S.[d]	37	1.9[c]	0.06[c]	34	1.8[c]	0.06[c]
Ascorbate fortified M.F.S.[d]	34	1.8[c]	0.06[c]	32	1.7[c]	0.05[c]
Iron fortified M.F.S.[d] without 5-CH₃-THF and FA	34	1.8[c]	0.06[c]	30	1.6[c]	0.05[c]
Iron and ascorbate fortified M.F.S.[d]	30	1.6[c]	0.05[c]	27	1.4[c]	0.04[c]
Iron fortified M.F.S.[d]	26	1.4[c]	0.04[c]	22	1.2[c]	0.04[c]

[a] 100% air saturated distilled water contains 8.38 ppm O₂.
[b] Calculated using the expression, (ppm O₂)/32.
[c] Values subject to considerable over-estimation due to the high solids concentration in these systems (Strang, 1981).
[d] M.F.S. denotes model food system.

DISCUSSION

Thermal stability of FA and 5-CH3-THF

The thermal stability of FA was found to be high in this study but less than the stability reported by other researchers. The 75–95% retention of FA, after processing at 100°C for 2 hr in all the model systems, compared with the greater than 95% retentions observed, after processing at 100°C for 2 hr at neutral pH, by Colman et al. (1975) and Paine-Wilson and Chen (1979). The slightly lower thermal stability of FA could be explained by the use of 0.1M potassium phosphate in all the model systems since O'Broin et al. (1975) found FA to be less stable in phosphate buffers. The thermal stability of FA in the model systems at 120°C and 140°C was also found to be very high, however the data obtained could not be compared since no previous research has investigated the thermal stability of FA or 5-CH3-THF at these elevated temperatures. Ristow et al. (1982b) provided evidence that FA does not interact with the components of the model system during thermal processing to form complexes which might affect its bioavailability. The combined results of these studies and that of Colman et al. (1975) support the efficacy of FA fortification prior to food processing.

The thermal stability of 5-CH3-THF was found to be very high in this study and considerably higher than the stabilities reported by other researchers. The 86–91% retention of 5-CH3-THF after processing at 100°C for 2 hr in all model systems was in marked contrast to the 10–20 min half-life of 5-CH3-THF at 100°C and neutral pH reported by Cooper et al. (1978), Chen and Cooper (1979), Paine-Wilson and Chen (1979), and Ruddick et al. (1980). O'Broin et al. (1975) and Ruddick et al. (1980) observed extensive losses of 5-CH3-THF after 2 hr at room temperature and 40°C respectively. 5-CH3-THF stability data at temperatures above 100°C can be estimated from research involving the processing of milk since Shin et al. (1975) reported that 92% of cow's milk folacin is in the 5-methyl form. The 88–94% retention of 5-CH3-THF, after processing at 120°C for 20 min in all the model systems, compared with the 50% retention reported by Ford (1967) for the sterilization of cows' milk at 111°C for 20 min. Ford et al. (1969) and Burton et al. (1970) reported an approximate 80% retention of folacin activity after the UHT processing of cows' milk.

FA and 5-CH3-THF are degraded by oxidative mechanisms requiring the presence of molecular oxygen (Chen and Cooper, 1979). Therefore, the high stabilities of FA and especially 5-CH3-THF are thought to be primarily due to the low oxygen partial pressures within the model systems, which were a consequence of the negligible headspace gas in the retort pouches and the consumption of oxygen by the model system components. Both FA and 5-CH3-THF were found to be most stable in the iron fortified model systems in which the oxygen partial pressures were the lowest, while they were least stable in the unfortified model systems in which the oxygen partial pressures were correspondingly the highest.

The approximate molar oxygen concentrations in all the model systems were in the range 0.04–0.06 μmol/g. The corresponding molar folacin concentrations were 0.02 μmol/g, which indicates that molecular oxygen was probably partially limiting in these model systems. Limiting oxygen concentrations would help explain the unexpected high thermal stability of 5-CH3-THF, and could also account for the higher than first order degradation kinetics observed for 5-CH3-THF and FA in the model systems. Ruddick et al. (1980) calculated second order rate constants for 5-CH3-THF degradation at 20°C at oxygen concentrations of 9.6 and 14.4 ppm respectively. The oxygen

concentrations in the model systems were approximately 1.5–2.0 ppm which would also partially explain why the thermal stability of 5-CH3-THF was found to be considerably higher in this study compared with the thermal stabilities quoted by Ruddick et al. (1980). The limited oxygen hypothesis is also supported by Ford (1967), Ford et al. (1969), and Burton et al. (1970) who found the thermal stability of milk folacin to be markedly enhanced when the oxygen partial pressure within the milk was limited. Furthermore, Chen and Cooper (1979) reported that 5-CH3-THF was considerably stabilized when heated at 100°C under a nitrogen atmosphere. Chen and Saad (1981) also found that nitrogen-packing enhanced folacin retention in Egyptian mulukhiyah during storage. The high stability of 5-CH3-THF could also be partially explained by the presence of ascorbate and lactose in the model food systems. These reducing agents would enhance the stability of 5-CH3-THF by helping it to be maintained in the reduced THF form. The protective effect of ascorbate on the stability of 5-CH3-THF has been previously demonstrated by Ford (1967), Chen and Cooper (1979), and Ek and Magnus (1980). The presence of antioxidant(s) in the polypropylene copolymer pouch film could possibly enhance the stability of 5-CH3-THF, if free radical mechanisms were involved, although the role of phenolic antioxidants has not been examined.

Kinetic modeling of FA and 5-CH3-THF degradation in the model systems was not possible since the data obtained were not sufficient for determination of a reaction order. Also, statistical analysis of the variation in folacin stability observed between the different model systems was not possible since the data obtained did not justify the application of non-linear statistical design. Nevertheless, the data obtained in this study strongly indicate the consistent protective effects of both iron and ascorbate on the thermal stability of FA and 5-CH3-THF in the model systems.

Folacin degradation products

The degradation products of FA were tentatively identified as pt-6-COOH and pABG. This finding is supported by Stokstad et al. (1948) who reported that FA is oxidatively cleaved between C-9 and N-10 to yield these nutritionally inactive derivatives. The observation that pABG and pt-6-COOH were not found as degradation products of 5-CH3-THF supports the hypotheses that substitution at the N-5 position of THF stabilizes the pteridine ring moiety, thereby making the folacin molecule very resistant to oxidative cleavage (Rabinowitz, 1960; Stokstad and Thenen, 1972). In contrast, THF is very susceptible to oxidative cleavage, which produces various pterins and pABG (Reed and Archer, 1980).

Gupta and Huennekens (1967) reported that 5-CH3-DHF is readily reduced to 5-CH3-THF upon the addition of a reducing agent, such as mercaptoethanol or ascorbate. The addition of these reducing agents is necessary to adequately protect the labile 5-CH3-THF remaining after thermal processing. However, a falsely high stability of 5-CH3-THF could be observed if the 5-CH3-DHF, formed during the oxidative degradation of 5-CH3-THF, was reduced back to 5-CH3-THF during the assay procedure. This possible error in the quantitation of 5-CH3-THF by HPLC was determined to be negligible since the TLC analysis of the model systems, without using ascorbate during and after thermal processing, showed that 5-CH3-DHF was not a significant degradation product of 5-CH3-THF. The importance of 5-CH3-DHF assessment is illustrated by recent observations which indicate approximately 60-80% molar folacin activity of dietary 5-CH3-DHF in chick bioassays (Ristow et al., 1982a). Further research

is required for the identification of the biologically inactive derivatives of 5-CH$_3$-THF degradation.

Oxygen partial pressure measurements

The various model system components all had the ability to reduce the oxygen partial pressure in their respective systems even at room temperature. The oxygen partial pressure, as opposed to the oxygen concentrations, of a solution is not affected by an increased solute concentrations (Strang, 1981). Therefore the decrease in the oxygen partial pressure must have been due to the chemical consumption of molecular oxygen, a lack of adequate equilibrations between the oxygen in the separate model systems and oxygen in the retort pouch headspace gas, or a change in the atmospheric pressure. The latter case was an insignificant consideration in this study but the lack of equilibration between the oxygen in the separate model systems and oxygen in the headspace gas was observable from the oxygen partial pressure measurements on the distilled water and 0.1M potassium phosphate systems. The oxygen partial pressures of these systems would be expected to remain constant after thermal processing and subsequent cooling since they contain no component that could cause the chemical consumption of molecular oxygen. However, a 10% decrease in the oxygen partial pressure was observed in both processed model systems. This was probably due to the fact that oxygen in solution is less soluble at increasing temperatures and therefore during thermal processing a small headspace may have been formed within each retort pouch. Insufficient time may have been allowed for the equilibration between the model system oxygen and headspace oxygen after subsequent cooling of the retort pouches before oxygen partial pressure measurements.

The findings that iron, ascorbate, and folacin compounds all had the ability to reduce the oxygen partial pressure within their respective systems was expected since ferrous iron oxidizes, in the presence of water, to ferric iron; ascorbate is oxidized to dehydroascorbate; and, FA and 5-CH$_3$-THF are both degraded by oxidative processes requiring the presence of molecular oxygen. It was found that the iron fortified model systems assumed lower oxygen partial pressures than the combined iron and ascorbate fortified model systems. This surprising observation can be explained by the probable chelation of iron by ascorbate, which could lessen the oxygen consumption ability of each.

The findings that lactose and potassium caseinate both had the ability to reduce the oxygen partial pressure within their respective systems, was slightly unexpected. Certain amino acid residues of potassium caseinate are susceptible to oxidation. For example, cystine may be oxidized to cysteic acid while methionine may be oxidized to methionine sulfoxide. These oxidations would not be extensive; however, slight oxidation would significantly reduce model system oxygen levels because of the high molar ratios present. Lactose is relatively inert to oxidation however its carbonyl group may be selectively oxidized to a carboxyl group to form its aldobionic acid, lactobionic acid (Webb and Johnson, 1965), with the resulting consumption of molecular oxygen. Although this reaction is unlikely, especially at room temperature, oxidation of only a small fraction of the total lactose would significantly reduce the oxygen partial pressure within the model systems since, on a molar basis, lactose is approximately 250 times more abundant than molecular oxygen.

In summary the thermal stability of FA and 5-CH$_3$-THF has been shown to be markedly enhanced by a variety of factors which reduced dissolved oxygen concentrations in model food systems. The results of this study indicate that the retention of FA added in fortification, and 5-CH$_3$-

THF, the predominant naturally occurring folacin vitamer, would be enhanced by packaging and processing under low oxygen conditions. The addition of ferrous iron and/or ascorbic acid at levels routinely used in fortification of foods such as infant formulas also improves the thermal stability of folacin.

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Relationships Between Hydrophobicity and Foaming Characteristics of Food Proteins

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―――――――――ABSTRACT―――――――――

Hydrophobocity measured fluorometrically for food proteins and pure proteins using cis-parinaric acid as a hydrophobic probe had significant correlations to foaming capacity when the proteins in solution were unfolded by heating in boiling water in the presence of 1.5% dodecyl sulphate prior to fluorometric measurement. Hydrophobicity measured without unfolding, which had previously shown a significant correlation to emulsification, was not significantly correlated with foaming capacity. Two highly significant regression equations were generated: one included hydrophobicity and dispersibility and the other, hydrophobicity and viscosity as the independent variables. High hydrophobicity and viscosity and moderate dispersibility were associated with optimum foaming capacity. There was a significant negative relationship between foam stability and charge density. Hydrophobicity and viscosity were also important in foam stability.

INTRODUCTION

PROTEIN FOAMS are important for several categories of foods including meringues, souffles, whipped toppings, chiffon desserts and leavened bakery products. Due to the importance of protein foams in the food industry, a large number of empirical studies have been carried out to examine the foaming behavior of proteins under a variety of conditions (Cherry and McWatters, 1981; Kinsella, 1976). These studies have demonstrated that numerous factors including pH, temperature, the presence of salts, sugars and lipids and the protein source, affect the foaming behavior of proteins. Many other studies have concentrated on the relationship between foaming properties and the physical and chemical properties of surfaces, e.g., surface pressure, surface concentration, surface elasticity and surface viscosity (Graham and Phillips, 1976; Phillips, 1981). Few attempts have been made to elucidate the foaming mechanism of a protein solution based on its properties in the bulk phase; and such attempts have not been very successful. However, a thorough understanding of foaming in these terms should be of value to food technologists as it should provide a good basis for control of the performance of proteins as foaming agents in foods.

We have been investigating the relationship between protein surface hydrophobicity (S_O) and emulsifying capacity and found good correlations (Kato and Nakai, 1980; Nakai et al., 1980). The S_O was fluorometrically determined using cis-parinaric acid (cis-PnA) as a hydrophobic probe according to the method of Sklar et al. (1977). This close relation can be explained by easier transfer of more hydrophobic protein molecules to the oil/water interface, thereby preventing the coalescence of oil droplets. Since foaming is also a phenomenon which occurs at the interface, which in this case is air/water instead of oil/water, a similar explanation may apply. According to Phillips (1981) the dynamic dilatational modulus:

$$\epsilon = -A(d\pi/dA) \qquad (1)$$

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which is the change in the surface pressure ($d\pi$) caused by a relative change in surface area (dA/A), is important for foamability as well as foam stability. Since the surface pressure is the decrease in surface tension produced by adsorbed protein films at the surface and the adsorption is due to their amphiphilic nature, a close relation between the hydrophobicity and foaming ability of proteins can be expected. Horiuchi et al. (1978) were able to correlate the foam stability of five enzyme hydrolyzed proteins with the content of surface hydrophobic regions of the molecules which was fluorometrically measured using 8-anilinonaphthalene sulphonate as a hydrophobic probe.

A number of workers have shown that protein solubility makes an important contribution to the foaming behavior of proteins. Hermansson et al. (1971) found that the foaming capacity of fish protein concentrate was provided by the soluble protein, which accounted for 1% of the total protein. Wang and Kinsella (1976) found that for alfalfa leaf protein the pH-foaming capacity curve paralleled its pH-solubility profile.

Theory suggests that net proton charge should influence the adsorption of proteins at the air-water interface (Cherry and McWatters, 1981). Sato and Nakamura (1977) observed that when the net charge on egg white proteins was increased by succinylation their foaming capacity was enhanced. Similar results have been observed by Miller and Groninger Jr. (1976) for fish protein concentrate and by Franzen and Kinsella (1976a, b) for soy and leaf proteins.

The foaming characteristics of protein solutions should be influenced by their viscosity (Bikerman, 1973). However, experiments in which the relationship between foaming and solution viscosity was examined, using additions of inert materials to alter the viscosity, have yielded conflicting results. Sato and Hayakawa (1979) found that the foaming capacity of yeast protein was enhanced when solution viscosity was increased by addition of polyethylene glycol or methylcellulose but foam stability was unaffected. Mita et al. (1977) observed an increase in the stability of gluten foams when solution viscosity was increased by addition of sucrose.

This paper presents a study of the foaming capacity and foam stability of a variety of food related proteins in relation to physicochemical properties measured in bulk solution to support a new interpretation of the foaming mechanism of proteins in solution.

MATERIALS & METHODS

Material

Bovine serum albumin #4488, ovalbumin #1319 and lysozyme #1782 were purchased from ICN Nutritional Biochemicals, Cleveland, OH. β-Lactoglobulin #L-6879 from cow's milk, ovomucoid #T-9253, trypsin #T-8128 pancreatic type II, ribonuclease-A #R-5000, conalbumin #C-0755 type I from chicken white and porcine pepsin #P-7012 were obtained from Sigma Chemicals, St. Louis, MO. B-Casein was separated from skim milk by urea fractionation (Aschaffenburg, 1963) and purified by DEAE cellulose chromatography (Thompson and Pepper, 1964). κ-Casein A was prepared from skim milk by the method of Zittle and Custer (1963).

Since the amount of a protein required for the experiments carried out in this study was large, it was impossible to use many

pure protein samples because of their cost. Crude protein samples were also used to increase the degrees of freedom in multiple regression analysis, thus improving the accuracy of the analysis. Soy protein isolates (95% protein, General Mills, Minneapolis, MN; Promin D, 90% protein, Central Soya, Chicago, IL), pea protein isolate (M412-046, Century cultivar, 85% protein, POS Pilot Plant, Saskatoon, Sask; Pro-pulse W100, 90% protein, Griffith Lab., Scarborough, Ont.) and sunflower concentrate (75% protein, Dr. Sosulski, Univ. Saskatchewan, Saskatoon, Sask.) were used for this purpose. Acid solubilized gluten was prepared from vital gluten (Whetpro 75%, Industrial Grain Products, Thunder Bay, Ont.). The method of Wu et al. (1976) was followed using 50% acetic acid. Protein was precipitated at pH 4.5. The dried sample had a protein content of 94%. Canola protein isolate (88% protein) was prepared from defatted Canola meal (Canbra Foods, Lethbridge, Alberta) as described by Nakai et al. (1980). cis-Parinaric acid was purchased from Molecular Probe (Plano, TX). Sodium lauryl sulfate (SDS) was the specially pure grade from BDH Chemicals, Vancouver, B.C.

Methods

Enzyme hydrolyzed proteins were prepared by incubating 200 ml of a 10% dispersion of protein with 2 ml of Alcalase 0.6L (Novo Ind., Copenhagen, Denmark) at 50°C for 4 hr. The pH of the mixture was maintained at 8.0 throughout the incubation. The hydrolyzed product was freeze-dried after heating the neutralized solution to 90°C.

Foaming properties of proteins were measured using a column aeration apparatus similar to that of Waniska and Kinsella (1979). A 100 x 2 cm glass column fitted with a sintered glass disc (pore size 40–60 μm) at the bottom was used. Air was sparged through 15 ml of 0.1% protein solution for 2 min at a flow rate of 35 ml/min. The volume of foam was recorded as an index of the foaming capacity of the protein. The time (t min) required for the foam to collapse to half its maximum volume (V_m ml) was measured. Foam stability was defined as ($2t/V_m$ x 50) which is the time for 50 ml of foam to collapse when the collapsing velocity is $V_m/25$ ml/min.

Protein hydrophobicity was determined by the method of Kato and Nakai (1980) after modifications. A 1.5% protein solution dissolved in 0.1M phosphate buffer, pH 7.0, containing 0.002% SDS was diluted with the same buffer to make a series of protein solutions ranging from 0.002–0.1% protein. Then, 10 μl of cis-PnA (3.6 x 10^{-3}M in absolute ethanol containing 10 μg/ml of BHA to prevent oxidation) was added to a 2 ml aliquot of each solution. An Aminco Bowman spectrofluorometer, No. 4-8202 was used to measure the relative fluorescence intensities of the cis-PnA-protein conjugates. The measurements were made at an excitation wavelength of 325 nm and an emission wavelength of 420 nm. The instrument was standardized so that a mixture of 2 ml decane and 10 μl of cis-PnA had a fluorescence intensity of 45%. Surface hydrophobicity (S_O) was measured as the intital slope of the curve of % fluorescence intensity vs % protein plot. The initial 1.5% protein solution was heated for 10 min in boiling water in the presence of 1.5% SDS prior to dilution to make a series of protein solutions for measuring an exposed hydrophobicity (S_e).

Dispersibility was measured as follows: A 10 ml aliquot of 1% protein dispersion in 0.1M phosphate buffer, pH 7.0, was stirred at setting #3 on a Fisher Thermomix magnetic stirrer for 10 min. The sample was centrifuged at 27,000 x g for 30 min and the protein content of the supernatant determined in a Technicon Autoanalyzer II after digesting according to the procedure of Concon and Soltess (1973).

Viscosity of 0.1% protein solutions was determined using an Ostwald viscometer. Net proton charge on the proteins was measured by hydrogen ion titration in 6M guanidine hydrochloride according to the method of Nozaki and Tanford (1961).

Nonprotein N was determined as follows: 10 ml of a 1% protein dispersion in phosphate buffer was centrifuged at 27,000 x g for 30 min. Three ml of 20% trichloroacetic acid was added to a 2 ml aliquot of the supernatant then the mixture was centrifuged at 27,000 x g for 30 min. N content of the supernatants before and after the addition of trichloroacetic acid was determined as described above for dispersibility determination. Nonprotein N% was calculated as the ratio of the two N values.

The three dimensional plot was done using UBC Surface program entitled 'Surface visualization routines' on an Amdahl 470V/8 computer. The method of Fujii and Nakai (1980) was used for data transformation for linearization.

RESULTS & DISCUSSION

Relationship between molecular flexibility and protein foam

The ratio of number of SS linkages/mol wt (SS/M) was used as an index of molecular flexibility. For 11 model proteins, it was found that all flexible proteins showed good foaming characteristics while the more rigid proteins, i.e., lysozyme, ovomucoid and ribonuclease, showed poor foaming properties (Fig. 1). There was a significant negative linear correlation ($r = -0.806$, $P < 0.01$) between foaming capacity (FC ml) and SS/M with a regression equation:

$$FC = 128.9 - 25.05 \, SS/M \qquad (2)$$

This relationship indicates that it is important for protein molecules to be flexible enough to spread out at the air/water interface to stabilize fresh air cells, thus preventing the collapsing of foams. Graham and Phillips (1976) observed that the flexible random coil protein β-casein had good foaming capacity, while lysozyme and bovine serum albumin, which have more rigid molecules exhibited lower foaming capacity. They later determined that although the rate of surface denaturation and surface pressure development could not be related to intrinsic flexibility, the structures formed by these proteins at the air-water interface reflect the intrinsic flexibilities of their molecules (Graham and Phillips, 1979).

Relationship between protein hydrophobicity and foaming properties

The importance of hydrophobicity for protein foaming properties was demonstrated by a significant ($r = 0.823$, $P < 0.01$) relation between the foaming capacity of the model proteins and the average hydrophobicity, $H\phi_{avg}$, of Bigelow (1967) as seen in Fig. 2. The regression equation obtained was:

$$\ln(FC + 30) = 0.0041 H\phi_{avg} + 0.0393 \qquad (3)$$

It has been reported that the S_O shows a significant correlation with two surface properties, i.e., emulsifying
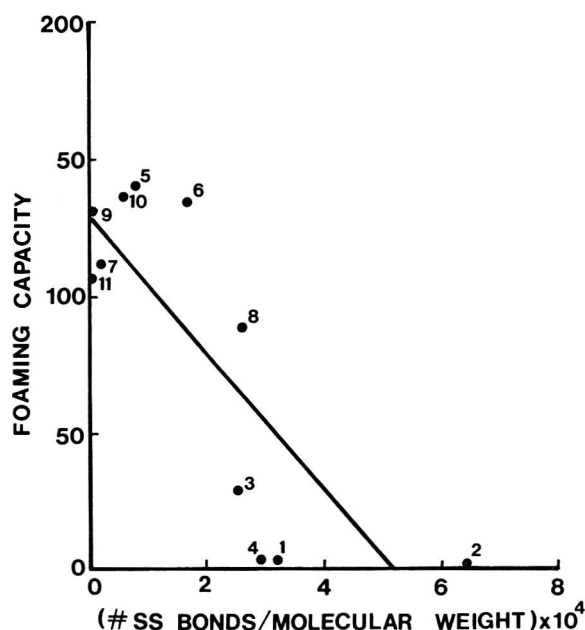
Fig. 1—Relationship between foaming capacity* and molecular flexibility measured as SS bonds/molecular weight. (1) Ribonuclease, (2) ovomucoid, (3) trypsin, (4) lysozyme, (5) pepsin, (6) conalbumin, (7) ovalbumin, (8) bovine serum albumin, (9) κ-casein, (10) β-lactoglobulin, (11) β-casein. *Each data point is the average of three determinations.

capacity and interfacial tension (Kato and Nakai, 1980). Since foaming capacity and foam stability are also surface properties of proteins, their relationship to S_O was expected. In the method of Kato and Nakai (1980) the test was carried out in the presence of 0.002% SDS which gave better correlation with surface properties than without SDS. However, no significant relationship was found between the surface hydrophobicity of proteins and their foaming capacity. These results suggest that the proteins are extensively un-

coiled at the air/water interface; more extensively than at the oil/water interface. Thus a measure of the total hydrophobicity, e.g., $H\phi_{avg}$, rather than surface hydrophobicity should have a more highly significant correlation with foaming capacity.

Since the amino acid composition is not always available for calculation of $H\phi_{avg}$ of proteins, denaturation conditions combining SDS and heat treatments were investigated to reproduce the $H\phi_{avg}$ values using cis-PnA fluorometry. Heating protein samples for 10 min in boiling water in the presence of 1.5% SDS was found to give a significant correlation (r = 0.820, P < 0.01) with $H\phi_{avg}$ (Fig. 3). This result indicates that the S_e measurement can be an index of the total hydrophobicity or the average hydrophobicity of Bigelow (1967). The S_e showed a significant correlation (r = 0.820, P < 0.01) with foaming capacity (Fig. 4) with the regression equation:

$$\ln(FC + 30) = 0.0014 S_e + 3.52 \qquad (4)$$

Lysozyme was omitted from plotting since its behavior was unusual. Similarly, Steinhardt et al. (1977) reported an extraordinary solubilization effect of lysozyme-SDS complex on a water-insoluble dye, dimethylaminoazobenzene.

The modified fluorescence probe method for measuring S_e values was applied to the crude protein samples. Since not all crude protein samples were completely soluble, the dispersibility (d%) was taken into account in the correlation study as a potential influencing factor.

Backward stepwise regression analysis was applied to the foaming capacity of all 19 protein samples, including model protein and crude protein samples, as functions of S_e and d. The best R^2 of 0.772 (P < 0.01) was obtained with the regression equation of:

$$FC = 260.4 \ln S_e - 0.301 S_e + 158.6 \ln d - 2.724 d - 1820 \qquad (5)$$

The beta values of S_e, d, $\ln S_e$ and $\ln d$ were 1.75, 1.61, 2.44 and 1.92, respectively, indicating that hydrophobicity was slightly more important than dispersibility in the regression equation for foaming capacity.

Fig. 5 shows the three dimensional plot of the relationship between the foaming capacity of proteins and their
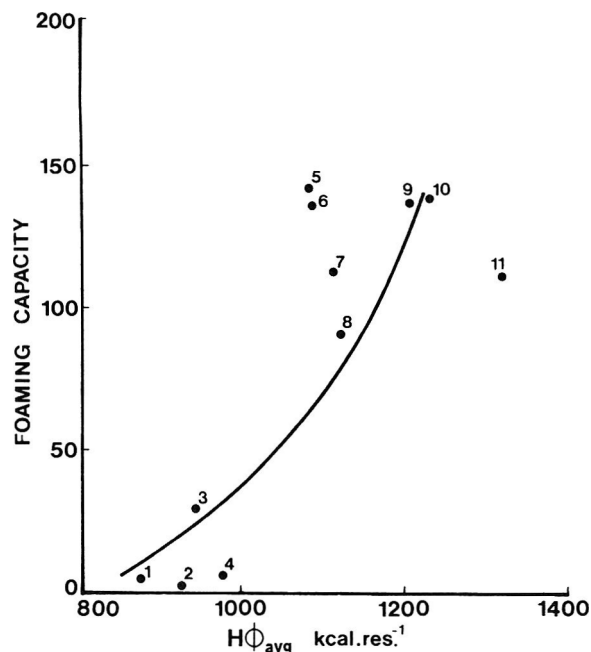
Fig. 2—Relationship between foaming capacity and Bigelow's average hydrophobicity ($H\phi_{avg}$). Sample identification is the same as in Fig. 1. The regression curve was linearized.
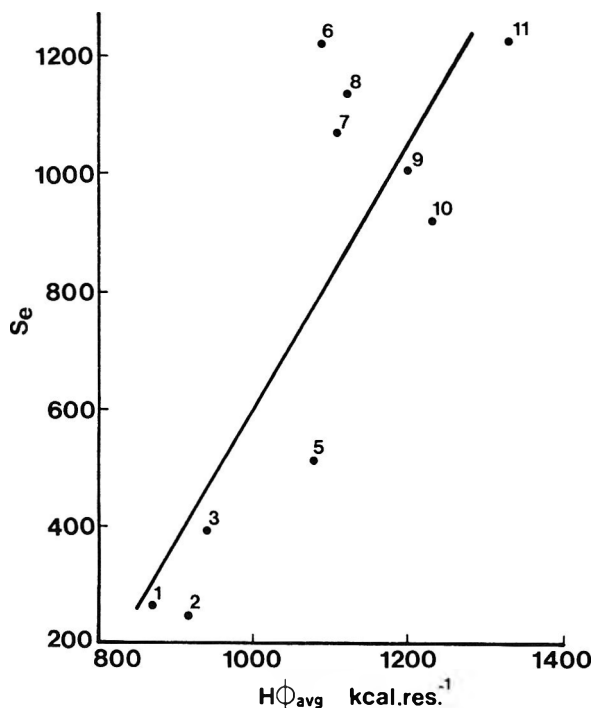
Fig. 3—Relationship between hydrophobicity (S_e)* measured after exposure to 100°C for 10 min in the presence of 1.5% SDS and Bigelow hydrophobicity ($H\phi_{avg}$). Sample identification is the same as in Fig. 1. The regression curve was linearized.
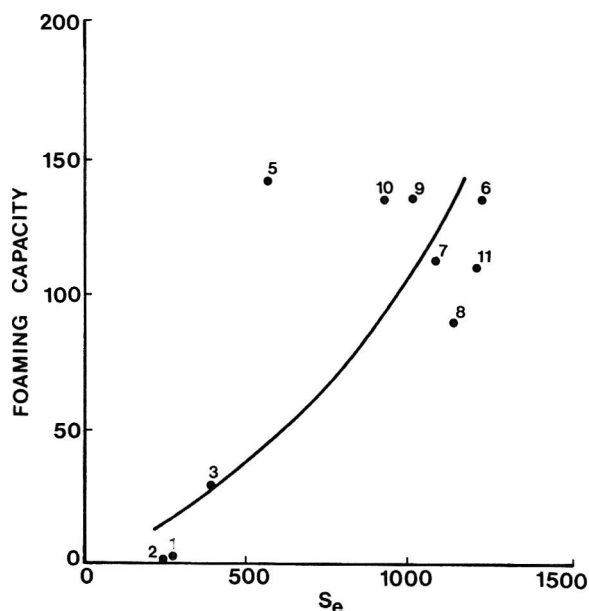
Fig. 4—Relationship between foaming capacity and hydrophobicity (S_e) measured after exposure to 100°C for 10 min in the presence of 1.5% SDS. Sample identification is the same as in Fig. 1. The regression curve was linearized.
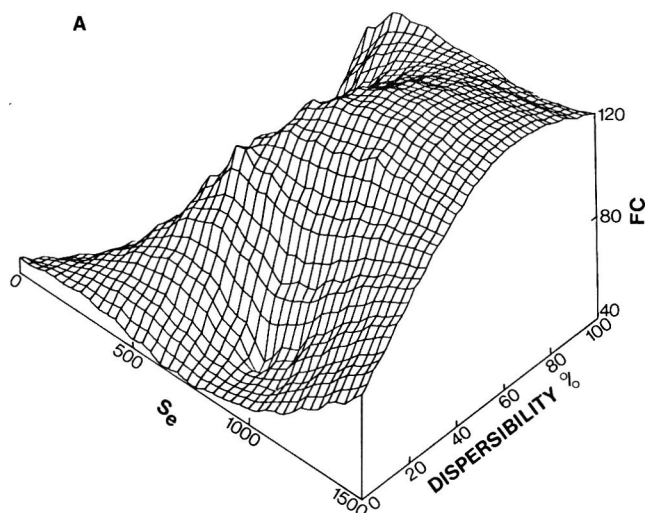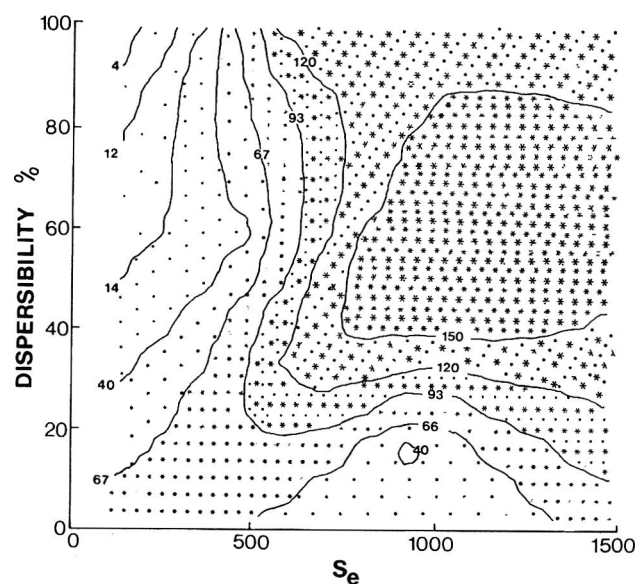
Fig. 5—Three dimensional plot (A) and its contour (B) of the relationship between foaming capacity, hydrophobicity and dispersibility. From 19 data.

hydrophobicity and dispersibility. The optimum foaming capacity appears to be associated with dispersibility above 40% and hydrophobicity values above 700. This suggests that hydrophobicity of 700 or above are associated with the good balance of hydrophilic and hydrophobic groups necessary for effective stabilization of air bubbles. Regardless of their degree of dispersibility, proteins with low hydrophobicity showed poor foaming capacity.

Good foaming capacity was exhibited by proteins with poor dispersibility of approximately 20% and hydrophobicity values above 500. This was probably due to stabilization of foams by protein particles present in the medium. It is well known that some particles can stabilize foams (Bikerman, 1973). It seems that particles lying in the air/water interface serve as a physical barrier to bubble coalescence.

When the foam stability (FS min) was plotted against $H\phi_{avg}$ (Fig. 6), the correlation coefficient after linearization (r = 0.807) was significant (P < 0.01) with a regression equation of:

$$\ln(FS + 0.18) = 0.0102H\phi_{avg} - 9.791 \qquad (6)$$

Importance of charge density for foaming

Net proton charge measured by titration in 6M guanidine hydrochloride showed good agreement with the calculated values in most cases (Table 1). Two exceptions were pepsin and trypsin. The measured charge for trypsin was −11 while the calculated charge was +7, probably because of the presence of impurities in the samples. In the case of pepsin the measured charge was −27 while the calculated charge was −32. This discrepancy resulted from the use of the literature value, or pH 1.0, for the isoelectric point of pepsin in the calculation of net proton charge (Fruton, 1972). The pH of deionized pepsin solution, or pH 3.0, was used as the isoionic point of pepsin in the measurement of net proton charge. It is interesting to note that while the measured isoionic point of pepsin is pH 1.0, the isoionic point calculated from the amino acid composition of pepsin, assuming a pK_a of 4.0 for the side chain carboxyl groups, is pH 3.0 (Fruton, 1972). It has been suggested that this discrepancy may be due to anions adsorbed on the protein during isoionic measurement or to the presence of abnormally acidic carboxyl groups.
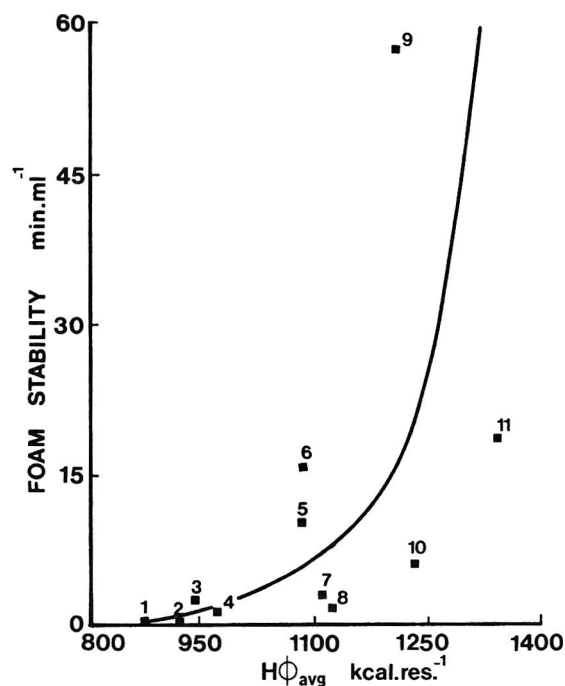
Fig. 6—Relationship between foam stability* and Bigelow hydrophobicity ($H\phi_{avg}$). Sample identification is the same as in Fig. 1. The regression curve was linearized. *Each data point is the average of triplicate determinations.

No good correlation was observed between the foaming capacity of the proteins and their charge density. For further confirmation of the effect of charge density, the foaming capacity of model proteins were measured at various pH's and ionic strengths. Analysis of variance indicated that over the range tested, ionic strength had no significant effect on foaming capacity (Table 2). For determining the pH effect, the pH range of protein solutions was kept between 5 and 9 to avoid unnecessary acid and alkali denaturation of proteins. Analysis of variance indicated that pH had a significant effect (P < 0.01) on foaming

capacity (Table 3). The proteins showed a tendency toward decreased foaming capacity as their net charge increased.

These results indicate that although the charge density of proteins affects their foaming ability, its influence may not be great. However, at high charge densities brought about by high pH, foaming capacity was affected as seen for pepsin in Table 3.

There was a significant correlation ($r = 0.725$, $P < 0.01$) between the foam stability of the model proteins and the inverse of their charge density (c units/residue). The regression equation was:

$$FS = 19.72c^{-1} - 2.11 \qquad (7)$$

As shown in Fig. 7, foam stability increased markedly at low charge density. This suggests that inter-molecular repulsion between protein molecules at the interface had an important destabilization effect on foams.

Relationship between solution viscosity and foaming properties

Properties such as molecular flexibility, size, degree of hydration and the extent of intermolecular association should influence the foaming properties of proteins. Since the viscosity of a protein solution is a function of molecular size, shape, flexibility, degree of hydration and intermolecular interactions (Tanford, 1961; Yang, 1961), the correlation of viscosity (η Pa·s) with foaming capacity (Fig. 8) was calculated for 16 protein samples. There was a significant correlation ($r = 0.849$, $P < 0.01$) with the regression equation of:

$$FC = 23388 \eta - 2622 \qquad (8)$$

Three of the crude protein samples, i.e. sunflower isolate, Canola isolate and Pro-pulse, were outlying, and therefore eliminated from the regression analysis. They had low viscosity of approximately 1.09 Pa·s but with high foaming capacity. A possible reason may be that these samples contained insoluble particles which had negligible effects on bulk viscosity but were capable of stabilizing foams.

The correlation of foaming capacity of proteins to their hydrophobicity, dispersibility and viscosity was calculated. Since there was a high degree of collinearity between viscosity and both hydrophobicity and dispersibility, as indicated by the significant ($P < 0.05$) correlation coefficients of 0.612 and 0.565, respectively, ridge regression analysis was carried out as recommended by Newell and Lee (1981). In the presence of the viscosity parameter, the contribution of dispersibility to the variability became nonsignificant, therefore, dispersibility was eliminated from the regression analysis. The best regression equation obtained was:

$$FC = 1493 \eta + 25.93 \ln S_e - 1775 \qquad (R^2 = 0.779, P < 0.01) \qquad (9)$$

Viscosity was more important than $\ln S_e$ in the equation as indicated in the beta values of 0.57 and 0.25, respectively.

Table 1—Net proton charge on the model proteins at pH 7.0

Protein	Calculated value[a] (H⁺/mole)	Measured value[b] (H⁺/mole)
Ribonuclease	+3	+3
Ovomucoid	−9	−8
Trypsin	+7	−11
Lysozyme	+7	+7
Pepsin	−32	−27
Conalbumin	−7	−5
Ovalbumin	−13	−12
Bovine serum albumin	−18	−16
κ-Casein	−3	−1
β-Lactoglobulin	−9	−8
β-Casein	−4	−3

[a] Calculated as described by Nozaki and Tanford (1967) using the pK$_a$s of titrable groups given in Table 3 of the same paper and the amino acid composition of the proteins.
[b] Each data point is the average of duplicate determinations

Table 2—Effect of ionic strength on foaming capacity[a]

Protein	Ionic strength		
	0.01	0.05	0.20
Ribonuclease	2 ± 1	2 ± 1	3 ± 1
Ovomucoid	12 ± 1	12 ± 1	13 ± 1
Lysozyme	2 ± 1	2 ± 1	3 ± 1
Pepsin	144 ± 3	147 ± 3	149 ± 3
Conalbumin	141 ± 3	142 ± 2	141 ± 2
Ovalbumin	48 ± 2	100 ± 2	122 ± 2
Bovine serum albumin	95 ± 4	103 ± 4	98 ± 2
κ-Casein	134 ± 2	132 ± 2	135 ± 2
β-Lactoglobulin	138 ± 2	135 ± 3	134 ± 4
β-Casein	104 ± 1	109 ± 2	108 ± 2

[a] Each data point is the average of triplicate determinations

Table 3—Effect of pH on foaming capacity[a]

Protein	pH		
	5	7	9
Ribonuclease	2 ± 1	2 ± 1	6 ± 1
Ovomucoid	97 ± 1	12 ± 1	11 ± 1
Lysozyme	3 ± 1	2 ± 1	8 ± 1
Pepsin	151 ± 2	147 ± 3	34 ± 1
Conalbumin	146 ± 2	143 ± 2	162 ± 3
Ovalbumin	140 ± 3	100 ± 2	42 ± 2
Bovine serum albumin	97 ± 1	101 ± 4	95 ± 2
κ-Casein	0 ± 0	134 ± 2	144 ± 4
β-Lactoglobulin	127 ± 2	135 ± 3	138 ± 2
β-Casein	0 ± 0	109 ± 2	107 ± 1

[a] Each data point is the average of triplicate determinations

Fig. 7—Relationship between foam stability and charge density.* Sample identification is the same as in Fig. 1. The regression curve was linearized. *Each data point is the average of duplicate determinations.
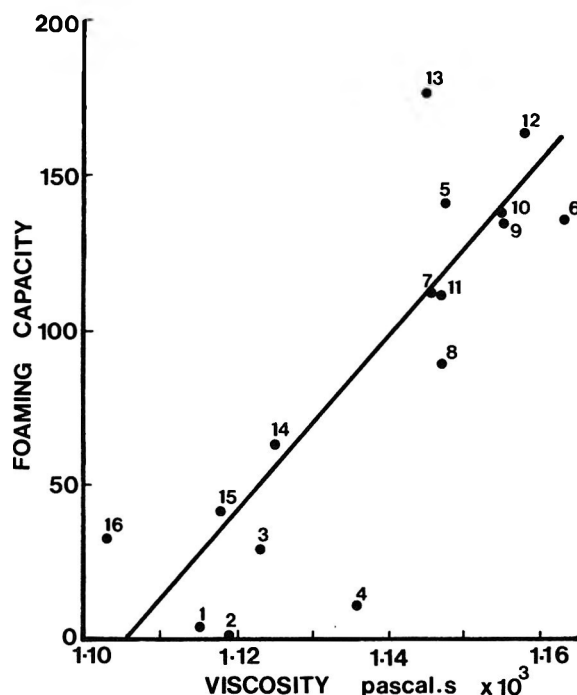
Fig. 8—Relationship between foaming capacity * and bulk viscosity **
(1) Ribonuclease, (2) ovomucoid, (3) trypsin, (4) lysozyme, (5)
pepsin, (6) conalbumin, (7) ovalbumin, (8) bovine serum albumin,
(9) κ-casein, (10) β-lactoglobulin, (11) β-casein, (12) whole casein,
(13) gluten, (14) pea isolate, (15) soy isolate, (16) Promine-D.
*Each data point is the average of triplicate determinations. **Each
data point is the average of duplicate determinations.
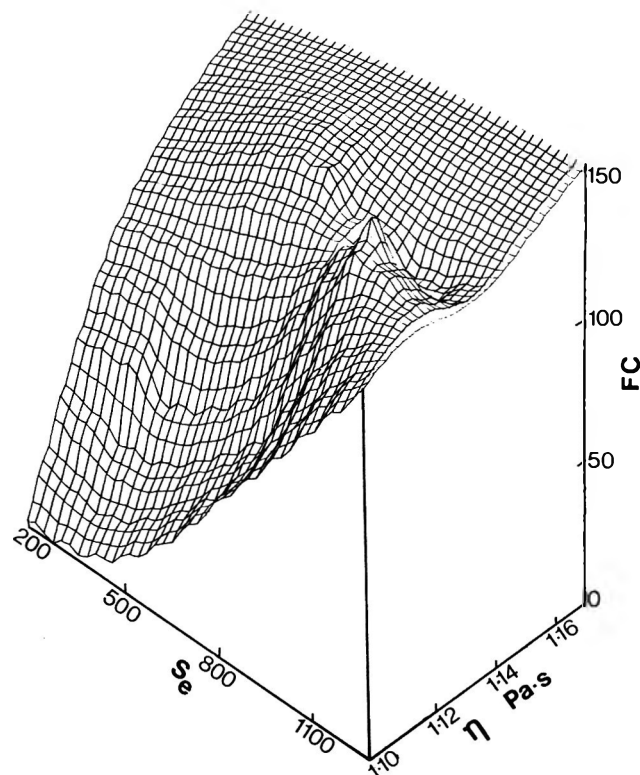
Fig. 9—Three dimensional plot of foaming capacity against hydro-
phobicity and bulk viscosity. From 19 data.

As seen in the three dimensional plot (Fig. 9), the foaming capacity increased with hydrophobicity (S_e) and viscosity.

Bikerman (1973) reported that solution viscosity was related to foam stability determined by drainage methods. There was also a significant correlation in our case as seen in Fig. 10. The regression equation was:

$$\ln(FS + 0.3) = 130.4\eta - 148.03 \qquad (r = 0.855, P < 0.01)$$
$$(10)$$

When backward regression analysis was applied to the same data using S_e, c and η as independent variables, S_e and η were rejected in the final regression equation obtained, implying that charge density is a controlling factor for foam stability although contributions may also be made by hydrophobicity and viscosity.

Effects of hydrolysis of crude protein samples on foaming capacity

Six crude protein samples were hydrolyzed to increase the nonprotein N level from below 5% to 27–40%. Hydrolysis resulted in an increase in dispersibility and a decrease in hydrophobicity for some samples (Table 4). The decreased hydrophobicity may have been caused by increased charge density on the peptides as a result of hydrolysis. Low foaming capacity observed (Table 4) may be due to a decrease in viscosity and hydrophobicity.

In general, the volume of foams of whipped hydrolysate is greater than that of parent protein, but the foam stability usually decreases by proteolysis (Richardson, 1977). Foam volume increases initially by limited proteolysis; however, continued proteolysis resulted in decrease.

Discrepancy of our results may be due to excessive proteolysis or a difference in the method for measuring the foaming capacity. An aeration method was used in our study for measuring the foaming capacity to reduce costs of expensive pure protein samples since the method requires
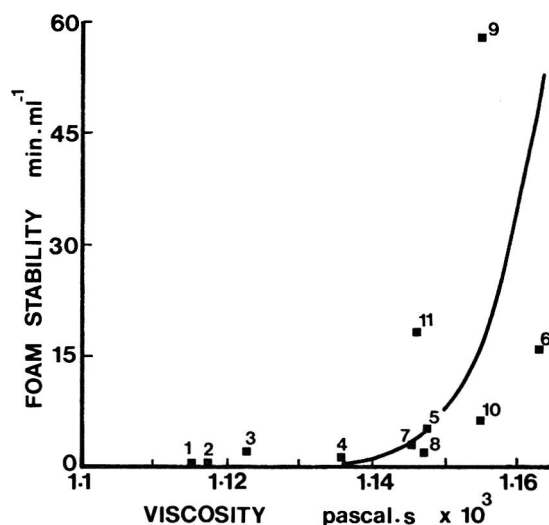
Fig. 10—Relationship between foam stability and bulk viscosity.
Sample identification is the same as in Fig. 1. The regression curve
was linearized.

low concentrations of proteins for the maximum foaming capacity (Waniska and Kinsella, 1979).

To obtain the best foaming properties the selection of proper hydrolysis conditions may be crucial for the best balance among solubility, hydrophobicity and viscosity, which is closely related to the extent of hydrolysis. More work is needed for full explanation of these relationships.

Cherry and McWatters (1981) stated that the proteins in the liquid film should (1) be soluble in the aqueous phase, (2) readily concentrate at the liquid-air interface, and (3) denature to form cohesive layer possessing sufficient viscosity and mechanical strength to prevent rupture and coalescence of air cells.

Table 4—Effect of enzyme hydrolysis on foaming capacity (FC), hydrophobicity (S_e) and dispersibility (d)

	untreated			hydrolyzed		
Sample	FC[a]	S_e[b]	d[b] (%)	FC[a]	S_e[b]	d[b] (%)
Pea	61 ± 4	277	36	0 ± 0	280	49
Sunflower	150 ± 3	597	31	2 ± 1	582	63
Canola	157 ± 3	950	44	51 ± 4	500	70
Casein	163 ± 4	725	53	2 ± 1	302	64
Promine-D	33 ± 3	927	13	5 ± 1	432	55
Soy isolate	41 ± 2	822	12	14 ± 2	485	57

[a] Each data point is the average of triplicate determinations
[b] Each data point is the average of duplicate determinations

For quick concentration and subsequent denaturation at the surface, the protein should be flexible, structurally less-ordered and, above all, hydrophobic. The proteins undergo conformational changes at the surface by unfolding to expose more hydrophobic regions of the molecules that facilitate the association of the polypeptides. This increases the viscosity of lamellae, thereby intensifying the foam stability. However, excessive self-association would result in the corruption of foam due to the loss of elasticity of lamellae.

This will explain the importance of molecular rigidity, solubility, hydrophobicity and viscosity in the foaming mechanism of proteins in solution. This paper reports the relationships of these properties of food proteins to their foaming properties.

Although, several hypotheses have been submitted to elucidate the foaming mechanism of a protein solution on the basis of its properties in the bulk phase, they lack substantial supporting data. Our results may provide data to these theories thus inducing a new interpretation to the protein foaming phenomenon.

In conclusion, a total hydrophobicity or the hydrophobicity of exposed or unfolded protein could be measured by the fluorometry using cis-PnA as a probe. This hydrophobicity in conjunction with viscosity and dispersibility could account for a major portion of the variability of foaming capacity of proteins. The surface hydrophobicity, which was important for emulsification, could not explain the difference in foaming capacity of proteins. High charge density on a protein destabilized protein foams. These results indicate the importance of hydrophobicity for determining the foaming properties of protein solutions.

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Thermal Destruction of 5-Methyltetrahydrofolic Acid in Buffer and Model Food Systems

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─────────ABSTRACT─────────

Thermal kinetic data (rate constants, k, and activation energies, Ea) for 5-Methyltetrahydrofolic acid (5-CH_3-H_4PteGlu) were determined in citrate buffers (pH 3–6) and in model food systems between 100° and 140°C. As pH increased from 3.0 to 6.0, the rate constants decreased as the temperature increased from 100° to 130°C, the rate constants increased. Ea values were 19.0, 17.0, 19.7 and 19.8 kcal/mole at pH 3, 4, 5 and 6, respectively. In the model food systems, the Ea values (kcal/mole) were 7.85 in apple juice and 10.6 in tomato juice. When dissolved oxygen content was reduced to 5.3 ppm, the stability of the 5-CH_3-H_4PteGlu was increased substantially.

INTRODUCTION

REPORTS of folacin deficiency in different groups in the population (Chanarin, 1969; Vandermark and Wright, 1972; Nutrition Canada, 1975; Hoppner et al., 1977) coupled with demands by food processing engineers for data to help them design processing systems, has emphasized the need for data on destruction of folates in foods during processing.

Folates in biological materials exist as reduced polyglutamyl derivatives, mainly tetrahydropteroylglutamate (H_4PteGlu), 5-formyltetrahydropteroylglutamate (5-CHO-H_4PteGlu). 10-formyltetrahydropteroylglutamate (10-CHO-H_4PteGlu) and 5-methyltetrahydropteroylglutamate (5-CH_3-H_4PteGlu). In plant materials, the major form of folate has been shown to be 5-CH_3-H_4PteGlu (Dong and Oace, 1973; Chan et al., 1973; Clifford and Clifford, 1977; Stokstad et al., 1977). Thermal stability of these forms has also been shown to differ greatly, with H_4PteGlu being extremely labile (Chen and Cooper, 1979) and 5-CHO-H_4Pte-Glu the most stable (Paine-Wilson and Chen, 1979).

Stability of 5-CH_3-H_4PteGlu has been studied under various conditions. At room temperature, O'Broin et al. (1975) reported a half life of 91 hr at pH 6 and 119 hr at pH 7. Greatest stability was observed under alkaline conditions. Cooper et al. (1978) reported that over 90% of 5-CH_3-H_4PteGlu loss occurred within 65 minutes of heating at 100°C. The half life was 21 minutes in aqueous solution at pH 7.0. However, Chen and Cooper (1979) reported a half life of 8.77 min in universal buffer at pH 7.0 at 100°C. Buffer ions have been shown to affect the stability of 5-CH_3-H_4PteGlu. O'Broin et al. (1975) also observed that at pH 6 and 7, 5-CH_3-H_4PteGlu was less stable in phosphate buffer.

Various studies have shown that the primary inactivation of folate compounds during food processing is oxidative (Ford et al., 1969; Rolls and Porter, 1973; Burton et al., 1970). Chen and Cooper (1979) observed that 5-CH_3-H_4PteGlu was considerably stabilized when heated at 100°C under nitrogen atmosphere. Ruddick et al. (1980) reported that the presence of oxygen was necessary for the degradation of 5-CH_3-H_4PteGlu.

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Work reported on thermal stability of 5-CH_3-H_4PteGlu has been done at temperatures far below those used in the processing industry, and since it has also been reported that kinetic data obtained from studies using buffer systems may not be applicable to food systems, it is worthwhile to study the stability of 5-CH_3-H_4PteGlu in buffer systems as well as model food systems at temperatures above 100°C.

MATERIALS & METHODS

Sample preparation

5-Methyltetrahydrofolic acid. DL-N-5-CH_3-H_4PteGlu (sodium salt, 90% purity) was obtained from Sigma Chemical Company (St. Louis, MO) and stored in a freezer at −20°C. When needed, 11.1 mg was dissolved in 5 ml of cold water (4°C) and 1 ml of this solution was diluted to 100 ml and stored in 5 ml aliquots in a freezer at −20°C, in the dark. When needed, 1 ml of the stock solution was diluted to 10 ml with citrate buffer of desired pH. The final concentration was 1.99 μg/ml.

Model food samples were prepared as described by Mnkeni and Beveridge (1982).

Thermal destruction of 5-CH_3-H_4PteGlu

Thermal destruction was done as described by Mnkeni and Beveridge (1982). For anaerobic destruction of 5-CH_3-H_4PteGlu, one ml of the prepared stock solution was diluted with 9 ml of citrate buffer or juice, and nitrogen was bubbled through the solution for 30 min in a glove box (Fisher Scientific) which had previously been flushed with nitrogen. The dissolved oxygen in the solution after flushing for 30 min was determined by using a Dissolved Oxygen Analyzer (New Brunswick Scientific, NJ). The amount of oxygen remaining after flushing was 5.3 ppm. Filling and sealing the capillary tubes was done in the glove box.

Assay of folates

Microbiological method using *L. casei* was used to assay for folacin, as described by Waters and Mollin (1961), with modifications as described by Tamura et al. (1972). Sterilization was done by filtering the solutions through a millipore filter type HA 0.45 μm. Growth of the microorganism was measured turbidimetrically at 660 nm on a Beckman A25 spectrophotometer equipped with a sipper cell. Growth was ccompared to a standard curve of *L. casei* growth in medium containing 5-CH_3-H_4PteGlu as the sole source of folate available to the organisms.

Calculations

Average values of two triplicate samples were fitted by first order rate equation,

$$-kt = 2.303 \log_{10} \frac{C}{C_0}$$

where k = first order rate constant;
  t = time;
  C_0 = concentration at t = 0; and
  C = concentration at time t.

by linear regression, and slope of the line used to determine k. Linear regression was also used to determine the relationship between reaction rates and temperature. Activation energies (Ea) were obtained from the slopes of lines relating ln k to 1/T where T = absolute temperature. A covariance technique (Ostle, 1975) was used to test slope equality. The analysis was performed on the IBM 3780 computer.

RESULTS & DISCUSSION

THE PLOT of the logarithm of percent $5\text{-}CH_3\text{-}H_4PteGlu$ retained versus time of heating at 100°C in buffer system is shown in Fig. 1. Straight lines were also obtained at 110, 121 and 130°C. The linearity of the curves indicate that $5\text{-}CH_3\text{-}H_4PteGlu$ degradation in citrate buffer could be described by first order reaction kinetics in agreement with Chen and Cooper (1979) or by pseudo first order kinetics (Ruddick et al., 1980).

Table 1 shows the effect of pH and temperature on the rates of the destruction for $5\text{-}CH_3\text{-}H_4PteGlu$. The rate constants at pH 5.0 were not significantly different from those at pH 6.0 ($p \geqslant 0.05$), except at 121°C, indicating that at lower pH values the rates of destruction are much faster compared to high pH values, in agreement with Paine-Wilson and Chen (1979) and O'Broin et al. (1975). The rate constants obtained in this study are in good agreement with values reported by Paine-Wilson and Chen (1979) – 0.254 min^{-1} at pH 3; 0.207 min^{-1} at pH 4; 0.110 min^{-1} at pH 5, and 0.103 min^{-1} at pH 6.0 – in Universal buffer. In citrate buffer pH 3.0 they reported a value of 0.083 min^{-1}. It has been shown that buffer ions affect the stability of $5\text{-}CH_3\text{-}H_4PteGlu$. Chen and Cooper (1979) reported a rate in constant of 0.032 min^{-1} in aqueous solution at pH 7.0 at 100°C, while Ruddick et al. (1980) reported a value of 0.108 min^{-1} in phosphate buffer pH 7.3.

As expected, the rates of destruction increased with temperature (Table 1). No data were collected for pH 3 and 4 at 130°C, because destruction was too fast to be measured by the present technique. Destruction was essentially complete within the first minute of the experiment.

Activation energies obtained as described before (Fig. 2) are 19.0, 17.0, 19.7 and 19.8 kcal/mole at pH 3, 4, 5 and 6. The slopes of the lines in Fig. 2 are not significantly different ($p \geqslant 0.05$), indicating that the activation energies are not significantly different. This suggests that the mechanism of destruction does not change over the pH range of 3–6. The Ea values obtained in this study are higher than values reported in the literature. Chen and Cooper (1979) reported an Ea value of 9.5 kcal/mole at temperatures ranging from 65–100°C, while Ruddick et al. (1980) working at a similar temperature range, reported an Ea value of 7.1 kcal/mole. The differences between the values obtained in the present study and the literature values could be due to the different temperature ranges employed, since an Ea value of 9.58 kcal/mole (Table 6) was obtained at pH 3.4 over the temperature range of 50–70°C.

Destruction of $5\text{-}CH_3\text{-}H_4PteGlu$ in limited oxygen (5.3 ppm)

The presence of oxygen has been implicated as a factor in the degradation of folates during heating (Herbert, 1968; Larrabee et al., 1961, Gupta and Huennekens, 1967).

Fig. 1—Rate of destruction curves for $5\text{-}CH_3\text{-}H_4PteGlu$ in citrate buffer at 100°C. Correlation coefficients, 0.94 at pH 6, 0.99 at pH 5, 0.96 at pH 4 and 0.98 at pH 3 are significant (p > 0.01).
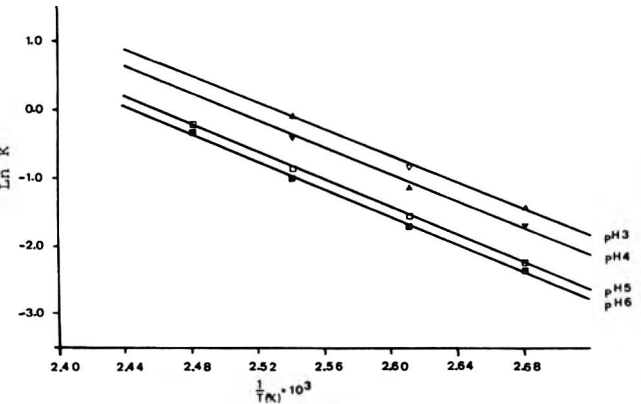
Fig. 2—Arrhenius plot for thermal degradation of $5\text{-}CH_3\text{-}H_4PteGlu$ in citrate buffer. Correlation coefficients, 0.99 at pH 3, 0.97 at pH 4, 0.99 at pH 5, and 0.98 at pH 6 are significant (p > 0.01). The slopes of the lines are not different (p > 0.05).

Table 1—Effect of temperature and pH on the rates of destruction of $5\text{-}CH_3\text{-}H_4PteGlu$ in citrate buffer

Temp °C	Rate constants (min^{-1})			
	pH 3.0	pH 4.0	pH 5.0	pH 6.0
100	0.243(0.98)[a] ±0.024[c]	0.192(0.96) ±0.027	0.110(0.99) ±0.007	0.104(0.94)[b] ±0.004
110	0.448(0.96) ±0.050	0.318(0.94) ±0.064	0.215(0.97) ±0.033	0.188(0.96) ±0.016
121	0.927(0.98) ±0.048	0.635(0.97) ±0.058	0.432(0.98) ±0.049	0.366(0.93) ±0.059
130	–[d]	–	0.808(0.94) ±0.038	0.783(0.96) ±0.043

[a] Correlation coefficients (in parenthesis) are significant ($p \geqslant 0.01$).
[b] Rate constants underlined by the same line are not significantly different ($p \geqslant 0.05$).
[c] 95% confidence interval.
[d] No data collected.

Temp °C	Rate constants (min^{-1})			
	pH 3.0	pH 4.0	pH 5.0	pH 6.0
100	0.106(0.95)[a] ±0.013[c]	0.092(0.96) ±0.012	0.072(0.97)[b] ±0.007	0.059(0.97) ±0.010
110	0.133(0.96) ±0.027	0.128(0.97) ±0.024	0.094(0.95) ±0.029	0.081(0.98) ±0.019
121	0.215(0.97) ±0.038	0.180(0.98) ±0.036	0.152(0.95) ±0.030	0.137(0.95) ±0.031
130	0.420(0.95) ±0.033	0.313(0.96) ±0.035	0.286(0.97) ±0.031	0.223(0.97) ±0.027
140	—	—	0.357(0.96) ±0.049	0.312(0.94) ±0.047

[a] Correlation coefficients (in parenthesis) are significant (p≥0.01).
[b] Rate constants underlined by the same line are not different (p≥0.05).
[c] 95% confidence interval.

Table 3—Activation energies for the degradation of 5-CH3-H4PteGlu in unlimited and limited oxygen

pH	Activation energy (kcal/mole)	
	Unlimited O$_2$	Limited O$_2$
3	19.0 [a]	13.6
4	17.0	11.8
5	19.7	13.2
6	19.8	13.3

[a] Activation energy values bracketed by the same line are not significantly different (p≥0.05).

The destruction rate curves for the 5-CH$_3$-H$_4$PteGlu in 100°C in citrate buffer with limited oxygen are shown in Fig. 3. The correlation coefficients obtained by applying first order reaction equation to the data (Table 2) are quite high, so the degradation reaction could be described by first order reaction equation. Ruddick et al. (1980) reported that the 5-CH$_3$-H$_4$PteGlu degradation reaction could be described by the second order reaction equation in limited oxygen. However, this seems not to apply here, probably because of the high temperatures employed here compared to those employed by Ruddick et al. (1980).

At all temperatures, the rate constants at pH 3 differed significantly (p < 0.05) from those at pH 4, 5 and 6 (Table 2). The rate constants at pH 4 were not significantly different (p ≥ 0.05) from those at pH 5.0, except at 110°C; also the rate constants at pH 5.0 were not significantly different from those at pH 6.0, except at 130°C. This generally indicates that the effect of pH is reduced when the dissolved oxygen is limited. Comparing the rates of destruction of 5-CH$_3$-H$_4$PteGlu in limited and unlimited oxygen (concentration of oxygen in water at room temperature) indicates that the rates of destruction are lower in limited oxygen, in agreement with Chen and Cooper (1979) and Ruddick et al. (1980).

The Ea values obtained in the two systems are shown in Table 3. Ea values in limited oxygen are significantly lower (p ≤ 0.05) than the values obtained in unlimited oxygen. These values suggest that the mechanism of degradation reaction is not the same in the two systems. In air or oxygen, 5-CH$_3$-H$_4$PteGlu is converted to 5-CH$_3$-H$_2$PteGlu (Larrabee et al., 1961). The mechanism and products of degradation of 5-CH$_3$-H$_4$PteGlu in limited oxygen have not been identified, so further work on this point may be of interest. Interestingly, although not statistically significant, lower values of Ea were obtained at pH 4 in both systems.
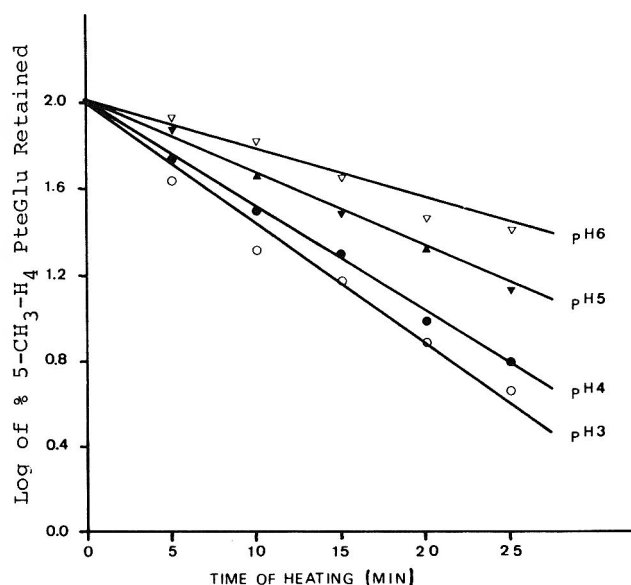
Fig. 3—Rate of destruction curves of 5-CH$_3$-H$_4$PteGlu in buffer with limited oxygen at 100°C. Correlation coefficients, 0.97 at pH 6, 0.97 at pH 5, 0.96 at pH 4 and 0.95 at pH 3 are significant (p > 0.01).

Destruction of 5-CH$_3$-H$_4$PteGlu in model food systems

It has been reported that kinetic data for thermal destruction of vitamins obtained from buffer systems may not be applicable to food systems (Lathrop and Leung, 1980). Data on ascorbic acid destruction has shown differences within foods as well as between foods and buffer systems (Heulin, 1953; Blaug and Hajratwala, 1972).

The destruction rate curves for 5-CH$_3$-H$_4$PteGlu in tomato juice and apple juice are shown in Fig. 4 and 5. The high correlation coefficients (Tables 4 and 5) indicate that over the temperature range studied, the kinetics of 5-CH$_3$-H$_4$PteGlu destruction in model food systems were consistent with first order kinetics. The destruction of the 5-CH$_3$-H$_4$PteGlu in the apple juice was too fast at 100°C to be followed by the present technique (100% destruction occurred within the first minute), so the experiment had to be done at lower temperatures. This was not expected, since in citrate buffer (pH 3.0) and tomato juice (pH 4.3) the compound could withstand temperatures of up to 130°C.

The rates of destruction 5-CH$_3$-H$_4$PteGlu in apple juice and tomato juice were higher than in the buffer systems

Table 4—Rates of destruction of 5-CH₃-H₄PteGlu in tomato juice (pH 4.3), in citrate buffer (pH 4 and 5) and in tomato juice with limited oxygen (5.3 ppm)

System	Rate constants k(min⁻¹)c			
	100°C	110°C	121°C	130°C
Citrate buffer pH 4.0	0.192(0.96)[a] ±0.027[b]	0.318(0.94) ±0.064	0.635(0.97) ±0.58	–
Tomato juice pH 4.3	0.374(0.99) ±0.048	0.508(0.95) ±0.106	0.792(0.92) ±0.072	1.065(0.93) ±0.058
Tomato juice pH 4.3 (Limited O₂)	0.160(0.98) ±0.032	0.259(0.97) ±0.041	0.353(0.95) ±0.024	0.488(0.98) ±0.062
Citrate buffer pH 5.0	0.110(0.99) ±0.007	0.215(0.97) ±0.033	0.432(0.98) ±0.049	0.808(0.94) ±0.038

[a] Correlation coefficients (In parenthesis) are significant (p⩾0.05).
[b] 95% confidence interval.
[c] The rate constants in citrate buffers pH 4 and 5 are repeated here to facilitate comparison.
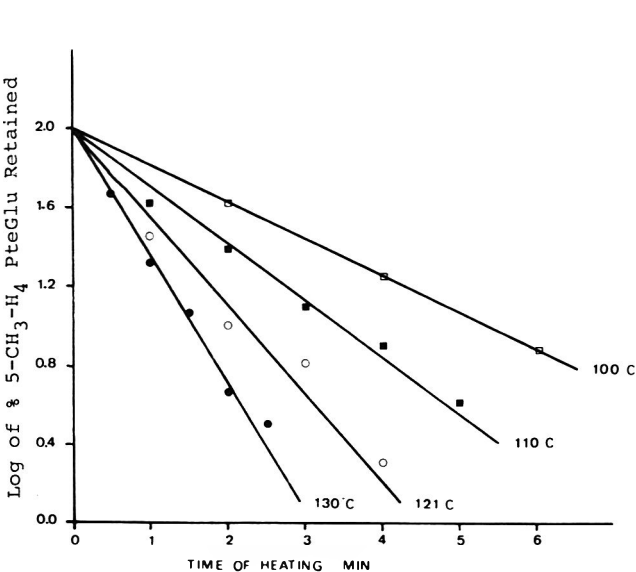
Fig. 4—Rate of destruction curves for 5-CH₃-H₄PteGlu in tomato juice, pH 4.3. Correlation coefficients, 0.99 at 100°C, 0.95 at 110°C, and 0.93 at 130°C are significant (p > 0.01) and the correlation coefficient 0.92 at 121°C is significant (p > 0.05). The slopes of the lines at 100 and 110°, 110° and 121° are not different (p > 0.05).

Table 5—Rates of destruction of 5-CH₃-H₄PteGlu in citrate buffer (pH 3.4), apple juice (pH 3.4) and in apple juice (pH 3.4) with limited oxygen (5.3 ppm)

System	Rate constants k(min⁻¹)		
	50°C	60°C	70°C
Citrate buffer pH 3.4	0.015(0.98)[a] ±0.004[c]	0.022(0.96)[b] ±0.006	0.036(0.97) ±0.008
Apple juice pH 3.4	0.123(0.96) ±0.032	0.193(0.95) ±0.039	0.249(0.97) ±0.047
Apple juice pH 3.4 (Limited O₂)	0.089(0.99) ±0.014	0.126(0.92) ±0.023	0.200(0.89) ±0.031

[a] Correlation coefficients (In parenthesis are significant (p⩾0.05)
[b] Rate constants bracketed by the same line are not significantly different (p⩾0.05)
[c] 95% confidence interval

Fig. 5—Rate of destruction curves for 5-CH₃-H₄PteGlu in apple juice, pH 3.4. The correlation coefficients, 0.96 at 50°C, 0.95 at 60°C and 0.97 at 70°C are significant (p > 0.01).

Table 6—Activation energies for the destruction of 5-CH₃-H₄PteGlu in different systems

System	Activation energy[a] (kcal/mole)
Apple juice pH 3.4	7.85(0.99)
Citrate buffer pH 3.4	9.58(0.99)
Tomato juice pH 4.3	10.6(0.99)
Apple juice (Limited Oxygen)	9.48(0.98)
Tomato juice (Limited Oxygen)	10.8(0.99)

[a] Activation energies are not significantly different (p⩾0.05).

(Tables 4 and 5). The high rates of destruction in the food systems could be due to unknown reactions between the 5-CH₃-H₄ PteGlu and some components of the food system. At high temperatures (121–140°C), the apple juice turned brown in color, probably due to browning of the sugars in the juice, but at low temperatures (50–70°C) no apparent change in color was observed. The 5-CH₃-H₄PteGlu might have reacted with the products of the degradation of the

sugars. Treatment of the food system to release the native folates might have affected the composition of the food systems, or an important component in the food systems, which could have stabilized the $5\text{-}CH_3\text{-}H_4PteGlu$, might have been removed with the native folates during the Dowex treatment. Other vitamins, such as thiamin (Feliciotti and Esselen, 1957) and pterolyglutamic acid (Mnkeni and Beveridge, 1982) have been shown to be more stable in food systems than in buffer solutions.

Reducing the amount of dissolved oxygen in the tomato juice reduced the rates of destruction significantly at all temperatures (Table 4), but in the apple juice significant difference was only observed at 60°C (Table 5). Activation energies obtained in the different systems are shown in Table 6. The values are not significantly different, indicating that the mechanism of destruction in the food systems does not change, although the rates of destruction were significantly reduced.

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Fiber Contents of Selected Raw and Processed Vegetables, Fruits and Fruit Juices as Served

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─────────ABSTRACT─────────

Pectin and neutral detergent fiber (NDF) contents were investigated for commercially processed foods as served: 10 vegetables, 10 fruits, and 8 fruit juices. Fiber content of fruits ranged from 0.7%–4.5% NDF and 0.28%–0.48% pectin; juices ranged from 0.1%–0.22% NDF and 0.2%–0.39% pectin. Fiber in frozen, canned and cooked vegetables ranged from 1%–4.4% NDF and 0.5%–1% pectin. In general, there is no evidence that any of the food preparation methods was superior. There appears to be no great loss of fiber when food is prepared by typical home methods or commercial processing.

INTRODUCTION

ALTHOUGH the role of fiber in the diet has received much attention recently (Inglett and Falkenhag, 1979), relatively little data exist on the individual fiber constituents of fruits and vegetables (Harland and Prosky, 1979). Progress has been impeded largely by lack of agreement on the definition of dietary fiber and on the analytical methodology necessary to characterize dietary fiber components.

The crude fiber method used in the past to characterize the fiber contents of fruits and vegetables is known to give falsely low values because of varying losses of fiber components including cellulose, lignin, hemicellulose and water soluble fiber (Van Soest, 1966). Detergent fiber methods (Van Soest, 1963; Goering and Van Soest, 1970) estimate cellulose, lignin and hemicellulose but certain water soluble gums, mucilages and pectins are not included (Baker et al., 1979). The American Association of Cereal Chemists has adopted as an official method a neutral detergent fiber (NDF) procedure which utilizes amylase digestion to remove starch that might otherwise adhere to the fiber (AACC, 1970).

Although no official methods are available for measuring water soluble fiber, procedures for measuring pectin, a major component of dietary fiber, are reasonably advanced (Southgate, 1969).

The purposes of this study were to determine insoluble neutral detergent fiber (NDF) and soluble pectin contents of selected fruits and vegetables in the table-ready state, i.e., raw, home-cooked and commercially processed, and to determine the effect of cooking on the fiber contents of several products. NDF and pectin were chosen as estimates of the fiber content because they are more rapid analyses than the detailed assay methods for the individual components of dietary fiber (see Anderson and Clydesdale (1980)). The data presented should be valuable as a guide to the effects of normal cooking and processing on the fiber content of fruits and vegetables, and for inclusion in tabulations of nutritional data such as USDA Handbook #8.

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MATERIALS & METHODS

Sample procurement and preparation

The study consisted of two phases: in the first phase, consumer packs of fruit juices, canned and frozen fruits and frozen vegetables were procured from retail or commercial sources and analyzed for their fiber content in the "as consumed" state. The second phase of the study was more extensive in that bulk quantities of seven products were procured and subsamples were taken and subjected to commercial canning, freezing and home cooking methods. The raw, cooked and commercially processed samples were analyzed for fiber content and appropriate comparisons made to determine if the preparation procedures produced any changes in fiber content.

Juice. The following juices were purchased at local markets as frozen concentrates: apple, grapefruit, grape and tangerine. The lemon and lime juices were purchased as bottled juices made from concentrates. Bottled and chilled single strength orange juice was purchased at a local outlet and retail units of papaya nectar were procured from a commercial packer.

All the juices were assayed as obtained without any sample preparation other than mixing. The frozen concentrates were allowed to thaw slightly, to make sampling easier. All samples were placed in Whirl-pak bags and frozen until analyzed.

Fruits. The following samples were obtained in consumer size cans from a commercial packer: blackberries, blueberries, boysenberries, gooseberries, strawberries, red raspberries and applesauce. All the berries were packed in heavy syrup except gooseberries which were in light syrup. Six #303 cans of berries were composited as a single sample in a food processor. All samples were placed in Whirl-pak bags and frozen until analyzed.

The following frozen samples were obtained as retail size units from local stores or commercial packers: loganberries, red sour cherries, boysenberries, blackberries, blueberries and melon balls. The fruits were individually composited in a food processor while still frozen. As little thawing as possible was allowed before compositing and storage.

Frozen vegetables. The following vegetables were procured as frozen, retail size units from local outlets or commercial packers: green beans, sweet peas, chopped spinach, brussels sprouts, chopped broccoli, broccoli spears, cut corn, winter squash and rhubarb. All vegetables were analyzed as the original frozen sample and as a "home-prepared" sample. The frozen vegetables were allowed to thaw until individual pieces could be separated. A composite of each sample was formed and then ground in a food processor. The samples were placed in Whirl-pak bags and frozen until analyzed.

A detailed description of the procedures used for the home prepared samples are included in Table 1. The samples were stirred during cooking as appropriate. Each cooked sample consisted of the

Table 1—Summary of cooking treatment for frozen vegetables

Sample	Amount sample cooked (oz)	Water added (ml)	Cooking time (min)	Drain cooked sample
Green beans	9	80	10	no
Peas	10	60	4	no
Chopped spinach	10–12	80	2	no
Brussels sprouts	8–10	80	10	yes
Chopped broccoli	10	60	3	yes
corn	10	60	2	no
Broccoli spears	8–10	120	8	yes
Winter squash	12–14	15	10	no

contents of three saucepans, which were composited in a food processor and placed in Whirl-pak bags and frozen until analyzed.

Rhubarb was prepared in a different manner: one-fourth cup of sugar was added to one-fourth cup of water and brought to a boil in a saucepan. The burner heat was reduced to low, and the syrup boiled for 2 min. One and one-fourth cups of rhubarb were added, and the rhubarb cooked for 8 min. The contents of several saucepans were composited in a food processor as a single sample. The samples were placed in Whirl-pak bags and frozen until analyzed.

For the second phase of the study, NFPA procured raw, frozen, and canned samples from commercial processors. If the processor did not have freezing facilities, blanched samples were obtained and packed in freezer containers and frozen. If the processor did not have canning facilities, raw samples were canned in NFPA's pilot plant, using a commercial process. The products sampled were apples, sweet potatoes, spinach, peas, summer squash, lima beans and carrots. Three separate samples of each item were obtained; in some cases, the samples came from crops maturing at different times during the growing season. An attempt was made to have the individual sample as uniform as possible so that when subsamples were taken, the natural variation would be minimized. Before being prepared, each subsample was washed and rinsed in distilled deionized water and any imperfections or extraneous materials were removed. After preparation, all samples were placed in Whirl-pak bags and frozen until analyzed. A detailed description of the cooking procedures used are included in Table 2. The samples were stirred during cooking as appropriate. Each cooked sample consisted of the contents of three or more saucepans, which were composited in a food processor and placed in Whirl-pak bags and

frozen until analyzed. Raw, frozen and canned unheated (drained) samples were composited and frozen as previously described.

Analytical methods

Insoluble fiber was estimated using the Schaller modification (AACC, 1970) of the Neutral Detergent Fiber procedure of Goering and Van Soest (1970). Pectin was estimated using a modification of Southgate's Total Dietary Fiber procedure (Southgate, 1969).

The analytical scheme used for pectin is shown in Fig. 1 and includes methanol extraction for sugar removal, an ether extraction for fat removal, and amylase treatment for starch removal. The pectin was extracted by the EDTA-Pectinase treatment of McCready and McComb (1952) and determined colorimetrically using the m-hydroxydiphenyl method of Blumenkrantz and Asboe-Hansen (1973).

RESULTS & DISCUSSION

TABLE 3 contains NDF and pectin data for the fruit juice samples. Most of the juices contained only minimal amounts of insoluble fiber. The papaya nectar contained more pulpy material and showed a higher fiber content. Much of the insoluble fiber from the fruit is removed during the juice extraction or finishing operation.

The amount of pectin in fruit juices averaged about 0.3% and was generally higher than the insoluble fiber content. Some removal of pectic substances may occur during the juice clarification process. Fruit juice is a class of food in which the pectin content exceeds the insoluble fiber content.

Table 4 shows the NDF and pectin contents of selected frozen and canned fruits. The NDF values ranged from 0.7—4.5% and were generally higher in the berries than in the fruits. The variability of the insoluble fiber data for the

Table 2—Summary of cooking treatments for products in phase 2

Sample/cooking method	Amount of sample cooked (oz.)	Water added (ml)	Cooking time (min)	Drain cooked sample
Apple				
Boiled	16	20	10	no
Microwave Cooked	16	10	6	no
Frozen, Boiled	10	15	3	no
Canned, Heated	16		4	no
Sweet Potatoes				
Boiled	42	950	35	yes
Microwave Baked	42		12	no
Frozen, Baked	18		35	no
Canned, Baked	18		30	no
Spinach				
Boiled	16		5	no
Microwave Cooked	16		9	no
Frozen, Boiled	10	30	8	no
Canned, Heated	16		2	yes
Peas				
Boiled	16	30	7	no
Microwave Cooked	16		7	no
Frozen, boiled	10	15	4	no
Canned, Heated	16		2	yes
Squash				
Boiled	16	20	13	yes
Microwave Cooked	16		7	yes
Frozen, Boiled	10	5	7	yes
Canned, Heated	16		2	yes
Lima Beans				
Boiled	16	100	25	no
Microwave Cooked	16	100	15	no
Frozen, Boiled	10	100	18	no
Canned, Heated	16		2	yes
Carrots				
Boiled	16	75	13	no
Microwave Cooked	16	30	10	no
Frozen, Boiled	10	30	14	no
Canned, Heated	16		2	yes
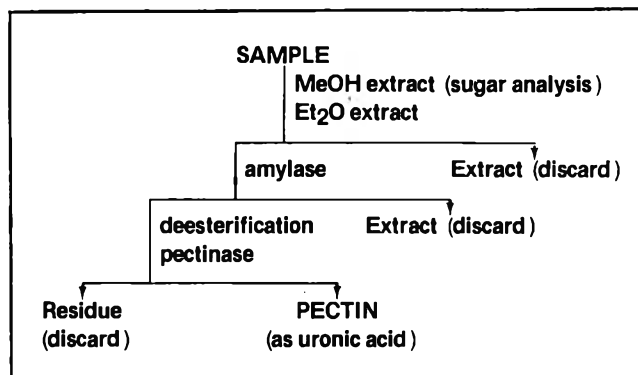
Fig. 1—Analytical scheme used for pectin analysis.

Table 3—Fiber content of fruit juices

Product	Number of samples	NDF[a] (%)	Pectin[a] (%)
Concentrate			
Apple	3	<0.1	0.2 ± 0.05[b]
Grapefruit	3	<0.1	0.3 ± 0.15
Grape	3	0.1 ± 0.05	0.4 ± 0.10
Tangerine	3	0.2 ± 0.15	0.4 ± 0.05
Bottled juice			
Lemon	6	0.1 ± 0.10	0.2 ± 0.05
Lime	3	<0.1	0.3 ± 0.05
Orange	3	0.2 ± 0.05	0.2 ± 0.01
Fruit nectar			
Papaya	3	0.3 ± 0.05	0.3 ± 0.10

[a] On wet weight basis
[b] Mean ± 1 standard deviation

berry samples is somewhat higher than expected because the small seeds tended to settle rapidly from the mixture and a homogeneous subsample was difficult to obtain.

The amount of pectin in the fruits ranged from 0.3–0.8% with most samples being between 0.4–0.5%. As in most foods, the pectin content is lower than the NDF.

Three types of berries were analyzed as frozen and canned samples. There appears to be little difference between either NDF or pectin content due to the type of processing. Direct comparisons should not be made because the samples were not from the same lot of fresh fruit.

The NDF and pectin content of selected frozen vegetables, before and after mild cooking, are shown in Tables 5 and 6, respectively. Table 5 lists the NDF content and percent retention for nine vegetables. Percent true retention was calculated using the following formula (Murphy et al., 1975).

$$\% \, TR = \frac{\text{Nutrient in Cooked Food}}{\text{Nutrient in Raw Food}} \times \frac{\text{Wt of Cooked Food}}{\text{Wt of Raw Food}} \times 100$$

This formula accounts for moisture gains and losses *during cooking*. The percent true retention is 100 if there is no real gain or loss of the nutrient *during cooking*. The NDF content of the uncooked vegetables ranged from 1.2% for rhubarb to 3.9% for peas, and the cooked vegetables, ranged from 1.0–4.1%. In general, the cooking procedures did not significantly alter the fiber content of the vegetables. The cooked vegetables contained as much insoluble fiber on a gram per 100 gram basis as did the frozen product. When allowing for changes in moisture content during cooking, the percent retention values approximate 100%, indicating that the NDF was well retained. There appears to be some minor loss of NDF in Brussels sprouts and broccoli; some or all of this loss, however, may be due to draining the cooked sample. These were the only vegetables drained, and the only ones demonstrating a loss. We suspect that the loss of NDF is due to loosening or physical breaking of the insoluble fiber into smaller particles which are washed away during draining.

Peas and corn appear to have increased their NDF content during cooking. Matthee and Appledorf (1978) suggest that an increase in NDF, ADF and cellulose (observed on a dry weight basis) might be a result of liberation of cellulose; Herranz (1981) also observed this phenomena. It is significant to note, however, that longer cooking times were reported in both articles. We did not investigate this phenomena further.

Table 6 lists the pectin content and retention values for these vegetables before and after cooking. Pectin in the frozen samples ranged from 0.4% in corn to 1.4% in Brussels sprouts and was similar in the cooked samples. None of the samples showed significant changes in pectin content during cooking, although the retention values for broccoli (a drained sample) appear to be somewhat lower. The high retention value for corn indicates that there was a significant increase of pectin in the cooked sample.

The high pectin retention for the cooked rhubarb may be due to the large quantity of sugar added during preparation. The analytical procedure was not designed for such high sugar levels. The neutral detergent fiber and pectin contents of the products in phase 2 are shown in Tables 7 and 8, respectively. Each of the entries is the mean of three separate samples, analyzed in duplicate.

Table 7 lists the NDF content and percent retention values of the fruit and six vegetables prepared in seven ways. The NDF contents of the raw vegetables range from 1.1% in squash to 4.1% in lima beans.

On a wet weight basis, preparation methods appear to have little effect on the NDF content of the vegetables or fruit. The NDF value for raw spinach is not representative of the cooked samples because the cooked samples were trimmed more closely than the raw. This produced an exaggerated loss of NDF in the cooked samples. In a few cases, on the basis of the percent retention data, small gains or losses of fiber due to cooking were observed. Some of the loss of insoluble fiber in the summer squash is due to draining the cooked samples. Also, in the lima beans and carrots, an increase in the NDF content was observed.

Table 4—Fiber content of fruits

Product	Number of samples	NDF[a] (%)	Pectin[a] (%)
Frozen Fruits			
Loganberries	3	4.4 ± 0.2[b]	0.4 ± 0.1
Melon balls	3	0.7 ± 0.2	0.8 ± 0.1
Red sour cherries	3	0.8 ± 0.1	0.5 ± 0.1
Blackberries	3	4.4 ± 0.2	0.6 ± 0.3
Blueberries	3	2.1 ± 0.6	0.4 ± 0.1
Boysenberries	3	3.6 ± 0.6	0.3 ± 0.1
Canned Fruits			
Gooseberries	3	2.1 ± 0.2	0.3 ± 0.1
Strawberries	3	1.3 ± 0.4	0.4 ± 0.1
Red raspberries	3	2.8 ± 0.2	0.5 ± 0.2
Blackberries	3	4.5 ± 0.3	0.5 ± 0.1
Blueberries	3	1.7 ± 0.2	0.4 ± 0.1
Boysenberries	3	3.8 ± 0.2	0.3 ± 0.1
Applesauce	6	1.4 ± 0.4	0.3 ± 0.1

[a] On wet weight basis
[b] Mean ± 1 standard deviation

Table 5—Neutral detergent fiber content and retention for frozen and cooked vegetables

Product	Number of samples	NDF[a] (%) Frozen	NDF[a] (%) Frozen cooked	Retention (%)
Rhubarb	3	1.2 ± 0.1[b]	1.0 ± 0.1	106
Green beans	3	1.8 ± 0.1	1.5 ± 0.1	92
Peas	3	3.9 ± 0.4	4.1 ± 0.4	116*
Spinach	3	2.1 ± 0.3	2.1 ± 0.3	98
Brussels sprouts	3	2.3 ± 0.2	1.8 ± 0.2	80*
Chopped broccoli	3	2.3 ± 0.2	2.2 ± 0.2	82*
Broccoli spears	3	2.1 ± 0.1	2.2 ± 0.1	93
Corn	3	2.1 ± 0.2	2.1 ± 0.3	115*
Winter squash	3	1.8 ± 0.1	1.7 ± 0.4	91

[a] On wet weight basis
[b] Mean ± 1 standard deviation [*Significantly different (95% confidence level) from 100]

Table 6—Pectin content and retention for frozen and cooked vegetables

Product	Number of samples	Pectin[a] (%) Frozen	Pectin[a] (%) Frozen cooked	Retention (%)
Rhubarb	3	0.7 ± 0.1[b]	1.0 ± 0.2	202
Green beans	3	1.0 ± 0.1	0.9 ± 0.1	95
Peas	3	0.7 ± 0.1	0.6 ± 0.1	98
Spinach	3	1.1 ± 0.3	1.1 ± 0.2	101
Brussels sprouts	3	1.4 ± 0.1	1.4 ± 0.1	105
Chopped broccoli	3	1.0 ± 0.1	1.1 ± 0.1	91
Broccoli spears	3	1.0 ± 0.3	1.0 ± 0.2	90
Cut corn	3	0.4 ± 0.1	0.4 ± 0.1	121*
Winter squash	3	0.6 ± 0.1	0.7 ± 0.1	101

[a] On wet weight basis
[b] Mean ± 1 standard deviation [*Significantly different (95% confidence level) from 100]

Table 7—NDF content for vegetables cooked by various methods

Cooking method	Apple NDF[a]	(% R)	Carrots NDF[a]	(% R)	Lima beans NDF[a]	(% R)	Peas NDF[a]	(% R)	Spinach NDF[a]	(% R)	Summer squash NDF[a]	(% R)	Sweet potatoes NDF[a]	(% R)
Raw	2.3±0.6[c]	—	1.8±0.4	—	3.7±0.2	—	3.4±0.7	—	3.2±0.6	—	1.1±0.2	—	2.1±0.7	—
Boiled	1.9±0.4	86	1.9±0.3	109	4.1±0.6	117*	3.4±0.6	101	2.1±0.2	61*	1.1±0.1D[b]	80	1.7±0.3	81
Microwave Cooked	2.4±0.8	101	1.9±0.3	93	4.4±0.5	116*	4.2±0.5	116	1.9±0.2	52*	1.0±0.1D	74*	1.9±0.4	83
Frozen	2.0±0.7	—	1.4±0.1	—	4.3±0.3	—	3.8±0.3	—	2.1±0.2	—	1.2±0.3	—	1.7±0.4	—
Frozen, Boiled	1.9±0.5	105	1.8±0.1	134*	4.0±0.3	107	3.3±0.3	85*	2.2±0.3	96	1.3±0.2D	79*	1.5±0.2	92
Canned, Unheated	1.8±0.6	—	1.1±0.2D[b]	—	4.2±0.4D	—	4.4±0.4D	—	2.9±0.5D	—	1.1±0.3D	—	1.3±0.2	—
Canned, Heated	2.0±0.8	121	1.7±0.2D	141*	4.0±0.4D	94	4.2±0.6D	90	1.6±0.5D	36*	1.5±0.1D	107	1.2±0.4	88

[a] In percent by wet weight
[b] D = Drained sample
[c] Mean ± 1 standard deviation
*Significantly different (95% confidence level) from 100

Table 8—Pectin content for vegetables cooked by various methods

Cooking method	Apple Pectin[a]	(% R)	Carrots Pectin[a]	(% R)	Lima beans Pectin[a]	(% R)	Peas Pectin[a]	(% R)	Spinach Pectin[a]	(% R)	Summer squash Pectin[a]	(% R)	Sweet potatoes Pectin[a]	(% R)
Raw	0.4±0.1[c]	—	1.0±0.3	—	1.2±0.2	—	0.8±0.1	—	0.8±0.1	—	0.6±0.1	—	0.8±0.2	—
Boiled	0.5±0.1	153	1.1±0.2	109	1.0±0.1	91	0.8±0.1	104	0.8±0.1	96	0.5±0.1D[b]	66*	0.8±0.2	103
Microwave Cooked	0.5±0.1	131	1.2±0.1	109	1.2±0.2	99	0.8±0.1	92	0.8±0.1	90*	0.5±0.1D	72*	0.8±0.1	91
Frozen	0.5±0.1	—	—	—	—	—	—	—	—	—	—	—	0.8±0.1	—
Frozen, Boiled	0.4±0.1	89											0.8±0.1	97
Canned, Unheated	0.4±0.1	—											0.7±0.1	—
Canned, Heated	0.5±0.1	116											0.6±0.1	89

[a] In percent by wet weight
[b] D = Drained sample
[c] Mean ± 1 standard deviation
*Significantly different (95% confidence level) from 100

The pectin content and percent retention values during cooking for the same products are shown in Table 8. The data show no readily apparent loss of pectin during cooking. Squash was the only exception; this is probably due to the draining of the cooked samples. Overall, pectin appears to be better retained during simulated home cooking than NDF.

CONCLUSION

THIS PAPER has listed the fiber values for many fruit and vegetable products; several of these can be significant sources of fiber in the normal diet. In general, there is no evidence that any of the food preparation methods was superior. There appears to be no great loss of fiber when food is prepared by typical home methods or commercial processing.

Fruit juices were found to have measurable amounts of pectin but little insoluble fiber. The method of processing had little effect on the fiber content of fruits and vegetables. However, samples that were drained after preparation usually demonstrated some loss of fiber. The soluble fiber (pectin) was better retained than the insoluble fiber (NDF) which occasionally exhibited some minor loss.

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Studies on Blackening of Pepper (*Piper nigrum*, Linn) During Dehydration

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———————————ABSTRACT———————————

Histochemical studies carried out in fresh green pepper berries at different stages of maturity showed that phenolic compounds distributed throughout the berries at a very young stage were confined to the epicarp and mesocarp alone at full maturity. The blackening that occurs in pepper on drying or on injury also showed a similar distribution pattern. Flavanols were not found in the young stage but appeared in the innermost cells of mesocarp covering endocarp after fertilization. Spores of *Glomerella cingulata*, present even in healthy pepper berries, were found to be the source of phenolase enzyme taking part in the blackening. Phenols in pepper were enzymatically oxidized and gave rise to black color when the cells were disturbed by dehydration or maceration.

INTRODUCTION

THE SKIN of the black pepper (*Piper nigrum*. Linn) is green at the time of harvest. On drying the berries turn black. In many plant products, food browning is found to occur by the phenolase catalyzed oxidation of colorless phenolic substrates followed by different secondary reactions (Mathew and Parpia, 1971). Such enzyme catalyzed oxidative reaction on polyphenols has been found to be the cause for the formation of black color of tea. In the case of pepper, however, no scientific investigations on the blackening have been carried out. This paper records the histochemical studies carried out on fresh green pepper berries with a view to studying the blackening of the berries during dehydration.

MATERIALS & METHODS

THE FRESH PEPPER BERRIES belonging to different varieties like *Karimunda, Velliyaramunda, Madamankotta, Chumala, Cheriyakaniakadan* and *Panniyur* I were obtained from local gardens (Pepper fruits are botanically classified as drupes, though popularly called berries). The stages of maturity mentioned are as described by Sumathikutty et al. (1979). Dehydrated green pepper berries (Lewis and Krishnamurthy, 1980) used in the studies were made in the laboratory by initially blanching in boiling water for 15 min and subsequent drying in a cross flow drier at 50°C.

The sections of berries were made with a freezing microtome at a thickness of 30μ. The sections were treated by dipping in each of the specific phenolic reagents described. The Folin-Denis reagent (phosphomolybdic tungstic acid solution), prepared as described in AOAC (1975), was diluted by adding 2 parts of water. The sections were dipped in this reagent followed by saturated sodium carbonate solution for 2 min for development of color. Bis-diazotized benzidine (Roux and Maihs, 1960) was prepared by mixing 2 parts of benzidine solution in hydrochloric acid and 3 parts of 10% aqueous sodium nitrite solution. Potassium ferricyanide-ferric chloride reagent was obtained by mixing equal parts of 0.3% aqueous solutions of potassium ferricyanide and of ferric chloride, and vanillin-hydrochloric acid reagent by adding to 2 parts of 10% alcoholic vanillin 1 part of concentrated hydrochloric acid (Mathew and Govindarajan, 1964). The color produced by Folin-Denis reagent was found not to be retained permanently while in all other cases the color developed was permanent. An untreated freshly cut section was used as the control.

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Culturing fungal spores from pepper berries was done after slicing, surface-sterilizing in 1% mercuric chloride for 10 min and washing thrice in sterile distilled water. Surface sterilized pepper slices were transferred to potato-dextrose-agar plates. Fungal growth was separated by inoculating needle using sterile diluents and cultured on potato-dextrose-agar by "pour plate technique" (Collins and Lyne, 1976). The isolated colonies were maintained on potato-dextrose-agar and Czapek-Dox agar (Harrigan and McCance, 1976) for further use.

The microscopic studies were carried out using a PZO Warzawa biological microscope, MB 10. Centrifugation of the macerate was done in a laboratory BGH Roto-Uni centrifuge. Scanning electron micrographs were taken with a Jeol 80 model scanning electron microscope.

RESULTS & DISCUSSION

The sections of pepper at pin-head stage (very young stage, when small undeveloped berries are known as pin heads) and mature stage are represented in Fig. 1. Sections at pin-head stage on treatment with Folin-Denis reagent, developed intense blue color (indicative of the phenolic group) throughout the section. Vascular bundles were also intensely colored blue. Control section remained pale white in color. With increasing maturity, intensity of the blue color with the Folin-Denis reagent decreased and was least in the fully mature stage, at the time of harvest. At full maturity the blue color was seen only in the skin (epicarp and mesocarp), the inner core (endocarp) being devoid of it. Complete absence of phenol in the inner core, as shown by absence of reactivity with the Folin-Denis reagent, coincided with structural specialization including formation of clearly differentiated skin and core. Oil cells also showed their appearance in the mesocarp (Fig. 2).

With bi-diazotized benzidine the mode of color reaction was essentially as above (Fig. 3). The characteristic brown color, developed as a result of coupling of the diazo group of the reagent and phenol in the berries, was slightly more prominent in the epicarp and the outer periphery of the endocarp. The color also developed well in the sections. Potassium ferricyanide-ferric chloride reagent, which reacts with phenolic reducing groups giving Prussian blue color, gave a more or less similar pattern of reactivity as the Folin-Denis reagent and bis-diazotized benzidine.

These reactions clearly showed the distribution of phenolic compounds in pepper berries. At pin-head stage, the phenolic compounds are distributed uniformly throughout the berry. At harvest mature stage phenolic compounds are present only in the epicarp and mesocarp while the endocarp is free from phenolic compounds. When treated with vanillin-hydrochloric acid (Fig. 4) (Mathew and Govindarajan, 1964), no trace of characteristic pink color was seen in the pin-head stage. This reagent specifically reacts with undeactivated phloroglucinol or resorcinol moieties available in flavanols, producing deep pink color. From immature stage onward, the innermost 2 to 3 layers of cells of mesocarp covering the endocarp were pink in color when treated with vanillin-hydrochloric acid, indicating the presence of flavanols. Accumulation of flavanols in these cells was not observed before fertilization and therefore can be taken as a post fertilization change.
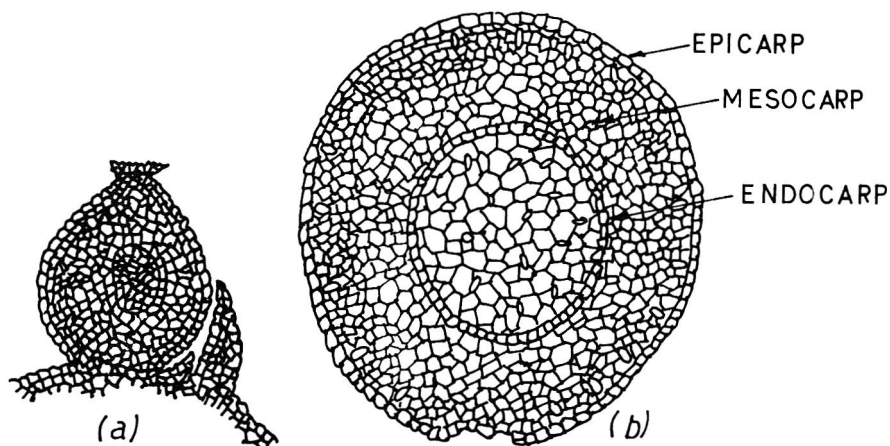
Fig. 1—A section of pepper at (a) pin head stage and (b) mature stage.

EPICARP
MESOCARP
ENDOCARP

(a)
(b)
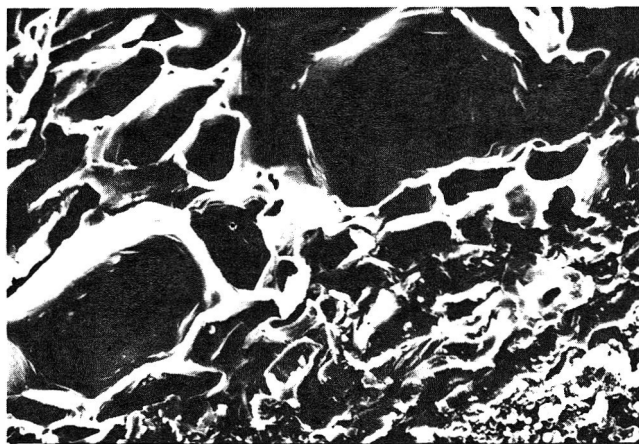
Fig. 2—Oil cells in the mesocarp at mature stage.

Substrates for blackening

When blackening of the sections was observed, it was found that at the pin-head stage as well as in the mature stage the first region to undergo blackening was the innermost layers of the mesocarp. Vacular traces supplying the spherical berries traverse through this region from the spike axis to the stylar end in close proximity to the endocarp. Fully blackened sections showed that blackening followed the same pattern as the distribution of phenols as indicated by bis-diazotized benzidine and Folin-Denis reagent. The only minor deviation from the phenol reactivity was that the cell walls distinctly appeared darker than the cellular cavities, and in the pin-head stage there was slightly more darkening in the skin than the inner core. In the mature stage, as could be expected, there was intense blackening in the skin; the inner core being devoid of blackening. Since vascular traces carry total polyphenols in them, when they were cut or injured, phenolic compounds oozed out and spread in the surrounding area. Vascular traces which traverse the inner most layers of mesocarp account for the localization of substrate for browning in the skin of the pepper fruit. During drying, turgidity of the cells is lost and phenolic extractives spread outwards along with the movement of moisture from inside to outside.

Polyphenol oxidase source

All healthy pepper berries from the variety of *Piper nigrum* that were examined, showed the presence of tiny globular spores having vigorous vibratory motion. On centrifugation of the macerate of fresh green pepper berries, the fraction containing the spores separated at around 2000 rpm (425 x *g*). This fraction produced brown color with extract of a blanched pepper indicating capacity to produce enzymatic change with the substrate present in pepper.

The pepper berries were surface-sterilized and the spores in them were cultured and subcultured four times in Czapek-Dox medium. The culture was found to be a mixture of *Colletotrichum* species, *Glomerella cingulata*, and *Fusarium oxysporum*. Spores of *Glomerella cingulata* were seen in all varieties of *Piper nigrum* studied, but *Fusarium oxysporum* spores did not occur in all the pepper berries examined. *Glomerella cingulata*, which is the perfect stage of *Colletotrichum* species (Westcott, 1960; Bessey, 1961) has been reported to cause *Pollu* disease (Nambiar, 1977)
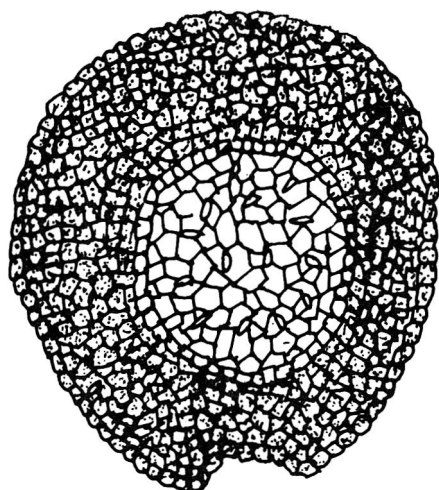
Fig. 3—A section of mature pepper reacted with bis-diazotized benzidine.
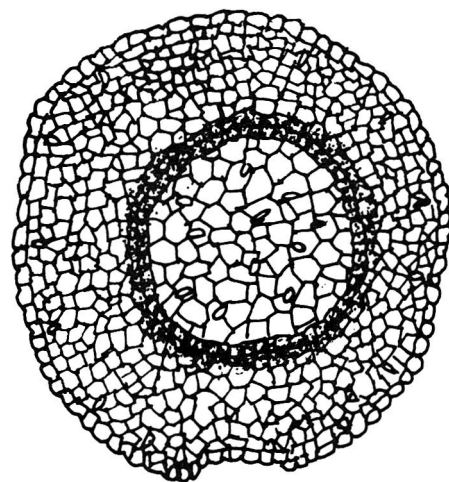
Fig. 4—A section of mature pepper reacted with vanillin-hydrochloric acid.
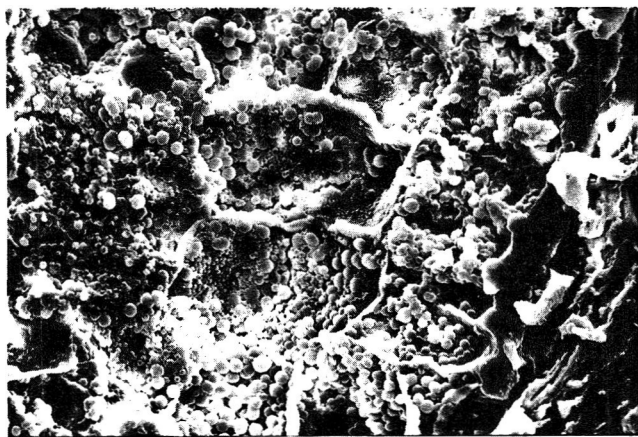
Fig. 5—A section of mature pepper showing fungal spores.
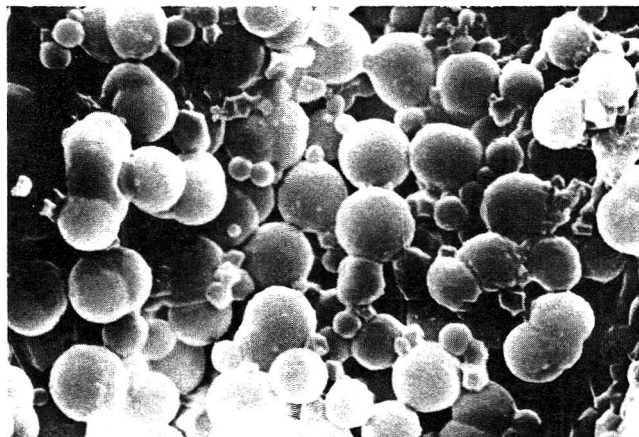
Fig. 6—A magnified portion of Fig. 5.

in pepper. It is not known whether this fungus has a symbiotic relationship with *Piper nigrum*, apart from its pathogenicity. The mycelial mat (in nutrient broth) which was grown from the spores obtained from pepper berries, was washed well with glass distilled water and acetone-dried (Colowick and Kaplan, 1955) using pre-chilled acetone in a Buchner filter and kept overnight in the glass distilled water at 5°C to extract the crude enzyme. The crude enzyme thus prepared, did not react with bis-diazotized benzidine or Folin-Denis reagent, showing that it is devoid of any reactive phenolic moieties. However, when added to an extract of pepper blanched at 100°C to inactivate enzyme and in the presence of phosphate buffer pH 7.0 at an optimum temperature of 73–78°C, the crude enzyme extract resulted in the formation of black color. At room temperature (28–30°C) there was no brown color formation. Neither the extract of blanched berries nor the crude enzyme, incubated separately at pH 7.0, showed any tendency to blacken. The crude enzyme extract, however, turned the green color of blanched berries (e.g. dehydrated green pepper) black. Heating at 100°C for 15 min effectively arrests the blackening. Addition of sodium metabisulphite, which is a well known phenolase inhibitor (Mathew and Parpia, 1971), also arrested the blackening reaction of fresh green pepper berries. With the addition of sodium metabisulphite to the pepper extract the motile spores of *Glomerella cingulata* present in it became immobile and clumped together. The experiment clearly showed that the fungal spores were the source of polyphenolase in the pepper berries. O-Dihydroxy polyphenolase activity has been reported in *Glomerella cingulata* (Walker and McCallion, 1980). No other source of phenolase was evident in the pepper berries and the entire enzyme activity appeared to be accounted for by the fungus. A possible partial contribution of enzymatic activity by yeast has been indicated in the tea fermentation, especially in the formation of better flavor (Pederson, 1979). Since no variety of pepper free from the spores of *Glomerella* is available it could not be differentiated from the enzyme system of the pepper tissues, if a phenolase system is present in the tissues of pepper. As could be seen from the scanning electrom micrographs (Fig. 5 and 6) innumerable spores occupy the inner core of healthy and mature pepper berries. As has been seen in Fig. 2, the oil cells occur mainly in the skin. In an in vivo system the spores from the fungus acted as an excellent enzyme source. Unlike most

other enzymes which lose their activity at high temperatures, this enzyme from the spores in pepper is peculiar in two respects: (1) optimum activity at a high temperature of 73–78°C; (2) heating for 10–15 min in boiling water is necessary for complete inactivation. The blackening of pepper therefore is clearly a polyphenolase catalyzed reaction. The phenolase enzyme source is the spores of *Glomerella cingulata* present in the berries. This is, therefore, an exmple of enzymatic browning, wherein the enzyme is not a part of the plant material itself but of alien origin.

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Changes in Chemical Constituents of Kiwifruit During Post-Harvest Ripening

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ABSTRACT

Kiwifruit (*Actinidia chinensis* Planch, Hayward cultivar) were ripened at 20°C under a stream of water-vapor saturated air containing 5 ppm ethylene gas. A remarkable rapid softening in the texture and rising of the soluble solids were observed in 24 hr. The total acidity, starch and amylose content decreased during ripening. Both fructose and glucose increased from 2.7% to 5.0% after 5 days of ripening. Sucrose content increased from 0.45% to 2.22% on the second day, then decreased to 1.19% after 5 days. No significant change in chlorophyll and total solids were observed throughout the experiment. There were stepwise decreases in the L and b_L values during ripening. The ascorbic acid content decreased from 210 to 190 mg per 100g fresh fruit after 5 days of ripening.

INTRODUCTION

THE KIWIFRUIT (*Actinidia chinensis* Planch) has a bright emerald green flesh covered with a thin furry skin. It has a characteristic attractive flavor when at the proper ripeness stage. The fruit is cultured principally in New Zealand and exported. In recent years, however, it has also been cultivated in Australia, California, the Mediterranean countries, and Japan. General informal concerning history, taxonomy, cultivar and culturing of kiwifruit was described by Schroeder and Fletcher (1967) and Magoon (1979). The physical and chemical changes in kiwifruit under various conditions were reported by Wright and Heatherbell (1967), Harris et al. (1972), Pratt and Reid (1974) and Mitchell et al. (1979).

Heatherbell (1975) analyzed the sugar and nonvolatile organic acids in kiwifruit by gas-liquid chromatography. Simmons (1978) studied the procedures for dried and canned kiwifruit. Heatherbell et al. (1980) also developed the process of a new fruit wine from kiwifruit. Wildman and Luh (1981) reported on the effect of sweetner types on quality of kiwifruit nectar.

In this work, the physical and chemical changes in ripening of kiwifruit in the presence of 5 ppm ethylene gas at 20°C were investigated.

MATERIALS & METHODS

Kiwifruit

Sixty kg of kiwifruit (Hayward cultivar) were harvested at the mature green stage in October 1980, and were stored at 1°C for experiments. The average weight, size and total solids of the kiwifruit used in the experiment are presented in Table 1.

Ripening the kiwifruits

Twelve kg of selected kiwifruit were placed in glass containers in a room kept at 20°C. A stream of water-vapor saturated air containing 5 ppm ethylene was passed over the fruit at a rate of 50 ml/min. The control sample (12 kg) was treated under the same condition except that no ethylene gas was used in the aeration process.

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Firmness

The fruits were peeled in a 10 x 10 mm area on both cheeks. The firmness of individual fruit was determined with a U.C. firmness tester with a 5/16 inch (0.794 cm) plunger. The average of 12 readings at 20°C was reported.

Sample preparation

Kiwifruit puree was prepared for analysis as follows unless described otherwise: The kiwifruits were hand peeled, followed by removal of seeds and cores. The products were homogenized at 1°C for 3 min in a Waring Blendor. The puree was frozen immediately in sealed jars at −26°C. The frozen puree was kept at −26°C in sealed glass jars.

pH Value and titratable acidity

A Corning Model 12 glass electrode pH meter was used to determine the pH value. Ten grams of the puree were titrated with 0.1N NaOH to pH 8.0. The results were expressed as percent citric acid.

Total solids

The AOAC (1980) vacuum oven drying method was used with slight modification. Five grams of sample were weighed accurately (±0.0001g) into aluminum dishes using a Sartorius 2400 balance. The aluminum dish containing diatomaceous earth was dried and weighed prior to adding the sample. The loss in weight after vacuum drying at 70°C for 6 hr was determined.

Starch and amylose

The Anthrone reagent method and iodine colorimetric method described by McCready et al. (1950) were employed to determine starch and amylose contents respectively.

A Perkin-Elmer's Model Coleman 575 spectrometer was used. Amylose content was measured at 660 nm, and total starch at 630 nm.

Soluble solids

The soluble solid was measured with a Zeiss Opton refractmeter at 20°C. The results are reported in degree Brix at 20°C.

HPLC for sugar determination

The HPLC described by Hunt et al. (1977) and Wildman and Luh (1981) was used to determine the sugars in kiwifruit with some modifications. A Waters Associates Chromatograph equipped with a Model 6000-A solvent delivery system, a Model R401 refractometer detector, a U6K universal injector and variable speed Omniscribe recorder was used. The column was a 30 cm x 4 mm i.d. stainless steel tube packed with μ-Bondapak-carbohydrate (Waters Associates). The precolumn was packed with CO-PELL PAC (Whatman).

Table 1—Properties of fresh kiwifruit used in the ripening study

Weight [Average of 12 fruits], g	69.60 ± 6.30[a]
Height, cm	5.67 ± 0.29
Major axis, cm	4.76 ± 0.25
Minor axis, cm	4.19 ± 0.22
Pressure test [0.794 cm plunger], lb	13.82 ± 0.12
pH	3.26
Acidity [% as citric acid]	1.46
Total solids, %	15.90
Soluble solids [°Brix at 20°C]	8.53

[a] ± : Standard deviation

The eluent was acetonitril (Burdric & Jackson high purity grade) and distilled water (85/15, v/v). Acetonitrile was filtered through a 0.5 μm flouropore filter. Distilled water was purified through a millipore-Q system. The solvent mixture was degassed for 1 min under vacuum with shaking.

Chromatographic standards were fructose, glucose, sucrose, and maltose. Standard sugar solutions of 1, 2, 3, 4 and 5% (w/v) were prepared. A calibration curve was obtained for each of the four sugars. Sugars in the samples were quantified by comparing peak areas of the samples to those of the sugar standards.

Five grams of the puree were refluxed with 45 ml of 90% (v/v) ethyl alcohol containing a small amount of CaCO3 for 1 hr at 80°C on a water bath. The mixture was filtered through Whatman No. 1 filter paper into a round bottom flask, and the residue was washed with 80% ethyl alcohol. The filtrate was evaporated to about 5 ml under vacuum at 30°C in a Buchis model Rotavapor-R unit, and diluted to 10 ml in a volumetric flask. Finally the solution was filtered through a 0.5 μm celotate filter (Millipore Corp.), using a Swinnex syringefilter. The injection volume was 10 μl in all cases.

Chlorophyll

The AOAC (1980) spectrophotometric method was used for determination of chlorophyll.

A Perkin-Elmer model Coleman 575 spectrophotometer was used to measure the absorbance at 660 and 642.5 nm respectively.

Results were reported as total chlorophyll, chlorophyll a, and chlorophyll b using the Comar and Zscheile equations (1942).

Color measurement

The color of the kiwifruit puree during ripening were measured in a cylindrical sample cup 5.6 cm in diameter. The depth of the sample was 2 cm. The sample cup was made of plastic with a colorless bottom plate 1.5 mm thick. A light-trap can coated inside with black paint was used to prevent light from entering the sample part. The illuminated area was elliptical 4.1 x 4.4 cm. Standard color plate No. $C_2 1062$ having reflectance values L = 64.8, a = 14.3, b = 6.4 was used as a reference.

The color determination are reported as L (brightness), a (green to red) and b (yellow to blue) values.

Ascorbic acid

The hydrazine colorimetric method was used to determine the ascorbic acid (Roe et al., 1948). Five grams of peeled kiwifruit were macerated in a mortar with 40 ml of 5% meta-phosphoric acid and glass beads. After adding 155 ml of meta-phosphoric acid, the product was centrifuged. The supernatant was used to determine the total ascorbic acid and dehydro-L-ascorbic acid. L-ascorbic acid content was obtained by the difference between total ascorbic acid

and dehydro-L-ascorbic acid. The results are expressed as mg per 100g sample.

RESULTS & DISCUSSION

Ripening

Firmness, one of the indicators for maturity level was influenced greatly by the presence of ethylene gas.

A rapid softening in texture was observed when the kiwifruits were exposed to ethylene gas at the 5 ppm level for 24 hr (Fig. 1). The firmness decreased from 13.8 lb to 6.1 lb on the firmness gauge at 20°C in 24 hr. However, very little change was observed in the control sample. The pressure test of the control sample was 9.4 lb even after 20 days at the sample temperature. Results indicate that ripening of kiwifruit was stimulated strongly by ethylene gas at the 5 ppm level.

Ethylene was shown to be produced naturally in plant tissue from glucose, linoleic acid and methionine (Yang, 1980). It is known to act as a plant hormone which controls the ripening process. The effect of ethylene on initiation of fruit ripening was described by Biale (1964), Pratt and Goeschl (1969) and Yang (1980). There are two principal concepts about fruit ripening. First, ripening occurs due to the increase in cell permeability which leads to the random mixing of enzymes and substrates present in the tissue (Bain and Mercer, 1964; Sacher, 1966). Secondly, the ripening process is at the final stage of differentiation process which is under genetic control. It involves the programmed synthesis of specific enzymes required for ripening (Frenkel et al., 1968; Hulme et al., 1968). Its effect on kiwifruit ripening was clearly demonstrated in this work.

Pratt and Reid (1974) reported that kiwifruit is clearly climacteric. They stated that the changes of respiration rates after harvest correspond with ethylene gas throughout the growing season. Wright and Heatherbell (1967) reported that kiwifruit is a climacteric fruit through the study of its physiological changes during post harvest ripening. In the present work, the response of kiwifruit to ethylene gas at the 5 ppm level was clearly observed.

Total acid and pH

There was a decrease in total acids and rise of pH value during ripening in the presence of 5 ppm ethylene (Fig. 2). However, no remarkable changes were observed in the control sample. Total acid content of kiwifruit was reported to range between 1.0–1.5% as citric acid (Heatherbell et al., 1975; Mitchel et al., 1979; Widlman and Luh, 1981; Wright and Heatherbell, 1967).

The total acidity of the kiwifruit ranged from 1.28–1.44%. The organic acids were metabolized by the fruit during the ripening process, resulting in decrease in total acidity and rise in pH value.

Starch

Starch is a common polysaccharide present in fruits such as apple, banana, mango, etc. It has been reported by Wright and Heatherbell (1967) that kiwifruit contains 5–8% starch. Pratt and Reid (1974) reported that young kiwifruit has a high starch content.

The changes in starch content of the kiwifruit during ripening at 20°C in the presence of 5 ppm ethylene was followed (Table 2). It was observed that about 70% of the decrease in starch occurred during the first 2 days of post-harvest ripening.

In the control sample, a much slower decrease in starch content was observed as compared with the ethylene-treated fruit.

The amylose content in the starch was about 50% throughout the experiment, except that some decrease was observed
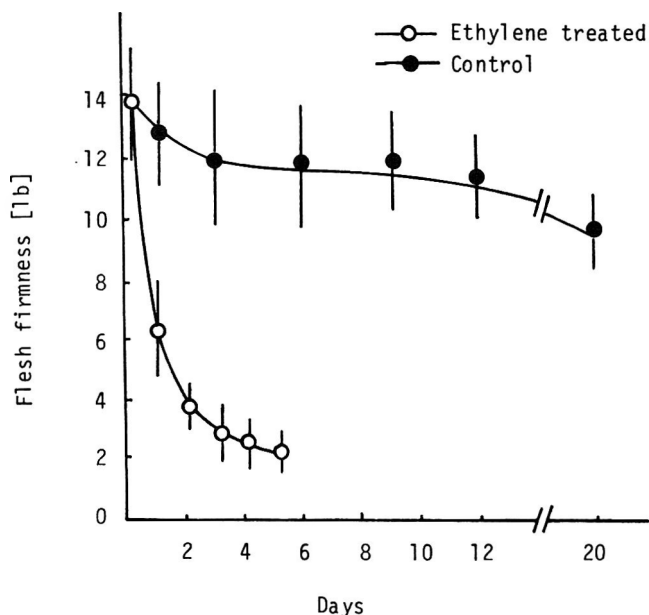
Fig. 1—Changes of firmness during ripening of kiwifruit (average of 12 fruit).

in the ethylene treated samples after 3 days of ripening.

It appears that starch is a reserve food in the kiwifruit. When the ripening process begins, the starch is converted into sugars.

Soluble and total solids

The change in carbohydrate components, especially the shift from starch to various sugars is one of the important phenomena observed in ripening of kiwifruit. The change of soluble solids content in kiwifruit during post-harvest ripening is presented in Fig. 3.

In the ethylene-treated fruit, the soluble solids increased from 8.5 to 12.4° Brix in 2 days at 20°C. This change represents almost 70% of the total change. However, Wright and Heatherbell (1967) reported that the change of soluble solids in the fruits treated with ethylene gas at 500 ppm and 20°C was not remarkable as compared with the control sample. The discrepancy may be explained by the difference in harvest maturity of the kiwifruit, or in the concentration of ethylene gas used in the investigations. It may be concluded that the harvest maturity and initial soluble solids content of the kiwifruits influence greatly the change in soluble solids during ripening. Results obtained here are in agreement with the finding of Pratt and Reid (1974) who reported that the response of the kiwifruit to ethylene gas was dependent on its maturity stage.

In the control sample, a much slower increase in soluble solids content was observed. The Brix Reading was 12.7° Brix after ripening at 20°C for 20 days, and was approaching optimum eating quality.

It was observed that the changes in soluble solids were accelerated by the presence of ethylene gas.

Total solids contents were in the range 15.8—16.2% throughout the experiment and no remarkable changes were observed during ripening process.

Analysis of sugar by HPLC

The changes in sugars of kiwifruit during ripening at 20°C with or without 5 ppm ethylene gas were determined by HPLC. The results are shown in Table 3. The presence of fructose, glucose, sucrose and an unknown compound (peak #4) was observed at all stages of ripening.

Heatherbell (1975) reported the presence of glucose, fructose, sucrose, and a trace amount of sorbitol in matured kiwifruit by gas-liquid chromatography. In the present work, however, sorbitol was not detected at any stages. An unknown substance (peak #4) was observed which has a retention time lying between those of sucrose and maltose. It seems to be a disaccharide, but its identity is not known at the present time.

The total sugar content increased almost in parallel with the Brix value. Fructose and glucose did not increase during the first day of ripening, but gradually increased in the ethylene-treated kiwifruit. Sucrose increased during 2 days of ripening, and then decreased gradually thereafter. In the control sample, sucrose increased during the first day.

Afterwards, it was maintained almost at the same level.

According to Heatherbell (1975), the levels of fructose, glucose and sucrose were 33.2%, 49.3% and 17.5%, respectively (2:3:1), in mature fresh kiwifruit.

In the present study, fructose and glucose in both control and treated samples were present at the almost same level throughout the experiment. However, the level of sucrose in treated samples changed with the ripening process. It increased after 24 hr, then decreased gradually after 5 days of ripening. There was no maltose in the kiwifruit at any stages of ripening, indicating that starch was not converted into maltose during ripening.

In earlier stage of ripening, it appears that biochemical changes through the pathway of sucrose synthesis occurred in which starch was acted on by the enzyme phosphorylase. In the second stage, sucrose seems to be inverted into glucose and fructose by the enzyme invertase.

Chlorophyll and color

The changes in chlorophyll content during ripening of kiwifruit are shown in Table 4. The activation of chloro-
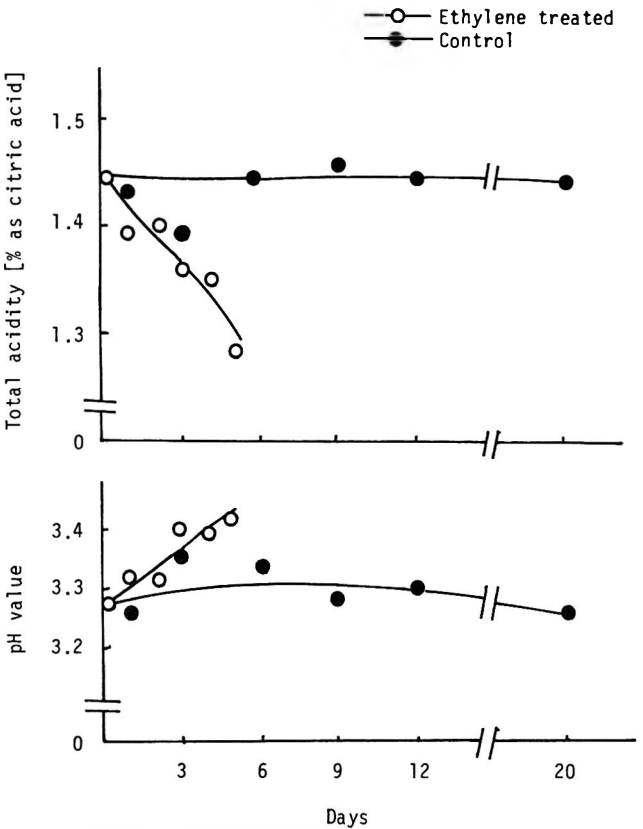
Fig. 2—Changes in total acid and pH value during ripening.

Table 2—Changes of starch and amylose during ripening of kiwifruit

Treatment		Initial	1	2	3	4	5		Days
Ethylene 5 ppm at 20°C	Starch, %	4.63	3.44	1.99	1.12	0.72	0.58		
	Amylose, %	2.29(49.5)[a]	1.76(51.2)	1.02(51.3)	0.50(44.6)	0.24(33.3)	0.20(34.5)		
		Initial	1	3	6	9	12	20	Days
Control	Starch, %	4.63	4.49	4.21	4.34	3.85	3.99	2.43	
	Amylose, %	2.29(49.5)	2.24(49.9)	2.19(52.0)	2.24(51.6)	2.04(52.4)	2.13(53.4)	1.25(51.3)	

[a] ( ) : Amylose % in starch

phylase in apple and banana during climacteric stage results in decomposition of chlorophyll a and b (Looney and Patterson, 1967). In this work, however, no significant changes in chlorophyll a and b were observed in both ethylene treated fruit and control samples.

The changes of Hunter color difference meter readings of the kiwifruit during ripening are presented on Table 5.
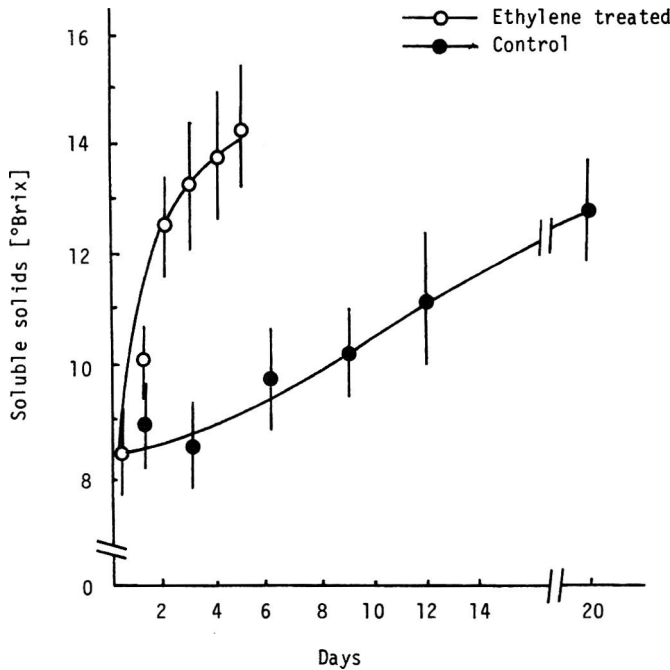
Fig. 3—Changes of soluble solids during ripening of kiwifruit.

The L value decreased remarkably during ripening of ethylene-treated fruit. The decrease may be correlated with the decrease in starch content.

The negative a_L value (greenness) increased slightly in the earlier stage and decreased again during further ripening. These results are coincident with the change of chlorophyll content. The decrease in b_L value results in the increase of a_L/b_L value which means the rise of greenness and this phenomenon agrees with the appearance to visual observation. The control fruit shows a similar trend to the ethylene-treated fruit regarding L and a_L values, and no change in b_L value was observed. The results indicate that chlorophyllase was not activated by ethylene, and the color appearance in kiwifruit was influenced largely by the starch content and the decrease in b_L value.

Ascorbic acid

The change in ascorbic acid content of kiwifruit during ripening are presented in Fig. 4.

Ascorbic acid contents of kiwifruit were reported to be in the range 30–110 mg per 100g fruit by Beutel et al. (1976) and Magoon (1979). Schroeder and Fletcher (1967) found the presence of 300 mg ascorbic acid per 100g fruit depending on the season when the fruits were harvested.

In this work the ascorbic acid content of the fruit prior to ripening was 215 mg per 100g fruit.

During ripening the ascorbic acid content decreased slightly in both ethylene-treated fruit and the control. It appears that the ascorbic acid content of the kiwifruit may vary with growing conditions and the degree of ripeness.

CONCLUSION

KIWIFRUITS harvested at the green mature stage can be ripened at 20°C under a water-saturated stream of air in the

Table 3—Analysis of sugar by HPLC in ripening kiwifruit in the presence of 5 ppm ethylene at 20°C (%)

Treatment	Sugar	Initial	1	2	3	4	5		Days
Ethylene 5 ppm at 20°C	Fructose	2.77(46.8)[a]	2.69(37.5)	3.47(37.3)	4.15(41.4)	4.47(42.3)	4.88(44.1)		
	Glucose	2.67(45.2)	2.69(37.5)	3.61(38.8)	4.15(41.4)	4.60(43.5)	5.00(45.2)		
	Sucrose	0.47(8.0)	1.79(25.0)	2.22(23.9)	1.72(17.2)	1.50(14.2)	1.19(10.7)		
	Peak #4	trace	trace	trace	trace	trace	trace		
	Total	5.91	7.17	9.30	10.57	10.57	11.07		
		Initial	1	3	6	9	12	20	Days
Control	Fructose	2.77(46.8)	2.77(43.9)	2.93(44.7)	3.09(45.0)	3.17(43.3)	3.64(44.1)	4.12(43.5)	
	Glucose	2.67(45.2)	2.70(42.7)	2.78(42.4)	3.05(44.5)	3.34(45.7)	3.80(46.1)	4.30(45.4)	
	Sucrose	0.47(8.0)	0.85(13.4)	0.84(12.9)	0.72(10.5)	0.80(11.0)	0.81(9.8)	1.05(11.1)	
	Peak #4	trace	trace	trace	trace	trace	trace	trace	
	Total	5.91	6.32	6.55	6.86	7.31	8.25	9.47	

[a] ( ) : % in total

Table 4—Chlorophyll contents in kiwifruit during ripening (%)

Treatment	Type of chlorophyll	Initial	1	2	3	4	5		Days
Ethylene ppm at 20°C	a	3.31	3.17	3.17	3.32	3.33	2.52		
	b	2.86	2.81	2.84	2.93	2.84	1.60		
	Total	6.17	5.98	6.01	6.25	6.17	4.12		
	a/b	1.16	1.13	1.12	1.14	1.17	1.58		
		Initial	1	3	6	9	12	20	Days
Control	a	3.31	3.21	3.30	3.37	3.37	3.13	3.13	
	b	2.86	2.82	2.95	2.81	3.02	2.69	2.84	
	Total	6.17	6.03	6.25	6.18	6.39	5.82	5.97	
	a/b	1.16	1.14	1.12	1.20	1.16	1.16	1.10	

Table 5—Changes of Hunter color difference meter readings of kiwifruit during ripening

Treatment		Initial	1	2	3	4	5		Days
Ethylene	L	52.3	51.8	43.4	35.9	30.5	32.2		
5 ppm	a_L	− 6.9	− 7.7	− 8.8	− 7.6	− 6.6	− 6.3		
at 20°C	b_L	25.9	25.3	25.0	19.2	15.6	14.1		
	a_L/b_L	− 0.27	− 0.30	− 0.38	− 0.39	− 0.42	− 0.44		

Treatment		Initial	1	3	6	9	12	20	Days
Control	L	52.3	52.3	51.2	51.5	49.8	48.9	46.7	
	a_L	− 6.9	− 7.2	− 8.1	− 8.1	− 7.8	− 7.9	− 7.5	
	b_L	25.9	25.4	25.5	25.9	25.9	25.2	24.0	
	a_L/b_L	− 0.27	− 0.28	− 0.31	− 0.31	− 0.31	− 0.32	− 0.31	

presence of 5 ppm ethylene gas in 2–5 days. The ethylene-induced ripening was accompanied by a decrease in pressure test, acidity, starch and amylose content, and an increase in soluble solids, sugars and pH value. The changes in fructose and sucrose content in the fruit during ripening were related to the decrease in starch through biochemical changes. Chlorophyll content was not affected by the ripening process. The decrease of L and b_L value was observed during ripening. The ascorbic acid content of the kiwifruit decreased from 210 mg per 100g to 190 mg in the ripening process.

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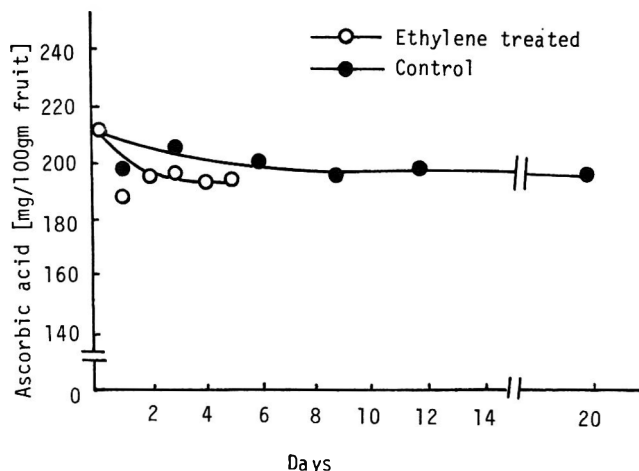Yang, S.F. 1980. Regulation of ethylene biosynthesis. HortScience 15: 238.

Fig. 4—Changes of ascorbic acid during ripening of kiwifruit.

Determination of Total Glycoalkaloids (TGA) in Dehydrated Potatoes

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————————————ABSTRACT————————————

A method was developed to optimize TGA extraction for dehydrated potato products. Maximum TGA content was obtained from uncooked dehydrated powder with hydration ratio (powder:water) of 1:4 (by weight) for the freshly prepared powder. Powders prepared from precooked potatoes required a hydration ratio (1:5) for optimum TGA extraction. For powders that were stored for 4 months, 1 hr of hydration was necessary for maximum TGA extraction. In all varieties tested the TGA content of powders was slightly less than the fresh tissue but these differences were not statistically significant. However, significant varietal differences were observed. The Chipbelle and Katahdin varieties were significantly higher in TGA content than Rosa and Pontiac varieties.

INTRODUCTION

GLYCOALKALOIDS (TGA) are a class of naturally occurring toxicants present in potato tubers. The primary constituents of this class of compounds in potatoes are α-solanine and α-chaconine, which are glycosides of the aglycone soladine. Ingestion of potatoes with high glycoalkaloid content (>30 mg/100g) was implicated in numerous outbreaks of poisoning and few instances death in humans and farm animals (Bomer and Mattis, 1924; Wilson, 1959; McMillan and Thompson, 1979). Because of the possible toxic effects as well as contributory to an undesirable bitter flavor, current standards for consumption define total glycoalkaloid (TGA) values greater than 20 mg/100g of raw potato as unsafe (Jadhav and Salunkhe, 1975).

Glycoalkaloids are formed and concentrated in the parenchyma cells of the periderm and cortex of the tubers and in areas of high metabolic acitvity such as eye regions (Wolf and Duggar, 1940; Lampitt et al., 1943). The TGA content of the tuber depends on the cultivar, maturity, environmental factors, and stress (Sinden and Webb, 1974).

Among the processed potato products developed recently, dehydrated potatoes are one of the most popular next to chips and french fries, and the production is still increasing. Also fabricated potato chips and French-fried potatoes, which are made from dehydrated potato powders, are beginning to appear on the market in increasing volume. It has been reported that both α-solanine and α-chaconine are stable to high temperatures (Jadhav and Salunkhe, 1975) upon instantaneous exposure, but the time factor was not considered. It is possible that removal of water from potatoes by different processing methods could elevate TGA concentrations and thereby create a health hazard as well as undesirable flavor (Sizer et al., 1980).

Smittle (1971) demonstrated the effect of extraction technique on glycoalkaloid analysis. A bisolvent extraction technique (Wang et al., 1972) employing a mixture of methanol and chloroform (2:1, v/v) was shown to extract higher amounts of glycoalkaloids than that obtained from Soxhlet extraction using acetic acid in ethanol, and this method has been widely used by many researchers. Baker et

al. (1955) reported that the extraction of glycoalkaloids was incomplete unless the freeze-dried powder was first reconstituted with water before extraction with acidified alcohol. Bushway et al. (1980a) and Maga (1980) determined glycoalkaloids in potato meals and extruded potato flakes using bisolvent extraction technique but without hydration.

A preliminary study in this laboratory on "aged" dehydrated potatoes has shown that the potato powder needs to be rehydrated with water before bisolvent extraction to determine the TGA content.

It has been shown that granules that have been "aged" at room temperature for 5 or more months, resorbed water much slower than newly produced potato granules (Ooraikul, 1977). The difference between the new and aged granules in their water readsorption properties is thought to be due to the molecular rearrangement and probably interaction of starch, protein, sugar and pectin during storage (Ooraikul et al., 1981).

This study was undertaken in order to develop a method for optimum extraction of TGA from dehydrated potatoes.

MATERIALS & METHODS

POTATOES of the Katahdin and Superior varieties were grown at the Cornell Research Farm at Riverhead, Long Island, NY; the varieties Rosa, Pontiac, and Chipbelle were grown at the Cornell Research Farm at Freeville, NY. The tubers were stored at 5°C in the dark for 4 months prior to the study.

Potatoes of comparable size were selected from each variety in order to limit variations resulting from size differences. Tubers were cut longitudinally from bud to stem end in order to include both the apical (bud) and basal (stem) portions, and slices were subsequently separated into cortex and pith sections. Cortex tissue including the periderm was used in the study since this is the area highest in glycoalkaloid content. The cortex tissue was freeze-dried in a Stokes freeze dryer and ground in a Wiley Mill through a 40 mesh screen. Part of the powder was analyzed immediately and part was stored 4 months under nitrogen prior to analysis. Moisture content of the potatoes was determined simultaneously. Commercially processed potato granules were purchased locally in a supermarket.

Total glycoalkaloid (TGA) determination

Analyses of fresh tubers were made using the modified titration method (Bushway et al., 1980b; Fitzpatrick et al., 1978) as controls for freeze-dried powder) and blended in a Waring Blendor (500 mL capacity) with 100 mL of bisolvent mixture (methanol:chloroform, 2:1) for 10 min at 18,300 rpm, followed by vacuum filtration using a Buchner funnel fitted with Whatman No. 1 filter paper. The residue was washed with bisolvent mixture and the filtrate was brought to a final volume of 200 mL. A 50 mL aliquot was placed in a 250 mL round-bottom flask and concentrated to 5–10 mL using a rotary evaporator. Ten mL of 0.2N HCl was added to the concentrate followed by sonication for 5 min in an ultrasonic cleaner. The flask was rinsed with 5 mL of 0.2N HCl and centrifuged at 38,000 x g at 6°C for 15 min. The supernatant was transferred to a 250 mL Erlenmeyer flask to which 25 mL of concentrated ammonium hydroxide was added to precipitate the glycoalkaloids. The basic solution was placed in a 70°C water bath (20–30 min) and then refrigerated overnight. The precipitate was collected by centrifugation at 38,000 x g for 15 min at 6°C and washed once with 2% solution of ammonium hydroxide. The pellet was dissolved in 5 mL of the tetrahydrofuran-water-acetonitrile (50:30:20 v/v) mixture once the ammonia vapors had dissipated (overnight at room tem-

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perature). This mixture was centrifuged at 12,000 x g for 5 min and an aliquot of 2 mL was taken from the supernatant and placed in a glass scintillation vial, evaporated to dryness using air and nitrogen. This solution was then titrated according to the comprehensive method of Fitzpatrick et al. (1978).

To determine the effect of rehydration in water (26°C) on the extraction of glycoalkaloids in freeze-dried potato powder as well as potato granules, more bisolvent mixture was added (i.e. equal to the volume of water added for hydration) to the controls than the ones rehydrated to keep the total volume constant. The amount of water added was calculated using the original moisture content of raw potatoes (powder:water, 1:4) in order to determine the effect of quantity of water on TGA extraction. Three different hydration ratios (1:2, 1:4 and 1/6 w/w) were used.

In order to study the effect of rehydration time on the extraction of glycoalkaloids, the freeze-dried powder was reconstituted in water for varying lengths of time (0, 1, 2 and 4 hrs.) prior to extraction with bisolvent mixture (Fitzpatrick et al., 1978).

Statistical analysis

Completely randomized design was employed, and statistical significance of the data was determined using two factor analysis of variance with two or three or four levels for each factor with protected LSD test described by Steel and Torrie (1960).

RESULTS & DISCUSSION

THE EFFECT of hydration on the extraction of TGA from dehydrated Katahdin potatoes and commercial potato granules was highly significant ($p < 0.01$) (Fig. 1). Unless precautions are taken in the extraction step a recovery of only 14–45% resulted. The amount of water to be added (1:4, powder:water) was calculated using the original moisture content of raw potatoes. However, in the case of commercial granules which are precooked, the amount of water needed for hydration (1:5, powder:water) is higher.

Since hydration was very important in the extraction of TGA, the effect of quantity of water was investigated. Three different powder:water hydration ratios (1:2, 1:4, 1:6) were used. In both Katahdin and Chipbelle powders, TGA recovery increased significantly ($p < 0.05$) with increasing amounts of water up to the ratio 1:4 (Fig. 2). With the hydration ratio 1:6 there was a tendency for the bisolvent mixture to separate into layers when the extract stood for a few hours, and this could possibly cause loss of TGA (Fitzpatrick et al., 1978; Mackenzie and Gregory, 1979). Therefore, it was concluded that a hydration ratio (1:4) was optimum for freeze-dried powders.

The effect of hydration time on extraction of TGA from dehydrated potatoes was also investigated. No significant differences ($p < 0.05$) were observed with different hydration times when freshly prepared Katahdin, Superior, and Chipbelle powders were used (Fig. 3). Ooraikul (1977) reported that fresh granules reconstitute quickly and that granules stored for at least five months at room temperature reconstitute much more slowly. During the process of aging physicochemical changes take place within the granules. French (1950) reported that gelatinized potato starch retrograded even at low moisture content (about 7%) in the granules. Interaction between proteins and reducing sugars to form melanoidins is also possible.

Therefore, for optimum extraction of TGA from "aged" dehydrated potatoes, the proper hydration time should be determined. The TGA extracted from Katahdin and Superior aged powders was significantly higher ($p < 0.05$) at 1 hr hydration as compared to that which was hydrated and analyzed immediately (Fig. 4). This trend was also observed for the Chipbelle variety, but the differences were not significant.

The TGA content of dehydrated powders following hydration (1:4, powder:water) was not significantly different from that of cortex tissue of fresh whole potatoes (Fig. 5). Although there was a tendency in all four varieties for the

TGA content of powders to be lower, these differences were not significant. However, varietal differences were significant ($p < 0.05$). The TGA contents of Chipbelle and

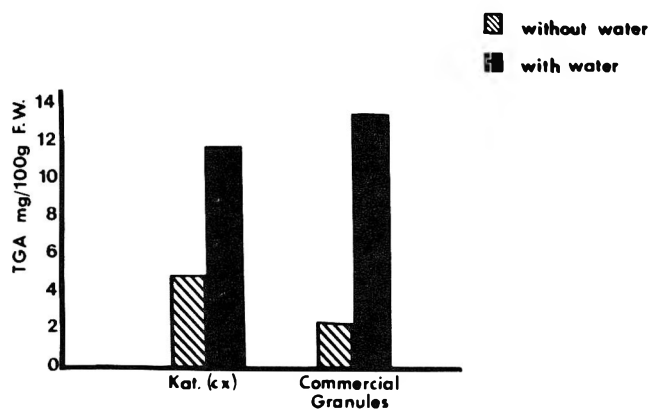Fig. 1—Effect of hydration on the extractable TGA from Katahdin potato powder (F.W. basis) and commercial potato granules (mg/100g of the granules).
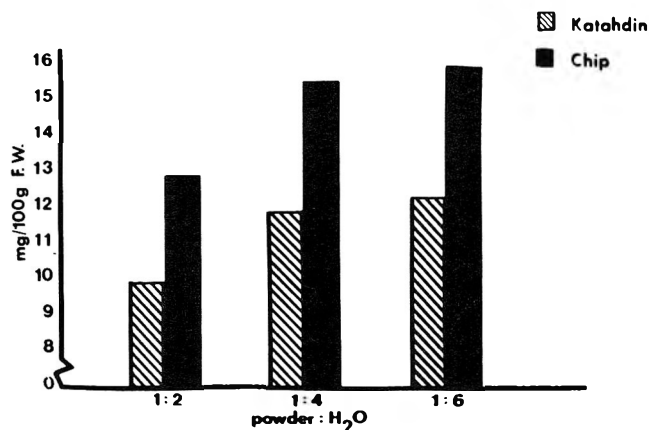
Fig. 2—Effect of hydration ratio (powder:water) on the extractable TGA from Katahdin, Superior and Chipbelle potato powders.
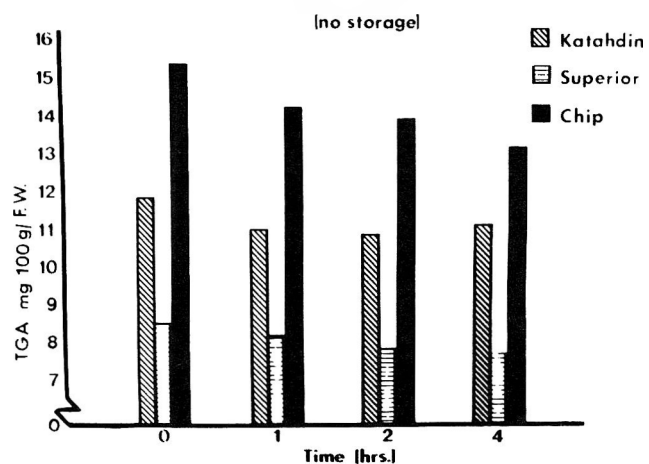
Fig. 3—Effect of hydration time (hr) on the extractable TGA from Katahdin, Superior and Chipbelle potato powders without storage.

CX POWDER
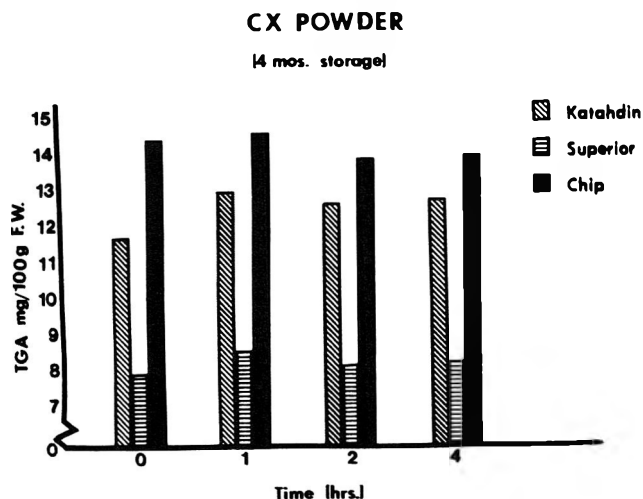
(4 mos. storage)

Fig. 4—Effect of hydration time (hr) on the extractable TGA from Katahdin, Superior and Chipbelle potato powders after 4 months storage.

GLYCOALKALOIDS

(c x)
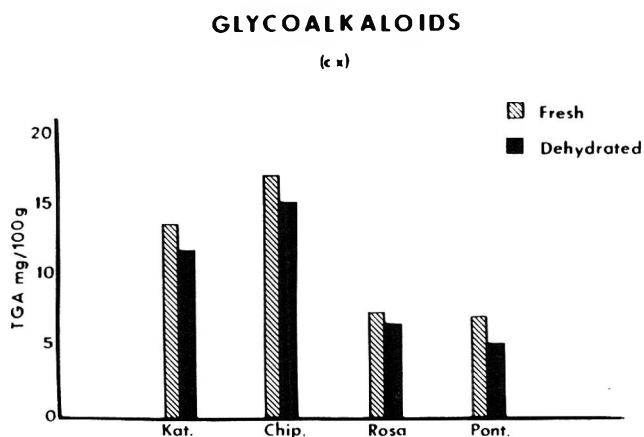
Fig. 5—Comparison of TGA content in fresh and dehydrated potatoes of the varieties Katahdin, Chipbelle, Rosa and Red Pontiac.

Katahdin potatoes were significantly (p < 0.01) higher than Rosa and Red Pontiac varieties.

Maga (1980) reported that low moisture (38% and 25%) and high extraction temperatures (130° and 160°C) could have destroyed TGA by physical or thermal degradation during the extrusion of potato flakes. Since high temperature and low moisture conditions during extrusion reduce the amount of bound water present in the final product, this could reduce the TGA recovery by bisolvent mixture. Possibly hydration before extraction with bisolvent mixture would have increased the recovery of TGA.

CONCLUSIONS

FOR OPTIMUM EXTRACTION of total glycoalkaloids (TGA) dehydrated potatoes should be hydrated with water before extraction. The time needed for hydration depends on the age of the dehydrated powder. Freshly prepared powders require less time than those which have been stored. The hydration ratio for maximum TGA extraction was (powder:water, 1:4) for uncooked and (1:5) for cooked potato products. Processed potato products such as chips and French fries which are also low in moisture content may need to be hydrated before extraction in order to obtain the optimum extraction of TGA. The method presented in this study offers a convenience for the processor or researcher who may not be able to analyze potatoes in the fresh form.

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Almond Nutmeat Moisture and Water Activity and its Influence on Fungal Flora and Seed Composition

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————————————ABSTRACT————————————

Higher moisture in almonds, a possible result of rain during harvest, was studied. Water activity (a_w) was positively correlated with moisture, increased reducing sugar and free fatty acid, and negatively correlated with total fat of almond kernels. These parameters indicate increased metabolism in the moist kernel and may be the cause of "concealed damage" a defect evidenced by brown centers in heated almonds. An almond water sorption isotherm has been developed and compared to mathematical equations. Fungal growth occurred at a_w 0.75 and above. Time for visible growth development was related to a_w and moisture. Fungal growth on the kernel depended upon a_w and time. Static flora did not change at a_w 0.70 and below. *Aspergillus glaucus* group was most frequently isolated at a_w 0.75–0.80 and other storage fungi more frequently as the a_w increased to 0.9. *A. niger* was the most frequent isolate.

INTRODUCTION

NEARLY ALL OF THE U.S. production of almonds is from California. They are one of the more important horticultural crops because they are high priced and because about 2/3 of the $600 million dollar crop is exported, mainly to Western Europe.

Moisture is important to almonds in several ways. Rain during harvest can cause molding of almonds still on the tree and possibly physiological changes after they have been knocked to the ground. Moist nuts are susceptible to insect invasion and damage, and during storage, nutmeats that are too wet can mold. The texture of the almond is sensitive to changes of moisture; almonds that are too wet become soft and pliable while those too dry become hard and brittle. Therefore kernel moisture and water activity must be controlled.

Water activity (a_w) is more closely related to the physical, chemical and biological properties of foods than is total moisture content (Rockland, 1980). To relate a_w and moisture in almonds a water sorption isotherm has been developed. It is compared with two other almond isotherms (Beuchat, 1978; Phillips et al., 1979) and mathematical functions are tested for goodness of curve fit.

Increased interest in a_w as a measure of food stability prompted us to examine the influence of higher than normal a_w in almonds. The chemical composition of almonds changes with a_w in the higher humidity region; almond mycoflora also changes with the a_w. These two changes, physiological and microbial, may be related to an almond defect known as "concealed damage," an internal defect characterized by brown centers in nutmeats after roasting. In extreme cases a bitter flavor is associated with this browning. The objective of this research was to determine of increased moisture in mature nutmeats was the cause of this "conceal damage."

MATERIALS & METHODS

WHOLE NUTMEATS from lots of Nonpareil almonds were obtained from processors or local stores. Equilibration of moisture in nutmeats was done at 25° ± 0.1°C. Saturated salt slushes (Stoloff, 1978), each with 30g salt in crystallizing dish, were placed in the bottoms of 1-gallon widemouth jars with 150g almonds to allow equilibration to different values of a_w. The tightly sealed jar lids were fitted with 40 × 100 × 2 mm plastic paddles hung from fishing barrel swivels and the jars placed in a rotary 25°C incubator shaker for at least 1 month operating at 100 rpm. With this arrangement the paddles hang and the jars move to provide constantly stirred air to aid equilibration of the internal moisture. Water activity was measured at 25°C using a Beckman Hygroline instrument calibrated against salt slushes.

All data are presented on a dry weight basis. Except where noted, all analyses were run using AOAC (1980) methods. Fungal counts were perfromed using dichloran-rose bengal-chlortetracycline agar (King et al., 1979) or dichloran-glycerol medium (Hocking and Pitt, 1980). Surface spread plating was used with 0.1 ml inoculum and 0.1% peptone diluent. Incubation of plates was at 25°C for 4 days. Fungal identifications were based upon standard taxonomic studies.

RESULTS & DISCUSSION

Almond water sorption isotherm

The curve in Fig. 1 represents an absorption water sorption isotherm above about a_w 0.5 with points below being desorption equilibrium values. It represents points from several studies where almond a_w was equilibrated over salt slush or where a_w was measured directly on almond samples. A total of 36 analyses were made on moisture of almonds equilibrated over salt slushes and 15 different moisture analyses were on almonds with a_w measured, for a total of 47 points plotted in Fig. 1. Measured and equilibrated a_w values are combined because both showed a similar relationship with moisture (significant at $p <$
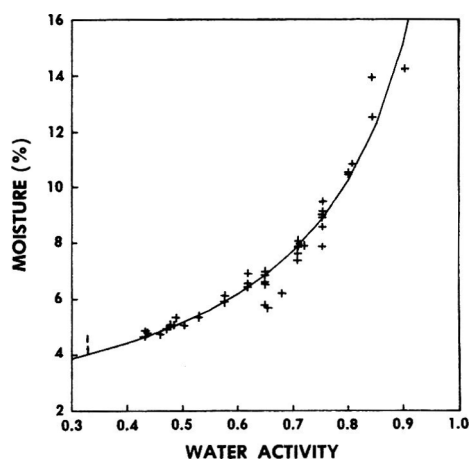

Fig. 1—Experimental data on moisture and a_w activity of whole Nonpareil almonds equilibrated over salt slushes or measured. The fitted curve was from the reciprocal linear formula, moisture = $1/[0.340 + (-0.299 \times a_w)]$.

Table 1—Coefficients of determination and parameter estimates for equations tested to fit data on almond moisture sorption curve.

Name	Equation[a]	Parameter estimates			Coefficient of Determination (r^2)
		a	b	c	
Hailwood and Horrobin	$M = [(a/a_w) + b - ca_w]^{-1}$	−0.00429	0.367	0.329	0.951
Reciprocal linear	$M = (a + b\,a_w)^{-1}$	0.340	−0.299		0.951
Halsey	$M = (-a/\log a_w)^{1/b}$	11.859	1.718		0.942
Oswin	$M = a(a_w/1 - a_w)^b$	5.126	0.494		0.939
Iglesias and Chirife	$M = (e_1/2) - \left\{(e_2)^2/[2e_1(2e_2 + 1)]\right\}$ where $e_1 = e^{a+ba_w}$ and $e_2 = e^{a+0.5b}$	1.321	2.383		0.898

[a] Boquet et al. (1978, 1979)

Table 2—Kendall rank corrlation coefficients, significance from zero probability (p<0.05), and number of analyses on selected analyses of almonds

Measurement	Moisture	Equilibrated a_w	Both a_w	Fat
Free fatty acid	0.65 0.0001 21	0.73 0.0001 18	0.68 0.0001 21	−0.44 0.006 21
Reducing sugar	0.37 0.04 17			

Table 3—Composition of four samples of roasted almonds from two varieties with differing amounts of concealed damage and a control sample that had not been rained on during harvest

Almond variety and condition	Reducing sugar (%)	Total sugar (%)	Carbohydrate (%)
1. Mission—Concealed damage	2.52	2.68	9.29
2. Mission—No visible concealed damage, sorted from (1) above	0.21	4.09	11.19
3. Nonpariel—Concealed damage	0.51	5.02	13.45
4. Nonpariel—No visible concealed damage sorted from (3) above	0.32	6.11	14.74
5. Nonpariel—Supreme grade (Control)	0.08	5.14	12.78

0.0001). Water activity of 0.7 represents approx. 7.7% moisture. The a_w of retail packaged almond kernels is about 0.47–0.50 corresponding to a moisture of about 4.8%.

The data shown in Fig. 1 were analyzed against several mathematical formulae used to describe similar water sorption isotherms (Boquet et al., 1978, 1979). Table 1 lists the coefficients of determination and parameter values for each formula tested. All five formulae have high coefficients of determination meaning a large percentage of the moisture variability among samples is explained by the equation defining the relationship with a_w. Visually lines generated from the Oswin and Iglesias and the Chirife equations do not fit as well as do the other formulae particularly at the ends. Although Boquet et al. (1978) found these two formulae to be the best fit, we did not, possibly because they restricted their data to a_w data below 0.8. The first three equations in Table 1 gave a good fit to our data but the reciprocal linear has the advantage of being

mathematically simpler. These equations can be used to predict storage conditions needed to prevent microbial growth. Also they can be used to predict the a_w of a formulated food containing almonds.

Two other reports of water sorption isotherms for almonds (Beuchat, 1978; Phillips et al., 1979) have curves that are similar in shape to Fig. 1 but both are lower on the moisture scale at a given a_w. To examined this difference we adjusted the a_w of two lots of almonds to about 0.5 and 0.7 and dried them by three techniques; 5 hr at 100°C under vacuum on ground nutmeats (AOAC) by us, 24 hr at 70°C under vacuum on ground nutmeats (Beuchat, 1973), and 96 hr at 86°C with whole nutmeats (Phillips, 1979). The three methods were characterized by different apparent moisture at the same a_w. The whole nutmeat curve had the lowest apparent moisture and a slope statistically different by analysis of covariance. The two drying methods using ground nutmeats had similar slopes and the AOAC method gave statistically higher apparent moisture. This emphasizes the importance of drying method on apparent moistures and argues for using standardized methods when establishing water sorption isotherms.

Effect of a_w and water on compositional changes during storage

Lots of almonds were also analyzed for carbohydrate, total sugar, reducing sugar, fat, and free fatty acid as well as moisture and a_w. These results were correlated with moisture, a_w (both measured and equlibrated), and storage time. The significant correlations (Kendall rank correlation coefficient; Noether, 1971) are listed in Table 2.

Reducing sugars and free fatty acid both increased with increasing moisture. Free fatty acids also increased with the equilibrated a_w. Crude fat decreased as free fatty acids increased as indicated by the negative correlation. These data suggest that metabolic activity increases with increased moisture. The metabolic activity increase is probably due to the nutmeat respiration or other enzymatic activity but we cannot rule out microbial metabolism as a source of this increase in metabolic products (Schwimmer, 1981).

The almond industry term "concealed damage" describes a defect in almonds characterized by an internal browning (brown centers) in the nutmeats especially noticeable after roasting. Sometimes there is bitter flavor associated with extreme cases of such damage.

Two samples of almonds that had "concealed damage" were separated into nutmeats that were visibly good or damaged and chemically analyzed. The results of analysis of the separated nutmeats and an undamaged control sample are shown in Table 3. Samples showing "concealed damage" had higher reducing sugar content and lower total sugar and carbohydrate than comparable almonds with normal colored nutmeats. Similar finds have been reported

Table 4—Predominate flora identified after equilibration of almonds over salts of a known a_w

a_w	Time & amt of growth	Predominate flora
0.98	15 days heavy growth	*Pencillium implicatum, Cladosporium cladosporioides, Aspergillus aculeatus*
0.93	15 days heavy growth	*A. cristatus, A. ruber, Alternaria* spp.
0.90	15 days heavy growth	*A. wentii, P implicatum, Rhizopus nigricans*
0.84	15 days light growth	*A. repens, A. ruber, P. implicatum, A. niger*
	35 days light growth	*A. montevidensis, A. umbrosus, A. repens, A. amstelodami*
0.80	35 days very light growth	*A. repens, A. montevidensis, A. chevalieri*
0.75	79 days very light growth	*A. cristatus, A. niger*
0.70	423 days no visible growth	*P. rugulosum, A. repens, A. ruber*

for macadamia nuts with brown centers (Prichavudhi and Yamamato, 1965).

We postulate that when an almond seed gets wet it starts to germinate and breakdown of protein, oligosaccharides and lipids occurs for nutrition of the growing plant. When the nutmeat is heated during field drying in the sun or during subsequent processing the monosaccharides, amino acids and other nutmeat constituents give rise to the browning reaction evidenced in "concealed damage." The correlations we found between moisture and increase in free fatty acids and reducing sugars are consistent with this hypothesis.

Mold flora

The time for visible mold to appear was related to moisture and a_w (Fig. 2). No mold growth was observed after 1½ yr on almonds stored at a_w 0.70 although a viable but static flora was present. The lowest a_w level where mold growth was observed was 0.75 (about 8.5% moisture) after 79 days storage. Mold growth at a_w 0.90 was evident after 18 days. Mold counts, initially at 1.7×10^3/g, increased with time when almonds were equilibrated over salts of a_w 0.75 or greater. At a_w 0.81 the count reached 2.3×10^6/g in 27 days while the count at a_w 0.75 remained nearly constant at 5.8×10^3/g. The mold flora that grew also changed with different a_w as shown in Table 4. At the higher a_w values there was growth of several fungi that grow well under moist conditions, while at lower a_w the *Apsergillus glaucus* group predominated in the several samples examined. Below a_w 0.75 the fungal flora were stable, did not grow, and were primarily *A. niger* group with several other of the fungi listed in Table 2 present. Overall *A. niger* was the most frequently isolated. As mentioned earlier mold growth may produce enzymes that could contribute to the "concealed damage" of almonds. However, "concealed damage" seems to take place faster than mold growth occurs.

Almonds are harvested by shaking from the trees, then are allowed to dry on the ground. Sometimes they are rained on before they can be picked up and they absorb water. This research shows that "concealed damage" may be a result of the absorbed water causing an increase in the metabolism of the nutmeat or of the fungi present. This increase in metabolism produces monomers, for example reducing sugars, that result in a Maillard type browning reaction when the nutmeats are heated.
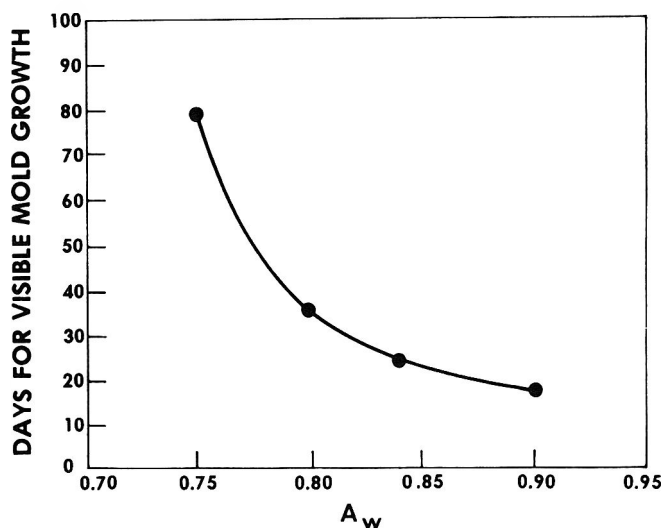
Fig. 2—Time for appearance of visible mold growth on almonds equilibrated at different water activities.

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A Diffusion Model with a Concentration-Dependent Diffusion Coefficient for Describing Water Movement in Legumes During Soaking

KENNETH H. HSU

──────────ABSTRACT──────────

A mathematical model based on Fick's diffusion equation with a concentration-dependent diffusion coefficient was proposed to describe the absorption of water by legumes. The equation was solved by using a numerical scheme. Parameter study was performed. The model is capable of predicting sigmoidal-shaped water uptake curves common to many legumes. Differences are discussed between the surface resistance boundary condition used in this study and the radiation boundary condition widely used for dehydration calculations. The validity of using the proposed model to described the water uptake of legumes was verified by the good fit between the experimental and theoretical curves.

INTRODUCTION

PROCESSING OF CEREALS and legumes often requires that the seeds be hydrated first to facilitate the consecutive extraction or cooking. Thus penetration of water into these materials is of theoretical and practical interest to processing industries.

Mechanism of water penetration into seeds was the subject of many autoradiographic studies (Butcher and Stenvert, 1973; Jackson and Varriano-Marston, 1980; Moss, 1977; Stenvert and Kingswood, 1976, 1977). It had been demonstrated that diffusion in the solid endosperm is the main mechanism that controls the rate of absorption in seeds regardless of the mode of entry of the moisture. Quantitative analysis of water diffusion in seeds, under the absorption condition, was first performed by Becker (1959, 1960), utilizing a simplified solution for solids of arbitrary shape, which he derived from Fick's diffusion equation. The uptake of water in the initial period of tempering of wheat can be described by the solution he introduced, and the diffusivity in this period can be considered constant. Chung et al. (1961) and Fan et al. (1962), by analogy, extended the equation derived by Becker to represent the volume increase of wheat, corn and sorghum during steeping. With slight modification, Becker's equation also had been applied to describe the kinetics of hydration for paddy rice during saoking (Bandyopadhyay and Ghose, 1965) and parboiling (Bandyopadhyay and Roy, 1976). Bakshi and Singh (1980) modified Fick's diffusion equation for a spherical body to incorporate a reaction term for the prediction of water uptake during the parboiling of rice. In all these cases, a constant diffusion coefficient was used without introducing too great an error in their results.

Other work, however, had shown that the diffusivity of water in cereals and oil seeds often are dependent upon the moisture content of the seeds. Chittenden and Hustrulid (1966) reported that the mean diffusivity of shelled corn varied linearly with the initial moisture content; they concluded that the actual diffusivity should depend also on moisture content at any point within the kernel. Shaykewich and Williams (1971) found that water diffusivity of rapeseeds increased drastically with the time of immersion in water. Hsu (1981) demonstrated that, during the soaking of

soybeans, the water diffusivity is strongly dependent upon the moisture content of the seeds and that the diffusion equation with constant diffusivity is inadequate in describing the water absorption curve. The applicability of diffusion equation with a constant diffusion coefficient to the sorption of soybeans also was questioned by others (Singh, 1972).

Quantitative analysis of water diffusion in cereals and legumes having concentration-dependent diffusion coefficient is scarce. Whitaker et al. (1969), using the technique of finite-difference, introduced a scheme for the solution of a nonlinear diffusion equation. They solved the equation for the case where the diffusion coefficient is a linear function of the concentration and tested the results over a model system under dehydration conditions with some success. The acceptability of this solution to the water uptake of cereals or legumes, however, was not tested.

It is, thus, the purpose of this study to theoretically develop a model for the prediction and analysis of water uptake in seeds where the diffusion coefficient is concentration dependent and to verify this model experimentally.

THEORY

BY ASSUMING that (1) the seeds are spherical, (2) diffusion takes place only in the radial direction, and (3) the effect of volume change due to absorption is negligible, one can use the diffusion equation in spherical coordinates provided by Crank (1975):

$$\frac{\partial C}{\partial t} = \frac{1}{r^2}\frac{\partial}{\partial r}(r^2 D \frac{\partial C}{\partial r}) \qquad (1)$$

where D is dependent only on concentration C.

Previous results (Hsu, 1981) on the absorption of water by soybeans had indicated that the diffusivity may vary exponentially with the moisture content of the beans; thus, it could be taken that:

$$D = D_0 e^{\kappa C} \qquad (2)$$

We will assume that the moisture is evenly distributed in the seeds, initially, or

$$C = C_0 \text{ for all } r, t = 0 \qquad (3)$$

and the boundary conditions are:

$$\frac{\partial C}{\partial r} = 0 \text{ at } r = 0, t > 0; \text{ and} \qquad (4)$$

$$C = (1 - e^{-\beta t})C_1 + C_0 e^{-\beta t} \text{ at } r = 1, t > 0 \qquad (5)$$

where C_1 is the saturation concentration.

The first boundary condition represents the symmetry of the system, and the second boundary condition defines the concentration change on the surface of the seed to be a first order process with a rate constant β. Preliminary studies assuming instantaneous saturation at the surface upon the immersion of seeds in water (i.e., $C = C_1$ at $r = a$) had produced absorption curves that overpredict the amount of uptake in the initial stages of soaking of soybeans. Most legumes and oilseeds contain waxy materials on their seed coats, which act as barriers to moisture movement. Varriano-Marston and Jackson (1981) also have shown that most water enters black beans at the hilum and is transported to the preiphery of the cotyledon. If this mode of water penetration were common to other legumes, the assumption of instant surface saturation might not be realistic. Eq. (5) describes a condition in which the surface concentration is allowed to increase from C_0 to C_1 over time. On writing this expression, we are assuming that the surface concentration

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change can be rapid but not instantaneous. The rate at which this change is accomplished is directly proportional to the value of the parameter β, as is shown in Fig. 1. Similar boundary conditions were used successfully to describe diffusion of organic vapors in glassy polymers (Long and Richman, 1960).

By making the following substitutions:

$$C^* = \frac{\bar{C} - \bar{C}_0}{C_1 - C_0}; \qquad R = \frac{r}{a}$$

$$T = \frac{D_0' t}{a^2}; \qquad D = \frac{D}{D_0'}$$

$$D_0' = D_0 e^{\kappa C_0}; \qquad k = \kappa(C_1 - C_0) \tag{6}$$

$$B = \frac{\beta a^2}{D_0'}$$

Eq. (1) to (5) can be expressed in dimensionless forms as:

$$\frac{\partial C^*}{\partial T} = \frac{\partial}{\partial R}\left(D\,\frac{\partial C^*}{\partial R}\right) + \frac{2}{R}\left(D\,\frac{\partial C^*}{\partial R}\right); \tag{7}$$

$$D = e^{kC^*} \tag{8}$$

$$C^* = 0, \text{ for all } R, \text{ at } T = 0; \tag{9}$$

$$\frac{\partial C^*}{\partial R} = 0, \text{ at } R = 0; \text{ and} \tag{10}$$

$$C^* = 1 - e^{-BT}, \text{ at } R = 1; \tag{11}$$

respectively. On introducing

$$S = \int_0^{C^*} D\,dC^* = \frac{1}{k}(D-1) = \frac{1}{k}(e^{kC^*} - 1) \tag{12}$$

Eq. (7) is further simplified to

$$\frac{\partial S}{\partial T} = D\left(\frac{\partial^2 S}{\partial R^2} + \frac{2}{R}\,\frac{\partial S}{\partial R}\right) \tag{13}$$

with initial and boundary conditions:

$$S = 0, \text{ for all } R, \text{ at } T = 0 \tag{14}$$

$$\frac{\partial S}{\partial r} = 0, \text{ at } R = 0; \text{ and} \tag{15}$$
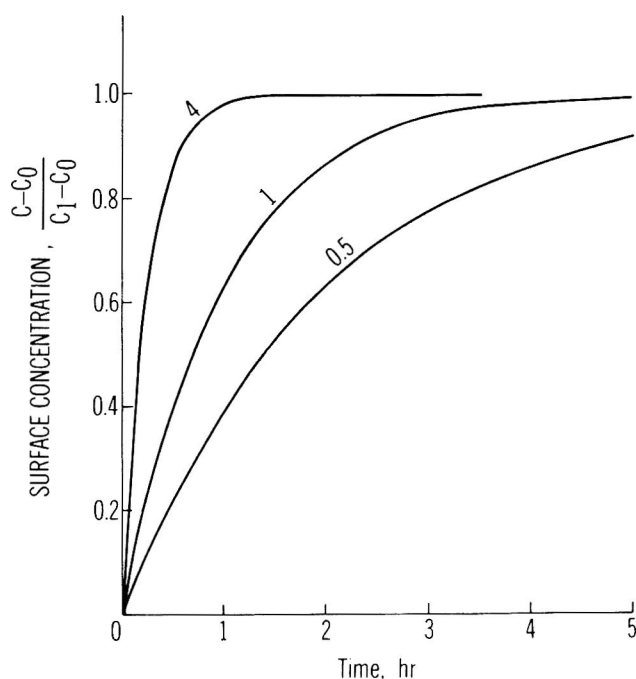
Fig. 1—Effect of parameter β on the change in the surface concentration with time.

$$S = \frac{1}{k}(e^{k(1-e^{-BT})} - 1), \text{ at } R = 1 \tag{16}$$

Eq. (13) is nonlinear and can best be solved numerically.

SOLUTION

NUMERICAL METHODS for the solution of nonlinear diffusion equations along with some calculated results were discussed by Crank (1975). In this study, a finite-difference method will be employed to solve the concentration-dependent form of the diffusion equation. One can visualize the sphere as being made up of n spherical shells with equal thickness, SR. The concentrations at the interfaces of these shells will be designated as S_i ($i = 1, \ldots, n+1$), with S_1 at $R = 0$ and S_{n+1} at $R = 1$. Thus,

$$\delta R = \frac{1}{n}, \text{ and}$$

$$R_i = (i - 1)\,\delta R, \qquad i = 1, \ldots, n+1$$

Discretization of the diffusion equation can be achieved by making the following approximations for the first- and second-order derivatives (Crank, 1975).

$$\frac{\partial S}{\partial R} = \frac{S_{i+1} - S_i}{\delta R} \tag{17}$$

$$\frac{\partial^2 S}{\partial R^2} = \frac{S_{i+1} - 2S_i + S_{i-1}}{(\delta R)^2} \tag{18}$$

with these substitutions, as well as that of Eq. (12), Eq. (13) becomes

$$\frac{dS_i}{dT} = (kS_i + 1)\left[\frac{S_{i+1} - 2S_i + S_{i-1}}{(\delta R)^2} + \frac{2}{(i-1)\delta R}\left(\frac{S_{i+1} - S_i}{\delta R}\right)\right] \tag{19}$$

with the initial and boundary conditions:

$$S_i = 0, \text{ for all } R, T = 0, i = 1, \ldots, n+1; \tag{20}$$

$$S_1 = S_2, @ R = 0; \text{ and} \tag{21}$$

$$S_{n+1} = [e^{k(1-e^{-BT})} - 1]/k, @ R = 1 \tag{22}$$

Eq. (19) is descriptive for any i th position except the two extremes; i.e., $R = 0$, and $R = 1$. At the center of the sphere, the symmetry of the system required that the two terms in the square brackets be zero; thus,

$$\frac{dS_1}{dT} = 0 \tag{23}$$

At the surface, dS/dT can be obtained simply by differentiating Eq. (23) with respect to T.

$$\frac{dS_{n+1}}{dT} = Be^{[k(1-e^{-BT}) - BT]} \tag{24}$$

There is now a set of (n+1) ordinary differential equations, as the result of discretization, which need to be solved simultaneously. The widely available software package GEAR (Hindmarsh, 1974) can be used for such calculations.

Actual concentration at a given position, C_i, can be determined from the calculated S_i according to Eq. (12). This concentration, however, is difficult to measure in the laboratory. An experimentally more verifiable quantity (i.e., fraction of total absorption, M_t/M_∞) will be used as a measure of the extent of diffusion. This quantity, M_t/M_∞, is defined as:

$$\frac{M_t}{M_\infty} = \frac{\int_0^1 C^* 4\pi R^2 dR}{\int_0^1 4\pi R^2 dR} \tag{25}$$

and it is evaluated by the application of Simpson's rule:

$$\frac{M_t}{M_\infty} = (\delta R)^3 \left\{ [\sum_{i=1}^{m} 4 C_{2i}^* (2i-1)^2 + 2C_{2i-1}^* (2(i-1))^2] + C_{2m+1}^* (2m)^2 \right\}$$

(26)

where m = n/2 = 10

MATERIALS & METHODS

Beans

Soybeans used were of Amsoy 71 cultivar produced as certified seeds from the 1974 crop. Faba beans were from Kuwait; they were screened first to obtain a relatively uniform size. The average diameter of each bean was determined as the arithmetic mean of the longest and shortest dimensions. For soybean, the average diameter was determined to be 0.714 cm; and for faba bean, it was 1.07 cm.

Water absorption

Ten grams of beans were placed in a water-proof plastic sample pouch and left to equilibrate in a temperature-controlled water bath to the test temperature. Beans then were transferred to a strainer and reimmersed into the water. After a preset time period, they were removed from water, superficially dried with facial tissues, and weighed. The weight gain was calculated as the difference between the measured weight at a given time and the original weight. Solids lost during soaking were ignored in this study because (1) the soaking time was relatively short, and (2) the amount of water absorbed was much greater than the amount of solid leached. Fractional absorption, M_t/M_∞, was determined as:

$$\frac{\text{weight gain after time t}}{\text{total weight gain}}$$

RESULTS & DISCUSSION

CALCULATIONS were performed by using the following values for constants: k = 1.0 and B = 286.2; time increment of 1×10^{-4} and spacial mesh of 20 (n = 20) were used also. The concentration profiles are shown in Fig. 2 with the dimensionless time, T, as the parameter. With the given constants, the concentration at the surface was shown to increase as time increased and came within 1% of saturation concentration at T = 0.02. Concentration at the center did not reach any significant level until time passed T = 0.03, that means a core should be evident up to this instant. As time progressed, the center concentration continued to increase, and the concentration gradient should continually decrease.

The effects of parameters D_0, κ, and β on the shape of the absorption curve are depicted in Fig. 3, 4, and 5, respectively, for the case in which the initial concentration is zero ($C_0 = 0$). The saturation concentration and the radius used for this parametric study are 1.00 g/g solid and 0.535 cm, respectively.
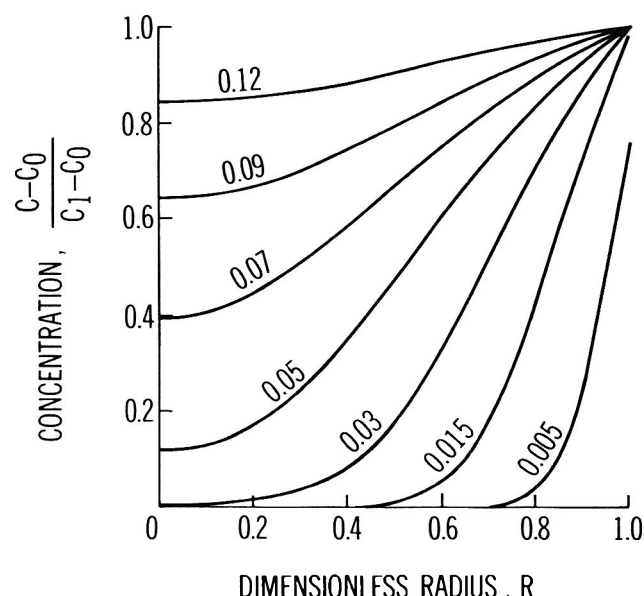
Fig. 2—Concentration profiles during the absorption of water. Numbers on curves are values of dimensionless time, T.
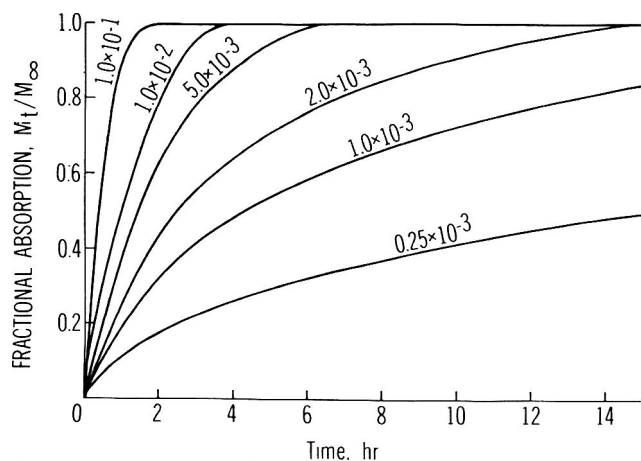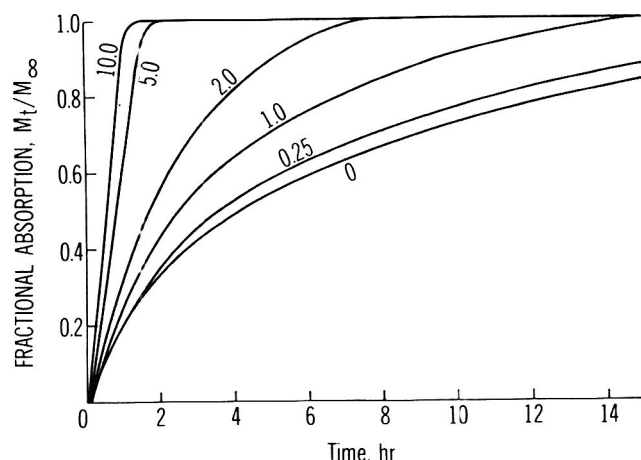
Fig. 3—Effect of parameters D_0 on the water absorption rate, at constant κ and β values. Numbers on curve are values of D_0.

Fig. 4—Effect of parameter κ on the water absorption rate, at constant D_0 and β values. Numbers on curves are values of κ.
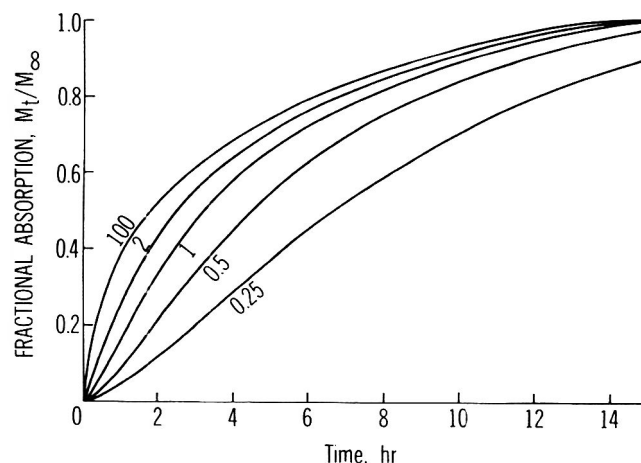
Fig. 5—Effect of parameter β on the water absorption rate, at constant D_0 and κ values. Numbers on curves are values of β.

Since the diffusion coefficient is dependent both on the parameters D_0 and κ as described in Eq. (2), any increase in their values will bring about an increase in the rate of uptake and, hence, a reduction of time required in reaching the maximum absorption. This effect is well demonstrated by the shift of the absorption curves to the left in Fig. 3 and 4. The effect of the parameter κ on the absorption curve is in addition to that due to the parameter D_0. At $\kappa = 0$, the system reduces to one of constant diffusivity, and rate of absorption is determined solely by the parameter D_0.

However, as κ takes on a positive value, the diffusion coefficient increases from its original D_0 value exponentially as absorption progresses, achieving high rates of uptake even into regions of low concentration driving force. This extended high uptake rate increases the steepness of the absorption curve. It could be expected that as the value of κ increases the steepness of the absorption curve will increase accordingly, as is shown in Fig. 4.

The effect of parameter β on the absorption curve is shown in Fig. 5. At high values, surface concentration approaches saturation very quickly (Fig. 1), and the absorption curve should approach that for the case of instant surface saturation. This is verified by Fig. 5; at $\beta = 100$, the absorption curve actually overlapped that for instant surface saturation. As value of β decreases, the delay of saturation at the surface further restricts the movement of water into the beans, which is reflected by the reduction of uptake of water in the initial stage of soaking. However, as time progresses, the surface becomes increasingly saturated, and the diffusion inside of the solid again becomes the only

resistance to absorption. This means that the effect of β on the absorption curve should be more pronounced at the initial stage rather than at the later stages of absorption. As a result, inflection points will appear on the absorption curves, particularly at low β values (Fig. 5), and the curves will become sigmoidal.

The boundary condition (Equation 11) used in this study differs from the radiation boundary condition (i.e., Bi $(1-C^*) = \partial C^*/\partial R$ @ $R = 1$), widely used under drying conditions, in that the surface concentration is strictly a function of time and not dependent upon the concentration gradient at the surface. Figure 6 shows the change of surface concentration with time for the two cases in discussion. Initially, the surface concentrations are increasing at relatively the same rate. As more water diffuses into the beans, the concentration gradient at the bean surface decreases, and the difference in the rate of surface concentration change becomes evident. It becomes increasingly more difficult for the gradient-dependent surface concentration to attend saturation as diffusion progresses. Fig. 7 shows the resultant absorption curves for the two cases in which the surface conditions are as presented in Fig. 6. After one hour of soaking, the water uptake predicted by using Eq. (11) as a boundary condition is higher than that predicted by employing the radiation boundary condition. Also, the absorption curve simulated by using the radiation boundary condition does not show any point of inflection, which has been observed in the water absorption curve of various legumes. At very high values of either B or Bi, the absorption curves approach that for instant surface saturation, as can be expected.

The proposed model could be verified by comparing the actual and predicted absorption curves. The diffusion model was programmed in a computer to simulate water uptake of legumes. It is obvious that, to simulate water
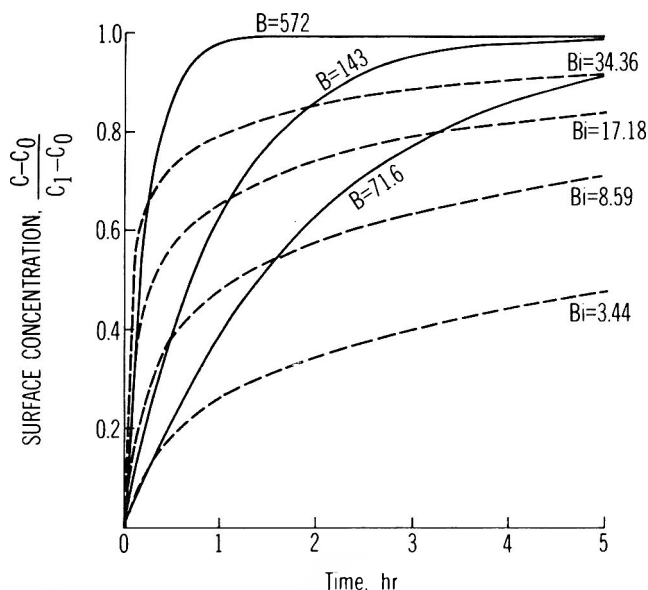
Fig. 6—Changes in surface concentration with time, as specified by the radiation boundary condition and Eq. (11).
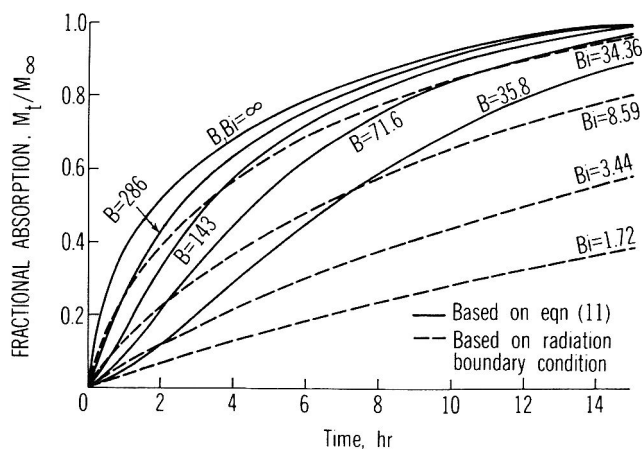
Fig. 7—Theoretical absorption curve simulated by using the boundary conditions as specified in Fig. 6.

Table 1—Values of the diffusion model parameters for various legumes

	Temp. (°C)	C_1^a (g/g dry solid)	C_0^a (g/g dry solid)	$D_0 \times 10^3$ (cm²) hr	κ (g solid / g H₂O)	β (1/hr)	ESS[b]
soybean	30	1.425	0.55	2.139	0.647	1.563	0.0002
faba bean	30	1.116	0.076	2.593	1.149	0.583	0.0022
Calif. small white bean[c]	40	—	0	18.49[d]	6.853[d]	0.652	0.0099

[a] C can be expressed as g/g solid since volume change was assumed negligible.
[b] Error sum of squares; 10 observations for each calculation.

[c] Data taken from Fig. 1 of Kon (1979).
[d] These values represented D_0 and κ, respectively. Because the initial moisture content for the Calif. small white bean was not provided, true values for D_0 and κ could not be calculated.

Fig. 8—Comparison of actual and theoretical absorption curves for various legumes.

NOMENCLATURE

a = Radius, cm
B = Dimensionless constant, $\beta a^2/D_0'$
Bi = Mass Biot number, dimensionless, ah/D_0'
C = Moisture concentration, g/g
C_1 = Saturation moisture concentration, g/g
C_o = Initial moisture concentration, g/g
C^* = Dimensionless moisture concentration
D = Diffusion coefficient, cm^2/hr
D_0 = Diffusion coefficient at zero moisture concentration, cm^2/hr
D_0' = Constant as defined by Eq. (6), cm^2/hr
D = Dimensionless diffusion coefficient
h = mass transfer coefficient, cm/hr
k = Dimensionless constant
n = Number of shells
r = Radial distance, cm
R = Dimensionless radial distance
S = Transformed concentration, dimensionless
t = Time, hr
T = Dimensionless time
β = Constant as defined by equation (5), hr^{-1}
κ = Constant as defined by equation (2), g/g

absorption, some estimates of the parameters κ, D_0 and β are needed. The best estimates of these parameters could be obtained by employing a sequential pattern search method to locate a set of parameters that would provide a minimal error sum of squares between the experimental and theoretical curves. In this study, the simplex search method (Fan et al., 1969; Spendley et al., 1962) was utilized. The values of parameters determined by the procedure were summarized in Table 1 for soya-, faba-, and California small white beans. The theoretical and actual absorption curves for these legumes were depicted in Fig. 8 for comparison. It could be seen that the theoretical curves fitted the experimental ones very well, indicating the accuracy with which the proposed diffusion model could predict the water uptake of legumes. This observation was supported by the low error sum of squares as shown in Table 1.

Water can penetrate more readily through the hull of soybean than it can that of faba- or California small white bean. This slow surface penetration of faba-, and California small white beans by water, as indicated by their sigmoidal absorption curves, was accurately reflected in low β values for these legumes. Food scientists who work with legume processing have long realized that beans with high moisture content hydrate more rapidly than those with low moisture (Smith and Nash, 1961; Crean and Haisman, 1963). Results of this investigation substantiated those reported observations. According to Eq. (2), water diffusivity, and hence rate of water uptake, increases with the water content of the bean if the parameter κ is positive. Table 1 shows that values of κ for all beans studied were indeed positive. The results also indicated that the diffusion coefficient of California small white bean was more dependent on the moisture content than either that of soya- or faba bean.

CONCLUSION

(1) Concentration dependence of the diffusion coefficient of some legumes can be adequately expressed in exponential forms.

(2) The boundary conditions to the diffusion equation introduced in this study can better describe the surface concentration changes under the absorption condition.

(3) The proposed model has been demonstrated to be accurate in describing the water absorption of various legumes.

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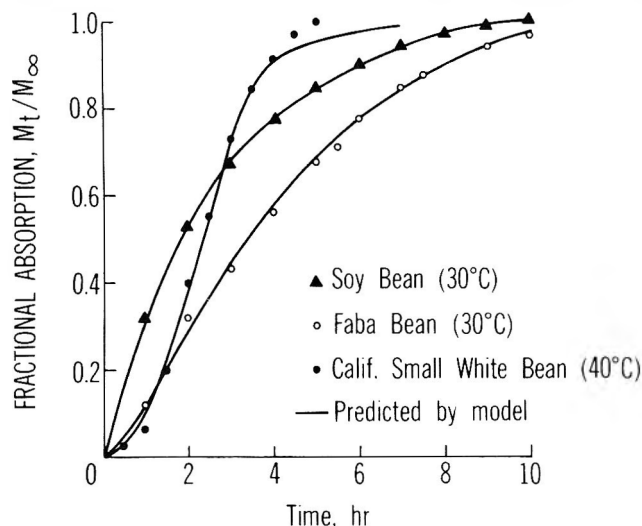Hsu, K.H. 1981. Water uptake of soybeans during the soaking treatment. Presented at the 66th Annual Meeting of AACC, Denver, CO, Oct 25—29.
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The Cause of Reduced Cooking Rate in *Phaseolus vulgaris* Following Adverse Storage Conditions

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————————ABSTRACT————————

The interrelationship between reduced cell separation rate, reduced imbibition value and reduced pectin solubility was investigated with reference to reduced cooking rate in *Phaseolus vulgaris* also termed the hardbean phenomenon. It was found that reduced imbibition value and reduced pectin solubility can both cause a reduction in the rate of cell separation during cooking of beans and hence an increase in their cooking time and that these two factors act synergistically. Accompanying symptoms are solute leakage during soaking due to membrane breakdown, phytin catabolism and pectin demethylation, all of which are key factors in the development of hardbean.

INTRODUCTION

LEGUMES PREDOMINATE in rural diets in Latin America because they are cheap and plentiful. They are high in protein (Bressani et al., 1961) as well as good sources of minerals (de Moraes and Angelucci, 1971) and some vitamins (Bressani et al., 1954; Bunnel et al., 1965). Some 30% of the dietary protein usually comes from legumes (Tandon et al., 1957), but they are susceptible to hardening from a variety of causes rendering them less edible since excessive cooking of beans reduces their nutritive value (Bressani et al., 1963). The hardbean or hard-to-cook phenomenon, therefore, is of great importance nutritionally and also has commerical and political consequences. The causes of reduced bean cooking rate has been variously cited in the literature as due to reduced water uptake (El-Tabey and Youssef, 1979), hardshell (Snyder, 1936), phytin deficiency and pectin calcification (Mattson, 1946/7). This study was undertaken to investigate the properties of hardbeans caused by incorrect storage and to determine its basis.

MATERIALS & METHODS

Biological material

Seeds of the black bean *Phaseolus vulgaris* var S-19-N were obtained from Dr. Ricardo Bressani, INCAP, Guatemala. Sub lots were grown in the Durham University heated glasshouses (21°C) in sterilized soil in 6" pots, one seed per pot for 12 wk under natural summer daylight. The beans were harvested once the pods had dried and turned light brown and stored at 4°C and low relative humidity until required. The moisture content at harvest was approx 10%. A supply of hard beans was obtained by storing half the crop at 34 ± 1°C at 70–75% relative humidity for 6 months.

Cooking rate

Whole beans were soaked for approx 18 hr at room temperature in distilled water, boiled and tested for softness at intervals by squeezing each bean individually between forefinger and thumb. A bean was considered cooked if the cotyledons yielded to only slight pressure. If the beans did not soften, they were boiled until they did. To determine the total cooking time of a sample (CT_{100}) the number of cooked beans at each interval was expressed as a percentage of those investigated and from these figures the CT_{100}

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in minutes of a batch of 100 black beans determined; for example, using a sample of 50 beans:

Time (min)	No. cooked	% Cooked	CT_{100}	
0	0	0	–	
20	18	36	36 x 20 min	= 720
35	48	96	(96–36) x 35 min	= 2100
45	50	100	(100–96) x 45 min	= 180
				3000

The CT_{100} of this sample is defined as 3000 min.

Imbibition value and leakage

The water-holding capacity, or imbibition value (I.V) of the beans after 18 hr soaking was determined as (wet weight)/(dry weight), taking into account the moisture content of the beans. To measure leakage from naked cotyledons the imbibing solution was allowed to evaporate, dried in an oven at 105°C for 2 hr and cooled in a desiccator, weighed and expressed at mg g^{-1} dry weight (d.wt) of bean tissue.

Osmotic restriction of imbibition value (I.V)

Batches of 65–70 beans were soaked overnight in sucrose solutions ranging in concentration from 0–1.75M. The gross I.V of the bean batches was measured and then the beans were boiled in fresh solutions of sucrose of the same concentration. The cooking time was determined and the I.V after cooking also measured.

Induction of leakage in control soft beans

Control soft beans were shelled and treated with hot 80% ethanol for 18 hr. The beans were then allowed to dry down to approx 10% moisture content and then soaked and cooked as normal.

Analysis of phytin

This was essentially according to the technique of Makower (1970), substituting Fe for Ca and Mg within the phytin and thus determining the phytin levels by measuring the Fe present at 384 nm using a Perkin Elmer 403 atomic absorption spectrophotometer after digesting the residue in 1.5 ml conc H_2SO_4 and 0.9 ml H_2O_2.

Quantitative analysis of calcium and magnesium adhering to the cell wall

The technique devised was as follows: 110 mg samples of freshly ground bean meal were washed in 8 ml 0.33M mannitol to remove membrane bound organelles without rupture and the tissue centrifuged down for 4 min at 2000 rpm. The supernatant was discarded and the residue washed in 8 ml 1.0M mannitol followed by three further washes in 8 ml deionized water, one wash in acetone and three washes in deionized water. Microscopic examination proved that only starch grains and cell wall fragments remained.

The residue was digested as for phytin analysis (though the very low solubility of $CaSO_4$ produced by the wet ashing of the tissue may influence the quantitative nature of this analysis) and analyzed for Ca and Mg on a Perkin Elmer 403 atomic absorption spectrophotometer at wavelengths of 211 nm and 285 nm, respectively. Each element was expressed as μg Ca/Mg g^{-1} d.wt whole tissue.

Further analysis proved there to be no significant contamination of the cell wall samples with Ca.Mg phytate.

Extraction and analysis of uronic acids

Pectin levels were determined by measuring the levels of their main component unit galacturonic acid, as uronic acid.

Cold water soluble uronic acids were extracted by soaking approx 4g of whole bean cotyledons for 18 hr in approx 100 ml distilled water at room temperature. The cotyledons were drained and then boiled for 20 min to remove the hot water soluble uronic acids. After draining, or if tissue breakdown was extensive, centrifuging, the residual tissue was then refluxed at 85–90°C in 150 ml 0.5% ammonium oxalate while continuously stirred. After 5 hr the suspension was allowed to cool and then centrifuged. The residue was washed with water and the oxalate soluble extracts bulked.

The residue was finally stirred for 15 min in 50 ml 0.05N NaOH at room temperature to remove the insoluble uronic acids, centrifuged, washed in 0.05N NaOH and the extracts bulked. Analysis of the uronic acids was according to the technique of Blumenkrantz and Asboe-Hansen (1973) except that o-hydroxyl diphenyl was used instead of m-hydroxy diphenyl.

Nonaqueous extraction of whole starch grains

The technique adapted from the industrial wet bench method was as follows: 40g of shelled black beans were macerated in ethanol until the suspension consisted of isolated starch grains and broken cells. The volume was made up to 1L with ethanol and while continuously stirred was poured at a rate of 1 ml sec^{-1} down a polished aluminum channel (3.36 m x 5 cm x 2.5 cm) sloping by 1.6%. As the tissue macerate ran down the channel the tissue particles sedimented out. Any clumps of cells settled out within the first cm,
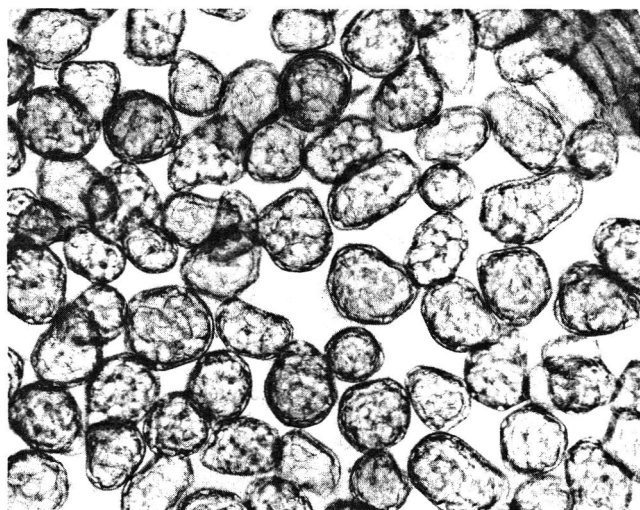
Fig. 1—Light micrograph of cells from a soft bean cotyledon after 30 min boiling.
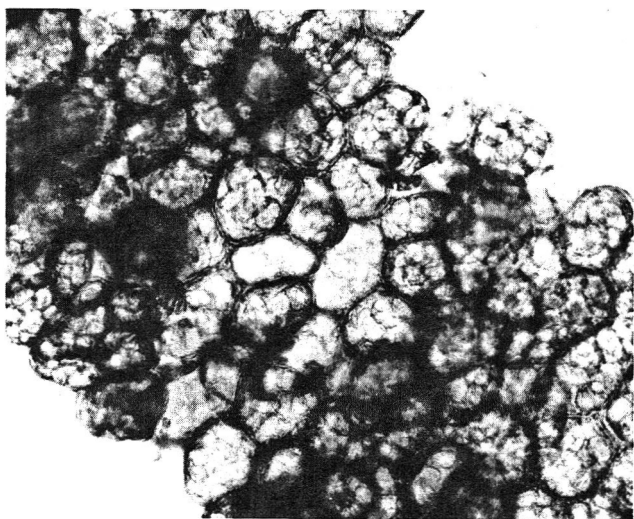
Fig. 2—Light micrograph of cells from a hard bean cotyledon after 30 min boiling.

starch grains settled out mainly in the first 40 cm and large cell debris, mainly cell walls, settled out in 1.5 m.

By re-running all the sediment which deposited between 5 cm and 60 cm four or five times it was possible to obtain a pure preparation of starch grains which were allowed to dry overnight at room temperature before moisture content determination.

The swelling power of the starch grains was determined according to the technique of Schoch (1964).

Determination of pectin esterification

This was performed according to the technique of Reeve (1959) which involved substituting methyl ester groups in the pectin chain with a hydroxylamine/iron complex; thus measuring the iron content indicated the level of pectin methyl esterification. The method was adapted for macerated tissue. The iron content of the samples was measured, after acid digestion of a known dry weight of tissue, on a Perkin Elmer 403 atomic absorption spectrophotometer.

RESULTS & DISCUSSION

RESULTS are presented in Fig. 1 through 8 and Tables 1 to 3. These demonstrate that hardbean is a textural problem defined as the failure of beans to soften sufficiently during the normal cooking process: it develops in beans stored with elevated moisture contents at high temperature, i.e. the two conditions act synergistically. The deterioration in texture quality is due to the failure of the cotyledon cells to separate during cooking as demonstrated in the light micrographs of cooked soft and hardbean tissue (Fig. 1 and 2). It was also noted that the imbibition value (I.V) of the hardbeans (1.88) was much lower than that of the soft beans (2.17). This result takes into account the presence of a layer of free water which developed between the cotyledons and testa of hardbeans due to the reduced expansion of the hardbean cotyledons. This nonimbibed water in hard beans may account for the disparity of views concerning the imbibition capacity of hardbeans (see for example Molina et al., 1975; Quenzer et al., 1978). If the cause of
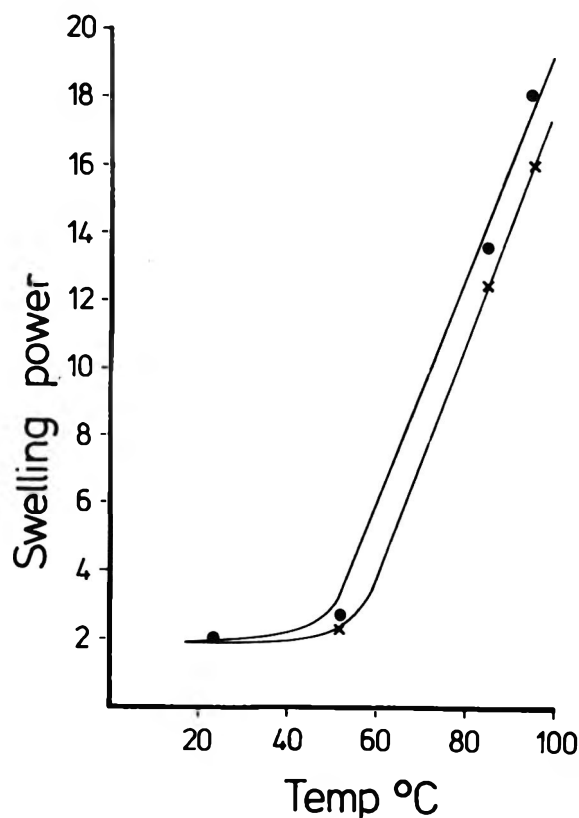
Fig. 3—Swelling power of extracted whole starch grains: ● Hardbean starch, x Soft bean starch.
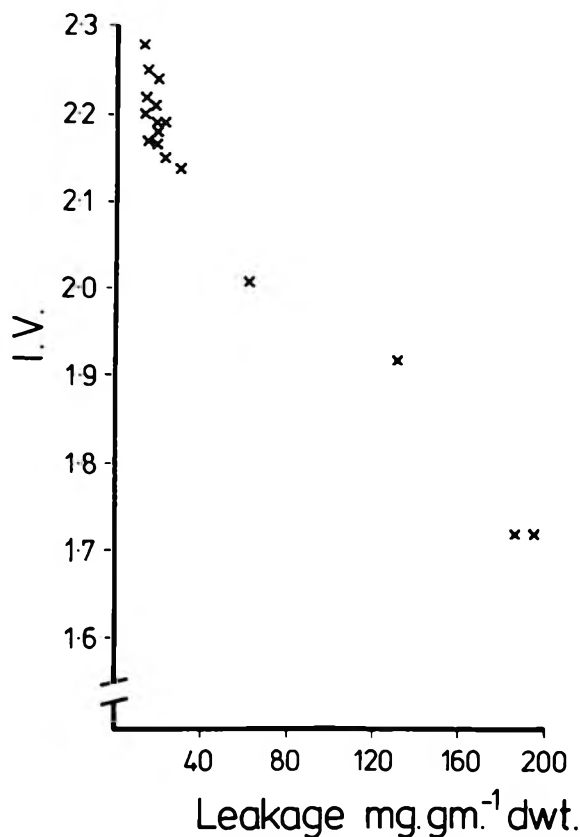
Fig. 4—Correlation between imbibition value and leakage in rehydrated beans.

Fig. 5—Influence of sucrose solutions on the imbibition value of black beans.

Fig. 6—Influence of sucrose solution on the cooking rate of black beans. The sub lot of Phaseolus vulgaris used here had a control CT_{100} of 2000 min as did all later generations of Black Beans grown at Durham.

the hardbean phenomenon was due to a reduction in the rate of cotyledon cell separation then this was due to either reduced middle lamella (pectin) solubility, or reduced imbibition value leading to a reduced turgor pressure which failed to force the cells apart, or both of these factors to differing degrees.

The imbibition value in hardbeans was not due to impermeability of the testa (i.e. hardshell) and probably not due to a change in the swelling power of starch grains (Fig. 3) which on isolation was found to be greater in hardbean starch grains although it is possible that starch grains in situ do not behave similarly. Rather, the cause of the reduced imbibition value was found to be caused by leakage of solutes from the cotyledons, during imbibition, which was nearly ten times higher in hardbeans (195 mg g^{-1}) than soft beans (21 mg g^{-1}). A correlation coefficient of -0.99 was found between leakage and imbibition value (Fig. 4). This may also partially account for the lower nutritive value of hardbeans (Molina et al., 1975). Ching and Schoolcraft (1968) noted leakage during imbibition of poorly stored crimson clover seed (Trifolium incarnatum) and related this to membrane degradation. Priestley and Leopold (1979) postulated that the increase in soybean (Glycine max) moisture content due to high relative humidity during storage permitted the activity of phospholipid hydrolyzing enzymes, but that membrane repair mechanisms were not operable as the beans were not fully hydrated, resulting in net membrane decay. In beans whose imbibition values had been artificially reduced by sucrose solutions (Fig. 5), the cooking time increased (Fig. 6) as the imbibition value decreased. Fig. 7 displays the negative correlation between imbibition value and cooking time. The imbibition value of the bean samples after softening were all greater than 2.38 (Table 1) which suggested that the beans had to absorb at least 1.4 times their own weight in water to develop sufficient turgor pressure to force the cells apart; the imbibition value of hardbeans never exceeded 2.10.
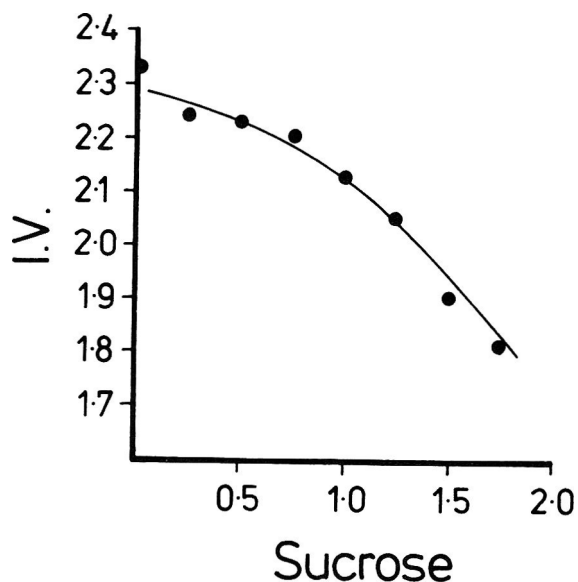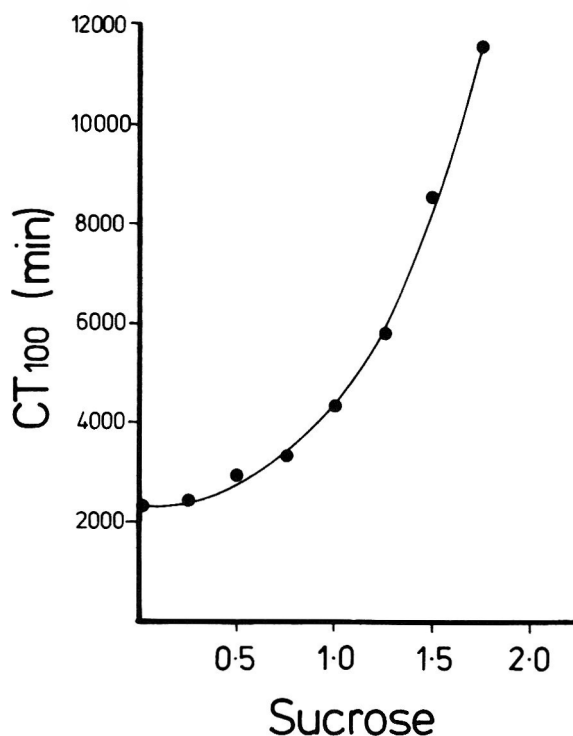
Treatment of shelled control soft bean cotyledons with hot 80% ethanol induced membrane breakdown and hence leakage of solutes, thus mimicing the cause of reduced imbibition value in hardbeans. These beans were dried and then imbibed and cooked as normal. The imbibition value was 1.78, similar to that of hardbeans, yet the CT_{100} was 3500 min, closer to that of soft control beans (3000 min) despite the imbibition value only reaching 2.15 on softening, thus the imbibition value was not the sole or prime influence on the rate of cell separation during softening.

Pectin solubility (Table 2) was much lower in hardbeans coinciding with an increase of 44% and 69% of pectic cal-

cium and magnesium, respectively. Table 3 shows that the degree of pectin esterification dropped from 51% to 15% and the quantity of calcium magnesium phytate within the beans from 29 to 18 mg g^{-1} d.wt.

It was postulated that the reduced pectin solubility was due to phytin breakdown releasing calcium and magnesium which formed cation bridges within the pectinaceous middle lamella, thus desolubilizing it. This was facilitated by pectin

Table 1—Imbibition value (I.V) of beans before and after cooking in sucrose solutions[a]

Sucrose [M]	I.V before cooking	I.V after cooking
0	2.24	2.44
0.25	2.24	2.38
0.50	2.23	2.43
0.75	2.20	2.47
1.00	2.13	2.46
1.25	2.05	2.43
1.50	1.90	2.43
1.75	1.80	2.45

[a] Samples consisted of between 65 and 70 beans and each were measured in bulk. The I.V before cooking was reduced by soaking in the sucrose solutions shown. I.V after cooking were all taken within 5 min of cooking.

Table 2—Solubilities of hard and soft bean pectin during sequential extraction (Measured as uronic acid mg g^{-1} d.wt[a]

SEQUENCE		Soft (Control) Bean	Hard Bean
	Cold water soluble	0.4	1.7
	Hot water soluble	13.0	3.0
	Ammonium oxalate soluble	18.0	12.5
	NaOH soluble	1.5	8.5
	Total	32.9	25.7
	Insoluble	—	7.2

[a] Values average of three replicates (values varied ± 2.5%)
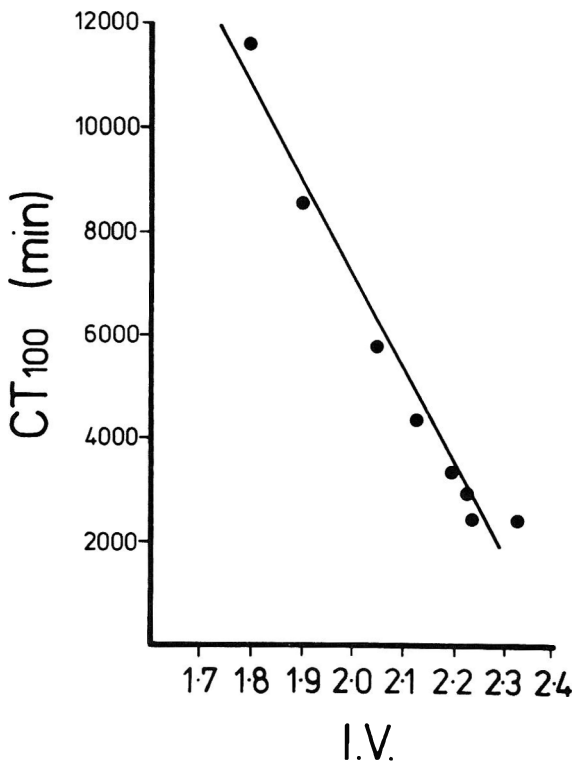
de-esterification which created more free carboxyl sites. Buescher et al. (1976) investigated the hardcore phenomenon in cold stored potatoes and reported that the hardening of patches in the tuber was due to a decrease in the levels of water soluble pectin, due to binding of phenolic substances and cations by pectin chains.

Incubating soft control beans with 0.03M CaCl$_2$ at pH 7.0 increased the cooking time by 60% which supported the hypothesis of bivalent cation involvement. The addition of pectin methyl esterase to the incubating solution allowed more pectin calcification to occur and increase the cooking time further (Fig. 8). This proved that the increased levels of pectic calcium and magnesium in the hard beans was partly responsible for increasing the cooking time of the beans. When this induced pectin calcification was combined with the effects of induced leakage (also Fig. 8) then the beans failed to soften at all supporting the suggestion that hardness is due to a combination of pectin calcification and reduced turgor pressure.

It is reasonable to conclude therefore that the increase in moisture content due to high relative humidity during storage, is one of the key factors in the initiation of hardening. It permits restricted metabolism which leads to membrane breakdown which in turn causes reduced leakage and imbibition value, also allowing access of bivalent cations from hydrolyzed phytin to the pectin. The influence of elevated moisture content in hard beans was previously reported by Burr et al. (1968) and Muneta (1964).

Table 3—Analysis of phytin levels, pectin esterification and cell wall cation levels[a]

	Soft (Control) bean	Hard bean
Ca µg g^{-1}	120	173
Mg µg g^{-1}	210	354
Phytin mg g^{-1}	29.1	18.6
Pectin esterification	51%	15%

[a] The Ca, Mg and phytin levels were each an average of three replicates. The cell wall cation levels were expressed as µg Ca/Mg adhering to the cell wall per gram dry weight of whole bean meal (values varied less than ± 5%).

Fig. 7—Correlation between imbibition value and cooking time of black beans.
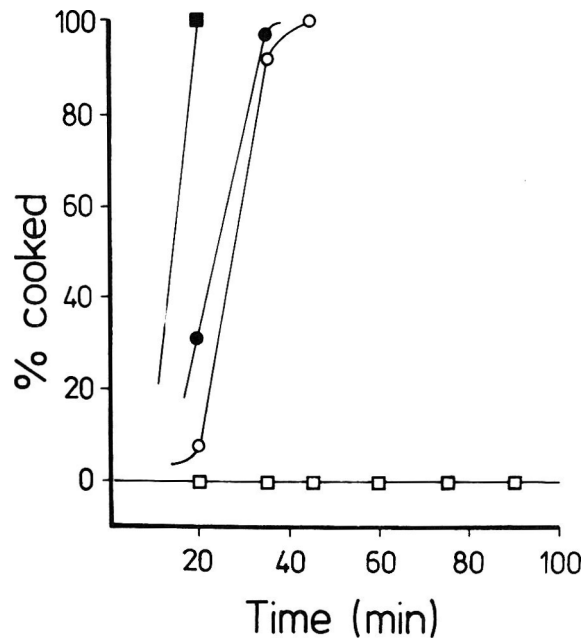
Fig. 8—Induction of hardness in soft black beans: ■ Control, ● 18 hr imbibition in 0.03M CaCl$_2$ pH 7.0, ○ 18 hr imbibition in 0.03M CaCl$_2$ pH 7.0 with pectin methyl esterase, □ 24 hr imbibition in 0.03M CaCl$_2$ pH 7.0 with pectin methyl esterase after pretreatment with hot 80% ethanol to induce leakage.

Characteristics of California Navel Orange Juice and Pulpwash

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——————— ABSTRACT ———————

California navel orange juice, two-stage pulpwash and concentrated orange juice obtained from a commercial processing plant during the 1979—80 processing season were analyzed by chemical, physical and microbiological methods. Statistically significant differences ($P < 0.01$) were found between single-strength orange juice and pulpwash in the concentration of nitrate, sulfate, phenolics, carbohydrates, protein, pectin, UV/VIS absorption characteristics, minerals and carotenes. Seasonal variations were found in carbohydrates, organic acids, minerals, microbiological data, UV/VIS absorption characteristics, carotenoids, protein and phenolics. This report outlines methods of analysis, summarizes analytical results and discusses application of these findings to the detection of pulpwash adulteration of orange juice.

INTRODUCTION

DETECTING ADULTERATION of orange juice has long been of interest to citrus processors, users and regulatory officials. Adulteration can range from simple addition of water, sugar and citric acid to more sophisticated methods involving addition of pulpwash solids. The Code of Federal Regulations §146.146 (1977) specifies that in the preparation of frozen concentrated orange juice "a properly prepared water extract of the excess pulp so removed (from the juice) may be added." This implies that only a limited amount of pulpwash may be added back to orange juice, i.e., only juice washed away from ruptured juice sacs that have been screened out of a given amount of orange juice may be returned to that juice. It has been generally accepted that a juice meeting the Federal Regulation could contain 6—10% pulpwash (Petrus and Attaway, 1980). This regulation is almost impossible to enforce since no generally applicable method currently exists for determining the amount of pulpwash in frozen concentrated orange juice. A method that may be useful for estimating the pulpwash content of Florida juices, has recently been proposed (Petrus and Attaway, 1980).

Many reports have been published on the compositional changes that occur in orange juice in relation to season, cultivar and geographical origin. Several recent reviews have been published on this subject (Jorgensen, 1971; Petrus and Vandercook, 1980). Most of the studies comparing orange juice and pulpwash have dealt with Florida fruit (Olsen et al., 1958; Huggart et al., 1959; Rouse et al., 1959; Sawyer, 1963; Attaway et al., 1972). A study was undertaken to provide a broad base of compositional data on orange juice and two-stage pulpwash solids for early, middle, and late season California navel oranges. This is a report of experimental details and analytical results. Statistical treatments of the data by multiple regression, chi-

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square and discriminant analysis procedures are reported in a separate paper (Vandercook et al., 1983).

MATERIALS & METHODS

THIRTY SAMPLES of natural-strength orange juice and pulpwash and 10 samples of concentrated orange juice were collected from the Sunkist processing plant in Ontario, CA during the '79-'80 navel season. Juice was extracted on model 700 extractors (Brown International Corp.) using 120 pounds pressure and 3/16" clearance. Orange juice samples were taken after seeds and membranes had been removed with a 0.010" screen. The pulp was washed in a two-stage counter-current process in which two paddle finishers (model 202, Brown International Corp.) act as liquid-solid separators. Screen size was 0.010" and the paddle had a 1/2" pitch, turning at 650 rpm with a 1/8" clearance. Samples of natural-strength orange juice and pulpwash were collected on five consecutive days during the early (December, '79), middle (March '80), and late (May-June, '80) season. For each set of samples, single-strength orange juice (SSOJ) and pulpwash (PW) were collected from the same lot of fruit. The early and late season samples came from the San Joaquin Valley; the mid-season samples represent a mixture of San Joaquin and southern California fruit. In addition, orange juice concentrate samples were taken during the early and middle season representing average production for each of the five sampling days. The concentrates, processed in a manner consistent with Federal Regulation, contained small amounts of pulpwash. Samples were subdivided, stored at $-10°C$ and analyzed as soon as possible after thawing to minimize enzymatic changes. All analytical results, except degrees Brix (°B), were calculated on the basis of $11.8°B$ for ease of comparison. The reported degrees Brix are those of the natural strength samples.

The mean, standard deviation and coefficient of variation was determined for each analytical parameter. Analysis of variance was performed to detect differences due to season and/or type of product.

°Brix

The dissolved solids, expressed as °Brix, were determined by measurement of refractive index at 20°C. The values were corrected for citric acid content (Stevens and Baier, 1939).

Acids

Titratable acids (total acids) were determined by direct titration of a 10 ml aliquot to pH 8.1. Results were expressed as citric acid in accordance with the method reported by the International Federation of Fruit Juice Producers (IFFJP Method No. 3, 1968). Citric acid was determined colorimetrically by reaction of the barium salt of citric acid with diazotized sulfanilic acid (IFFJP Method No. 22, 1973). D-isocitric acid was determined enzymatically (Boehringer Mannheim, 1980). L-malic acid was determined enzymatically (IFFJP Method No. 21, 1964).

Carbohydrates

Glucose, fructose, and sucrose were determined by HPLC (Palmer, 1975). Total sugars and reducing sugars were determined by the automated procedure of Vandercook et al. (1975). Pectin concentrations were determined by a modified version of the IFFJP method (No. 26, 1964). Following extraction of water-soluble pectin, the insoluble pectin was extracted with alkali. The values reported as alkali-soluble pectin, therefore, include oxalate-soluble pectin.

Formol number

The formol value was determined after adjusting 25 ml of sam-

ple to pH = 8.1 with 0.25N NaOH. Following the addition of 10 ml of a 35% formalin solution, the liberated acidity was determined by titration to pH = 8.1 (IFFJP Method No. 30, 1965) and expressed in ml of 0.1N-alkali/100 ml of juice.

Amino acids

Total amino acids were determined by an automated, ninhydrin based method (Vandercook et al., 1975). Results were expressed in meq/100 mL. Individual free amino acids were measured by AAA Laboratories (Seattle, WA) with a Dionex D-500 amino acid analyzer. Results were expressed in μmoles/ml. The analysis sequence was that reported in Dionex application Report No. 2, (Pickering, 1978). The samples were clarified before analysis by centrifugation at 50,000 x g for 20 min followed by filtration through a Metricel GA-8 membrane filter. Proline (μmoles/ml) was also determined colorimetrically using the acidic ninhydrin method reported by Ting and Rouseff (1979).

Protein

Values for protein were determined by the micro Kjeldahl procedure of the IFFJP (Method No. 28, 1965).

Vitamins

Ascorbic acid was determined by a modified version of the 2,6-dichloroindophenol titration method (AOAC, 1975). The titration endpoint was measured amperometrically to allow the use of automated equipment. Thiamin was analyzed by the fluorometric procedure of the AOAC (1975). Total carotenoids were determined photometrically by a modification of the IFFJP method (No. 44, 1972) using an ethanol/hexane extract rather than a methanol/petroleum ether extract. Beta-carotene was determined after separation by column chromatography on aluminum oxide (AOAC, 1975).

Phenolics

Total phenolics were estimated via a coupling reaction with diazotized sulfanilic acid and reported as mg phenol/100 ml (Vandercook et al., 1975). Bioflavonoids were measured photometrically (Davis, 1947) and reported as mg hesperidin/100 mL.

Spectral properties

The visible (443 nm) and ultra-violet (325,280 nm) absorption of dilute samples were measured by the method described by Petrus and Attaway (1980).

Microbiological assay

The automated assay of Vandercook et al. (1980) was used. This is a rapid micro-determination of orange juice authenticity based on the rate of pH change due to acid production by a massive inoculation of Lactobacillus plantarum. The rate of pH change is proportional to juice concentration. Results are expressed as the ratio of the rate of pH change of sample to that of a standard at an equivalent °Brix.

Ash

The ash content was determined according to the IFFJP method (No. 9, 1962). Alkalinity was determined by titration (AOAC, 1975). The results were expressed as alkalinity number (ml of 0.1N NaOH/g of sample).

Minerals

Sodium, potassium, magnesium and calcium were determined by a modified version of the atomic absorption method of McHard (1976). Following dilution to 50 ml with deionized H_2O, the digests were analyzed with either an Instrumentation Laboratories Model 351 or Model 951 spectrophotometer.

Sulfate

After precipitation as barium sulfate, the sulfate content was determined gravimetrically according to the procedure of the IFFJP (Method No. 36, 1973).

Nitrates

A modified version of the method reported by Kamm et al.

(1965) was used. The modified method involves reduction of nitrate to nitrite and the use of N(1-napthyl) ethylene-diamine·2 HCl as a coupling agent to produce a colored compound which can be determined colorimetrically. Copies of the method are available upon request.

RESULTS & DISCUSSION

EARLY, MIDDLE, AND LATE season navel orange juice and pulpwash have been characterized by multiple chemical and physical parameters. The means, standard deviations and ranges for the parameters, except free amino acids, are presented in Table 1 for both single-strength orange juice and pulpwash. Table 2 presents mean values for early, middle, and late season samples.

Comparison of SSOJ and PW

Carbohydrates. Simple sugars were analyzed by two methods. Values for total sugars (and reducing sugars) measured by reaction with alkaline potassium ferricyanide were consistently higher than those determined by HPLC due to the nonspecificity of the ferricyanide reaction. Curl and Veldhuis (1948) reported the proportion of sucrose, glucose and fructose in Florida Valencia orange juice to be 2:1:1, and the HPLC results in Tables 1 and 4 support similar pattern for California navel orange juice and pulpwash. In more recent experiments with Florida orange juice, Ting and Attaway (1971) found concentrations of glucose and fructose remained relatively constant as the season progressed while sucrose increased. In our study (Table 2) sucrose increased from early to mid-season but decreased in late season samples. Mid-season values for glucose and fructose were significantly lower than for early or late season samples. Pulp extracts have been reported (Sawyer, 1963; Attaway et al., 1972) to contain a lower sucrose content than orange juice but essentially the same concentrations of glucose and fructose as orange juice. Our results agree with the reported trend, although we found higher sucrose contents than did the earlier studies.

The pectic substances of orange juice have been extensively investigated by a variety of methods: gravimetric (precipitation with ethyl alcohol, or as calcium pectate) and colorimetric (carbazole reaction). Concentrated orange juice from Florida contained pectin values (colorimetric) of 280-610, 330-860, and 710-1240 mg/kg at 11.8°B for water-soluble, base-soluble, and total pectin, respectively (Rouse et al., 1974). Our results revealed lower water soluble and total pectin values for single-strength navel juice (Table 1) and navel concentrate (Table 4) than those reported by Rouse et al., (1974). While this difference may be due to varietal differences, the pectin content of citrus juices has been shown to depend on extractor and finishing pressures (Vandercook et al., 1966; Atkins and Rouse, 1953) and on the pulp content of the finished product (Iranzo et al., 1977). In agreement with Iranzo et al. (1977), the concentration of total pectin in California navel PW was roughly double that of SSOJ.

Because concentrations are similar, our results indicate measurement of simple sugars will not distinguish PW from SSOJ. High pectin content may indicate the presence of PW in orange juice. However, the broad ranges that seem to characterize pectin content dilute the value of pectin measurements as a singular tool for detecting or estimating PW content in orange juice.

Acids. Organic acid content in California and Florida orange juice ranged from 2.2—15.5 g/L for citric acid and from 0.6—3.1 g/L for malic acid (Vandercook, 1977a). The citric and malic acid concentrations for navel orange juice were well within these ranges. Sawyer (1963) reported lower citric acid contents in PW than in SSOJ, but we found similar concentrations of citric acid in both PW and

SSOJ. A significant seasonal difference was observed in the concentration of citric acid, isocitric acid and total acidity for both SSOJ and PW (Table 2).

Easily measured organic acids of navel orange juice offer no aid in detecting pulpwash. The ratio of citric to isocritic acid has been used to detect adulteration of orange juice by dilution or addition of sugars and citric acid. A recent review (Petrus and Vandercook, 1980) cites key isocitrate references and summarizes the reported ratio ranges. The citric/isocitric acid ratios found for navel orange juice (Table 1) fit within the reported ranges for juices from other orange varieties.

Amino acids. Using the then recently developed ion-exchange method, Clements and Leland (1962) reported a preliminary survey of the amino acids present in navel orange juice. Table 3 lists the mean seasonal values for a more complete survey of the amino acids of California navel juice using the greater resolution possible with the lithium citrate buffer system. Comparison of the navel juice results (Table 3) to a summation (Vandercook, 1977b) of published amino acid values for the dominant orange varieties reveals that the navel results generally fit the summation ranges. However, the levels of alanine, arginine and glutamic acid in navel orange juice exceed the cited ranges (Vandercook, 1977b).

When analysis of variance was applied to the amino acid data, the following patterns emerged. First, three of the seven dominant amino acids display significant seasonal

Table 1—Analytical values[a] for navel single-strength orange juice and navel pulpwash

Analysis	Single strength			Pulpwash		
	Mean	Standard deviation	Range	Mean	Standard deviation	Range
°Brix	13.5	0.8	11.9–14.6	7.4	2.8	2.7–11.1
Carbohydrates						
Glucose (g/100 ml)[b]	2.42	0.21	2.10-2.73	2.48	0.44	1.89–3.55
Fructose (g/100 ml)[b]	2.57	0.11	2.38–2.74	2.64	0.28	2.30–3.39
Sucrose (g/100 ml)[b,g]	4.49	0.53	3.67–5.64	3.96	0.59	2.49–4.79
Total sugar (g/100 ml)[b]	9.48	0.28	8.85–10.29	9.08	0.44	6.69–11.73
Total sugar (g/100 ml)[c]	12.6	0.3	11.6–12.8	12.1	1.3	10.2–14.3
Reducing sugar (g/100 ml)[c]	8.7	0.4	8.0–9.4	8.3	0.7	7.2–9.4
H₂O-sol. pectin (mg/L)[g]	192	85	67–340	447	207	117–866
NaOH-sol. pectin (mg/L)[g]	374	156	247–800	721	517	108–1623
Total pectin (mg/L)[g]	569	222	340–1096	1168	607	343–2349
Acids						
Total acids (g/L)	7.5	2.2	5.0–11.1	7.8	2.6	5.3–15.1
Citric acid (g/L)	9.6	2.9	6.7–15.1	8.9	2.3	6.6–13.4
Isocitric acid (mg/L)	76.7	24.2	49.7–122	75.6	32.0	47.7–158
Citric/isocitric ratio[d]	100	6	91–100	107	10	86–127
Malic acid (g/L)	0.89	0.28	0.58–1.45	0.91	0.38	0.48–1.99
Amino acids						
Total amino acids[e]	3.31	0.54	2.37–4.64	3.28	0.58	2.34–4.45
Formol number[e]	24	3	16–31	26	3	21–31
Proline (μmole/ml)[f]	13.7	3.0	9.2–9.6	14.4	2.5	9.3–18.8
Minerals						
Ash (g/L)	3.47	0.65	2.51–4.78	3.89	1.09	3.13–6.49
Na (mg/L)[g]	6.0	2.0	4.01–10.3	24.0	17.7	9.0–75.0
K (mg/L)[g]	1732	143	1479–2090	2025	495	1537–3462
K/Na ratio	312	79	170–450	109	47	46–190
Mg (mg/L)[g]	101	8	87–114	117	15	100–155
Ca (mg/L)[g]	129	35	91–188	275	189	132–806
Alkalinity of ash[e,f]	15.1	1.2	11.7–16.5	11.5	2.9	5.23–14.2
Vitamins						
Vitamin C (mg/L)	555	64	445–688	571	80	439–725
Thiamin (mg/L)	0.758	0.078	0.638–0.959	0.821	0.079	0.701–1.003
Total carotenes (mg/L)[g]	6.34	3.02	1.40–10.4	4.81	2.46	0.337–8.01
β-carotene (mg/L)	0.132	0.079	0.040–0.372	0.088	0.046	0.008–0.208
UV/VIS						
VIS – 443 nm[e]	0.148	0.05	0.067–0.254	0.113	0.05	0.031–0.268
UV – 325 nm[e,g]	1.32	0.21	1.02–1.63	1.62	0.30	1.28–2.44
UV – 280 nm[e,g]	2.60	0.61	1.77–3.82	3.10	0.56	2.28–4.41
Miscellaneous						
Nitrate (mg/L)[g]	3.5	1.0	1.2–5.3	26.5	19.3	8.7–81.9
Sulfate (mg/L)[g]	72	12	45–87	104	24	76–160
Hesperidin[e,g]	365	78	260–545	486	71	343–885
Total phenolics[e,g]	168	18	143–193	211	37	160–296
Protein (g/100 ml)[g]	0.76	0.07	0.67–0.92	0.66	0.11	0.45–0.83
Microbiological[e]	1.04	0.29	0.69–1.58	1.12	0.67	0.65–3.39

[a] Except for initial °Brix values, all other values are listed on 11.8° basis.
[b] Determined by HPLC.
[c] Determined by the procedure of Vandercook et al. (1975)
[d] Ratio of total acids to isocitric acid.

[e] See experimental section for units.
[f] Determined by the colorimetric procedure of Ting and Rouseff (1979).
[g] Parameter for which a significant difference (P < 0.01) is observed between SSOJ and PW.

trends. Arginine (P < 0.005) increased while aspartic acid (P < 0.025) and asparagine (P < 0.005) decreased as the season progressed. Alanine, proline, serine and γ-aminobutyric acid did not vary with season. Second, there is no significant difference (P > 0.1) in amino acid composition and concentration, between SSOJ and PW. This position is supported by the lack of a significant difference (P > 0.01) between SSOJ and PW in total amino acids (ninhydrin method) and in formol number.

The proline contents of SSOJ and PW, estimated by the colorimetric method of Ting and Rouseff (1979), were consistently higher than concentrations determined by the ionexchange method. Ninhydrin response from trace peptides and basic amino acids such as arginine (Niedmann, 1976) may account for the discrepancy.

In agreement with Niedmann (1976), measurement of the major orange juice amino acids is not useful for detecting pulpwash, but can play a role in detecting adulteration by dilution. The formol value, as a reflection of amino acid content, provides a quick check for simple dilution and in tandem with measurement of proline and ammonia, can help detect sophisticated dilution (Wallrauch, 1980).

Minerals. Benk (1965) reported ranges for sodium, potassium, calcium and magnesium of 0.4–2.0, 114–193, 5.4–15, 6 and 7.3–15.3 mg/100 ml, respectively, in the juice of oranges from different geographical locations. Ting et al. (1974) reported ranges of Ca (3.4–20.9 mg/100 ml) and Mg 93.4–16.4 mg/100 ml) for Florida orange juice. We found the levels of Na, K, Mg and Ca in SSOJ to be within these published ranges. Additionally, the

Table 2—Seasonal variation in analytical mean value[a] for navel single-strength orange juice and navel pulpwash

Analysis	Single strength			Pulpwash		
	Early	Middle	Late	Early	Middle	Late
°Brix	12.7±0.8	13.5±0.6	14.2±0.3	5.2±3.1	7.2±1.2	9.8±1.5
Carbohydrates						
Glucose (g/100 ml)[b,g]	2.40±0.16	2.21±0.11	2.67±0.11	2.20±0.21	2.33±0.25	2.92±0.44
Fructose (g/100 ml)[b,g]	2.62±0.10	2.45±0.06	2.64±0.08	2.56±0.11	2.48±0.19	2.86±0.35
Sucrose (g/100 ml)[b,g]	4.52±0.09	5.02±0.42	3.92±0.19	3.93±0.26	4.41±0.51	3.51±0.60
Total sugar (g/100 ml)[b,g]	9.54±0.12	9.68±0.20	9.23±0.13	8.69±0.19	9.22±0.32	9.29±0.46
Total sugar (g/100 ml)[c,g]	12.5±0.2	12.9±0.4	12.5±0.2	11.2±1.5	13.2±0.8	11.9±0.3
Reducing sugar (g/100 ml)[c]	8.8±0.4	8.3±0.2	8.8±0.2	7.8±0.8	8.4±0.4	8.8±0.5
H₂O-sol. pectin (mg/L)[g]	108±27	188±68	280±42	299±190	499±221	542±148
NaOH-sol. pectin (mg/L)[g]	280±31	303±33	538±180	317±274	749±530	1098±444
Total pectin (mg/L)[g]	388±29	491±51	818±111	616±232	1248±376	1640±296
Acids						
Total acids (g/L)[g]	9.7±1.7	7.2±0.4	5.4±0.3	10.7±2.6	7.0±0.6	5.8±0.4
Citric acid (g/L)[g]	12.7±2.7	8.8±0.7	7.1±0.4	11.5±2.1	8.1±0.7	7.1±0.6
Isocitric acid (mg/L)[g]	107±13	70.2±2.8	53.4±3.0	102±15	61.8±4.5	51.5±3.5
Citric/isocitric ratio[d]	94±4	103±7	102±5	95±6	113±9	112±4
Malic acid (g/L)	0.89±0.29	0.91±0.30	0.88±0.33	1.01±0.59	1.05±0.22	0.70±0.16
Amino acids						
Total amino acids[e]	3.36±0.52	3.42±0.69	3.14±0.48	3.44±0.64	3.55±0.55	2.85±0.33
Formol number[e]	25±3	21±3	23±2	27±2	24±2	24±3
Proline (μmole/ml)[f]	13.2±2.6	14.0±3.5	14.0±3.3	15.5±2.7	14.6±2.3	12.9±2.2
Minerals						
Ash (g/L)[g]	4.27±0.33	3.17±0.14	2.97±0.32	5.09±1.20	3.26±0.10	3.35±0.12
Na (mg/L)[g]	8.0±2.3	4.7±0.5	5.2±0.6	42.5±20.5	17.5±3.5	12.3±4.2
K (mg/L)[g]	1841±144	1761±57	1592±84	2517±588	1775±141	1783±201
K/Na ratio	250±94	380±46	306±22	65±17	108±26	154±41
Mg (mg/L)[g]	108±7	101±3	93±5	128±22	115±10	108±5
Ca (mg/L)[g]	174±12	99±6	115±13	477±220	172±30	183±38
Alkalinity of ash[e,g]	15.6±0.8	14.8±0.6	15.0±2.0	8.6±3.2	12.5±0.7	13.3±1.2
Vitamins						
Vitamin C (mg/L)[g]	618±42	551±43	495±32	651±51	578±34	483±33
Thiamin (mg/L)	0.73±0.13	0.78±0.04	0.77±0.02	0.81±0.12	0.82±0.06	0.84±0.05
Total carotenes (mg/L)[g]	2.60±1.44	8.56±0.61	7.87±1.72	2.10±2.32	6.72±0.90	5.59±0.64
β-carotene (mg/L)	0.143±0.130	0.114±0.035	0.138±0.044	0.076±0.078	0.084±0.022	0.104±0.0
UV/VIS						
VIS − 443 nm[e,g]	0.128±0.046	0.126±0.031	0.190±0.052	0.083±0.033	0.098±0.020	0.158±0.0
UV − 325 nm[e,g]	1.42 ± 0.12	1.06±0.05	1.48±0.12	1.83±0.38	1.35±0.06	1.70±0.11
UV − 280 nm[e,g]	3.13±0.50	1.91±0.13	2.75±0.25	3.44±0.61	2.55±0.23	3.31±0.31
Miscellaneous						
Nitrate (mg/L)	2.6±1.2	3.8±0.9	4.1±0.4	27±13	37±30	16.4±3.9
Sulfate (mg/L)	66±18	71±10	77±9	121±41	93±12	99±8
Hesperidin[e,g]	408±105	341±45	348±83	641±147	444±36	375±30
Total phenolics[e]	176±20	158±18	169±13	230±59	204±11	200±24
Protein (g/100 ml)[g]	0.79±0.05	0.78±0.08	0.70±0.02	0.71±0.05	0.73±0.06	0.52±0.05
Microbiological[e,g]	1.33±0.28	1.04±0.06	0.75±0.06	1.70±0.97	0.86±0.14	0.81±0.10

[a] Values are listed on an 11.8° Brix basis.
[b] Determined by HPLC.
[c] Determined by the procedure of Vandercook et al. (1975).
[d] Ratio of total acids to isocitric acid.
[e] See experimental section for units.
[i] Determined by the colorimetric procedure of Ting and Rousell (1979).
[g] Parameter for which a significant (P < 0.01) seasonal variation was observed.

values for Na, K, Mg, Ca, and ash show a significant (P < 0.01) seasonal dependence, declining as season progresses.

The mineral content of PW was higher (P < 0.01) than in SSOJ. Average values, in mg/L, for the water supply during the study were: Na (13), K (2), Mg (10) and Ca (51). Considering the mean values of Na, K, Mg and Ca for SSOJ and PW, the mean °Brix (7.4) and comparison °Brix (11.8) for PW, and the large ranges displayed by PW it appears that the elevated levels of Na and Mg in pulpwash are a reflection of pulpwashing water with a lesser contribution from the pulp. The K and Ca differences are too large to reflect a major water influence and must reflect some leaching of K and Ca from pulp (membrane, albedo) by the acidic juice during the pulpwashing. In a comparative study of OJ and PW prepared from Florida oranges, the K/Na ratio was substantially lower for PW (102) than for SSOJ (512) (Attaway et al., 1972). We found mean values of 109 (PW) and 312 (SSOJ) for the K/Na ratio.

Increased minerals content can be an indication of pulpwash. However, the wide range of minerals shown by PW, the varied impact of the water used in pulpwashing and the seasonal variation suggest that measurement of minerals should only be one part of a pulpwash detection scheme.

Vitamins. No significant differences were noted for the vitamin C and thiamin contents of SSOJ and PW. Vitamin C concentration decreased as the season progressed, but thiamin concentrations remained unchanged. Total carotene contents were lower in PW than in SSOJ while β-carotene concentration was not significantly different for SSOJ and PW. The total carotene concentrations generally increased in SSOJ and PW as the season progressed, whereas the β-carotene levels remained relatively constant through the season. These vitamin components of orange juice have no value in detecting pulpwash.

UV/VIS absorption characteristics. A spectral method to detect PW in Florida orange juice based on the sum of absorbances at three wavelengths (443, 325, 280 nm) and the ratio of absorbances at two wavelengths (443/325) has been reported (Petrus and Attaway, 1980). To begin testing the applicability of the Florida based method to California juices we carried out the indicated spectral measurements. A significant difference (P < 0.01) was noted between SSOJ and PW for absorbance values at 325 and 280 nm. For both SSOJ and PW, absorbance at each of the three wavelengths varied (P < 0.01) with season. Additionally,

the absorbance sum for navel orange juice and PW differs from the sum established for Florida orange juice. Average absorbance sums reported by Petrus and Attaway (1980) for Florida were 2.22 (at 11.8°B) for orange juice while PW gave a higher sum of 3.48. The mean absorbance values in Table 1 sum to 4.07 for SSOJ and 4.83 for PW while absorbance values derived from concentrated juice (Table 4) give a sum of 3.67; A part of the lower absorbance sum for concentrate, versus SSOJ, comes from a lower absorbance at 280 nm. Hesperidin, a major contributor to absorbance at 280 nm is a flavanone of low solubility that crystallizes during concentration of juice. Because the absorbance sums for navel juices are more like sums for Florida PW, navel orange juices will appear to contain PW when evaluated by the Petrus and Attaway method (1980) using Florida based constants. A larger pool of absorbance data from navel juices, like that amassed in the Florida study, is needed to establish the statistical base that may allow the Petrus/Attaway (1980) method to be adapted to California/Arizona navel juice.

Miscellaneous. Mean nitrate levels of 3.5 and 26.5 mg/L and sulfate levels of 72 and 104 mg/L were found for SSOJ and PW, respectively. These significant differences reflect nitrate (16 mg/L) and sulfate (15 mg/L) concentrations for the water used in the pulpwashing operation.

Protein content of PW was found to be statistically lower than that of SSOJ and a significant seasonal decrease in protein was observed for both SSOJ and PW.

The microbiological assay did not show any significant differences between SSOJ and PW. Metabolic rates of *Lactobacillus plantarum* were the same in both SSOJ and PW and the metabolic rate decreased for both SSOJ and PW as the season progressed.

The total phenolic content of the PW samples was significantly (P < 0.01) higher than that of the SSOJ samples. This trend was confirmed by the Davis (1947) values and agrees with the higher phenolic content of pulpwash reported by Rouse et al. (1959). No clearly defined seasonal trend was apparent for the phenolic content of SSOJ and PW.

Comparison of SSOJ and concentrated orange juice

Concentrate samples summarized in Table 4 were gathered only during early and mid-season and contain PW

Table 3—Amino acid concentrations for early, middle, and late season navel single-strength orange juice and navel pulpwash expressed in μmoles/ml (mean values)

Amino acid	Single strength				Pulpwash			
	Early	Middle	Late	Mean[a]	Early	Middle	Late	Mean[a]
Alanine	1.66±0.39	1.65±0.41	1.93±0.36	1.75±0.38	1.77±0.35	1.42±0.22	2.00±0.32	1.73±0.37
Arginine	3.13±0.39	5.02±0.52	6.45±0.66	4.87±1.49	3.36±0.35	4.50±0.74	6.59±0.76	4.82±1.51
Aspartic acid	2.78±1.34	1.82±0.26	1.90±0.69	2.17±0.10	3.39±0.57	1.47±0.28	2.29±0.48	2.38±0.91
Glutamic acid	0.82±0.29	0.65±0.05	0.63±0.11	0.70±0.20	1.09±0.13	0.61±0.07	0.64±0.10	0.78±0.25
Glycine	0.25±0.05	0.19±0.03	0.27±0.04	0.24±0.05	0.24±0.05	0.17±0.03	0.28±0.04	0.23±0.06
Histidine	0.060±0.01	0.070±0.02	0.125±0.02	0.085±0.04	0.090±0.030	0.070±0.010	0.136±0.020	0.099±0.036
Isoleucine	—	0.045±0.008	0.058±0.020	0.052±0.016	—	0.039±0.005	0.054±0.013	0.046±0.012
Leucine	—	0.047±0.014	0.069±0.025	0.058±0.021	—	0.053±0.017	0.075±0.032	0.064±0.027
Lysine	0.17±0.03	0.24±0.03	0.30±0.04	0.24±0.06	0.18±0.03	0.24±0.04	0.32±0.04	0.247±0.069
Phenylalanine	0.10±0.05	0.08±0.02	0.15±0.03	0.11±0.04	—	0.08±0.02	0.16±0.03	0.12±0.05
Proline	11.1±2.0	12.8±2.5	11.9±3.0	11.9±2.4	12.7±1.8	11.5±1.6	12.3±1.6	12.2±1.7
Serine	2.47±0.54	1.76±0.44	1.58±0.27	1.94±0.57	2.54±0.28	1.46±0.23	1.59±0.14	1.86±0.54
Threonine	0.21±0.04	0.13±0.03	0.16±0.03	0.17±0.04	0.18±0.03	0.12±0.04	0.17±0.02	0.16±0.04
Tyrosine	—	0.042±0.012	0.051±0.009	0.047±0.016	—	—	—	—
Valine	0.183±0.109	0.128±0.030	0.171±0.031	0.161±0.063	0.250±0.083	0.129±0.021	0.181±0.033	0.187±0.067
Asparagine	4.77±1.88	2.78±0.36	2.29±0.58	3.28±1.54	4.74±1.18	2.57±0.39	2.32±0.59	3.21±1.35
Glutamine	0.53±0.36	0.39±0.04	0.38±0.13	0.43±0.22	0.42±0.10	0.44±0.06	0.41±0.14	0.42±0.10
γ-aminobutyric acid	2.88±0.46	2.66±0.35	3.50±1.02	3.01±0.73	3.28±0.47	2.52±0.42	3.54±0.68	3.11±0.67
Total	—	—	—	31.2	—	—	—	31.7

[a] Mean of early, middle, and late values

as allowed by Federal Regulation. The concentrate samples do not represent the same lots of fruit as the SSOJ and PW samples (see Materials & Methods). Nevertheless, the concentrate results (Table 4) are in agreement with the total season SSOJ results (Table 1) and provide confirmation for the general magnitude of the SSOJ results displayed in Table 1.

To see if any of the constituents (pectins, uv/vis, minerals) that tend to differentiate PW from SSOJ reflect in the concentrate values, the early plus mid-season SSOJ data and the concentrate data were evaluated by analysis of variance. Six of the analytical constituents revealed significant difference between SSOJ and concentrate.

Table 4—Analytical values [a] for navel concentrate

Analysis	Mean	Standard Deviation	Range
°Brix	66.3	1.34	63.4–67.5
Carbohydrates			
Glucose (g/100 ml)[b]	2.37	0.16	2.12–2.59
Fructose (g/100 ml)[b]	2.48	0.17	2.20–2.74
Sucrose (g/100 ml)[b]	4.50	0.24	4.19–4.86
Total sugar (g/100 ml)[b]	9.36	0.19	8.51–12.8
Total sugar (g/100 ml)[c]	12.6	0.4	11.6–12.8
Reducing sugar (g/100 ml)[c]	8.2	0.4	7.5–8.7
H_2O-sol. pectin (mg/L)	358	167	177–594
NaOH-sol. pectin (mg/L)	264	31	215–301
Total pectin (mg/L)	622	174	403–839
Acids			
Total acids (g/L)	8.4	1.6	6.6–10.9
Citric acid (g/L)	10.9	2.1	8.3–13.7
Isocitric acid (mg/L)	87.3	22.5	63.8–124
Citric/isocitric ratio[d]	101	9	86–113
Malic acid (g/L)	1.08	0.28	0.67–1.52
Amino acids			
Total amino acids[e]	3.06	0.33	2.33–3.51
Formol number[e]	24	2	22–26
Proline (μmole/ml)[f]	11.7	1.7	8.9–14.3
Minerals			
Ash (g/L)	3.44	0.20	3.21–3.83
Na (mg/L)	11.2	4.0	5.6–18.5
K (mg/L)	1814	402	1631–1973
K/Na ratio	182	69	106–330
Mg (mg/L)	104	8	92–119
Ca (mg/L)	145	35	100–183
Alkalinity of ash[e]	15.8	1.3	14.3–18.5
Vitamins			
Vitamin C (mg/L)	575	24	540–627
Thiamin (mg/L)	0.723	0.087	0.544–0.820
Total carotenes (mg/L)	7.57	2.54	4.61–11.88
β-carotene (mg/L)	0.262	0.135	0.127–0.538
UV/VIS			
VIS – 443 nm[e]	0.124	0.013	0.107–0.143
UV – 325 nm[e]	1.23	0.17	1.03–1.52
UV – 280 nm[e]	2.32	0.42	1.84–2.90
Miscellaneous			
Nitrate (mg/L)	8.7	1.4	6.4–10.5
Sulfate (mg/L)	62	9	47–75
Hesperidin[e]	387	70	296–613
Total phenolics[e]	172	14	148–195
Protein (g/100 ml)	0.77	0.06	0.73–0.90
Microbiological[e]	1.17	0.28	0.82–1.54

[a] Except for initial °Brix values, all other values are listed on an 11.8° Brix basis.
[b] Determined by HPLC.
[c] Determined by the procedure of Vandercook et al. (1975).
[d] Ratio of total acids to isocitric acid.
[e] See experimental section for units.
[f] Determined by the colorimetric procedure of Ting and Rouseff (1979).

The colorimetric values for reducing sugars (P < 0.01) and total sugar (P < 0.05) were lower in concentrate while β-carotene was higher (P < 0.05) in concentrate than is SSOJ. These patterns were not indicated by our SSOJ and PW results and do not appear to be linked to the presence of small amounts of PW in the concentrates.

The UV/VIS absorbance values for the concentrates were not significantly different than the SSOJ values. The potential contribution, from PW, to these values may be lost in the concentration step. Some hesperidin (major uv absorption near 280 nm) crystallizes on interior surfaces of the evaporator during juice concentration and is depleted in the final concentrate.

Elevated levels of water-soluble and alkali-soluble pectin seen in PW (Table 1) suggest that pectin levels could be elevated in the concentrate samples. The water-soluble pectin level in the concentrates was higher (P < 0.01) than in the single-strength juices but the levels of alkali-soluble pectin were similar in both SSOJ and concentrate. The possible increase in both pectin forms from PW may be overcome in the concentration process. Concentration conditions (pH, heat) should allow solubilization of protopectin that would drop the level of alkali-soluble pectin and give the increased level of water-soluble pectin that was noted.

The most direct reflection of PW emerged for Na and NO_3; both values are elevated (P < 0.01) in the concentrate samples. Sodium and nitrate are characteristically low in SSOJ and their increased levels in concentrate comes from the water that was used in pulpwashing.

CONCLUSIONS

DIFFERENCES between California navel two-stage pulpwash and single-strength orange juice are relatively small although statistically significant in some cases. Many of the parameters evaluated were found to be seasonally dependent. No significant differences were found between SSOJ and concentrated orange juice containing small amounts of PW. The problems of detecting small quantities of PW in concentrated orange juice are compounded by the wide natural variations in amounts of the components studied.

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Sugar Analysis of Fruit Juices: Content and Method

B. W. LI and P. J. SCHUHMANN

————————————ABSTRACT————————————

The sugar contents of 186 samples of 11 different juices from a nation-wide sampling were determined by the use of a total sugar analyzer (TSA), a gas-liquid chromatograph (GLC) or both. Values for total sugar ranged from <3% in most tomato juices to >15% in some lemonades on a ready-to-serve basis. Agreement between the two methods was satisfactory.

INTRODUCTION

SUGAR is the major constituent of most fruit juices. A number of publications have reported either individual or total sugars in some of these beverages. Paul and Southgate (1978) listed the total sugar content of five juices sampled in Great Britain. Birkhed et al. (1980), in a Swedish study aimed at disclosing the sources of sugar in the diet for purposes of a dental caries study, determined the content of individual sguars in six beverages including three fruit drinks; Fitelson (1970) examined a number of fruits and fruit concentrates by gas-liquid chromatography to establish the characteristic peaks associated with natural fruits as an aid to detection of adulteration in certain commercial products. Hurst et al. (1979) included juices among a variety of food items they analyzed for sugars by high-performance liquid chromatography. Data were obtained for most common fruit juices but no attempt was made to assess a range or variability related to sampling. Southmayd (1970), Wrolstad and Shallenberger (1981) presented data from the literature on free sugar and sorbitol content of nine fruits. They include data on different varieties from many sources using different methods of analysis. So far, no data are available on the sugar content of fruit and vegetable juices sampled throughout the United States. The increased interest of nutritionists in the role of sugars in the diet has created a need for such information. The Nutrient Composition Laboratory of USDA, in order to provide adequate nutrient data for food composition tables, undertook a national sampling of fruit and vegetable juices. The nine fruit and two vegetable juices included in the study account for the major part of consumption of this type of beverage in the country. From preliminary studies, we found that the amount of each individual sugar often varies between containers of a given product, while the total sugar content remains nearly constant (Li et al., 1982). This report gives the mean value and range of total sugar in each type of juice, as determined by a total sugar analyzer (TSA), comparison of some of these data with that obtained by gas-liquid chromatography (GLC) and a brief description of the analytical methods.

MATERIALS & METHODS

Samples

Juices were procured in two different major supermarkets in each of five metropolitan areas, Atlanta, Boston, Chicago, Houston

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and Los Angeles, during the months of July, August, and September, 1980. The detailed sampling scheme will be described elsewhere (Holden et al., 1983). These were variously canned, bottled, chilled (in either bottles or cartons) and frozen concentrates. Concentrates were shipped in dry ice and were not used unless they were frozen upon arrival. Chilled juices were shipped by air in iced insulated containers. All samples were processed as soon as possible upon arrival. A sample number was assigned to each container, the concentrates were reconstituted and the contents of each container divided into several subsamples and then stored below −15°C until analyzed.

To determine the stability of sugars in frozen samples of reconstituted or ready-to-serve juices, we resampled and analyzed each of eight types of juice after they had been stored at −15°C for at least 18 months. The data (GLC) indicated that neither the total nor the individual sugars had changed.

Sample preparation was made with the assumption that at natural strength, the sugars in all juices are completely in solution. Frozen subsamples were thawed and well mixed and then centrifuged at 1000 rpm for 10 min. The clear supernatant solution was suitable for sampling for either the TSA or GLC procedure. The former required dilution with water to prepare a solution containing approximately 50 μg/ml of sugar. The GLC sample of 0.02 ml or 0.05 ml was measured directly into a sample vial.

Standards and reagents

Standard sugars (Sigma Chemical) for both methods were dried under vacuum over P_2O_5 before use. For TSA analysis, standard solutions were prepared of glucose (60 μg/ml), sucrose (60 μg/ml), and glucose plus sucrose (30 μg/sugar/ml). For GLC analysis, 0.5 ml aliquots of a standard solution containing 1.0 mg/ml of fructose, glucose and sucrose were dried and stored below −10°C until needed.

Reagents for preparing the trimethylsilylated oxime derivatives were: pyridine (Burdick & Jackson Laboratories, Inc., Muskegon, MI) containing 25 μg/ml of dydroxylamine hydrochloride (Fisher Scientific Co., Silver Spring, MD) and 2 mg/ml β-phenyl-D-glucopyranoside, (Sigma Chemical Co.); hexamethyldisilazane (PCR Research Chemicals, Inc., Gainesville, FL); trifluoroacetic acid (Aldrich Chemical Co., Inc., Milwaukee, WI). They were used as purchased. The color reagent for TSA analysis, p-hydroxybenzoic acid hydrazide (Aldrich Chemical Co.) was prepared as a 0.33M stock solution in 0.5N HCl (reagent grade). This was diluted with 1.0N NaOH, 150 ml to 1000 ml, just before use.

TSA analyses

The total sugar values were obtained with a flow injection system, refered to as a total sugar analyzer (TSA) (Fig. 1). The TSA system was developed and tested in this laboratory (Stewart, 1979). The sampling portion includes a sampling pump (Polystaltic pump, Buchler Instruments, Inc., Fort Lee, NJ), an auto sampling device (Model 2, Technicon, Tarrytown, NY) and an injection valve (Valco Instruments, CV-6-OHPa-N-60, Houston, TX). The flow through system is controlled by means of three minipumps (Milton Roy, 196-0066 series, Laboratory Data Control, Riviera, FL). Detection is accomplished by means of a 20-μl flow cell and tungsten lamp (Schoeffel Instrument Co., Westwood, NJ) and a photomultiplier-microphotometer. J10-222A. (Am. Inst. Co., Silver Spring, MD). The system is equipped with an Omni-Scribe recorder (Houston Instruments, Houston, TX) and an Autolab Minigrator (Spectra Physics, Santa Clara, CA).

Similar automated colorimetric systems for sugar analysis have been described (Mundie et al., 1976; Hudson et al., 1976). Procedures used in this report have been presented elsewhere (Li et al., 1982) Duplicates were run of each sample. The standard solutions
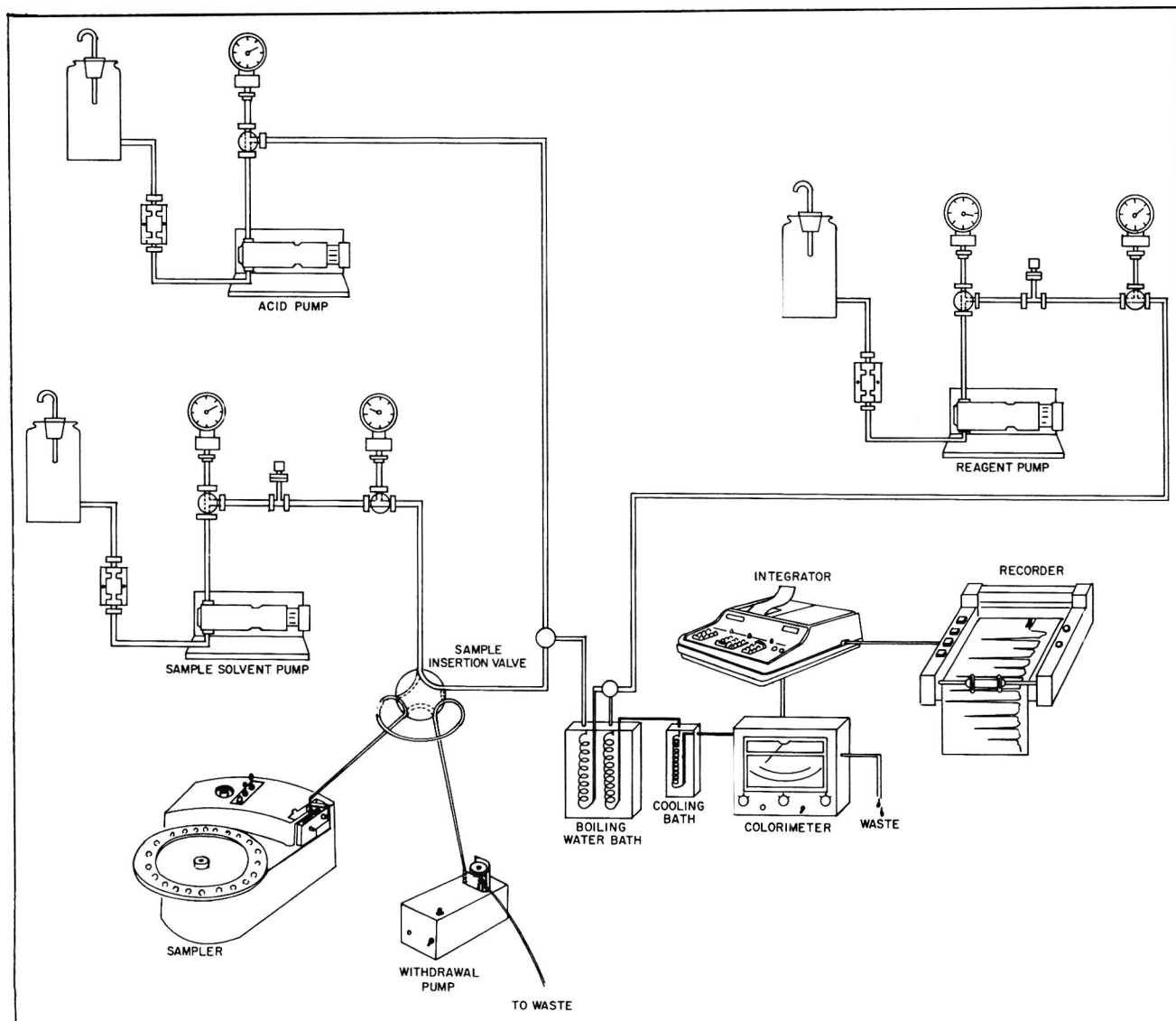
Fig. 1—Schematic of the automated total sugar analyzer.

were run before, after and interspersed with each set of samples. The TSA system was set to monitor the absorbance at 400 nm and to make 80 measurements/hour.

GLC analyses

The gas chromatographic measurements were obtained using a Hewlett-Packard 5840A, equipped with an autosampler (HP-7672A) and a hydrogen flame detector. The column (6' x 1/8"SS) was packed with SP-2250 on 80/100 mesh Supelcoport. Operating conditions were: injection port 200°C, detector 300°C, helium carrier flow of 30 ml/min, hydrogen flow of 40 ml/min and air flow of 300 ml/min. The column temperature was programmed from 170°C to 300°C at 10°/min. The injection volume was 1 μl. Derivatives were prepared of duplicate samples and of standards and analyzed as described by Li and Schuhmann (1981).

RESULTS & DISCUSSION

THE SUGAR CONTENT of 186 juices is shown in Table 1. The standard deviation reflects the overall variation among brands and samples. The precision of the colorimetric measurement is better than 1.0% at the 50 μg level. The mean coefficient of variation for duplicate samples of juice is 1.3%. It can be seen that the juices from different brands and different parts of the country did not vary greatly in total sugar content. The mean value of sugar for all the fruit juices was between 7% and 15% and the range did not exceed this with the exception of one lemonade at 15.3%.

Table 1—Sugar content of juices

Juice	No. of samples	Total sugar (g/100ml)	
		Mean + S.D.	Range
Apple	19	11.30 ± 1.12	9.12 — 13.50
Citrus Blend	7	10.60 ± 0.23	10.30 — 11.00
Cranberry	10	13.50 ± 0.47	12.90 — 14.20
Grape	9	13.70 ± 0.56	13.00 — 14.90
Grapefruit	15	7.50 ± 0.49	6.95 — 8.86
Lemonade	29	11.20 ± 1.41	9.66 — 15.30
Orange	41	10.40 ± 0.44	9.31 — 11.30
Pineapple	7	12.50 ± 0.64	11.20 — 13.40
Prune	10	13.10 ± 0.63	12.00 — 14.20
Tomato	28	2.76 ± 0.48	2.22 — 4.11
Vegetable	11	3.22 ± 0.21	3.00 — 3.74

The tomato and vegetable juices average between 2% and 4% total sugar.

Sugar content of samples, which contain either fructose, glucose, and/or sucrose, can be determined rapidly with an automated flow injection system. Quantitation of total sugar depends on the formation of a colored product formed between p-hydroxybenzoic acid hydrazide and the carbonyl group of the sugars. When these are the only sugars present, analysis by TSA is suitable because sucrose is hydrolyzed quantitatively during the acid hydrolysis step and the color
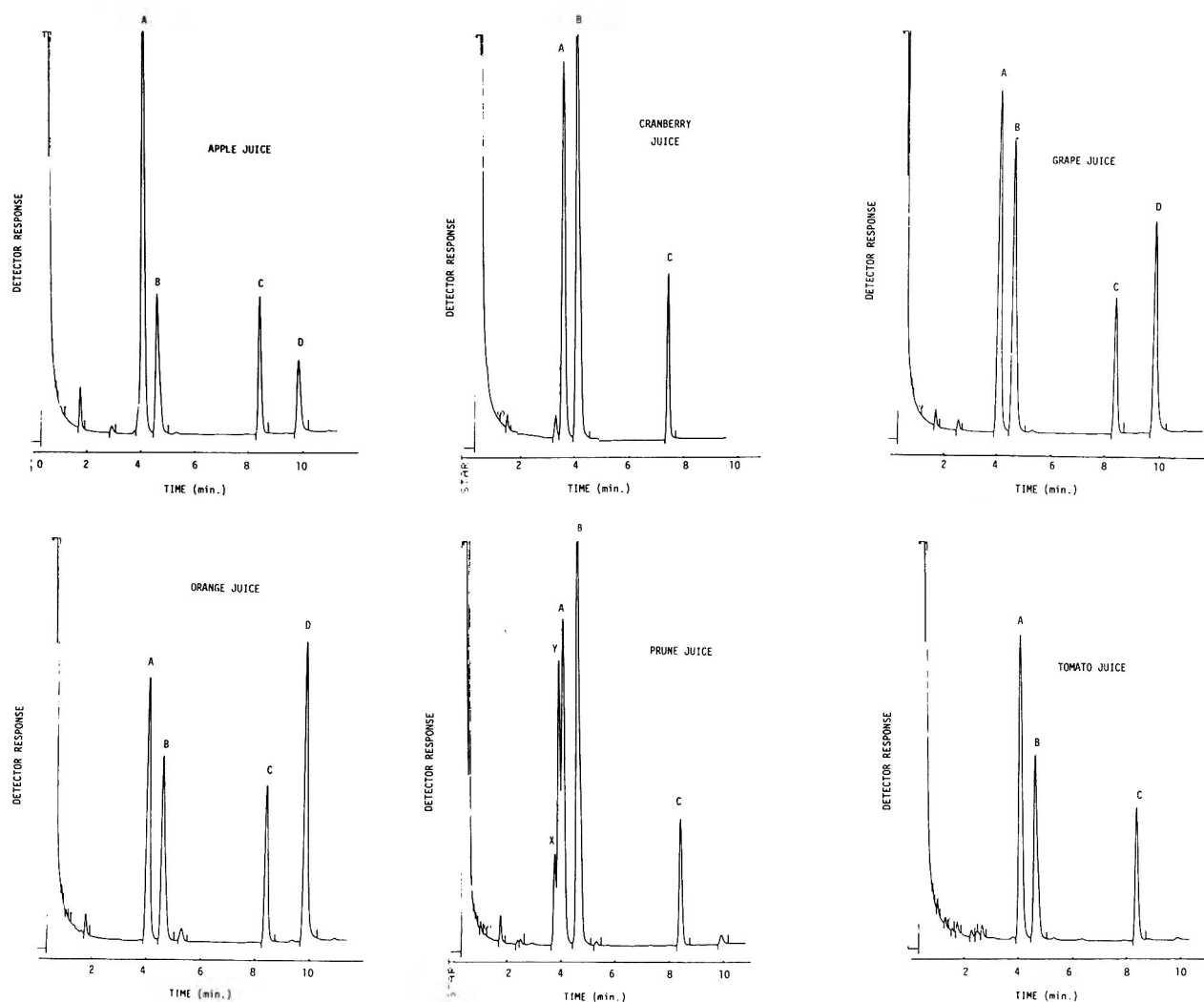
Fig. 2—Gas chromatograms of TMS derivatives of sugars in fruit juices. Column: SP-2250 on 80/100 mesh Supelcoport, 6' x 1/8" stainless steel; temperature: 170–300°C at 10°/min; flow rate: 30 ml/min, He; detection: FID. A. fructose, B. glucose, C. β-phenyl-D-glucopyranoside, D. sucrose, X. unknown, Y. sorbitol.

Table 2—Sugar content of juices—A comparison of methods

	Total sugar (g/100ml)		
Juice	TSA[a]	GLC[b]	TSA/GLC
Apple	12.40	12.10	1.02
Cranberry	13.80	13.40	1.03
Grapefruit	7.22	7.55	0.96
Lemonade	10.70	10.30	1.04
Orange	10.30	9.98	1.03
Tomato	2.50	2.48	1.01

[a] Total sugar analyzer, value expressed as total monosaccharides.
[b] Gas-liquid chromatograph; value expressed as sum of individual sugars.

Table 3—Individual sugar content of juices (GLC Analysis)

Juice	No. Samples	Fructose	Glucose	Sucrose
		(g/100 ml) ± S.D.		
Apple	6	6.39 ± 0.95	3.09 ± 1.40	1.06 ± 0.33
Lemonade	13	2.52 ± 0.89	2.75 ± 1.00	5.38 ± 2.30
Orange	16	3.03 ± 0.15	2.22 ± 0.33	5.25 ± 0.25
Tomato	12	1.68 ± 0.26	1.19 ± 0.27	N.D.[a]

[a] Not detectable

response for fructose and glucose is the same within the experimental error of the method.

A comparison of the total sugar obtained by the TSA with the sum of sugars obtained by GLC for one sample of each of six juices is shown in Table 2. Although there appears to be a bias tending to give higher values with the TSA, the agreement is generally good. The relatively high value for grapefruit juice by GLC we have found is caused by the coelution of fructose and citric acid. It is possible to separate these two derivatives on a column packed with SE-52 stationary phase, but we have not had the opportunity to recheck all the juices using this column. Both methods are subject to possible interferences from other constituents.

For example, sorbitol, a major component in prune juice, may or may not be resolved from fructose on a gas chromatograph, depending on the relative comcentration of these two compounds. While sorbitol and citric acid do not react with the color reagent in the total sugar analyzer, ascorbic acid does give a color response which is 13% that of glucose (Blakeney and Mutton, 1980). Since the level of ascorbic acid in fruit juice, as reported in the literature is 10-50 mg/100g (Paul and Southgate, 1978), the resultant color would not be measurable at the dilutions used for the sugars.

The GLC technique gives qualitative information not available with the TSA. Examples of average values for individual sugars in four juices are presented in Table 3. We also include gas chromatograms of six of the juices in Fig. 2. The chromatograms show a number of minor peaks asso-

Statistical Evaluation of Data for Detecting Adulteration of California Navel Orange Juice

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ABSTRACT

Compositional data from California early-, mid-, and late-season navel orange juice, concentrate and two-stage pulpwash were analyzed by various statistical techniques (ratios, regression, chi-square, linear combination, and discriminant analysis) for detecting adulteration. These techniques were evaluated according to applicability for detecting three types of adulteration: (1) addition of sugar and/or citric acid, (2) addition of excess or unauthorized pulpwash, and (3) addition of other constituents intended to mask the dilution. Most parameters were significantly affected by time of harvest following commercial maturity. Two-stage pulpwash showed small but significant differences from corresponding single strength juice. Product differences were of about the same magnitude as the differences due to harvest date.

INTRODUCTION

THERE IS CONSIDERABLE world-wide interest in analytical methods to detect orange juice adulteration. Recent reviews (Jorgensen, 1971; Mears and Shenton, 1973; Petrus and Vandercook, 1980) cover hundreds of papers published on the subject. Many of these included statistics for interpreting and evaluating juice content or authenticity. Many authors claimed or implied some degree of advantage for one method over those published previously. It has been virtually impossible to compare proposed procedures since most studies have been limited in parameters measured, seasons, varieties and geographical origins.

Three common types of adulteration will be considered: (1) simple dilution with sugar-water and perhaps acid, (2) adulteration with citrus by-products such as peel extracts and excess or unauthorized pulpwash, and (3) a combination of the first two and various chemicals in an attempt to mask dilution or circumvent standard authenticity tests. The second type is complicated by the fact that some

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governmental bodies (U.S. Federal) permit the addition of solids washed from the pulp of a batch of fruit to be added back to the concentrator input stream while others (Florida) prohibit the addition of any solids from pulp-washing. The pulp-washing process can have a significant effect on the quality and composition of the product. There are currently no precise standards for pulpwash.

We began a comprehensive regional study on the composition of orange juice (OJ) and pulpwash (PW). The first phase involved single strength orange juice, concentrate, and pulpwash solids (two-stage process) from early-, mid-, and late-season California navels. Samples were analyzed by 45 chemical, physical, and microbiological tests. The experimental details are reported separately (Park et al., 1983). This is an evaluation of the analytical results and the analysis of the data by various statistical treatments.

MATERIALS & METHODS

FORTY SAMPLES of California navel orange juice, concentrate, and pulpwash from early-, mid-, and late-season were analyzed for a variety of constituents. These constituents included individual compounds and group totals for sugars, acids, amino acids, minerals, phenolics, and vitamins. Details of the sampling and analytical methods for the tests, as well as means, standard deviations, and ranges are reported elsewhere (Park et al., 1983). Raw analytical data are available on request. The analysis of variance, linear and multiple regression procedures were taken from Snedecor (1956). The chi-square technique was according to Lifshitz et al. (1974). The discriminant analysis procedure was adapted from Steiner (1949) and Cooley and Lohnes (1971). Combinations and ratios of the amino acid concentrations (proposed in the literature) were calculated.

Calculations were done on a Hewlett Packard 1000 computer and an Apple II Plus computer. The programs in BASIC are available on request.

RESULTS & DISCUSSION

A SUMMARY of the analytical results including the overall means, standard deviations and coefficients of variation along with means for each season of harvest and product type are presented elsewhere (Park et al., 1983). The evaluation scheme used to subdivide the data into manageable groupings is outlined in Fig. 1. The preliminary statistical

Table 1—Correlations between selected parameters for California navel orange juice ($|r| > 0.4$ significant at 0.01).

	SO$_4$	Ca	Mg	K	Na	Ash	Alk. of ash	Total acid.	Citric acid	Isocitric acid	Arginine	Total Phenols
Calcium	0.72											
Magnesium	0.58	0.83										
Potassium	0.67	0.92	0.77									
Sodium	0.74	0.95	0.81	0.87								
Ash		0.72	0.60	0.66	0.62							
Alkal. of Ash	−0.75		−0.68	−0.72	−0.81	−0.58						
Citric acid		0.80						0.86				
Isocitric acid				0.58		0.52		0.71	0.79			
Arginine						−0.53		−0.70	−0.73	−0.75		
Formol No.	0.50	0.55				0.48						
Ascorbic			0.52	0.56	0.52	0.69		0.54	0.57	0.67	−0.80	
Total Phenols	0.72	0.80	0.68	0.65	0.76	0.56	−0.77					
UV325	0.54	0.68	0.53	0.48	0.59	0.57	−0.57					0.68
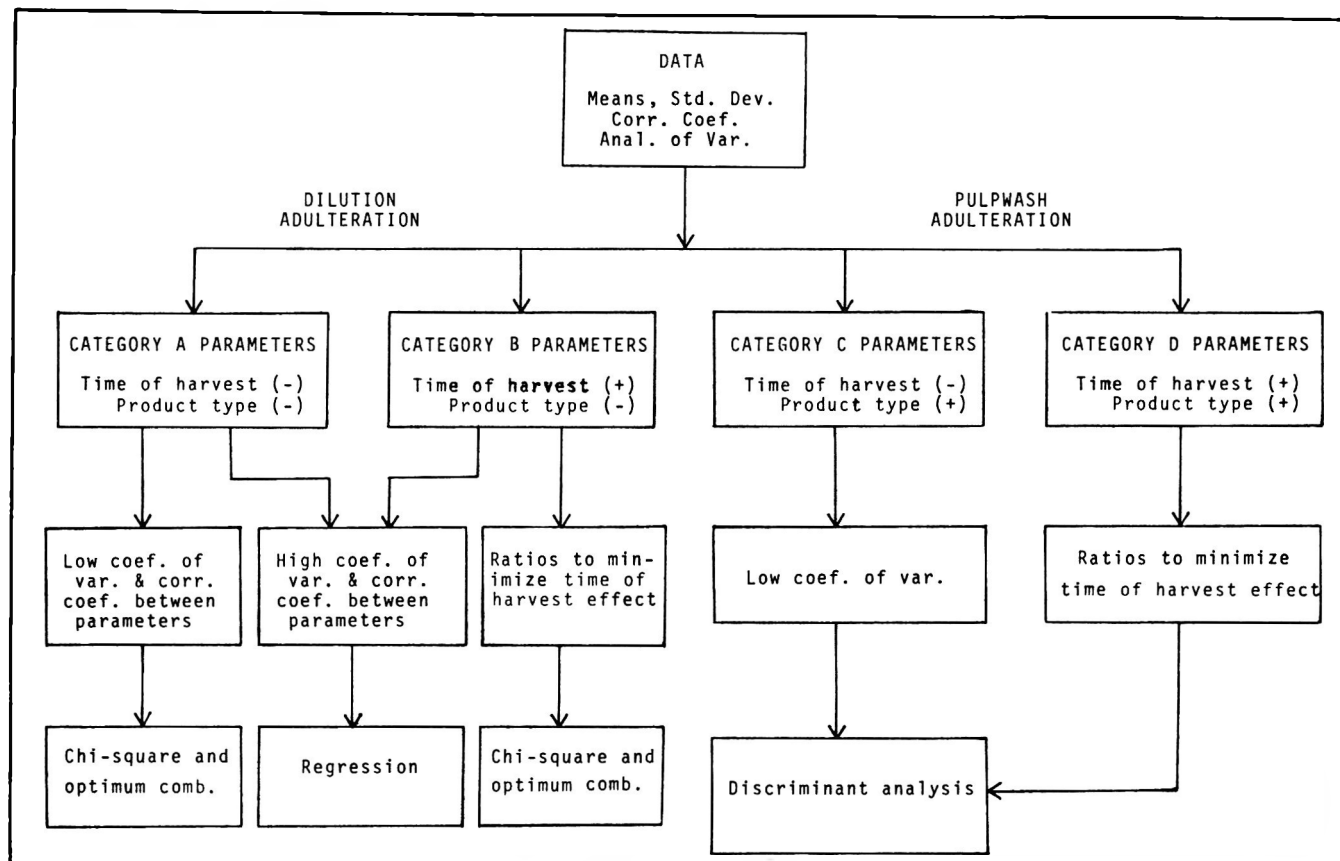
Fig. 1—Data evaluation flow diagram.

Table 2—Parameter categories according to significance (P < 0.01) of seasonal and processing effects

Significant effects due to:	A	B	C	D
Time of harvest	none	yes	none	yes
Product type	none	none	yes	yes
	Reducing sugars	Total sugars	Nitrate	Sucrose
	Thiamin	Glucose	Sulfate	Water-sol. pectin
	Malic acid	Fructose	Total phenolics	Base-sol. pectin
	β-carotene	Citric acid		UV(325) abs.
	Formol no.	Ash		UV(280) abs.
	Total amino acids	VIS(443) abs.		Davis
	Proline	Microbiological		Alkal. of ash
		Ascorbic acid		Sodium
		Isocitric		Potassium
		Total acid		Magnesium
				Calcium
				Total carotenes
				Protein

treatments of the complete data set, after adjusting to 11.8° Brix, consisted of determining the mean, standard deviation, and coefficient of variation for each parameter. The correlation coefficients between all parameters were calculated, and selected coefficients greater than 0.40 (P < 0.01) are listed in Table 1. Each parameter was placed into one of four categories based on an analysis of variance: (A) no significant (P > 0.01) effect due to either time of harvest or product type; (B) significant (P < 0.01) effect due to time of harvest only; (C) significant (P < 0.01) effect due to product type only; and (D) significant (P < 0.01) effect due to both time of harvest and product. The parameters classified by category are listed in Table 2.

The suitability of each general parameter category for adulteration type I (dilution) and type II (citrus by-product addition) are indicated in the second grouping of boxes in

Fig. 1. Parameters in category A (without any harvest time or product effects) with a small variance would seem ideal for use in detecting dilutions. Likewise, parameters in category C (with a product effect only) would be ideal for use in detecting pulpwash addition. However, most of the parameters have a strong effect due to time of harvest and fall into categories B or D (Table 2). The type of statistics used in evaluating the juice depends on the type of adulteration suspected and the analytical capabilities of the laboratory.

The chi-square analysis (Lifshitz et al., 1974), discriminant analysis (Cooley and Lohnes, 1971) and the "optimum combination" (Steiner, 1949) are best suited to parameters with relatively low coefficients of variation and a low correlation between parameters. On the other hand, regression analysis (Snedecor, 1956) requires a high correlation

between the dependent parameters and the independent parameters and a fairly wide spread of the data (high coefficient of variation). Parameters in categories B and D were combined as ratios in an attempt to minimize effects due to time of harvest. Some of the more useful ratios are listed in Table 3. The first six ratios essentially eliminated the time-of-harvest effects from the individual parameters. The last three ratios show enhanced differences due to product type. These ratios were used in a discriminant analysis along with other category C parameters to estimate the presence of excess pulpwash.

The "optimum combination" method of Steiner (1949) established a linear combination of parameters, the sum of which has a lower variance than any of the parts. All possible combinations of category A parameters, taken three

and four at a time, were tested by this method. The nine category A parameters were also tested as a group by the technique and parameters which made a negligible contribution were eliminated. A few of the best combinations are listed in Table 4. The combinations most sensitive to dilution included thiamin, isocitric acid and the formol index which have relatively low coefficients of variation.

Lifshitz et al. (1974) compared a chi-square approach for estimating adulteration in Israeli lemon juice with multiple regression for the same variables and demonstrated that chi-square was superior. This method was applied to all combinations of the variables taken three or four at a time. For the sake of comparison between the chi-square and optimum combination methods the same sets of parameters were considered. The results are shown in the last column of Table 4. Comparing the number of rejected samples of pure and adulterated juices we see that the optimum combination method was slightly more sensitive to adulteration, for these data. Of the pure samples rejected, both were PW for the optimum combination method, whereas the chi-square approach rejected mostly juices.

A multiple regression approach similar to that of Rolle and Vandercook (1963) for lemon juice and Coffin (1968) for orange juice was applied to the current data. All possible combinations were determined on three independent variables at a time with total sugars as the dependent variable. In most cases the standard deviation of Y (total sugars) independent of the X's was slightly lower than for Y alone, but none of the relationships were significant.

The data was evaluated by a discriminant analysis to detect pulpwash in orange juice. Category C parameters and selected ratios from Table 3 were used. As examples, a few of the better discriminant functions are listed in Table 5. In general, more than three variables resulted in a poorer separation of the groups. Function 1 showed a difference between the pure group means that was 12 times the standard deviation. With this function a theoretical addition of 20% pulpwash (of average composition) to the individual juice samples resulted in 9 of 15 samples falling outside a

Table 3—Ratios of parameters which minimize effects due to time of harvest and emphasize product effects

	Mean		F-Value	
Ratio	SSOJ	PW	Product	Time of harvest
Davis/protein	486	753	22**	4
Davis/sodium	70	27	40**	1.7
W-S pectin[a]/Alk. of ash[b]	250	691	18**	0.2
B-S pectin[c]/Alk. of ash	24	59	11**	2
Calcium/sodium	22	12	46**	1
Protein/magnesium	7.9×10^{-3}	5.9×10^{-3}	40**	3
UV325[d]/sodium	0.25	0.10	80**	12**
Sodium/magnesium	0.059	0.20	62**	17**
Alk. ash/sodium	2.87	0.76	131**	10**

**Significant at $P < 0.01$ level.
[a] Water-soluble pectin
[b] Alkalinity of ash
[c] Base-soluble pectin
[d] Absorbance at 325 nm (1:20 dilution)

Table 4—Comparison of optimum combination (Steiner, 1979) and chi-square (Lifshitz et al., 1974) methods of adulteration detection on selected parameters of California oranges

Optimum combination function	Opt. comb. mean and coef. of var.	No. of samples rejected out of 40 (P.05)				Chi-square coefficients[e]
		Opt. comb.		Chi-square		
		Pure	20% Adul.	Pure	20% Adul.	
$C_1 = 93.2$(Thiamin)+ 3.51(Formol) + 0.67(Citr/Isocitr.)	$237 \pm 6.75\%$	1	35	3	29	$x^2 = 3.67\, y_1^2 + 0.00323\, y_2^2$ $+ 0.000102\, y_3^2 - 0.0132\, y_1\, y_2$ $- 0.000254\, y_1\, y_3 + 0.000332\, y_2\, y_3$
$C_2 = 82.4$(Thiamin) +3.48(Formol) +0.65(Citr/Isocitr.) +1190(Microbiol/Isocitr)	$244 \pm 6.75\%$	1	36	5	27	$x^2 = 4.11\, y_1^2 + 0.000323\, y_2^2$ $+ 0.000103\, y_3^2 + 5340\, y_4^2$ $- 0.0276\, y_1 y_2 + 0.000646\, y_1\, y_3$ $- 97.3\, y_1\, y_4 + 0.000334\, y_2\, y_3$ $- 0.284\, y_2\, y_4 - 0.0983\, y_3\, y_4$

[a] Where $y_i = X_i - \mu_i$ = deviation of individual sample value (parameter i) from mean.
Parameters are the same order as in the first column.

Table 5—Discriminant analysis of naval orange juice and pulpwash

	Parameter coefficients				Means		PW — SS
	W-S pectin						
Function	Alk. Ash	Na/K	Na/Mg	No3	SS	PW	σ_{ss}
1	0.0288	—	7.09	0.0076	0.800	2.74	12.0
2	0.0313	—	7.35	—	0.82	2.69	10.9
3	0.0297	781	−28.2	—	1.28	3.85	7.17
4	0.0391	946	−34.7	−0.030	1.46	3.93	5.81

two standard deviation range. Multiple sampling of a suspected population would increase the probability of detecting differences. Most of the parameters useful in the discriminant functions are minerals which may be associated with the wash water, and accumulate in the concentration step. Thus, it could be misleading to use a pulpwash detection method which depends on variable water quality. Common manufacturing procedures in California (based on Federal standards) result in a pulpwash content of less than 10% pulpwash solids in the final concentrate. A two-stage pulpwash blended according to Federal standards should yield a finished product well within the natural range of orange juice composition.

Recently Petrus and Attaway (1980) published a spectrofluorometric and spectrophotometric method to detect PW in Florida orange juice. Fluorescence spectra from a high resolution instrument were used to qualitatively determine the presence of pulpwash while UV and visible absorbance were used to estimate the amount of pulpwash. A spectrofluorometer of the required sensitivity was not available to us; however, in this case the classification of the samples was known. The UV and visible determinations were made according to the Petrus method..The absorbance sums and ratios are listed in Table 6 along with the corresponding average values reported for Florida fruit. The relatively differences between the absorbance sums and ratios for orange juice and pulpwash were similar for both regions, but the absolute values were quite different. Furthermore, the time of harvest affected both parameters causing the calculated juice percentage (Petrus equations) in single strength orange juice to change. Based on our study

spectral data from California navel oranges appear qualitatively similar to that from Florida reported by Petrus and Attaway but so quantitatively different as to preclude direct application of the Petrus equations.

A third type of adulteration involves addition of a combination of ingredients in attempt to mask dilution. This type, which might be called a sophistication, would probably be the most difficult to detect. One approach to detecting sophistication is to analyze many constituents and establish critical concentration ranges (Beilig et al., 1977). Another is to consider concentration ratios of constituents. The total and individual amino acids have been widely used to help establish authenticity of juices (Koch, 1979; Wallrauch, 1980a).

Some proposed combinations and ratios along with current values for California navel samples are presented in Table 7. Wallrauch (1974) proposed the ratio of the formol index to proline for detecting manipulation of total amino acids. Formal index/proline ratios for navel orange juices are relatively constant and similar to those reported for laboratory prepared Greek, Italian, Spanish and Israeli oranges juices. Zamorani et al. (1973) suggested the sum of proline and arginine divided by the sum of asparagine and aspartic acid would be characteristic of the citrus fruit. They reported approximate values of 3, 1, and 1/3 (weight basis) for Italian cultivars of orange, grapefruit and lemon, respectively. (On a molar basis orange would be 2.85.) They noted an increase in this ratio as the fruit ripened. The ratios for navel PW and SSOJ were equal, and both were higher than Zamorani reported (Table 7). The "Zamorani ratios" calculated on orange juice amino acid data from

Table 6—Absorbance sums and ratios for California navel orange juice, concentrate and pulpwash and comparison with corresponding Florida values

Sample	Absorbance sums[a]	Absorbance ratio[b]
Calif. navel:		
Early Conc.	4.18 ± 0.31	0.084 ± 0.006
SS	4.68 ± 0.46	0.090 ± 0.031
PW	5.35 ± 0.98	0.046 ± 0.019
Mid Con	3.17 ± 0.14	0.122 ± 0.006
SS	3.10 ± 0.19	0.119 ± 0.025
PW	3.99 ± 0.30	0.072 ± 0.015
Late SS	4.42 ± 0.39	0.127 ± 0.027
PW	5.17 ± 0.45	0.092 ± 0.033
Florida c.		
FCOJ	2.22 ± 0.15	0.144 ± 0.026
PW	3.48 ± 0.44	0.048 ± 0.020

[a] Sum of absorbances at 443 nm, 325 nm, 280 nm (1:20 dilution with ethanol)
[b] Ratio of absorbance at 443 nm to 325 nm.
[c] Mean values from Petrus and Attaway (1980) calculated to 11.8° Brix.

Table 8—Comparison of selected amino acid ratios of California navel oranges and Brazilian oranges[a]

Denominator	Numerator			
	ASP	SER	ALA	PRO[b]
SER	1.2 (1.7)			6.5 (5.8)
ASN	0.69 (0.74)	0.6 (0.43)		4.0 (2.5)
GLU	3.1 (2.8)	2.5 (1.7)		
GLY	9.9 (11.5)		7.7 (5.2)	54 (37)
γ-AB	0.76 (0.94)		0.58 (0.42)	4.0 (3.1)
ARG	0.41 (0.52)			2.5 (1.7)

[a] Wallrauch (1980b). Brazilian values are in parentheses.
[b] Proline in the current navel study was determined with an amino acid analyzer. Brazilian values were by a manual method (Wallrauch, 1974).

Table 7—Comparisons of reported values of various amino acid combinations and ratios with values from California navel products

Combination	Values reported originally	Units	SSOJ	PW	Early	Mid	Late
Formol index/Proline[a]	19 ± 5	Pro. (g/L)	20.0	21.7	22.6	19.1	20.0
(PRO + ARG)/(ASP + ASN)[b]	2.85	mmol/L	3.29	3.40	2.02	3.93	4.08
Percent minor amino ac. (VAL, MET, ILE, LEU, TYR, PHE)[c]	SS 1.5	mol%	1.4	1.1	1.1	1.1	1.4
	PW 3.7						
Percent major amino ac. (SER, PRO, γ-AB, ARG)[d]	64–83%	mol%	70	70	64	73	73
γ-Aminobutyric acid	2.0 –5.7	mmol/L	3.0	3.1	3.1	2.6	3.5
Arginine	2.7 –5.1	mmol/L	4.8	4.8	3.4	4.8	6.5
ARG/γ-AB[e]	0.71–1.49	mmol/L	1.65	1.59	1.07	1.84	1.95

[a] Wallrauch (1974)
[b] Zamorani et al. (1973)
[c] Gherardi et al. (1976)
[d] Habegger and Sulser (1974)
[e] Vandercook and Price (1974)

Table 9—Comparison of adulteration detection sensitivities with the optimum combination (Steiner, 1949) and the chi-square (Lifshitz et al., 1974) methods using ratios of various navel orange juice parameters

Parameter sets	Coef. for opt. comb.	Opt. comb. mean and coef. of var.	Number of samples rejected out of 40 (P.05)				Coef. for chi-square[a]
			opt. comb.		chi-square		
			Pure	20% Adul.	Pure	20% Adul.	
Formol/UV$_{325}$	2.26						$\chi^2 = 0.00255y_1{}^2 + 0.248y_2{}^2$
Formol/Proline	17.3						$+ 6.34y^2{}_3 + 0.00214y_1y_2$
Glucose/Fructose	243	272 ± 6.0%	3	40	2	38	$+ 0.020y_1y_3 - 0.089y_2y_3$
Formol/UV$_{325}$	1.78						$\chi^2 = 0.00284y_1{}^2 + 9.22y_2{}^2$
Glucose/Fructose	286						$+ 1.48y_3{}^2 - 0.0381y_1y_2$
Glucose/Sucrose	−28.7	285 ± 6.2%	2	40	2	32	$+ 0.0418y_1y_3 - 4.13y_2y_3$
Formol/T. Phenolics	166						$\chi^2 = 58.5y_1{}^2 + 0.249y_2{}^2$
Formol/Proline	15.6						$+ 6.49y_3{}^2 - 0.511y_1y_2$
Glucose/Fructose	225	263 ± 6.4%	3	39	2	34	$- 6.78y_1y_3 - 0.0682y_2y_3$

[a] Where $y_i = x_i - \mu_i$ = deviation of individual sample value (parameter i) from mean. Parameters are in same order as in the corresponding parameter set in the first column.

Table 10—Linear relationships between selected parameters with a potential for detecting sophistication in orange juice

Function	Correlation
Arginine = 14.3 − 0.0178 (Ascorbic)	−0.803
Arginine = 7.75 − 0.320 (Tot. acid)	−0.700
Alkal. Ash = 17.0 − 0.0168 (Ca)	−0.797
Alkal. Ash = 16.2 − 0.172 (Na)	−0.812
Total phenols = 149 + 0.201 (Ca)	0.800
Na = −3.72 + 0.0950 (Ca)	0.950
Mg = 88.0 + 0.0822 (Ca)	0.828
K = 1420 + 2.48 (Ca)	0.925
K = −262 + 20.7 (Mg)	0.767

Niedmann (1976), Kock (1979), and Wallrauch (1980a) fell in the range 2.0–2.4 (molar). The navel data showed major seasonal effects reflecting an increase in arginine. Gherardi et al. (1976) suggested that the sum of the minor amino acids (VAL, MET, ILE, LEU, TYR, PHE) as a percentage of the total would be indicative of added pulp extract. They reported 1.5% and 3.7% for orange juice and pulpwash, respectively. Table 7 shows the difference in these percentage values between navel juice and two-stage pulpwash is small. Habegger and Sulser (1974) proposed a ratio of the sum of the major amino acids (PRO, ARG, γ-AB, SER) to the total amino acids to help establish authenticity. Table VII indicates navel juice and PW fall within the reported range. Vandercook and Price (1974) reported γ-aminobutyric acid and arginine values and their ratios for Florida FCOJ and PW and for California Valencia orange juice. In that study, Florida PW was higher in γ-aminobutyric acid and lower in arginine than FCOJ. A similar trend was reported by Neidmann (1976). The California navel orange juice in the present study did not show this trend. The seasonal increase in arginine was apparent in both studies and has been reported before (Zamorani et al., 1973; Wallrauch, 1980a).

Wallrauch (1980a) studied the amino acid composition of Brazilian orange juice as a function of season and reported a series of ratios which could be used to help establish authenticity. The corresponding ratios of the navel juices were within the ranges Wallrauch reported (Table 8).

Ratios of other potentially useful constituents were evaluated as means of detecting adulterants. For example, substances added to increase the formol number would affect ratios of the formol number and other constituents. Table 9 presents a few selected ratios and their evaluation by the "optimum combination" (Steiner, 1949) and chi-square (Lifshitz et al., 1974) statistical approaches. The number of pure and adulterated (mathematically adjusted) samples rejected out of the total sample set (40) was similar.

The ratios K:Ca and K:Mg have recently been proposed as indices of adulteration (Benk, 1980; Wallrauch, 1980b). The mean and standard deviation for California navel oranges were 12.5 ± 3.9 and 18.2 ± 2.0, respectively. Benk reported ranges for K:Ca of 8.8–27.0 and for K:Mg of 9.6–22.7. Wallrauch reported ranges of 18.4–35.4 and 15.4–21.6, respectively. Ranges that broad could only detect an extreme level of adulteration. However, there are some potentially useful correlations between minerals (Table 1). A few selected relationships, in the form of linear regression equations, are presented in Table 10. They could be useful in verifying authenticity of a sample where a few of the analytical values are outside the normal range.

CONCLUSIONS

DATA FROM THESE EXPERIMENTS, although limited, provide valuable insights into relationships between composition, time of harvest and product type. Comparisons of California navel data with published results demonstrate the general similarity among varieties of oranges grown in different regions. California navel orange juice, compared with two-stage pulpwash product, had many statistically significant, but relatively small, compositional differences. In most cases these differences were about equal or less than those due to time of harvest.

Probably the greatest benefit from these experiments will be guidance in selecting analytical parameters in future adulteration detection studies. These results make it possible to focus on parameters most suitable for a given type of suspected adulteration. Also, when one or more parameters are outisde the "normal" range these results can help determine if the discrepancy might be explained by seasonal differences.

Finally, the need for several new lines of research is indicated. For example, the composition of two-stage pulpwash in some cases is different than the corresponding single strength orange juice. The compositional changes due to multistage pulpwashing processes need to be assessed.

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A Research Note
Preference for Commercially Processed Dill Pickles in Relation to Sodium Chloride, Acid, and Texture

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ABSTRACT

Commercially processed dill pickles from 15 companies were evaluated for levels of NaCl, acetic acid, and texture by objective measurements and for preference by a consumer panel. Although differences in preference were significant, the concentrations of NaCl and acid, or the values of texture were not significantly related to these differences. The results indicated that NaCl in processed dill pickles could be reduced from present levels without affecting sensory preference for the product.

INTRODUCTION

PROCESSED DILL PICKLES are cucumbers (*Cucumis sativus* L.) that have undergone lactic acid fermentation (curing) in a salt brine and have been processed or preserved in a vinegar solution containing dill and sometimes other spice flavorings (Anonymous, 1981). U.S. Standards for "good flavor" of processed dill pickles require equilibration to at least 0.60% acid as acetic acid and $10-19°$ salometer (Almanac, 1981), which would represent $2.60-5.00\%$ NaCl at 15.6°C (ISC, 1972). In unpasteurized pickled products, minimum acid levels are critical for product preservation while minimum NaCl levels are probably more important for flavor than for preservation (Bell et al., 1972; Jones and Harper, 1952). In view of the present concern for sodium levels in foods (White, 1981), many food companies are re-appraising the concentrations of NaCl that are necessary to maintain consumer acceptance of their products.

The present study was undertaken to determine if a relationship existed between consumer preference and NaCl, acetic acid, or texture characteristics of commercially processed dill pickles.

MATERIALS & METHODS

Samples

Commercially processed whole dill pickles packed in jars ranging in size from 24–32 oz were obtained from 15 companies. A consumer panel evaluated the samples for preference and analytical methods were used to evaluate the samples for NaCl, acetic acid and texture characteristics.

Sensory evaluations

Preference was determined by use of the 9-point hedonic scalar technique (ASTM, 1972). The scale ranged from "like extremely" (9) to "neither like nor dislike" (5) to "dislike extremely" (1). A total of 315 volunteers were obtained as consumer laboratory panelists who were either students or staff members from the University of Arkansas. They consisted of anyone who liked dill pickles and were available in the laboratory on the day of testing.

A partially balanced incomplete block design was used for order and design of sample presentation (Sokal and Rohlf, 1969). Daily order of sample presentation was randomized. Two different samples of pickles were presented individually to one panelist. Each pickle sample was paired with each of the other fourteen samples, three times. It was presented as the first sample to one panelist,

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as the second sample to the next panelist, and a random order was used for the third presentation. Therefore, preference scores for each pickle sample are an average of 42 individual judgements.

All samples were served at ambient temperature. Whole pickles were cut to remove the ends and then cut in half and left in jar liquid until served. The samples were coded with a three-digit random number. All panelists were instructed as to the nature of the test prior to tasting, and instructed to clear their palate with an unsalted cracker and water between sample presentations. Panelists were seated in fully lighted isolated booths and evaluated samples in the mid-morning and mid-afternoon. Panelists rated their preference for each sample on a separate score card and included comments if they so desired.

Objective evaluations

Brine in six jars from each company was pooled for the NaCl and acetic acid evaluations. Sodium chloride concentration of the brine was determined by titration with a standardized mercuric nitrate solution (Sigma, 1976). Acetic acid in the brine was determined by titration with 0.1N NaOH and phenolphthalein indicator.

Ten whole pickles from each company were randomly selected from a composite sample of six jars for texture evaluations. Texture was evaluated with a U.C. Fruit Firmness Tester (Buescher et al., 1979). Results are an indication of the force required to penetrate the tissue and are reported as Newtons (N).

Data analysis

Preference scores were analyzed by analysis of variance and means were separated by Duncan's multiple range test (Sokal and Rohlf, 1969). Mean preference scores were correlated with values obtained for the NaCl, acetic acid, and texture evaluations.

Table 1—Relationship of preference scores[a] to sodium chloride, acetic acid, and texture measurements[b] of commercially processed dill pickles

Sample	Preference score[c]	NaCl (%)	Acetic acid (%)	Texture (N)[d]
1	6.7a	3.68	0.82	80.1
2	6.7a	3.57	0.81	71.2
3	6.4a,b	3.66	0.78	71.2
4	6.3a,b,c	2.97	0.54	71.2
5	6.1a,b,c,d	3.14	0.59	71.2
6	6.1a,b,c,d	3.69	0.71	71.2
7	6.0a,b,c,d	3.54	0.66	75.6
8	5.8a,b,c,d	3.65	0.71	71.2
9	5.8a,b,c,d	3.99	0.65	75.6
10	5.6b,c,d,e	3.06	0.65	62.3
11	5.4c,d,e,f	3.06	0.73	71.2
12	5.3c,d,e,f	3.96	0.53	48.9
13	5.3d,e,f	2.60	0.81	71.2
14	4.8e,f	3.81	0.99	66.7
15	4.6f	2.22	0.66	75.6
r[e]	1.000	0.377	0.224	0.115

[a] Each preference score is based on a mean of 42 individual judgments. Mean values followed by common letter(s) are not significantly different (p < 0.05).
[b] Value for objective measurements based on one composite sample; therefore statistical analysis among means was not appropriate.
[c] 9 = like extremely; 5 = neither like nor dislike; 1 = dislike extremely.
[d] Newtons of force required to penetrate the tissue.
[e] Correlation coefficients (r) between preference scores and NaCl, acetic acid, or texture.

A Research Note

Production and Quality of Cheddar Cheese Manufactured from Whole Milk Concentrated By Reverse Osmosis

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ABSTRACT

Pasteurized whole milk was concentrated by reverse osmosis (RO) on a pilot plant scale. The retentate was then used to produce cheddar cheese following the traditional method but using 50% less starter and 60% less rennet. The biochemical composition of the RO cheese was close to that of ordinary cheddar. The resulting non uniformity of the fresh curd as well as the granular texture of the cheese were probably due to the high lactose content of the retentate. Contamination of the milk from bacteria already present in the reverse osmosis system caused the high coliform level of the cheese.

INTRODUCTION

THE UTILIZATION of concentrated milk in cheese making (Maubois and Mocquot, 1975; Mathews et al. 1976; Jepsen 1977) began with the adoption of the M.M.V. method by Maubois and Mocquot (1971). Most of the studies involved milk concentrated by ultrafiltration. Up to now, no attempt has been made to utilize whole milk concentrated by reverse osmosis (RO) for cheese making although RO concentrated skimmed milk for ice cream manufacturing produced encouraging results (Bundgaard, 1974). This note discusses the composition and the bacteriology of RO cheddar cheese with respect to ordinary cheese made with the same original milk.

MATERIALS & METHODS

THREE MODULES type B1 Paterson Candy International (PCI) pilot unit, equipped with cellulose acetate membrane type T1/12 W were installed in a regular cheese plant in order to reflect actual operating conditions encountered in the cheese industries. Fresh whole milk pasteurized at 72°C for 15 sec was supplied by La Cooperative Agricole du Bas Saint-Laurent, Canada.

The effects of pressure, temperature and concentration on the permeate flux were investigated and the results reflected the works reported by Donnely et al. (1974), Skudder et al. (1977), Abbott et al. (1979) and De Boer and Hiddink (1980).

The milk was concentrated by a factor of 2, at 20°C and 4 MPa

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inlet pressure. The retentate was pumped into an open vat where it was submitted to the usual cheddar making sequences as published by Davis (1981) but with 50% less starter. This choice was purely arbitrary; pretests on small scale with the same retentate showed that 40% of the usual amount of rennet was necessary to obtain, in 25 min, the adequate firming of the curd.

Samples of milk and cheese were analyzed by methods of AOAC (1970) for: total solids, proteins, milk fat, ash and lactose. Total bacteria plate count, coliforms and fecal coli were evaluated by methods specified by the Canadian Health Protection Branch (1979). The Acid Degree Value (ADV) was measured by a method described by APHA (1970).

RESULTS & DISCUSSIONS

TABLE 1 shows the analysis of the milk, whey and cheddar cheese produced by the plant. It can be seen from the table that the concentration factor was identical for all milk constituents (proteins, milk fat and lactose) in the retentate (about x 2.1). The Acid Degree Value (ADV) did not increase appreciably during the membrane processing, that is, the nature of the fat did not change.

The regular plant cheddar contained 32.3% milk fat and 27.0% protein while for the case of fresh RO cheese the values are 30.6% and 26.8%, respectively. The most important difference between these two cheddar cheese resides is their lactose concentration; the lactose content of the RO cheese is above the accepted limit. This could account for the nonuniform granular texture obtained with the fresh RO cheese. The resulting defective texture could also be due to the make procedure since the basic structure of cheese is laid down earlier during the curd firming (Green et al., 1981) or to the amount of whey protein trapped in the RO cheese (Olsen and Price, 1970). As for the wheys, the RO cheese whey contains twice as much protein and nearly five times as much fat as the ordinary cheese whey.

The bacteriology of the final product is shown in Table 2. This table contains also the specific bacteria count for sanitation water, the starting milk, the concentrate and the cheese. It can be observed that there exists no real increase in the total bacteria plate count when the concentration factor is taken into account. There were no fecal coli present in the milk or in the cheese. Coliform infected the RO cheese (1.5×10^5 bacteria per gram) and lactobacilli level increased by a factor of one thousand with respect

Table 1—Composition (%w/w) of milks, cheeses, curds and wheys[a]

	Total solids	Fat	Proteins	Lactose	Ash	Acid degree value[b]
Starting milk	11.86 ± 0.06	3.68 ± 0.14	2.98 ± 0.20	3.90 ± 0.48	0.67 ± 0.04	0.66 ± 0.02
Retentate	25.62 ± 0.77	7.54 ± 0.28	6.60 ± 1.04	8.20 ± 1.50	0.92 ± 0.15	0.73 ± 0.05
RO cheese						
— curd		30.55 ± 1.43	26.80 ± 1.05	2.20 ± 0.75	4.04 ± 0.74	
— whey	15.48 ± 0.68	1.20 ± 0.21	1.66 ± 0.17	7.40 ± 1.24	1.16 ± 0.08	
Plant cheese						
— curd		32.74 ± 4.96	27.00 ± 1.97	0.63 ± 0.28	3.84 ± 0.27	
— whey	6.94 ± 1.10	0.27 ± 0.07	0.80 ± 0.21	3.60 ± 1.71	0.60 ± 0.04	

[a] Based on four samples. Pasteurized whole milks: Feed flow rate = 0.25 kg s^{-1}; Feed temperature = 20°C; Inlet pressure = 4 MPa. The curd was sampled just after pressing.
[b] Defined in the test as the amount of 1N KOH required to titrate 100g of fat.

	Mean Total Bacteria Plate count (per ml)	Lactobacilli (per gram of 20 days old chees	Coliforms (per ml)	Fecal coli (per ml)
Starting milk	4.0×10^3		< 18	< 18
Concentrated milk (C x 2)	1.1×10^5		1.3×10^3	< 18
RO cheese		2.2×10^8	1.6×10^5(a)	< 2
Plant cheese		1.2×10^5	< 1.8(a)	< 2
Sanitation water (11 days old)	4.6×10^6		1.6×10^5	45

(a): Values are given per gram of cheese

to the plant cheese. This type of bacteria was already attached inside the RO equipment (1.6×10^5 coliforms in the sanitation water). Although both the membrane side and the shroud side of the RO equipment were kept in a solution of 10 ppm chlorine after cleaning (Bell Chemical, 1981), the coliform level of the resulting RO cheese was too high to satisfy the Canadian Regulations (Elliot, 1978).

This first attempt at cheddar cheese manufacturing from RO concentrated whole milk shows that 60% less rennet than usual, is adequate, for a concentration factor of two. Lactose could be a problem but further investigations will corroborate or contradict the amount of lactose in the final curd. The quality of the curd cheese will help to determine the required amount of starter. The bacteriology of the products should be improved significantly if stricter sanitary measures are taken regarding the RO system.

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RESULTS & DISCUSSION

SENSORY EVALUATIONS indicated that preference was significantly different ($P < 0.05$) for processed dill pickles among certain companies (Table 1). Mean preference scores ranged from 4.6–6.7 while NaCl, acetic acid, and texture values varied from 2.22–3.99%, 0.53–0.99%, and 48.9–80.1N, respectively. The mean preference scores were not significantly correlated with values of NaCl, acetic acid, or texture.

Mean preference scores from 5.8–6.7 were not significantly different. This range included pickles with 2.97–3.99% NaCl, 0.54–0.82% acetic acid, and 71.2–80.1N for texture. These results indicated that a range of NaCl and acid concentrations may be suitable for obtaining the highest preference ratings and that NaCl levels may be reduced without reducing consumer preference.

Sensory preference evaluations of commercially processed dill pickles apparently are not simply related to a single factor such as salt, acid, or texture but include their combination along with the spice formulation. Reduction of NaCl in processed dill pickles to levels lower than present levels without affecting "good flavor" may be possible by altering spice formulations.

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A Research Note
Antioxidant Activity in Dried Orange

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————————ABSTRACT————————

The antioxidant properties of ground, dried orange when incorporated into ground beef were studied. The 2-thiobarbituric acid test indicated that at the 3% and 7% levels, by weight, dried orange reduced rancidity development in both frozen and unfrozen cooked meats but not in raw meats. In a second test photometric measurements showed carotene bleaching and thus oxidation to be reduced by a methanol extract of the dried orange. Compounds responsible for antioxidant activity were not identified.

INTRODUCTION

THE POTENTIAL of dried orange flour as an antioxidant merits consideration. A high percentage of orange is left in the form of pulp and peel after the juice is extracted. Some researchers are experimenting with these by-products for use as a filler in various foods. The results may be promising since the flour is palatable, high in fiber, and hydrophillic (Belshaw, 1978).

Although few researchers have considered the antioxidant properties of the orange, they contain several compounds having the potential for antioxidant activity. Flavnoid compounds occur in the albedo or the orange (Albrigo and Carter, 1977) and have been investigated as potential antioxidants (Ramsey, 1962; Pratt, 1963; McClure, 1975; Hammerschmidt and Pratt, 1978). The orange also contains organic acids such as malic, citric, and ascorbic which are capable of antioxidant activity (Chipault, 1962; Nagy, 1980 1980). Various polyphenolic acids and their derivatives which also occur in oranges (Kefford and Chandler, 1970) have been shown to have antioxidant activity (Pratt, 1963; Cofer, 1964). The purpose of this research was to determine whether dried orange could retard rancidity in the products to which it was added.

MATERIALS & METHODS

Sample preparation

Five dozen Hamlin oranges, *Citrus Sinesis* (L.) Osbeck (Swingle and Reece, 1967) were juiced, coarsely ground and dried under vacuum at 80°C for approximately 12 hr. The dried lots were finely ground (mesh #16) and all of the lots were mixed and stored at 10°C throughout the experiment which lasted approximately 3 months. Meat loaves were prepared by combining 50g ground beef and 0.5g salt with 3% or 7% dried orange by weight. Some samples were baked immediately in foil-lined loaf pans at 217°C for 15 min. The samples to be tested uncooked were wrapped in foil and stored in the refrigerator at 10°C or the freezer at −15°C. Unfrozen samples were held at −15°C for 3 or 6 wk and then thawed in the refrigerator for 24 hr. After thawing, the samples were held in the refrigerator an additional 24 hr prior to the 1-day test and then five additional days prior to the 6-day test. A total of six loaves was used for one test run. The entire procedure was carried out three times.

Chemical analyses

Ten-gram samples were removed from each loaf for the 2-thiobarbituric acid (TBA) test (Tarladgis et al., 1960). A second test for

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antioxidant activity was carotene bleaching in a linoleic acid-carotene emulsion (Hammerschmidt and Pratt, 1978). Methanol-orange extracts were prepared by soaking 40g of dried, ground orange in 200 ml of methanol overnight. The extract was boiled 5 min and the filtrate was evaporated on a rotary evaporater at 40°C to a final volume of 10 ml. The orange-methanol extract was held at −20°C. Prior to testing, 10 ml of methanol were added to the extract and the mixture was refiltered through Whatman #42 paper. The emulsion on which the carotene-methanol extract was tested was prepared by dissolving approximately 1 mg carotene in 10 ml chloroform. One ml of the carotene-chloroform solution was added to 20 mg purified linoleic acid and 200 mg Tween 40 in a boiling flask. A roatry evaporator at 50°C removed the chloroform and 50 ml of distilled water were swirled into the mixture to form an emulsion. Five ml of the emulsion were combined with 0.2 ml of the orange-methanol extract. The concentration of the carotene and the orange flour was 0.01 mg and 0.4g respectively in 5 ml of the emulsion. The control sample contained 0.2 ml methanol. The emulsion mixture minus carotene served as a blank for spectrophotometric readings at 470 nm. Stoppered tubes were placed in a 50°C water bath and readings were taken at 15-min intervals for 120 min. Antioxidant activity was represented as percentage or original carotene remaining with time. Statistically significant differences were determined by analysis of variance using a randomized block design.

RESULTS & DISCUSSION

THE ANTIOXIDANT ACTIVITY of dried orange in raw and cooked unfrozen ground beef is shown in Fig. 1. The raw samples exhibited no significant differences in TBA number when compared with the control; however, in cooked samples the addition of dried orange at 3% and 7% significantly reduced the TBA numbers at 3 and 6 days of storage. There was no significant difference between 3% and 7% levels.

A similar trend was seen in samples which were frozen and then held in refrigerated storage. Dried orange was ineffective in producing significant differences in TBA numbers in uncooked frozen samples. In the cooked samples dried orange did not produce significantly different TBA values at the 0 time or after frozen storage for 3 or 6 wk duration. However, thawing for 24 hr and storing in the refrigerator for 6 days resulted in significantly lower TBA numbers in the samples containing dried orange at 3% or 7% levels. The difference between 3% and 7% orange again was not significant.

Fig. 2 shows the effect of the methanol extract of dried orange in the linoleic acid-carotene emulsion. The methanol control was bleached rapidly, representing oxidation. On the other hand, the sample with the orange lost less than 10% of the carotene through oxidation during the entire 2-hr period.

From the results of this study it appears that dried orange has antioxidant activity in both a meat system and a carotene-linoleic acid system. The finding in the present study of greater oxidation in cooked meat than raw is in agreement with a recent study by Cross et al. (1979) in which they found cooked beef patties consistently had higher TBA numbers than uncooked. The length of time for frozen storage did not affect the oxidation of the samples. It is possible that the freezing period was not long enough for any changes to occur. Since there was no significant difference in effectiveness as an antioxidant between levels
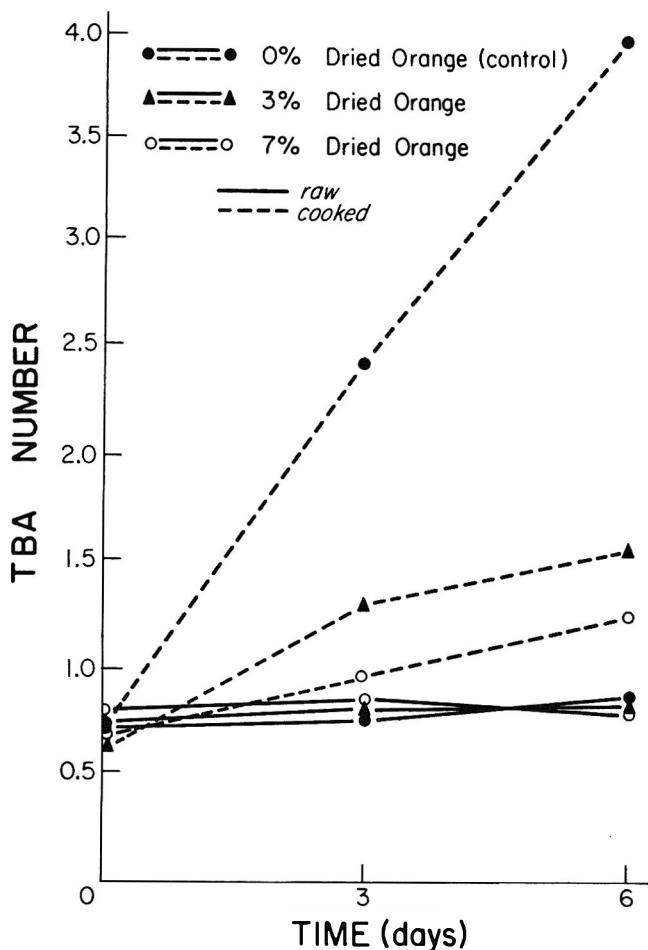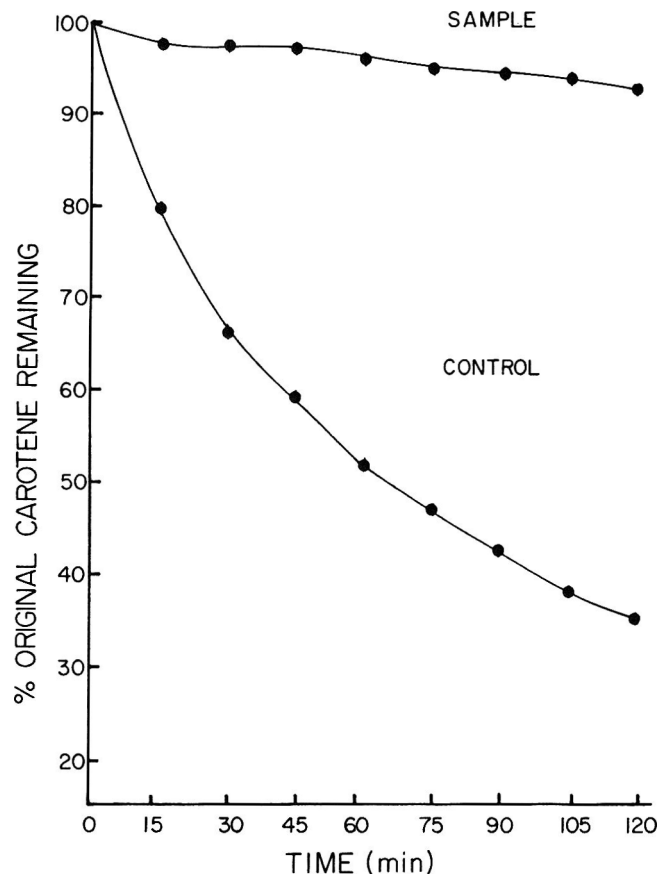
Fig. 1—TBA numbers in unfrozen ground beef.

Fig. 2—Antioxidant activity measured by bleaching of a linoleic acid-carotene emulsion.

of 3% and 7% dried orange, it is possible that a lower level could be as effective as the ones tested.

There are many possible compounds which could contribute to the antioxidant activity of dried orange. Further research is needed to isolate the compounds responsible and to determine the individual contribution of each one to the antioxidant effect as a whole.

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A Research Note
Debittering Citrus Juices with β-Cyclodextrin Polymer

P. E. SHAW and C. W. WILSON, III

──────────ABSTRACT──────────

A β-cyclodextrin polymer was used to remove the bitter substances limonin and naringin from aqueous solution and from filtered orange and grapefruit juices using batch and continuous flow column treatments. Other components such as naringenin 7β-rutinoside, coumarins and flavonoids were removed, but the total soluble solids (°Brix), total acidity and ascorbic acid content of the juice were unchanged. The polymer could be regenerated by extraction with an organic solvent.

INTRODUCTION

SOME NAVEL ORANGE and grapefruit juices have high levels of the bitter substances limonin or naringin (in grapefruit) which adversely affect the juice quality. A simple, inexpensive way to decrease levels of these substances in juices is needed to make them more acceptable to consumers.

Reduction in limonin content in orange juice was achieved by adsorption on cellulose acetate gels (Chandler and Johnson, 1974), by use of immobilized enzymes to convert the limonin to nonbitter compounds (Maier et al., 1977), and by several other methods summarized by Maier et al. (1977). Konno et al. (1981, 1982) reported a decrease in bitter flavor when they added β-cyclodextrin to Iyo orange, grapefruit and citrus natsudaidai juices. However, they presented no analytical evidence to indicate decreased limonin or naringin content in the treated juices.

Solms and Egli (1965) used β-cyclodextin and epichlorohydrin to prepare cyclodextrin polymers. These polymers form inclusion complexes with many organic compounds because the "doughnut" shape of the monomeric cyclodextrin unit permits inclusion in the open "hole" if the organic molecule will fit (Hirotsu et al., 1982). Such polymers have potential to selectively remove specific components from foods, but their use for this purpose has not been previously reported.

We report use of the insoluble β-cyclodextrin polymer described above to remove naringin and limonin from grapefruit juice and limonin from navel orange juice by a procedure that permits regeneration of the polymer.

MATERIALS & METHODS

Preparation of samples

Canned grapefruit juice and fresh Florida navel oranges were purchased from a local market. The juice from nine oranges (990 ml) was first heated to 80°C to convert all limonin precursor to limonin. Both the grapefruit and orange juices were clarified by centrifugation at 9000 rpm (Sorvall GSA rotor) for 30 min followed by filtration of the supernatant through a 0.45 μm Millipore filter in order to permit analysis by high-performance liquid chromatography (HPLC) and to prevent plugging of the gravity flow columns. Solutions of naringin (naringenin 7β-neohesperidoside) and naringenin 7β-rutinoside were prepared by dissolving the crystalline com-

pounds in water. Limonin solutions were prepared by dissolving limonin crystals (8 mg) in 2 ml of acetonitrile and diluting the solution to 1000 ml for the debittering experiments or in absolute ethanol (0.1 μg/10 μl) for thin-layer chromatography (TLC).

Preparation of β-cyclodextrin polymer

β-Cyclodextrin (25g, Sigma Chemical Co., St. Louis, MO) was polymerized using the procedure of Solms and Egli (1965). The polymer (36 g) was ground in a micro Wiley mill fitted with a 40-mesh screen. The fines were then removed by twice stirring the ground polymer with 200 ml portions of water for 15 min and decanting the cloudy supernatant after settling. The residue was stirred with 200 ml of acetone, filtered and dried in air to give 34g of polymeric resin.

Analytical methods

HPLC was performed with a Perkin-Elmer Model Series 2 pump and Model LC–85 variable-wavelength detector, a Hewlett-Packard 3390A integrator, using a 6 μl or 20 μl loop, and a Perkin-Elmer HS-5 C18 (5 μm) column (15 cm) with a Brownlee RP-18 (5 μm) guard column (4 cm). For studies on aqueous solutions, limonin, naringin and naringenin 7β-rutinoside were determined using a mobile phase of acetonitrile-water (75:25 v/v) at 207 nm and a flow rate of 1.0 ml/min. Naringin and naringenin 7β-rutinoside in filtered grapefruit juice were determined on the column at 280 nm by the procedure of Fisher and Wheaton (1976). Limonin in filtered juice was determined by the TLC procedure of Tatum and Berry (1973). Ascorbic acid in filtered orange and grapefruit juices was quantified by diluting each sample 1:1 with 12% metaphosphoric acid using the column at 245 nm following the procedure of Shaw and Wilson, 1982.

Batch and column debittering procedures

For the batch procedure, 1g of β-cyclodextrin monomer or polymer was added to 55 ml of filtered juice or aqueous standard solution and the resulting mixture was stirred magnetically for 60 min. The polymer was removed on a coarse sintered glass Buchner funnel under reduced pressure. For regeneration, the polymer was resuspended in 25 ml of organic solvent, stirred 30 min, and filtered. In the column procedure, 1g of polymer was packed with water in a glass column 18 mm in diameter and 90 ml of filtered juice or aqueous solution was passed through at a rate of 3–5 ml/min. Fractions (10 ml each) were collected and the column was treated with 25 ml of organic solvent (5 ml/min) to regenerate the column by eluting the retained components. Further extraction of the polymer afforded little additional removal of retained compounds. HPLC or TLC procedures determined levels of limonin, naringin and naringenin 7β-rutinoside in the fractions.

RESULTS & DISCUSSION

β-CYCLODEXTRIN POLYMER was effective in removing the bitter compounds limonin and naringin from aqueous solutions and in selectively removing them from filtered citrus juices by batch and continuous flow processes (Table 1). In the batch process, 1g of polymer reduced limonin in 55 ml of aqueous standard solution from 8 to 2 ppm in 15 min. In filtered navel orange juice limonin was reduced from 8 to 4 ppm in the same time. Similar treatment of grapefruit juice reduced limonin from 3 to 1 ppm. Naringin content in aqueous solution decreased from 436 to 161 ppm in 15 min, and in filtered grapefruit juice from 432 to 207 ppm. Nonbitter naringenin 7β-rutinoside was removed from solution at a faster rate than limonin or narin-

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Table 1—Reduction in bitter components in juices and model systems by treatment with β-cyclodextrin polymer

Sample	Batch Concn. in ppm at (min):			Column Concn. in ppm in fraction no.:									
	S[a]	15	60	S[a]	1	2	3	4	5	6	7	8	9
Limonin in:													
Water	8	2	2	8	0.4	0.4	0.4	0.5	0.5	0.7	0.9	1.2	2.3
Navel orange juice	8	4	4	8	N[b]	N	1	2	3	4	4	5	6
Grapefruit juice	3	–	1	3	N	N	N	N	1	1	1	1.5	2
Naringin in:													
Water	436	161	135	400	19	27	40	52	67	82	123	175	218
Grapefruit juice A[c]	432	207	216	432	30	117	160	238	268	346	333	359	359
Grapefruit juice B[d]	432	294	324										
Naringenin 7β-rutinoside in:													
Grapefruit juice A[c]	164	79	82	164	7	18	30	44	56	75	75	82	82
Grapefruit juice B[d]	164	69	77										

[a] S = concentration at start of experiment.
[b] N = not detected by TLC (< 1 ppm).
[c] Fresh polymer used.
[d] Regenerated polymer used.

gin in the grapefruit juice sample. The capacity of the resin to remove these substances appeared to be reached in 15 min, since no appreciable further decrease was noted up to 60 min. When an equivalent amount of the monomer was used in the aqueous solutions, no measureable decrease in limonin or naringin content was seen after 60 min.

The polymer used in the batch process was regenerated by an organic solvent wash. Naringin was extracted with methanol and limonin with ethanol or acetonitrile. Greater than 78% of the adsorbed bitter components were removed by this procedure except limonin from navel orange juice, where 50% was removed. Regenerated polymer was used to debitter a second batch of grapefruit juice (sample B in Table 1). The regenerated polymer was less effective in removing naringin, but was equally effective in removing naringenin 7β-rutinoside.

In continuous flow column studies (Table 1), β-cyclo-dextrin polymer was highly effective initially in removing both limonin and naringin from aqueous solutions and from citrus juices. However, the polymer gradually lost its capacity to remove these compounds. The column could be regenerated by elution with 25-ml portions of ethanol or acetonitrile for limonin and methanol for naringin (61–78% removal). Both substances could also be removed from the polymer by elution with water, but relatively large volumes (>160 ml) were required, probably because of their low solubilities in water.

Citrus juices contain many compounds that may compete with the bitter compounds for inclusion in the polymer and reduce the capacity of the polymer to remove limonin and naringin. Flavonoids (in navel orange) and coumarins (in grapefruit) were observed on TLC plates when the procedure of Tatum and Berry (1973) was used to determine the amounts of limonin released during regeneration. However, the more desirable components of the juice, e.g., sugars, organic acids, vitamin C, which must be retained for

a debittering process to have significant potential, did not appear to be affected by the polymer treatment. Total soluble solids (°Brix), titratable acidity and ascorbic acid content remained unchanged. Since the polymer was insoluble in water, acetone, methylene chloride, methanol, tetrahydrofuran and dimethylformamide, it probably did not add anything to the juice.

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Raoult's Law, Water Activity and Moisture Availability in Solutions

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————————ABSTRACT————————

A rationalization of Raoult's law has led to the conclusion that water activity (a_W) is a joint solution property of vapor pressure, solute and solvent concentrations. It is shown from this that a_W is not a measure of the absolute value of the mole fraction of water as indicated by Raoult's law but a measure of only a fraction of the mole fraction of water remaining free in solution available and unbound to solute molecules. The law is shown to overestimate this water activity (a_W) at all dilutions by an amount equal to the product of the mole fraction of solute and the lowered relative vapor pressure the solute generates in solution.

INTRODUCTION

A PRIMARY CONCERN in a newly formulated food is to prevent the growth of microorganisms and one practical way to do this is to control the availability of moisture which is believed to be measured by its water activity.

A method often used to estimate these water activities is the Raoult's law equation for ideal solutions empirically put forward as a result of experimental measurements. This law is suggested (Ross, 1975, Bone et al., 1975; Bone, 1973) for calculating, for example, the water activities of simple binary solutions for use in the Ross (1975) equation where literature values of water activity for certain food ingredient concentrations are lacking.

But the equation is only applicable to dilute solutions and its use in the investigation of practical problems at concentrations of most interest in the food industry is limited (Money and Born, 1951). The usefulness of this law in the food industry may, no doubt, be considerably enhanced if it could apply at lower water activities. Ideally Raoult's law should apply over the full range of pressures and its limited range of application supports the view (Fried, Hameka and Blukis, 1977) that it does not define ideal or dilute solutions exactly. It is the purpose of this note to suggest a rationalization of the Raoult's law equation to define more accurately available moisture or a_W in solutions and in foods.

MATERIALS & METHODS

AS MOLECULES are dissolved into a pure water solution they bind water molecules around them in a hydration sphere (Labuza, 1975). Eventually enough of the molecules may be bound to affect the equilibrium evaporation rate of water at the air liquid interface. If measured it is found that the vapor pressure is decreased. This decrease is generally believed to be described by Raoult's law equation for ideal solutions which empirically states that

$$p/p_0 = a_W = \frac{55.5}{55.5 + m_s} \tag{1}$$

where p_0 = saturated vapor pressure of water molecules; p = partial vapor pressure of the solution measured at the same temperature as p_0; a_W = water activity; m_s = the molal concentration of a kg of water. Ideally this equation should apply over the entire range of

pressures when a plot of estimated a_W vs m_s is made but Fig. 1 shows that the law is only obeyed at low solute concentrations; at higher solute concentrations the law breaks down and the curve deviates more and more strongly in the direction of higher water activities or low concentrations. In other words the mole fraction of water which from Eq. (1) equals the water activity of an ideal solution is overestimated at high solute concentrations.

If the mole fraction of water is now expressed in terms of the mole fraction of solute, Eq. (1) becomes:

$$a_W = 1 - \frac{m_s}{m_s + 55.5} \tag{2}$$

If from Eq. (1) the mole fraction of water overestimates the water activity of an ideal solution at high solute concentrations, then the second term on the right hand side of Eq. (2) which represents the mole fraction of solute molecules in the solution must be underestimated at high solute concentrations. It may be observed of the latter fraction that the presence of an m_s term in the denominator results in a smaller fraction than in its absence. It is to be expected therefore that by dropping this term from the denominator of the mole fraction of solute a desired diminution in the calculated value of the water activity at high solute concentrations should result. Dropping this term simplifies Eq. (2) to:

$$a_W = 1 - \frac{m_s}{55.5} \tag{3}$$

It is usual in physical chemistry books for Eq. (3) to be derived, again from Eq. (2), but on the basis that in very dilute solutions the magnitude of m_s is small compared with 55.5. If this latter derivation is correct, a plot of estimated a_W from Eq. (3) vs its corresponding m_s should give a straight line limited in range to dilute solutions corresponding to high water activities but as may be observed (Fig 1, b) the plot applies over both dilute and concentrated solutions which may cover the entire range of pressures in agreement with our assumptions.

The amount of overestimation of the water activity of a solution calculated with Raoult's law Eq. (1) over that calculated with the new Eq. (3) amounts to

$$(\frac{m_s}{m_s + 55.5}) (\frac{m_s}{55.5}).$$

But since from Eq. (3) $m_s/55.5 = 1 - a_W$ the above overestimation equals the product of the mole fraction of solute and the lowered relative vapor pressure of the solution or

$$\frac{m_s}{m_s + 55.5}(1 - a_W).$$

Hence expressed in mole fractions, Eq. (3) is equivalent to:

$$a_W = \frac{55.5}{m_s + 55.5} - \frac{m_s}{m_s + 55.5}(1 - a_W) \tag{4}$$

Eq. (4) explains that a_W of a solution is approximately equal to Raoult's prediction only when the parameter is close to unity. It also indicates that at all solution concentrations a solute reduces the mole fraction of water by an amount equal to the product of its mole fraction and the lowered relative vapor pressure it generates in solution. Eq. (4) thus clearly indicates that a_W measures that fraction of the mole fraction of water not balanced by a corresponding mole fraction of solute molecules. In other words, a_W measures available mole fraction of water in solution. The a_W of pure water ($m_s = 0$) from the above equation is unity while when m_s is as high as that of the solvent water ($m_s = 55.5$) the available mole fraction of water and therefore the a_W of the solution is zero.

But in solutions containing 55.5 moles of solute and therefore having zero a_W, the component weights of solute and therefore the

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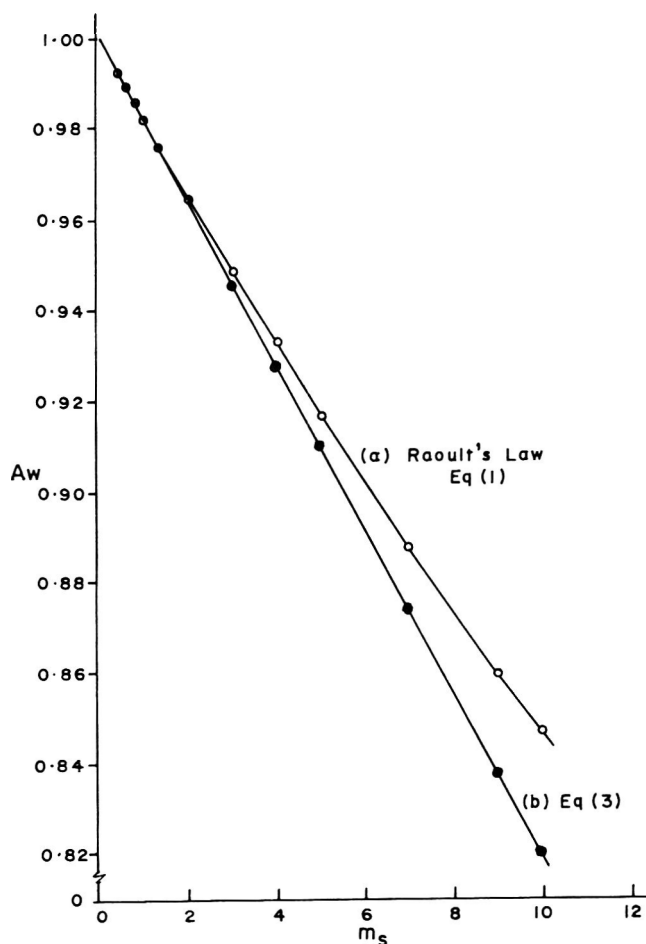
Fig. 1—Deviation of Raoult's law from the ideal plot given by Eq. (3).

than with percent moisture content of the solution. The use of the term "water activity" as a measure of available moisture in solutions and in foods therefore appears to be justified contrary to reservations of Mossel (1975) and Tracy (1975). These observations support those made by Scott (1953) and Christian (1981) that even though percent water content of a given solution or food may be high, not all of it may be available for microbial growth. Microorganisms therefore exhibit growth optima at very high a_w in response to diminishing available mole fraction of water or a_w than to diminishing moisture content.

In conclusion, Raoult's law is shown to apply approximately at very high a_w or very low solute concentrations. At all concentrations a_w is shown to be a joint solution property of vapor pressure, solute and solvent concentrations. It is not defined as an absolute measure of the mole fraction of water indicated by Raoult's law but as a measure of a fraction of the mole fraction of water remaining free in solution, available and unbound to solute molecules. Its effects influence at various levels the physical, chemical and biological properties of systems reacting to it as is illustrated by Labuza's (1970) food stability map. As a measure of available moisture we agree from this discussion with Van den Berg and Bruin (1981) that a_w does not act solely through the gas phase as vapor pressure and that its practical effects cannot be separated from the nature of the substances adjusting it.

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moisture contents of the solutions may be different due to differences in molecular weights of solutes. Thus, for example, solutions of NaCl and sucrose containing 55.5 moles of solute and therefore having zero a_w may be calculated to contain 24% and 5% water (wet basis), respectively. These calculations indicate that water activity correlates better with residual or available mole fraction of water

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A Research Note
Effect of Grain Size on Degree of Milling, Color and Cooking Time of Sorghum

R. B. H. WILLS and M. R. ALI

—————————ABSTRACT—————————

Sixteen colored and white hybrid sorghum cultivars were size-graded and the three major sizes, 4.00–4.74 mm, 3.35–3.99 mm and 2.80–3.34 mm, dehulled for 60 sec. Within a cultivar the degree of milling (loss of unbroken kernel during milling) varied between individual sizes and was lower for the smaller grain. The pericarp content increased 2.80 > 4.00 > 3.35 mm+ grain. The color of ground pearled grain of 4.00 mm+ and 3.35 mm+ sizes was similar to that of white cultivars but 2.80 mm+ grain had a higher color absorption due to some residual pericarp. The cooking time ranged from 54 min to 64 min and was shorter for the smaller grain.

INTRODUCTION

SORGHUM GRAIN is composed of different layers which have been described elaborately by Rooney (1973), Sullins and Rooney (1975), Hulse et al. (1980) and Hosney et al. (1974). Some important factors are that the pericarp and testa of the grain contain the coloring matter and tannin, the aleurone layers have high quality proteins and vitamins and the peripheral endosperm contains the highest concentration of proteins. Abrasive dehulling employs a rough surface to progressively remove the surface layer of the grain and is used to remove the pigmented layers. Ali and Wills (1980) showed that cooking time of whole grain was influenced by the degree of milling while Desikachar (1974) claimed that the presence of peripheral endosperm on the cooked grain was poorly accepted. Hence the degree of milling is the important determinant of eating quality. Wills and Ali (1982) showed that grain size affects the milling properties and hence the recovery of the unbroken grain. In this paper we examined a range of sorghum cultivars to determine whether grain size affected the milling properties, and appearance and cooking time of the milled grain.

MATERIALS & METHODS

THIRTEEN COMMERCIAL hybrids and three experimental hybrids were obtained from sorghum breeding research stations and graded into three sizes: 4.00–4.79 mm (designated as 4.00 mm+), 3.35–3.99 mm (3.35 mm+) and 2.80–3.34 mm (2.80 mm+). Average weight of grain and pericarp content was measured according to Ali and Wills (1980). Five grams of each of these sizes were dehulled for 60 sec in a Kett Electric Pearler. The unbroken grain with the germ still attached were separated and the loss of weight was termed as scouring and is used as the measure of degree of milling. The cooking time of the milled grain was determined according to the method of Ali and Wills (1980). The unbroken kernels were ground in a Krupp coffee grinder and the reflectance absorbance was measured in a Beckman Reflectance Spectrophotometer at 450 nm.

RESULTS & DISCUSSION

THE PERICARP CONTENT of the unmilled grain of 16 cultivars is given in Table 1. Grain of 2.80 mm+ contained the highest pericarp content while 4.00 mm+ grain had more pericarp than 3.35 mm+ grain. While 2.80 mm+ grain had the thickest pericarp, the degree of milling of this sized

grain was the lowest. Degree of milling of different sized grain of 16 cultivars that had been milled 60 sec is also presented in Table 1. The difference in degree of scouring of 4.00 mm+, 3.35 mm+ and 2.80 mm+ varied from 8.6 to 11.8%, 6.7 to 12.7%, and 4.5 to 10.8%, respectively, between the cultivars. Within the same cultivar the degree of milling was lowest in the 2.80 mm+ grain and was generally higher in 4.00 mm+ grain than in 3.35 mm+ grain. The aim of milling is to remove only the bran layers with minimum loss of endosperm and the time required for 2.80 mm+ grain to be milled to the ideal stage is therefore longer than that required for other sized grain. Overall, 4.00 mm+ grain had a higher pericarp content than 3.35 mm+ grain but also tended to have a faster rate of milling although individual cultivars varied somewhat from the mean. Since all the sorghum grain examined contained a range of grain size it would seem that no batch of sorghum will be milled satisfactorily unless the grain is first size-graded and the individual size milled separately for different times. If the whole grain is milled together for a fixed time there will be a percentage of either undermilled grain with not all the pericarp removed or overmilled grain with the loss of saleable endosperm.

The effect of undermilling of small grain is seen in the color reflectance measurements (Table 2). The unmilled color grain gave reflectance values of 0.32–0.35 units. The values for dehulled grain of 4.00 mm+ and 3.35 mm+ size ranged from 0.18–0.23 which were similar to the values obtained for the white cultivars after milling. However, the 2.80 mm+ grain produced higher readings which ranged from 0.20–0.27, indicating the retention of some original pigment.

The milled grain of six cultivars (YN332, K21621, K21637, NK280, Dorado and SM8) was cooked and the cooking time for each cultivar was 4.00 mm+ > 3.35 mm+ > 2.80 mm+ and averaged 63 min, 58 min and 55 min,

Table 1—Pericarp content and degree of milling (d.m.) of different sizes of sorghum cultivars (g/100g)

Cultivar	4.00 mm+		3.35 mm+		2.80 mm+	
	Pericarp	d.m.	Pericarp	d.m.	Pericarp	d.m.
YN332	8.8	11.2	9.6	11.5	10.1	10.0
K21621	6.8	8.8	5.5	6.7	8.0	6.3
K21637	6.4	10.4	5.9	8.3	7.7	7.6
NK280	9.4	11.8	7.6	11.4	12.4	10.8
Dorado	7.0	11.6	6.3	8.5	10.9	6.1
SM10	7.8	8.6	6.6	9.9	8.9	5.9
Leader	6.4	10.1	6.1	8.2	9.3	7.3
Goldrush	7.9	11.1	6.5	10.7	9.1	7.4
C43	8.0	9.9	7.6	11.6	8.8	7.8
F64A	7.9	11.1	6.8	12.1	9.9	7.1
5161	—[a]	—	6.0	9.1	9.8	6.2
SM8	—	—	6.3	9.6	10.4	8.4
Sunlover	—	—	6.3	9.2	8.9	7.1
C42T	—	—	6.0	11.1	7.8	4.5
Pacific 001	—	—	9.0	12.7	11.4	6.5
Texas610SR	—	—	8.2	11.0	10.2	10.5

[a] Negligible grain of this size present

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Cultivar	Grain color	Color					
		4.00 mm+		3.35 mm+		2.80 mm+	
		Undehulled	Dehulled	Undehulled	Dehulled	Undehulled	Dehulled
YN332	White (pearly)	0.25	0.19	0.25	0.19	0.25	0.21
K21621	White (dull)	0.28	0.20	0.28	0.19	0.28	0.21
K21637	Red (dark)	0.35	0.21	0.33	0.23	0.37	0.27
NK280	Bronze	0.33	0.22	0.33	0.23	0.32	0.25
Dorado	Bronze	0.32	0.20	0.32	0.21	0.32	0.27
F64A	Brpmze	0.32	0.18	0.32	0.17	0.32	0.20
SM8	Bronze	—	—	0.32	0.19	0.32	0.25

respectively. Thus if sorghum grain is not size graded before milling and cooking, the use of constant cooking time will result in the undermilled 2.80 mm+ grain being overcooked before the bigger grain is cooked to an acceptable texture. In addition, some of the 2.80 mm+ grain burst during cooking; an effect attributed by Desikachar (1974) to the grain being undermilled.

The efficient use of sorghum grain for human food will currently necessitate the segregation into different sizes which will be dehulled for different times. The use of white grain can solve the color problem but the main purpose of dehulling is to remove the pericarp which, besides coloring materials contains tannin, wax, etc. (Chibber et al., 1978). Cultivar breeding programs have been efficient in increasing the yield but little attention has been paid to the quality of the grain. The development of cultivars with a uniform grain size would allow sorghum to be more readily used for human food.

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A Research Note
Color Changes in Blue Crabs *(Callinectes sapidus)* During Cooking

B. H. HIMELBLOOM, J. E. RUTLEDGE, and S. L. BIEDE

——————————ABSTRACT——————————

The change from the blue-green color to the red-orange color of Blue crabs was monitored during cooking using a Hunterlab Color Difference Meter set in the L, a, b scale. Changes in the "a" readings were correlated with the changes in internal body temperature. A cooking temperature of 100°C resulted in maximum red-orange color development after 30 sec with no significant changes occurring subsequently. The internal body temperature of the crabs was shown to be only slightly above the initial temperature when maximum color development occurred. The results of this study show that color changes in Blue crab do not serve as an adequate index of proper cooking.

INTRODUCTION

ASTAXANTHIN, a carotenoid, is responsible for the pigmentation of crabs and other shellfish (Fox, 1976). The blue-green color is produced by an astaxanthin-protein linkage; thus, when heat is applied to the crab, the protein is denatured and the familiar red-orange color appears (Fox, 1976). Many consumers mistake the change from the raw, blue-green color to the red-orange color of the crabs as an indicator of adequate cooking. One seafood cookbook for example, recommends cooking crabs until they turn red (Stanforth, 1969). This could result in consumer health problems from improper cooking, if time-temperature relationships are not adequate.

The objective of this study was to evaluate the influence of various water temperatures and time periods on the color changes which take place in Blue crabs during cooking.

MATERIALS & METHODS

BLUE CRABS *(Callinectes sapidus)* were commercially harvested from Vermillion Bay, off the Louisiana coast during April and May, 1979. The live crabs were delivered to the Louisiana State Univ., Food Science Dept. and placed in a refrigerated cooler (4°C) for at least 3 hr to allow for ease of handling.

Shell color measurements

Crabs were divided into groups of six and individually subjected to water temperatures of 76.6, 82.2, 87.8, 93.3, and 100°C for time periods of 0.5, 1, 2, 4, 8, and 16 min. A total of 180 crabs were used for the 30 treatments.

Before cooking, the color of each crab was measured with a Hunterlab Tristimulus Colorimeter (Model D25M-9), fitted with an M type reflectance sensor, using the Hunterlab "Lab" scale. The "a" scale which measures redness appeared appropriate for measuring crab color changes during cooking. A 2-inch circular area of the crab, located within the four ridges of the carapace directly anterior to the internal body cavity, was measured. After each measurement the crab was rotated 90° and the resulting four readings averaged. Following the heat treatment, the crabs were placed in an ice bath to lower the body temperature and thus minimize any additional color changes. The crabs were wiped dry and the resulting color was measured by the procedure described above.

Heat penetration

Five crabs, varying in weight from 120–347g, were cooked in water at 100°C for 15 min. Internal temperatures were measured using two 20 gauge, Teflon-insulated, copper-constantan duplex wire thermocouples located in the central body cavity of each crab. Thermocouples were positioned by insertion through the cartilage dorsal to the swimming fin and secured by attachment to the swimming fin. The slowest heating thermocouple was used to indicate body temperature. The temperature of the water surrounding the crabs was measured with similar thermocouples whose lead wires were installed through the retort shell using a 1-inch stuffing box (O.F. Ecklund, Inc., Cape Coral, FL). Temperatures were recorded with a 12-point, stripchart recorder (L&N Model-W). All processing was done in a Dixie retort (Model RDT13) equipped with pneumatic instrumentation suitable for processing with a water or steam cook.

Timing for the heat penetration started when the crabs were placed in boiling water. An excess of water was employed so that the addition of the crabs did not reduce water temperature.

RESULTS & DISCUSSION

THE ABSENCE of red color is shown by the low "a" values recorded at 0 time; however, as crabs were heated the astaxanthin-protein linkage was broken and the typical red-orange color developed. Values on the "a" scale of 15 or above were considered typical of the red-orange color of cooked crabs. The largest mean "a" value (21.93) was observed after subjecting the crabs to 100°C water for 16 min. The rate of color development was dependent on time and temperature of exposure (Fig. 1). Crabs placed in water at 100°C had a mean "a" value of 15.27 after only 30 sec of exposure; full color development was not seen in crabs heated in 93.3 and 87.8°C water until after 4 and 8 min, respectively. Crabs heated in 76.6°C water had a mean "a" value of only 10.7 after 16 min, thus, never reaching full color development.

Even though heating crabs in water at 100°C accelerated the color changes, longer exposures at this temperature do not greatly enhance the color. Hence, crabs cooked in boiling water (100°C) for 30 sec generally appear the same as one cooked for 16 min.

The rate of heat penetration in crabs cooked in water at 100°C is seen in Fig. 2. The low initial temperatures are due to cooler storage (4°C) prior to cooking. The largest crab (347g) was slowest in heating. After 1 min in the 100°C water the internal temperature was 16.1°C or below for all crabs regardless of size, thus, still showing effects of refrigerated storage. However, as seen in Fig. 1, the astaxantin-protein complex was partially denatured by this time resulting in the red-orange shell color. It is apparent, therefore, that color development does not serve as an adequate index of proper cooking. The sharp rise in the "a" values after only 30 sec of exposure at 100°C is indicative of the rapid break-down of the astaxanthin-protein linkage, and was followed by a gradual increase in "a" values through 8 min.

Schultz (1980) reported a 7-log reduction in *Vibrio cholerae* if the internal temperature in Blue crabmeat homogenates reach 70°C, and was maintained at that temperature for 1 min. This particular organism is fairly heat sensitive and has been implicated in a few foodborne
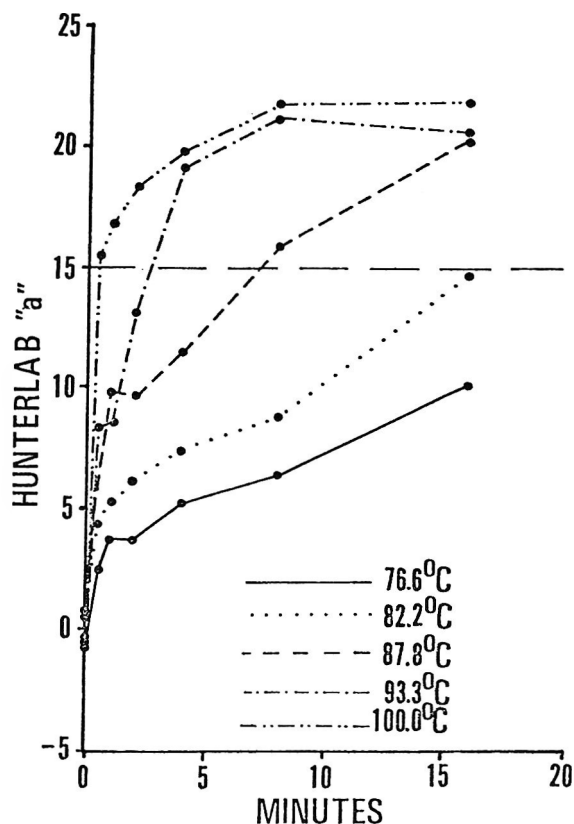
Fig. 1—Influence of cooking time and temperature on the development of red color in Blue crabs as monitored by changes in the Hunterlab "a" values. (Temperatures correspond to cooking water temperature.)

outbreaks in Louisiana (Center for Disease Control, 1978). The largest crab used in this study (347g) did not reach an internal temperature of 71°C until it had been held in
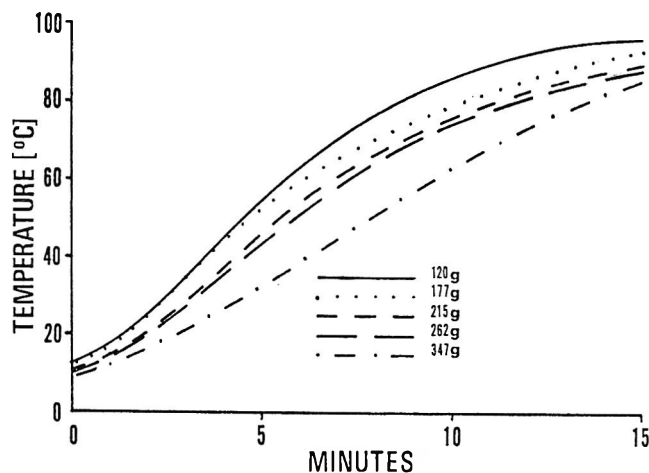

Fig. 2—Heat penetration curves for various sized Blue crabs processed in water at 100°C. (Temperatures were measured in the internal body cavity.)

boiling water (100°C) for greater than 11 min (Fig. 2); however, maximum red color had developed much sooner.

Based on this study, color changes during cooking of Blue crabs cannot be relied upon as an adequate index of proper cooking. Processors and consumers should, therefore, be cautioned not to use red color development during cooking as a gauge of doneness.

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ciated with each juice. Some of these have been identified as low molecular weight acids. They are characteristic of the juices. Because of the need to restrict TSA analyses to fructose, glucose, and sucrose, the GLC is a necessary complementary method for unknown materials and gives as well a quantitative check on the *total* sugar values.

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A Research Note
Nutrient Composition of Historical Canned Food Samples

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ABSTRACT

Several historical food samples were analyzed for nutrient and mineral composition. The samples included a 40-yr old can of sweet corn, relish found aboard the sunken U.S.S. Monitor and seven samples recovered from the sunken steamboat Bertrand. The nutrient content of the canned corn compared favorably with reported values, with the exception of lower amounts of ascorbic acid. Significant levels of riboflavin, niacin, carotene and protein were found in the pickle relish. The samples recovered from the Bertrand contained calcium levels comparable to today's products, but the sodium levels were higher and potassium levels were lower.

INTRODUCTION

IN THE PAST FEW YEARS, the National Food Processors Association (NFPA) has had the opportunity to examine and analyze for nutrient content, several historical food samples. They included a 40-yr old can of corn; pickle relish found aboard the sunken U.S.S. Monitor; and several canned products recovered from the steamboat, Bertrand.

The can of corn was found in 1974 in the basement of a home in California and had been packed in 1934 by the Faribault Canning Company. NFPA received the can intact, opened it and analyzed it for sterility, metals and nutrient content.

In 1865, the steamboat Bertrand snagged and swamped while heavily laden with provisions destined for Fort Benton, Montana, and the gold mining camps. It was uncovered in 1968 under 30 ft of silt about 25 miles north of Omaha, Nebraska. Among the food items recovered from the Bertrand were almonds, catsup, brandied fruits, honey, oysters, tomatoes, spices and jelly (Petsche, 1974). In 1974, NFPA received several samples from the Bertrand which were analyzed for nutrient content.

In 1862, the U.S.S. Monitor sank in a storm off Cape Hatteras, North Carolina, less than a year after its launching (Gorman, 1980). In August 1979, underwater archaeological crews from the North Carolina Division of Archives and History found the first artifacts from the ship. Later, a sealed Mason jar of pickle relish was recovered, and in 1980, NFPA was asked to analyze a portion of the relish.

MATERIALS & METHODS

ALL PROXIMATE and vitamin analyses were conducted according to AOAC procedures (AOAC, 1970 or 1975). Mineral determinations were made by atomic absorption and atomic emission spectroscopy using a Varian 1100 or a Perkin-Elmer 560 atomic absorption spectrophotometer. Lead and cadmium determinations were made by anodic stripping voltametry using a PAR 174 polarographic unit combined with an ESA 2014 cell unit. Phosphorus was determined by the AOAC (1970) molybdovanadate colorimetric procedure.

RESULTS & DISCUSSION

The 40-yr old canned corn was found to be commer-

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cially sterile, and the kernels appeared much like recently canned corn. No significant amount of ascorbic acid was found (Table 1), but many of the other nutrients compared favorably with the values for corn reported by Watt and Merrill (1963) in Handbook #8. The iron and vitamin A content were nearly twice the level reported in Handbook #8, while the solids, ash, protein and calcium values were similar.

In addition, the corn was analyzed for six minerals for which Handbook #8 has no analytical data, although it does contain estimated or imputed data for sodium and potassium. The lead content of the corn was 0.37 ppm. This compares well with the average lead content for fruits and vegetables packed in 1974, the year the corn was analyzed (Elkins, 1981).

The nutrient composition of the pickle relish recovered from the U.S. Monitor is contained in Table 1. Watt and Merrill (1963) reported imputed values for proximate constituents, calcium and iron in sour pickle relish, and these values were similar to those obtained for the historical sample.

It was difficult to identify visually the ingredients in the pickle relish. The vitamin A content of 565 I.U./100g suggested that there were other ingredients in addition to cucumbers. Pepper seeds were found in the relish, and peppers could have been the source of vitamin A. Little ascorbic acid and thiamin were found, but significant levels of riboflavin and niacin remained.

Nutrient data for seven canned products recovered from the Bertrand are contained in Table 2. No ascorbic acid was found in the mixed vegetables or brandied peaches, but 7.3 mg/100g of ascorbic acid were found in the catsup sample. The analysis was conducted using the indophenol titration method, and several metals such as iron, tin and copper could have interfered if present in significant

Table 1—Constituents of canned corn and Monitor relish

Constituent	40-yr old butter kernel	Monitor relish
Ascorbic acid (mg/100g)	<1	<1
Vitamin A (I.U./100g)	543	565
Protein (%)	2.4	1.0
Solids (%)	21.9	7.0
Ash (%)	0.8	2.2
Iron (mg/100g)	1.1	2.2
Calcium (mg/100g)	3.7	36.8
Sodium (mg/100g)	152	681
Potassium (mg/100g)	253	255
Copper (mg/100g)	0.04	*
Manganese (mg/100g)	0.16	*
Lead (ppm)	0.37	*
Cadmium (ppm)	0.01	*
Thiamin (mg/100g)	*	<0.005
Riboflavin (mg/100g)	*	0.07
Niacin (mg/100g)	*	0.5
Calories/100g	*	22
Fat (%)	*	0.6
Carbohydrates (%)	*	3.1

* Not analyzed due to sample and time limitations

Table 2—Nutrients in Bertrand samples

Constituent	Oysters	Plum tomatoes	Mixed vegetables	Brandied peaches	Catsup	Red peppers	Honey
Ascorbic acid (mg/100g)	*	1.5	<1.0	<1.0	7.3	*	*
Vitamin A (I.U./100g)	*	160	*	*	*	4252	*
Protein (%)	5.9	*	*	*	1.6	<0.1	0.6
Solids (%)	*	*	*	*	12.0	18.0	*
Iron (mg/100g)	1.6	1.1	1.3	1.5	*	*	*
Zinc (mg/100g)	0.8	2.5	10.8	0.3	*	*	*
Manganese (mg/100g)	0.2	0.1	0.2	0.2	*	*	*
Copper (mg/100g)	0.7	11.3	4.4	0.7	*	*	*
P_2O_5 (mg/100g)	202.6	15.2	10.8	21.5	*	*	*
Sodium (mg/100g)	395	346	191	1	*	*	*
Potassium (mg/100g)	98	57	30	69	*	*	*
Calcium (mg/100g)	22.5	27.0	13.6	7.0	*	*	*
Ash (%)	2.4	1.4	0.5	0.2	*	*	*

* Not analyzed due to sample and time limitations

quantities. Although the layers of silt covering the steamboat provided a relatively inert atmosphere, it is unlikely that this amount of ascorbic acid would remain after 109 years.

The plum tomato and red pepper samples were analyzed for vitamin A content and significant quantities of carotene were detected. The red peppers contained approximately one half of the vitamin A value of 9,590 I.U./100g reported for red peppers in Handbook #8 (Watt and Merrill, 1963). Handbook #8 does not have an entry for plum tomatoes but does report the vitamin A content of tomatoes as 900 I.U./100g. The plum tomatoes recovered from the Bertrand contained approximately 18% of this level.

The protein levels in the catsup, red pepper and honey samples were comparable to those reported by Watt and Merrill (1963), but the oysters contained 70% of the protein level expected.

Percent ash and mineral content of four of the Bertrand samples are presented in Table 2, and the data for several of the minerals were not available in Handbook #8 for direct comparison. The oysters contained less iron than the 5.6 mg/100g reported in Handbook #8, while the plum tomatoes contained twice as much iron as the 0.5 mg/100g reported for canned tomatoes, and the brandied peaches contained 1.51 mg/100g of iron compared to the 0.3 mg/100g reported for canned peaches in heavy syrup. All calcium values were comparable to today's products, and in general, the potassium values were lower.

These samples offered a unique look at food consumed a hundred years ago and the quality of the processing techniques used in that era. That these samples contained many nutrients speaks well for the processing capabilities and techniques used and attests to the importance of the storage environment for nutrient retention.

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A Research Note
Salt Content of Selected Snack Foods

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————————————ABSTRACT————————————

The salt content of 15 snack foods was analyzed using autoanalysis and titration methods. The salt content was alarmingly high in certain snack foods. However, intake is dependent on the quantity of the snack foods consumed and the frequency of consumption. Higher salt values were obtained by autoanalyzer. Variability was observed in the values obtained by both methods. Autoanalysis has good potential for use in rapid salt determination, particularly for use in quality control and sodium labeling.

INTRODUCTION

SALT (sodium chloride) is the most common source of sodium in the American diet. Sodium is found in a variety of foods, including meats, fish, dairy products, and vegetables, and it is added to the processed foods for various reasons (Marsh et al., 1980; IFT, 1980). There are several less obvious sources such as baking powder, baking soda, MSG (monosodium glutamate), and other intentional additives.

More attention has been recently focused on dietary sodium in the causation and control of hypertension than on any other nutrient (National Dairy Council, 1981). Certain health problems such as hypertension have been associated with excessive intake of sodium in diets (Dahl et al., 1972). According to the Food and Nutrition Board (1980), the estimated safe and adequate daily dietary intake of sodium is 1100–3300 mg (2.8–8.4g sodium chloride) for healthy adults. The most effective way suggested to maintain sodium intake within the range is to reduce the amount of salt added at the table or in cooking and to reduce moderately the selection of obviously salty foods.

The contribution of snack foods to the sodium intake is very controversial, largely because of the lack of data. In a study, it was found that many of the foods eaten as snacks that make a significant contribution to the sodium intake of teenagers were foods that are not typically labeled as "snack foods" (Morgan and Bundy, 1981). Evening snacks contributed more to the daily sodium intake than morning and afternoon snacks consumed by college students. Sodium was taken in more from regular meals than snack foods (Khan, 1982). Significant differences were found between brands and within brands of selected canned vegetables (Sinar and Mason, 1975). Variations were also found in the sodium contents of frozen vegetables having various brands and even between two samples of the same vegetables (Thomas and Lewis, 1954). Similar variations in sodium contents were found in ready-to-serve foods (Holinzer et al., 1966).

With the recent focus on sodium labeling, it becomes imperative to use a relatively easy and rapid method which can be used by quality control units in the food industry. Also indigenous sources of sodium and its relation to the

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salt content becomes confusing. Although there are different analytical methods for the determination of salt content in foods, there are very few published results pertaining to their performance and accuracy.

The primary objective of this study was to provide data on the salt content of selected common snack foods. The data obtained by two rapid methods of salt determination (autoanalyzer and titration) used in this study also were comparatively evaluated.

MATERIALS & METHODS

FIFTEEN different types of snack foods were purchased from local retail markets. Common names of the snack foods are used in order to avoid identity of their brand names. All these snacks were manufactured by nationally known industries.

For salt determination by autoanalyzer, Dicromat® Salt Analyzer Model 1-10 (Diamond Crystal Salt Co.) was used. Procedure outlined by the manufacturer was used for analysis. To 20-g samples of the snack foods, 200 ml distilled water (nano-free) were added and allowed to soak for 3 min, while stirring occasionally. The contents were filtered and poured through the reservoir and probe of the salt analyzer. The automatic read-out was recorded, which directly gave the percent salt content of the solution.

For titration, the silver nitrate method was used. Ten milliliters of the filtrate obtained in the earlier procedure were pipetted into a 150 ml beaker. Approximately 16 drops of 5% potassium dichromate solution were added to the filtrate. The filtrate was then titrated against 0.1N silver nitrate solution. Salt content was calculated as follows:

$$\% \text{ salt} = \text{"x" ml of 0.1N silver nitrate} \times 0.585$$

At least three replicates of each sample were analyzed by both methods.

RESULTS & DISCUSSION

THE SALT CONTENTS of the snack foods are presented in Table 1. The maximum and minimum salt contents and the differences in mean values are presented (Table 2) in order to illustrate the variability between samples. The independent precision of the two methods employed was confirmed by the uniform results obtained among determinations of the same sample. Variability in sodium content between similar products and even among same samples were reported earlier (Sinar and Mason, 1975; Thomas and Lewis, 1954; Holinzer et al., 1966). It should be noted that these values represent salt content per 100g of the snack foods and the quantities of these snacks consumed may be much less than 100g. The mean values ranged from 273 mg/100g to 2,370 mg/100g by titration and from 300 mg/100g to 2,483 mg/100g by autoanalysis. In view of the safe and adequate daily dietary intake of sodium of 1,100–3,300 mg (2.8–8.4g sodium chloride) these snack foods may contribute to a considerable amount of sodium intake. However, much depends on the amount of snack food consumed and the frequency of consumption.

Pretzel twists contained the highest amount of salt compared to the other snacks analyzed in this study. Toasted corn, puff balls, corn-flavored chips, and saltines contained above 1,000 mg of salt content per 100g. The data obtained on salt content will definitely be useful, since these types of data are virtually nonexistent. It is relatively easy to grade

Table 1—Salt content of snack foods as determined by titration and autoanalyzer

| Snack foods | Salt content[a] (mg/100g) | |
	Titration	Autoanalyzer
Corn chips	741 ± 77	767 ± 24
Taco tortilla chips	868 ± 14	950 ± 0
Toasted corn	1,307 ± 37	1,433 ± 24
Salted peanuts	595 ± 14	667 ± 47
Granola snack bar	273 ± 14	300 ± 41
Corn-flavored snacks	1,014 ± 27	1,117 ± 24
Pretzel twists	2,370 ± 145	2,483 ± 155
Nachos chips	546 ± 77	617 ± 47
Puff balls	1,190 ± 28	1,267 ± 24
Sunflower seeds	721 ± 30	783 ± 24
Tortilla chips	468 ± 41	525 ± 35
Saltines	1,794 ± 37	1,867 ± 24
Nacho cheese chips	292 ± 41	317 ± 24
Salty rye crackers	819 ± 0	883 ± 24
Wheat crackers	566 ± 170	650 ± 71

[a] Mean values ± standard deviation.

Table 2—Percent differences and ranges in salt contents of snack foods as determined by titration and autoanalyzer

| Snack foods | Salt content (mg/100g) | | | | Percent difference between mean values |
| | Titration | | Autoanalyzer | | |
	Min	Max	Min	Max	
Corn chips	673	848	750	800	3.5
Toasted corn	1,258	1,346	1,400	1,450	9.6
Salted peanuts	585	614	600	700	12.1
Pretzel twists	2,253	2,574	2,350	2,700	4.8
Granola bar	263	293	250	350	9.9
Wheat crackers	380	790	600	750	14.8
Puff balls	1,170	1,229	1,250	1,300	6.5
Sunflower seeds	673	731	750	800	8.6
Corn-flavored chips	995	1,053	1,100	1,150	10.2
Regular tortilla chips	410	497	475	550	12.2
Saltines	1,755	1,843	1,850	1,900	4.1
Nachos regular chips	439	614	550	650	13.0
Nachos cheeze chips	263	351	300	350	8.6
Salty rye crackers	819	819	850	900	7.8
Taco tortilla chips	848	878	950	950	9.4

and label low sodium products than those containing high levels of sodium, since it is difficult to define the "high" level.

The percent difference between the two methods is also presented in Table 2. Higher values were consistently recorded by autoanalysis compared to the titration method. These higher values may be attributed to the fact that the salt analyzer used in this study measured the conductivity (electrolyte in solution) and so it would also respond to indigenous electrolytes, other than chloride. However, this difference may not be significant in relation to the wide variation of salt in these products. Variability was noted in the values obtained by the analyses of same snacks and even among same samples. The differences in both methods ranged from 3.5% to 14.8%.

The advantages noted by the use of autoanalyzer were: (a) the method is very easy and convenient, (b) it can be used without any special training, (c) no special elaborate sample preparations are needed, (d) is economical compared to other methods, and (e) is very rapid.

Data obtained in our study are more useful from the point of the salt content in snack foods, rather than the accuracy of any method. The data are indicative of the alarmingly high level of salt in certain snack foods. Also, variability may be expected among samples and this point needs consideration. Further studies are needed to provide additional data on the salt content of various foods.

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A TLC-Fluorescent Method of Detecting and Evaluating Individual Antioxidative Components

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―――――――――― ABSTRACT ――――――――――

A new method was developed to detect and to evaluate the amount and/or activity of individual antioxidative components extracted from plant materials or food products. The antioxidative components were separated on a fluorescent TLC plate and visualized by spraying with a tocopherol-stripped soybean oil solution followed by exposing to UV irradiation in a TLC-viewing UV cabinet. Antioxidative components on the plate appeared after 1–3 hr of UV irradiation as fluorescent spots on a dark background, and their amounts and/or activity can be evaluated from the area and/or the fluorescence-persisting time of the respective spots.

INTRODUCTION

WHEN ANTIOXIDATIVE COMPONENTS extracted from plant materials or food products are separated on a TLC plate, they can be visualized by a number of chemical or physical methods. However, only a few of them are specific for antioxidative compounds. The carotene-linoleic acid coupled spray of Philip (1974) is not only specific but also capable of evaluating the activity of individual antioxidative components (Hammerschmidt and Pratt, 1978). Liu and Chen (1980) sprayed the TLC plate with a solution of methyl esters of fatty acids and exposed it to UV light. After an appropriate time of UV irradiation the plate was sprayed with a peroxide reagent, N,N-dimethyl-p-phenylenediammonium dichloride (Knappe and Petri, 1962), to develop light yellow spots on a purple background. In this paper we introduce a new method which is simple and capable of detecting and evaluating individual antioxidative components separated on a TLC plate, and is therefore particularly useful in screening antioxidative components extracted from plant materials or food products.

MATERIALS & METHODS

TLC plate

Silica gel 60 F254 pre-coated TLC aluminum sheets (Merck Art. 5554) were used after reactivation at 105°C for 30 min.

TLC-viewing UV cabinet

A TLC-viewing UV cabinet, such as the Chromato-Vue cabinet Model CC-20 of Ultra-Violet Products, Inc. or the UV Betrachter 29050 of CAMAG, was used. The cabinet has short wave (254 nm) and long wave (366 nm) UV lamps at the top.

Soybean oil solution for spray or dipping

A soybean oil solution of about 3% concentration in n-hexane was freshly prepared and treated with active carbon (Merck, charcoal activated GR, Art. 2186) to remove tocopherols, and stored in a refrigerator.

Operational procedures

Dry the fluorescent TLC plate on which antioxidative components have been separated. Spray it (2–6 times) evenly with, or dip

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it (5 sec) in the freshly prepared soybean oil solution. Immediately place the plate in the UV cabinet and start short wave UV (254 nm) irradiation. Observe and sketch, or photograph, the zero time UV absorption pattern of the chromatogram, and continue observation of the fluorescence changes under UV light. Record the time of background darkening which accompanies the appearance of fluorescent spots. Mark or photograph the fluorescent spots that appear on the chromatogram, and continue observation of the fluorescence changes under UV light. Record the time of disappearance of each fluorescent spot for comparison of fluorescence-persisting time for activity evaluation.

RESULTS & DISCUSSION

An experiment with the oleoresin of betel pepper rhizomes

The oleoresin prepared from betel pepper (*Piper betle* L.) rhizomes was subjected to TLC separation with benzene: ethyl formate:formic acid (75:24:1, v/v), and then to detection and evaluation of individual antioxidative components by the proposed method. The results are illustrated in Fig. 1. At zero time (plate a) there were quite a few spots with light blue or dark appearance observed on a bright fluorescent background. After 1–3 hr, depending on the quality and quantity of the oil applied and on the temperature of UV irradiation (plate b), the entire background gradually turned dark while the spots of antioxidative compounds remained unchanged, resulting in the appearance of bright fluorescent spots on a dark background. After prolonged exposure to UV irradiation (plates c, d and e), the fluorescent spots became smaller and smaller and finally disappeared, depending on the amount and/or the antioxidative activity of each component. It is noteworthy that the fluorescent spots which appeared after exposure to UV irradiation (plate b) did not necessarily coincide with the dark spots observed at zero time of UV irradiation (plate a).
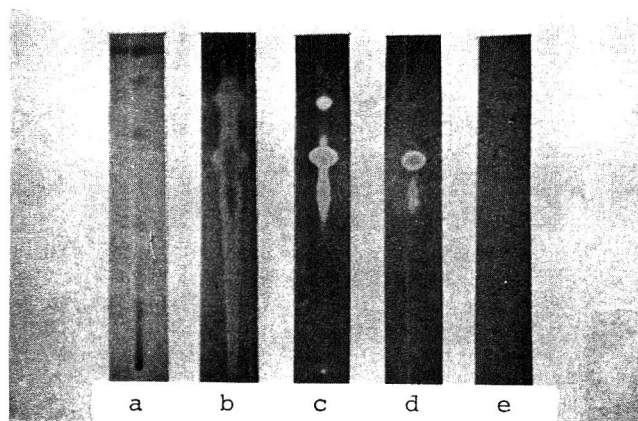

Fig. 1—Thin-layer chromatograms of antioxidative components of the oleoresin obtained from betel pepper rhizomes, as observed by the proposed fluorescent detection method at different stages of UV irradiation. Plate: Silica gel 60 F254 pre-coated TLC aluminum sheet; Solvent: Benzene:ethyl formate:formic acid (75:24:1, v/v) (a) At zero time, (b) after about 2 hours, and (c), (d) and (e) at different stages after prolonged time of UV irradiation.

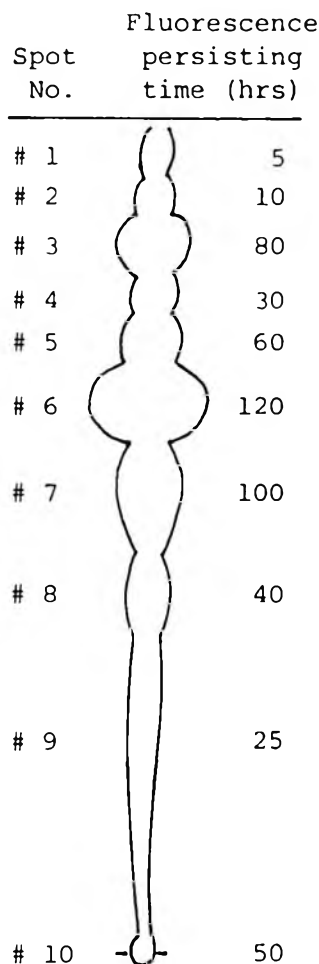| Spot No.	Fluorescence persisting time (hrs)
# 1	5
# 2	10
# 3	80
# 4	30
# 5	60
# 6	120
# 7	100
# 8	40
# 9	25
# 10	50

Fig. 2—Thin-layer chromatogram and fluorescence-persisting times of antioxidative components of the oleoresin obtained from betel pepper rhizomes, as observed and measured by the proposed TLC-fluorescent method. (See Fig. 1 for the plate and the solvent.)

The results of quantitative evaluation of the antioxidative components by the proposed method are illustrated in Fig. 2. The relative amounts of individual antioxidative components in the oleoresin can be roughly estimated from spot areas, while their activity can be evaluated from the fluorescence-persisting time shown at the right side of each spot. It is apparent from these data that the #6 component is the major antioxidative component of betel pepper rhizomes, and that the #7 and the #3 components are the second and the third important ones, respectively. These compounds can then be eluted separately from new chromatograms for further purification and identification.

Comparison between the methods of antioxidant detection on TLC plates

This TLC-fluorescent method for specific detection of antioxidants on TLC plates was compared with those of Philip (1974) and Liu and Chen (1980). The main differences are as follows: (1) Philip's method and our method needs only one spray, while the method of Liu and Chen needs two; (2) The fat oxidation in Liu and Chen's method and our method is induced by UV irradiation, while that in the method of Philip is induced by daylight; (3) In the methods of Philip and Liu and Chen a chromogenic spray is needed and the color observation is subject to disturbance by the presence of colored compounds, while our method needs no chromogenic reagent and the fluorescence observation is not disturbed by colored compounds; (4) In Philip's method and our method the color of fluorescence observation can be conducted continuously on one chromatogram, while in the method of Liu and Chen several chromatograms are needed to spray intermittently for antioxidant detection; (5) Philip's method and our method are capable of evaluating the activity of individual antioxidative components, while the method of Liu and Chen is not. The proposed method, therefore, has some advantages over the other two methods in detecting and/or evaluating antioxidative components separated on TLC plates.

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A Research Note
Volatile Flavor Components of Nira (*Allium tuberosum* Rottl.)

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─────── ABSTRACT ───────

The flavor components of Nira (*Allium tuberosum* Rottl.) have been studied by a combination of gas chromatography and mass-spectrometry. Twenty-nine compounds were identified in the oil obtained from extraction of the steam distillate of Nira. The identified compounds include 7 sulfides, 2 ketones, 18 alcohols and 2 esters. The main volatile components were dimethyl disulfide and dimethyl trisulfide.

INTRODUCTION

Allium tuberosum Rottl. is a perennial plant cultivated in Japan. This plant has been used as a vegetable and is called "Nira" in Japanese.

The characteristic odors of *Allium* species are due to the occurrence of sulfur-containing flavor components. The flavor precursors were shown to be S-alk(en)yl-L-cystine S-oxides [alk(en)yl:propyl; *trans*-1-propenyl; allyl; methyl] (Stoll and Seebeck, 1948, 1949a,b; Schwimmer and Weston, 1961; Schwimmer and Guadagni, 1968). The constituents of *Allium cepa* L. (onion) have been studied intensively by Bernhard (1968), Brodnitz et al. (1969, 1971), Boelens et al. (1971) and Kameoka and Demizu (1979). A number of investigators have reported on the volatile flavor constituents of other *Allium* species, such as garlic (Brodnitz et al., 1971; Freeman and Whenham, 1975), chive (Wahlroos and Virtanen, 1965), leek (Schreyen et al., 1976) and caucas (Nishimura et al., 1971). Constitution, formation and origin of *Allium* flavor components were also reviewed by Johnson et al. (1971) and Schwimmer and Friedman (1972).

This paper deals with characteristic flavor components of the neutral fraction of Nira.

MATERIALS & METHODS

Sample preparation

55 kg of fresh Nira cultivated at Miyazaki-prefecture in Japan was purchased in May, 1981. The leaves were chopped and placed in a 30L stainless vessel for steam distillation. After steam distillation of the leaves for 8 hr, the steam volatile oil was obtained by extraction of the distillate (30L– with ether and evaporation under N_2. The yield was 7.22g (0.013%) of a dark brown oil with a strong sulfur odor. The volatile oil was treated with 5% $NaHCO_3$ solution, then 5% NaOH solution, and separated into three fractions: neutral (4.37g), phenolic (1.79g) and acidic (0.98g).

Adsorption chromatography

Fractionation of the neutral compounds into sulfur compounds and nonsulfur compounds was carried out by liquid-solid chromatography. Using a column filled with silica-gel, the neutral fraction (4.37g) was divided into two fractions by elution with hexane and ether (350 mL each). The solvents were allowed to evaporate under N_2. Oils obtained from the hexane fraction and the ether fraction weighed 3.42g and 0.85g, respectively. Each oil was analyzed by gas chromatography.

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Gas chromatography (GC)

GC was performed with a Simadzu GC-4C PTF type gas chromatograph equipped with a flame ionization detector and a linear temperature programmer. A S.C.O.T. column was used: 40m X 0.3 mm (i.d.) Thermon-600T glass capillary column; temperature programmed from 80–210°C at 4°C/min; injector and detector temperature 250°C; carrier gas N_2, flow rate 40 mL/min. Values of the peak areas were calculated by a Simadzu C-RIA Chromato-Pack.

Gas chromatography–Mass spectrometry (GC-MS)

GC-MS was carried out on a Simadzu LKB 9000 instrument. A 3m glass packed column (3 mm i.d.), coated with PEG-20M (3%), temperature programmed from 80–200°C at 5°C/min, with He as carrier gas, was used. Operating conditions for the GC-MS were: separator oven 250°C; ion source 210°C; ionization voltage 70 ev; ion accelerating voltage 3500V.

RESULT & DISCUSSION

THE NEUTRAL FRACTION was roughly separated by the method of adsorption chromatography into two fractions: sulfur compounds and nonsulfur compounds. Fig. 1 shows gas chromatograms of sulfur compounds fraction [1] and nonsulfur compounds fraction [II], respectively. The sulfur compounds fraction gave the characteristic Nira odor. The volatile compounds were identified by comparison with mass spectra and gas chromatographic retention times of authentic compounds. The components identified are presented in Table 1 and the peak numbers refer to those of Fig. 1.

Sulfur compounds are the main constituents of the volatile oil since they were represented by relatively large gas chromatographic peaks. Diallyl sulfide, dimethyl disulfide, methyl allyl disulfide, dimethyl trisulfide, diallyl disulfide, methyl allyl trisulfide, and dimethyl tetrasulfide accounted for 74.6% of the total peak area of the neutral fraction. In particular, sulfides containing a methyl group, such as dimethyl disulfide (Peak 1) and dimethyl trisulfide (Peak 11), compose 48.5% of the neutral fraction. Diallyl sulfide (Peak 2) and diallyl disulfide (Peak 7), which had a garlic-like odor, were present in small quantities in Nira. Bernhard (1970), using gas chromatography, has analyzed the constitution of many *Allium* species. He concluded that the flavor components of *Allium tuberosum* Rottl. mainly consisted of methyl allyl disulfide and dimethyl disulfide. Bernhard (1970) also reported that diallyl disulfide and methyl allyl disulfide represented the major volatile compounds in garlic (*Allium sativum*). Thiols and thiosulfonates, known components of onion oil, were not detected in Nira.

All sulfur compounds identified in this study can be postulated as enzymatic decomposition products of sulfoxide amino acids. The presence of sulfides in *Allium* and their biosynthesis from S-alkyl-L-cystine S-oxides have been thoroughly discussed by Brodnitz et al. (1969).

Many alcohols were identified as trace constituents. Since sulfides are present in large quantities, the aroma of monoterpene alcohols, such as linalool (Peak 26), α-terpineol (Peak 41), nerol (Peak 45) and geraniol (Peak 47), is masked by the odor of the sulfides. These alcohols have not been reported in the literature on *Allium* species.
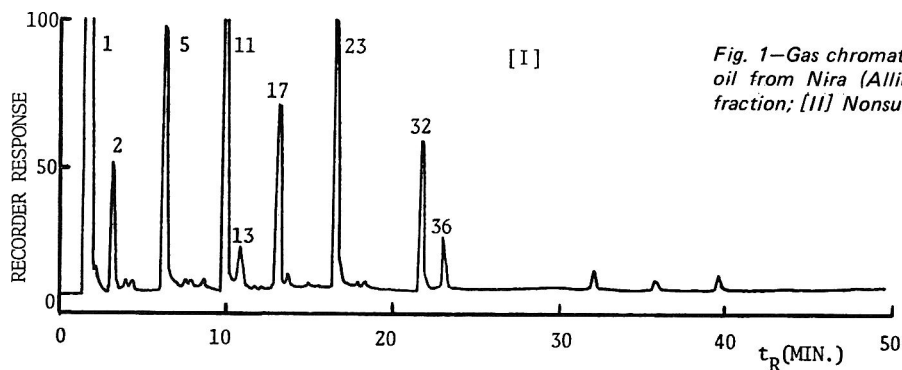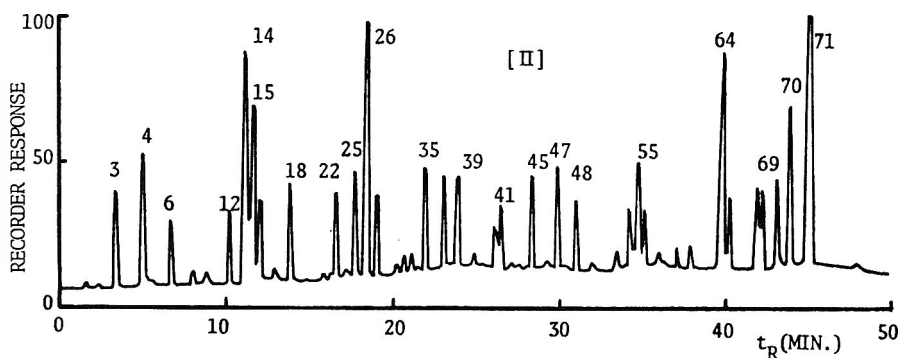
Fig. 1—Gas chromatograms of neutral fraction of the steam volatile oil from Nira (Allium tuberosum Rottl.). [I] Sulfur compounds fraction; [II] Nonsulfur compounds fraction.

Table 1—Volatile flavor components of neutral fraction from Nira

Peak no.	Component	Peak area (%)
*1	dimethyl disulfide	32.3
*2	diallyl sulfide	2.3
3	1-butanol	0.1
*5	methyl allyl disulfide	7.9
10	1-hexanol	**tr.
*11	dimethyl trisulfide	16.2
14	cis-3-hexen-1-ol	0.8
15	trans-2-hexen-1-ol	0.3
16	cis-2-hexen-1-ol	**tr.
*17	diallyl disulfide	2.7
18	2-undecanone	0.1
22	1-octen-3-ol	0.1
*23	methyl allyl trisulfide	9.6
25	1-octanol	0.1
26	linalool	0.9
*32	dimethyl tetrasulfide	3.6
35	1-nonanol	0.2
39	2-tridecanone	0.1
41	α-terpineol	**tr.
42	1-decanol	**tr.
45	nerol	0.2
47	geraniol	0.3
48	1-undecanol	**tr.
52	1-dodecanol	**tr.
58	1-tridecanol	**tr.
61	1-tetradecanol	**tr.
64	α-cadinol	0.8
69	methyl oleate	0.1
70	methyl linoleate	0.7

* sulfur compound
** tr. < 0.05%

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A Research Note
Effect of Marination upon Mineral Content and Tenderness of Beef

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————————ABSTRACT————————

This study compared marinated and nonmarinated beef to determine differences in semimembranosus muscle tenderness and selected mineral content. The minerals analyzed were iron, zinc, magnesium and sodium. Marination resulted in a significantly more tender muscle, as determined by shear values and trained panel evaluations. Mineral content was significantly affected by marination with substantial losses of iron, zinc, and magnesium and increased sodium. Marination would be a feasible method of enhancing tenderness of more economical cuts of beef; however, the subsequent loss and/or gain of minerals should be considered.

INTRODUCTION

MARINATION of meat cuts is a practical method of tenderizing that has been used for many years. Recent cookbook publications contain an unusually large number of meat recipes utilizing less expensive meat cuts and/or marination techniques. Commercial dry-mix, as well as, bottled marination preparations are available in the supermarket. Increased costs of meat, particularly beef, has forced many consumers to select more inexpensive, less tender cuts, use various methods of tenderizing, select less costly alternate products or decrease meat consumption to balance the home budget. The effect of marination upon the nutritional content of beef muscle is not well documented by research, with only sparse and conflicting reports made about the effect on tenderness (Hamm, 1960; Hamm and Deatherage, 1960; Wierbicki et al., 1957; Swift and Berman, 1959; Webb et al., 1967; Harrell et al., 1978; Griswold, 1955a, b; Roberts, 1965; Lind et al., 1971; Wenham and Locker, 1976). Since beef, as well as other meat products, is a major contributor of certain minerals in the diet, a study was conducted to determine the effect of marination upon selected mineral (iron, zinc, magnesium, and sodium) retention and tenderness.

MATERIALS & METHODS

TEN SIMILARLY CROSSBRED CATTLE (Hereford, Angus, Brahman and Charolais) were raised by the Louisiana State Univ. Dept. of Animal Science. Five of the animals were forage-fed (bermuda and rye grass pasture) beginning at 9 months of age and five of the animals were fed on forage plus a concentrated grain ration 198 days prior to slaughter. All animals were slaughtered at a live weight of 465–517 kg at 18–24 months of age for forage-fed beef, and 441–472 kg at 15–18 months of age for limited grain-fed beef. The right side of each carcass was aged 6 days at 1–2°C at which time the semimembranosus (SM) muscle was removed and cut into steaks 2.54 cm thick. Subcutaneous fat in excess of 0.64 cm was removed and steaks were wrapped and frozen for approximately 6–8 wk at 0°C.

The SM muscles were used to determine cooking losses, mineral analyses, pH, shear values and trained taste panel evaluations. Shear

values, using a 1.27 cm core, were determined on the Warner-Bratzler Shear instrument. The SM muscles were randomly assigned to one of three treatments: control (untreated), acid or beef-flavored marination. The composition of marinades are illustrated in Table 1. Sodium content was calculated from food composition tables and food manufacturer analysis to be 4.9 gm per liter for both marinades.

All SM muscles were thawed 24 hr by refrigeration at 1–2°C and weighed. Those samples to be marinated were pierced three to four times on both sides and immersed completely in solution for 24 hr at refrigeration temperatures, 1–2°C. All muscles were then reweighed, roasted at 177°C to an end internal temperature of 70°C, reweighed and evaluated. Shear values, pH and cooking losses were determined.

Mineral analyses were determined by atomic absorption spectrophotometry at the appropriate wavelength for each mineral (Kowalezuk, 1970; Freeland and Cousins, 1976; Haeflein and Rasmussen, 1977).

A multi-factor analysis of variance was used to determine significance of differences in means of cooking losses, pH, shear values, tenderness, iron, magnesium, sodium and zinc between and within treatments of the SM muscles (Steel and Torrie, 1960).

RESULTS & DISCUSSION

Effect of marination

The feed management system did not have an effect upon mineral content of the SM muscles. Therefore, the analyses presented are based on combined forage and limited grain-fed beef. Marination resulted in distinct changes in the surface appearance of the cooked muscles which exhibited a gray exterior color and a more natural pink interior color. The cooked acid-marinated muscle had an iridescent sheen on the surface reported by other investigators (Wenham and Locker, 1976).

The mean values for pH, cooking losses, shear values, tenderness and mineral content are summarized in Table 2.

Table 1—Composition of marinades

Ingredient	Weight (g)	pH
Acid		
Lime juice	180	
Deionized water	120	
Sugar	80	
Oil	15	
Salt	5	
	400	2.56
Beef-flavored		
Deionized water	392	
Beef flavor cubes: salt, hydrolyzed vegetable protein, corn syrup solids, sugar, beef fat, monosodium glutamate, dextrose, onion powder, water, garlic powder, caramel color, natural flavorings, disodium guanylate, oisodium inosinate, partially hydrogenated vegetable oi , artificial color	8	
	400	5.49

Variables	Untreated	Acid-marinated	Beef flavored-marinated	n[a]
pH[b]	5.7	4.7[f]	5.6	40
Cooking loss (%)[c]	24.45	24.67	27.45[f]	20
Shear (kg)[b]	5.18	4.19[f]	4.37[f]	20
Tenderness[b,d,e]	4.12	5.39[f]	5.00[f]	20
Zinc content[c,e] (mg/100g)	3.37	2.55[f]	2.80[f]	20
Iron content[c,e] (mg/100g)	3.72	3.66	2.97[f]	20
Magnesium content[b,e] (mg/100g)	22.46	17.76[f]	19.49	20
Sodium content[b,e]	62.37	142.36[f]	190.76[f]	20

[a] n = number of samples per treatment (10 animals × cooked state × 2 replications)
[b] Significant at $P < 0.01$
[c] Significant at $P < 0.05$
[d] Hedonic scale 1—8, 8 = most tender
[e] Determined on a wet weight basis
[f] Treatments significantly different from untreated samples

Acid marination resulted in a significantly lower ($P < 0.01$) pH, with negligible differences in the pH of the untreated and beef-flavored marinated samples. Beef-flavored marination resulted in greater cooking losses. There were significant differences ($P < 0.01$) found in both shear force and tenderness scores due to marination treatment. Both the acid and beef-flavor marinated muscles were rated more tender with lower shear values than untreated muscles. There was close agreement between shear values and panel scores for tenderness. The tenderizing effect of marination agrees with the findings of Weir (1960), who suggested both salt and weak acid as tenderizing agents. Of the three treatments, acid marination resulted in the most tender muscles which is contrary to the earlier findings of Griswold (1955a). This effect may be due to some increased hydration of the muscle fiber, as well as some solubilization of the collagenous tissue. It is apparent that the increased tenderness is not attributed solely to change in pH value.

There were differences in the content of all minerals analyzed because of marination treatments. Acid-marination resulted in a significant ($P < 0.01$) decrease in magnesium content as compared to the untreated samples. There was a significant ($P < 0.01$) increase in sodium content as a result of both marinations. Sodium, being an extracellular ion, was easily absorbed by the muscle during marination. Sodium value more than doubled with acid marination and tripled with beef flavor marination. The higher values for beef flavor marination may be attributed to interaction of sodium chloride with recipe ingredients and increased muscle fiber permeability. Marination resulted in consistently lowered values of iron, magnesium and zinc. The decrease in mineral content (zinc and magnesium) was greater with acid marination; however, the iron content was lowest with beef-flavored marination. The acid apparently had a stabilizing effect upon the form of iron, retaining more than zinc or magnesium. Since iron, magnesium and zinc are primarily intracellular ions, the loss is attributed to leaching of the ions from the muscle during marination.

This study suggests to the practicing dietitian, nutritionist or consumer that marination can be successfully used to enhance tenderness of less-tender beef thus reducing beef cost. The consumer would be well advised to note, however, that marination can result in a substantial loss of selected trace minerals for which meat is a major source, and an increase in sodium content. If marination is to be used without the loss of trace elements, an acceptable marinade should be used which could be consumed with meat or used in other dishes, such as sauces, gravies or soups. For those persons who are controlling sodium intake for dietary or preventive health measures, more careful screening of the marinade recipe ingredients or labeling contents of commercial preparations would be beneficial to avoid excessive intakes of sodium. Marinades can be made without added sodium that are appealing to the sensory mechanisms.

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Ms received 12/27/82; accepted 1/4/83.

ERRATA NOTICE

J. Food Sci. 47(6): 1757–1762 + 1766 (1982). Effect of auger- and press-type mechanical deboning machines on selected characteristics of mechanically deboned poultry by M.G. Mast, T.G. Uijttenboogaart, A.R. Gerrits, and A.W. deVries. In Table 3, page 1759, all values for "mois-ture:protein" and "moisture:fat" must have the colon replaced by a period; for example, 4:3 should read 4.3. Also, in Table 4, page 1760, the emulsifying capacity for Paoli MDP should be 153.7 instead of 53.7.

J. Food Sci. 47(6): 1965–1972 (1982). Gel characteristics – Structure as related to texture and waterbinding of blood plasma gels by Anne-Marie Hermansson. On page 1968, Figures 7 and 8 were reversed. The correct figures with appropriate captions follow:
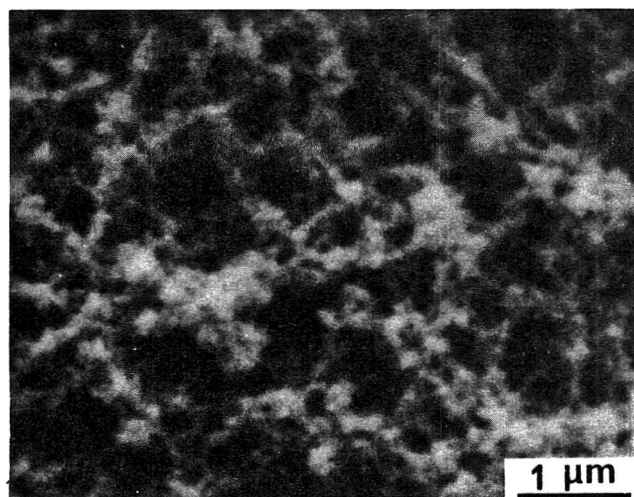
Fig. 7—SEM micrograph of a gel made at pH 9 and heated to 92°C at a magnification of 15,000x.
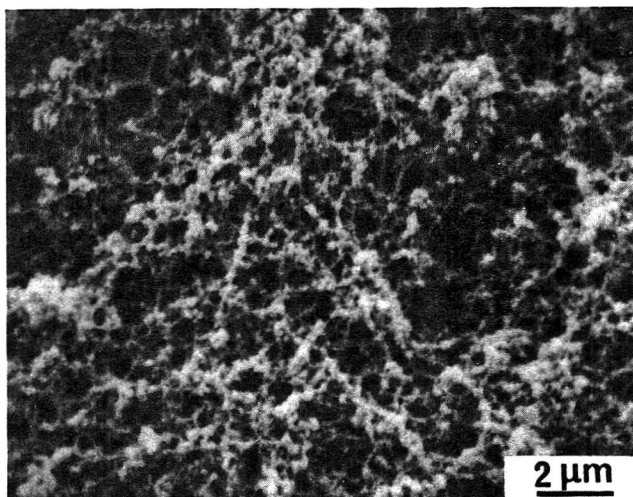
Fig. 8—SEM micrograph of a gel made at pH 7 and heated to 77°C at a magnification of 5,000x.

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