5 September-October 1987
Volume 52, No. 5

Coden: JFDAZ 52:1121–1444
ISSN: 0022-1947

JOURNAL OF FOOD SCIENCE

Basic Research, Applied Science and Engineering

A PUBLICATION OF
THE INSTITUTE OF FOOD TECHNOLOGISTS

Research Notes

AUTHOR INDEX
Volume 52: Number 5

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September-October 1987
Volume 52, No. 5

JOURNAL OF FOOD SCIENCE

ISSN: 0022-1947

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Interactions in Protein/Polysaccharide/Calcium Gels

V.M. BERNAL, C.H. SMAJDA, J.L. SMITH, and D.W. STANLEY

ABSTRACT

Protein-polysaccharide-calcium gels were prepared using combinations of crude myofibrillar protein, whey protein concentrate, sodium alginate, methylcellulose, low methoxyl pectin, carrageenan and $CaCl_2$. The nature of the interactions taking place in the gels and the effect of freezing or heating on viscosities and water losses were investigated. In most cases the stability of the gels was increased by freezing and lowered by heating. Myofibrillar protein with added whey protein concentrate formed stronger gels with the polysaccharide gums than did the myofibrillar protein extract alone. With the exception of methylcellulose gels, electrostatic interactions seemed to be the main forces involved in the formation and stability of the gels.

INTRODUCTION

INTERACTIONS between polysaccharides and proteins occur widely in biological systems. Their interaction in food systems often plays a role in determining the functional properties of these systems (Stainsby, 1980). Understanding the mechanisms involved in the interactions between proteins and polysaccharides and the way in which these interactions are affected during processing is important when these components are added into foods to improve their functional properties (Ledward, 1979; Stainsby, 1980). Although the evidence seems to indicate that the major forces responsible for these interactions are electrostatic in nature (Imeson et al., 1977), other interactions such as hydrogen, hydrophobic or covalent bonds may also be significant in the stabilization of these complexes. Before the potential of polysaccharides as ingredients in protein foods can be fully realized their impact on functional properties must be understood.

When foods containing protein or polysaccharides are heated, gelation often takes place. This process involves the orderly interaction of macromolecules to form a three-dimensional matrix structure. In polysaccharide gels such an ordered network will be formed through specific and regularly interrupted chain-to-chain interactions between two or more aligned chain segments (Dea, 1982). Because these interactions involve relatively weak forces (e.g., hydrogen and ionic bonds), cooperative association of a large number of interactions will be necessary to obtain a firm polysaccharide gel structure. In the case of protein gels, the molecules may be native or denatured and the formation of covalent crosslinks is not essential (Van Kleef, 1986). However, most food protein gels involve heat denaturation followed by aggregation. An ordered structure will be promoted if aggregation is relatively slower than denaturation, allowing the denatured molecules to orient themselves in a systematic fashion prior to aggregation (Hermansson, 1978). Thus, conditions that retard intermolecular interactions will result in a more homogeneous and regular network and consequently a stronger gel. Protein denaturation usually increases the availability of potential interaction sites and the flexibility of the protein chains. The formation of protein gels, either pure or mixed, will involve the same interactions that stabilize the native protein structure (i.e., hydrogen, hydrophobic and electrostatic bonds). The nature of these forces and the effect that processing (freezing, heating) may have on them will influence the rheological properties of the gel. While electrostatic and hydrogen bonds will become weaker upon heating, disulfide bridge formation will be promoted and hydrophobic interactions will be enhanced. The opposite effects are observed upon cooling.

Whether a specific polysaccharide could form a heat-induced mixed gel with a food protein would depend on its ability to interact with the protein. Interaction may be enhanced if the forces that stabilize the polysaccharide gel structure are compatible with those present in the food protein gels. The use of model systems where the composition can be controlled and the interactions between the constituent molecules can be evaluated may provide a more meaningful understanding of the correlation between the functional properties of the system and the effects observed during the manufacture and preparation of a specific food product. The nature of the forces involved in the interactions which lead to the formation and stabilization of a gel can be investigated through the addition of agents that promote or prevent these interactions. High salt concentrations weaken electrostatic interactions; by suppressing electrostatic repulsion, some salts may enhance hydrophobic interactions (Cheftel et al., 1985). Three salts that can be used to study protein-polysaccharide interactions are NaCl, NaSCN and urea. NaCl and NaSCN have charge neutralization effects; NaSCN can also destabilize hydrophobic interactions but only at high concentrations (>1.0M). Urea disrupts electrostatic and hydrogen bonds and influences water structure, increasing the solubility of hydrophobic amino acids and, therefore, affecting hydrophobic interactions (Cheftel et al., 1985). Propylene glycol is another reagent that affects water structure; it may disrupt hydrophobic forces and promote hydrogen and electrostatic bonds (Utsumi and Kinsella, 1985). Mercaptoethanol reduces the inter- and intramolecular disulfide bonds that are frequently involved in heat-induced protein-protein interactions. The effects of these reagents on the viscosity and water holding properties of the protein-polysaccharide gels should provide some insight into the interactions involved in the formation and stabilization of gel systems. It may be possible to utilize the information generated by this type of research to formulate easy to form protein-polysaccharide gels that have enhanced thermal stability (to both freezing and heating), high adhesiveness and other desirable characteristics (Mohamed and Stainsby, 1984). Thus, the objectives of this study were: (1) to determine the nature of the protein-polysaccharide interactions occurring in mixed gels formed by combining polysaccharides and $CaCl_2$ with meat proteins and, in some instances, with whey protein concentrate, and (2) to study the effects of freezing and heating on these interactions.

MATERIALS & METHODS

A CRUDE MYOFIBRILLAR PROTEIN preparation (MP) was obtained as follows. Beef muscle obtained immediately after slaughter was trimmed, ground, mixed with Guba-Straub solution (1:1:1 mixture of 0.3M KCl, 0.1M KH_2PO_4 and 0.05M K_2HPO_4, pH 6.5, 4°C; 100g muscle/300 mL solution) and homogenized at 4°C for 15 min in a Waring Blendor. The suspension was allowed to settle overnight at 4°C. The precipitate was collected by centrifugation at 5780 × g for 1 hr at 0°C; pellets were redissolved by adding a small amount of 0.6M KCl and brought to pH 7.0 with 0.1M NaOH. Equal volumes

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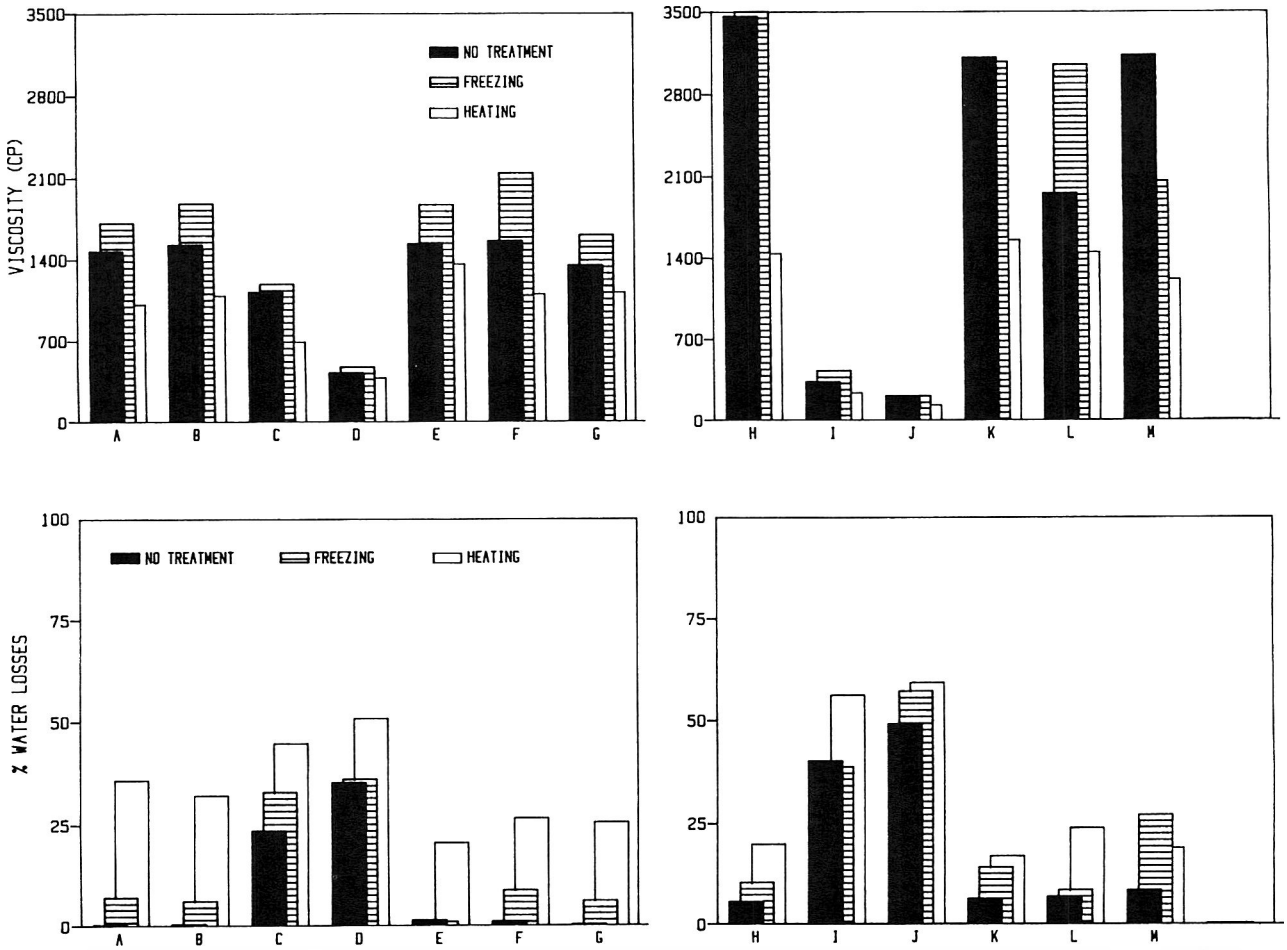

Fig. 1—Viscosity and water losses of high viscosity sodium alginate/$CaCl_2$ gels. (A) control (no protein added); (B) myofibrillar protein (MP); (C) MP + NaCl; (D) MP + NaSCN; (E) MP; pl propylene glycol; (F) MP + mercaptoethanol; (G) MP + urea; (H) whey protein concentrate (WPC); (I) WPC + NaCl; (J) WPC + NaSCN; (K) WPC + propylene glycol; (L) WPC + mercaptoethanol; (M) WPC + urea.

of extract slurry and glycerol were mixed thoroughly and then pressed through two layers of cheesecloth. The filtrate was refiltered by gravity through four layers of cheesecloth and stored at −18°C for the duration of the study. Myofibrillar protein pellets were obtained by ultracentrifugation at 64000 × *g* for 30 min at 4°C.

Genugel carrageenan (exact composition unknown) and Genu low methoxyl pectin (34% esterification, 16% amidation) were obtained from Foodpro National Inc. (Lachine, PQ). High and low viscosity sodium alginates were obtained from Kelco (Division of Merck Co. Inc., Rahway, NJ). Methylcellulose was obtained from Dow Chemical Co. (Midland, MI). Whey protein concentrate (WPC), was obtained from New Zealand Milk Products Inc. (Petaluma, CA). Analytical grade NaCl, NaSCN, urea and propylene glycol were obtained from Fisher Scientific (Fair Lawn, NJ). Mercaptoethanol (MSH) was obtained from Kodak (Eastman Kodak, Rochester, NY). All other reagents used were analytical grade or better.

Solutions of the polysaccharide gums (1 % w/v) were prepared with deionized water in which 0.1% w/v $CaCl_2$, protein (either 1% MP or a 0.5% MP/0.5% WPC mixture), and the specific stabilizer/destabilizer reagent had been previously dissolved. The calcium/polysaccharide ratio in each solution was 1:10 (w:w). The addition of protein was followed immediately by the addition of polysaccharide. The sols were homogenized for 5 min in a Sorvall omnimixer and allowed to gel at room temperature (22°C). Some of the samples had no protein or reagents added; protein-containing gels will be referred to as mixed gels.

Viscosity was measured at room temperature using a Haake Rotovisco RV3 rotational viscometer (Haake Inc., Saddle Brook, NJ) equipped with an RV 467 (MV II) rotor at 64 rpm. Water losses were determined as percents from the volumes measured before and after centrifugation of 10 ml aliquots of each sample at 41000 × g for 15 min at 22°C.

Initial viscosity and water loss measurements were taken after the gels had been covered and aged at room temperature (22°C) for 2-3 hr. After measurement, the gels were placed in a −18°C freezer for 3 hr, removed and thawed at room temperature. Viscosity and water loss of these samples (denoted as FT values) were measured at least 1 hr after equilibration. Portions of FT samples were heated to 80°C in a water bath, held at this temperature for 10 min and cooled to room temperature. Viscosity and water loss of these samples (denoted HT values) were taken at least 1 hr after equilibration.

To study the nature of the forces involved in the intermolecular interactions in the system, each stabilizer/destabilizer reagent (0.05M NaCl, 0.05M NaSCN, 10% propylene glycol, 0.02 M mercaptoethanol or 0.05M urea) was added to a gel prepared from each of the polysaccharide-protein combinations used (1% MP or 0.5% MP - 0.5% WPC). The viscosity and water loss of these gels were evaluated as described above. Analysis of variance and Duncan's multiple-range test were performed using the SAS (1982) statistical procedures.

RESULTS & DISCUSSION

THE RESULTS indicated that, with the exception of the methylcellulose (MC) gels, electrostatic forces seemed to be the main forces involved in the formation and stability of the protein-polysaccharide gels studied. These interactions likely involve negatively charged carboxyl groups in the polysaccharide and positively charged side chains of the amino acids in the protein (Imeson et al., 1977; Ledward, 1979).

Alginates formed the most viscous gels; these gels are known to be thermostable (King, 1984). Figure 1 shows the results obtained with the high viscosity sodium alginate (HVA). In the gels containing 1% MP, significant ($P<0.01$) viscosity decreases were produced by NaSCN, NaCl and urea; initial significant ($P<0.01$) water losses were produced only by NaCl and NaSCN. Significant water losses were observed in all gels

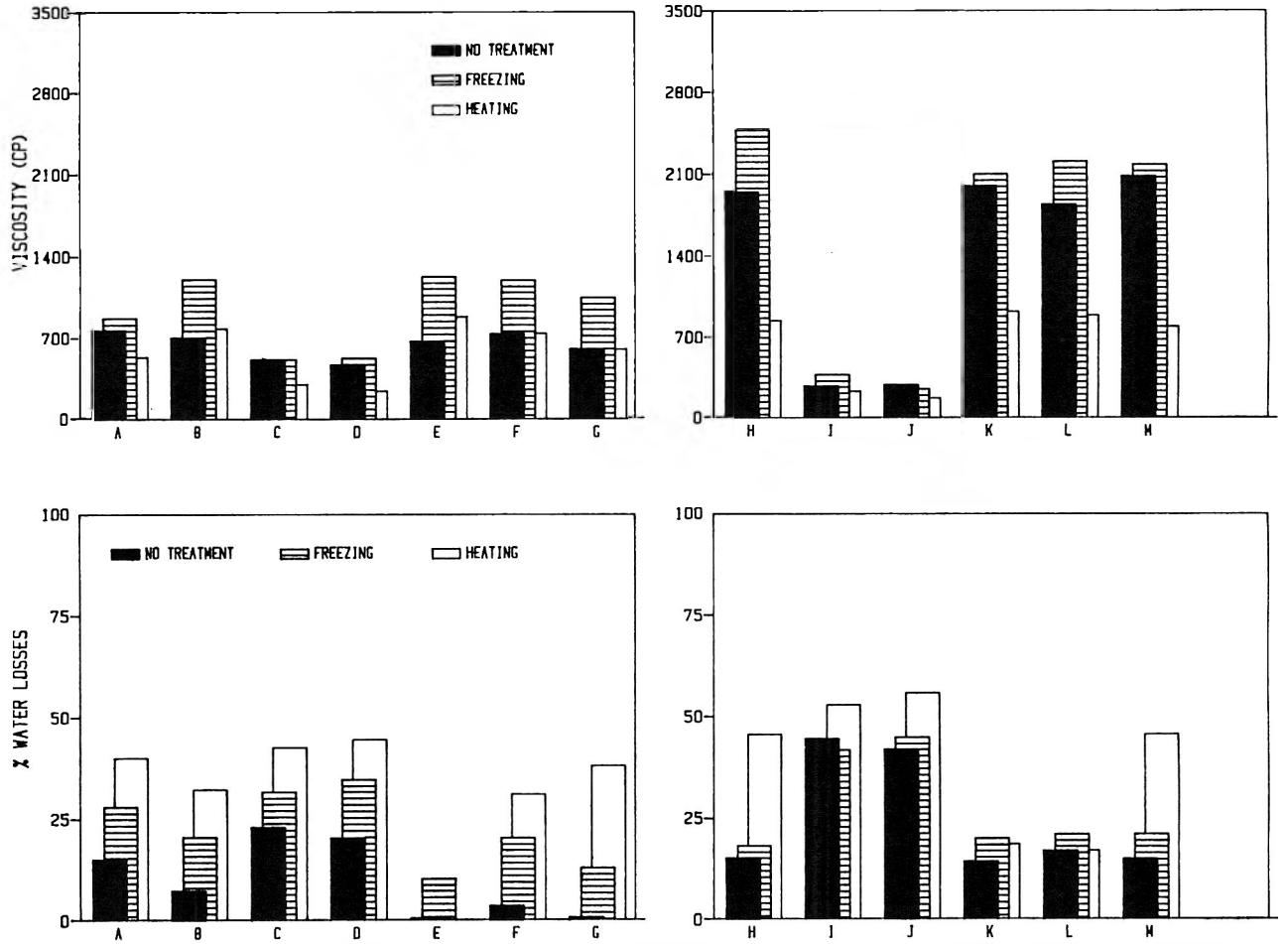

Fig. 2—Viscosity and water losses of low viscosity sodium alginate/$CaCl_2$ gels. (A) control (no protein added); (B) myofibrillar protein (MP) (C) MP + NaCl; (D) MP + NaSCN; (E) MP + propylene glycol; (F) MP + mercaptoethanol; (G) MP + urea; (H) whey protein concentrate (WPC); (I) WPC + NaCl; (J) WPC + NaSCN; (K) WPC + propylene glycol; (L) WPC + mercaptoethanol; (M) WPC + urea.

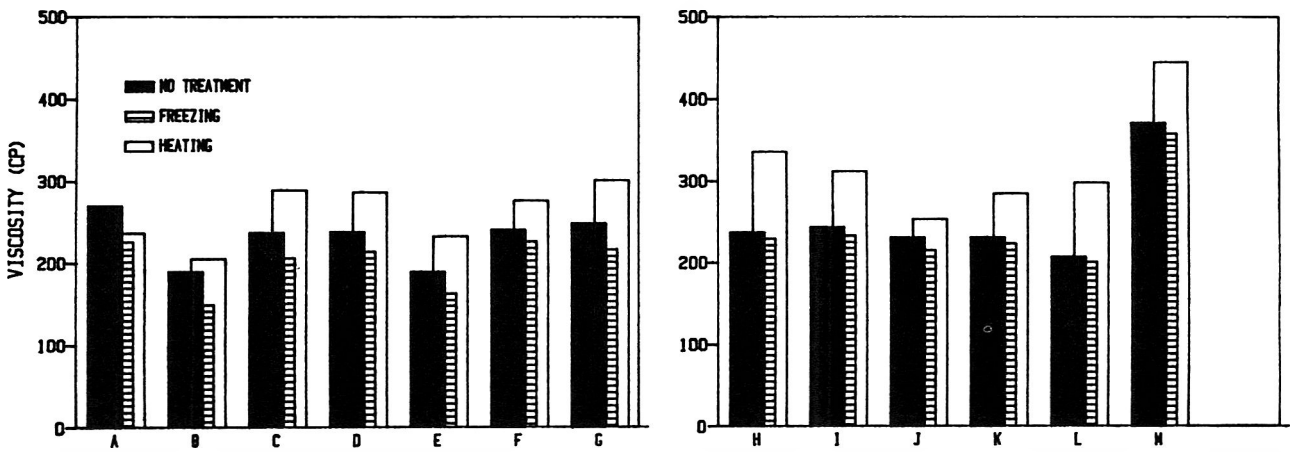

Fig. 3—Viscosity of methylcellulose/$CaCl_2$ gels. (A) control (no protein added); (B) myofibrillar protein (MP); (C) MP + NaCl; (D) MP + NaSCN; (E) MP + propylene glycol; (F) MP + mercaptoethanol; (G) MP + urea; (H) whey protein concentrate (WPC); (I) WPC + NaCl; (J) WPC + NaSCN; (K) WPC + propylene glycol; (L) WPC + mercaptoethanol; (M) WPC + urea.

after heating. With the exception of the gels containing NaCl and NaSCN the replacement of 0.5% MP by 0.5% WPC in the HVA gels produced an overall increase in viscosity and minor, though significant ($P<0.01$), water holding improvements. The detrimental effects of NaCl and NaSCN were more enhanced in the MP/WPC gels. The values obtained for the low viscosity alginate (LVA) samples are shown in Fig. 2. Absolute viscosity values were lower ($P<0.01$) than for the HVA samples; these increased after freezing and were lowered after heating. The effects exerted by the added chemical reagents on these gels seemed similar for both alginates. The major changes in the properties of all the mixed alginate gels were affected by NaSCN and NaCl indicating that the formation of these gels likely involved electrostatic interactions.

The behavior of MC gels was different from that of the alginates (Fig. 3). The addition of both protein components as well as the other chemical reagents decreased the viscosity of MC; significant ($P<0.01$) viscosity increases were produced

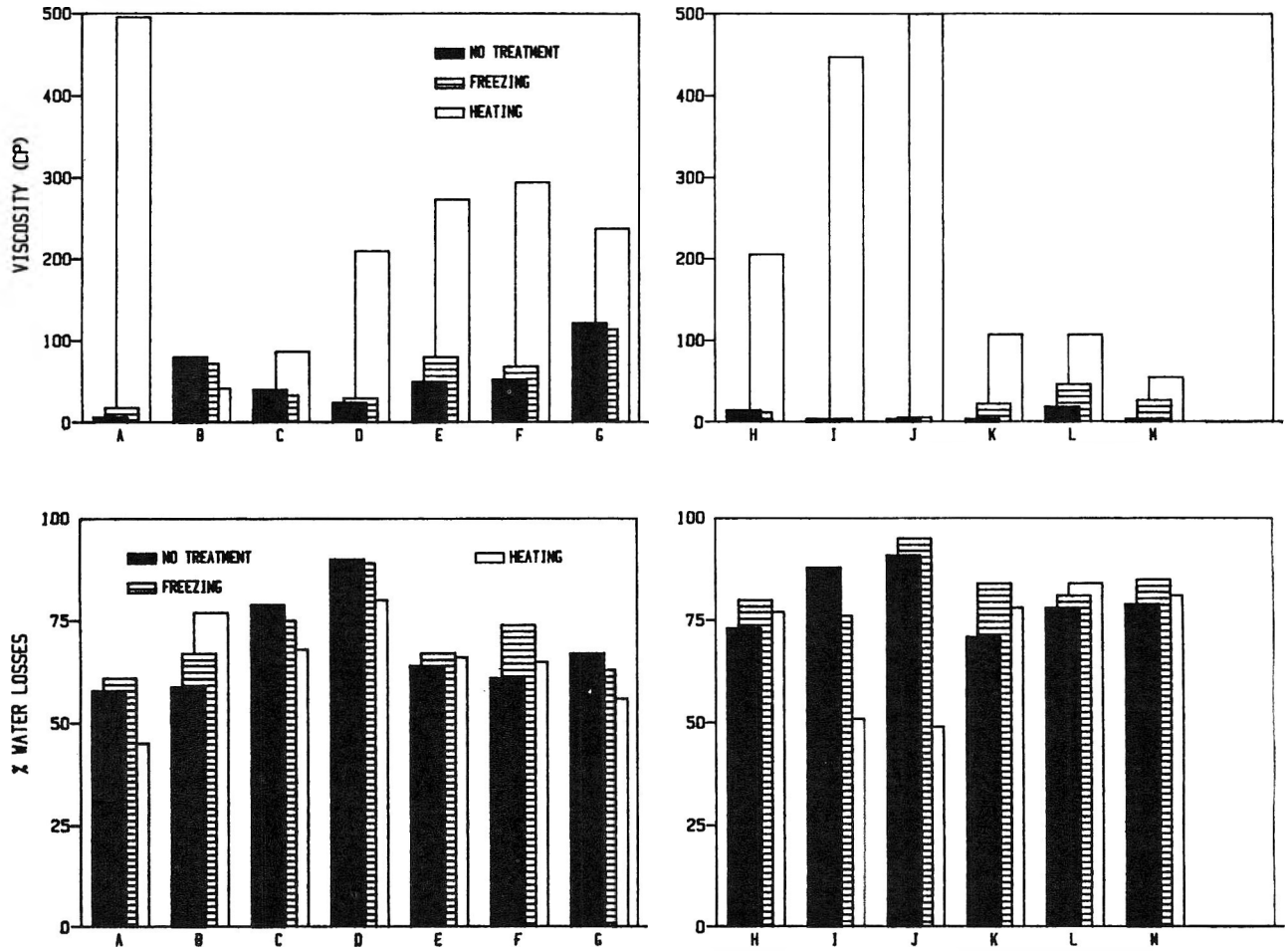

Fig. 4—Viscosity and water losses of low methoxyl pectin/$CaCl_2$ gels. (A) control (no protein added); (B) myofibrillar protein (MP); (C) MP + NaCl; (D) MP + NaSCN; (E) MP + propylene glycol; (F) MP + mercaptoethanol; (G) MP + urea; (H) whey protein concentrate (WPC); (I) WPC + NaCl; (J) WPC + NaSCN; (K) WPC + propylene glycol; (L) WPC + mercaptoethanol; (M) WPC + urea.

only by urea in the WPC/MP gels. In the MP gels, urea, MSH NaCl, and NaSCN increased viscosity ($P<0.01$). In the WPC/MP gels, viscosity was decreased by MSH. The viscosity of all MC gels, significantly ($P<0.01$) lower than that of the alginates, was significantly ($P<0.01$) decreased by freezing and increased upon heating. The control MC gel (no protein added) was the only treatment in which viscosity was higher prior to heating. Both urea and heat unfold the protein structure, probably due to weakening of hydrophobic forces (Lapanje, 1978), and their effect may be mainly exerted on the protein components of the gels, modifying their ability to participate in any interaction with the polysaccharides. Remarkably stable water holding properties were observed in these gels; no water was lost with any of the chemical or temperature treatments. This behavior of the MC gels seemed to indicate that the main forces responsible for their stabilization were not of electrostatic nature.

The initial viscosity of LMP gels containing MP was reduced ($P<0.01$) by the reagents added, with the exception of urea (Fig. 4). Significant ($P<0.01$) increases were observed upon heating in the control (no protein added), the 1% MP gels containing MSH, PG, urea and NaSCN, and in the MP/WPC gels containing no reagent, NaSCN and NaCl. Mitchell et al. (1978) pointed out that this property of LMP to thicken only upon cooling after heat treatment would allow enhanced heat penetration and be an advantage when using LMP as a thickener in canned foods. The water holding properties of these gels were inferior to those of the alginates and MC gels.

Carrageenan formed weak gels having extremely low viscosity and water-holding capacity (Fig. 5). The MP gels had significantly ($P<0.01$) higher viscosities than the control gels. The viscosities of these gels were lowered ($P<0.01$) after freezing and heating. The MP/WPC gels had lower ($P<0.01$) initial viscosities, but these were significantly ($P<0.01$) increased by freezing and sometimes by subsequent heating. Although carrageenan is capable of interacting with kappa-casein (Stainsby, 1980; Modliszewski, 1984), interaction between carrageenan and the proteins used in this study was not apparent. The carrageenan preparation used may have had a composition similar to that cited by Whistler and Daniel (1985) for a commercial carrageenan mixture: i.e., high proportions of kappa (gelling) and lambda (nongelling) carrageenans and a low proportion of iota (gelling) carrageenan. This, coupled with the presence of calcium and sodium (both destabilizers of the predominant carrageenan presumably present) in the gels, could account for failure of the carrageenan preparation to gel. Carrageenan has been used to produce a gelled brine in canned meat products; however, probably due to poor compatibility between carrageenan and meat proteins, the gel forms mostly on the surface and not throughout the product (Modliszewski, 1984). Although carrageenan may help to improve the water holding properties of meat emulsions, it perhaps does so by holding water in the intersticial spaces of the gel network rather than by true interactions with the proteins in the formation of the network.

In protein/polysaccharide/calcium systems the components could interact in more than one way. Both the protein and the polysaccharide could interact on their own, with calcium ions, or with each other, with or without calcium involvement (Hughes et al., 1980). Calcium bridges would maximize interactions between negatively charged molecules and might improve gel firmness and stability. Antonov et al. (1985) were able to pre-

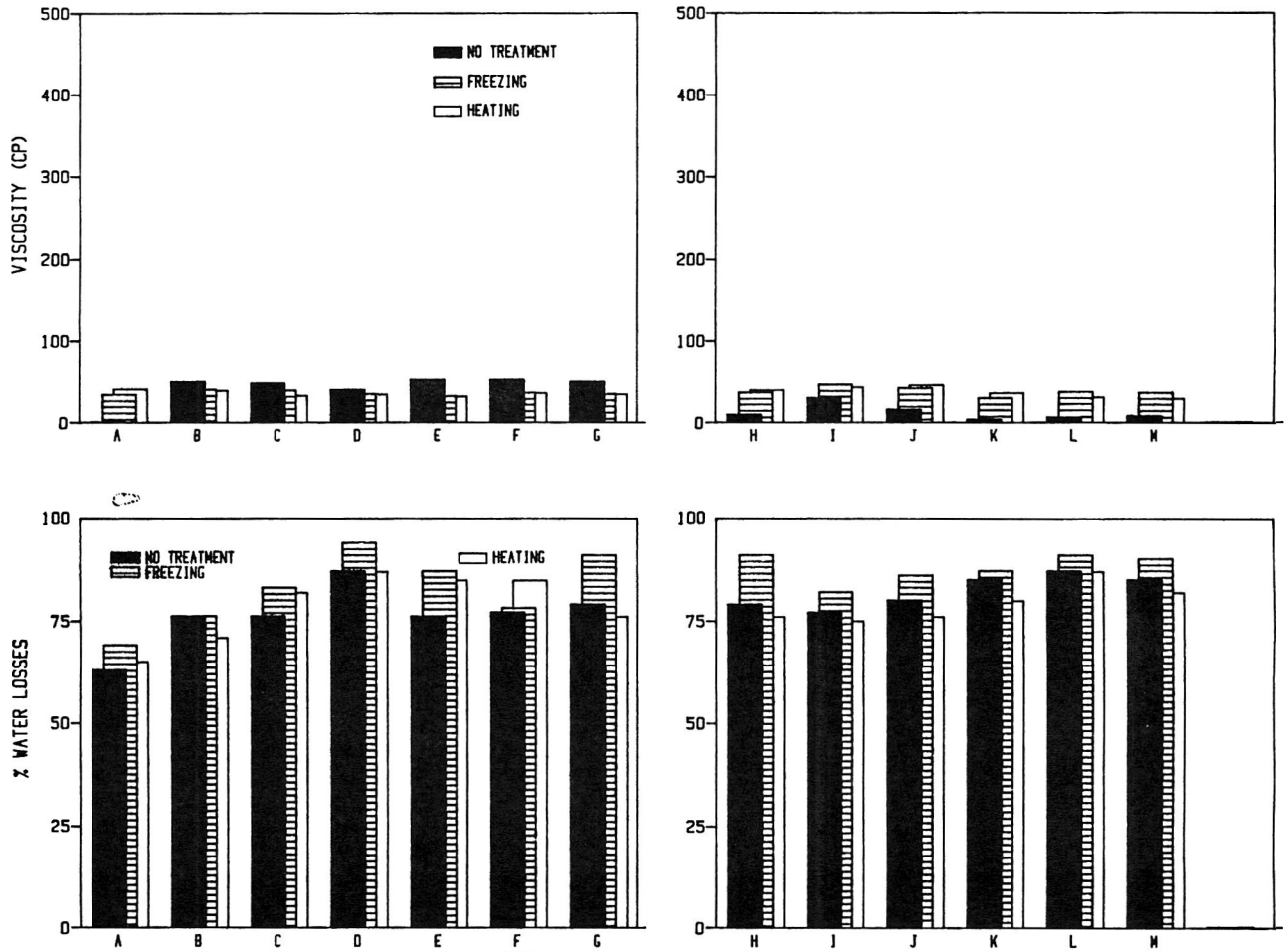

Fig. 5—Viscosity and water losses of carrageenan/$CaCl_2$ gels. (A) control (no protein added); (B) myofibrillar protein (MP); (C) MP + NaCl; (D) MP + NaSCN; (E) MP + propylene glycol; (F) MP + mercaptoethanol; (G) MP + urea; (H) whey protein concentrate (WPC); (I) WPC + NaCl; (J) WPC + NaSCN; (K) WPC + propylene glycol; (L) WPC + mercaptoethanol; (M) WPC + urea.

pare protein-polysaccharide fibers from solutions of casein and sodium alginate coagulated into a bath containing 10% $CaCl_2$ and 5% NaCl; whether the matrix fibers were formed from mixed gels of calcium caseinate and calcium alginate or from complex protein-polysaccharide gels formed with or without the participation of calcium ions was not determined. In some instances, interactions between proteins and polysaccharides seemed to occur both before and after heat treatment. Protein-protein associations at temperatures in the 30–35°C range would occur primarily through noncovalent hydrophobic forces (Liu et al., 1982; Lanier et al., 1982), while during heating, gel structures may be stabilized by the development of disulfide and other covalent bonds. The same may be true in mixed protein-polysaccharide systems. Although protein denaturation during heating would be expected to increase the number of groups in the protein available for interaction, resulting in more stable gels, this effect was not observed. Extensive protein-protein interactions during protein aggregation would prevent the interaction between proteins and polysaccharides. Although reactions involving disulfide groups may be expected to be the main form of protein aggregation, these were not apparent. Thus, if protein aggregation occurred it must have involved other forms of interaction.

Whey protein addition generally resulted in an increase in viscosity in the gels studied in this work. The higher viscosities observed in most of the WPC-containing gels may be the result of the ability of this protein to interact with both MP and polysaccharides, with or without calcium involvement. It is known that if WPC is heat denatured prior to its addition to a meat protein system, extensive whey protein aggregation occurs and the possibilities for interaction between the proteins during the heating of the meat system are drastically reduced (Hermansson, 1975). On the other hand, when whey proteins are concentrated by non-denaturing means (i.e., ultrafiltration), they may be able to interact more extensively with the other components of the gel. In addition, the concentration of calcium ions is known to affect the thermal gelation of membrane-processed WPC (Kohnhorst and Mangino, 1985); high calcium levels may produce excessive protein-protein aggregation that inhibits gelation and reduces gel strength, while a lower calcium content may increase the hardness of the gel through enhanced but moderate crosslinking. These authors also pointed out that although disulfide bond formation may contribute to gel formation, hydrophobic interactions may be more important.

Water may be held within the gel through capillary mechanisms or by hydrogen bonding with positively and negatively charged groups within the gel structure. Since water binding involves electrostatic and hydrogen bonds, it should not be surprising to find that the water holding properties of the gels were affected to the greatest extent by those reagents and treatments known to disrupt these bonds. The effect of freezing on the viscosity of the gels could be partially explained if water exiting during freezing was not able to penetrate the gel structure upon thawing.

Although it was not possible to establish the specific molecular organization of the mixed gel systems studied, some insight into the forces responsible for their formation and their stability during freezing, thawing and heating was gained. Care must be exerted when trying to extrapolate the results obtained with model systems to a more complex system like the one existing in processed food products. The ultimate usefulness

—Continued on page 1136

Influence of Lipid Composition on the Water and Fat Exudation and Gel Strength of Meat Batters

R.C. Whiting

ABSTRACT

Blends of lard with eigh: separate fats or oils (70:30 w/w) were prepared and used as the fat component of meat batters. After cooking, the batters were analyzed for water and fat exudation and gel strength. None differed significantly from the control of 100% lard. Positive correlation coefficients, however, were significant between percent monounsaturated triglycerides and water exudation, polyunsaturated triglycerides and fat exudation and saturated triglycerides and gel strength. Addition of lecithin, cholesterol or methyl palmitate to the lard increased fat exudation and decreased gel strength. Sodium laurate had the opposite effect whether added to the lard or to the aqueous phase.

INTRODUCTION

LOWERING SALT LEVELS in comminuted meat products to reduce dietary sodium intake necessitates a stringent process control to avoid failure of the meat batter. This requires more complete knowledge of the factors that lead to a successful product.

The physical structure of meat batters has been described both as an emulsion and as a suspension of solid fat particles (Swasdee et al., 1982; Honikel, 1983). If it is a true emulsion, the chemical composition of the lipid phase would be expected to affect the physical properties of the batter. If the batter is a suspension, the hardness and melting point of the fat would be the major controlling parameters.

Initial studies on the role of lipid composition in emulsion stability utilized the liquid oil-meat extract model system of Swift et al. (1961). Twenty-six fat and oil samples were tested for emulsification ability (Christian and Saffle, 1967). The magnitude of the differences found between the lipids was small. They noted that more short-chain and monounsaturated triglycerides were emulsified than were other triglycerides. Frazen and May (1968), however, found no difference between various blends of corn oil with coconut oil, linseed oil or lard.

Studies using a meat batter also gave equivocal results. Rendered beef and pork fats were fractionated into four portions having widely differing melting characteristics (Swift et al., 1968). The most stable batters were produced in the portion with the highest melting temperature. Chicken frankfurters made with chicken, beef or pork fat or cottonseed oil were firmest with beef fat or cottonseed oil when tested at ambient temperature but not when heated (Baker et al., 1969). Townsend et al. (1971) found that frankfurters made with beef fat, pork fat and cottonseed oil had similar physical properties, although beef fat required a higher chopping temperature. Histological examination of these frankfurters showed the hardness of the fats was related inversely to the ease of dispersing the fats into a fine, stable emulsion (Ackerman et al., 1971). Chattoraj et al. (1979) reported that the poor dispersibility of sheep and goat fat was improved by mixing with 50% peanut oil. Lee et al. (1981a, b) concluded the emulsion stability and physical properties of the frankfurter were directly related to the fat hardness and melting properties.

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The purpose of this work was to determine whether the lipid phase affects the functional properties of a meat batter. The forming and cooking of the batters closely modelled commercial manufacturing and this work would determine the batter stability through the heat setting of the gel. Composition of the batters was changed from the standard formulation and the resulting changes in the three functional properties of the batter (water exudation, fat exudation and gel strength) were monitored. The understanding of the physiochemical nature of a meat batter might lead to improvements in the physical characteristics of batters made with reduced levels of salt.

MATERIALS & METHODS

Materials

Fresh beef bottom rounds and pork back fat tissues were obtained from local abattoirs. The beef was trimmed of fat and gross connective tissue and stored at 1°C. Both fresh and frozen adipose tissue was used. Samples were analyzed for protein, fat, and moisture by Tecator Kjeldahl, Soxhlet and oven drying methods, respectively (AOAC, 1984). The beef lean and pork fat were ground separately through a 3/16 in. (5 mm) plate prior to use.

Fat was rendered from ground pork adipose tissue by heating on a hot plate under a stream of nitrogen up to a maximum temperature of 125–145°C. The liquid lard was filtered through glass wool and blended while liquid (ca 100°C) with other natural fats and oils at a ratio of 70:30 (w/w). The blends were cooled at room temperature (22°C) before storing at 1°C. Cocoa butter was obtained from Cortes Hnos & Co., Cpor A (Dominican Republic); coconut oil from Industrial Products Group, Stokely Van-Camp Inc. (Indianapolis, IN); olive oil from Filippo Berio (Lucca, Italy); tallow and hydrogenated tallow from Michigan Shortening Co. (Detroit, MI); commercial sunflower oil from Wesson; and safflower oil from Hollywood Health Foods. Butter oil was obtained by melting butter and decanting off the fat through glass wool to remove precipitated proteins. Other lipids were reagent grade except for commercial soybean lecithin. These were blended into a liquid lard in quantities from 1 to 5% (w/w).

Formulation and processing

Procedures for making batters and analyzing for fat and water exudations and gel strength were similar to those used by Whiting (1984). The standard batter formulation of 180g contains 95g lean beef, 49g pork lard, 32g ice and 3.6–5.0g NaCl. The batter composition would be approximately 12% protein, 25% fat, 59% water, and 2.0–2.8% salt. All ingredients were added to the chopping bowl of a food processor (Cuisinart CFP-9) and chopped with brief interruptions to scrape the sides of the bowl and to measure the temperature until 16.0 ± 0.5°C was reached (ca 85 sec). The pH of the uncooked batters was measured by insertion of a combination electrode and automatic temperature compensator directly into the batter.

Three 30 ± 0.1g aliquots of the batter were weighed into 50 mL glass centrifuge tubes (i.d. 2.5 cm) and centrifuged at 200 x *g* for 10 min. The centrifuge tubes were stoppered and placed into a 70–75°C water bath to cook for 30 min. Immediately after removal from the water bath, the water and fat exudates were decanted into calibrated conical 15 mL centrifuge tubes for measurement.

The gel remaining in the centrifuge tube was allowed to cool to room temperature. Gel strength was determined by placing the centrifuge tube vertically in a rack placed on the platen of an Instron Universal Testing Machine, forcing a 1/4 in. (0.64 cm) diameter, flat-bottomed rod through the gel at 50 mm/min and recording the maximum force (grams) of the initial penetration.

Analytical procedures

Thermal properties of the fat blends were determined on a differential scanning calorimeter (Perkin Elmer 990 Thermal Analyzer). The first warming curve of a 30 mg sample was measured by heating from -10°C to 70°C at a rate of 5°C/min. The percent melted fat at 16°C was approximated by dividing the area of the calorimeter curve below 16°C by the total area and multiplying by 100.

The iodine number of the fat blends was determined by the Wijs method (AOAC, 1984). The hardness of the blends was measured by keeping the samples that had cooled overnight at 1°C on ice until immediately before measurement on the Instron. A 1/4 in. (0.64 cm) diameter flatbottom plunger was forced into the fat sample at 100 mm/min. The peak force (grams) of the initial penetration was recorded.

Statistical analyses

Three analytical tests were averaged from each batter to make a single replicate. The data were analyzed by analysis of variance using Duncan's multiple range test and Dunnett's test to compare each treatment to the control, both at the 95% probability level (Steel and Torrie, 1960). Simple correlation coefficients were also calculated using each blend as a data point ($n = 9$).

Results & Discussion

THE WATER EXUDATION TEST measured the tendency of a frankfurter batter to lose water during smokehouse cooking (Meyer et al., 1964; Townsend et al., 1968). Fat exudation indicated whether a batter would fail resulting in fat caps inside the casing or fat losses when reheated by the consumer. Penetration force was a simple measure for the strength and elasticity of the heat-set gel. These three functional properties also give an indication of the biochemical interactions of the meat proteins, specifically protein-water, protein-lipid, and protein-protein, respectively.

The standard salt level (2.2%) was varied from 2.0–2.8% for different batches of meat so that the control batch exhibited measurable water and fat exudations. Changes in lipid composition could then show an increase or decrease in the exudations.

The thermal characteristics of the blends containing 30% natural lipids in lard show a wide variety of melting behaviors (Fig. 1). The first heating curves were used as they would reflect most closely the phase changes of fat during the commercial frankfurter manufacture (Rossell, 1967). However, rendered pork fat does not have thermal behavior identical to pork adipose tissue (Swift et al. 1968; Townsend et al., 1968). Also, rendered fats are generally known to be inferior to adipose tissue for making meat batters (Honikel, 1983).

Peaks indicating phase changes between -10° to 5°C were unsaturated triglycerides, and transitions above 20°C were from saturated triglycerides. Composition of the blends was calculated from data in USDA (1979) Handbook 8-4 (Table 1). Safflower and sunflower oils were high in polyunsaturated triglycerides, olive oil contains large amounts of monounsaturated fats (C18:1); coconut oil has lauric acid (C12:0); butter oil contained triglycerides of short chain fatty acids; coconut, cocoa butter, tallow and hydrogenated tallow were higher in saturated triglycerides than lard. The iodine numbers of the blends reflected the compositions; lard had a normal value of 65 (Dugan, 1971), the blends high in polyunsaturated lipids (safflower and sunflower) and monounsaturated lipids (olive) had higher values. The blend with hydrogenated tallow has the lowest iodine number.

The percent lipids in the blends melted at 16°C ranged from 16 to 75%. For lard and most blends, the 16°C chopping temperature was between the two major endothermic peaks. Pork fat does not liquify below 20°C (Honikel, 1983). Townsend et al. (1968) noted that the instability of emulsions comminuted to temperatures above 18.5°C coincided with the onset of melting of the high-melting portions of the fats.

The hardness was also related to the composition, thermal behavior and iodine number, but the range was much greater. At 1°C the safflower, sunflower, and olive oil blends were very soft and nearly liquid, whereas coconut oil, cocoa butter and hydrogenated tallow blends were very hard.

Despite large differences in composition and physical characteristics of the blends, the water and fat exudations and gel strength did not differ (Dunnett's test $p \geq 0.05$) from the 100% lard control. Although fat exudation was higher for the highly unsaturated blends (safflower, sunflower and olive), differences from the lard control were nonsignificant. However, the correlation coefficient between percent saturated fats and fat exudation was -0.78 and between percentage polyunsaturated fats and fat exudation was 0.64, both significant ($p \geq 0.5$) (Table 2). This resulted because the fat compositions of the blends were used when calculating the correlation coefficients, whereas they were not for the analysis of variance. The correlation coefficients were therefore, more sensitive measures of an effect of lipid composition. Because compositions were expressed as percents of the three classes of triglycerides, if one of the three components were positively correlated,there would be a tendency for another to be negatively correlated. The iodine number was also significantly correlated to fat exudation. In emulsions containing 22% fat, Swift et al. (1968) found the high melting fractions (saturated triglycerides) produced more stable emulsions.

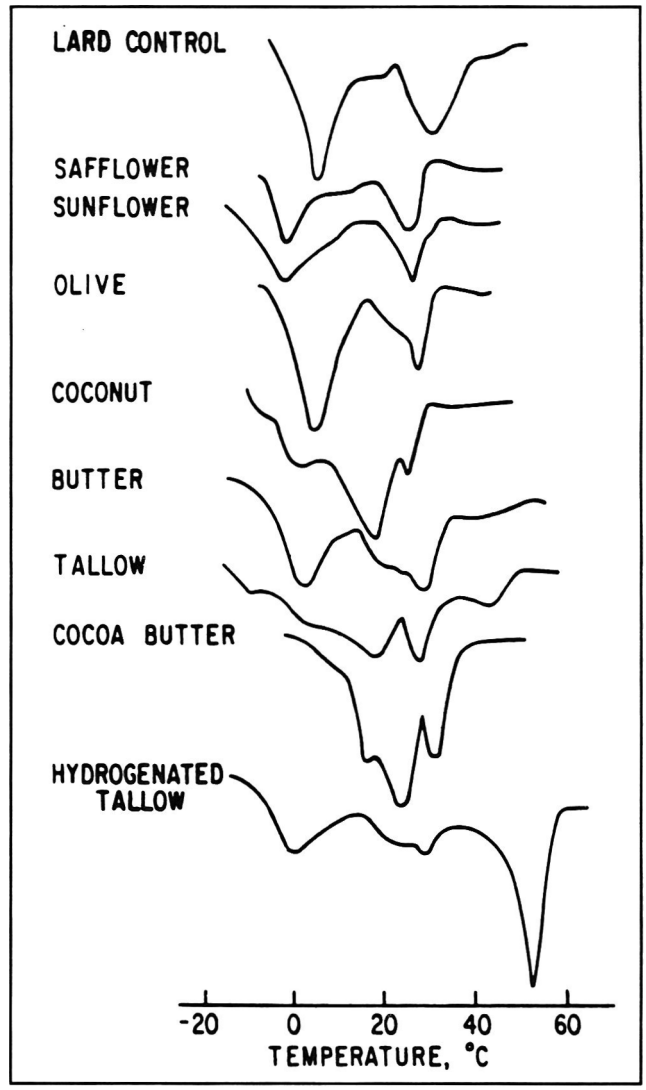

Fig. 1. Thermal behavior of blends of 30% fats or oils in lard. Endothermic peaks point downward.

Gel strengths of batters made from any blend did not differ from those made from lard. However, cooked batters made with hydrogenated tallow had greater gel strength than those made from the three highly unsaturated blends (Duncan's multiple range test) (Table 1). Significant correlation coefficients were also found between percent saturated fats and gel strength ($r = -0.91$) and between polyunsaturated fats and gel strength and ($r = -0.68$). That gel strength emulsion stability varied with the fat hardness agreed with studies by Lee et al. (1981a, b). The correlation coefficient between gel strength and fat hardness in this study was 0.82. These observations are consistent with the model of a batter being primarily a suspension of fat particles in a viscous protein matrix and not a true emulsion. Studies have found poorer emulsion stability when the melting point was too high (Ackerman et al., 1971; Chattoraj et al., 1979) but this may reflect a limitation of the equipment's ability to chop the fat into sufficiently small particles.

The effect of additions of relatively small amounts of pure lipids to the lard on the batters is shown in Table 3. The thermal transitions

Table 1—Properties of natural fat blends and the functional properties of meat batters made from the blends

	100% Lard (Control)	Blended fat							
		Safflower	Sunflower	Olive	Coconut	Butter	Cocoa butter	Tallow	Hydrogenated tallow
Calculated composition[a]									
% Saturated	41	31	31	32	55	48	47	44	58
% Monounsaturated	47	36	38	56	34	42	44	46	33
% Polyunsaturated	11	31	29	10	8	9	9	9	8
Iodine Number	65	102	82	78	47	62	55	54	50
% Melted at 16°C	63	62	75	68	62	47	16	45	24
Hardness (g)	273	20	27	23	1773	617	2647	987	3350
Water Exudate[b] (% batter)	9.1	9.4	7.6	12.6	9.4	8.3	10.9	7.4	7.9
Fat Exudate[b] (% batter)	0.6	4.8	6.1	6.7	0.3	0.8	1.9	0.5	2.0
Gel strength[b] (g)	490	189	218	222	497	445	515	503	600

[a] USDA (1979)

[b] All water exudates, fat exudates and gel strengths are not significantly different from lard control ($p > 0.05$) ($n = 3$, each in triplicate).

Table 2—Simple correlation coefficients between triglyceride composition and functional properties

	Correlation coefficient			
	Hardness	Water exudate	Fat exudate	Gel strength
% Saturated	0.84	−0.28	−0.78	0.91
% Monounsaturated	−0.43	0.59	0.23	−0.34
% Polyunsaturated	−0.54	−0.18	0.64	−0.68
Iodine Number	−0.73	0.20	0.77	−0.82

$r \geq 0.63$ for $p \leq 0.05$ ($n = 9$)

were nearly identical (data not shown), and the percent lipid melted at 16°C was relatively consistent. Hardness was also much more uniform then before. The 5% tristearin did decrease the percent melted fat and increase hardness, while addition of 1.25% methyl linoleate plus 1.25% methyl linolenate had the opposite effect. Lecithin, cholesterol and methyl palmitate were generally detrimental to the functional properties. Only sodium laurate improved them.

Frazen and May (1968) noted that concentrations of phospholipid above 150 mg per 100 mg protein in an oil-extracted protein model system increased the amount of oil emulsified. Our lecithin concentrations were less than 5 mg/100g. Meyer et al. (1964) using a similar model system found lecithin did not improve the emulsion stability. Emulsifiers with either high or low hydrophilic-lipophilic balance values (HLB) all decreased stability. Honikel (1982, 1983) reported lecithin had a detrimental effect on cooking losses in Brühwurst mixtures.

In this experiment the physical characteristics of the blends were similar, yet large differences were found in the cooked batters and indicated that the chemical properties of minor components of the lipids would affect the quality of the meat batter. These results suggested interactions between lipids and proteins occurred, indicating, in part, an emulsion.

Because addition of sodium laurate improved the batter, further experiments were conducted with it. Different techniques for adding laurate did not affect laurate's ability to improve functionality (Table 4). All laurate additions were equivalent to 1.4% of the batter. Water exudation for the rendered fat control and the adipose tissue control were the same, but the fat exudation was much greater for the rendered fat control. As expected, the gel strength was less in the rendered fat batter (Honikel, 1983). The water losses and fat exudations were nearly eliminated with any of the laurate additions. Laurate added to the adipose tissue treatments produced the greatest gel strength. Interestingly, laurate was equally as effective when added to the aqueous phase as when added as part of the lipid phase (rendered fat). Therefore, in subsequent tests laurate was added as a dry ingredient to formulations using adipose tissue.

Table 5 shows the effect of differing sodium laurate concentrations in the batter on the three functional properties. There was a 2.5% salt control and a 1.5% reduced salt series with 0–1.4% laurate (0–5% lipid).

Water exudation doubled with reduction in salt, and 0.14% laurate reduced the exudation to an amount equivalent to the 2.5% salt level. Greater laurate additions nearly eliminated water exudation. The effect of laurate on fat exudation was similar; all three laurate additions did not differ significantly from the 2.5% salt control. The gel strength was reduced by the lower salt levels, but the laurate restored it. The gel strength of the reduced-salt batter was different from the 2.5% control at the 94% confidence level and the two higher laurate additions were greater ($p \leq 0.05$) than the 1.5% salt without laurate batter. Unfortunately, an objectionable soapy flavor was noted by the author with all concentrations of laurate used. Although no nitrite or spices which might mask the flavor were included in these batters, batters containing laurate were judged as unlikely to be acceptable at concentrations needed to be effective.

Two other fatty acids, caprylic (C8:0) and myristic (C14:0), were tested to determine whether they might be more effective than laurate (C12:0) and thereby, usuable at lower concentrations (Table 6). Sodium laurate was added at 0.2% and the others were added at equal molarity as fatty acids. Surprisingly, neither was able to improve the three functional properties above the 2.0% salt reference as laurate did. The pH of the raw batters showed the laurate salt increased the pH and the acid form of the other two decreased the pH, but the changes were not considered sufficiently great to account for the observed results. Meyer et al. (1964) commented without presenting data that 3% oleic acid aided emulsification and binding in a sausage emulsion. It is doubtful that such a high concentration of any free fatty acid would be sensorily acceptable.

Triglycerides having increased hardness improve the water and fat exudation and gel strength of meat batters, assuming adequate chopping and dispersion of the fat. This suggested that the batter was a solid suspension with the protein matrix entrapping the fat particles before and after thermal setting of the gel. The gel had to retain the fat when it liquified during the smokehouse cooking and reheating before consumption. The additions of pure lipids suggested that at relatively high concentrations an emulsification effect might also oc-

Table 3—Characteristics and functional properties of meat batters made with lard containing selected lipid components

	Lard (Control)	5% Glycerol	1% Crude lecithin	5% Cholesterol	5% Tristearin	5% Stearic acic	5% Sodium laurate	5% Methyl palmitate	2.5% Methyl linoleate and Methyl linolenate
Iodine #	61	54	67	63	63	76	60	—	71
% Melted at 16°C	56	52	53	60	48	51	52	54	64
Hardness (g)	177	173	137	177	503	150	213	137	68
Water exudate (% batter)	10.6	8.6	17.0[a]	13.5	12.8	11.8	1.6*	11.6	8.4
Fat exudate (% batter)	3.8	0.8	14.6*	14.0*	6.4	4.3	2.1	13.3*	3.3
Gel strength (g)	416	516	186*	157*	336	359	467	154*	365

* Within each row, Dunnett's $p \leq 0.05$ from control ($n = 3$, each in triplicate).

[a] Significance $p \leq 0.06$.

Table 4—Functional properties of meat batters with different sodium laurate additions

	Adipose tissue (control)	Laurate powder added at start of chopping	Laurate pre-mixed into ground adipose tissue	Rendered fat	Rendered fat containing sodium laurate
Water exudate (% batter)	12.3	0.0*	0.1*	12.6	0.2*
Fat exudate (% batter)	0.6	0.0	0.0	4.0*	0.0
Gel strength (g)	437	666*	613*	314	461

* Within each row, Dunnett's $p \leq 0.05$ compared to adipose tissue control ($n = 3$, each in triplicate).

Table 5—Effect of sodium laurate additions on the functional properties of meat batters

% NaCl	2.5	1.5	1.5	1.5	1.5
% Na laurate (% batter)	0	0	0.14	0.56	1.4
Water exudate (% batter)	10.7	16.5*	8.8	0.9*	0.1*
Fat exudate (% batter)	0.3	1.0*	0.5	0.0	0.0
Gel strength (g)	608	419[a]	528	734	643

* Within each row, Dunnett's $p \leq 0.05$ 2.5% salt control unless specified ($n = 3$, each in triplicate).
[a] Significance $p \leq 0.06$

Table 6—Functional properties of meat batters after additions of caprylate, laurate, and myristate

	Control	Sodium laurate[a]	Caprylic acid	Myristic acid
pH	5.41	5.58*	5.37*	5.39
Water exudate (% batter)	7.6	0.8*	8.6	7.0
Fat exudate (% batter)	0.4	0.0*	0.3	0.2
Gel strength (g)	529	592	549	532

* Within each row, Dunnett's test $p \leq 0.05$ ($n = 3$, each in triplicate).
[a] Na laurate added at 0.2% of the batter, the others at equal molarity to laurate.

cur. However, these lipids also affected water binding and gel strength implying that their action was also on the gel matrix and not exclusively at the lipid--aqueous phase boundry.

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I thank J.W. Hampson (ERRC,ARS, USDA, Philadelphia, PA 19118) for invaluable assistance in procuring samples and conducting the thermal analyses.

Portions of this paper were presented at the 46th Annual Meeting of the Institute of Food Technologists, Dallas, TX, June, 1986.

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Influence of Various Salts and Water Soluble Compounds on the Water and Fat Exudation and Gel Strength of Meat Batters

R.C. WHITING

ABSTRACT

Meat batters were prepared in which the sodium or chloride from salt was replaced by other ions. Then the functional properties of the batters were determined by measuring water and fat exudation, and gel strength. Generally cations from groups IA and IIA of the periodic table equalled or surpassed the batters made with sodium only, whereas other cations decreased water binding. Of the anions, bromide, ortho- and pyrophosphates, and citrate increased water retention. Zinc chloride increased fat exudation greatly. Magnesium chloride and sodium pyrophosphate increased the gel strength. Magnesium and calcium chlorides made good batters although they caused a drop of approximately 0.25 pH units. Sodium thiosulfate, sodium borohydride, starch, sucrose, glycerol, arginine and urea improved the water binding and gel strength, while nonionic detergents, monoglycerides and alcohols were very detrimental.

INTRODUCTION

FOR PRODUCTION of successful meat batters with good water and fat binding and optimum texture, the myosin or actomyosin proteins must be suspended-solubilized, denatured and then aggregated by heat to form a gel structure (Gaska and Regenstein, 1982; Acton and Dick, 1984; Ziegler and Acton, 1984). With insufficient suspension-solubilization, there is not enough protein available to form the gel. If extensive aggregation precedes or accompanies denaturation, a floc or precipitate will form. The slower the aggregation step relative to denaturation, the finer and more oriented the gel network will be. Excessive loss of water or fat resulting in a mushy or mealy texture may result from failure to form a gel or from formation of an excessive number of interprotein bonds that decrease the water binding ability and the capillary spaces that trap water. A successful gel is a balance between protein-water and protein-protein interactions. Protein gels were observed to form when conditions changed from promoting solubility to promoting either insolubility or aggregation (Hegg, 1982). Salt is added as the solubilizing agent for the proteins in meat batters; generally a 4.0% brine is necessary to insure a good batter (Acton et al., 1983). Therefore, reducing the contribution by meat products to the dietary sodium necessitates an increased knowledge of the factors important in creating a good meat batter.

Some effects resulting from changing the lipid phase components of the meat batter were noted (Whiting, 1987). Triglyceride composition had only a small influence; lecithin, cholesterol and methyl palmitate, a detrimental influence; and sodium laurate, a beneficial influence on the functional properties. In this paper, various salts and nonionic water soluble substances were added to meat batters to: (1) determine which salts were beneficial or detrimental, (2) examine other water soluble compounds that might affect the batter, (3) provide some insight into the chemical processes that formed and stabilized the batter, and (4) find possible alternatives to sodium chloride.

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MATERIALS & METHODS

BEEF BOTTOM ROUND and pork adipose tissue were obtained from local abattoirs and stored at 1°C until used. All procedures for proximate analyses of the beef and pork, making the batter, measuring the batter's water binding, fat binding and gel strength and statistical analyses were as reported (Whiting, 1987). The standard batter formulation of 180g contained 95g lean beef, 49g pork adipose tissue, and 32g ice. The 0.45M NaCl was calculated on the aqueous phase and corresponded to 2.0% salt based on the entire batter or 3.1% brine. This slightly low salt level was chosen to form a marginal batter which could show either positive or negative changes. All ingredients were added to the chopping bowl of a food processor (Cuisinart CFP-9) and chopped with brief interruptions to scrape the sides of the bowl and to measure the temperature until 16.0 ± 0.5°C was reached. The pH of the uncooked batters was measured by insertion of a combination electrode and automatic temperature compensator directly into the batter.

Three 30 ± 0.1g aliquots of the batter were weighed into 50 mL glass centrifuge tubes (i.d. 2.5 cm) and centrifuged at 200 x *g* for 10 min. The centrifuge tubes were stoppered and placed into a 70–75°C water bath to cook for 30 min. Immediately after removal from the water bath, the water and fat exudates were decanted into calibrated conical 15 mL centrifuge tubes for measurement.

The gel remaining in the centrifuge tube was allowed to cool to room temperature. Gel strength was determined by placing the centrifuge tube vertically in a rack placed on the platen of an Instron Universal Testing Machine, forcing a 1/4 in. (0.64 cm) diameter, flat-bottomed rod through the gel at 50mm/min, and recording the maximum force (grams) of the initial penetration.

The salt additions replaced a molar equivalent amount of NaCl. Nonionic compounds replaced an equivalent weight of water to keep protein, fat and salt levels equal. Level of salts were chosen, in part, to minimize resulting pH changes.

Concentrations were calculated based on the aqueous phase of the batter (i.e., 136g). Additions in Table 4 were dissolved in water, neutralized with 0.1M HCl or NaOH, if necessary, and chilled before incorporation into the batter, unless specifically indicated. Many of these compounds were tested *only* to observe their effects on a meat batter. These experiments *should not* be interpreted to imply safety or legality in food products.

RESULTS & DISCUSSION

THE WATER EXUDATION TEST predicts the batter's loss of water during commercial smokehouse processing (Meyer et al., 1964) and is related to the meat protein's water binding ability. The fat exudation measures the batter's ability to bind and emulsify the fat. Failure to bind fat would result in fat caps during smokehouse processing and excessive fat loss when reheated by the consumer. The penetration force measures the strength of the protein-protein interactions after heat gelation which would be perceived as a frankfurter's firmness.

The effects replacement of NaCl by equivalent molar amount of various salts had on the pH and three functional properties of the meat batter are listed in Table 1. Average values for functional properties of the NaCl controls are typical (Whiting and Miller, 1984; Whiting, 1984, 1987). The pH values are normal for beef and close to the point of batter failure with this salt concentration (Whiting, 1984). Fat exudations were

Table 1 — Effect of selected salts on the functional properties of a meat batter

Salt[a]	M	pH	Water exudate (% batter)	Fat exudate (% batter)	Gel strength (g)
NaCl-Control	0.45	5.59	10.0	0.10	470
NH_4Cl	0.15	5.63	6.0	0.07	530
KCl	0.15	5.65	8.0	0.17	470
LiCl	0.15	5.61	8.0	0.13	550
$MgCl_2$	0.15	5.36	0.0*	0.00	630
$CaCl_2$	0.15	5.33	2.7*	0.00	710*
$CaCl_2$	0.013	5.58	6.3	0.03	540
CuCl	0.014	5.54	19.7*	0.20	490
$ZnCl_2$	0.013	5.43	28.0*	6.60*	430
$FeCl_3$	0.0043	5.44	19.0*	0.70	360
$LaCl_3$	0.0045	5.46	17.3*	0.43	340
$AlCl_3$	0.0044	5.43	19.3*	0.47	390
$SnCl_4$	0.0044	5.31	21.7*	2.00	350
EDTA	0.016	5.41	20.0*	0.57	400
NaBr	0.15	5.63	4.7*	0.10	520
$NaNO_2$	0.15	5.65	8.0	0.07	460
Na_2SO_4	0.013	5.61	6.0	0.07	530
Na_2HPO_4	0.013	5.86*	1.7*	0.03	660
$Na_4P_2O_7$	0.0075	5.77	0.0*	0.00	950*
Na acetate	0.013	5.70	5.0*	0.20	540
Na citrate	0.022	5.78	3.0*	0.10	580

* Means in a column having an asterisk are significant from NaCl control by Dunnett's Test ($p \leq 0.05$)

[a] Salts replace an equal molar quantity of NaCl

generally low in this series, and therefore, only salts which caused detrimental changes in the batter could be identified.

The first set of chloride salts (elements in group IA and IIA of the periodic table and ammonium) were generally equal to or better than the NaCl they replaced. All tended to reduce water exudation and most increased the gel strength relative to the control, although not always significantly. The 0.15M $MgCl_2$ and $CaCl_2$ improved the batter despite decreasing the pH more than 0.2 pH units, a pH change which would be expected to decrease quality or even cause a failure of the batter (Whiting, 1984).

The second set of salts include transition metal chlorides having valences from one to four. These were added in low amounts, replacing very little NaCl, because of their strong ability to lower the pH when the cation binds to the proteins (Whiting and Richards, 1978). Except for stannous chloride, the pH decreases were not greater than with magnesium and calcium chloride in the first set of salts. These metal chlorides were quite detrimental to all three functional properties. Water exudates were generally doubled over the controls. Fat exudations were not as consistent, but addition of zinc caused a large exudation. With such large exudations, gel strengths were not decreased as much as expected, but gels lost their elastic nature and became mushy and mealy.

Addition of disodium EDTA was expected to improve the functional properties by chelating the detrimental metal cations, but the opposite was observed. This form of EDTA did lower the pH but probably it chelated the beneficial and more prevalent magnesium and calcium ions (USDA, 1980).

The last set of additions was sodium salts which tended to raise the pH slightly and decrease the water losses. Phosphates greatly increased the gel strength as well. The beneficial effects of phosphates in meat systems are well documented although the mechanism is still debated. Trout and Schmidt (1984) showed evidence that it was a combination of pH and ionic strength; others (Hamm, 1970) believe phosphates selectively bind to the proteins and increase solubilization and water holding capacity. The sodium sulfate at concentrations equivalent to the sodium orthophosphate did not improve the water binding or gel strength as much. Sodium citrate had a significant beneficial effect on water binding and, to a lessor extent, the gel strength. Its action may be attributed to the increase in pH, increased ionic strength from polyvalency or chelation of cations.

Attempts have been made to fit the effects of ions in meat systems to the Hofmeister series of chaotropic ions (Gortner and Gortner, 1953; Swift and Sulzbacher, 1963, Regenstein, 1984). The Hofmeister series is a ranking based on ability to floculate proteins (Gortner and Gortner, 1953), and this ability may or may not aid binding and gelation depending on the strength of the ion-protein interaction and on the effect of this interaction on protein solubility and aggregation upon heating (Ziegler and Acton, 1984). Wierbicki et al. (1957) found calcium and magnesium chlorides reduced the amount of juice expressed from cooked meat relative to that from sodium chloride or potassium chloride. However, Regenstein (1984) reported the expressible moisture from a cod fish loaf was greater with calcium and magnesium chlorides and showed sodium chloride and iodide to be somewhat better than nitrite or sulfate. Whiting and Richards (1978) found divalent copper and zinc chlorides had a detrimental effect on chicken frankfurters. Polyvalent cations are considered to form salt bridges between adjacent proteins and reduce water binding by tightening the protein matrix. Monovalent copper, however, behaved similarly.

Sodium, potassium, magnesium and calcium chlorides were tested for their batter forming ability when added as the only salt. Table 2 shows results of the four salts at equal ionic strengths. In this series, potassium was poorer ($p \leq 0.05$) than sodium in all three properties. Magnesium was very effective in improving water binding, while calcium was not ($p \leq 0.05$) different from sodium. This reinforces the conclusions drawn from Table 1 and indicates magnesium, calcium and potassium chlorides could functionally substitute for sodium. Cost, flavor and toxicity problems may restrict or prevent their use.

The separate influences of pH and cations were examined (Table 3). The pH of the batters was altered by additions of small amounts to 1N NaOH or HCl at the beginning of the chopping. Lowering the pH by 0.20 pH units to 5.61 was detrimental to all three functional properties. Adding 0.0043M ferric chloride reduced the pH 0.20 units and had an equally detrimental effect on the functional properties. Addition of ferric chloride and raising the pH 0.20 units back to that of the sodium chloride control only partially restored the water and fat exudates and gel strength. Magnesium chloride appeared to act independently of pH. Batters with an addition of 0.19M magnesium chloride were equal to or better than the controls even with the 0.26 pH units decrease. Raising the pH back to 5.80 did not improve the gel strength of the batter.

Table 2—Functionality of meat batters made with NaCl, KCl, $MgCl_2$ or $CaCl_2$ as the sole added salt

Salt	M	pH	Water exudate (% batter)	Fat exudate (% batter)	Gel strength (g)
NaCl	0.45	5.72	7.3[b]	0.17[a]	660[bc]
KCl	0.45	5.80	10.0[c]	0.23[b]	560[a]
$MgCl_2$	0.15	5.48	2.7[a]	0.00[a]	590[ab]
$CaCl_2$	0.15	5.52	7.0[b]	0.00[a]	700[c]

n = 2

[a-c] Means in a column with different superscripts are significantly different by Duncan's multiple range test ($p \leq 0.05$)

Table 3—Effect of salt and pH on the functionality of meat batters

Salt	Treatment	pH	Water exudate (% batter)	Fat exudate (% batter)	Gel strength (g)
NaCl		5.81[c]	3.0[ab]	0.07[ab]	540[bc]
NaCl	Reduced pH	5.61[b]	12.7[c]	0.27[bc]	360[a]
$FeCl_3$		5.60[b]	11.7[c]	0.33[c]	360[a]
$FeCl_3$	Raised pH	5.83[c]	5.3[b]	0.17[abc]	460[b]
$MgCl_2$		5.53[a]	0.3[a]	0.00[a]	550c
$MgCl_2$	Raised pH	5.80[c]	0.0[a]	0.00[a]	550[c]

n = 3

[a-c] Means in a column with different superscripts are significantly different by Duncan's multiple range test ($p \leq 0.05$)

Table 4—Effects of various compounds on the functionality of a meat batter[a]

	pH	Water exudation	Fat exudation	Gel strength
Control	(5.58)	(10.3%)	(0.50%)	(458g)
5% Sucrose	1.00	0.57	0.31	1.06
5% Glycerol	1.00	0.43*	0.53	1.14
6.6% Potato Starch[b]	0.99	0.07*	4.53	1.05
0.25% SDS	1.01	1.17	1.06	0.92
0.25% Triton X-100	1.01	2.36*	15.2*	1.06
0.66% Monoglyceride[b]	1.00	2.16*	22.0*	0.96
0.66% TWEEN 80[b]	1.00	2.96*	54.0*	1.20[d]
0.56% Ethanol	1.00	0.96	1.04	0.89
1.4% Ethanol	1.00	1.06	1.65*	1.03
1.4% Isopropanol	1.00	0.93	1.13	1.29
5% Methanol	1.01	2.11*	6.25*	1.00
0.66% H_2O_2	1.00	1.04	1.36	0.92
0.66% Na Thiosulfate	0.99	0.25*	0.78	1.10
0.25% 2-Mercaptoethanol	1.00	1.11	1.06	0.92
0.1% Na Borohydride[b]	1.14*	0.26*	1.00	1.02
0.4% N-ethylmaleimide[b]	1.00	1.21	1.35	1.05
0.4% Cystine[b]	1.00	0.97	1.41	0.86
5% Dioxan	1.01	0.54	0.81	1.48[c]
4.5% Trifluoroacetic acid	1.01	0.64	0.94	1.42
0.5% Urea	1.00	0.82	0.73	0.98
2.0% Urea	1.01	0.39*	0.27*	1.21
5.0% Urea	1.02*	0.00*	0.00	1.24
0.32% Arginine·HCl[b]	1.00	0.96	0.84	0.90
0.65% Arginine·HCl[b]	1.00	0.89*	0.61*	0.92
1.3% Arginine·HCl[b]	1.02	0.63*	0.36*	0.92
0.25% Na Citrate[b]	1.01	0.72	1.00	1.00
0.50% Na Citrate[b]	1.03*	0.52	1.40	1.41*

[a] Values are ratio of treatment to respective control values. Averages of the control values are in parentheses. *Values in a column with an asterisk are significantly different from their respective control by Dunnett's Test. ($p \leq 0.05$) n = 3 or 4.
[b] Added as powder to batter.
[c] Probability of being the same as the control by Dunnett's test is 0.06.
[d] Mealy texture.

amounts of selected compounds to the batter formulations. Because this table combines a series of experiments made with different batches of meat, the average values for the controls (NaCl only) are given and the values for the treated batches are expressed as the ratio with their respective control. The pH of meat batches varied from 5.3 – 5.9, for example. Within each experimental group, the treated batters were statistically compared to their control by Dunnett's (Steel and Torrie, 1969) test ($p < 0.05$). Although several compounds significantly changed the pH, these changes were small.

Sugars and complex carbohydrates can bind water, stabilize hydrophobic interactions and increase the thermal stability of globular proteins. Sucrose had only a nonsignificant tendency to reduce water and fat exudation. Glycerol significantly decreased the water exudation agreeing with recent findings on frankfurter texture by Lacroix and Castaigner (1985). Potato starch effectively reduced the water exudation. Fat exudations were frequently small in control batters; slightly greater exudations in treatments, therefore, resulted in large ratios even though fat exudation was not excessive.

Nonionic detergents were quite detrimental to the batter. Triton X-100 and TWEEN 80 greatly increased water and fat exudation. The TWEEN 80 gel was very dry and mealy. Van Eerd (1971) used the extracted protein-oil emulsification model to place the HLB values of meat proteins at approximately 14. TWEEN 80 should be a good emulsifier for this HLB value. Van Eerd (1971) found a modest improvement in emulsion stability with it in an oil model system. These data suggested meat batters were more like a gel than an emulsion. An ionic detergent, sodium dodecyl sulfate, did not have a significant effect. The sulfate group may make it too hydrophilic to act as an emulsifier, as sodium laurate was shown to be in the previous paper (Whiting, 1987). The commercial monoglyceride was also detrimental to both water and fat exudations. Honikel (1982) reported brühwurst with mono- or diglycerides or their lactic or citrate esters had only a slight effect on jelly or fat deposits.

Water has a high solvent polarity (Snyder and Kirkland, 1974) with a great ability for dipole interaction and capability to both accept and donate protons. Alcohols reduce the dielectric constant of water and reduce the ability of water to interact with protons. This effect increases with concentration and increasing chain length of the alcohol. They generally act as denaturants to proteins. Methanol (5%) and ethanol (1.4%) were detrimental to the batter's water and fat exudations. Lower ethanol concentrations and 1.4% isopropanol had no effect. Denaturation of the proteins during chopping was detrimental to the meat batter.

Samejima et al (1981) concluded that the heat-induced gelation requires aggregation of the head portion of myosin by formation of disulfide bonds. Kim et al. (1986) used cystine, Na borohydride and N-ethylmaleimide to indicate a role of sulfhydral groups in fish protein gelation at low temperatures (surimi). In our experiments, disulfide oxidation by H_2O_2, retention of sulfhydryls and reduction of disulfides by mercaptoethanol or blockage of sulfhydryls by N-ethylmaleimide had no effect. Promotion of disulfide interchange by cystine also had no effect. Reducing agents, Na thiosulfate and Na borohydride, significantly reduced water exudation. These are general reducing agents and with an absence of any affect from mercaptoethanol or N-ethylmaleimide likely do not involve the disulfides but instead the general reducing potential of meat. Prerigor and fresh meat have better functionality than stored meat, this data suggesting that the greater reducing potential was a factor.

Dioxane and trifluoroacetic acid (neutralized with NaOH before being added to the batter) have high hydrogen boncing abilities and showed a tendency to improve the water binding and gel strength. Urea solubilizes proteins by disrupting hydrogen bonds and stabilizing hydrophobic and peptide groups. The pH was slightly increased but probably not enough to account for the significantly improved water and fat retention observed with addition of 2 and 5% urea. Arginine at 0.65% and higher reduced fat and water exudations. Its hydrophilic side chain can be involved in electrostatic and hydrogen bond formation. These data suggested the importance of denaturing the suspended-dissolved proteins before initiating the protein-protein interactions that form the gel.

Citrate was found to significantly increase the gel strength and to a lesser extent water binding confirming the observation on Table 1. The 0.5% addition raised the pH slightly in a batch of meat having the lowest pH.

This work suggested several alternatives for improving meat batter when made with reduced sodium chloride. Group IA and IIA cations and phosphate, acetate and citrate anions can partially substitute for NaCl. Solubilities must be considered. Calcium and magnesium salts and phosphates, for example, are not very soluble when added together. Starches and other carbohydrates will reduce water exudation. The feasibility of arginine and urea is unknown; however, Stansby et al. (1968) noted that dogfish, which are used for human consumption, contain more than 1% urea.

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Extended Shelf Life of Frankfurters and Fish Frankfurter-Analogs with Added Soy Protein Hydrolysates

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ABSTRACT

Incorporation of a low salt protein hydrolysate into a sausage batter significantly extended the shelf life of the product stored at 25°C. This temperature was chosen for saving the energy costs for refrigeration. The supplemented frankfurters ($a_w = 0.92$) had CFU/g 1/5000 and 1/1000 of that for the unsupplemented controls ($a_w = 0.97$) after a 10-day aerobic and anaerobic storage at 25°C, respectively. However, bacterial counts of fish frankfurter-analogs treated similarly were reduced only slightly. Differences in flavor and texture between the supplemented and unsupplemented frankfurters were nonsignificant. Supplemented fish frankfurter-analogs were found to be significantly softer than the unsupplemented controls.

INTRODUCTION

WATER ACTIVITY of most of the meat products is comparatively high. However, the multiplication of microorganisms in meats depends not only on a_w but also on other factors, such as storage temperature, pH, redox potential (Eh), nitrite level and the competitive flora. In most instances the shelf life of a meat product is regulated by a combined effect of several microbial inhibitory factors (Leistner, 1974). However, a major predictor of the shelf life of a meat product is the a_w value. For the preservation of meat products, NaCl has been used as an a_w-lowering agent or humectant. To decrease a_w to a level at which most microorganisms are inhibited ($a_w < 0.92$), higher than the normal 2–3% NaCl would be required. Guilbert et al. (1981) reported the use of commercial protein hydrolysates prepared by acid hydrolysis as humectants in meat paste products; however, the a_w-lowering effect of these hydrolysates could be attributed largely to NaCl. Although commercial enzymatic hydrolysates were found to be effective in a_w-lowering, bitterness was detected in the meat products in which these hydrolysates were blended (Guilbert et al., 1981). A nonbitter NaCl-free soy protein hydrolysate which was effective in reducing a_w in aqueous solutions and ground beef was developed (Vallejo-Cordoba et al., 1986).

The objectives of this study were to assess the effectiveness of this nonbitter NaCl-free soy protein hydrolysate in extending the shelf life of frankfurters and fish frankfurter-analogs when stored at room temperature for saving the energy cost, and to compare flavor, color and texture between frankfurters or analogs with and without protein hydrolysate supplementation.

MATERIALS & METHODS

Materials

The soy protein hydrolysate was prepared by the acid-enzymatic hydrolysis of Vallejo-Cordoba et al. (1986). Frankfurter sausage ingredients and cellulose casings (2 cm i.d.) were obtained from Intercontinental Packers Ltd., Vancouver, B.C. Fresh ling cod (Ophiodon elongatus) was purchased from MacMillan Fisheries (Vancouver, B.C.). The spice and cure mixtures used for the fish frankfurter-analogs were the same as those used for frankfurter sausages. Paprika and red beet powder used for the fish frankfurter-analogs were purchased from the local market. The bacteriological media used was PCA (plate count agar), a product from Difco Laboratories (Detroit, MI). All other chemicals were reagent grade.

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Frankfurter sausage preparation

Frankfurter sausages were prepared by using a sausage batter provided by a local meat processor which uses the basic formulation shown in Table 1. The sausage batter was divided into two portions, one was supplemented with 15% hydrolysate (pH 5.9) and the other was left as the control (pH 6.5). Sausage batters were mixed for 10 min, stuffed into cellulose casings and tied into 10 cm links. Frankfurters were smoked in a humidity controlled commercial smoking house. The smoking schedule was as follows: 71°C (30 min), 82.2°C (45 min) and 93.3°C (15 min). Smoked frankfurters were showered with cold water and kept at 4°C overnight. Casings were aseptically removed and sausages were placed individually in Ziploc freezer bags or Cryopak bags for storage under aerobic or anaerobic (vacuum pack) conditions at 25°C. Cryopak bags were sealed with a Multivac vacuum sealer (Sepp Haggenmuller KG, Allgau, W. Germany). Approximately 15 samples from each lot were frozen until they were served for the sensory tests.

Fish frankfurter-analog preparation

Frankfurter-analogs were prepared by using the basic formulation shown in Table 2. Fresh ling cod fillets were ground through the coarsest plate (0.9 cm ID) of a Hobart grinder and washed with two changes of iced water. The mince was then stirred into the water and allowed to settle, after which the water was drained from the mince. The drained mince was placed in a cheese press and pressure was applied for approximately 3 min until most of the water was squeezed out from the mince. The pressed mince was frozen for three days at −40°C. Partially thawed mince was ground again and mixed with the rest of the ingredients using a blade Hobart mixer. The mixture was divided into two portions; one was supplemented with 15% hydrolysate (pH 5.87) and the other was left as the control (pH 6.39). Both fish batters were mixed for 10 min, stuffed into cellulose casings and tied into 10 cm links. Frankfurter analogs were steam-cooked for 5 min to reach an internal temperature of 80°C which was maintained for 30 min. After cooking, the samples were showered with cold water and refrigerated overnight at 4°C. Casings were removed and the frankfurter-analogs were placed individually in Ziploc freezer bags or Cryopak bags for storing at 25°C under aerobic or anaerobic (vacuum pack) conditions.

Table 1—Frankfurter formula[a]

Ingredients	Composition (%)
Pork trims	12.02
Mechanically deboned fowl (containing 3% NaCl)	25.06
Beef by-products	27.76
Pork fat	17.17
Corn starch	4.75
Wheat flour	1.02
Spice mixture	1.56
Binder	1.28
Sodium chloride	1.17
Liquid smoke	0.10
Sodium erythorbate	0.08
Cure mixture (containing 6.4% $NaNO_2$ + 93.6% NaCl)	0.26
Water	7.77

[a] This formula contains 2.17% NaCl

Table 2—Fish frankfurter-analog formula[a]

Ingredients	Composition (%)
Fish meat (cod)	94.13
Spice mixture	1.60
Sodium Chloride	2.26
Paprika	1.00
Beet powder	0.60
Liquid smoke	0.10
Cure mixture (containing 93.6% NaCl)	0.26
Ascorbate	0.05

[a] This formula contains 2.5% NaCl

Determination of water activity and moisture of frankfurters and fish frankfurter-analogs

Water activity of triplicate samples of frankfurters and frankfurter-analogs were determined at 25°C after equilibration for 1 hr by using a Hygroskop Rotronic DT DMS (Rotronic Ag, Zurich). Moisture was determined in triplicate by drying in a vacuum oven until constant weight was reached at 95-100°C (AOAC, 1980). The least significant differences between the control and the supplemented products were determined according to Steel and Torrie (1960).

Sensory analyses

Scheffe's paired comparison test (Larmond, 1977) was carried out to investigate flavor, texture and color differences between 15% hydrolysate supplemented and unsupplemented frankfurters or fish frankfurter-analogs. Thawed frankfurters were broiled for 7 min and served in pairs to 10 panelists in partitioned booths. The panelists (6 females and 4 males) were graduate students and lab technicians, age 23–35. They were asked to mark from "extremely firmer than" to "extremely softer than" the controls for texture, from "like extremely more than" to "dislike extremely more than" the controls for flavor evaluation and from "extremely lighter than" to "extremely darker than" the controls for color. These results were subjected to one-way analysis of variance.

Total plate counts

After predetermined storage times, samples were aseptically removed from duplicate pouches of the same lot for microbiological analyses. A 10g sample was homogenized for 30 sec with 90 mL 0.1% peptone solution in a stomacher. The bacteriological media used was PCA (plate count agar). The method used was the Spiral Plate method of Gilchrist et al., (1973); plates were incubated at 35°C for 48 hr. The colony forming unit (CFU) counts were an average of counts on three plates and expressed as CFU per gram of sample.

Determination of nonammonia amino nitrogen as an estimation of biogenic amines

Samples (25g) of frankfurters or frankfurter-analogs which were obtained after sampling at predetermined times (day 0 and 8 or 10) during storage at 25°C were blended with 75 mL 5% (w/v) trichloroacetic acid (TCA) as recommended by Murray and Gibson (1972). The homogenates were centrifuged and the supernatants were filtered through Whatman #1 filter paper. The clear filtrates were kept under refrigeration until assayed for total nitrogen (TVN), trimethylamine (TMA) and amino nitrogen. TVN and TMA in the filtrates were determined by the microdiffusion technique of Beatty and Gibbons (1937) as modified by Bryant et al. (1973). Ammonia was calculated by subtracting TMA from TVN. Ammonium chloride and trimethylamine hydrochloride dissolved in 5% TCA were used as standards.

Amino nitrogen in the filtrates was determined by the trinitrobenzenesulfonic acid (TNBS) method of Camargo et al. (1979) as modified by Kwan et al. (1983). Glycine dissolved in 5% TCA was used as standard.

Nonammonia amino nitrogen was calculated by subtracting the ammonia nitrogen from the total amino nitrogen. Nonammonia amino nitrogen is an indirect estimation of the biogenic amine content in the samples.

RESULTS & DISCUSSION

Water activities of frankfurters and fish frankfurter-analogs

Water activities and moisture of frankfurters and frankfurter-analogs are summarized in Table 3. Water activity of the supplemented sausages decreased significantly ($P<0.05$) to 0.921. Water activity of frankfurter-analogs was significantly ($P<0.05$) decreased by the addition of 15% hydrolysate; however, they were still higher than that of the meat sausages.

The a_w-lowering effect of the hydrolysate in these products may be due to an osmoregulatory effect of free amino acids, low molecular weight peptides and carbohydrates as discussed in the previous paper (Vallejo-Cordoba et al., 1986). This hydrolysate was found to decrease the a_w of distilled deionized water to 0.948 when used at a 20% (w/w) level (Vallejo-Cordoba et al., 1986). However, hydrolysate supplemented products had lower moisture contents due to increased solids in the formulation and therefore, this factor also could have contributed to the effectiveness of the hydrolysate in reducing water activity.

Keeping quality of frankfurters and fish frankfurter-analogs

Total plate counts are summarized in Tables 4 and 5. Storage tests of frankfurters clearly showed improved shelf stability as a result of the addition of 15% hydrolysate.

Supplemented sausages stored under aerobic conditions were microbiologically stable for at least 42 days, whereas the unsupplemented sausages reached unacceptable CFU/g values after 10 days and showed distinct signs of spoilage. The unsupplemented sausages had approximately 5000 and 1000 times more CFU/g than the supplemented sausages after a 10-day aerobic and anaerobic storage, respectively.

The keeping quality of fish frankfurter-analogs was determined after an 8 day storage at 25°C (Table 5). The vacuum packaged, unsupplemented frankfurter-analogs showed bacterial numbers of 60 times more than the supplemented analogs. The hydrolysate was not as effective as in meat sausages since the water activity and moisture of the fish gel (control) was higher than for meat sausages. Thus, even the supplemented analogs had a water activity (0.956) at which most microorganisms could multiply. Further work is required in the development of shelf stable fish products. The improved shelf stability of frankfurters and fish frankfurter-analogs cannot be attributed only to the added soy hydrolysate and its effect on water activity and moisture content, but also to its slight pH reducing effect. The pH of the frankfurters and the frankfurter analogs was not adjusted after the addition of the hydrolysate.

Table 3—Water activities and moisture contents of supplemented and unsupplemented frankfurters and fish frankfurter-analogs (Mean ± SD)

	Frankfurters		Fish Frankfurter-analogs	
Samples	a_w[a]	Moisture[a] (%)	a_w[a]	Moisture[a] (%)
Unsupplemented (control)	0.966 ± 0.010	51.57 ± 1.07	0.977 ± 0.002	77.57 ± 0.65
15% hydrolysate supplemented	0.921 ± 0.019*	50.05 ± 0.27	0.956 ± 0.005*	69.17 ± 0.11*

[a] n = 3

* Significantly different from the control (within columns) according to the least significant differences test.

Table 4—Total plate counts of unsupplemented and 15% hydrolysate supplemented frankfurters stored at 25°C

Storage condition	Sample	CFU/g Day 1	Day 10	Day 18	Day 25	Day 42
Aerobic	Unsupplemented	10^3	30×10^6	66×10^6	24×10^7	19×10^6
	Supplemented	10^3	70×10^2	27×10^3	10^3	11×10^3
Anaerobic	Unsupplemented	10^3	22×10^6	89×10^5	26×10^6	42×10^6
	Supplemented	10^3	18×10^3	40×10^3	45×10^4	74×10^4

Table 5—Total plate counts of unsupplemented and 15% hydrolysate supplemented fish frankfurter-analogs stored at 25°C

Storage condition	Sample	CFU g Day 1	Day 8
Aerobic	Unsupplemented	$<10^2$	26×10^6
	Supplemented	$<10^2$	11×10^5
Anaerobic	Unsupplemented	$<10^2$	42×10^6
	Supplemented	$<10^2$	74×10^4

Nonammonia amino nitrogen as an estimation of biogenic amines

Chemical tests such as the determination of biogenic amines, ammonia and total volatile nitrogen were used as spoilage indices in meat and fish products (Sayem-El-Daher et al., 1984; Hui and Taylor, 1983; Bryant et al., 1973). In this study, chemical tests were carried out to further confirm the presence of spoilage in the frankfurters stored at 25°C as indicated by microbiological analyses. An attempt was made to determine nonammonia amino nitrogen which would provide an indirect estimation of biogenic amines in the samples by using two simple tests (TNBS and microdiffusion). Nitrogen increases of aerobically packaged frankfurters and fish frankfurter-analogs during storage at 25°C are presented in Table 6. These chemical indices of spoilage were used only for products packaged under aerobic conditions since vacuum packaged products did not show nitrogen increases even though bacterial numbers had increased (Tables 4 and 5). The reason for this different behavior might be that biogenic amines and ammonia were produced by the action of specific bacteria, i.e., putrefactive bacteria which grow under aerobic conditions (Gill, 1982). Therefore, the increase in CFU/g of vacuum packaged frankfurters might have been due to non-putrefactive bacteria.

Smaller nitrogen increases for the supplemented sausages (Table 6) indicated that bacterial growth was suppressed and so were bacterial numbers (Tables 4 and 5) upon the addition of 15% hydrolysate. Increases of nonammonia amino-N in the supplemented frankfurters were approximately 1/9 of those of the unsupplemented controls; these results agreed with the decrease in CFU/g in the supplemented sausages to 1/5000 of that for the unsupplemented samples. In contrast, increases of non-ammonia amino-N for the supplemented fish frankfurter-analogs were only approximately half those for the unsupplemented controls. These results further confirmed the small reduction to only 1/60 CFU/g in the supplemented fish frankfurter-analogs.

Total plate counts and chemical indices have shown that the addition of the soy protein hydrolysate improves the stability of frankfurters stored at 25°C. However, even frankfurters with a water activity of 0.92 may allow the growth of Staphylococcus aureus; under anaerobic conditions this organism is reported to be inhibited at $a_w < 0.91$ (Scott, 1953). Therefore, the frankfurters prepared in this study cannot be categorized under 'storable' products (no refrigeration required). According to Rodel (1975), 'storable' products have an $a_w \leq 0.95$ and pH ≤ 5.2 or $a_w \leq 0.91$ or pH ≤ 5.0; perishable meat products have either $a_w \leq 0.95$ or pH ≤ 5.2 and must be stored at or below 10°C and easily perishable meat products have $a_w > 0.95$ and pH > 5.2 and must be stored at or below 5°C.

The frankfurters prepared in this study show a potential for further development in a new generation of foods called "Shelf-Stable Products." These products would have an a_w between 0.90 and 0.95 which require additional "hurdles" to insure microbiological stability of the product. Microbial "hurdles" are defined as intrinsic and extrinsic factors in foods that affect survival and/or ability of a specific microflora to grow and produce specific effects in the food. This "hurdle effect" described by Leistner (1985) include the possible use of one or more of the following factors to insure stability of the product: a_w, pH, redox potential (Eh), thermal process, preservatives and competitive microflora.

Sensory analyses

Paired comparison flavor, texture and color scores of supplemented frankfurters and fish frankfurter-analogs are presented in Table 7. No significant differences ($P > 0.05$) in flavor or texture were observed between the supplemented and unsupplemented frankfurters. Supplemented sausages were found to be significantly ($P < 0.05$) darker than the unsupplemented ones. However, this problem may be solved by using soy protein concentrates; soy flour results in a hydrolysate rich in sugars which cause increased browning during processing. The soy flour protein hydrolysate used in this study contained 34.81% total carbohydrates as previously reported by Vallejo-Cordoba et al. (1986).

No differences ($P > 0.05$) in flavor or color were detected between the supplemented and unsupplemented fish frankfurter-analogs. The supplemented analogs were found to be significantly ($P < 0.05$) softer than the unsupplemented controls. Graininess of the supplemented analogs was a major criticism. It appeared that the hydrolysate interfered with proper gel formation of fish proteins.

In summary, the shelf life of hydrolysate supplemented frankfurter sausages was extended. Energy costs can be saved by storing hydrolysate supplemented frankfurters at room temperature. To achieve the same protection against microbial growth by using NaCl, higher concentrations than the normal

Table 6—Increases of amino nitrogen, total volatile nitrogen (TVN), ammonia, trimethylamine (TMA) and nonammonia amino nitrogen of frankfurters and fish frankfurter-analogs during storage at 25°C under aerobic conditions[a]

Frankfurter	Nitrogen increase[b] mg amino-N/100g	mg TVN/100g	mg NH_3-N/100g	mgTMA-N/100g	mg non-NH_3 amino-N/100g
Unsupplemented	445	65	46	19	400
Supplemented	59	13	13	0	45
Unsupplemented analogs	1200	347	303	44	897
Supplemented analogs	471	58	40	18	430

[a] Frankfurters and frankfurter-analogs were stored for 10 and 8 days, respectively.
[b] Average of triplicate analyses

Table 7—Paired comparison flavor, texture and color scores[a] of frankfurters and fish frankfurter-analogs supplemented with 15% soy hydrolysate

Samples	Flavor[b]	Texture[c]	Color[d]
Frankfurters	−0.5	0	0.7*
Fish frankfurter-analogs	−0.4	1.7*	0.05

[a] Scoring, compared to unsupplemented sausages
[b] 4 = like extremely more, 3 = like very much more, 2 = like moderately more, 1 = like slightly more, 0 = no difference, −1 to −4 = dislike
[c] 4 = extremely firmer, 3 = very much firmer, 2 = moderately firmer, 1 = slightly firmer, 0 = no difference, −1 to −4 = softer
[d] 4 = extremely lighter, 3 = very much lighter, 2 = moderately lighter, 1 = slightly lighter, 0 = no difference, −1 to −4 = darker
* Significant difference ($P < 0.05$), one-way ANOVA

practice would be required. Although the shelf-life of hydrolysate supplemented fish frankfurter-analogs was not as good as the frankfurter counterparts, it does not preclude the possibility of preparing shelf-stable products with texture similar to the unsupplemented control by reducing the moisture content of the products.

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Ms received 12/24/86; revised 3/17;87; accepted 4/9/87.

Paper NO. 49 presented at the Annual Conference of the Canadian Institute of Food Science and Technology, Toronto, ON, June, 1985. This work was carried out according to the terms of contract 04SB.01916-EP22 of the ERDAF Program for Agriculture Canada.

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of food ingredients in these products can be proven only through their direct application.

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Ms received 1/12/87; revised 4/4/87; accepted 4/27/87.

This work was supported in part by the Natural Sciences and Engineering Research Council of Canada, Canada Packers Inc., and the Ontario Cattlemen's Association.

Gamma-Irradiation of *Clostridium botulinum* Inoculated Turkey Frankfurters Formulated with Different Chloride Salts and Polyphosphates

SHAI BARBUT, ARTHUR J. MAURER, and DONALD W. THAYER

ABSTRACT

The effects of gamma-irradiation doses (0, 0.5, and 1.0 Mrad) on *C. botulinum* toxin production in turkey frankfurters formulated with three different chloride salts (NaCl, KCl, and $MgCl_2$) at isoionic strength (equal to 2.5% NaCl) and three types of phosphates added to 2.0% NaCl frankfurters were studied. The use of 2.5% NaCl together with 0.5 or 1.0 Mrad was substantially more effective at inhibiting botulinal toxin production when frankfurters were incubated at 27°C than the combination of irradiation with KCl or $MgCl_2$ (40, 9, and 4 days, respectively, when treated with 1 Mrad). Phosphate addition revealed that 0.4% sodium acid pyrophosphate addition was the most inhibitory for botulinal toxin production followed by hexametaphosphate and tripolyphosphate addition.

INTRODUCTION

SODIUM REDUCTION in the North American diet has been recommended as one of the steps to reduce hypertension and symptoms associated with coronary heart diseases and renal failure (Pearson and Wolzak, 1982). Epidemiological studies related to animals and humans, strongly suggest that the consumption of sodium by some consumers should be curtailed to reduce the development of high blood pressure (Sebranek et al., 1983). Processed meat products contribute an average of about 15% to the total estimated dietary sodium chloride (NaCl) intake. However, even though a large portion of the salt in our diet (25–35%) is added at home (IFT, 1980), the consumer seems to be more concerned with the salt content in prepared food items purchased from a store.

Salt added to processed meat products has three major functions: solubilize salt soluble proteins to provide desired texture upon heating, inhibit bacteria growth including pathogens, and provide and enhance flavor (Ingram and Kitchell, 1967). Various ways to reduce sodium content of meat products have been under investigation during the past few years and were reviewed by Maurer (1983) and Terrell (1983) in poultry and red meat products, respectively. Four main approaches were mentioned: reduction of NaCl only; substitution with other chloride salts; addition of other ingredients like phosphates; and/or alteration of processing techniques.

Seman et al. (1980) reported that 50% replacement of the 2.5% NaCl with potassium chloride (KCl) was acceptable in bologna. In addition the replacements of the 1.25% NACl with 50% KCl or 50% magnesium chloride ($MgCl_2$) plus 0.4% phosphate addition was also found acceptable in this type of product. Hand et al. (1982) found that up to 35% of the total 2.5% NaCl in beef/pork or turkey frankfurters can be replaced by KCl or $MgCl_2$ without changing the overall acceptability of the freshly made product; however, after 6 wk of refrigerated storage the two NaCl replacement treatments were inferior to the 2.5% NaCl treatment in overall acceptability.

The information in the literature suggests that NaCl, even though indirectly, still plays a major role in the preservation of various foods including processed meat products. The published research, however, is too limited to provide answers to the large number of questions that may be raised in relation to partial or total replacement of NaCl and its antimicrobial activity in processed foods (Sofos, 1984). The effects of NaCl on inhibiting *C. botulinum*, for example, in processed meat products was demonstrated by Pivnick and Barnett (1965), Tanaka (1982), and Barbut et al. (1986a). They all reported that NaCl concentrations which fall within the acceptable sensory levels had an affect on botulinal toxin development under abused temperature conditions.

The effect of phosphate on improving physical and sensory characteristics of reduced salt meat products was studied by various researchers (Swift and Ellis, 1956; Knipe et al., 1985; Sofos, 1986). It was reported that phosphates, such as tripolyphosphate (TPP), hexametaphosphate (HMP) and sodium acid pyrophosphate (SAPP), can improve the texture and flavor of reduced salt comminuted meat products containing 2.0% or 1.5% NaCl (Seman et al., 1980; Sofos, 1986; Barbut et al., 1987). Phosphate effects were claimed by some to be due to a pH shift or an increase in non-specific ionic strength, whereas others claimed a specific effect where pyrophosphate, for example, was observed to help in dissociating the actomyosin to actin and myosin (Tonomura et al., 1967; Sofos, 1986).

Tompkin (1984) in his review on phosphates stated that "the antimicrobial potential of phosphates has not been fully explored and only limited information is available." Nelson et al. (1983) showed that 0.4% SAPP was more effective than TPP and HMP at the same pH when used together with 0.26% potassium sorbate and 40 ppm nitrite to inhibit *C. botulinum* in chicken frankfurters. Barbut et al. (1986b) showed that in turkey meat emulsions SAPP was more effective in inhibiting botulinal toxin production when it was compared to HMP and TPP, however, without sorbate.

The use of food irradiation is a promising way to help preserve heat sensitive foods. A scientific status summary by the Institute of Food Technologists (IFT, 1983) reviewed the efforts made in this area during the last three decades. Radiation can be used to replace some food preservatives. Wierbicki and Brynjolfsson (1979), for example, reported that gamma-irradiation was used successfully to substitute for most of the nitrite in ham, bacon and corned beef. They reported that those products were found to be indistinguishable in flavor from commercially produced counterparts. Radiation dose effects expressed as D-values for *C. botulinum* inactivation was summarized by Kreiger et al. (1983).

The objectives of this study were to investigate the antibotulinal potential of gamma-irradiation in doses up to 1 Mrad when NaCl was totally replaced by two other chloride salts and when three polyphosphates were used in a reduced salt product.

MATERIAL & METHODS

Treatments

Eighteen frankfurters treatments (Table 1) were prepared. Three different chloride salts (NaCl, KCl and $MgCl_2$) at equal ionic strengths (IS) equivalent to 2.5% NaCl, and three types of polyphosphates TPP, HMP, and SAPP added to 2.0% NaCl frankfurters were compared in

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Table 1—Effects of radiation dose on C. botulinum toxin production in turkey frankfurters formulated with different chloride salts and polyphosphates

Trt. no.	Trial	Salt Type	Salt IS[a]	Salt %	PO_4[b] 0.4%	Dose Mrad	pH	Days at 27°C: 1	2	3	4	5	6	7	8	9	10	11	12	13	15	20	30	40	50
								(No. of toxic samples out of 3)																	
1	A	NaCl	0.42	2.5		0	6.53	—[f]	—	—	—	3													
	B						6.52	—	—	—	3														
2	A					0.5	6.55	—	—	—	—	—	—	—	—		—		—		—	—	—	—	—
	B						6.50	—	—	—	—	—	—	—	—		—		—		—	—	—	2	
3	A					1.0	6.55	—	—	—	—	—	—	—	—		—		—		—	—	—	—	—
	B						6.52	—	—	—	—	—	—	—	—		—		—		—	—	—	—	
4	A	KCl	0.42	3.13		0	6.60	—	—	1	2	3													
5	A					0.5	6.59	—	—	—	3														
6	A					1.0	6.58	—	—	—	—	—	—	—	—	1	3								
7	A	$MgCl_2$	0.42	1.33		0	6.43	—	—	—	3														
8	A					0.5	6.45	—	—	—	—	3													
9	A					1.0	6.45	—	—	—	1	1	3												
10	A	NaCl		2.0	TPP[c]	0	6.71	—	—	—	3														
11	A				TPP	0.5	6.71	—	—	—	3														
12	A				TPP	1.0	6.73	—	—	—	—	—	1	1	1	1	1	3							
13	A	NaCl		2.0	HMP[d]	0	6.52	—	—	—	3														
14	A				HMP	0.5	6.54	—	—	—	—	1	—	—	—	—	—	—	3						
15	A				HMP	1.0	6.55	—	—	—	—	—	—	—	—	—	—	—	8	3					
16	A	NaCl		2.0	SAPP[e]	0	6.30	—	—	—	—	—	—	—	—	—	3								
17	A				SAPP	0.5	6.32	—	—	—	—	—	—	—	—	—	—		—		—	—	—	1	—
18	A				SAPP	1.0	6.31	—	—	—	—	—	—	—	—	—	—		—		—	—	—	—	—

[a] IS = Ionic strength.
[b] PO_4 = Phosphate.
[c] TPP = Tripolyphosphate.
[d] HMP = Hexametaphosphate.
[e] SAPP = Sodium acid pyrophosphate.
[f] No toxin sample out of the three checked was found.

an irradiation experiment. Only the 2.5% NaCl treatment, which represents the most commonly used NaCl level in this type of product (Maurer, 1983; Sofos, 1983), was tested twice in two different trials.

Ingredients and product manufacturing

Mechanically deboned turkey meat (MDTM) with 14.0% protein, 13.5% fat, 70.5% moisture and 1.2% ash (AOAC, 1975) was used. The common ingredients in the frankfurters were 2.0% corn syrup solids, 1.0% dextrose, 0.25% white pepper, 0.07% nutmeg, 0.05% sodium erythorbate, 0.015% sodium nitrite and 0.5% liquid smoke (Milwaukee Seasoning Laboratories, Germantown, WI). NaCl (Columbus Chemical, Inc., Columbus, WI), KCl, $MgCl_2$ (Fisher Scientific) TPP, HMP, and SAPP (Stauffer Chemical, Inc., Washington, PA) concentrations varied among treatments. Product manufacturing, *C. botulinum* inoculation and pH measurements of the cooked products are described in a previous publication (Barbut et al., 1986b).

Spore inoculum

The *C. botulinum* spore inoculum used was a composite of equal numbers of five strains each of type A and B (56A, 62A, 69A, 77A, 90A, 53B, 113B, 213B, 13983B, and Lamanna — akra B). The inoculum concentration was 4.5–5.0 x 10^2 spores/g of frankfurter emulsion. All raw batters were inoculated with the same volume of spore suspension.

Irradiation

Frankfurters were vacuum packaged individually followed by washing the packages with 400 ppm chlorine solution and ethanol. The frankfurters were kept in a cooler and irradiated on the following day at doses of either 0.5 or 1.0 Mrad at a temperature of 2 ± 1°C (by immersing the packages in slushed ice and water) in a self-contained, Cesium-137 irradiator with a strength of 132,000 Ci at the Eastern Regional Research Center, USDA, Philadelphia, PA. A dose rate of 10 krad per minute was used in the experiment. Ferrous sulfate/cupric sulfate ($FeSO_4/CuSO_4$) dosimeters were used to monitor the absorbed doses.

Toxicity testing and spores enumeration

Botulinal toxicity presence using the mouse bioassay test (three packages sampled/treatment/observation), confirmatory testing and spore enumeration by the 5-tube most probable number (MPN) method are also described in previous publication (Barbut et al., 1986b).

RESULTS & DISCUSSION

Chloride salts

The antibotulinal effects of the three chloride salts (NaCl, KCl, $MgCl_2$) added at the same IS of 0.42 (equivalent to 2.5% NaCl) coupled with irradiation treatments are presented in Table 1. The results clearly showed that NaCl was the most inhibitory salt to *C. botulinum* toxin production after exposure to 0.5 or 1.0 Mrad of gamma-irradiation. This was a significant finding considering the trend on the market today for sodium reduction/replacement in processed food products. Both KCl and $MgCl_2$ have been reported in the literature to have the potential to partially replace NaCl in meat products (Seman et al., 1980; Hand et al., 1982).

In the nonirradiated treatments NaCl delayed toxin production by at least one day more than either KCl or $MgCl_2$. The results are in agreement with Barbut et al. (1986a) who reported NaCl to be the most inhibitory salt for *C. botulinum* toxin production in poultry frankfurters when NaCl, KCl and $MgCl_2$ were compared. It was shown in that study (Barbut et al., 1986a) that at higher concentrations two of the salts were even more inhibiting; at an IS of 0.68 (equivalent to 4.0% NaCl), the salts were ranked as NaCl > KCl >> $MgCl_2$ and delayed toxin production up to 30, 16 and 3 days, respectively.

At the 2.5% NaCl level, 0.5 Mrad was sufficient to inhibit toxin production up to 40 days in the second trial and at least up to 50 days (experiment was terminated) in the first trial. Under the conditions provided for *C. botulinum* growth in this experiment (27°C, anaerobic environment, heat shock due to cooking and high inoculation levels), a one month delay in toxin production can be considered very effective. These results represent a delay in toxin production of at least 10 fold (in both trials a and b) due to the 0.5 Mrad treatment. At the 1.0 Mrad level, no toxin was detected throughout the entire experiment when 2.5% NaCl was used.

Total replacement of NaCl with KCl coupled with irradiation treatment was not as effective in delaying botulinal toxin pro-

duction. Exposure of KCl containing frankfurters to 0.5 Mrad delayed toxin production by one day as compared to the non-irradiated sample. Application of 1.0 Mrad to the KCl containing frankfurters delayed toxin production up to the 9th day, which represents a threefold improvement. This improvement was not as effective as the irradiated NaCl treatment where toxin production was inhibited for a minimum of 40 days when 1.0 Mrad was used.

No marked improvement over the nonirradiated treatment was observed when the $MgCl_2$ frankfurters were irradiated with 0.5 or 1.0 Mrad. Heinis et al. (1977) and Ma-lin and Beuchat (1980) reported that the presence of magnesium ions in media and/or foods actually improved recovery of heat- or cold-injured *V. parahaemolyticus*. Similarly, Hughes and Hurst (1976) showed that Mg^{++} was required for repair of heat injured *S. aureus*.

Phosphates

Three types of phosphates which are commonly used in the meat industry were added to the 20% reduced NaCl poultry frankfurter (2.0% NaCl) and evaluated for their antibotulinal effect. Comparing the non-irradiated treatments indicated that only SAPP was effective in delaying toxin production. Both the TPP and HMP treatments became toxic after the 4th day, whereas the SAPP treatment inhibited toxin production until the 10th day. This is in agreement with previously reported results (Barbut et al., 1986b). Nelson et al. (1983) reported a similar trend when comparing the three phosphates in the presence of sorbate.

Exposure of the reduced salt frankfurters containing TPP to 0.5 Mrad did not improve the antibotulinal effect; exposure to 1.0 Mrad did delay toxin detection by 2 days as compared to the non-irradiated control (6 vs 4 days) and all the TPP samples were toxic by the 11th day. In frankfurters containing 2% NaCl + HMP and treated with 0.5% Mrad, one toxic sample was found on the 5th day and all samples were toxic by the 12th day; with 1.0 Mrad all HMP containing frankfurters were toxic by the 13th day.

The SAPP treatment was more effective than either the TPP or the HMP treatments in delaying toxin production. SAPP containing frankfurters treated with 0.5 Mrad delayed toxin production up to 40 days compared to 10 days without irradiation. No toxic samples were found when the SAPP treatment was exposed to 1.0 Mrad. Inhibition of botulinum toxin production in the 2% NaCl + 0.4% SAPP treated with 1.0 Mrad was equivalent to 2.5% NaCl treated with 1.0 Mrad.

Additional information obtained in this study dealing with estimating the number of survived spores (in few of the treatments) by the MPN method suggested that *C. botulinum* spores were inactivated or injured due to gamma-irradiation exposure, and their ability to grow/recover mainly depended on the type salt present in the media. Neither the 0.5 Mrad nor the 1.0 Mrad treatment was sufficient to totally inactivate all the C. botulinum spores. The lowest number recorded was 10 spores/g after exposure to 1 Mrad in the 2.5% NaCl treatment, suggesting that in each of the treatments there were still enough viable spores that had the potential to grow. Sublethal damage, as a result of heat, chemical or irradiation, will results in spores which are more sensitive than uninjured spores to selective agents such as: NaCl (Chowdhury et al., 1976), antibiotics (Rowley et al., 1983) and nitrites (Roberts and Ingram, 1966). In this study it was observed that *C. botulinum* spores were inactivated or injured by exposure to gamma-irradiation, and their ability to recover and produce toxin was directly related to the type of salt in the meat system. NaCl at a concentration of 2.5% exhibited the best *C. botulinum* inactivation when coupled with irradiation, followed by KCl and $MgCl_2$. When phosphates were added to the reduced NaCl treatments, only SAPP demonstrated a marked antibotulinal activity which was further enhanced when coupled with gamma-irradiation treatment.

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Ms received 1/9/87; revised 4/8/87; accepted 4/25/87.

Research supported by the College of Agricultural and Life Sciences, and the University-Industry Research Program, Univ. of Wisconsin, Madison, WI 53706, and the ARS, USDA, Philadelphia, PA 19118.

We express our appreciation to the Food Research Institute, University of Wisconsin-Madison for *C. botulinum* toxin testing facilities and to the Wisconsin Turkey Federation for donating meat and materials.

Smokehouse Process Conditions for Meat Emulsion Cooking

G. S. MITTAL, C. Y. WANG, and W. R. USBORNE

ABSTRACT

The effects of various rates of increase of the smokehouse temperature (ST) (0.42 to 0.98°C/min) and relative humidity (SRH) (1.1 to 3.9%/min) on the meat emulsion product qualities were investigated. The optimum process conditions were calculated based on the water holding capacity (WHC), emulsion stability (ES), shrinkage, textural parameters and sensory attributes. Least shrinkage was obtained when the product was processed under rapid temperature and relative humidity increase. The WHC was higher when the product was processed at a lower ST. The most stable product was obtained at the lowest ST and the highest SRH. Cohesiveness and elasticity were not affected by ST and SRH, brittleness was unaffected by SRH, and hardness was not influenced by ST. The lowest hardness, chewiness and gumminess were obtained at the highest SRH.

INTRODUCTION

THERE ARE SEVERAL OPERATIONS accompanied with the smoking and cooking of the meat emulsion. The sequential and systematic organization of these operations is known as the thermal schedule. The following is a brief review of the various stages of meat emulsion thermal processing: (1) **Drying.** Kramlich et al. (1973) reported that drying is closely related to skin formation which affects shrinkage and to surface conditions of the product which affect smoke depositing and color development; (2) **Smoking.** The primary purposes of smoking meat are the development of flavor and color, protection from oxidation and preservation; (3) **Resting.** The purposes of the resting period are to allow smoke penetration into the product and the settlement of the mist; (4) **Cooking.** Cooking has tremendous effects on the final product characteristics. In general, the effects of cooking on the emulsion products are (a) the destruction of micro-organisms and improvement of storage life, (b) the denaturation and coagulation of the meat proteins, (c) improvement of the peelability of the final product, (d) stabilization of the red color in cured meat and (e) modification of the product texture; (5) **Chilling.** The purposes of chilling using a cold water shower are to wash off excess smoke and to make the sausages mechanically stable.

Little work has been done in the area of optimization of thermal processing schedule for meat emulsion products. The computer controlled smokehouse systems offer potential for the optimization of thermal processing schedule. High temperature and high humidity were reported to have an adverse effect on emulsion stability, texture and color development (Saffle et al., 1967; Monagle et al., 1974). Simon et al. (1965) reported that high relative humidity during emulsion cooking reduced the toughness--firmness of the final product. Various investigators (Monagle et al., 1974; Townsend et al., 1971; Saffle et al., 1967) suggested that a steady rate of increase in the smokehouse temperature resulted in a more acceptable product. Monagle et al. (1974) started the cooking cycle at 55°C and increased the temperature by 5° to 6°C every 10 min to 83°C. With this cycle, better scores were obtained for both product color and texture acceptability, but greater shrinkage was also observed compared to other processing schedules. Saffle et al. (1967) showed that low relative humidity cooking resulted in better texture and color development but greater shrinkage.

This study was conducted (1) to determine the effects of various rates of increase of the smokehouse temperature and relative humidity on the meat emulsion product qualities and (2) to develop optimal process conditions based on the acceptable product qualities.

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Table 1—Process variables and their levels

	Level				
Variable	−1.41	−1	0	1	1.41
ST, °C min	0.42	0.50	0.70	0.90	0.98
SRH, %/min	1.09	1.50	2.50	3.50	3.91

Table 2—Formulation of raw emulsion

Composition	mass (g)
Lean beef (21.4% protein, 6.2% fat)	980
Pork fat (2.0% protein, 88.2% fat)	515
Ice	825
NaCl, (2.25%)	54.5
Sucrose (0.5%)	12.1
Black pepper (0.33%)	8.00
Coriander (0.06%)	1.45
Nutmeg (0.07%)	1.70
Garlic powder (0.06%)	1.45
Mace (0.11%)	2.66
Sodium nitrate (200 ppm)	0.485
Erythorbate (550 ppm)	1.33

MATERIALS & METHODS

Experimental design

The two variables investigated were the rate of increase of the smokehouse temperature (ST) and the rate of increase of the smokehouse relative humidity (SRH) during cooking. A rotatable central-composite design with two replications was used. Table 1 shows the process design variables and their levels.

Product preparation

Lean beef and pork fat were supplied by the Meat Laboratory of the University of Guelph. Lean beef was ground twice through a 4.8 mm plate and pork fat once through a 12.7 mm plate. The meat and fat were weighed into the proper amounts (table 2), packaged, frozen and stored at -20°C until required for the experiments. Packages were thawed at 2°C for 48 hr before use. The lean beef, curing agents and half of the ice were placed in a chopper (Eduard Mueller, Gmbh, Germany, Type MTZ 10/70) and chopped for 60 bowl revolutions at a speed of 13 rpm. The rotational speed of the chopper was then increased to 25 rpm. Seasonings, pork fat and the rest of the ice were added to the meat batter and chopped for another 140 bowl revolutions. The end temperature of the emulsion was kept below 17°C.

The emulsion was transferred to an hydraulic stuffer (Mainca, type EB25, Canada Compound Co., Toronto) for extrusion into 20-21 mm

Table 3—A thermal schedule with the setting of ST = 0.7°C/min and SRH = 2.5%/min

Steps	1	2	3	4	5	6	7	8	9
Process	Drying	Liquid smoking	Resting			Cooking			Showering
T (°C)	60	--	--	57	64	71	78	85	--
RH,%	--	--	--	45	70	70	70	70	100
Air changes per min	17	0	0			17			0
Duration (min)	20	10	10	10	10	10	10	10	15

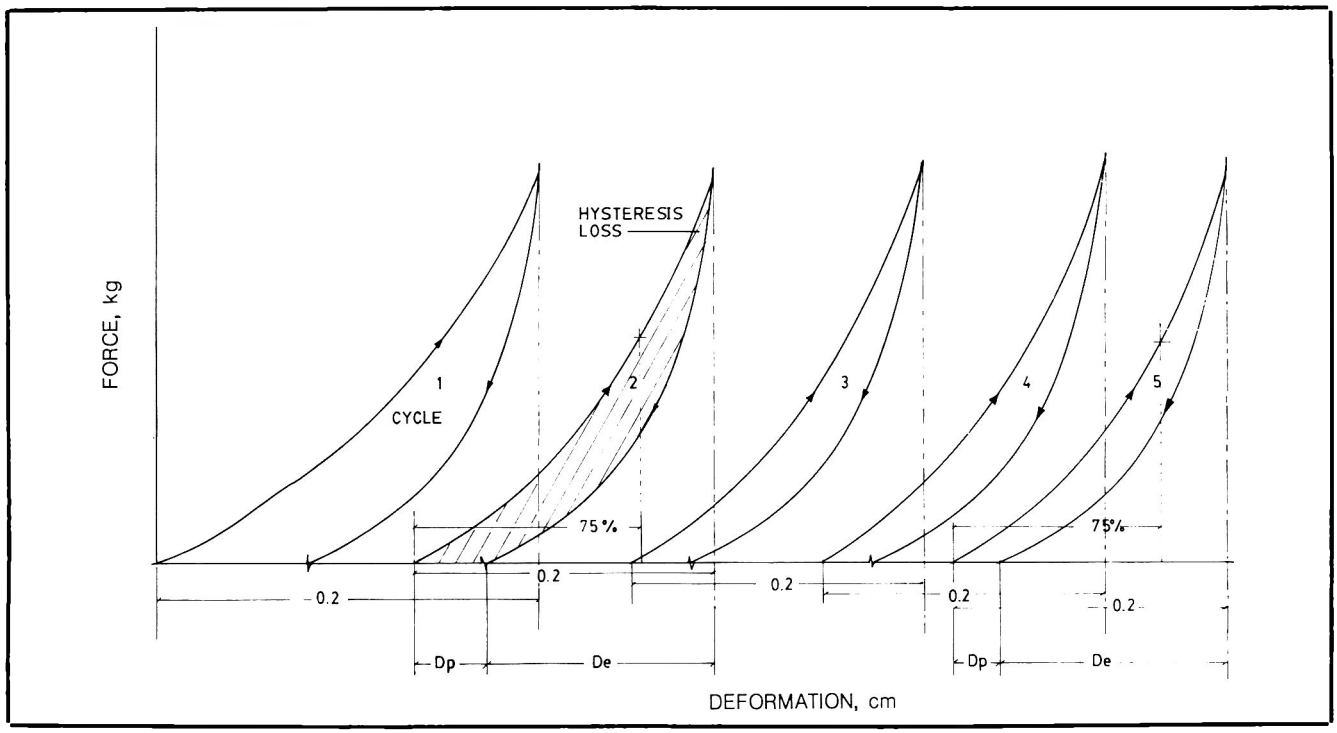

Fig. 1—A typical force-displacement curve obtained from the multi-cycle compression test

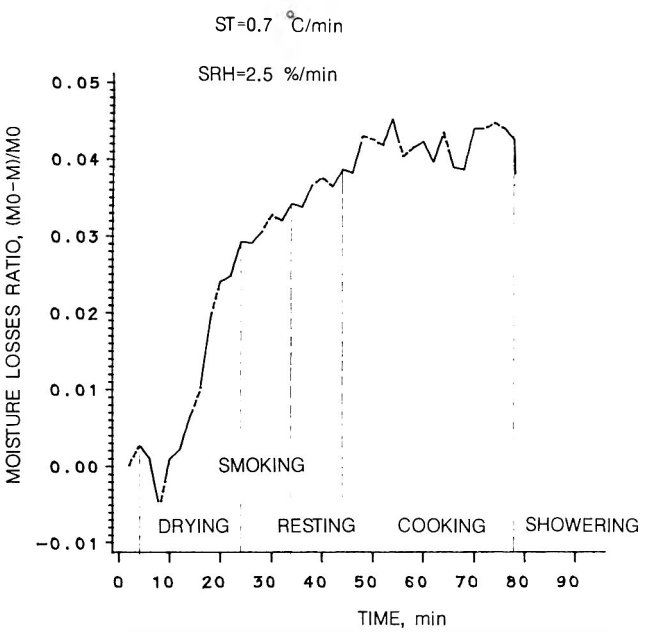

Fig. 2—Moisture loss history during thermal processing of wieners

No-Jax casings. The wieners were linked using a hand linker (Koch Supplies., Kansas City, MO). The length of each link was about 15 cm. The sausages were hung on a smoke house truck and stored for 24 hr at 2°C.

For each treatment, two T-type thermocouple probes were inserted at the center and 7 mm from the center of the encased sausage with

Table 4—Correlations between shrinkage, CS, WHC, CS and processing variables[a]

Variable	ST	SRH	MC_f
SH1	-0.73 (**)	-0.18 (0.58)	-0.29 (0.37)
SH2	-0.23 (0.48)	-0.26 (0.41)	-0.35 (0.27)
SH4	0.53 (0.07)	-0.11 (0.73)	-0.10 (0.76)
WHC	-0.20 (0.17)	0.18 (0.22)	-0.51 (**)
ES	0.05 (0.66)	-0.38 (***)	-0.40 (**)
CS	-0.03 (0.89)	-0.12 (0.56)	0.38 (0.07)

[a] Values in brackets are the probability of the hypothesis that there is no correlation
** $0.001 < p < 0.05$
*** $p \leq 0.001$

the help of a rubber stopper. The ends were sealed with vacuum grease and secured with a cotton thread. A data logger (Fluke Model B 2008, John Fluke Mfg. Co. Mountlake Terrace, WA) was used to record the sausage temperatures, dry and wet bulb temperature of the smokehouse and mass of the sausages using two load cells.

The total mass of the emulsion for each treatment was divided into three portions for mass measurements. Two of these portions were used for continuous mass monitoring by means of two load cells. The mass of these portions were recorded prior to and after cooking using a balance.

The emulsion was cooked in a microprocessor controlled KSI single truck batch smokehouse (Knud Simonsen Industries, Rexdale, Ontario). A conventional vertical air flow of 610 m/min (17 air changes/min) was used. An example of a thermal processing schedule is shown in Table 3. The cooking was terminated when the internal temperature

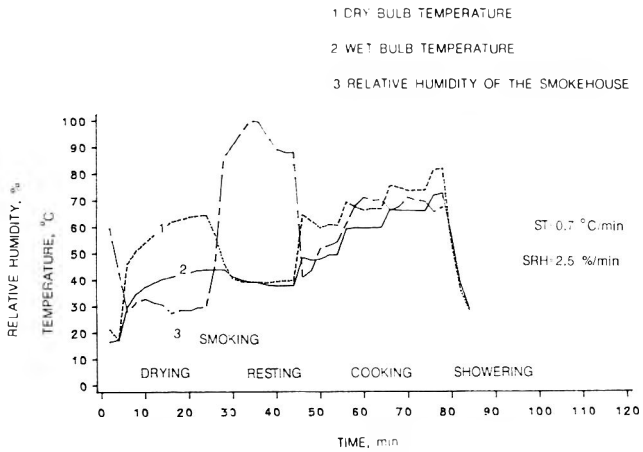

Fig. 3—Smokehouse temperature and relative humidity at ST = 0.7°C/min and SRH = 2.5%/min

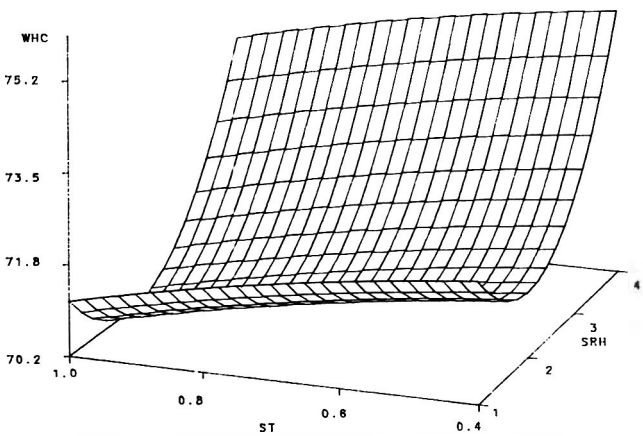

Fig. 4—Response surface of WHC as a function of ST and SRH at MC_f = 60%

of the product reached 70°C. The wieners were weighed, placed in the cooler for 24 hr at 2°C, weighed again, peeled, vacuum packaged, and stored at 2°C until the following analyses were completed.

Water holding capacity (WHC)

The method used was that of Wierbicki et al. (1957) with the following changes: (1) 20g sample was weighed into a plastic tube (29 mm o.d.) with a conical end, (2) cooling after heating was avoided to prevent fat solidification and (3) the heating period was increased to 45 min to ensure equilibrium temperature of the sample. The WHC was calculated by the following equation:

$$WHC = (\text{total moisture} - \text{free moisture})100/\text{total moisture}$$

Emulsion stability (ES)

The method of Saffle et al. (1967) was used with the following modifications: (1) a centrifuge tube (29 mm o.d.) with a round bottom replaced the Paley bottle, (2) the melted fat along with the water was poured into a dish and the mass of fat was recorded after drying for 12 hr at 100°C and (3) four replications of each sample were analyzed. The ES was then calculated:

$$ES = (\text{fat released})100/\text{sample mass}$$

Textural analysis

The following methods were used for textural parameter measurements: (1) textural profile analysis, (Bourne, 1978), (2) elastic texture parameter analysis (Segars et al., 1974), and (3) Warner-Bratzler (W.B.) shear force (Voisey et al., 1975). Two to three samples with skin were cut from the center of the wieners for these tests.

Textural profile analysis was performed using an Instron Universal testing machine (Model 1122) with 20 cm/min and 5 cm/min for the chart and cross-head speeds, respectively. Each specimen was compressed twice using a 5 cm diameter uniaxial flat plate to 75% of weiner's initial height. The textural parameters were calculated from the textural profiles (Bourne, 1978).

Elastic textural parameters were obtained using the same Instron machine with chart and cross-head speeds of 30 cm/min and 1 cm/min, respectively. Each sample was compressed 20% of the already deformed length before the cross-head reversed direction and returned to its zero strain position and repeated five times. Figure 1 is a typical force-deformation response from this test (Segars et al., 1974). The following elastic parameters were calculated:

(1) $DE = D_c/(D_c - D_p)$;

(2) AMD = a ratio of stress to strain at 75% compression in the second cycle;

(3) AME = similar to AMD, but calculated from the fifth cycle;

(4) C20 = stress at 20% compression from the second cycle;

(5) SE = strain energy/sample volume, i.e., the area under the force-deformation plot from the second cycle;

(6) $MHL = (A_L\text{-}A_{(L)})100/A_L$ from second cycle.

The W.B. shear force (SHEAR) was determined using a single blade W.B. shear press set at 10% sensitivity.

Cook test

Five to six wieners were weighed and place in 1000 ml boiling water. The wieners were removed from the heat when boiling resumed, then allowed to stand for 10 min, drained, and placed in a Mason jar for cooling for 20 min. Final mass was recorded, and results were expressed in percent water loss.

Table 5—Regression models for shrinkage, WHC, ES, CS and textural parameters

$SH1 = 14.99 - 25.94\ ST + 15.50\ ST^2$, $R^2 = 0.77$	(1)
$SH2 = 24.26 - 42.90\ ST - 0.33\ SRH + 29.63\ ST^2$, $R^2 = 0.86$	(2)
$WHC = 3.63\ MC_f - 3.95\ SRH - 0.04\ MC_f^2 - 1.41\ ST^2 + 0.98\ SRH^2$, $R^2 = 0.99$	(3)
$ES = -5.1\ ST - 1.89\ SRH + 0.56\ MC_f - 0.01\ MC_f^2 + 2.23\ ST.SRH$, $R^2 = 0.99$	(4)
$CS = -12.51\ ST - 2.48\ SRH + 0.003\ MC_f^2 - 0.24\ SRH^2 + 4.76\ ST.SRH$, $R^2 = 0.96$	(5)
$BRIT = 46.20\ ST + 4.35\ MC_f - 0.06\ MC_f^2 + 2.78\ SRH^2 - 18.65\ ST.SRH$, $R^2 = 0.98$	(6)
$HARD = 1.55\ ST - 12.26\ SRH + 4.11\ MC_f - 0.04\ MC_f^2 + 1.80\ SRH^2$, $R^2 = 0.99$	(7)
$COH = -0.018\ SRH + 0.002\ MC_f + 0.015\ ST.SRH$, $R^2 = 0.98$	(8)
$ELAS = -0.044\ SRH + 0.016\ MC_f - 1.7E-4\ MC_f^2 + 0.020\ ST^2 + 8.74E-3\ SRH^2$, $R^2 = 0.98$	(9)
$CHEW = -0.574\ SRH + 0.182\ MC_f - 0.002\ MC_f^2 + 0.488\ ST.SRH$, $R^2 = 0.95$	(10)
$GUM = -1.08\ SRH - 0.18\ MC_f + 2.43\ ST^2$, $R^2 = 0.97$	(11)
$SHEAR = 0.52\ SRH + 0.30\ MC_f - 0.003\ MC_f^2 - 0.46\ ST.SRH$, $R^2 = 0.99$	(12)
$DE = 0.025\ MC_f - 0.0002\ MC_f^2 - 0.083\ ST^2 - 0.005\ SRH^2 + 0.05\ ST.SRH$, $R^2 = 0.99$	(13)
$AMD = 1.72E3\ MC_f + 4.00E4\ ST^2 + 4.74E3\ SRH^2 - 3.24E4\ ST.SRH$, $R^2 = 0.98$	(14)
$AME = -2.48E5\ ST + 3.33E3\ MC_f + 1.53E5\ ST^2 - 9.33E2\ ST.SRH$, $R^2 = 0.98$	(15)
$C20 = 521.1\ MC_f + 1.23E4\ ST^2 + 1.44E3\ SRH^2 - 9.73E3\ ST.SRH$, $R^2 = 0.98$	(16)
$SE = 43.91\ MC_f + 1343.97\ ST^2 + 144.42\ SRH^2 - 968.48\ ST.SRH$, $R^2 = 0.98$	(17)
$MHL = -1.10\ ST + 1.73\ MC_f - 0.015\ MC_f^2 - 0.26\ SRH^2$, $R^2 = 0.99$	(18)

Sample size: 48 for WHC, DE, AMD, AME, C20, SE and MHL; 12 for SH1 and SH2; 24 for CS; 71 for BRIT; and 72 for rest of the dependent variables.
Level of significance (P > F): 0.0013 for SH1, 0.0008 for SH2, and 0.0001 for all other regression models.

Table 6—Duncan's multiple range test for ES[a]

ST, C min	0.98	0.50	0.90	0.70	0.42
ES, % fat release	7.16	7.14	6.70	6.50	6.21
SRH, % min	1.50	1.09	2.50	3.50	3.91
ES, % fat release	7.37	6.85	6.61	6.47	5.85

[a] Any two means underscored by the same line are not significantly different from each other at P≤0.05

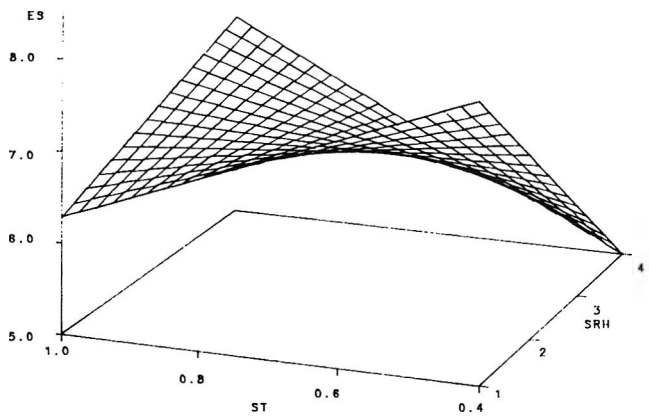

Fig. 5—Response surface of ES as a function of ST and SRH at MC_f = 60%

Sensory evaluation

The test panel was composed of 9 to 10 untrained judges. The samples were the same used for the cook test. Each judge evaluated color, texture, flavor and overall acceptability of the product. A semi-structured scale was used consisting of 15 cm long horizontal lines (Stone et al., 1974). The judges evaluated the product by placing a mark between the two ends which represented the most desirable and undesirable attributes. The results were obtained by measuring the distance from the left (most undesirable) side of the scale to the judge's rating in cm.

Statistical analyses

Statistical analyses of the data were conducted using SAS - Statistical Analysis System (SAS, 1982) on IBM 3081 D mainframe computer. For regression analyses GLM (General Linear Modeling) and STEPWISE procedures of the SAS were used. The GLM procedure uses the principle of least squares to fit a fixed-effects linear model. The procedure performs both univariate and multivariate analyses, including analysis of variance. The STEPWISE procedures was used for data promoting insight into the relative strengths of the relationships between proposed independent variables and a dependent variable. The significance level for entering a variable in a model was 0.50. The significance level for a variable for staying in the model was 0.10. SAS procedure CORR was used to compute univariate descriptive statistics and correlation coefficients between variables. Analysis of variance and Duncan Multiple Range test were computed with SAS procedure of ANOVA.

RESULTS & DISCUSSION

Shrinkage

The overall product shrinkage measured immediately after cooking (SH1) varied from 3.7 to 6.6% with a mean of 4.8%. The correlations between SH1 and ST and SHR were -0.73 and -0.18, respectively (Table 4). The negative correlations suggested that least shrinkage was obtained when the product was processed under rapid temperature and relative humidity increase. Similar results were reported by Saffle et al. (1967). In general, moisture evaporated most rapidly during drying, and moisture loss rate remained relatively constant during cooking. Condensation was observed for the first 5 to 10 min. About 1% moisture was lost during smoking, and an additional 0.5 to 3% moisture loss was observed during cooking. Over 50% of the total moisture loss occurred during the drying stage (Fig 2); further moisture loss was reduced due to skin formation. Both drying and smoking helped in skin formation (Kramlich et al., 1973). A regression model was obtained to predict SH1 as a function of ST (Table 5). The analysis of variance showed an insignificant effect of SRH on shrinkage.

The shrinkage after 24 hr storage (SH2) was not significantly correlated with ST or SRH (Table 4), and a second order relationship existed between these variables (Table 5). The response surface plotted with the help of the model showed that the optimal processing conditions for minimum shrinkage were at ST = 0.72°C/min and SRH = 3.9%/min.

The overnight storage shrinkage (SH4 = SH2 - SH1) was highly correlated (r = 0.53) with ST (table 4), which indicated that inadequate skin was formed at a high ST. Fully developed skin will reduce moisture loss during storage due to decrease in moisture diffusivity.

Figure 3 shows the smokehouse temperatures and relative humidity during sausage cooking for one experimental condition. Similar plots were observed for other test conditions.

Water holding capacity

The mean of the WHC was 73.3% with a s.d. of 2.3%. The ANOVA showed that the effect of the ST and SRH was significant at 80% confidence level. The Duncan's multiple range test indicated that there was significant difference in WHC only for the products processed at the two extreme ST levels (0.41 and 0.98°C/min). The WHC was significantly correlated negatively with the final moisture content (MC_f).

A multiple regression model was obtained (Table 5), and the response surface of WHC as a function of ST and SRH is shown in Fig. 4. This figure shows that a concave downward

Table 7—Correlation among the TPA parameters, SHEAR and sensory attributes[a]

Variable	COLO	TEXTU	FLAV	OVAL	MC_f	SHEAR
HARD, N	−0.27	−0.43	−0.35	−0.34	−0.35	0.04
	(0.20)	(**)	(0.09)	(0.10)	(0.10)	(0.84)
COH	0.16	0.04	0.14	0.16	0.05	−0.45
	(0.45)	(0.85)	(0.50)	(0.46)	(0.82)	(**)
ELAS, cm	−0.02	−0.14	−0.53	−0.27	−0.18	−0.04
	(0.94)	(0.51)	(**)	(0.20)	(0.40)	(0.85)
CHEW, N.cm	0.02	−0.26	−0.40	−0.21	−0.23	−0.23
	(0.93)	(0.22)	(**)	(0.32)	(0.28)	(0.26)
GUM, N	−0.01	−0.25	−0.12	−0.10	−0.15	−0.34
	(0.95)	(0.24)	(0.57)	(0.65)	(0.49)	(0.10)
SHEAR, N	−0.03	0.08	0.19	0.43	−0.29	—
	(0.88)	(0.71)	(0.39)	(**)	(0.16)	—

[a] Values in brackets are the probability of the hypothesis that there is no correlation
** $p < 0.05$

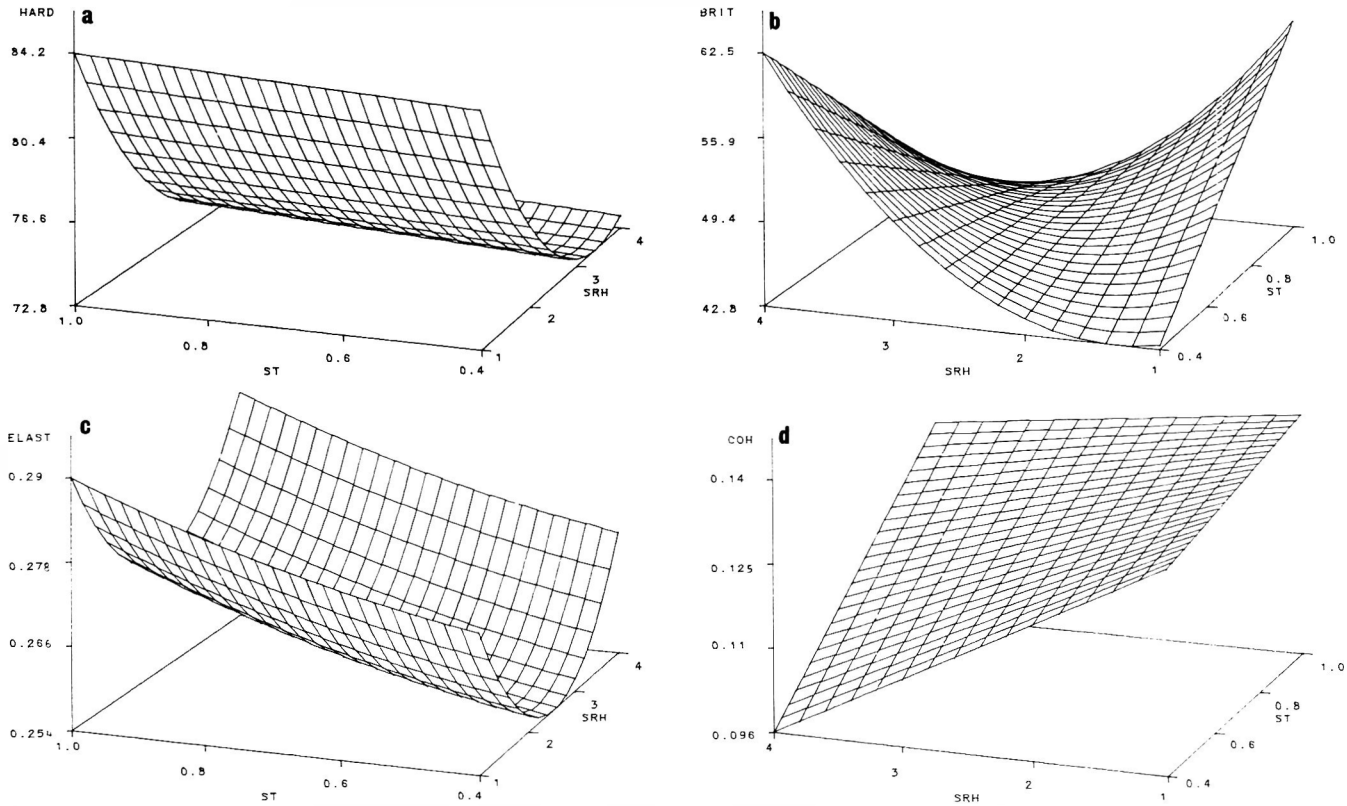

Fig. 6—Response surfaces of texture profile analysis parameters as a function of ST and SRH at MC_f = 60%; (a) Hardness, (b) Brittleness, (c) Elasticity, and (d) Cohesiveness

Table 8—Correlations among elastic textural parameters, processing variables and sensory attributes[a]

Variable	COLO	TEXTU	FLAV	OVAL	MC_f	ST	SRH
DE	−0.07	−0.06	−0.25	−0.31	0.10	0.04	0.33
	(0.74)	(0.79)	(0.24)	(0.14)	(0.64)	(0.80)	(**)
AMD	0.16	−0.40	−0.01	0.09	0.20	−0.24	0.10
	(0.46)	(-**)	(0.97)	0.69	(0.36)	(0.10)	(0.52)
AME	0.21	−0.41	0.12	0.15	0.16	−0.38	0.10
	(0.34)	(**)	(0.57)	(0.49)	(0.46)	(**)	(0.49)
C2O	0.20	−0.44	0.10	0.15	0.11	−0.28	0.13
	(0.36)	(**)	(0.66)	(0.47)	(0.61)	(**)	(0.38)
SE	0.21	−0.40	0.06	0.19	0.11	−0.23	0.15
	(0.34)	(**)	(0.75)	(0.38)	(0.61)	(0.11)	(0.32)
MHL	−0.17	0.16	0.02	0.01	−0.16	0.02	−0.33
	(0.44)	(0.46)	(0.95)	(0.97)	(0.45)	(0.90)	(**)

[a] Values in brackets are the probability of hypothesis that there is no correlation
** $p \leq 0.05$

relationship existed between the WHC and SRH with the minimum WHC and SRH = 2%/min. A liner relationship was observed between WHC and ST, although the effect of ST on WHC was relatively slight.

Emulsion stability

The ES varied from 5.4 to 9.8% fat released. The ES was significantly affected by SRH (Table 4). The Duncan's multiple range test (Table 6) showed that the product was most stable when processed at the lowest ST (0.42°C/min) and the highest SRH (3.9%/min), but most unstable at the high ST and SRH. A high SRH did not result in an unstable product because the ultimate RH achieved during cooking (<70%) was not high enough to cause such instability. A regression model of the ES as a function of ST, SRH, and MC_f is given in Table 5. Figure 5 illustrates the response surface of ES as a function of ST and SRH at MC_f = 60%. This shows a saddle type behavior; maximum fat was released at the minimum and maximum values of ST and SRH.

Cook shrinkage (CS)

There were not significant correlations among CS, ST and SRH (Table 4). The ANOVA showed that CS was not affected by ST and SRH. However, CS was positively correlated with MC_f; and at higher moistures greater moisture losses resulted during the cook test. A regression model is given in Table 5. The response surface of CS was similar to Fig. 5 obtained for ES. The maximum values existed at the minimum and maximumvalues of ST and SRH.

Textural profile analysis (TPA)

The ANOVA showed that none of the textural parameters obtained from TPA were influenced by ST. However, HARD and GUM were significantly correlated with SRH. This indicated that the body firmness was mainly affected by the relative humidity. The Duncan's tests indicated that COH and ELAS

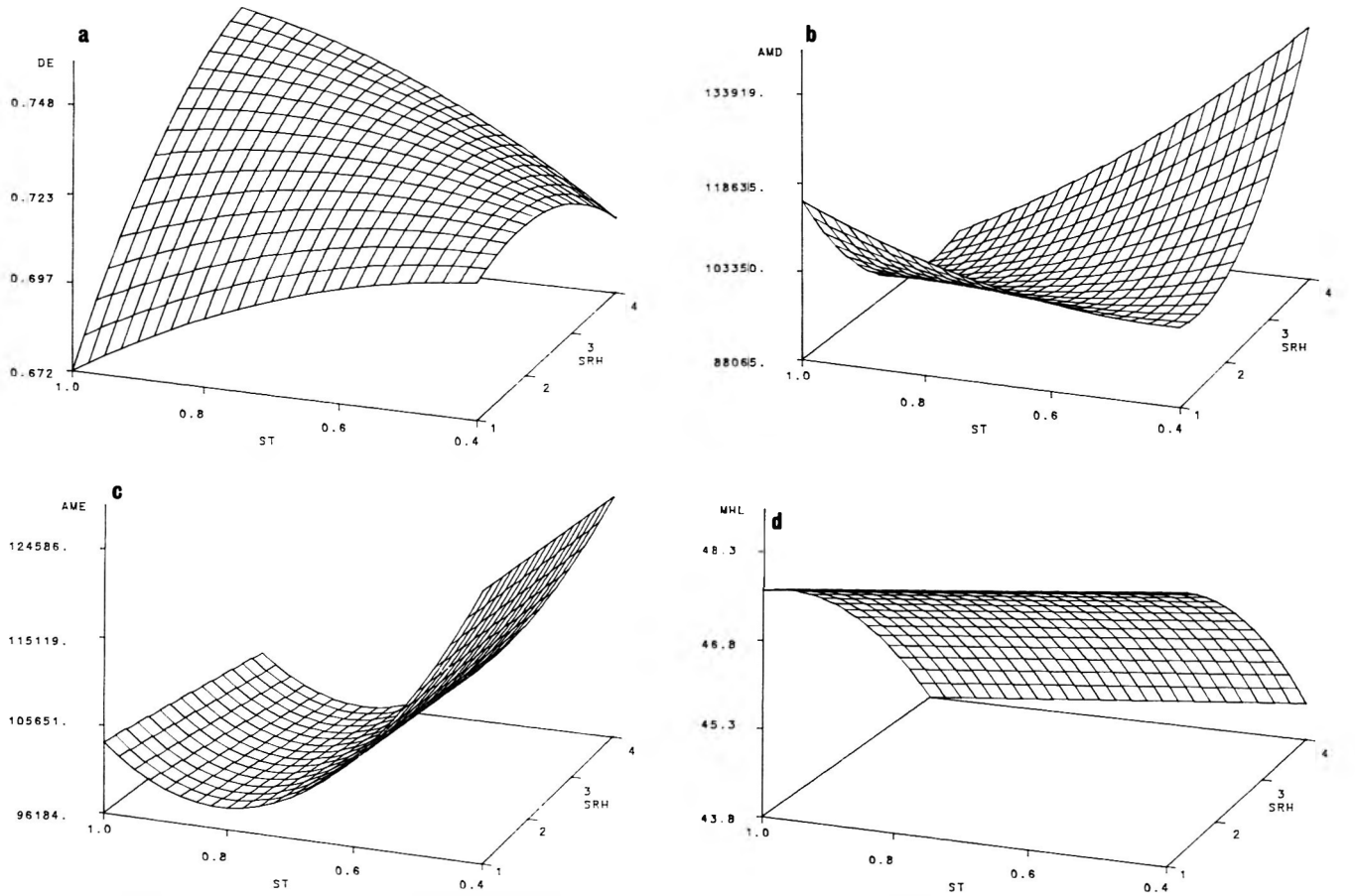

Fig. 7—Response surfaces of elastic textural parameters as functions of ST and SRH at MC, = 60%; (a) Degree of elasticity, (b) Apparent modulus of deformability, (c) Apparent modulus of elasticity, and (d) Mechanical hysteresis loss

Table 9—Optimum processing conditions for minimum ES, SH2, CS and maximum WHC

Variable	ST	SRH	Optimal value
SH2, % moisture loss	0.72	3.50	7.60
ES, % fat released	0.50	3.50	5.80
CS, % losses	0.50	3.50	2.40
WHC, % moisture retained	0.50	3.50	73.4
Y (optimum)	0.58	3.50	**

** WHC = 73.3 % moisture retained
ES = 6.0 % fat released
SH2 = 8.2 % moisture loss
CS = 2.7 % losses
bounds: 0.5 < ST < 0.9 °C/min
1.5 < SRH < 3.5 %/min

were not affected by ST and SRH; BRIT was not affected by SRH and HARD by ST. The lowest HARD, CHEW and GUM values were obtained at the highest SRH. Similar results were given by Simon et al. (1965) who reported that toughness-firmness was reduced by raising the smokehouse RH.

HARD was the only parameter that had significant negative correlation with the sensory texture score (Table 7) indicating that the panelists liked a more tender product. Significant correlations between FLAV and both ELAS and CHEW were also observed.

Multiple regression models for these textural parameters as functions of ST, SRH and MC_f are listed in Table 5. The response surfaces of HARD, BRIT, ELAS, and COH as functions of ST and SRH at MC_f = 60% are shown in Fig. 6, a to d. The COH showed linear response surface, and similar responses were observed for CHEW and GUM. Higher order response surfaces were observed for the behavior of HARD, BRIT and ELAS. The minimum values of HARD and ELAS were obtained at SRH between 2.5 and 3.5%/min. A saddle shaped response surface was observed between BRIT and process variables.

Warner-Bratzler shear press

The W.B. shear (SHEAR) did not show significant correlation with product firmness or hardness (Table 7). This agrees with the findings of Voisey et al. (1975). SHEAR, however, was significantly correlated with the sensory overall acceptability. Similar findings were reported by Fox et al. (1983) who found that SHEAR provided a good indication of the skin firmness, but not of the body firmness. Furthermore, SHEAR was significantly correlated with COH (r = 0.45) and ST (r = 0.27). The Duncan's multiple range test showed that the SHEAR value was maximum when the product was processed under the lowest ST. Thus, the sausage skin was better formed under a low heating rate and panelists preferred this type of product. The regression model for SHEAR as a function of ST, SRH and MC_f is given in Table 5.

Elastic textural parameters

Six elastic textural parameters (DE, AMD, AME, C20, SE and MHL) were calculated from the five-cycle compression test. The AME and C20 were significantly correlated with ST; DE and MHL highly correlated with SRH (Table 8). Since AMD, AME, C20 and SE had significant negative correlations with the sensory texture score, a less elastic product was considered better by the panelists. These four parameters were positively correlated with the overall-acceptability score, however, the correlations were poor. The Duncan's tests showed that DE and MHL were not influenced by ST; and AMD, AME, C20 and SE were least affected by SRH. Again AMD, AME, C20 and SE were significantly affected by ST. These parameters are related to the force and energy required to deform the product under elastic deformation. The highest values of these four parameters were obtained at the lowest ST (0.42°C/min). Both MHL and DE were highly affected by SRH. As

Table 10—Optimal values of the textural parameters and processing conditions

Variable	ST °C/min	SRH %/min	Minimum	ST °C min	SRH % min	Maximum
HARD, N	0.50	3.41	73.14	0.90	1.50	80.3
COH	0.50	3.50	0.11	0.90	1.50	0.14
ELAS, cm	0.50	2.53	0.27	0.90	3.50	0.29
CHEW, N.cm	0.50	3.50	1.91	0.90	1.50	2.86
GUM, N	0.50	3.50	8.48	0.90	1.50	11.61
SHEAR, N	0.90	1.50	5.78	0.50	3.50	6.64
BRIT, N	0.50	1.68	44.44	0.50	3.50	53.68
DE	0.90	1.50	0.70	0.90	3.50	0.74
AMD, Pa	0.90	3.10	9.09E4	0.50	3.50	1.15E5
AME, Pa	0.80	3.50	9.66E4	0.50	1.50	1.13E5
C20, Pa	0.90	3.00	2.79E4	0.50	3.50	3.50E4
SE, J m^3	0.90	3.00	2.41E3	0.50	3.50	3.04E3
MHL, %	0.90	3.50	44.90	0.50	1.50	47.90

bounds: 0.5 < ST < 0.9 °C min
1.5 < SRH < 3.5 %/min

SRH increased, DE increased but MHL decreased. Both parameters had poor correlations with the sensory scores.

Regression models for these elastic parameters as a function of ST, SRH and MC_f are given in Table 5. To visualize the effects of ST and SRH on these parameters, three dimensional plots were drawn at MC_f = 60% (Fig. 7, a to d). The AMD, AME, C20 and SE, which were significantly correlated with sensory texture score, were shown to be concave upward, while DE and MHL were concave downward. The maximum value of DE and MHL occurred at SRH of about 1.5 and 3.0%/min, respectively. The response surfaces of AMD, SE and C20 were alike. The minimum values of AMD, SE and C20 occurred at the specific values of SRH depending on ST. The AME was highly affected by ST and reached a minimum at ST = 0.8° C/min.

Sensory evaluation

All products were equally acceptable by the untrained sensory panel. The Duncan's test showed that there were significant differences in sensory scores among the panelists, which may be due to an untrained panel.

Optimization

A multidimensional optimization program developed by Mittal and Usborne (1986) was used. This was based on the algorithm of Hooke and Jeeves (1961) which determined the global minimum and maximum values of dependent variables and associated independent variable values. The optimal processing conditions obtained were within the bounds of 0.5 to 0.9°C/min and 1.5 to 3.5%/min for ST and SRH, respectively. An arbitrary objective function was estimated to simultaneously determine the minimum CS, SH2 and the maximum WHC and ES. These four response variables were assumed to be equally important, i.e., Y = ES + SH2 + CS - WHC. Based on the individual regression models, objective function and the optimization program, the optimal values of these parameters were caluculated (Table 9) along with their associated conditions. Thus, optimum process conditions can be calculated for any desired objective function. However, experimental verification of the optimum process conditions is recommended.

The global maxima and minima of the textural parameters of the TPA, SHEAR, and the elastic textural parameters were calculated from their response surfaces (Table 10). An objective function including all these variables has not yet been developed because more information relating the textural parameters to each other and to sensory is necessary.

CONCLUSION

THE PRODUCT PROCESSED at the highest SRH (3.9%/min) and an ST of 0.5 to 0.7°C/min usually resulted in a better product with respect to emulsion stability, WHC and shrinkage. The optimal process conditions to obtain minimum fat release, cook shrinkage and shrinkage during smokehouse processing and maximum WHC were ST = 0.58°C/min and SRH = 3.5%/min. Based on the response surfaces, global maxima and minima of individual textural parameters and related process conditions were calculated.

The present knowledge allows the determination of the maximum and minimum values of individual parameters, however, an overall objective function relating various functional properties has yet to be developed.

SYMBOLS

A_L	Area under loading curve, N.cm
A_{UL}	Area under unloading curve, N.cm
AMD	Apparent modulus of deformability, Pa
AME	Apparent modulus of elasticity, Pa
ANOVA	Analysis of variance
BRIT	Brittleness, N
C20	Stress at 20% compression, Pa
CHEW	Chewiness, N.cm
COH	Cohesiveness
COLO	Sensory score for the product color, cm
CS	Consumer cook shrinkage, % loss
DE	Degree of elasticity
D_e	Elastic deformation, cm
D_p	Plastic deformation, cm
ELAS	Elasticity or springiness, cm
ES	Emulsion stability, % fat released
FLAV	Sensory score for the product flavor, cm
FP	Fat-protein ratio
GUM	Gumminess, N
HARD	Hardness, N
M	Mass of the product, kg
MC_f	Final moisture content of the product, % wet basis
MHL	Mechanical hysteresis loss, %
MO	Initial mass of the product, kg
OVAL	Sensory score for the product overall acceptability, cm
P	Probability
r	Correlation
R^2	Coefficient of determination
RH	Relative humidity, %
SE	Strain energy per unit volume, J/m^3

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An Investigation into Glucose as a Potential Precursor of N-Nitrosothiazolidine in Bacon

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ABSTRACT

The possible involvement of glucose in the formation of N-nitrosothiazolidine (NTHZ) in bacon was investigated by pumping pork bellies with brines containing added glucose (2 and 4%). Raw bacon samples processed with glucose had significantly lower levels of N-nitrosothiazolidine-4-carboxylic acid than the smoked control bacons. When the bacon samples with glucose were fried for 6 and 12 min, no NTHZ was detected in the fried products. N-Nitrosopyrrolidine (NPYR) levels in these samples were much lower than those in the fried control samples. Results of this study demonstrate that the contribution of glucose to NTHZ formation in bacon is minimal relative to that of formaldehyde in the wood smoke.

INTRODUCTION

IN RECENT YEARS, considerable attention has focused on the presence of N-nitrosothiazolidine (NTHZ) and N-nitrosothiazolidine-4-carboxylic acid (NTCA) in smoked cured meats (Pensabene and Fiddler, 1983a,b; Mandagere et al., 1984; Ikins et al., 1986). Sen et al. (1986) reported trace amounts of NTHZ in various smoked meat samples and a few smoked fish and considerably higher (up to 13,700 μg/kg) levels of NTCA. These investigators also provided evidence that NTCA will undergo decarboxylation during frying of bacon, and that the formation of NTHZ was dependent on frying temperature and frying time.

It is widely accepted that NTHZ and NTCA formation in smoked cured meats results from the interaction of formaldehyde in the smoke with cysteamine and cysteine, respectively, followed by nitrosation (Pensabene and Fiddler, 1985; Sen et al., 1985, 1986). Mandagere et al. (1984) speculated that formaldehyde may also be formed from the fragmentation of glucose during frying of bacon. This speculation was based on results of model system studies which showed that heterocyclic compounds including thiazolidine, 2-methylthiazolidine and 2-ethylthiazolidine are formed on heating a cysteamine/glucose model browning system (Sakaguchi and Shibamoto, 1978). The major objective of this study was to clarify the precursor role, if any, of glucose in NTHZ and NTCA formation in raw and fried bacon. A secondary objective was to confirm the inhibitory role of glucose on N-nitrosopyrrolidine (NPYR) formation in fried bacon.

MATERIALS & METHODS

Bacon processing

Fifteen skinned pork bellies were obtained from a local slaughterhouse within 24 hr postmortem and stored for no more than 2 days in a cooler at 2°C. The bellies were randomized into three groups with five in each. Bellies in group 1 were stitch pumped to 110% of their green weight with a brine containing 15% sodium chloride, 5% sucrose, 3.5% sodium tripolyphosphate, 1,200 mg/kg sodium nitrite, and 5,500 mg/kg sodium ascorbate, and smoked for 8 hr by a standard smoking process (Reddy et al., 1982). Bellies in groups 2 and 3 were stitch-pumped with brines containing 2% and 4% glucose instead of 5% sucorse, respectively. All other ingredients of the brine were similar to those in the control (group 1). The bellies were pumped to 110% of their weight and held at 2°C for 2 days. The glucose-treated samples were cooked in the smoke house at 58°C for 8 hr in the absence of wood smoke. The bacon samples were sliced and vacuum packaged and held at 2°C for 1 week. Two packages randomly selected from each belly were analyzed for NTHZ and NTCA. Two other randomly selected packages from each belly were fried in a preheated electric frying pan at a thermostat setting of 340°F (171°C). The control samples (group 1) were fried for 6 min (3 min per side) while the glucose samples were fried for 6 and 12 min (3 and 6 min per side, respectively). The fried samples were ground and analyzed for NTCA, NTHZ and other volatile N-nitrosamines.

N-Nitrosamine analysis

NTHZ and other volatile N-nitrosamines in raw and fried bacon were determined by the mineral oil vacuum distillation procedure described in detail by Ikins et al. (1986). All samples were analyzed in duplicate and precautions were taken to avoid artifactual N-nitrosamine formation during sample preparation (Ikins et al., 1986). Percent recovery of the added internal standard, N-nitrosothiomorpholine, was 86 ± 5%. N-Nitrosamine values were not corrected for recovery of the internal standard.

The NTCA contents of the bacon samples (raw and fried) were determined using the ethyl acetate extraction procedure described by Mandagere et al. (1986a). The average recovery of the internal standard (N-nitrosopipecolic acid) was 79 ± 4%. The gas chromatographic-Thermal Energy Analyzer conditions were as described by Mandagere et al. (1986a).

Statistical analysis

Statistical analyses of N-nitrosamine data were carried out according to the methods described by Gill (1978).

Safety note

N-Nitrosamines are potent carcinogens and should be handled with appropriate safety precautions.

RESULTS & DISCUSSION

N-NITROSAMINE DATA for the raw and fried bacon samples are summarized in Table 1. The raw bacon samples processed with glucose (groups 2 and 3) contained significantly lower ($p<0.01$) levels of NTCA compared to the smoked control samples. This clearly demonstrates that NTCA formation in bacon is primarily due to the smoking process. The very low levels of NTCA in the glucose-bacon samples likely arise from the residual smoke in the smoke house. Similar levels of NTCA were found in a limited number (3) of control bacon samples (i.e. containing sucrose) which were cooked simultaneously with the glucose samples in the smoke house. N-Nitrosodimethylamine (NDMA) and NPYR levels in all treatments were somewhat similar, the small differences most likely being due to compositional differences in the bellies (Pensabene et al., 1979).

NTCA levels in fried bacon decreased during the frying process. Mandagere et al. (1986b) have shown that approximately 2% of the NTCA present in raw bacon is converted to NTHZ during frying. NTCA was not detected in the bacon containing glucose, the limit for reliable detection being 5 μg/

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Table 1—N-Nitrosamine concentrations in bacon processed with two levels of glucose

Treatment group	N-Nitrosamine concentration (μg/kg)[d]			
	NDMA	NPYR	NTHZ	NTCA
Raw bacon				
(1) Control (0.5% sucrose)	2.5 ± 0.8	1.0 ± 0.2	1.9 ± 0.5	365 ± 16
(2) 0.2% glucose	3.1 ± 0.9	1.0 ± 0.3	ND[e]	8 ± 3[b]
(3) 0.4% glucose	3.8 ± 0.7	1.5 ± 0.7	ND	12 ± 3[b]
Fried bacon				
(1) Control (6 min)	2.1 ± 0.7	5.0 ± 1.7	4.5 ± 0.8	189 ± 8
(2) 0.2% glucose (6 min)	3.9 ± 0.9	2.5 ± 0.3[c]	ND	ND
(2) 0.2% glucose (12 min)	2.8 ± 0.6	1.0 ± 0.3[c,d]	ND	ND
(3) 0.4% glucose (6 min)	2.5 ± 0.8	2.6 ± 0.6[c]	ND	ND
(3) 0.4% glucose (12 min)	1.5 ± 0.5	ND[c,d]	ND	ND

[a] N-Nitrosamine levels represent average of two determinations, five bellies per treatment.
[b] Significantly different from control ($p<0.01$).
[c] Significantly different from control ($p<0.05$).
[d] Significantly different 6 min sample of the sample glucose concentration ($p<0.01$).
[e] ND, not detected; limit of detection (1 μg/kg for NTHZ; 5 μg/kg for NTCA).

kg. Added glucose (0.2 and 0.4%) in bacon did not appear to influence NTHZ formation during frying. Similarly, increasing frying time from 6 min to 12 min did not induce NTHZ formation which indicates that glucose fragmentation to formaldehyde is of minor importance. Sakaguchi and Shibamoto (1978) showed that model systems containing glucose and cysteamine produced large amounts of thiazolidine when refluxed for 2 hr at 100°C. If this reaction had occurred during the frying of bacon containing the added glucose, large amounts of thiazolidine would have been produced. Thiazolidine, because of its weak basicity, would have undergone rapid nitrosation to NTHZ (Coughlin, 1979). Since no NTHZ was detected in the glucose-treated bacon (limit of detection 1 μg/kg), the major pathway for NTHZ formation in bacon must involve formaldehyde in the wood smoke. Previous studies in our laboratory had also indicated that sucrose did not contribute to NTHZ formation in fried, unsmoked bacon (Ikins, 1986).

While the contribution of glucose to NTHZ and NTCA formation appears to be minimal, it has been established that glucose can undergo Maillard-type reactions with cysteamine (Bonner and Meyer Zu Reckendorff, 1961) and with cysteine (Schubert, 1939; Weitzel et al., 1959) to form 2-[D-glucopentahydroxypentyl-(1)]-thiazolidine and the carboxylic acid derivative, respectively. Coughlin (1979) reported that the D-glucose/L-cystcine adduct, being weakly basic, should be nitrosated quite rapidly in nitrite-containing foods and *in vivo* in the gastro-intestinal tract following ingestion of the adduct. The nitrosated compound is also a β-oxidized N-nitrosamine and many of these compounds are proximate carcinogens and/or mutagens (Coughlin, 1979).

To date, no nitrosated Amadori compounds have been isolated from food systems. Scanlan and Reyes (1985) reported that these compounds can be isolated and identified by a reverse phase high performance liquid chromatographic-thermal energy analyzer system. However, due to the basic incompatibility between the two systems, little progress has been made in studying the formation of nonvolatile nitrosated Amadori compounds in foods.

The addition of glucose to bacon had a marked influence on NPYR levels in the fried product (Table 1). Significant reductions ($P<0.05$) in NPYR levels were observed when the bacon samples were fried for 6 min. These results agree with the findings of Bailey and Mandagere (1980) and Theiler et al. (1984), who reported major reductions in NPYR formation in fried bacon cured with glucose and other reducing sugars. Theiler et al. (1984) initially hypothesized that the mechanism of reducing sugar inhibition involved the amine-sugar browning reaction (Maillard reaction) and that the products formed (Amadori compounds) were capable of competing with NPYR precursors such as proline in the nitrosation reaction. Results of further studies with the nonreducing sugars, α and β-methylglucose, did not support this hypothesis as NPYR levels were reduced to the same extent as with glucose. These investigators concluded that the classic Maillard reaction occurring between added reducing sugars and primary amines did not adequately explain the mechanism of inhibition.

N-Nitrosamine data in Table 1 also indicate that the NPYR levels in the glucose-bacon samples fried for 12 min were significantly lower ($p<0.01$) than those fried for 6 min. This difference could be due to volatilization of the NPYR formed and/or creation of additional secondary amino compounds as a result of the extended frying period. NDMA levels in the fried bacon were generally not affected by the presence of glucose in the bacon. Statistical treatment of the NDMA data is not reported due to the low levels of NDMA present, levels that can be affected by duration of frying, and by compositional differences between pork bellies (Pensabene et al., 1979).

In conclusion, it has been demonstrated that the contribution of glucose to NTHZ and NTCA formation in bacon is minimal relative to that of formaldehyde in the smoke. Addition of glucose to bacon, although effective in reducing NPYR formation during frying, has practical limitations because of its tendency to produce excessive browning in the fried product. Furthermore, formation of N-nitroso Amadori compounds is also undesirable because of their potential mutagenicity/carcinogencity.

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Effects of Preblending, Reduced Fat and Salt Levels on Frankfurter Characteristics

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ABSTRACT

Frankfurters were manufactured from preblended (PB) or nonpreblended (NPB) meats to contain traditional and reduced levels of salt (1.5, 2.0, or 2.5%) and/or fat (17% = low; 25% = high). Emulsions stability, color, Kramer shear and palatability were evaluated. Salt had a positive effect on emulsion stability ($P<0.05$). Low-fat frankfurters were darker, redder, less blue in color, drier and more resistant to shear than high-fat frankfurters. Low-fat franks containing 1.5% salt had a softer texture than those containing 2.0 or 2.5% salt. Preblending did not affect textural properties. With modification of the formulations, low fat-low salt franks can be manufactured.

INTRODUCTION

CONSUMER DESIRE for lower fat and lower salt levels in processed meats has increased since publication of the report of the U.S. Senate Select Committee on Nutrition and Human Needs (1977). The committee advocated reduction in fat and sodium intake as a part of the "Dietary Goals for the United States."

The effects of reduced salt (Seman et al., 1980; Puolanne and Terrell, 1983) and reduced fat (Carpenter et al., 1966; Cross et al., 1980; Valvano, 1983) on the textural and sensory properties of processed meat products have been investigated. These reductions have resulted in definite changes in product characteristics. However, a simultaneous reduction of both fat and salt levels has not been well characterized.

Certain processing technologies, such as preblending, affect the texture of meat products. Preblending is the addition of salt, nitrite and water to the meat 12 or more hr prior to sausage manufacture. Preblending enhances protein bind, color and water holding capacity (Acton and Saffle, 1969; Shannon, 1983). The combination of reduced salt and preblending may improve the bind and product texture of processed meat products over that of products containing normal levels of salt and fat. The objective of this study was to determine the effects of salt and/or fat levels, in conjunction with preblending, on the visual, textural and palatability characteristics of frankfurters.

MATERIALS & METHODS

Product manufacture

Meats (60% beef trimmings, 40% pork trimmings) were formulated to approximately 20 or 30% fat (low, high) using an Anyl-Ray machine (Kartridg Pak Co., Davenport, IA) and were assigned to a preblended (PB) or non-preblended (NPB) treatment. Raw materials were preblended with one-half of total sodium nitrite (78 ppm), the remainder was incorporated during frankfurter production, and all of the salt (sodium chloride) by mixing 5 min and storing at 0–2°C for 24 hr before manufacture. Non-preblended meats were manufactured in a similar manner except that the meat, salt and nitrite were mixed just prior to frankfurter manufacture. These two blending treatments were further subdivided into salt levels of either 1.5, 2.0, or 2.5%.

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The meats (PB or NPB) were mixed (10 min) with water, spices and the remaining nitrite (78 ppm) where required, and passed through a Hobart emulsion mill (Hobart Co., Troy, OH). The raw batters were stuffed (25 mm cellulose casings), linked (12–14 cm in length) using a Vemag Model 1000 stuffer (Reiser and Co., Boston, MA) and frankfurters from each treatment were randomly allotted to one of three holding times (1, 4 or 7 hr) in a 0—2°C cooler. After the designated holding time, the frankfurters were heat processed in a Mauer Rondair smokehouse (S&F Sheet Metal Co., Bronx, NY). The smokehouse processing cycle was: (a) reddening-heating: 10 min, 40°C, dampers open; (b) drying: 25 min, 54°C, dampers open; (c) smoking: 10 min, 54°C, dampers closed; and (d) cooking: 20 min, 78°C, dampers closed (to internal temperature 67°C). After heat processing, the frankfurters were cold-water showered, stored in a 0–2°C cooler (24 hr), peeled and vacuum packaged for subsequent analyses. The entire experiment was replicated twice.

Textural and palatability measurements

Raw frankfurter batter stability was analyzed for all formulations using the emulsion stability test (Townsend et al., 1968) after the holding periods of 1, 4, or 7 hr. Total, fat, gelatinous and proteinaceous losses were then expressed as a percent of raw sample weight. Tenderness of the finished frankfurters was examined by use of the Instron Kramer shear cell. Values for peak force and area under the curve were obtained using a 2500 kg load cell, load range of 0–100 kg, and chart speed of 20 mm/min. The finished frankfurters were analyzed for fat using the AOAC (1980) procedure and salt using a chloride ion electrode (Orion, Cambridge, MA).

Frankfurter samples used in taste panel analyses were frozen at −35°C for 3 wk. The samples were prepared by steeping the frankfurters in individual pans for 7 min in previously boiling water. Cooking loss was obtained from the difference in the weights of the uncooked and cooked frankfurters. Panelists were served two warm, 2.5 cm pieces of each sample and all treatment combinations were repeated twice. The samples were served to panelists under red overhead lighting and in individual panel booths. Taste panelists ($n=8$) evaluated each sample for exterior texture, interior texture, juiciness and saltiness using a 15 cm unstructured line scale with the origin and endpoint labeled with extremes for each trait (interior or exterior texture: soft to hard; juiciness: dry to juicy; saltiness: bland to salty). Ratings were expressed as the distance from the origin that a sample was rated.

Visual measurements

Vacuum packaged frankfurters (three packages per treatment) were placed in a retail display cabinet (107.6 lux, 12 hr on, 12 hr off, 2°C) for 6 wk. All samples were monitored for color on a weekly basis using the Hunter Color Difference Meter (Hunter Labs, Reston, VA).

Table 1—Emulsion stability of raw frankfurter batter as influenced by salt level.

Salt level	Losses[a] Fat	Gelatinous	Proteinaceous	Total
1.5%	1.38[b] (0.11)	16.68[b] (0.22)	1.10[b] (0.06)	19.15[b] (0.25)
2.0%	1.07[c] (0.08)	11.78[c] (0.33)	0.60[c] (0.05)	13.46[c] (0.36)
2.5%	0.70[d] (0.09)	7.76[d] (0.28)	0.27[d] (0.02)	8.74[d] (0.34)

[a] Losses expressed as a percentage of raw sample weight.
[b-d] Means within a column followed by different superscripts are different ($P<0.05$). Parenthetical values are standard errors.

Table 2—Hunter colorimeter L, a_L and b_L values of frankfurters as influenced by fat and salt levels

	Trait					
	L value		a_L value		b_L value	
Salt level	Low fat	High fat	Low fat	High fat	Low fat	High fat
1.5%	45.59[f] (0.31)	47.27[e] (0.13)	10.60[cd] (0.18)	10.32[e] (0.18)	11.61[g] (0.15)	12.22[d] (0.15)
2.0%	45.32[f] (0.29)	47.66[d] (0.12)	10.50[d] (0.13)	9.66[f] (0.13)	11.74[f] (0.17)	12.93[c] (0.17)
2.5%	44.80[g] (0.37)	49.43[c] (0.15)	10.73[c] (0.11)	8.87[g] (0.11)	11.96[e] (0.17)	12.93[c] (0.17)

[a] Low fat was 17.0% fat.
[b] High fat was 24.6% fat.
[c-g] Means within a trait followed by different superscripts are different ($P<0.05$).
Parenthetical values are standard errors.

Table 3—Hunter b_L values of frankfurters as affected by fat level and preblending

	Fat level[b]	
Blend treatment[a]	Low	High
NPB	11.59[e] (0.14)	12.71[c] (0.14)
PB	11.96[d] (0.13)	12.68[c] (0.13)

[a] NPB was not preblended. PB was preblended for 24 hr.
[b] Low was 17.0% fat. High was 24.6% fat.
[c-e] Means followed by different superscripts are different ($P<0.05$). Parenthetical values are standard errors.

Table 4—Kramer peak force and area under the curve for frankfurters as influenced by fat and salt levels

	Kramer peak force		Kramer area under the curve (cm^2)	
Salt level	Low fat[a]	High fat[b]	Low fat	High fat
1.5%	20.63[d] (0.61)	17.42[f] (0.43)	12.35[e] (0.31)	9.87[h] (0.22)
2.0%	24.24[c] (0.54)	18.76[e] (0.47)	13.96[d] (0.35)	10.64[g] (0.22)
2.5%	25.43[c] (0.51)	19.24[e] (0.57)	14.69[c] (0.39)	11.32[f] (0.26)

[a] Low fat was 17.0% fat.
[b] High fat was 24.6% fat.
[c-h] Means within a trait followed by different superscripts are different ($P<0.05$). Parenthetical values are standard errors.

Table 5—Taste panel scores for exterior and interior frankfurter texture as influenced by fat and salt level

	Exterior texture[a]		Interior texture[a]	
Salt level	Low fat[b]	High fat[c]	Low fat	High fat
1.5%	7.11[e] (0.30)	6.70[e] (0.33)	8.14[d] (0.32)	6.21[e] (0.25)
2.0%	8.86[d] (0.31)	7.09[e] (0.32)	8.59[d] (0.30)	5.73[e] (0.24)
2.5%	9.60[d] (0.28)	6.46[e] (0.29)	8.99[d] (0.25)	6.11[e] (0.22)

[a] Distance from origin of a 15 cm long, unstructured line with origin = soft and endpoint = firm.
[b] Low fat was 17.0% fat.
[c] High fat was 24.6% fat.
[d,e] Means within a textural trait followed by different superscripts are different ($P<0.05$). Parenthetical values are standard errors.

Two readings on each package were taken for L (lightness to darkness), a_L (redness to greeness) and b_L (yellowness to blueness) values. Saturation scores were calculated according to the method of MacDougall et al. (1985). At initial and biweekly periods, packages were removed from display and tested for residual nitrite levels (AOAC, 1980).

Statistical analyses

The data were analyzed with the Statistical Analysis System (Barr et al., 1976) utilizing a split-split-split plot experimental model, with fat level as the whole plot, and blend, salt and holding time as the respective split plots. For all parameters, holding time treatment was not significant ($P>0.05$). These data were pooled over holding time and reanalyzed as a split-split plot, thereby increasing the precision of the statistical analysis. Means were separated by use of the Duncan's multiple range test (Steel and Torrie, 1980). Where interactions did not exist, main effects were examined.

Table 6—Taste panel juiciness scores[a] of frankfurters as influenced by fat level and preblending treatments

	Fat level[b]	
Blend treatment[c]	Low	High
NPB	7.21[f] (0.23)	10.10[d] (0.20)
PB	7.62[f] (0.24)	9.32[e] (0.19)

[a] Distance from origin of a 15 cm long, unstructured line with origin = dry, and endpoint = juicy.
[b] Low was 17.0% fat. High was 24.6% fat.
[c] NPB was not preblended. PB was preblended for 24 hr.
[d-f] Means followed by different superscripts are different ($P<0.05$). Parenthetical values are standard errors.

Table 7—Taste panel scores[a] for frankfurter saltiness as influenced by preblend treatment, fat level and salt level

	Preblended treatment[b]			
	NPB		PB	
Salt level	Low fat[c]	High fat[d]	Low fat	High fat
1.5	5.18[k] (0.44)	7.20[hi] (0.44)	5.34[k] (0.40)	5.48[k] (0.39)
2.0	6.52[ij] (0.46)	9.36[ef] (0.44)	8.14[gh] (0.43)	8.31[fgh] (0.39)
2.5	9.11[efg] (0.38)	9.39[ef] (0.38)	9.46[ef] (0.44)	9.88[e] (0.29)

[a] Distance from origin of a 15 cm long, unstructured line with origin = bland and endpoint = salty.
[b] NPB was not preblended. PB was preblended for 24 hr.
[c] Low fat was 17.0% fat.
[d] High fat was 24.6% fat.
[e-k] Means followed by different superscripts are different ($P<0.05$). Parenthetical values are standard errors.

RESULTS

ALTHOUGH FORMULATED to contain 20 or 30% fat, the finished frankfurters actually contained 17.0 and 24.6% fat, respectively. This 7.6% difference translates into approximately 30% less fat in the low versus high fat frankfurters. These results highlight limitations of using estimates of raw material composition rather than chemical analysis for formulation. Salt levels in the finished product (1.6, 2.1 and 2.5%) were comparable to formulated levels (1.5, 2.0 and 2.5).

Emulsion stability

The stability of raw batters was evaluated using the emulsion stability test (Table 1). Salt was the only factor influencing

amount of fat, gel or proteinacious solid loss, with increased levels of salt decreasing the total loss from the batters. Neither fat level nor holding time showed a significant effect on emulsion stability in this study.

The 1.5% level of salt resulted in a significantly higher cooking loss (6.31 ± 0.63) than the 2.0% (1.69 ± 0.23) or the 2.5% (1.04 ± 0.12) levels. High levels of salt are known to increase the water binding properties of a product (Hamm, 1960; Knipe et al., 1985).

Visual characteristics

Franks created with preblended meats had slightly lower L values (47.27 ± 0.21) ($P<0.05$) than franks made with nonpreblended meats (46.10 ± 0.19), indicating a darker color. Similar results have been reported by Acton and Saffle (1969).

The effects of salt on color were moderated by the level of fat (Table 2). In general, high-fat frankfurters were lighter in color than low-fat frankfurters. For the high-fat franks, decreasing the level of salt resulted in a darker product. The reverse trend was noted for low-fat franks, where the lower salt levels resulted in lighter products. Low-fat franks also were more red and less yellow than high fat franks. For the low-fat frankfurters, increasing the salt level tended to increase the yellowness while having no consistent effect on redness. Redness was increased in the high-fat frankfurters by decreasing the level of salt, although yellowness was subsequently decreased.

Low-fat, preblended franks were slightly, but significantly ($P<0.05$), more yellow than high-fat, preblended franks (Table 3). Yellowness of high-fat franks was not influenced by preblending.

Textural and palatability characteristics

Low-fat frankfurters required greater force to shear than high-fat frankfurters (Table 4). Within each fat level, the 1.5% level of salt reduced the Kramer peak force below the other salt levels. Area under the curve, a measure of total work required to shear the product, decreased with decreasing levels of salt within each fat level.

In general, the taste panel found high-fat frankfurters softer in internal and external texture than low-fat frankfurters (Table 5). One exception was that the low fat-low salt franks were equivalent to all of the high-fat franks in exterior texture.

Taste panel juiciness scores were higher ($P<0.05$) for franks containing 2.5% (9.37 ± 0.17) and 2.0% (8.99 ± 0.19) salt than for those containing 1.5% salt (7.38 ± 0.23). Preblending reduced juiciness scores for high-fat frankfurters but not for low-fat frankfurters (Table 6).

Table 7 presents the three-way interaction of fat level by salt level by preblend treatment on saltiness scores of the franks. In almost every case, the panel was able to detect differences in the amount of salt incorporated into the formulation. For nonpreblended treatments with 1.5 or 2.0% salt, the high fat franks were perceived as more salty than their low fat counterparts. These differences were removed by preblending.

DISCUSSION

THE PRIMARY REASON for this research was to determine if low-fat and/or low-salt frankfurters could be produced that were similar in color and texture to frankfurters containing more traditional levels of fat and salt. Several observations may be made about these data. First, preblending has minimal effect on the color and texture of frankfurters. Second, in many cases, the low salt-low fat franks were the best low-fat alternative to the high fat-high salt franks. Color was slightly darker for these low fat-low salt frankfurters; however, this might be remedied by adjusting the formulation to include more pork. The difference in saltiness between the treatments might be compensated by changing the salt blend but yield differences would likely remain. Thus, it appears that low salt-low fat frankfurters can be manufactured with textural characteristics similar to frankfurters containing more traditional levels of fat and salt. Formulation changes would likely be needed to overcome differences in flavor and color.

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Ms received 12/15/86; revised 4/3/87; accepted 4/21/87.

Color of Black Salami Sausage: Dissociation of Heme from Myoglobin and Hemoglobin

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ABSTRACT

Dissociation curves determined spectrophotometrically for myoglobin and hemoglobin in citric acid buffers showed that the heme groups dissociated from the proteins at pH-values comparable to those normally found in dry sausages. The extent of dissociation parallelled the degree of denaturation of the proteins as measured by differential scanning calorimetry. Extraction of newly prepared black salami sausage mince with phosphate buffer removed all pigments, which could not be extracted from ripened sausage with pH of about 4.5. This was in agreement with the solubility properties of the hydrophilic pigments myoglobin and hemoglobin, and the hydrophobic properties of the heme pigment. These experiments showed that the color of black salami sausage was due to heme.

INTRODUCTION

IN THE PRODUCTION of fermented sausages, carbohydrates are used as fermentable substrates in sausage batters inoculated with starter cultures. Upon fermentation the production of mainly lactic acid lowers the pH to approximately 5 (De Ketelaere et al., 1974; Acton et al., 1977).

A number of methods have been used to obtain the characteristic red color of dry sausages (Karmas, 1977), lowering of pH and generation of nitric oxide are often utilized. It is generally accepted that the color formed is nitrosylmyoglobin and nitrosylhemoglobin (Karmas, 1977). However, acid denaturation of myoglobin and hemoglobin has been found to occur in the pH region 4–5, and this denaturation leads to detachment of the heme group (Steinhart et al., 1963; Allis and Steinhart, 1970). Fox and Thomson (1963) studied the formation of nitrosylmyoglobin and found that below pH 4.5 the spectrum of the final product was no longer that of nitrosylmyoglobin. At pH 4.0 the spectrum was that of nitrosylhemochrome, as reported by Hornsey (1956), or that of water-soluble, denatured nitrosylhemochrome (Fox and Thomson, 1963).

Previously it has been found that in salami sausage the hydrophobic compound nitrosylheme is formed (Slinde and Nordal, 1978). The aim of the present study was to show that heme dissociated from the globin moiety in black salami sausage, when pH was lowered to values generally found in ordinary salami. Black salami sausage is obtained when ascorbic acid and nitrite are omitted and blood is added.

MATERIALS & METHODS

THE DISSOCIATION of heme from horse myoglobin and bovine hemoglobin was measured spectrophotometrically in 50 mM citric acid buffer by following the decrease in absorbance at 409 nm for myoglobin and 406 nm for hemoglobin (Allis and Steinhardt, 1970). Denaturation of hemoglobin and myoglobin was measured using a Perkin Elmer DSC 2, as described by Hägerdal and Martens (1976).

Industrially produced Norwegian black salami (Stabbur) sausages were used in the experiments. The recipe (100 kg) consisted of: horse meat, 33.0 kg; cow meat, 25.0 kg; lard, 30.5 kg; blood, 5.0 kg; plus salt, spices, sugar, casing and starter culture, 6.5 kg. The ground meat was stuffed into fibre casings of 70 mm diameter. The ripening was carried out essentially as described by Skjelkvåle et al. (1974).

Extraction of hydrophilic color components of the sausages was done as described by Slinde and Nordal (1978). Ten grams sausage were homogenized with 40 mL 50 mM phosphate buffer, pH 6.9. The solution was cleared by centrifugation and the supernatant beneath the lipid layer was withdrawn. The extraction was repeated once and the amount of pigments was determined by scanning the Soret region, or the amount of heme was determined as pyridine hemochrome (Fuhrhop and Smith, 1975).

During the ripening period pH was measured in four different sausages after homogenization of a ten gram sample with 40 mL of distilled water.

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RESULTS

HEMOGLOBIN has a somewhat higher stability than myoglobin (Fig. 1), the detachment of the heme group starting at approximately pH 5.0. For both myoglobin and hemoglobin the degree of dissociation increased upon prolonged storage. This was most pronounced for hemoglobin.

The apparent heat of transition ($\triangle H_{app}$) of myoglobin decreased as the pH of the buffer decreased (Fig. 2). At a pH of 4.7 (Fig. 2) $\triangle H_{app}$ reached zero, indicating extensive unfolding of the globin chain which would favor detachment of heme. Similar results were obtained with hemoglobin.

During the ripening process lactic acid producing bacteria lowered pH as shown in Fig. 3A. The amount of hydrophilic color components extractable (Fig. 3B), i.e., myoglobin and hemoglobin, was found to parallel the pH decrease.

When black salami with a pH slightly below 5 was cut, a red core was found. The color of such a core was easily extracted by the hydrophilic buffer. The spectrum of the extract was that of reduced myoglobin and hemoglobin. When sliced black salami with a red core was exposed to light, it turned black rather quickly and the pigment was no longer extractable.

DISCUSSION

AS SHOWN in the present experiments both hemoglobin and myoglobin showed changes in absorption properties in the pH region generally found in ripened dry sausages. According to Steinhardt et al. (1963) and Allis and Steinhardt (1970), when ferrihemoglobin is acid denatured, migration of heme from its native site and protein unfolding occur simultaneously. The decrease in apparent heat of transition observed by differential scanning calorimetry upon pH lowering indicated unfolding of the globin chains (Fig. 2). This is in agreement with Bismuto et al. (1983), who found that the acid induced molecular transition of myoglobin unfolds the heme binding site of the molecule.

The extraction performed (Fig. 3B) showed that the amount of extractable hydrophilic pigment decreased to zero during the ripening period. The color of black salami was, therefore, due to a hydrophobic component: heme is such a pigment. It was thus concluded that in produced black salami the dissociation of heme from myoglobin and hemoglobin due to pH lowering gave rise to the black color. The red core found in black salami with a pH slightly below 5, indicated that the

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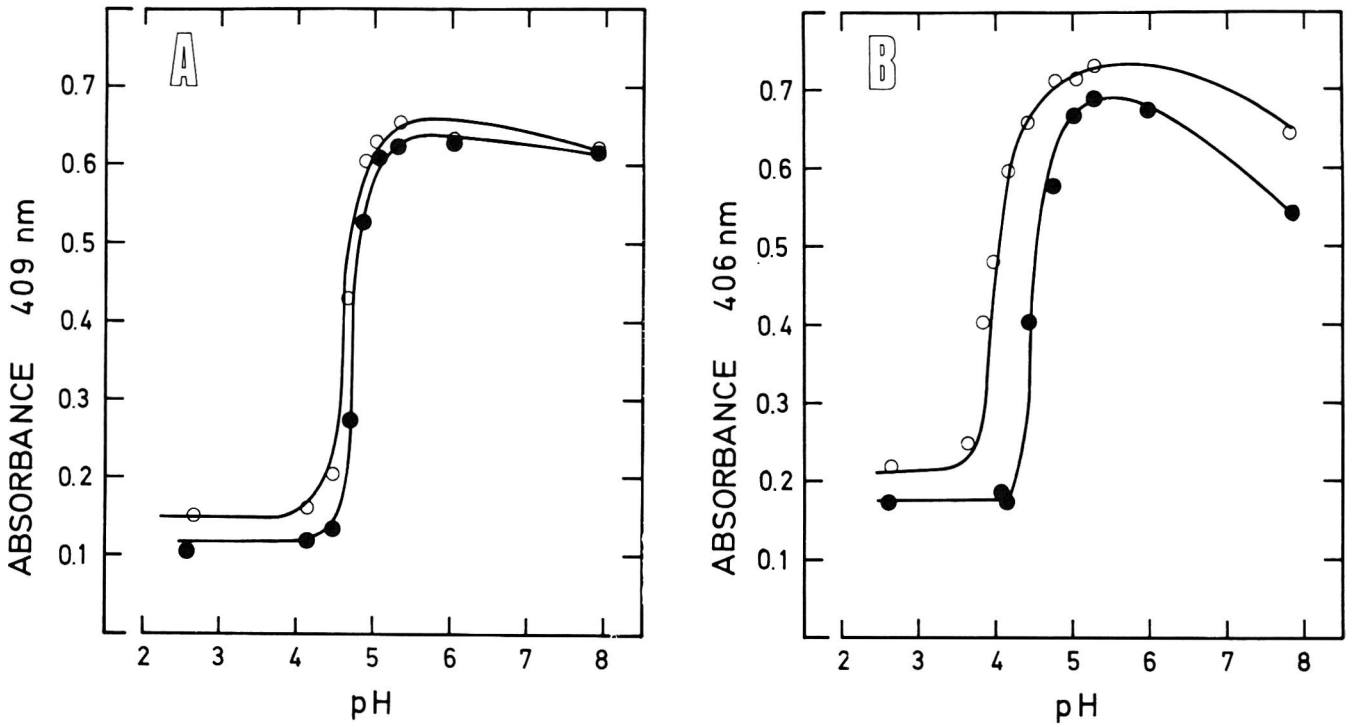

Fig. 1—Absorbance of horse myoglobin (A) and bovine hemoglobin (B) at different pH values: ○ immediately after mixing; ● after 24 hours; T = 25°C.

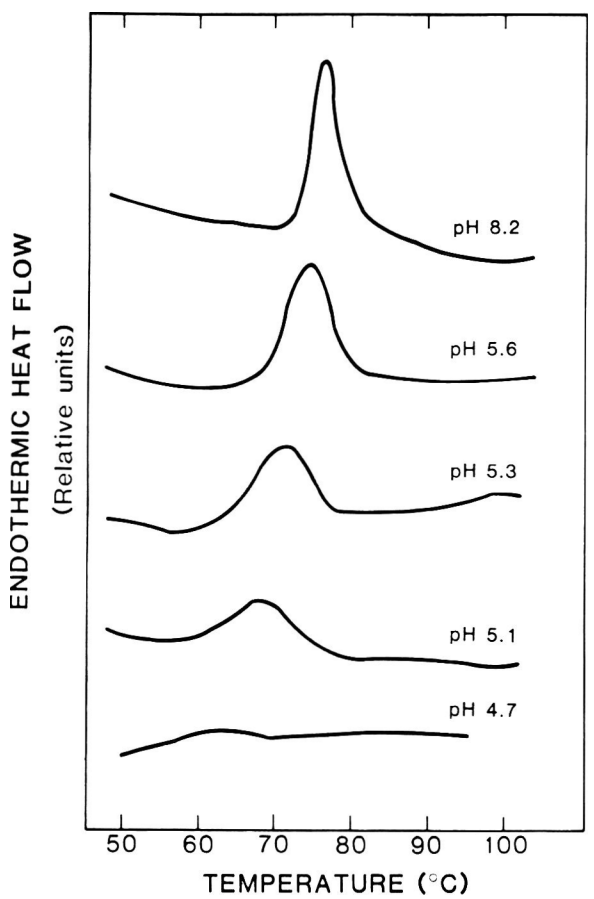

Fig. 2—Thermograms obtained by differential scanning calorimetry showing the apparent heat of transition (ΔH_{app}) of horse myoglobin. A 4.5% myoglobin solution in a 0.05M phosphate/citrate buffer was used; heating rate 10°C · min⁻¹.

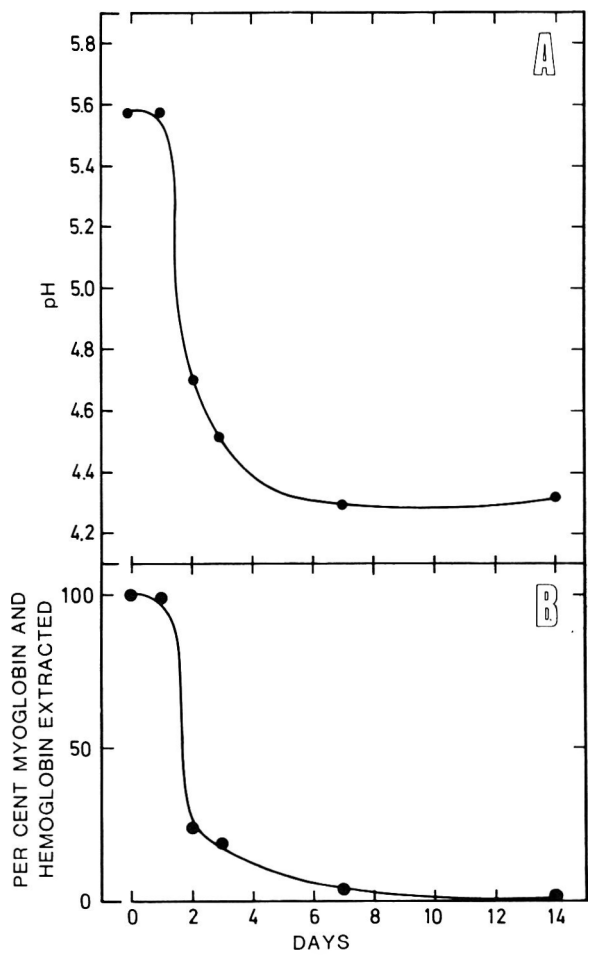

Fig. 3—(A) Decrease of pH, and (B) the relative amount of hemoglobin and myoglobin extracted from black salami sausage during the fermentation period.

milieu within the sausage was reductive and that the heme group of reduced hemoglobin and myoglobin was less susceptible to detachment.

In previous studies the dissociation curve of myoglobin shifted towards a higher pH value in the presence of ascorbate and nitrite (Slinde and Nordal, 1978). This indicated that the color component nitrosylheme was more hydrophobic than heme. Tarladgis (1962a, b) suggested that the cooked cured pigment was dinitrosylhemochrome. This idea was supported by Lee and Cassens (1976) who found that twice the amount of ^{15}N from nitrite was bound in heated as opposed to unheated preparations of cured myoglobin. Denaturation by acid or heat both released the heme group or nitrosylheme group in the presence of ascorbate and nitrite. The low pH persisting in salami favored generation of nitric oxide (Bonnett and Martin, 1976). The milieu present in salami sausage thus favored the formation of dinitrosylheme. Slinde and Nordal (1978), Tarladgis (1962a, b) and Lee and Cassens (1976) suggested that the color of cured salami is that of dinitrosylheme. These results favored the conclusion of the present work, that the color of black salami was that of heme.

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Ms received 10/26/86; revised 1/26/87; accepted 2/26/87.

The technical assistance of Tone Bergersen and Marit Knutsen Hårstad is greatfully acknowledged.

MEAT EMULSION COOKING--SMOKEHOUSE CONDITIONS. . .From page 1146

SH1	Product shrinkage measured immediately after processing, % loss
SH2	Product shrinkage measured after 24 hr storage at 2°C, % loss
SH4	Moisture loss during storage (SH2-SH1), % loss
SHEAR	Shear force recorded from W.B. Shear press, N
SRH	Rate of increase of smokehouse relative humidity, %/min
ST	Rate of increase of smokehouse temperature, °C/min
s.d.	Standard deviation
T	Temperature, °C
TEXTU	Sensory score for product texture, cm
TPA	Texture profile analysis
WHC	Water holding capacity, % moisture retained
Y	Objective function

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Ms received 8/29/86; revised 2/19/87; accepted 3/17/87.

The authors acknowledge the financial assistance provided by the Natural Science & Engineering Research Council, Ottawa, and the Ontario Ministry of Agriculture and Food.

Comparative Evaluation of Whey Protein Concentrate, Soy Protein Isolate and Calcium-Reduced Nonfat Dry Milk as Binders in an Emulsion-Type Sausage

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ABSTRACT

The effects of various nonmeat binders on the yield and textural properties of knockwurst, an emulsion-type sausage product, were investigated. Three whey protein concentrate levels, calcium-reduced nonfat dry milk and soy protein isolate and an all-meat control were evaluated. Whey protein concentrate proved to be a viable binder alternative for specific emulsion-type meat products by providing similar stability, textural and sensory attributes in comparison to equal levels of soy protein isolate and calcium-reduced nonfat dry milk.

INTRODUCTION

A UDSA Food Safety and Inspection Service rule issued in June of 1982, approved the use of whey protein concentrate (WPC) as a binder in sausages, at levels up to 3.5% (USDA, 1982). At the present time though, little information has been published with regard to the performance of WPC as a binder in sausage products. Morr (1979) proposed that whey proteins could be utilized in processed meat products to improve their water and fat binding properties without adversely affecting their flavor or textural properties. Lee et al. (1980) found that the use of WPC resulted in equal bind, increased juiciness and improved flavor in a meat loaf when compared to an equal level of nonfat dry milk.

There are different methods for making WPC, including electrodialysis, ion exchange, gel filtration, metaphosphate complexing, and reverse osmosis and ultrafiltration. When WPCs are prepared by different processes, they vary in chemical composition, nutritional quality and functionality (Casella, 1983). Swartz (1983) emphasized the need for careful testing of a particular type of WPC prior to its use in meat products due to these functionality differences resulting from processing variables.

The purpose of this study was to assess the potential of WPC, produced by reverse osmosis and ultrafiltration, as a binder in a finely emulsified sausage, specifically knockwurst. The performance of WPC was compared to that of soy protein isolate (SPI) and calcium-reduced non-fat dry milk (RNFDM), commonly used binders, to determine its potential use in meat products.

MATERIALS & METHODS

Meat preparation and processing

Three types of meat, beef (80% lean, 20% fat), pork (80% lean, 20% fat) and pork (50% lean, 50% fat), were used in the production of knockwurst (Table 1). The basic knockwurst formulation contained 25% water, 2.0% sodium chloride, 0.5% dextrose, 0.4% liquid flavoring, 0.06% sodium erythorbate and 0.015% sodium nitrite, expressed as a percentage of the meat block (Table 1). Four whey protein concentrate levels (WPC; Nutri-Pro 60N, Nutri-Search Company, Cincinnati, OH) (0, 1.75, 2.0 and 3.5% of the meat block), one level of calcium-reduced non-fat dry milk (RNFDM; Savortex, Express Foods, Louisville, KY) (3.5% of the meat block) and one level of soy protein isolate (SPI; Supro 620T, Ralston Purina, St. Louis, MO) (2% of the meat block) comprised the knockwurst treatments. Each treatment was formulated to contain 24% fat using varying amounts of the three different meat components. Lean meat components, water, salt, flavoring, dextrose, nitrite and erythorbate were chopped first in a Seydelmann 64L vacuum chopper (Robert Reiser and Co., Boston, MA) for 1 min at high speed before adding the WPC, hydrated SPI or RNFDM. After chopping for an additional 1 min, the last ingredient, fat pork, was added and chopping continued for another 1 min. After chopping, the treatments were sampled for stability analyses, mechanically stuffed into 34 mm collagen casings and linked. The knockwurst were thermally processed to an endpoint internal temperature of 68°C as follows: 1.5 hr at 54.4°C and 53% relative humidity (RH), 1.0 hr at 64.4°C and 32.5% RH, 1.75 hr at 76.7°C and 27% RH and 0.25 hr at 82.2°C and 25% RH. Smoke was generated during the last 3 hr of thermal processing. Two replications of the experiment were conducted, each on different days.

Table 1—Knockwurst formulation[a]

Ingredient	Amount
Lean pork (80% lean, 20% fat)	9.46 kg
Lean beef (80% lean, 20% fat)	2.27 kg
Fat pork (50% lean, 50% fat)	1.89 kg
Water	3.41 kg
Salt	268.8 g
Dextrose	66.8 g
Liquid seasoning	51.1 g
Sodium erythorbate	7.5 g
Sodium nitrite	2.1 g

[a] Individual test batches also contained 1.75% (238.4g), 2.0% (272.4g), 3.5% (476.7g) whey protein concentrate, 3.5% calcium-reduced nonfat dry milk NFDM (476.7g) or 2.0% soy protein isolate (272.4g), respectively.

Stability analyses

Cooked yields were determined for each treatment-replication combination. Total weights were recorded for each batch after stuffing and before being placed in the smokehouse. Batch weights were again recorded 24 hr after thermal processing. Cooked yields were calculated using the formula:

$$\text{Cooked yield} = \frac{\text{Cooked, chilled weight}}{\text{uncooked weight}} \times 100$$

Emulsion stability was determined using the procedure developed by Townsend et al. (1968). The temperature of a 34g sample was raised to 68.8°C during a time period of 1.5 hr. Protein, fat, gel water and total losses were measured.

Released fat (RF) and water binding capacity (WBC) were determined using a modified version of the procedure developed by Puolanne and Terrell (1983). Constant lengths of 34 mm collagen casing were weighed before stuffing. Stuffed weight was then recorded and emulsion weight determined by difference. A manually operated stuffer was used for these samples due to small sample size (approx. 400g). The knockwurst RF and WBC samples were then heat processed with the rest of the knockwurst. After 48 hr in a 2°C cooler, peeled weight and weight of fat physically separated from the stick were determined. Subsequent determination of added water, based on emulsion weight, allowed the determination of the weight of separated fat in g/100g batter (RF) and WBC (g/100g batter) for individual samples

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using the following formula:

$$WBC = \frac{\text{Emulsion wt (g)} - \text{Peeled wt(g)} - \text{added water (g)}}{\text{Emulsion wt (g)}} \times 100.$$

Instron textural analyses

A modification of the compression testing procedure first introduced by Friedman et al. (1963) was used. All samples were 1.5 cm thick with a 3.4 cm diameter. The knockwurst was tested both warm (60°C) and cold (3.5°C), in triplicate. The warm knockwurst samples were prepared by placing the links in boiling water, removing the container from the heat when boiling resumed and slicing after allowing the samples to steep in the hot water for an additional 10 min. Samples were held until tested in a double boiler placed in a 60°C water bath.

As described by Lesiak (1980), each sample was placed on a 15.2 x 21.6 cm platform and compressed 40% of its thickness (0.6 cm) with a 14.3 cm diameter plate. The sample was compressed two consecutive times. Hardness, elasticity, cohesiveness and chewiness were determined by methods described by Friedman et al. (1963) and Bourne (1968). Hardness is defined as the peak force of the first curve. Elasticity equals the distance of the base of the second curve. Cohesiveness is determined by dividing the area of the first curve by the area of the second curve. Chewiness is the product of hardness x elasticity x cohesiveness. Test parameters included a 500 kg load cell, a full scale load of 2 kg, a crosshead speed of 50 mm/min and a chart speed of 500 mm/min.

Sensory evaluation

Consumer panels, composed of college students and staff, evaluated each treatment in the two replicates of this study for flavor, texture, juiciness, aftertaste and overall acceptability. A seven-point hedonic scale was used (7 = like extremely; 4 = neutral; 1 = dislike extremely), and each treatment was assigned a random three-digit number. Two panel sessions were held for each treatment-replication with six samples evaluated per session. The knockwurst were evaluated warm 60°C, as they would normally be consumed. Panel size ranged from 33 to 36 individuals.

Statistical analyses

A randomized complete block design was used to analyze this study. Results of the various tests and sensory evaluation were analyzed using Duncan's new multiple range test to determine differences among treatment means (Steel and Torrie, 1980). Analysis of variance, treatment means and standard deviations were determined with the Statistical Analysis System (Barr et al., 1979).

RESULTS & DISCUSSION

EMULSION STABILITY DATA (Table 2) indicate that all binder treatments improved emulsion stability over the all-meat control, as evidenced by lower ($P<0.05$) values for total loss. Comparison of the 2.0% levels of WPC and SPI showed that knockwurst batter containing 2.0% WPC experienced lower ($P<0.05$) fat, gel water and total losses than the SPI. Thus, the 2.0% WPC produced a more stable batter than did the SPI. The most stable batter ($P<0.05$) was produced by 3.5% RNFDM. Batter containing 3.5% WPC was not different from that with 3.5% RNFDM for protein and fat losses, but greater ($P<0.05$) gel water and total losses were seen for that with the 3.5% WPC than the RNFDM. Since total loss is the summation of the other components, the major difference between batters containing 3.5% RNFDM and WPC was increased stability of the gel water component associated with the use of the RNFDM.

Within level of WPC, 0% (or control) consistently produced greater ($P<0.05$) losses than the other concentrations and thus a less stable system (Table 2). Further relationships with WPC were mixed for emulsion stability (ES), gel water, and total losses, while no differences ($P>0.05$) in ES fat or protein loss were found between the 1.75%, 2.0% and 3.5% concentrations of WPC. Future study should strive to determine the concentration of WPC, between 0% and 1.75%, at which a significant decline in ES fat and protein loss is registered, to establish a minimal concentration of WPC.

Released fat was determined by the physical removal and weight determination of fat from the surface of the cooked and cooled knockwurst. Knockwurst with 2.0% WPC had less ($P<0.05$) released fat than that with the same concentration of SPI (Table 3) and was thus in agreement with the ES result for fat loss. The 3.5% WPC and RNFDM treatments experienced no measureable release of fat (Table 3), in partial agreement with the result for ES fat loss which showed fat loss, but no difference between the two treatments. Within level of WPC, 0% was the only treatment which had measurably released fat, thereby illustrating that the addition of WPC increased fat bind. In this study, product that did not exhibit conspicuous amounts of surface fat but had an oily texture, which indicated a lesser degree of fat loss, was recorded as having no released fat since scraping the entire surface of the product would have resulted in the removal of other components in addition to fat. Future use of this test should include such a scraping in conjunction with laboratory analysis to determine the amount of fat in the scraping residue.

Water binding capacity, determined by subtracting the weights of the cooked, peeled product and added water from the original emulsion weight, indicates the ability of a formulation to retain the water inherent to the muscle. From this calculation it can be seen that decreases in WBC values indicate increases in actual WBC. No significant treatment differences were identified for WBC (Table 3). This was in disagreement with the ES gel water results, in which significant differences were found. This discrepancy may be due to the nature of the tests. The ES gel water test would be expected to show greater differences since it measures the bind of both inherent and added water, while the WBC test is sensitive to the loss of inherent water only.

No significant treatment differences for cooked yield were found (Table 3), which indicates that the addition of the various types and levels of binders provided no advantage in cooked yield over the control in this study.

Results of Instron compression of warm and cold knockwurst are presented in Table 4. An interaction ($P<0.05$) for hardness between temperature and treatment was observed. The treatments were dependent on either hot or cold temperature for different responses, with greater ($P<0.05$) hardness associated with the hot test temperature. An exception occurred in the case of the control where the responses to temperature

Table 2—Emulsion stability of knockwurst containing various types and levels of binders

Parameter	WPC[a] 0.0%	WPC[a] 1.75%	WPC[a] 2.0%	WPC[a] 3.5%	RNFDM[b] 3.5%	SPI[c] 2.0%	S.E.M.
Total loss (mL/34g)	4.67[d]	1.97[ef]	1.44[f]	2.51[e]	0.74[g]	2.23[e]	(0.29)
Protein loss (mL/34g)	0.33[d]	0.04[e]	0.06[d]	0.09[e]	0.02[e]	0.07[e]	(0.11)
Fat loss (mL/34g)	0.38[d]	0.13[e]	0.09[e]	0.04[e]	0.01[e]	0.28[d]	(0.07)
Gel water loss (mL/34g)	3.95[d]	1.80[fg]	1.29[g]	2.38[e]	0.71[h]	1.88[ef]	(0.53)

[a] WPC = Whey protein concentrate.
[b] RNFDM = Calcium reduced non-fat dry milk.
[c] SPI = Soy protein isolate.
[d-h] Means in the same row bearing different superscripts are significantly different ($P<0.05$).

Table 3—Cooking characteristics of knockwurst containing various types and levels of binders

Parameter	WPC[a] 0.0%	WPC[a] 1.75%	WPC[a] 2.0%	WPC[a] 3.5%	RNFDM[b] 3.5%	SPI[c] 2.0%	S.E.M.
Released fat (g/100g)	0.18[f]	0.00[d]	0.00[d]	0.00[d]	0.00[d]	0.07[d]	(0.03)
Water binding capacity (g/100g)	−.65[d]	−.55[d]	−0.60[d]	−.18[d]	−.61[d]	1.02[d]	(1.84)
Cooked yield (%)	84.76[d]	85.91[d]	85.59[d]	83.95[d]	85.38[d]	86.51[d]	(1.32)

[a] WPC = Whey protein concentrate.
[b] RNFDM = Calcium reduced nonfat dry milk.
[c] SPI = Soy protein isolate.
[d-f] Means in the same row bearing different superscripts are significantly different (P<0.05).

Table 4—Texture of knockwurst containing various types and levels of binders, as measured by Instron compression

Treatment	Hardness 3.5°C	Hardness 60°C	Cohesiveness 3.5°C	Cohesiveness 60°C	Elasticity 3.5°C	Elasticity 60°C	Chewiness 3.5°C	Chewiness 60°C
0.0% WPC[a]	7.53[e]	9.07[f]	0.62[e]	0.79[d]	58.0[d]	48.8[e]	268.6[g]	350.4[g]
	(0.97)	(1.25)	(0.03)	(0.03)	(3.9)	(4.8)	(37.1)	(76.6)
1.75% WPC	9.30[d]	11.55[e]	0.66[d]	0.79[d]	55.0[e]	51.5[de]	344.7[f]	474.1[f]
2.0% WPC	9.52[d]	11.77[e]	0.66[d]	0.81[d]	55.3[de]	50.7[de]	348.6[f]	480.2[f]
3.5% WPC	10.10[d]	15.53[d]	0.68[d]	0.79[d]	56.8[de]	55.0[d]	388.2[d]	682.2[d]
3.5% RNFDM[b]	9.57[d]	13.05[e]	0.66[d]	0.78[d]	57.0[de]	54.0[d]	360.8[ef]	554.2[e]
2.0% SPI[c]	11.10[d]	13.28[e]	0.62[e]	0.80[e]	55.8[de]	53.2[de]	383.3[de]	585.2[e]
S.E.M.	(0.99)	(2.07)	(0.02)	(0.03)	(2.8)	(4.4)	(40.8)	(122.6)

[a] WPC = Whey protein concentrate.
[b] RNFDM = Calcium reduced nonfat dry milk.
[c] SPI = Soy protein isolate.
[d-g] Means in the same column bearing different superscripts are significantly different (P<0.05). Means underscored by a common line are not significantly different.

were not significantly different. In contrast, Lesiak (1980) found that warm Polish sausage samples exhibited significantly (P<0.05) lower hardness values than cold samples.

The relative order of magnitude of hardness treatment means fluctuated with temperature. At both temperatures, knockwurst with 2.0% and 3.5% levels of WPC matched or surpassed the hardness of the 2.0% SPI and 3.5% RNFDM treatments, respectively, and the addition of the different types and levels of binders increased (P<0.05) product hardness, compared with the control. Within the hot test temperature, the 2.0% WPC and SPI treatments produced knockwurst hardness values that were not different and the 3.5% WPC treatment produced a harder (P<0.05) product than the 3.5% RNFDM. With WPC, 0% and 3.5% produced lower and greater product hardness, respectively, than the other concentrations. Within the cold test temperature, the hardness of knockwurst with 2.0% WPC and SPI, and 3.5% WPC and RNFDM treatments were not different. Within level of WPC, 0% produced lower (P<0.05) knockwurst hardness values than the other concentrations. Thus, for both temperatures and within concentration of WPC, the results of this study agreed with those of Swartz (1983) who stated that the addition of WPC to comminuted meat systems resulted in increased firmness, when compared to a meat control.

Significant interaction was also observed between temperature and treatment, for compression cohesiveness (Table 4). The treatments were again dependent on either hot or cold temperature for different responses, with greater (P<0.05) cohesiveness associated with the hot test temperature. Lesiak (1980), in work with Polish sausage, also observed that warm product exhibited greater (P<0.05) cohesiveness than cold samples.

As for hardness, the order of magnitude of cohesiveness treatment means fluctuated with temperature. Within the hot test temperature, no treatment differences were found. Thus, the WPC produced equally cohesive products in comparison to the SPI and RNFDM treatments and control. Within the cold test temperature, the 2.0% WPC treatment produced a more (P<0.05) cohesive product than the 2.0% SPI treatment. Cohesiveness values of the 3.5% WPC and RNFDM treatments were not different (P>0.05). Within the levels of WPC, 0% yielded a less (P<0.05) cohesive product than the other concentrations of WPC. Within both hot and cold test temperatures, knockwurst with equal concentrations of WPC matched or exceeded the cohesiveness of that with SPI and RNFDM.

No significant interaction, between temperature and treatment, was identified for compression elasticity or chewiness (Table 4). No elasticity treatment differences were identified between knockwurst containing 2.0% WPC and SPI, 3.5% WPC and RNFDM or the various concentrations of WPC (P>0.05). Thus, the addition of binders had no effect on the recovery of the product between successive compression cycles. Treatment differences (P<0.05), however were observed for compression chewiness. The 2.0% SPI treatment produced a more (P<0.05) chewy product than the 2.0% WPC; chewiness of the 3.5% WPC and RNFDM treatments was not different (P>0.05). Within level of WPC, the chewiness of knockwurst with 3.5% was greater (P<0.05) than that with the other concentrations. Products said to be more chewy, such as that with 2.0% SPI compared to the 2.0% WPC, would have increased resistance to destructive force.

Sensory panel evaluation data are shown in Table 5. The panelists consistently rated knockwurst containing 2.0% WPC more desirable (P<0.05) than that produced with 2.0% SPI, in agreement with Siegel et al. (1979) who also found that panelists discriminated against the flavor of products which contained SPI. In all cases but one (aftertaste, in which no significant treatment difference was detected), knockwurst containing the 3.5% RNFDM was rated more desirable (P<0.05) than that containing 3.5% WPC. However, knockwurst with 1.75% WPC and 3.5% RNFDM were not rated different (P>0.05) for any of the sensory attributes, which suggests that lower levels of WPC might result in better sensory response. The concentrations of WPC were also scored consistently, with the 0% rated as less (P<0.05) desirable than the other concentrations in each case. Swartz (1983) and Lee et al. (1980) also found that WPC improved the flavor, texture and juiciness of processed meat products, as compared to a meat control. The 1.75%, 2.0% and 3.5% concentrations of WPC were not significantly different form each other for any of the sensory parameters.

CONCLUSION

WHEY PROTEIN CONCENTRATE proved to be a viable binder alternative for specific emulsion-type meat products by

Table 5—Sensory panel evaluation scores for knockwurst containing various types and levels of binders

	WPC[b]				RNFDM[c]	SPI[d]	
Parameter[a]	0.0%	1.75%	2.0%	3.5%	3.5%	2.0%	S.E.M.
Flavor	4.18[g]	4.81[ef]	4.66[f]	4.66[f]	5.12[e]	3.93[g]	(1.04)
Texture	3.33[g]	4.72[ef]	4.43[f]	4.28[f]	5.00[e]	3.75[g]	(1.32)
Juiciness	3.45[g]	4.72[ef]	4.52[f]	4.25[f]	5.03[e]	3.25[g]	(1.14)
Aftertaste	3.97[g]	4.57[f]	4.52[f]	4.61[f]	4.87[f]	3.87[g]	(1.02)
Overall acceptability	3.63[g]	4.72[ef]	4.55[f]	4.48[f]	5.00[e]	3.64[g]	(1.06)

[a] Samples presented at 60°C using a hedonic scale of 1 = dislike extremely, 4 = neutral, 7 = like extremely.
[b] WPC = Whey protein concentrate.
[c] RNFDM = Calcium reduced nonfat dry milk.
[d] SPI = Soy protein isolate.
[e-g] Means in the same row bearing different superscripts are significantly different ($P<0.05$).

providing similar stability, textural and sensory attributes in comparison to equal concentrations of soy protein isolate and calcium-reduced nonfat dry milk.

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Ms received 12/24/86; revised 3/17/87; accepted 3/18/87.

Portions of this paper were presented at the 46th Annual Meeting of the Institute of Food Technologists, Dallas, TX, June 1986.
Reference to a firm or brand name does not imply endorsement by the U.S. Dept. of Agriculture to the exclusion of others of a similar nature.

Effect of Mixture and Storage on the Palatability of Beef-Turkey Patties

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ABSTRACT

Beef and turkey mixtures were formulated to contain 100/0, 80/20, 60/40, 40/60, 20/80 or 0/100% lean beef/lean turkey with a constant fat content. Sensory parameters and physical characteristics were measured to evaluate the product during storage. There were no differences ($P>0.05$) in beef flavor between 80% and 100% beef products or between 0%, 20%, 40% and 60% beef patties. Juiciness scores were increased by adding turkey while texture scores were decreased. Similarly, 80% and 100% beef patties had greater ($P<0.05$) hardness than other mixtures. Off-flavor scores were not affected by mixture although malonaldehyde content of cooked patties increased markedly as the percentage of turkey increased. In general, ground beef products containing between 20% to 80% turkey had similar sensory, storage and physical characteristics.

INTRODUCTION

TRADITIONALLY, ground meat products are made primarily from beef and pork. However, other meat animal species, such as poultry, can be used to make acceptable ground products. According to the USDA (USDA, 1985), turkey consumption per capita has increased 56.2% during the period from 1964 to 1984 and about half of the turkey consumed is in the form of further processed products. The most popular processed products are made from turkey breast and other white meat portions, resulting in an abundance of dark meat portions, such a thigh meat. In contrast to ground beef or pork, turkey thigh meat is typically very lean which makes it useful for formulating with pork or beef trim with high fat contents. It is also relatively inexpensive; therefore, ground meat products combining beef with turkey thigh meat could be made with a reduced fat content and/or at a lower cost while still maintaining nutritional quality and quantity of protein in the product. Moreover, poultry meat has a mild flavor so mixed species meat products would be expected to retain the characteristic flavor of the red meat component.

Several researchers have examined the use of beef mixed with turkey and other poultry meats in cured or sausage-type products. Dawson (1970) incorporated ground beef into fermented sausage made with either turkey or chicken. Fermented sausages containing 50% beef received the highest acceptance scores, although 100% chicken products were more tender than 100% turkey products or products made from beef combined with chicken or turkey. Dhillon and Maurer (1975a) evaluated summer sausage formulated with mechanically deboned chicken meat (MDCM) mixed with ground beef. Various combinations up to the 50% MDCM level resulted in an acceptable product with good color, firmness and texture.

In another experiment (Dhillon and Maurer, 1975b), summer sausages stored at different temperatures (20°C and 4°C) and formulated from 50% MDCM and 50% ground beef, 50% mechanically deboned turkey meat (MDTM) and 50% ground beef or from 100% ground beef were evaluated. The overall shrinkage observed was higher in sausages containing MDCM and MDTM than in the 100% ground beef sausages held at 20°C although weight loss in general was higher for sausages stored at 20°C than 4°C. Shear values and sliceability scores of sausage formulated with MDCM and MDTM increased with length of storage showing increased firmness, presumably related to drying of the products.

However, there is little information about uncured, ground products made from mixtures of beef and turkey meat. Therefore, the present study was designed to provide information on sensory properties, texture characteristics and lipid oxidation in ground meat products made with different mixtures of beef and turkey. The effects of frozen storage for up to 28 days and the effects of various cooking methods on sensory properties were also evaluated.

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MATERIALS & METHODS

Product preparation

Fresh, hand-deboned turkey thigh meat was purchased from a local processor. Beef trim and beef fat were obtained from the Univ. of Illinois Meat Science Lab. Lean turkey, lean beef and beef fat were coarsely ground through a 1.3 cm plate, sampled for composition analysis, vacuum packed and stored frozen at −20°C until used. The ground turkey thigh meat, ground lean beef and ground beef fat contained 6.9, 4.1 and 68.7% extractable lipid, respectively.

Six different mixtures of beef and turkey were prepared as shown in Table 1. Formulations were based on the content of fat-free muscle and the mixture was formulated to contain lean in proportions of 0/100, 20/80, 40/60, 60/40, 80/20, or 100/0% beef/turkey. These values are used throughout the paper as nominal designations of mixture composition and are an accurate indication of the proportion of lean muscle contributed by each species. Because fat added to the ground products was beef fat which contained some muscle, the 0% beef-100% turkey mixture contained some beef lean. The designation was left as 100% turkey for the sake of simplicity. A lower fat content was also accepted for this mixture in order to maintain the incremental increase in turkey muscle between different mixtures.

Each of the six batches was ground separately through a 0.48 cm plate, stuffed into 10.2 cm diameter casings with a hydraulic stuffer and frozen overnight at −20°C. The next day (day 0 of storage), the chubs were sawed into 2.5 cm thick patties and stored at −20°C until used. Taste panel evaluation, Instron texture measurements, proximate analysis and malonaldehyde content measurements were conducted after 0, 14, and 28 days of storage at −20°C. Patties were thawed overnight at 4°C before cooking.

Cooking procedures

On each test day, patties were cooked on Farberware open hearth grills and internal temperature was monitored using copper-constantan thermocouples with a Campbell CR5 Digital Temperature Recorder. Patties were turned at an internal temperature of 34°C and removed from the grill at an internal temperature of 68°C.

After 28 days storage, additional patties were also cooked in a microwave oven, a convection oven, and on a grill to evaluate the effect of cooking method on sensory parameters of the different mixtures. In all cases, the patties were cooked until the internal temperature reached 68°C.

Taste panel evaluation

The taste panel was composed of faculty members and graduate students with previous experience in evaluating the sensory parameters

Table 1—Composition of ground meat mixtures[a]

Nominal mixture[b]	Formulation (%)			Actual composition (%)			
				Uncooked		Cooked	
	Lean beef	Lean turkey	Beef fat	Fat	H_2O	Fat	H_2O
0% Beef-100% turkey	0	80.6	19.4	22.0	61.7	23.5	53.3[a]
20% Beef-80% turkey	6.0	64.5	29.5	30.6	55.0	25.4	51.8[b]
40% Beef-60% turkey	21.4	48.4	30.2	29.3	55.8	26.1	50.2[c]
60% Beef-40% turkey	36.8	32.2	31.0	30.0	54.7	24.6	50.5[bc]
80% Beef-20% turkey	52.2	16.1	31.7	29.7	54.5	25.4	49.7[c]
100% Beef-0% turkey	67.7	0	32.3	29.9	54.4	25.9	47.6[d]

[a] Since there were no significant mixture-by-storage interactions, values were pooled across storage times. Means for cooked composition having different superscripts are significantly different ($P<0.05$).

[b] Refers to the target percentage lean from each species in the mixture. Nominal values are also used throughout the text to identify treatments.

examined. Panel members were familiar with the evaluation form and had previously received at least one hour of instruction in its use. Panelists were seated in partitioned booths with red lighting in a room separate from the sample preparation room. Panelists were given water for rinsing and eight samples were evaluated during each tasting session. Samples were presented in random order within a replicate and 2 replications were completed. The sensory parameters evaluated, juiciness, beef flavor intensity, off-flavor intensity and texture, were scored on a 15 cm unstructured rating scale with anchors at each endpoint and a centerline. The verbal description of the parameters were 0 cm or left anchor = extremely dry, bland, intense or soft, and 15 cm or right anchor = juicy, intense, none or firm, respectively.

Instron compression measurements

Patties containing 20% and 80% beef which had been stored for 28 days were cooked on a grill and cooled at 4°C for 2 hours. Cores, 2.5 cm diameter, 2.5 cm thick, were removed from each patty. The force necessary to compress the samples by 75% of original thickness was measured twice on each core with a 1122 Universal Instron Machine. The Instron was equipped with a 500 kg compression load cell and had a crosshead descent speed of 100 mm/min, a chart speed of 100 mm/min and a load range of 5 kg. Hardness, measured by the height of the first compression force curve, was expressed as kg force (explicitly, kg × G). Cohesiveness was measured as the ratio of the area under the second compression force curve (A_2) to the area under the first curve (A_1) as described by Bourne (1978).

Thiobarbituric acid (TBA) assay

Raw and cooked patties from each mixture were pulverized in liquid nitrogen with a blender and the concentration of lipid oxidation products was determined using the TBA assay method of Witte et al. (1970) with malonaldehyde (MA) as a standard. The content of oxidation products was expressed as milligrams of MA per kg fat in the sample.

Proximate analysis

Fat and moisture contents were determined in triplicate using repeated chloroform:methanol (2:1) extraction and oven drying (Riss et al., 1983).

Statistical analysis

Treatments were arranged as a factorial design and data were analyzed using Statistical Analysis System (SAS, 1982) procedures. SAS General Linear Models (GLM) procedures were used for analysis of variance with a model having mixture and storage time as main effects and a mixture by storage interaction term. When a significant main effect existed, differences between treatments were determined using Duncan's Multiple Range Test. Data presented in tables demonstrating a main effect were pooled across the other treatment variable using the means output of the GLM procedure. The SAS Correlation procedure (CORR) was used to examine the relationship of mixture or storage to the various sensory and physical characteristics.

RESULTS & DISCUSSION

ANALYSIS OF VARIANCE indicated there were no significant interactions between mixture and storage effects so the main effect of each of these parameters was examined by pooling data across the second parameter. Sensory evaluation scores for different mixtures are shown in Table 2.

Among the four parameters tested, beef flavor had the largest range of difference between formulations and there was a clear trend of increased beef flavor with increased beef content. In spite of the wide range of beef flavor, the beef flavor scores of products containing from 40% beef up to 80% beef were not statistically different nor were scores for patties containing from 0% to 60% beef. Differences in beef flavor scores between the 0% and 100% beef patties and the other mixtures were larger than for products with 20–80% beef.

A variety of heat-induced reactions, including proteolysis of peptides, degradation of sugars, oxidation and decarboxylation of lipids, degradation of thiamine and ribonucleotides and interactions between sugars, amino acids and fats, may lead to the production of meaty flavors (Vanden Ouweland et al., 1978). However, the specific factors contributing to the unique species flavors are not well characterized. Studies on meat from various animals indicate the lean from different species gives rise to identical meaty flavors when heated, while the characteristic species flavor is present in the fat portion of the meat (Hornstein and Crowe, 1960; Wasserman and Talley, 1968). Therefore, the similarity of beef-flavor scores in products with intermediate mixtures is not surprising since all of the products in the present study contained relatively high levels of beef fat. In contrast to the results of our study, Baker et al. (1969) found no differences in flavor scores for chicken frankfurters containing different levels of beef fat, pork fat or cottonseed oil. Furthermore, Cross et al. (1980) observed no differences in beef flavor intensity of ground beef patties made with 16 to 28% fat, which suggests that a difference in fat content is not a major factor in the flavor intensity of cooked ground beef.

There were no significant differences observed in off-flavor among the various mixtures nor were there significant differences in juiciness among patties containing 0, 20, 40 or 60% beef. However, the juiciness scores tended to show a small numerical increase as the percentage of turkey in the mixture increased. This trend agreed with our proximate analysis of cooked products shown in Table 1. As the amount of turkey in the patties increased, the moisture retained during cooking increased. Similar results were obtained by Barbut et al. (1984).

Taste panel scores for texture were also affected by the proportion of beef and turkey. Texture scores were numerically more desirable as the percentage of beef increased in the patties (Table 2). However, comparison of pooled means indicated that the texture scores for the patties containing from 40% to 100% beef were not different from each other ($P>0.05$). This would agree with results from experiments comparing mixed species summer sausage in which there were no significant differences in texture scores in summer sausages formulated with 65% hand-deboned chicken meat and 35% beef, compared to 100% beef (Dhillon and Maurer, 1975b). Texture scores of summer sausage containing MDCM and beef, 50%:50%, or MDTM and beef, 50%:50%, were also similar (Dhillon and Maurer, 1975a).

Table 2—Main effect of mixture on taste panel scores for mixed, ground beef and turkey products[a]

Percent beef	Juiciness	Texture	Beef flavor	Off-flavor
0	11.70 ± 0.21[a]	8.14 ± 0.32[a]	6.75 ± 0.39[a]	13.32 ± 0.26[a]
20	11.31 ± 0.26[a]	8.62 ± 0.30[b]	8.21 ± 0.47[a]	13.61 ± 0.26[a]
40	11.07 ± 0.30[ab]	9.22 ± 0.31[bc]	9.05 ± 0.56[ab]	13.79 ± 0.24[a]
60	10.88 ± 0.35[ab]	9.34 ± 0.48[bc]	9.44 ± 0.51[ab]	13.48 ± 0.29[a]
80	9.76 ± 0.39[c]	9.41 ± 0.30[bc]	9.94 ± 0.37[bc]	13.24 ± 0.33[a]
100	10.23 ± 0.21[bc]	9.95 ± 0.15[c]	11.28 ± 0.28[c]	13.82 ± 0.25[a]

[a] Values are means ± SEM (n = 9). Since there were no significant mixture by storage time interactions, values were pooled across storage times. Means in columns having different superscripts are significantly different (P<0.05). Left anchor or 0 cm = extremely dry, bland, intense or soft, and right anchor or 15 cm = juicy, intense, none or firm, respectively.

The effect of storage on sensory parameters is shown in Table 3. Beef flavor, off-flavor and texture of mixed, ground beef and turkey patties were not affected by storage for 14 or 28 days at −20°C. The lack of change in flavor and off-flavor may be the result of the relatively short periods of storage time. Sim and Carlin (1968) observed no significant changes in flavor and off-flavor for turkey steak patties stored for 1 or 2 months at −18°C, but as the storage time increased to 4 months, the flavor scores decreased and off-flavor scores increased slightly. Martinson and Carlin (1968) also observed that flavor and off-flavor scores of precooked boneless turkey roasts stored at −18°C decreased more with storage between 2 and 7 months than between 2 weeks and 2 months.

Malonaldehyde contents were determined as an indicator of oxidative changes in samples. Overall, the concentration of MA prior to cooking increased with frozen storage (data not shown), although there was no effect (P>0.05) of storage on MA content of cooked patties. The relationship between turkey or beef content, MA content and cooking is shown in Fig. 1. Because MA values partly reflect lipid composition, data are presented as the ratio of turkey fat to beef fat in the mixtures, ranging from 0 for 100% beef patties to 0.29 for the 100% turkey lean patties. The concentration of MA for patties containing less than 80% beef (ratio of turkey fat to beef fat > 0.1) was higher after cooking while MA levels were slightly lower after cooking compared to patties containing 80 or 100% beef. The MA contents of cooked patties increased linearly with the increase in the ratio of turkey fat/beef fat while before cooking, the MA content of patties was relatively unaltered by the change in mixture. This may indicate that the turkey fat or oxidized products in the turkey fat served as a pro-oxidant during the cooking process (Verma et al., 1985). Enhanced oxidation in poultry during cooking was reported by Siu and Draper (1978) in a survey made of the MA content of 96 fresh and processed meat and fish samples. They observed that cooking led to substantial increases in MA content in some meats but not in others, including beef chuck steak and beef round steak. The oxidative deterioration of muscle lipids and concurrent development of off-flavor involves oxidation of unsaturated fatty acids, particularly polyunsaturated fatty acids which have three or more double bonds and are a component of phospholipids (Reineccius, 1979; Allen and Foegeding, 1981). Since poultry has more unsaturated fatty acids (Katz et al., 1966) than beef (Hood and Allen, 1971), it is more susceptible to oxidative degradation and pro-oxidant activity.

The correlation coefficient between concentration of MA and off-flavor scores was not significant (r = −0.35) and a similar lack of correlation between concentration of MA and off-flavor scores has been observed by others (Dawson and Schierholz, 1976; Dawson et al., 1975; Martinson and Carlin, 1968).

Hardness of cooked patties increased as the amount of beef in the mixture increased (Table 4). Hardness scores were negatively correlated with juiciness (r = −0.93) and positively with texture (r = 0.64). The negative correlation between hardness and juiciness suggests that as the products became harder, presumably by increased binding of the meat particles, they also became less juicy. Correlations between hardness and sensory texture were also observed by Lyon et al. (1980). Sofos and Allen (1977) reported a decrease in Instron hardness with the replacement of beef by pork or beef heart in frankfurters. However, they failed to find any correlation between hardness and sensory texture or moisture.

Cohesiveness did not differ (P>0.05) among the six different formulations (Table 4), and no correlations were found between cohesiveness value and sensory texture or juiciness. The lack of correlation between objective measures of cohesiveness and sensory parameters indicates that cohesiveness may not be a useful measurement of texture for mixed ground meat patties. A poor correlation between sensory evaluation of texture and cohesiveness of patties made from flake-cut, mechanically deboned poultry meat and structured soy protein fiber was also observed by Lyon et al. (1980).

The method used in cooking food may have a critical influence on sensory properties of food. Microwave ovens are becoming popular because of shortened cooking time (Cipra et al., 1971) and theoretical energy savings of as much as 65% compared to a convection oven (Snyder, 1978). However, ground beef or turkey was less acceptable when cooked in microwave ovens rather than conventional gas or electric ovens (Cipra et al., 1971; Cremer, 1982; Berry and Leddy, 1984) so we also examined the effect of cooking methods on sensory properties (Table 5). Analysis of variance indicated no significant interaction between mixture and cooking method interaction for any sensory parameter, thus, sensory scores were pooled across mixtures. The lack of interaction suggests a similar effect of cooking method on sensory characteristics for both ground beef and ground turkey.

Of the three cooking methods tested, grilling was found to be the most acceptable. Scores for juiciness and off-flavor of grilled patties were higher than for oven or microwave cooking, while texture and beef flavor intensity were not significantly different among the three methods (P<0.05). This was in contrast to results reported by Berry and Leddy (1984) where the effect of cooking method on beef-flavor intensity of ground beef patties was frying > convection oven roasting > microwave cooking. Off-flavor intensity scores were less desirable with convection oven or microwave cooking while mixtures

Table 3—Main effect of storage taste panel scores for mixed, ground beef and turkey products[a]

Day	Juiciness	Texture	Beef flavor	Off-flavor
0	10.54 ± 0.23[a]	9.04 ± 0.15[a]	8.92 ± 0.43[a]	13.73 ± 0.21[a]
14	10.71 ± 0.29[ab]	9.08 ± 0.29[a]	9.12 ± 0.46[a]	13.54 ± 0.18[a]
28	11.23 ± 0.21[b]	9.23 ± 0.30[a]	9.30 ± 0.47[a]	13.37 ± 0.18[a]

[a] Values are means ± SEM (n = 18) pooled across mixtures. Means in columns having different superscripts are significantly different (P<0.05). Left anchor or 0 cm = extremely dry, bland, intense or soft, and right anchor or 15 cm = juicy, intense, none or firm, respectively.

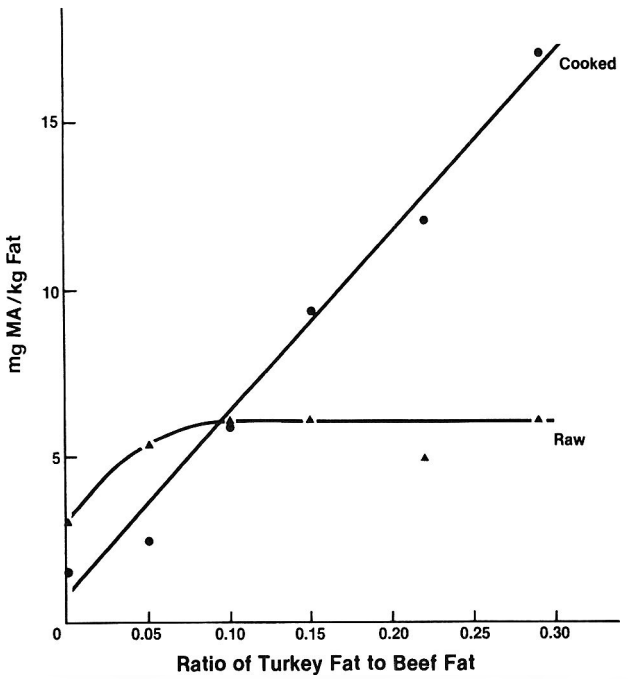

Fig. 1—Relationship of mixture expressed as the ratio of turkey to beef fat and malonaldehyde content before and after cooking.

Table 4—Physical properties of mixed beef and turkey patties cooked after 28 days of storage[a]

% Beef	Hardness (kg)	Cohesiveness
0	13.75 ± 0.11[a]	0.25 ± 0.01[a]
20	12.88 ± 0.09[a]	0.26 ± 0.02[a]
40	13.38 ± 0.05[a]	0.23 ± 0.02[a]
60	15.00 ± 0.17[a]	0.40 ± 0.10[a]
80	24.00 ± 0.10[b]	0.25 ± 0.04[a]
100	20.50 ± 0.28[b]	0.26 ± 0.04[a]

[a] Measurements made with an Instron Model 1122. Numbers are the means ± SEM (n=4). Means in columns having different superscripts are significantly different (P<0.05).

Table 5—Effects of cooking methods on sensory characteristics of mixed, ground beef and turkey patties[a]

Cooking method	Grill	Oven	Microwave
Juiciness	11.23 ± 0.27[a]	11.34 ± 0.30[a]	10.10 ± 0.26[b]
Texture	9.09 ± 0.36[a]	8.61 ± 0.42[a]	9.37 ± 0.62[a]
Beef flavor	8.92 ± 0.72[a]	7.88 ± 0.70[a]	7.53 ± 1.19[a]
Off flavor	13.17 ± 0.25[a]	11.14 ± 0.25[b]	12.07 ± 0.40[b]

[a] Values are means ± SEM (n = 4). Values were pooled across the two mixtures (20% and 80% beef) becuase there were no significant mixture by cooking method interactions. Means in rows having different superscripts are significantly different (P<0.05). Left anchor or 0 cm = extremely dry, bland, intense or soft, and right anchor or 15 cm = juicy, intense, none or firm, respectively.

cooked by microwave had lower juiciness scores than other cooking methods.

CONCLUSION

THE RESULTS of this study indicate that products made from different mixtures of beef, beef fat and turkey have similar palatability and physical characteristics. Furthermore, retention of quality during storage is not adversely affected by blending different species of meat. Beef flavor, off-flavor, juiciness and texture scores were not different between 100% beef products and products with 20% turkey lean nor between those with 40, 60 or 80% turkey lean. Neither beef flavor or off-flavor were affected in any blend by frozen storage for up to 28 days. This suggests that properly labeled products made with a blend of beef and turkey dark meat would be more acceptable than ground turkey alone.

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Ms received 8/25/86; revised 5/1/87; accepted 5/11/87.

Chemical and Instrumental Analyses of Warmed-Over Flavor in Beef

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ABSTRACT

Raw, freshly cooked, stored and recooked beef muscle samples were assessed by chemical, instrumental and sensory methods of analyses for flavor quality, with particular emphasis on warmed-over flavor (WOF). The character notes used by a trained sensory panel to describe WOF were cardboardy, rancid, stale, and metallic. Samples analyzed by direct gas chromatography utilizing either packed or fused silica capillary columns showed that compounds usually associated with lipid oxidation reactions could be used as marker compounds to follow the development of WOF. Of the many compounds that appeared to be markers, hexanal and 2,3-octanedione as well as total volatiles showed a highly significant degree of correlation when compared to sensory scores and 2-thiobarbituric acid (TBA) numbers. Many of the volatile compounds that were identified in WOF meat samples were also found in the distillates prepared for the TBA reaction.

INTRODUCTION

THE ACCEPTANCE of meat, poultry, and fish products depends to a large extent on the desirability of their flavor. Some of the factors that influence the flavor of these products include animal feed intake, metabolic processes, processing methods, storage conditions and sanitation. Some specific problems are oxidation reactions resulting during long term storage, forage fed off-flavors in beef and sheep, species and sex-specific flavors, undesirable flavors resulting from processing procedures such as irradiation and high temperature retorting, and, one of the most important of these problems, "warmed-over" flavor (WOF) in meats and poultry.

Tims and Watts (1958) first defined the rapid development of oxidized flavor in refrigerated cooked meats as WOF and described it as rancid or stale. Normally, the off-flavor became apparent within the first 48 hr after refrigeration at 4°C. This rapid development was the most pertinent factor that differentiated WOF from the ordinary rancidity flavors that develop during long term storage. In the past decade, WOF has been the subject of many reports including two comprehensive reviews (Pearson et al., 1977; Pearson and Gray, 1983). Many reports support the hypothesis that WOF is a result of lipid oxidation which is catalyzed nonenzymatically by metal ions, a topic recently reviewed by Love (1983). More specifically, the polyunsaturated fatty acids from polar lipids (particularly phospholipids) appear to be the primary substrate for the formation of oxidation reaction products that contribute to WOF, since the destruction of phospholipid is concomitant with WOF formation (Wilson et al., 1976; Pearson et al., 1977; Love, 1983; Igene et al., 1985; Willemot et al., 1985). Nevertheless, the precise mechanisms involving the formation of WOF have never been resolved. Almost all researchers in the area do agree that the WOF and classically described "rancidity" are definitely different. However, the flavor molecules causing both may be the same in varying intensities during the dynamic meat flavor changes leading to WOF. The primary purpose of this study was to conduct basic experiments that would correlate fundamental chemical and sensory changes observed in meat with character notes that may contribute to WOF. Other purposes were (1) to present an objective instrumental method to follow the development of WOF, (2) to identify volatile compounds that distilled during the TBA procedure.

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MATERIALS & METHODS

Meat preparation

Thirty-six top round roasts (semimembranosus and adductor muscles, 2–3 kg) were purchased from local supermarkets. In some cases, top rounds (7–9 kg each) were purchased from wholesale meat companies, packaged in Cryovac bags for storage and used within 10 days of slaughter. The meat was kept at 4°C if it were to be cooked within 2 days; otherwise, it was stored at −20°C. Microbiological assays were conducted for total aerobic plate count, coliforms, Salmonella, and Pseudomonas by a commercial laboratory. Microbial counts were within acceptable range. Roasts, averaging 2.3 kg, were cooked in a preheated oven at 177°C for 141 min until the internal temperature reached 71°C. After cooking, roasts were either cut into chunks (ca 6.4 cm cubes) or ground in a meat grinder. The cooked samples that were to be examined for development of WOF were wrapped in aluminum foil and refrigerated at 4°C for 16 hr, after which, the samples were reheated 10 min in a preheated oven at 70°C and analyzed. For storage tests, the meat was kept in the refrigerator for several days at 4°C prior to reheating.

Sensory analysis

An inhouse panel of 15 members was trained to recognize WOF by using difference tests—triangle tests, ranking tests and intensity rating tests. The warm, reheated ground roast beef and the freshly cooked ground beef samples were presented to the sensory analytical panel and the intensity of WOF was estimated on a 10 cm line scale. The anchors were no WOF and extreme WOF. Distances were measured from the anchor representing no WOF. The freshly cooked beef was always found to contain no WOF. The average difference determined between the samples was 6.23 (a rating that lies between moderate and much difference). In seven triangle tests, the panelists found differences ($P<0.05$) between freshly cooked ground beef and stored (1 day, 4°C). Thus, the response data indicated that the panel was operating at an acceptable performance level and within a small range of error and could differentiate between freshly cooked roast beef and that stored for 1 day at 4°C and reheated. The character notes most used to describe WOF in this model were cardboardy, rancid, stale and metallic. The warmed-over flavor characteristics in these roasts were calibrated for intensities according to the descriptors and beef patty standards of Johnsen and Civille (1986).

Chemical analyses

Volatile compounds in meat samples were separated by direct gas chromatography using an external closed inlet device (ECID) (Legendre et al., 1979) on either packed Tenax GC/PMPE columns (Dupuy et al., 1978) or on SE-54 capillary columns (Dupuy et al., 1985b). Identification of the compounds was accomplished with each system coupled to a Finnigan-MAT 4000 Gas Chromatograph/Mass Spectrometer/Data System. Volatile compounds in distillate of aqueous samples prepared for malondialdehyde (MDA) determinations were separated and identified by the method recently described (Vercellotti et al., 1985). In this method, 200 mL distillate were placed in an Erlenmeyer flask with a condenser topped with a standard taper Teflon stopper, which held a 9mm glass cartridge filled with Tenax-GC. This system was held under vacuum (5 psi) while heating from ambient to 60°C. The volatiles were stripped and adsorbed onto the Tenax-GC trap, which was then inserted into the external closed inlet device (ECID) attached to a gas chroma-

tograph and the volatiles thermally desorbed, as described recently (Dupuy et al., 1985a). MDA values of the experimental samples were determined by the distillation method of Tarladgis et al. (1960); 2 thiobarbituric acid (TBA) reaction products were measured at 532 nm with a Hewlett-Packard 8450-A diode array spectrophotometer. Protein determinations were by the method of Lowry et al. (1951).

Total lipids (organic solubles) in raw, freshly cooked and stored meat were determined by extracting with chloroform:methanol (3:1) using an Omnimixer to homogenize the sample (50 g with 200 ml solvent). The mixture was then put on a Buchner suction filter with a spun glass filter. The residue was then homogenized two more times with solvent as above. The combined organic filtrates were separated from water in a separatory funnel, and the lower layer drawn off and dried over sodium sulfate. The extract was concentrated in a preweighed round bottom flask on a rotary evaporator with an aspirator pump at diminished pressure (1 mm Hg). The residues were taken to dryness under vacuum at 40°C and then dried overnight to constant weight.

Experimental samples were extracted with cold (4°C) water filtered to obtain the soluble flavor compounds. A ratio of 1 part meat to 5 parts deionized water was used; homogenization was done in the Omnimixer cooled with an ice bath. The extracts were examined for flavor compounds by sensory and chemical analyses.

RESULTS & DISCUSSION

TO DEVELOP A WOF MODEL in beef that was consistent and representative of the flavor phenomenon, a large number of acceptable quality commercial beef samples were prepared under similar roasting conditions. For this purpose, the roasts were processed for sensory panel evaluation and also analyzed for malondialdehyde (MDA) formation and volatile compounds by direct gas chromatography. In all, some 230 separate raw and roasted beef samples were assayed by chemical, instrumental and sensory analyses. The raw samples were not evaluated by sensory analysis. The necessity to have cooked beef samples that were evaluated by a trained sensory panel required proper chemical correlation between biochemical and physical data and sensory data. The roast beef samples were compared for flavor aromatics, WOF characteristics and intensities according to the descriptors reported by Johnsen and Civille (1986). The 1 day storage of this ground roast beef produced essentially the same intensity of cardboard and painty off-flavors (intensity of 3 and 5, respectively, on a fifteen point scale) as the stored grilled meat patty standards evaluated in Johnsen and Civille (1986). The Johnsen and Civille (1986) methodology has proven very useful in this study and should set a working standard for future WOF studies.

Volatile compounds

The dynamic flavor deterioration process in meat creates essentially a new sample with time. Hence, aging studies on 36 original roast beef samples resulted in literally hundreds of samples that were assayed in this study. Direct gas chromatography (GC) is a powerful research tool for assessing food quality. The versatility of this method was recently reported in studies on WOF and vegetable oils (Dupuy et al., 1985a,b); food quality assessment (St. Angelo et al., 1985); Maillard reaction products (Bailey, 1985); carbonyl-amine reactions of carbohydrates in foods (Vercellotti et al., 1985); and geosmin in catfish tissue and pond water (Dupuy et al., 1986).

Meat samples, raw, freshly cooked and stored meat exhibiting WOF, were examined by direct GC with the results reported in Fig. 1-4. In Fig. 1, the direct GC volatile profiles of the samples were obtained by analysis on packed columns, whereas in Fig. 2, 3 and 4, the samples were analyzed on capillary columns. In Fig 1, the dashed line trace, which represents freshly cooked meat, indicates the volatile profile is very low. However, in meat samples stored for 2 days at 4°C and reheated, the volatile profile was increased. The compounds present in highest concentrations were hexanal, heptanal, pentanal, nonanal, 2-pentylfuran (products of lipid oxidation) and 2,3-octanedione. The concentrations of these

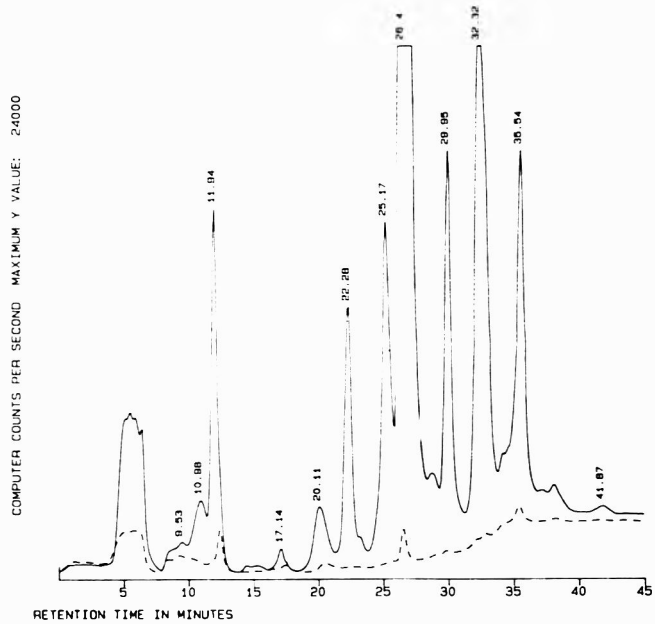

Fig. 1—Volatile profiles from freshly cooked (lower curve) beef and from roast beef stored at 4°C for 2 days (upper curve) and rewarmed. Volatiles were obtained on Tenax polyMPE packed columns. Major components, identified by direct GC MS, were propanal (11.94), pentanal (22.28), 3-hydroxy-2-butanone (25.17), hexanal (28.4), heptanal (29.95), 2,3-octanedione (32.32), and nonanal (35.54).

compounds increased with time over the next several days but at a lower rate. In Fig. 2, the volatile profile of freshly cooked meat obtained on capillary columns is better resolved and many more compounds are evident. The intensity of these compounds greatly increased on storage (Fig. 3-4). These studies show that the direct GC method for determining volatiles in meat samples could be used to evaluate quality. Furthermore, they showed that a GC with the ECID and capillary column gave better resolution and increased sensitivity than a GC equipped with the ECID and packed columns.

More than 150 compounds were identified by direct gas (fused silica capillary) chromatography/mass spectrometry (GC/MS) in the volatiles of the WOF roast beef. The majority of the compounds appeared to be at the ppb concentrations whereas others seemed to be in higher amounts. Compounds identified at the ppm level that appear to intensify during the development of WOF are listed in Table 1. Most of them appeared to be secondary reaction products from oxidation of polyunsaturated fatty acids. Of all the compounds observed, hexanal was the one which always had the highest intensity. It increased in concentration within hours after reheating and probably began to increase immediately after initial cooking. The compound, 3-hydroxy-2-butanone, was present in most of the samples analyzed. As beef is stored at refrigerated temperatures over several days, many of the compounds identified in Table 1 increase in their intensities. Many of these markers had positive correlations compared to meat samples experimentally identified as having WOF. However, their contribution to the odor/flavor notes of WOF remain unknown.

The correlation of sensory evaluation, MDA production and marker volatiles corresponding to WOF formation, was simplified by using only two of the compounds, hexanal and 2,3-octanedione, plus total volatile content. Mean sensory, chemical, and instrumental values of samples stored at 4°C for 1 day are shown in Table 2. Each sensory value represents the mean of 12 to 15 panelists. The overall mean from the one-day WOF beef was more than twice the value of that mean from freshly cooked beef. Other overall mean values also showed

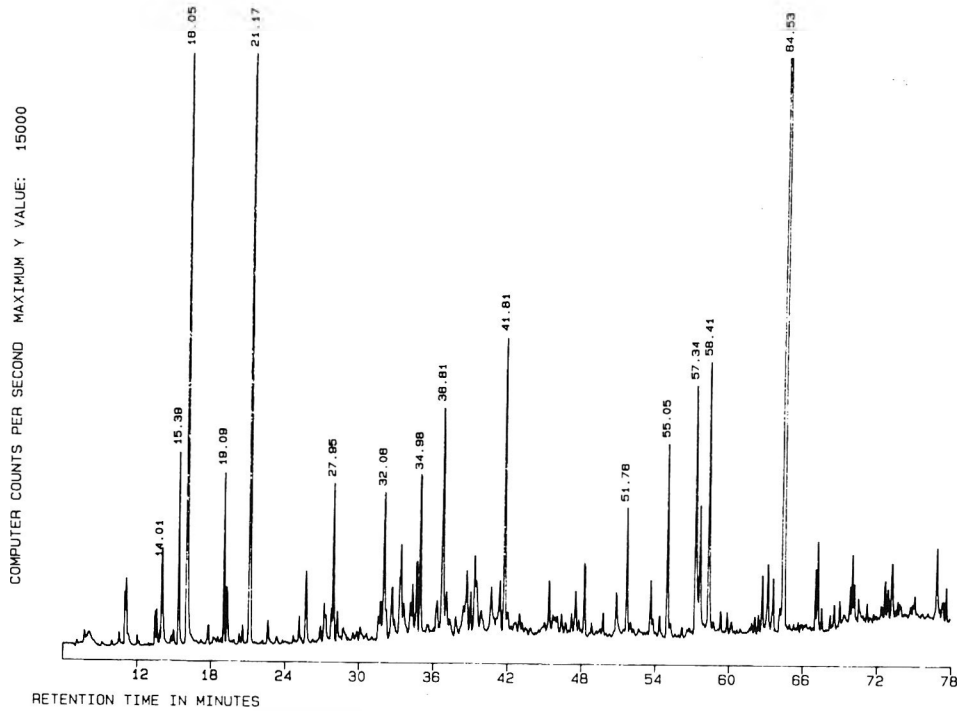

Fig. 2—Volatile profile from freshly cooked beef. Volatiles were obtained on direct capillary gas chromatography. Major components, identified by direct capillary GC/MS were pentanal (15.39), 3-hydroxy-2-butanone (18.05), hexanal (21.17), 2,3-octanedione (32.08), 2-pentylfuran (ca. 33), octanal (34.98) and nonanal (41.84).

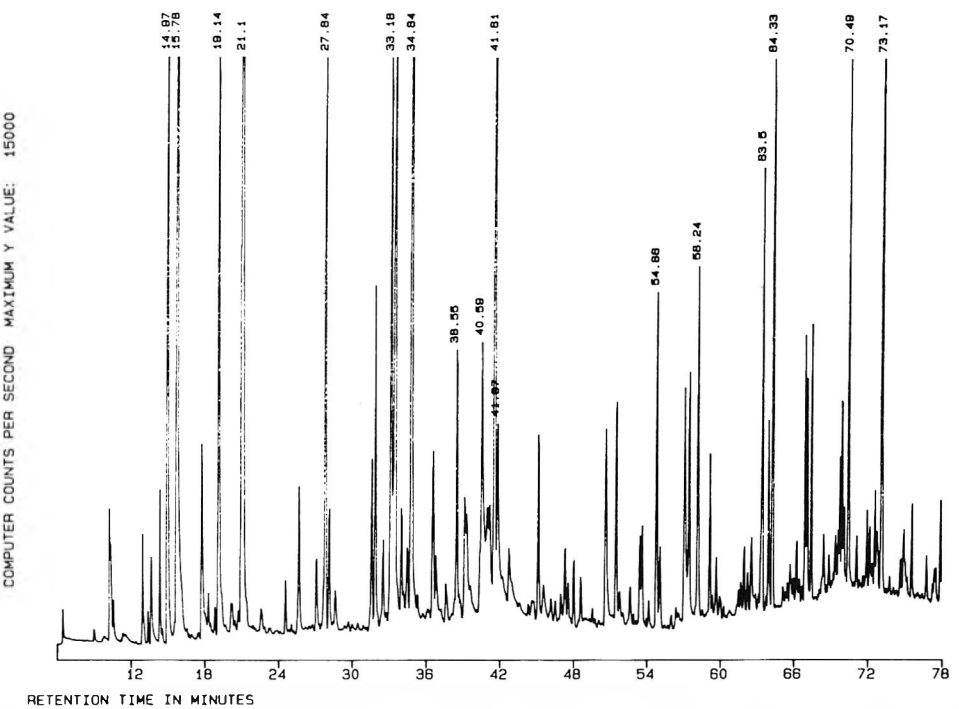

Fig. 3—Volatile profile from cooked beef stored at 4°C for 2 days, then rewarmed. Volatiles were obtained and identified similarly to those described in Fig. 2. Additional compound identified was t,t-decadienal (54.86).

larger increases. The correlation coefficients between sensory scores and chemical or instrumental values and between TBA and instrumental values showed very good agreement, ranging from 0.80 to as high as 0.92. The correlation coefficients for all four parameters were exceptionally high, when one considers that the meat samples were purchased over several months and were probably from various breeds of cattle. Similar correlations have not been made previously and should be invaluable in studies on the mechanism of formation of WOF.

When the specific character note, cooked beef brothy (CBB) was plotted as total volatiles, hexanal and TBA numbers in 22 samples, there was a negative correlation; i.e., as the intensity of the CBB character note decreased, the instrumental and chemical values increased. Likewise, when the painty (PTY) and cardboardy (CBY) character notes were measured in the same samples, a positive correlation was observed. For example, as those notes (PTY and CBY) increased with storage at 4°C, the instrumental and chemical values also increased.

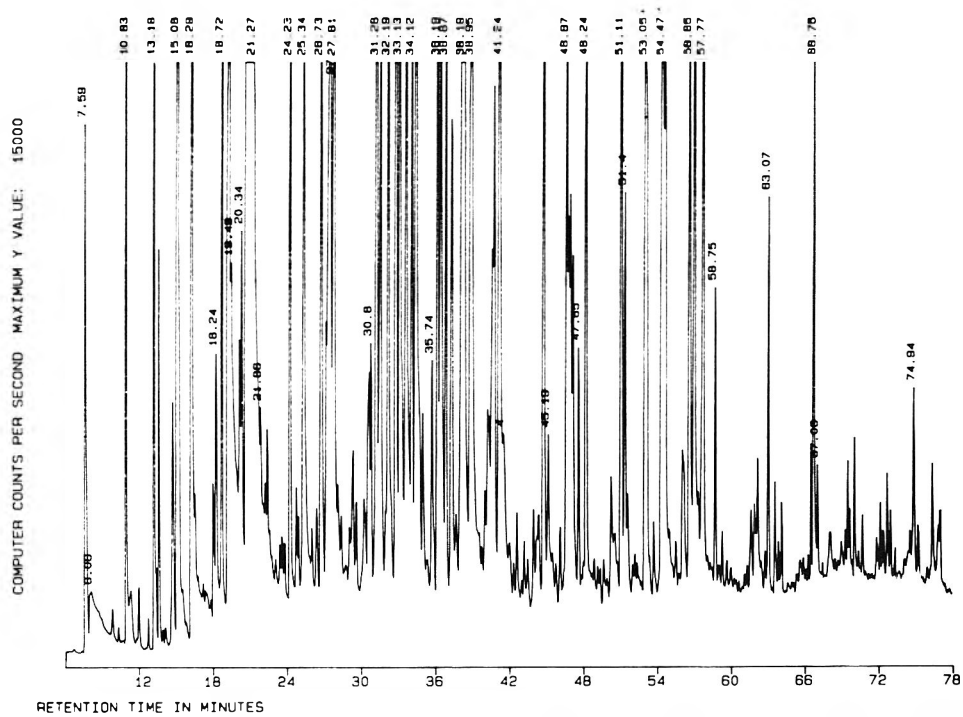

Fig. 4—Volatile profile from cooked beef stored at 4°C for 6 days, then rewarmed. Volatiles were obtained and identified similarly to those described in Fig. 2. Additional compound identified was t,c-decadienal (53.05).

Table 1—Compounds consistently found in roast beef volatiles by GC MS

Ethanol	2-Hexenal
2-Propanone	1-Hexanol
Pentane	2-Heptanone
Propanal, 2-methyl-	Heptanal
2,3-Butanedione	2-Heptenal
Hexane	Benzaldehyde
Methane, trichloro	1-Hepten-3-ol
Butanal, 3-methyl	2,3-Octanedione
Heptane	2-Octanone
Acetic Acid	Furan, 2-pentyl-
Pentanal	t,c-2,4-Heptadienal
2-Butanone, 3-hydroxy-	t,t-2,4-Heptadienal
Propanoic Acid	2-Octenal
Furan, 2,3-Dihydro-4-methyl	Nonanal
1-Pentanol	Propanoic Acid, 2-methyl-3-hydroxy
2,3-Butanediol	1-Dodecene
1-Heptene, 5-methyl-	2-Nonenal
Hexanal	2-Decenal
3-Octene	t,c-2,4-Decadienal
2-Pentenal, 2-Methyl-	t,t-2,4-Decadienal

Table 2—Mean sensory, chemical, and instrumental values of cooked beef

	Sensory score[b]	TBA[c]	Total volatiles[c]	Hexanal[c]	2,3-Octanedione[c]
Overall mean[a]					
Fresh	2.1	3.75	5.35	2.55	0.19
WOF	5.0	14.71	19.09	11.16	1.60
Correlation coefficients[d]					
r		0.80	0.84	0.80	0.81
r′		----	0.84	0.92	0.88

[a] n = 14
[b] Intensity of WOF; 0 = No WOF; 10 = extreme WOF.
[c] ppm, based on hexane equivalent for GC peaks.
[d] r = correlation coefficient between sensory scores and chemical or instrumental values; r′ = correlation coefficient between TBA and instrumental values.

Malondialdehyde content

Many variations of the TBA assay, including extraction and distillation methods, were tried when measuring malondialdehyde or other TBA reactive substances. The several hundred meat samples assayed for MDA as an end product of polyunsaturated fatty acid oxidation in this study showed a good correlation with the sensory panel judgments and with total volatiles and concentrations of hexanal and 2,3-octanedione (Table 2). The final method of choice, the distillation procedure, produced a clear distillate containing many aldehydes, which could react with the TBA reagent. Whereas there have been many compounds identified as being directly related to the reddish-pink TBA pigment formed, it was of interest to examine by GC the composition of the MDA distillate from the combined beef samples used in this study and to compare the results with volatile compounds found in aqueous extracts of cooked beef. Distillates from aqueous extracts of several beef samples were prepared for TBA assay of MDA. These distillates represented increasing MDA concentrations (raw, 0.2–0.4 ppm; fresh roast, 0.6–0.8 ppm; WOF, 6.0–8.0 ppm). However, other compounds that co-distill with MDA were not known. Using the direct GC/MS method and capillary columns, 73 compounds were identified in the distillates, with their concentrations increasing from raw to WOF samples closely paralleling rates of increase for the TBA values for MDA. An interesting finding in the distillates of all meat samples was that the 2,3-octanedione and hexanal were the principal peaks in the chromatograms with a close comparison in quantities involving higher aldehydes and hydrocarbons. The major compounds found in the distillates are listed in Table 3. The compounds that were in the highest concentration were hexanal, 2,3-octanedione, 2,4-decadienal and pentanal. According to Marcuse and Johansson (1973) 2,4-alkadienals are the principal type of compounds that absorb at 530 nm when the TBA reaction is run at 95°C. In the TBA method used in this study, 95–100°C was the temperature of choice. In the distillate fraction (Table 3), the alkadienals were present in high concentrations as determined by direct capillary GC/MS. Those compounds that had the highest ratio of concentration in WOF beef to fresh cooked beef were 2,4-heptadienal, 2-pentylfuran and pentanal, all secondary reaction products of linoleic acid oxidation (Frankel, 1986).

Prior to distillation, half of the combined aqueous extracts were analyzed by direct GC/MS (capillary column) and the identified compounds are listed in Table 3. Although there

Table 3—Major volatile compounds found in combined distillate and aqueous extracts of beef samples: raw, freshly cooked, cooked and stored for 1 day at 4 C

Name	Dis-:Raw area	Dis-:Fresh (F) area	Dis-:WOF (G) area	G:F	Ext-:Fresh (I) area	Ext-:WOF (J) area	J:I
1. Propanal, 2-methyl-	125	1871	10469	5.60	144	599	4.16
2. Butanal, 3-methyl-	54586	150	3658	24.39	282	1548	5.49
3. Pentanal	45319	10374	353114	34.04		42328	
4. Hexanal	190292	411296	4735950	11.51	91933	344168	3.74
Unknown	21664	51305	1170740	22.82	12802	50217	3.92
5. 2-Hexenal		1019	5578	5.47	35	158	4.51
6. 1-Hexanol	11926	29609	46812	1.58	1779	12924	7.26
7. 2-Heptanone	809	815	7937	9.74	48	477	9.94
8. Nonane	357	325	3205	9.86	264		
9. Heptanal	8919	11013	151518	13.76	1077	2395	
10. Acetic acid, pentyl ester			949		134	1467	10.95
11. Benzaldehyde	5634	33608	84831	2.52	1363	7560	5.55
12. 2,3-Octanedione	8718	77346	608456	7.87	10376	48106	4.64
13. Furan, 2-pentyl		52	2483	47.75	126	134	1.06
14. Octanal	2947	3488	19914	5.71	126	1166	9.25
15. 2,4-Heptadienal	1002	907	49401	54.47	217	1252	5.77
16. Nonanal	6840	1777	30367	17.09	163	1110	6.81
17. 2-Nonenal	4917	14304	96373	6.74	410	1705	4.16
18. Decanal	16598	1924	16596	8.63	202	702	3.48
19. 2,4-Decadienal (trans, cis)	2045	31273	54985	1.76	47	1622	34.51
20. 2,4-Decadienal (trans, trans)	15214	29784	476981	2.08	358	7009	19.58
pH	5.85	5.65	5.21	—	—	—	
Sensory score	—	1.76	5.64	—	—	4.13	
TBA values	0.85	3.44	12.19	—	1.31	17.99	

were not as many long chain fatty aldehydes as in the corresponding distillate samples, the 2,3-octanedione and hexanal peaks were prominent in the aqueous extract of WOF roast beef. The compounds with the highest ratios in comparing WOF to fresh cooked beef were the decadienals, also secondary reaction products of linoleic acid oxidation (St. Angelo et al., 1980). It is of interest to note that of the compounds that have been identified (Sinnhuber and Yu, 1977) as interfering in the TBA-malondialdehyde reaction, none was the major volatile component of fresh-cooked or WOF beef.

Heterocyclic compounds

During the course of the studies on volatile compounds, identification of volatiles that were typical of acceptable roast beef flavor by the direct capillary GC/MS method was difficult. Almost all of the compounds identified were from lipid oxidation type reactions. These compounds are generally not contributors to cooked beef flavor, which contains compounds such as isoxazoles, pyrazines, thiophenes, thiazoles, pyrroles, pyridines and furans (Mussinan et at., 1976). Furthermore, a synthetic mixture of the lipid oxidation products used as marker compounds for WOF and identified from WOF beef (Table 1) produced an odor reminiscent of a somewhat rancid linseed or neatsfoot oil but not roast beef with WOF. Also, as the samples were being evaluated for sensory notes, WOF notes strongly masked the aromas of good beef flavors as WOF intensified. These results suggested that in addition to the production of new products from reactive intermediates during WOF formation (i.e., lipid oxidation products) essential roast beef flavor components possibly underwent reactions and/or eventually diminished or degraded.

Therefore, experiments were conducted to generate and trap acceptable meat flavor compounds on porous organic polymers followed by thermal desorption, separation and identification by the direct capillary GC/MS method. Results are shown in Table 4. Many of these heterocyclics have been previously identified as contributors to beef flavor (MacLeod and Seyyedain-Ardebili, 1981; Hsu et al., 1982; Min et al., 1979; Baines and Mlotkiewicz, 1984; Galt and MacLeod, 1984; MacLeod and Ames, 1986). It should be further emphasized that these subtle flavor compounds are present at parts per billion levels in meat (Liu et al., 1987) and represent a delicate balance between desirable flavors and impalatable foods. On the other hand, the lipid oxidation products associated with WOF are present in parts per million and, thus, far outweigh the impact from the aromatics associated with good beef flavor. The differences between the concentrations of the two classes of compounds probably account for the difficulty in identifying by sensory analyses those compounds directly responsible for the desirable roast beef flavor.

Table 4—Beef flavor heterocycles identified from cooked meat concentrate using purge and trap system with GC-MS

1	2-Ethylfuran	30	4-Ethyl-2-methylthiazole
2	Dimethyldisulfide[a]	31	2-Ethyl-6-methylpyrazine
3	1H-Pyrrole	32	Trimethylpyrazine
4	4,5-Dimethyloxazole	33	2-Ethyl-5-methylpyrazine
5	2-Methylthiophene	34	2-Ethyl-5-methylthiazole
6	3-Methylthiophene	35	2-Ethyl-3-methylpyrazine
7	Thiazole	36	cis-2-Ethyldimethyl-1,3-oxathiane
8	2-Furancarbonitrile	37	5-Ethyl-4-methylthiazole
9	3-Methylisothiazole	38	2-Ethenyl-6-methylpyrazine
10	Dihydro-2-methyl-3 (2H)-furanone	39	2-Ethyl-2,6-dimethyl-1,3-oxathiane
11	4-methylthiazole	40	Dipropyldisulfide*
12	Methylpyrazine	41	1-Methylethylpropyldisulfide*
13	2-Furancarboxaldehyde	42	5-Ethyl-2,4-dimethylthiazole
14	1-Azabicyclo[2,2,2]-octane	43	3-Ethyl-2,5-dimethylpyrazine
15	2-Methylthiazole	44	2-Ethyl-3,6-dimethylpyrazine
16	Trimethyloxazole	45	2,3-Dihydro-4H-1-benzopyran-4-one
17	2,4-Dimethylthiophene	46	Isomer of compound 45
18	2,5-Dimethylthiophene	47	Bis-(1-Methylethyl)disulfide*
19	1-(2-Furanyl)ethanone	48	2-Propylthiophene
20	Ethyl pyrazine	49	2,5-Diethyl-4-methylthiazole
21	2,4-Dimethylthiazole	50	Thiazolidinethione
22	1-Ethyl-1H-pyrrole-2,5-dione	51	2-Methyl-3,5-diethylpyrazine

[a] Not heterocyclic compounds

Organic and aqueous extractables

Data showed that the organic extractable material increased after cooking (from 2.6% to 9%). These materials were most likely membrane phospholipids (freed from the matrix during

cooking), browning polymers, oxidation products and/or degradation products such as the Strecker type formed between amino acids and sugars. That phospholipids are a major contributor to WOF was shown by Igene and Pearson (1979) and Willemot et al. (1985).

To determine if raw, cooked chunk and WOF ground beef samples contained the WOF note in the organic soluble layer, the samples were first extracted with vegetable oil followed by centrifugation and extraction with chloroform:methanol (3:1). Headspace volatiles from the vegetable oil extract had an aldehydic, rancid, meaty odor but no WOF odor. The chloroform:methanol extract had a slight detectable beefy odor, more of a sweet acid odor, but no WOF odor.

Samples of raw and cooked roast beef that were extracted with water to obtain the soluble flavor compounds were examined sensorily and chemically. Sensory results indicated that the aqueous extract of the raw meat sample had a definite uncooked, bloody meat flavor. The fresh roast beef aqueous extract had a brothy, browned meat flavor, whereas the WOF roast beef extract was described with such terms as strong cardboard, sulfury or oxidized/rancid, in accord with the WOF descriptors of Johnsen and Civille (1986).

In summary, the significance of the above findings are manyfold. For example, instrumental analyses by direct GC using either packed or fused silica capillary columns can be used to assay for beef quality. This method of assessment should be applicable for any type of meat sample, ground, chunk, sliced, or minced, and raw or cooked (fresh or stored). Many compounds have been identified in the meat samples analyzed on packed and capillary columns. The sensitivity of the direct GC approach was greatly enhanced by interfacing the ECID with capillary columns. Whereas the packed columns are very practical when the intention is to follow the development of WOF, capillary columns offer the increased sensitivity needed to focus in on a particular volatile compound. Furthermore, the direct GC method is so sensitive, it can distinguish between high quality cooked beef samples and those that have been mixed with small (25%) quantities of beef containing WOF (Dupuy et al., 1987). Volatile secondary reaction products of lipid oxidation appeared to increase as WOF was developing. Some of these compounds, such as hexanal, pentanal and 2,3-octanedione could be used as markers to follow the development of WOF. The increase in many of these compounds paralleled the increase in TBA numbers. These increases correlated with sensory evaluations. The direct GC method can also be used to study the type of volatiles that co-distill with the compounds that give positive TBA numbers, and thus offers an insight to determine exactly which of the many compounds is directly responsible for them. WOF in roast beef, as recognized by sensory evaluation, is found in the aqueous extract and only to a lesser extent in the organic and oil extracts. The principal flavor volatiles of organic and oil extracts are carbonyl compounds which appear to evolve from lipid oxidation reactions. These compounds represent a reproducible factor in the off-flavor of roast beef and relate directly to the onset of WOF. WOF flavor in roast beef possibly results from oxidative degradations with concommitant production of new compounds which may mask normal, desirable beef flavors.

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Ms received 1/5/87; revised 3/23/87; accepted 3/23/87.

Identification and Antibiotic Resistance of Staphylococci and *Enterobacteriaceae* Isolated from Restructured and Conventional Steaks

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ABSTRACT

The identification and antibiotic resistance of staphylococci and *Enterobacteriaceae* isolated from restructured and conventional steaks were determined. Five *Staphylococcus* species, and seven species and three CDC Enteric Groups from the Family *Enterobacteriaceae* were identified. Sixty-seven percent of the 160 staphylococci were sensitive to the 13 antimicrobial agents tested. Antibiotic resistance was greater for staphylococci from restructured (46%) than from conventional (16%) steaks. Thirty-eight percent of the restructured and 15% of the conventional steak isolates were resistant to tetracycline. Sixty-three percent of the 97 *Enterobacteriaceae* were resistant to cephalothin, 10% were resistant to streptomycin and 11% were resistant to tetracycline. Less than 10% had multiple resistance.

INTRODUCTION

ANTIBIOTICS have been used in animal feeds since the early 1950s for growth promotion, improved feed efficiency and for control and prevention of diseases (CAST, 1981). It is well established that feeding antimicrobial agents causes marked increases in the proportion of antibiotic resistant bacteria in the intestinal tract (Langlois et al., 1978a, b; Linton et al., 1975; Smith, 1962, 1975; Smith and Crabb, 1957). During the 1960s scientists became concerned about the potential effect on public health of the use of subtherapeutic levels of antibiotics in animal feed (Braude, 1978; Swann, 1969; Van Houweling, 1972). Certain antibiotics are believed to contribute to the establishment of a reservoir in animals of drug resistant enteric pathogenic and/or nonpathogenic bacteria that may transfer their resistance to human or animal pathogens (Falkow, 1975; Smith, 1969). Because such pathogens would be able to survive in the presence of antibiotics to which they were resistant, it was theorized that effective treatment of disease caused by the antibiotic resistant pathogens would be more difficult or impossible.

The known outbreaks in which there is evidence linking the use of antibiotics in animal agriculture with diseases due to antibiotic resistant bacteria in humans have not implicated subtherapeutic use of antibiotics in animal feeds (Anderson, 1970; CAST, 1981; Cohen et al., 1977; Fish et al., 1967; Lyons et al., 1980; Rowe et al., 1979). However, several recent outbreaks of human salmonellosis have been linked to the consumption of meat from animals fed or treated with antibiotics (Fontaine et al., 1978; Holmberg et al., 1984a, b; Riley et al., 1983). Using plasmid profiles, Holmberg et al. (1984b) and Riley et al. (1983) attempted to establish a link between using antibiotics in animals and subsequent illness from consumption of meat from the antibiotic fed animals. These are purported to be the first definitive studies to directly link use of antibiotics in animals to human disease and to demonstrate that antimicrobial resistant bacteria in animals can be transmitted to and colonize in humans. However, the suspect organism, *Salmonella newport*, was not cultured from meat samples known to come from the suspect herd or from the barnyard premise (FNA, 1985). Many of the patients had been taking antibiotics for treatment of minor infections a few days prior to being hospitalized for salmonellosis (Holmberg et al., 1984b). Antibiotics can facilitate infections with resistant organisms because they inhibit the sensitive bacterial flora of the host, thus enabling the invading resistant strains to gain a foothold and proliferate.

The objectives of this study were to identify and determine the antibiotic resistance of staphylococci and *Enterobacteriaceae* isolated from restructured and conventional steaks manufactured from electrically stimulated, hot- and cold-boned carcasses.

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MATERIALS & METHODS

Source of isolates

The isolates used in this study were from restructured and conventional steaks described previously (Newsome et al., 1987). Three steers from the University of Kentucky beef herd had not been subjected to subtherapeutic levels of antibiotics. In cases where disease treatment was necessary, therapeutic doses of appropriate antibiotics were used according to manufacturers' directions. The degree of antibiotic exposure could not be determined for the seven steers purchased from the auction market. However, it was assumed that any antibiotic exposure was according to accepted practices.

The isolates examined in this study were obtained from Baird-Parker (BP) agar and violet red bile glucose (VRBG) agar plates used in a study to determine the microbiological quality of conventional and restructured steaks manufactured from electrically stimulated hot- and cold-boned carcasses (Newsome et al., 1987). After counts were recorded, five colonies were picked at random from BP and VRBG agar plates into Brain Heart Infusion (BHI) agar deeps, incubated overnight at 35°C and stored at 2°C until activated for testing. Cultures were activated by subculturing twice in BHI broth and streaking on TSA plates to check purity. Well-isolated colonies from TSA plates were used to prepare working cultures.

Staphylococci

The 200 isolates obtained from BP agar plates were gram stained (Doetsch, 1981) and tested for catalase activity and coagulase activity by the tube method (Smibert and Krieg, 1981). The following method was used to determine susceptibility to lysostaphin, bacitracin, and novobiocin. An 18-hr active culture was swabbed over the top one-half surface of an agar P (Phillips and Nash, 1985) plate and then streaked over the remainder of the plate to obtain well-isolated colonies. A sterile 6.5 mm blank disk (Difco) was placed firmly in the middle of the swabbed portion of the plate. Two drops of filter sterilized lysostaphin (100 μg/mL; Sigma Chemical Co., St. Louis, MO) were added to the disk. A 0.04 U bacitracin differentiation disk (Difco) was placed firmly onto the agar surface to one side of the lysostaphin disk and a 5 μg novobiocin sensitivity disk (Difco) was placed firmly on the other side of the lysostaphin disk. Plates were incubated upright at 35°C for 18 hr. Following incubation, diameters of zones of growth inhibition around the bacitracin and novobiocin disks were measured. A zone of growth inhibition around the lysostaphin disk denoted sus-

ceptibility (Lachica et al., 1971). Isolated colonies or less dense growth within the zone of growth inhibition was considered to indicate partial resistance to lysostaphin. Isolates were considered bacitracin resistant if the zone of growth inhibition was less than 10.5 mm in diameter around the bacitracin disk (Falk and Guering, 1983). Novobiocin resistance was indicated by a zone of growth inhibition equal to or less than 16 mm in diameter (Kloos and Jorgensen, 1985). Sensitivity to novobiocin was used as an aid in the identification of the isolates to species level by the Staph Trac System. Gram-positive, lysostaphin susceptible, bacitracin resistant isolates were considered to be staphylococci. Growth from the agar P plates was used to prepare the inocula used to identify these isolates to species level by the Staph Trac system (Analytab Products, Inc., Plainview, NY) according to the directions of the manufacturer. Susceptibility of the 160 isolates identified as *Staphylococcus* species to ampicillin (10 μg), chloramphenicol (30 μg), clindamycin (2 μg), methicillin (5 μg), erythromycin (15 μg), gentamicin (10 μg), nitrofurantoin (300 μg), kanamycin (30 μg), neomycin (30 μg), penicillin G (10 units), polymyxin B (300 units), tetracycline (30 μg) and vancomycin (30 μg) was tested by the method of Bauer et al. (1966).

Enterobacteriaceae

The 200 isolates obtained from VRBG agar were gram-stained (Doetsch, 1981) and tested for oxidase activity (Smibert and Krieg, 1981). The 97 isolates found to be gram-negative, oxidase-negative rods, were identified by the API 20E system (Analytab Products, Inc., Plainview, NY) according to the directions of the manufacturer. Susceptibility of the 97 gram-negative, oxidase-negative rods to ampicillin (10 μg), amikacin (30 μg), chloramphenicol (30 μg), carbenicillin (100 μg), cephalothin (30 μg), gentamicin (10 μg), kanamycin (30 μg), neomycin (30 μg), nalidixic acid (30 μg), streptomycin (10 μg), sulfisoxazole (250 μg), sulfamethoxazole with trimethorprin (SXT; 23.75/1.25 μg) and tetracycline (30 μg) was tested by the method of Bauer et al. (1966).

RESULTS & DISCUSSION

Species comprising the staphylococcal microflora

Of the 200 isolates obtained from BP agar, 40 isolates were not considered to be staphylococci since they were not gram-positive, catalase-positive, lysostaphin sensitive, bacitracin resistant cocci. None of the 160 staphylococci was coagulase-positive. Five different *Staphylococcus* species were identified for the 160 isolates (Table 1). *Staphylococcus epidermidis*, *Staphylococcus capitis* and *Staphylococci hominis* were isolated from both restructured and conventional steaks, while *Staphylococcus xylosus* and *Staphylococcus warneri* were isolated only from conventional steaks. Compared with conventional steaks, restructured steaks had a greater percentage of *S. epidermidis* (92 vs 83%), a lower percent *S. hominis* (1 vs 5%) and the same percent *S. capitis* (7%).

Staphylococcus epidermidis is one of the most prevalent species found on normal human skin (Kloos, 1980; Kloos and Wolfshohl, 1982; Lowy and Hammer, 1983) and is isolated in very small numbers from the skin of less than 5% domestic animals (Kloos, 1980). *Staphylococcus hominis* and *S. haemolyticus* also are frequently isolated from human skin, while *S. warneri*, *S. capitis* and *S. xylosus* are less frequently isolated from humans (Kloos, 1980). *Staphylococcus xylosus* is one of the major species found on the normal skin of cattle and other ungulates (Kloos, 1980). Since staphylococci are one of the major microbial groups inhabiting human skin, it is reasonable to expect that they would be found as part of the microflora of food products, such as restructured steaks, which require considerable human contact during preparation and processing. Therefore, it is not surprising that a greater percent *S. epidermidis* was observed in staphylococcal isolates from restructured than conventional steaks. On the other hand, conventional steaks contained greater percents of those species which are isolated less frequently from human skin. Differences observed in *Staphylococcus* species between restructured and conventional steaks appeared to reflect differences in their methods of preparation.

Table 1—Staphylococcus *species from restructured and conventional steaks identified by the Staph-Trac system*

Staphylococcus species	Type of steak: Restructured (86)[a]	Type of steak: Conventional (74)
	%	
S. epidermidis	92	83
S. capitis	7	7
S. hominis	1	5
S. xylosus	0	4
S. warneri	0	1

[a] Number of isolates.

Antibiotic restance of staphylococci

Antibiotic resistance of staphylococci from restructured and conventional steaks is shown in Table 2. None of the isolates was resistant to chloramphenicol, clindamycin, gentamicin, neomycin or vancomycin. In addition, none of the isolates from conventional steaks was resistant to amikacin, methicillin, erythromycin, kanamycin, penicillin G and polymyxin B. Except for tetracycline, resistance to the other agents was 3% or less. Three percent of the restructured steak isolates were resistant to nitrofurantoin compared with 1% of the conventional steak isolates. Tetracycline resistance was much greater for restructured steak isolates (38%) than for conventional steak isolates (15%).

Eighty-one percent of the conventional steak isolates were sensitive to all 13 antimicrobials compared with 53% of the restructured steak isolates. None of the isolates from conventional steaks was resistant to more than one agent, while 3% of the restructured steak isolates showed multiple resistance.

Differences in antibiograms of isolates from the two types of steaks may reflect a greater contamination of the restructured steaks with microorganisms of human origin. Environmental factors also may play a role if hides of cattle are contaminated with drug resistant microorganisms from the environment, since the carcasses and the meat from them may become contaminated during slaughter and subsequent preparation of the meat cuts. The greater amount of handling involved in preparation of restructured steaks compared with conventional steaks provides an excellent opportunity for contamination of these steaks with antibiotic resistant bacteria from the environment.

Antibiotic resistance of the five *Staphylococcus* species to the 13 antimicrobials is shown in Table 3. The one isolate identified as *S. warneri* was sensitive to all 13 agents, while the three *S. xylosus* strains were resistant only to tetracycline. The 11 *S. capitis* strains were resistant to three agents with 36% of the isolates showing resistance to nitrofurantoin and tetracycline and 9% showing resistance to kanamycin. The

Table 2—Antibiotic resistance of staphylococci isolated from restructured and conventional steaks

Antimicrobial agent	Disk conc	Type of steak: Restructured (86)[a]	Type of steak: Conventional (74)
		%	
Amikacin	30 μg	1	0
Chloramphenicol	30 μg	0	0
Clindamycin	2 μg	0	0
Erythromycin	15 μg	1	0
Gentamicin	10 μg	0	0
Kanamycin	30 μg	1	0
Methicillin	5 μg	1	0
Neomycin	30 μg	0	0
Nitrofurantoin	300 μg	3	1
Penicillin G	10 units	1	0
Polymyxin B	300 units	1	0
Tetracycline	30 μg	38	15
Vancomycin	30 μg	0	0

[a] Number of isolates

Table 3—Antibiotic resistance of Staphylococcus *species identified from restructured and conventional steaks*

Antimicrobial agent	Disk Conc.	*Staphylococcus* species: *S. epidermidis* (140)[a]	*S. hominis* (5)	*S. xylosus* (3)	*S. capitis* (11)	*S. warneri* (1)
		%				
Amikacin	30 μg	0	20	0	0	0
Chloramphenicol	30 μg	0	0	0	0	0
Clindamycin	2 μg	0	0	0	0	0
Erythromycin	15 μg	7	0	0	0	0
Gentamicin	10 μg	0	0	0	0	0
Kanamycin	30 μg	0	0	0	9	0
Methicillin	5 μg	0	20	0	0	0
Neomycin	30 μg	0	0	0	0	0
Nitrofurantoin	300 μg	1	0	0	36	0
Penicillin G	10 units	0	20	0	0	0
Polymyxin B	300 units	1	0	0	0	0
Tetracycline	30 μg	27	40	67	36	0
Vancomycin	30 μg	0	0	0	0	0

[a] Number of isolates

strains of *S. epidermidis* and *S. hominis* were each resistant to four agents.

The antimicrobial agents to which *S. epidermidis* was resistant are agents used in human and/or veterinary medicine. In addition to penicillin G and tetracycline, *S. hominis* was resistant to amikacin and methicillin which are used primarily in human medicine. *Staphylococcus xylosus* was resistant only to tetracycline which is used in both human and veterinary medicine. In addition to showing resistance to nitrofurantoin and tetracycline, *S. capitis* was resistant to kanamycin which is used predominantly in human medicine.

Various strains of *S. aureus* and *S. epidermidis* have been responsible for large numbers of bacteremia cases and hospital epidemics (Jaffee et al., 1980; Jessen et al., 1969; Lowy and Hammer, 1983). The outbreaks were attributed to the use of antimicrobials in hospitals and the presence of massive bacterial reservoirs which led to the development of resistant strains of staphylococci with different antibiotic resistance patterns (Jessen et al., 1969). Gill et al. (1983) reported that unlike nonclinical isolates, the antibiograms of *S. epidermidis* isolated from clinical specimens frequently showed multiple antibiotic resistance.

Most studies on antibiotic resistance of bacteria from food of animal origin have involved only gram-negative enteric, particularly *Escherichia coli* and *Salmonella* species. Kwaga and Adesujun (1984) studied the resistance of 229 *S. aureus* strains isolated from five ready-to-eat products in Nigeria. They found that 60% of the isolates were resistant to one or more antibiotics. Resistance to penicillin (41%), ampicillin (28.4%) and sulfisoxazole with trimethoprin (27.9%) was most frequently observed. Their results suggested that antibiotics were being misused by both medical and veterinary practices in Nigeria.

The mechanisms by which the interspecific transfer of antibiotic resistance occur among staphylococci has only recently been identified (Archer and Johnson, 1983; Forbes and Schaberg, 1983; Jaffee et al., 1980, 1982; Naidoo and Noble, 1978). Antibiotic resistance is transferred similarly in gram-negative bacteria. Plasmid transfer between staphylococci had traditionally been believed to require the participation of bacteriophage (Lacey, 1975).

Species comprising the gram-negative isolates

Of the 200 isolates obtained from VRBG agar, only 97 were gram-negative, oxidase-negative rods. The large number of gram-negative, oxidase-positive isolates obtained from VRBG was surprising since this medium contains bile salts and crystal violet to make it selective for gram-negative, oxidase-negative enterics. Incubation of the VRBG agar plates for 48 hr rather than 24 hr may have allowed growth of microorganisms inhibited during the initial 24 hr. The 97 oxidase-negative isolates consisted of 81 isolates from restructured steaks and 16 isolates from conventional steaks. As a result of the uneven distribution of isolates, no comparison was made of the species comprising isolates from the two types of steaks.

The majority of the oxidase-negative isolates from the steaks were identified by the API 20E system as *Hafnia alvei* (62%). The remainder of the isolates were identified as *Serratia liquefaciens* (10%), *Enterobacter agglomerans* (7%), *Enterobacter cloacae* (6%), *Escherichia coli* (6%), *Escherichia vulnaris* (2%), *Klebsiella oryzanae* (1%), CDC Enteric Group 19 (3%), CDC Enteric Group 1 (2%), CDC Enteric Group 11 (1%). The percentage of *H. alvei* and *S. liquefaciens* comprising the microflora of the steaks in this study was similar to the observations reported by Hanna et al. (1979), Kennedy et al. (1980), and Patterson and Gibbs (1977). Kennedy et al. (1980) inoculated vacuum packaged beef pieces with 2.0×10^3 *H. alvei* per mL. They observed that vacuum packaging did not provide a more favorable environment than aerobic packaging for survival of psychrotrophic *Enterobacteriaceae* such as *H. alvei*. However, *H. alvei* grew competitively with the resident microflora in the vacuum packaged beef and subsequently comprised a major part of the microbial count after 3 and 4 wk storage. *Hafnia alvei* is commonly found in water, soil, feces, on the hands of abattoir workers, in the air, on carcasses, in chill rooms, and on tables in boning rooms (Patterson and Gibbs, 1977). Since it is psychotrophic, it would be expected to be found in vacuum packaged meat stored at refrigerated temperatures.

Antibiotic resistance of *Enterobacteriaceae* isolates

Antibiotic resistance was not determined according to source of isolates, since the majority of the isolates were from restructured steaks. None of the 97 oxidase-negative isolates from VRBG agar was resistant to ampicillin, carbenicillin, nalidixic acid, SXT or sulfisoxazole (Table 4). Sixty-three percent of the isolates were resistant to cephalothin, while 11% were resistant to tetracycline and 10% were resistant to streptomycin. Two percent of the isolates were resistant to kanamycin, while 1% were resistant to amikacin, chloramphenicol, gentamicin and neomycin.

Twenty-five percent of the isolates were sensitive to all agents, while 61% were resistant to only one agent. Fourteen percent of the isolates had multiple resistance.

Approximately two-thirds of the cattle brought to market have been exposed to some form of antibiotics (Anonymous, 1985). Contamination and cross-contamination of carcasses with their intestinal contents can and often does occur at slaughter (Howe and Linton, 1976). It is probable, therefore, that a certain percentage of isolates from cattle will be resistant to one or more antibiotics, since selective antibiotic pressure increases antibiotic resistance of the fecal microflora (Hays and

Table 4—Antibiotic resistance of gram-negative, oxidase-negative isolates obtained from restructured and conventional steaks

Antimicrobial agent	Disk conc	Percent of isolates resistant to: (97)[a]
Amikacin	30 μg	1
Ampicillin	10 μg	0
Carbenicillin	100 μg	0
Cephalothin	30 μg	63
Chloramphenicol	30 μg	1
Gentamicin	10 μg	1
Kanamycin	30 μg	2
Nalidixic acid	30 μg	0
Neomycin	30 μg	1
Streptomycin	10 μg	10
SXT	23.75/1.25 μg	0
Sulfisoxazole	250 μg	0
Tetracycline	30 μg	11

[a] Number of isolates

Table 5—Antibiotic resistance of the gram-negative, oxidase-negative species identified from restructured and conventional steaks

Species	Number of isolates	Antimicrobial agent[a]								
		AM	C	CF	GM	K	N	S	SU	TE
		%								
H. alvei	59	0	0	87	17	0	0	83	0	83
S. liquefaciens	10	0	0	89	0	0	0	0	0	0
E. agglomerans	7	0	0	14	0	0	0	0	0	0
E. cloacae	6	0	17	17	0	17	17	17	33	17
E. coli	6	0	0	0	0	0	0	0	17	17
E. vulnaris	2	0	0	0	0	50	0	50	0	100
K. oryzanae	1	0	0	0	0	0	0	0	0	0
CDC Enteric Group 19	3	0	0	0	0	0	0	67	0	67
CDC Enteric Group 1	2	0	0	0	0	0	0	50	0	50
CDC Enteric Group 11	1	100	0	0	0	0	0	0	0	0

AM = 10 μg ampicillin; C = 30 μg chloramphenicol; CF = 30 μg cephalothin; GM = 10 μg gentamicin; K = 30 μg kanamycin; N = 30 μg neomycin; S = 10 μg streptomycin; SU = 250 μg sulfisoxazole; TE = 30 μg tetracycline

Muir, 1979; Gardner and Smith, 1969; Huber et al., 1971; Jarolmen et al., 1976; Langlois et al., 1984a,b; Linton, 1977). The majority of studies of antibiotic resistance of the microflora of food-producing animals have been done with poultry and swine. Caudry and Stanisick (1979) found that between 13 and 89% of *E. coli* from frozen chicken carcasses were resistant to at least one of the eight antibiotics studied and that multiple resistance was common. Harnett and Gyles (1985) also reported that multiple resistance was common among *E. coli* which they isolated from bovine and porcine carcasses. In studying the antibiotic resistance of coliforms isolated from freshly slaughtered poultry carcasses and from fresh, chilled poultry, Bensink and Botham (1983) observed that approximately 85% of 13,000 isolates examined were resistant to at least one antibiotic. This value is surprising since fewer antibiotics are now being used in the poultry industry than were used in the 1970s (Anonymous, 1985). Howe and Linton (1976) investigated the contamination of calf carcasses by *E. coli* from the intestinal contents at slaughter. They found that *E. coli* from these carcasses showed resistance to at least one antibiotic. CAST (1981) estimated that nearly 80% poultry, 75% swine and 75% dairy calves marketed or raised in the U.S. are fed antibiotics at some times during their life, while only 60% feedlot cattle are exposed to antibiotics. Therefore, it was not surprising that the microflora of cattle in this study showed less antibiotic resistance than observed for swine, poultry and calves in other studies.

Antibiotic resistance of the species identified are shown in Table 5. The one strain of *K. oryzanae* was sensitive to all agents, while the one CDC Enteric Group 11 strain was only resistant to ampicillin. *Enterobacter agglomerans* and *S. liquefaciens* were only resistant to cephalothin. *Enterobacteria cloacae* was resistant to the most agents. Six of the 10 species were resistant to tetracycline and four species were resistant to cephalothin.

The high resistance to cephalothin was surprising, since this antibiotic is used in human medicine and is not used for animals. Langlois et al. (1986) reported greater cephalothin resistance in a herd not exposed to antibiotics for over 13 yr for fecal isolates from swine on pasture than from swine in finishing units or farrowing units. Similar results were observed for swine born and raised on pasture without antibiotic exposure. Mamber and Katz (1985) suggested that perhaps enteric bacilli which colonize the intestinal tract of chickens were not necessarily selected for by antibiotic supplementation of animal feed. They suggested that colonization by and proliferation of antibiotic resistant strains may be dependent on their common presence in the environment from which they can colonize the intestine of newly hatched chicks. Wells and James (1973) observed a relatively high incidence of antibiotic resistance in nonantibiotic exposed pigs and reported that the acquisition of R factors by ingestion of vegetation containing antibiotic-producing microorganisms may be the reason.

The results indicated that the differences observed in *Staphylococcus* species between the restructured and conventional steaks reflected differences in their method of preparation. The antibiotic resistance patterns observed for *Staphylococcus* and *Enterobacteriaceae* suggested that antibiotic resistance was due to antibiotic use in both animals and humans and that environmental factors contributed to the spread of resistance organisms to the steaks.

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Kentucky Agricultural Experiment Station Journal Article No. 87-5-23.

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Ms received 1/13/87; revised 3/30/87; accepted 4/17/87.

This study was supported in part by Research Grant No. 82-CRSR-2-2051 from the U.S. Dept. of Agriculture and in part by a research grant from the American Meat Institute. Michigan Agricultural Experiment Station Journal Article No. 12201.

Modification of the Schricker Nonheme Iron Method to Minimize Pigment Effects for Red Meats

K. S. RHEE and Y. A. ZIPRIN

ABSTRACT

A colorimetric nonheme iron assay procedure for meat was modified to avoid pigment effects in determining the nonheme iron content of red meats. The modification consisted of mixing the red meat sample with $NaNO_2$ before incubation with an acid mixture to minimize the breakdown of heme pigments into nonheme iron, and inclusion of a second blank for the brownish color of the incubated liquid phase, in addition to the reagent blank.

INTRODUCTION

ACCURATE ESTIMATES of nonheme iron content of foods are important not only for evaluation of nonheme iron versus heme iron availability from foods but also for assessment of storage stability of certain foods. Nonheme iron has been reported to be a major catalyst of lipid oxidation in cooked meat (Sato and Hegarty, 1971; Love and Pearson, 1974; Igene et al., 1979).

Reported nonheme iron values for a beef muscle vary widely. For example, reported mean values for the nonheme iron content (wet weight basis) of beef longissimus dorsi muscles (raw) range from 1.80 μg/g muscle (Igene et al., 1979) to 8.4 μg/g (Schricker et al., 1982). Upon examination of the nonheme iron assay methods used in the two reports, it appeared that one method (Igene et al., 1979) might underestimate nonheme iron content of muscle tissues as a result of insufficient extraction/recovery of nonheme iron proteins (ferritin, transferin and others) from the muscle while the other method (Schricker et al., 1982) could overestimate the nonheme iron content of red meats because of pigment effects. The purpose of the present study was to develop a more accurate nonheme iron assay procedure for red meats by modifying the Schricker method through minimization of pigment effects.

MATERIALS & METHODS

Materials

Semimembranosus and longissimus dorsi muscles were dissected from beef top round and ribeye steaks (not frozen) purchased from a local supermarket and used immediately. Ground lean from the beef chuck was obtained from the Texas A&M University Meat Science & Technology Center, vacuum-packaged and stored for approximately 8 months at −20°C before analysis for nonheme iron.

Distilled, deionized water was used in preparing all solutions and for all phases where water was used. Iron stock standard (50 mg/dL in approximately 0.8N nitric acid) and myoglobin (metmyoglobin) were obtained from Sigma Chemical Company. All other chemicals used were of reagent grade.

Reagents

Acid mixture. HCl (6N) and 40% trichloroacetic acid (TCA) were mixed in equal volumes. When high blank values were observed due to contamination of TCA with iron, the HCl-TCA solution was treated with an anion exchange resin (e.g., BioRad Agl-X2).

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Bathophenanthroline disulfonate reagent. Bathophenanthroline disulfonic acid (sodium salt), 0.162g, was dissolved in 100 mL water and 2 mL thioglycolic acid (96–99%) added. The reagent was stored in an amber bottle.

Saturated sodium acetate solution. Sodium acetate (400g) was stirred with 500 mL water and, when the solution (initially very cold) was warmed to room temperature, more sodium acetate was added until crystals remained undissolved.

Color reagent. Water:saturated sodium acetate solution:bathophenanthroline disulfonate reagent (20:20:1) was prepared fresh each day.

Iron standards. The iron stock standard was diluted with the acid mixture to prepare standards of 0.5–5.0 μg Fe/mL.

$NaNO_2$ reagent. $NaNO_2$ solution, 0.39% (w/v), was prepared with water fresh each day.

Finalized modified procedure

A meat sample was ground (chopped) very finely using a food processor equipped with a stainless steel multi-purpose blade and a heavy-duty plastic work bowl. Five gram aliquots of ground meat (in triplicate) were weighed into test tubes (2.5 cm x 1.5 cm) with screw caps and the meat in each tube was mixed thoroughly with 0.2 mL $NaNO_2$ reagent (156 ppm $NaNO_2$ based on the meat weight). Fifteen mL of the acid mixture were added to each tube and the tube was tightly stoppered. The test tube contents were thoroughly mixed, incubated in a water bath-shaker at 65°C for 20 hr, vigorously shaken, and cooled to room temperature. One mL of the acidic liquid above the meat residue ("incubated liquid phase") was transferred to a small centrifuge tube and 5 mL color reagent added. The tube contents were thoroughly mixed and centrifuged at 3500 x *g* for 10 min. The absorbance of the supernatant was read at 540 nm against the reagent blank (1 mL acid mixture + 5 mL color reagent).

To determine the absorbance at 540 nm of the incubated liquid phase (with a brownish color) before adding the color reagent, one mL of the liquid phase and 5 mL water:saturated sodium acetate solution (21:20)—the one part bathophenanthroline disulfonate reagent used in preparing the color reagent was replaced by one part water for this solution— were mixed, centrifuged, and read at 540 nm against a blank made of 1 mL acid mixture (HCl-TCA) plus 5 mL water:saturated sodium acetate solution, 21:20 (the resultant reading was now called "liquid phase blank" value). The liquid phase blank value was then subtracted from the sample reading obtained in the previous step.

Using the Fe standard curve (0.5–5.0 μg Fe/mL), the iron content in 1 mL of the incubated liquid phase was determined. The nonheme iron content of the meat was calculated by the following equation:

$$\mu g \text{ nonheme iron/g meat} = \text{Fe conc of the incubated liquid phase } (\mu g/mL) \times (15 + 0.2 + \text{moisture content of 5g meat})(mL)/5(g)$$

Statistical analysis

When necessary, data were analyzed by analysis of variance and mean separation by the Student-Newman-Keuls' test (Steel and Torrie, 1980).

RESULTS & DISCUSSION

THE NONHEME IRON ASSAY procedure described originally by Schricker et al. (1982) involves incubation of a ground meat sample at 65°C with the acid mixture (HCl-TCA) and reaction of an aliquot of the liquid phase with the color reagent

(sodium acetate-bathophenanthroline disulfonate-thioglycolic acid), followed by reading the absorbance at 540 nm. The modification made in the present study included mixing the meat sample with 156 ppm $NaNO_2$ before adding the acid mixture in order to minimize the breakdown of heme-associated iron into nonheme iron under the incubation conditions (acid and temperature), and the use of the liquid phase blank (not a reagent blank) to measure the absorbance at 540 nm due to the color of the sample solution prior to the color development step.

A preliminary experiment was designed to determine whether the residual $NaNO_2$ present in the incubated liquid phase would interfere with the colorimetric assay of Fe. When the absorbance readings (at 540 nm) of 15 mL portions of the acid mixture containing known amounts of Fe (standard) were compared with those readings of the acid mixtures (15 mL) containing the same amounts of Fe plus 780 μg $NaNO_2$ (equivalent to 156 ppm $NaNO_2$ for 5g meat per assay tube), no differences were found, indicating that $NaNO_2$ had no effect on the Fe chromophore development.

To determine the optimum reaction time between meat heme pigments and $NaNO_2$, a batch of $NaNO_2$-added ground beef muscle (semimembranosus) was allowed to stand at 4°C. At various time intervals, between 0 and 24 hr, aliquots were removed and incubated with the acid mixture for nonheme iron assays. The amount of nonheme iron was essentially the same for the $NaNO_2$-treated subsamples held for different lengths of time, but was much higher when the $NaNO_2$ treatment was omitted (Table 1).

When ground beef muscle samples (with or without $NaNO_2$ treatment) were incubated at 65°C for 20 hr with the acid mixture, the incubated liquid phase above the meat residue was somewhat turbid and had a brownish color. The color of the incubated liquid phase apparently did not originate from meat pigments because the spectra (375–750 nm) of the incubated liquid phase did not reveal the presence of any form of myoglobin or hemoglobin. The characteristic peaks in the Soret area and in the visible for myoglobin and hemoglobin derivatives (Antonini and Brunori, 1971) were not observed in the spectra of the incubated liquid phase. Meat pigments (hemoproteins), if leached out of the meat into the liquid phase, would have been precipitated by TCA in the acid mixture.

When the liquid phase was reacted with the ninhydrin reagent (Bailey, 1967) at predetermined intervals during the 20 hr incubation period, absorbance readings for the amino acid chromophore increased with increasing incubation time (Table 2), indicating that the concentration of amino acids in the liquid phase increased as a result of meat protein hydrolysis by the acid mixture. When reacted with a phenol-H_2SO_4 reagent (Dubois et al., 1956), the acidic liquid phase after 0.5–20 hr of incubation gave rise to a strong positive reaction for the presence of simple sugars and related substances; the carbohydrate chromophore readings remained nearly constant for 0.5–2.5 hr of incubation, but decreased upon prolonging the incubation time (Table 2). Therefore, it was concluded that the brownish color of the liquid phase might be due primarily to the products of nonenzymatic browning reaction between amino acids and reducing sugars that were released from the meat into the liquid phase. In any case, the effect of coloration in the incubated liquid phase can be eliminated by subtracting the absorbance reading of the liquid phase blank from that of the liquid phase plus color reagent, as described in the "Materials & Methods" section.

Table 1—Nonheme iron values of meat[a] treated with $NaNO_2$ and held for various lengths of time prior to incubation with HCl-TCA solution

Holding time at 4°C (hr)	Nonheme iron[b] (μg/g meat)
0	3.25[d]
1	3.39[d]
2	3.25[d]
4	3.21[d]
24	3.36[d]
Without $NaNO_2$ treatment	4.54[c]

[a] Beef semimembranosus muscle (Sample No. 1)
[b] n = 3; with the liquid phase blank.
[c,d] Means within the column with different superscript letters are significantly different ($p<0.05$).

Table 2—Reaction of the incubated liquid phase for the presence of amino acids and sugars

Incubation time at 65°C (hr)	Ninhydrin reaction[a] (absorbance at 533 nm)	Phenol-H_2SO_4 reaction[b] (absorbance at 490 nm)
0	0.015	—
0.25	0.087	—
0.5	0.115	1.06
1.0	0.150	1.02
1.5	0.175	1.10
2.0	0.180	0.96
2.5	0.195	1.00
3.0	0.263	—
6.5	0.370	0.86
20.0	0.720	—

[a] 2 mL sample + 2 mL ninhydrin reagent; for the presence of amino acids.
[b] 2 mL sample + 0.05 mL 80% phenol + 5 mL conc H_2SO_4; for the presence of sugars.

Nonheme iron concentrations of several beef samples, determined with and without $NaNO_2$ treatment and with and without the liquid phase blank, are shown in Table 3. When determined without $NaNO_2$ treatment and without the liquid phase blank (the Schricker procedure), nonheme iron values of the beef samples were 43–93% higher than the values obtained with $NaNO_2$ treatment and with the liquid phase blank (the modified procedure). Nonheme iron values of the samples obtained without the liquid phase blank and with $NaNO_2$ treatment were 31–55% higher than those obtained with both the liquid phase blank and $NaNO_2$ treatment. The exact mode of action of $NaNO_2$ added to meat sample in the modified procedure is not known. However, it is presumed that the $NaNO_2$ addition might stabilize the porphyrin ring of meat heme pigments. Nitrite has been shown to prevent the release of heme iron as nonheme iron upon heating beef muscle pigment extracts (Chen et al., 1984; Morrissey and Tichivangana, 1985).

The effect of addition of purified metmyoglobin to ground beef muscle before incubation with the acid mixture on nonheme iron values was evaluated to establish that heme iron was not included in the measurement of nonheme iron in the modified procedure. As shown in Table 4, very little of the iron in metmyoglobin was measured as nonheme iron in the modified procedure. The nonheme iron value difference of 4.30 μg between meat alone (5g) and meat plus metmyoglobin (5g + 30mg), when determined by the modified Schricker procedure, was mostly due to "free" iron present in the metmyoglobin preparation purchased; when an aqueous solution containing 30 mg metmyoglobin was treated first with EDTA (2% in the solution) to chelate "free" iron and then with TCA (12.5% in the solution) to precipitate the hemoprotein metmyoglobin, followed by centrifugation, the supernatant contained 3.27 μg iron ("free" iron) when determined by atomic absorption spectrometry. Nonheme iron (Fe standard) added to ground beef muscle samples at levels of 2–6 μg per gram of meat was quantitatively recovered in the modified procedure (Table 5).

Since the nonheme iron concentrations of raw beef muscles as determined by the modified Schricker method were much lower than those obtained by the procedure of Schricker et al. (1982), but higher than the nonheme iron concentrations of beef muscles as reported by Igene et al. (1979) and Chen et al. (1984), i.e., 1.80 and 1.15–1.31 μg/g, respectively, an experiment was conducted where the nonheme iron content of a beef muscle sample was determined by the method of Igene et al. (1979), the procedure of Schricker et al. (1982), and the modified Schricker procedure. The procedure used by Chen et al. (1984) was not included because it is basically the same as that of Igene et al.

Table 3—Nonheme iron concentrations of beef muscles as affected by $NaNO_2$ treatment of meat and inclusion of the liquid phase blank

Beef muscle	Without $NaNO_2$ treatment		With $NaNO_2$ treatment	
	Without liquid phase blank[a]	With liquid phase blank	Without liquid phase blank	With liquid phase blank[b]
	Nonheme iron (μg g meat)[c]			
Semimembranosus (No. 1)	4.54[f]		5.04[e]	
Semimembranosus (No. 2)	4.58[d]	3.57[f]	4.20[e]	3.20[g]
Longissimus dorsi	4.60[d]	3.62[e]	3.77[e]	2.78[f]
Ground lean chuck	7.06[d]	5.90[e]	5.16[f]	3.85[g]
	Overestimation of nonheme iron content (%)			
Semimembranosus (No. 1)	94	40	55	0
Semimembranosus (No. 2)	43	12	31	0
Longissimus dorsi	67	30	36	0
Ground lean chuck	83	53	34	0

[a] Without $NaNO_2$ treatment and without liquid phase blank = the Schricker procedure (Schricker et al., 1982).
[b] With $NaNO_2$ treatment and with liquid phase blank = the modified procedure.
[c] n = 3.
[d-g] Means within the same row with different superscript letters are significantly different ($P < 0.05$).

Table 4—Effect of purified metmyoglobin (MetMb) added to beef muscle before incubation with the acid mixture on nonheme iron values

Method	Sample	Nonheme iron[a] (μg)	"Free" iron[b] (μg)
Schricker procedure	Meat (5g)[c]	36.51	
	Meat + MetMb (5g + 30mg)	47.19	
Modified Schricker procedure	Meat (5g)	16.35	
	Meat + MetMb (5g + 30mg)	20.65	
"Free" iron in proteinous solution[d]	MetMb (30mg)		3.27

[a] n = 5.
[b] n = 3.
[c] Semimembranosus (No. 3).
[d] The procedure consisted of: (1) separation of "free" iron by chelating with 2% EDTA, (2) precipitation of the hemoprotein MetMb with 12.5% trichloroacetic acid, (3) centrifugation, and (4) determination of iron in the supernatant by atomic absorption spectrometry.

Table 5—Recovery in the modified Schricker procedure of nonheme iron added to ground beef muscle

Sample	Nonheme iron (μg)[a]	Recovery of added Fe (%)
Trial 1		
Meat (5g)[b]	11.08	—
Meat (5g) + 10.7 μg Fe	21.90	101
Meat (5g) + 32.1 μg Fe	43.34	100
Trial 2		
Meat (5g)[c]	14.10	—
Meat (5g) + 9.1 μg Fe	22.92	97
Meat (5g) + 27.4 μg Fe	42.58	104

[a] n = 3.
[b] Semimembranosus (No. 4).
[c] Semimembranosus (No. 5).

(1979). Nonheme iron value was lowest by the procedure of Igene et al. (1979) and highest by the (original) Schricker procedure (Table 6). The procedure of Igene et al. (1979) apparently underestimated the nonheme iron content of a meat sample because it ignored nonheme iron present (probably in the form of iron-proteins) in the meat residue left after water extraction of sample. For the beef muscle sample used for data shown in Table 6, the muscle residue excluded in the nonheme iron assay procedure of Igene et al. (1979) contained 1.72 μg of iron per gram of the original meat sample when determined by the modified Schricker (present) procedure.

Table 6—Nonheme iron values of a beef semimembranosus muscle sample[a] as determined by different methods

Method	Nonheme iron[b] (μ g meat)
Schricker et al. (1982)	7.30[c]
Modified Schricker	3.27[d]
Igene et al. (1979)	1.16[e]

[a] Semimembranosus (No. 3).
[b] n = 3.
[c-e] Means within the column with different superscript letters are significantly different ($P < 0.05$).

In conclusion, the treatment of meat with nitrite and the inclusion of "liquid phase blank" are recommended when using the procedure of Schricker et al. (1982) to determine the amounts of nonheme iron in red meats. The moisture content of the meat sample must be known before the calculation of nonheme iron concentration, although Schricker et al. (1982) did not specify the need for consideration of the moisture content of meat in their nonheme iron assay procedure.

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Ms received 11/13/86; revised 3/20/87; accepted 3/23/87.

Technical article No. 21,202, Texas Agricultural Experiment Station. Supported in part by the National Live Stock and Meat Board, Chicago, IL and the Natural Fibers and Food Protein Commission of Texas, Dallas, TX. The authors appreciate the assistance of Wayne Elger for iron determinations by atomic absorption spectrometry.

the Tenax particles, while water not held by the sodium sulfate passed through to the dry ice condenser; nonvolatiles remained in the flask. Collection continued for 4 hr. This time period was based on the absence of detectable odor or visible water in the sample flask, attainment of constant weight of the flask and no further increase in recovered volatiles with increased collection time.

Volatiles analysis

The contents of the Tenax trap were analyzed by gas chromatography (GC) on a Shimadzu GC-9A instrument using a flame ionization detector (FID) with nitrogen as carrier gas at 30 mL/min. The Tenax trap was heated at 240°C for 20 min with carrier flow. Desorbed volatiles were carried to a loop of 1/16 in o.d. × 0.03 in i.d. (1.59 mm o.d. × 0.76 mm i.d.) tubing, where they were condensed in a dry ice/acetone bath. At the end of 20 min, the dry ice trap was removed and the loop heated for 12 min with a heat gun to vaporize sample volatiles, which were carried to the GC column. The oven was at 40°C for 5 min, increased at 5°/min to 150°C, then 10°/min to 200°C and held at 200°C for 5 min. The column was 6 ft x 1/8 inch o.d. (1.8 m x 3.2 mm o.d.) x 2.1 mm i.d. stainless steel packed with 3% OV-17 (® Ohio Valley Specialty Chemical Co.) on Chromasorb WHP 100/120 (Alltech Assoc., Deerfield, IL).

Volatiles identification

For peak identification by mass spectrometry (MS), the contents of the Tenax trap were eluted with hexane. The hexane solution was concentrated under a stream of air, then analyzed on a Hewlett Packard 5995 GC-MS. The column was 12 m × 0.2 mm i.d. fused silica coated with a 0.33 μm film of crosslinked methyl silicone. Carrier gas was helium at 1.5 mL/min. The oven was isothermal at 40°C for 5 min, then increased at 5°/min to 200°C.

Odor evaluation

Before skin removal, chickens were evaluated by a panel of nine (storage study) or ten (dose study) people, who rated odor on a 5-point scale, with 1 meaning very unacceptable, 3 meaning neutral and 5 meaning very acceptable. Off-odor identities were also noted.

RESULTS & DISCUSSION

IN COMPARING GC-FID chromatograms from the chickens irradiated at different doses, increasing dose caused an overall increase in the amounts of volatile material. Only a few peaks, however, increased regularly with dose. Variations in other peaks were minor, and perhaps due to sample variation not eliminated by our mixing procedure. There were linear increases in peak heights only for the peaks with retention times (RT) of 2.5 min, 4.8 min, 9.2 min and 11.8 min, as shown in Fig. 1. Regression analysis indicated positive slopes and correlation coefficients ($p<0.05$) for all four compounds. GC – MS analysis identified these as octane, 1-octene, hexanal and nonane, respectively. These have all been found in cooked chicken in previous work (Ramaswamy and Richards, 1982). These volatiles are also found in both cooked and irradiated beef (Merritt, 1972). Previous studies of irradiated chicken (Freeman et al., 1976; Merritt et al., 1985) identified a number of compounds, including octane and octene. We did not attempt identification of every peak in the irradiated samples, but all were apparently also present in the nonirradiated control. This is in general agreement with previous work (Merritt et al., 1975), which found that qualitatively the same volatiles occurred in both irradiated and control meat but that larger quantities were released from irradiated specimens.

Increasing radiation dose caused a decrease ($p<0.01$) in odor acceptability (Table 1). While all irradiated samples were rated lower than the control, only the highest dose, 1200 krad, received lower than the neutral rating of 3. Most of the panel were familiar with and able to identify, irradiation odor. Since the compounds identified can be formed by decomposition of fatty acid hydroperoxides, exclusion of oxygen during irradiation may inhibit their formation. This might also help decrease the irradiation off-odor of irradiated chicken, although we could not conclude that the off-odor was caused by these specific compounds.

The unstored 300 krad irradiated half chicken (Fig. 2, bottom) gave more volatiles than its nonirradiated control (Fig. 2, top), as in the dose study. Again, only a few peaks were markedly larger in the irradiated sample. These were assumed to be the same as those identified in the dose study, based on retention times. There was also a slight irradiation odor in the unstored 300 krad treated chickens (Table 2), as in the dose study. Similar differences in volatiles were observed between irradiated and nonirradiated chicken halves stored for 7 days, though the differences were less. The irradiated sample stored 7 days had a more acceptable odor than either its nonirradiated control or the unstored irradiated sample (Table 2). In the samples stored for 14 days, the nonirradiated half had a greatly increased level of volatiles (Fig. 3, top) compared to the irradiated half (Fig. 3, bottom). The irradiated sample stored for 14 days still had an odor about as acceptable as fresh chicken but its nonirradiated control was very unacceptable (Table 2). Both the increase in volatiles and the decrease in acceptability of the nonirradiated sample were almost certainly due to microbial growth. While microbe levels were not determined, previous studies on irradiated and control chicken (Freeman et al., 1976; Kahan and Howker, 1978) support this assumption. Microbial studies of this type of product are necessary, as there is concern that selective destruction of microorganisms would allow growth of pathogens without typical signs of food spoilage (FDA, 1986).

Since the three storage times used different chickens, sample variation may somewhat confound comparisons among times. Still, only small differences in amounts of volatiles were seen

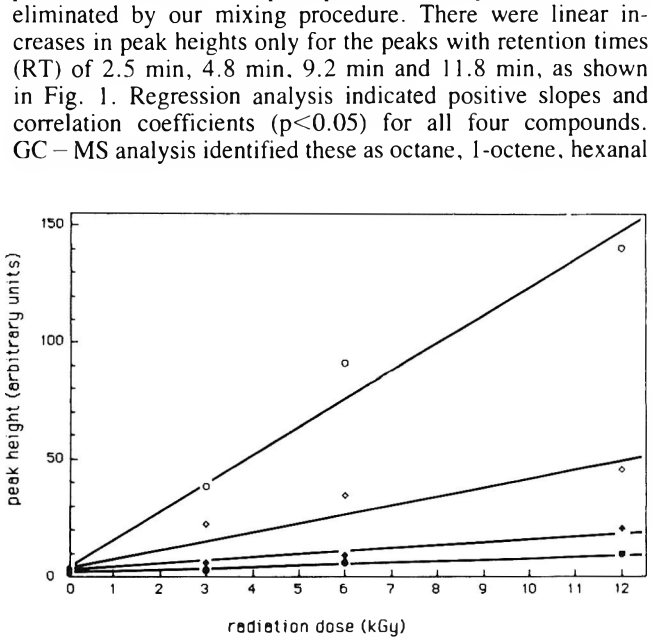

Fig. 1—Increases in volatile compounds with irradiation dose: ●, octane (RT = 2.5 min); ♦, 1-octene (RT = 4.8 min); ◇, hexanal (RT = 9.2 min); ○, nonane (RT = 11.8 min).

Table 1—Odor evaluation scores from dose study

Dose (krad)	Score[a]
0	4.80 ± 0.42[b]
300	3.60 ± 0.52[c]
600	3.10 ± 0.57[d]
1200	2.90 ± 0.74[d]

[a] Mean ± standard deviation (n = 10); 1 = very unacceptable, 5 = very acceptable.
[b-d] Pairs followed by the same letter are not significantly different ($p>0.05$).

Table 2—Odor evaluation scores from storage study

Storage (days)	Dose (krad)	Score[a]
0	0	4.56 ± 0.73[b]
0	300	3.22 ± 0.83[c]
7	0	3.33 ± 0.71[c]
7	300	4.44 ± 0.73[b]
14	0	1.56 ± 0.73[d]
14	300	4.22 ± 0.67[b]

[a] Mean ± standard deviation (n = 9); 1 = very unacceptable, 5 = very acceptable.
[b-d] Pairs followed by the same letter are not significantly different ($p>0.05$).

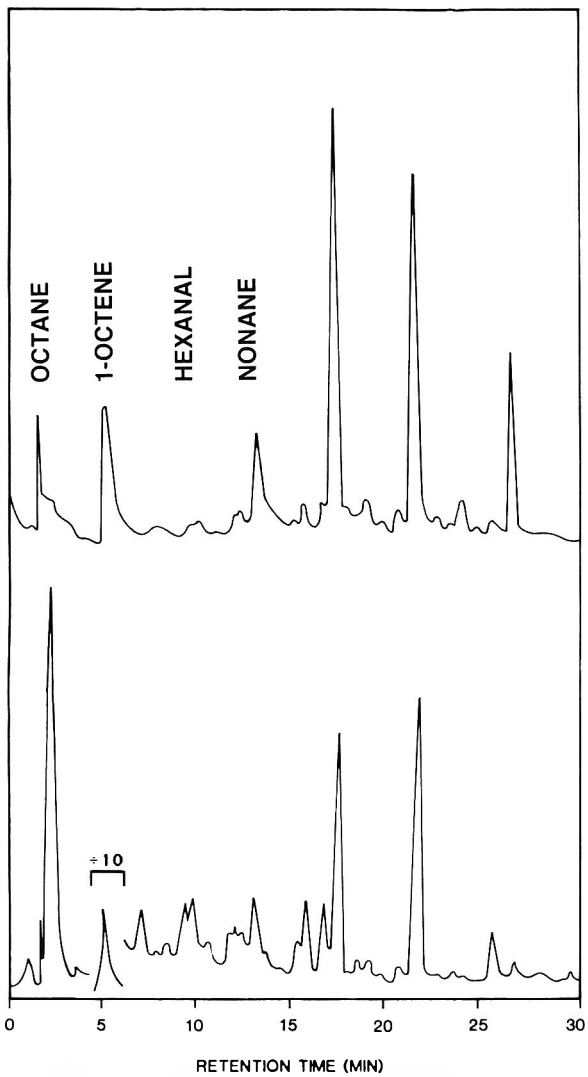

Fig. 2—GC-FID of unstored chickens. top: unirradiated, bottom: 300 krad.

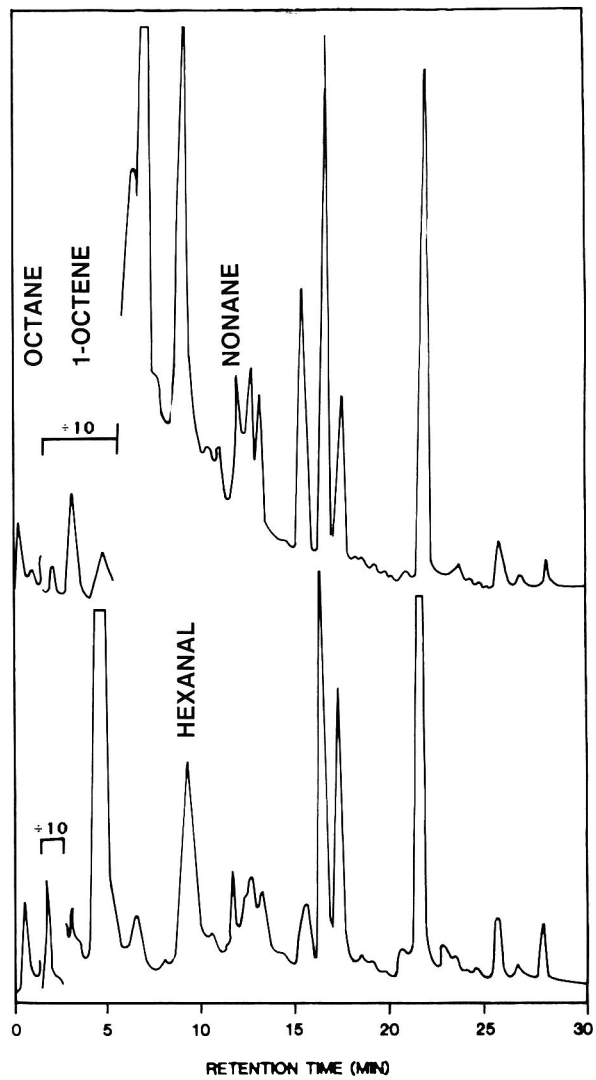

Fig. 3—GC-FID of chickens stored 14 days. top: unirradiated, bottom: 300 krad.

when the irradiated half chickens stored for 0, 7, and 14 days were compared with each other or with the 300 krad sample from the dose study. Some volatiles were found apparently in nonirradiated chickens stored for 7 days and 14 days, not seen in fresh chicken nor in irradiated chicken. Irradiated samples stored 7 and 14 days were rated almost as high as fresh non-irradiated chicken, suggesting dissipation of the irradiation odor and lack of microbial spoilage. Analysis of variance of the odor evaluation scores indicated a significant contribution to total variability both from irradiation and from storage and also from the interaction of these two effects.

In summary, the relationship among irradiation, storage, volatiles, and odor was complex. Treatment of fresh chicken with 300 krad allowed an acceptable product after extended refrigerated storage. Volatile compounds identified in irradiated samples were not uniquely products of irradiation.

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Ms received 12/15/86; revised 3/28/87; accepted 4/17/87.

Presented at the 46th Annual Meeting of the Institute of Food Technologists, Dallas, TX, June 1986.

This research was funded by the USDA-ARS, ERRC under a collaborative agreement with Drexel Univ.

The authors are grateful to Ronald K. Jenkins for technical assistance and to Donald W. Thayer and Eugen Wierbicki (deceased) for their useful suggestions.

Yields, Color, Moisture and Microbial Contents of Chicken Patties as Affected by Frying and Internal Temperatures

Y. H. YI and T. C. CHEN

ABSTRACT

Frozen, raw, battered and breaded chicken patties were tempered to an internal temperature of 1.1 ± 0.1°C. The patties were fried to three different internal temperatures (48.9°, 60.0° and 71.1°C) in each of three shortenings preheated to 168.3°, 179.4°, and 190.6°C. Yields and selected quality characteristics of the patties were measured. At the same internal cooking temperature, frying yield and moisture content of the patties were not different ($P > 0.05$). Higher frying temperature and longer frying time yielded lower Hunter "L" values and a tendency to produce higher Hunter "a" values. An interaction between frying temperature and internal temperature was significant for microbial counts. No difference ($P > 0.05$) in microbial count was observed when patties were fried to an internal temperature of 60.0° or 71.1°C.

INTRODUCTION

COOKING of meat brings about changes in its structure which depend on the method of cooking, composition of meat, and the degree of doneness. These changes have a profound influence on the palatability and yield of cooked meat and, thus, are the main concern of consumers (Mostert and Stadelman, 1964). In deep fat frying, the fat reacts with protein and carbohydrate components of the food, developing unique flavors and aromas which have definite appeal to consumers (Weiss, 1983).

Yields of products are of great concern to operators of fast food outlets and poultry product companies. Regardless of cooking method, meat shrinkage and weight loss occur. Love and Goodwin (1974) indicated that the percentage weight loss of precooked poultry parts was more for parts browned in the deep fat fryer at a lower temperature for a longer time than those parts browned at a higher temperature for a shorter time. Frying chicken patties to a satisfactory degree of doneness is difficult since patties often differ widely in composition, size, shape and ingredients used.

Information concerning the microbiological quality of precooked chicken products is limited. Chen et al. (1973) isolated micrococci and staphylococci from both hot water- and microwave energy-precooked chicken parts and from commercial ready-to-eat chicken products. Wang et al. (1976) reported that the log number of psychrophilic bacteria counts for commercial frozen fried chicken products ranged from 2.74 to 4.66/g with a mean value of 3.40/g.

Prefried, ready-to-eat, carry-out chicken products are very popular in the United States as substantiated by the tremendous increase in the sale of these products in the past several years (Thornton, 1983; Lovette, 1985a,b). A variety of frying temperature and frying time combinations for chicken parts has been suggested by many researchers (American Hospital Association, 1972; Thorner, 1973; Yang and Chen, 1979; and Weiss, 1983).

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Although a number of papers have been published on the effect of cooking time, degree of doneness, internal temperature on yield, moisture content, and microbiological quality of chicken parts, little or no study has been conducted on commercially produced chicken patties. It is considered important to evaluate the influence of frying to different internal temperatures on the quality of precooked poultry products (Sheldon et al, 1980). The objective of this experiment was to investigate the effects of alteration in frying temperatures and internal temperatures on the yield, color, moisture and microbial contents of commercial type chicken patties.

MATERIALS & METHODS

Chicken patties

Battered and breaded raw chicken patties from the same lot were obtained from a processing line in a commercial poultry product plant. The composition of raw patties was 39.3% breast meat with skin, 26.6% water and/or ice, 19.9% skin, 12.3% soy protein, 1.0% seasoning, and 0.3% polyphosphate. All ingredients were mixed in a Bold Blendor (Western Springs Engineering, IL). The patties were shaped in a Formax forming machine (Formax Inc., IL), battered, breaded, frozen by placing on dry ice and stored at −18°C in polyethylene poultry bags. Raw chicken patties had a mean moisture content of 65.14%.

The frozen patties, averaging 9.5 mm in thickness and 85.9 g in weight, were tempered in a refrigerator for approximately 50 min to an internal temperature of 1.1 ± 0.1°C. Copper constantan thermocouple wires connected to a Speedomax M Multipoint Recording Potentiometer (Leeds and Northrup Co., PA) were used for recording the frying shortening and internal patty temperatures. The patties were fried, four at a time, in a commercial fryer (J.C. Pitman & Son Inc., NH.) containing 29.5 kg preheated liquid shortening (Hunt-Wesson Foods, Inc., CA) at three different frying temperatures, 168.3°, 179.4°, and 190.6°C, to three different internal temperatures, 48.9°, 60.0° and 71.1 °C, respectively, before being removed from the oil. A total of nine combinations of frying temperatures and internal temperatures were fried in the same vat of shortening. Patties were handled care-

Table 1—Effect of frying and internal temperatures on yield, moisture content and microbial count of chicken patties[a,b]

Internal temp (°C)	Frying temperature (°C) 168.3	179.4	190.6
		Yield (%)	
48.9	101.3a	101.2a	99.8ab
60.0	97.9b	99.5ab	99.0b
71.1	98.4b	98.6b	98.1b
		Moisture content (%)	
48.9	47.9a	48.0a	48.1a
60.0	46.7ab	46.9ab	46.4ab
71.1	45.4b	45.4b	46.0ab
		Microbial count (log CFU/g)	
48.9	3.64e	2.44d	1.81abc
60.0	2.11abcd	2.19cd	2.27d
71.1	1.52a	1.75ab	1.66a

[a] For yield, mean of 12 observations; for moisture content, mean of 4 observations; and for microbial count, mean of 8 observations.
[b] Means, not followed by the same letter are significantly different ($P<0.05$).

Table 2—Hunter color values of chicken patties from different frying and internal temperatures[a,b]

	"L"			"a"			"b"		
Frying temp (°C)	168.3	179.4	190.6	168.3	179.4	190.6	168.3	179.4	190.6
Internal temp (°C)									
48.9	62.3a	58.4b	53.2d	1.49c	4.79bc	6.42ab	17.1a	18.4a	18.1a
60.0	57.5bc	54.7cd	49.3e	4.71bc	6.38ab	9.31a	19.1a	20.4a	18.8a
71.1	54.4cd	53.2d	47.9e	6.05ab	6.24ab	9.41a	20.2a	19.3a	19.5a

[a] Mean of 8 observations.
[b] Means within a color group not followed by the same letter are significantly different ($P < 0.05$).

fully to avoid any unnecessary contamination before, during and after the frying operation.

Yield and moisture content

Frying yield was obtained by dividing the fried patty weight by the raw patty weight and multiplying by 100. About 2g sample was taken from a whole ground patty and oven dried for 16–18 hr at 100–102 °C. The sample was cooled in a desiccator to room temperature and weighed. Weight loss was used to calculate the percentage of moisture content in the cooked product (AOAC, 1984).

Color measurement

The external color of fried patties was measured with a Hunterlab Model D-25 Color and Color Difference Meter (Hunter Associates Laboratory, VA). Standard plate number 6277, with a "L" value of 83.3, an "a" value of −4.9, and a "b" value of 25.7 was used for reference.

Total microbial counts

The fried patty samples were packaged, asceptically, in polyethylene poultry bags and stored in a 2–4 °C refrigerator. A 30g portion of a chicken patty, within 24 hr of frying, was removed, aseptically, and blended with 270 mL sterilized 0.1% peptone solution in a sterilized Waring Blendor at high speed for 1 min. Using the "pour plate technique" (APHA, 1976), serial dilutions were made and duplicates were plated with Plate Count Agar (Difco) and acidified Potato Dextrose Agar (Difco), for total psychrotrophic microbial and total fungal counts, respectively. The plates were incubated for 72 hr at 20°C to determine the total psychrotrophic counts. Mold and yeast counts were enumerated after 5 days of incubation at 25°C. The average number of colonies from the duplicate plates were reported as the number of colony forming units (CFU) per gram patty.

Statistical analysis

Experiment design was 3×3 factorial arrangement in a complete random design. The level of the first factor was frying temperature and the level of the second factor was internal temperature. Replicates for yields, moisture, microbial counts and color measurements were 12, 4, 8, and 8, respectively. Data were analyzed statistically by analysis of variance as described by Steel and Torrie (1980). Duncan's new multiple range test (Duncan, 1955) was used to separate the means when required.

RESULTS & DISCUSSION

AT 168.3°C FRYING TEMPERATURE, 80, 115, and 150 sec were required for patties to reach internal temperatures of 48.9°, 60.0°, and 71.1°C, respectively. At 179.4°C, the frying time was reduced to 70, 90, and 110 sec for internal patty temperatures of 48.9°, 60.0°, and 71.1°C, respectively. At 190.6°C frying temperature, the frying time was further reduced to 60, 80, and 100 sec for these same three internal temperatures. In general, the internal temperature of the patties increased approximately 11.1°C after removal from the fryer. In this study, only 4 patties were fried at a time to avoid any significant change in frying shortening temperature.

The effects of the interaction between frying temperature and internal temperature on yield and moisture content were significant (Table 1). Yield was improved ($P < 0.05$) when patties were fried at 168.3°C or 179.4°C to an internal temperature of 48.9°C as compared to those fried to an internal temperature of 71.1°C. When patties were fried at 190.6°C, there were no significant differences in yield or moisture content regardless of the internal temperature. There were no significant differences in yield or moisture content of patties fried to the same internal temperature regardless of frying temperature. It was concluded that internal temperature is the primary factor affecting the yield and moisture content rather than frying temperature. The increased cooking loss of meat products as the internal temperature or cooking temperature increased has been reported (Esselen et al, 1956; Marshall et al., 1960; Hoke and Kleve, 1966; Acton, 1972; and Batcher and Deary, 1975).

Lower frying temperature and lower internal temperature yielded patties with higher Hunter "L" values (increased lightness). Higher frying temperature and higher internal temperature had a tendency to produce higher Hunter "a" values (increased redness). No difference ($P > 0.05$) in Hunter "b" values was observed for the patties regardless of the frying and internal temperatures (Table 2). In order to get uniform brownness of the poultry parts at different frying temperatures, Love and Goodwin (1974) reported that it is necessary to employ a different browning time for each frying temperature.

An interaction between frying temperature and internal temperature was also significant for microbial counts. When patties were fried to an internal temperature of 48.9°C, there was a significant decrease in microbial counts as frying temperature increased. This effect was not significant when patties were fried to an internal temperature of 60.0° or 71.1°C (Table 1). An internal temperature of 71.1°C is required to be labeled "fully cooked", "ready-to-eat", "baked", or "roasted" poultry according to the USDA (1973). Raw patties from the same source had a mean total psychrotrophic count of log 5.1 CFU/g. The total fungal counts of the patty samples at zero days were low and less than 10 CFU per gram sample regardless of internal and frying temperatures. Dawson et al. (1975) reported that properly cooked meat products presented little health problems since total bacterial counts were low and essentially no coliforms survived the cooking process.

CONCLUSIONS

IN CHICKEN PATTY FRYING, the effects of the interaction between frying temperature and internal temperature on yields and moisture content were significant. The primary factor affecting the yield and moisture was the internal temperature rather than frying temperature. Higher frying and internal temperatures resulted in products with lower Hunter "L" and "a" values; while no effect on Hunter "b" values was observed. An interaction between frying and internal temperatures was also significant for microbial counts. No difference in microbial counts was recorded when patties were fried to an internal temperature of 60.0°C or 71.1°C.

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Ms received 10/14/86; revised 3/16/87; accepted 3/20/87.

Mississippi Agricultural & Forestry Experiment Station Fournal Series No. 6664.

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ual activities were measured when each proteinase was inhibited. In the cases of metallo- and aspartic proteinases, about 50% activity remained and for serine proteinase, 85% remained (Fig. 3).

Under the same conditions of inhibitors, residual activities of degradation of myofibril and collagen were measured. There were 34.6, 71.2, and 94.2% remaining activities of myofibril degradation, and 39.6, 71.2, and 93.3% remaining activities of collagen degradation by inhibiting serine-, metallo- and aspartic proteinase, respectively (Table 3). It was suggested that the contribution of serine proteinase to meat tenderizing ability was the highest among them.

To confirm the contribution of serine proteinase, the mode of degradation of myofibrils with the pure serine proteinase was investigated. Though myosin light chain was degraded more slowly by pure serine proteinase than by the crude enzyme, the degradation pattern of myofibrils resulting from the treatment with the pure serine proteinase was very similar to that with the crude enzyme (Fig. 4a,b).

An enzyme composition of raw soy sauce or culture fluid can be changed by using various molds (Tagami et al., 1977) or mutants (Nasuno and Ohara, 1971). So, raw soy sauce or culture fluid can be improved to be suitable as meat tenderizer.

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Ms received 12/31/86; revised 4/8/87; accepted 4/25/87.

The authors thank Professor S. Arai and Dr. Nishimura of the Univ. of Tokyo for valuable advice. The authors also thank Drs. Hayashi, Motai, Nakadai, and Shimizu for their helpful discussions.

Retro-Aldol Related Degradations of 2,4-Decadienal in the Development of Staling Flavors In Fried Foods

DAVID B. JOSEPHSON and ROBERT C. LINDSAY

ABSTRACT

2,4-Decadienal was degraded to 2-octenal and ethanal by a water-mediated alpha/beta double bond hydration, retro-aldol condensation reaction series. Subsequently, similar degradation of 2-octenal resulted in the formation of hexanal and ethanal. Two other significant compounds were tentatively identified as either 3-keto-*t*4-decenal and 3-keto-*c*4-decenal or *anti* and *syn* 2-carboxyaldehyde-5-pentyl-2,5-dihydrofuran. The rate of retro-aldol related degradations of 2,4-decadienal in aqueous model systems was independent of oxygen, but was greatly accelerated with heat. Decreases in concentrations of 2,4-decadienal isomers and some increases in hexanal and 2-octenal were noted in staling deep-fat fried chicken and fish during holding for 3 days at 6°C.

INTRODUCTION

THE 2,4-ALKADIENALS, including 2,4-dicadienal, are formed from the autoxidation of polyunsaturated fatty acids, and are generally considered to contribute desirably to the aromas of a variety of freshly-prepared foods (Patton et al., 1959; Mookherjee et al., 1965; Wilson and Katz, 1972; Harkes and Begemann, 1974; Janney et al., 1974; Nawar, 1985). However, because the 2,4-alkadienals are also found in foods with oxidized, stale, and warmed-over flavors (Nonaka and Pippen, 1966; Thomas et al., 1971; Dimick et al., 1972; Rao et al., 1976, 1978), they are frequently associated with undesirable flavors as well. In addition, the 2,4-alkadienals are susceptible to further degradation via classic Farmer autoxidation and photooxidative mechanisms (Farmer et al., 1943; Matthews, et al., 1971; Michalski and Hammond, 1972; Schierberle and Grosch, 1981; Grosch et al., 1981) which add to undesirable flavors through contributions of derived carbonyl compounds.

Although stale and warmed-over flavors of meats have been shown to be a manifestation of lipid autoxidation, the chemical mechanism for the formation of these flavors in reheated foods remains undetermined (Timms and Watts, 1958; Sato and Hegarty, 1971; Wilson et al., 1976; Ruenger et al., 1978; Igene et al., 1979; Igene and Pearson, 1979; Pearson and Gray, 1983). Freshly fried foods possess regions which exhibit low water activities that favor the formation of lipid oxidation products, but when foods are held water migrates into these regions causing a concurrent loss of crispness and development of staling flavors.

Retro-aldol condensation reactions following initial alpha/beta double-bond hydrations of 2-alkenals yield corresponding shorter-chain aldehydes and ethanal which nonoxidatively contribute to flavor changes in a variety of foods (Ohloff et al., 1985; Bauer and Garbe, 1985; Gracey et al., 1985; Josephson and Lindsay, 1986; 1987). The structurally related 2,4-alkadienals would be expected to undergo similar degradations, but the presence of an additional conjugated double-bond could alter the course of reactions. Therefore, the purposes of this research were: (1) to investigate the water-mediated retro-aldol type degradations of 2,4-alkadienals using 2,4-decadienal as a model compound: and (2) to compare concentrations of selected volatile carbonyls in freshly deep-fat fried battered chicken and fish to amounts found in samples that had been held refrigerated to determine changes that were occurring during a staling period.

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MATERIALS & METHODS

2,4-Decadienal model systems for retro-aldol type reactions

Effect of air. 2,4-Decadienal (*cis/trans* and *trans/trans* isomers; Bedoukian Research Inc., Danbury, CT; 600 ppm) was added with 2-ethyl-hexanol (Aldrich Chemical Co., Milwaukee, WI, internal standard, 400 ppb) to distilled water (500 mL, pH 7.5) which had been degassed (1 mm Hg, 2 hr) in a vacuum desiccator, and then flooded with nitrogen before continuously stirring for 1 hr at room temperature (21°C). After the 1 hr equilibration period the solution was divided equally with 250 mL placed under air. Both systems were then magnetically stirred continuously at room temperature (21°C), and samples (20 ml) were removed for gas chromatographic (GC) analysis from each at times 0, 4, 6, 12, 24, 48, 72, 96, and 120 hr. To achieve a nitrogen atmosphere after drawing out a 20 mL sample, the solution was placed under vacuum (2–3 mm Hg), and was then again flooded with nitrogen until a slight vacuum remained.

Effect of heat. 2,4-Decadienal (*cis/trans* and *trans/trans* isomers; 600 ppm) and 2-ethyl-hexanol (internal standard, 400 ppb) were added to degassed (1 mm Hg, 2 hr) distilled water (125 mL, pH 7.5) and stirred under nitrogen for 1 hr. This mixture was then heated over a 50 min period to approximately 70°C, and at 10 min intervals a 20 mL sample of the reaction mixture was removed for GC analysis. In order to maintain the nitrogen headspace, a 125 mL flask was employed that was fitted with a balloon cap and equipped with a side arm fitted with a stopcock which allowed samples to be drawn out (flask was inverted) without entry of air. Samples (20 mL) for GC analysis were drawn off into a glass stoppered flask from the heated solution at 0, 10, 20, 30, 40, and 45 min. The temperature of samples at withdrawal was measured, and then the mixture was sealed and rotated in a dry ice-methanol bath for approximately 15 sec.

Extraction and analysis of samples

Each sample (20 mL) was extracted with 2 mL hexane (HPLC grade; EM Science, Cherry Hill, NJ), and the extracts were concentrated under a stream of nitrogen at room temperature (21°C) to approximately 50 μL. Concentrates of volatile compounds in hexane were then analyzed using a Varian 1740 gas chromatograph equipped with an effluent splitter for simultaneous FID measurement and odor assessment of individual peaks. Separations involving odor evaluations were achieved using a 3 m × 2 mm i.d. glass column packed with 7% Carbowax 20M on Chromosorb W AW/DMCS with a temperature programming rate from 50°C to 220°C at 4°C/min.

Additionally, volatile compounds in hexane extracts were analyzed by capillary column gas chromatography in conjunction with mass spectrometry using a Carbowax 20 M (60 m × 0.25 mm i.d.) fused silica capillary column (J & W Scientific Inc., Rancho Cordova, CA) operated with helium carrier gas. A program rate of 50°C (5 min) to 140°C at 6°C/min followed by a rate of 10°C/min from 140°C to 220°C was employed.

Deep-fat fried chicken and fish

Fresh, uncooked chicken legs and breasts (2.5 kg) and frozen-then-thawed Alaskan pollack fillets (1.5 kg) were obtained from a retail supermarket. Each piece of chicken and fish was battered and breaded

by first brushing with fresh whipped egg, and then rolling in soda cracker crumbs to obtain a coating approximately 5 mm thick. Pieces were deep-fat fried (190°C) in 12L corn oil (Mazola Corn Oil; Best Foods, CPC International Inc., Englewood Cliffs, NJ) for 3 min, then the pieces were held at room temperature 2–3 min for temperature equilibration. This process was immediately repeated two additional times for a total cooking time of 9 min.

After frying, samples were cooled to about 70°C, and either 750–800g chicken (chunked; 25–35 cm^3) or 650–700g pollack (flaked) were each placed into 12L flasks fitted with nitrogen purging tubes. The remaining samples were refrigerated (6°C) for 3 days prior to reheating by microwave (4–5 min; 2450 MHz; Standard kitchen-type microwave oven; Litton Systems, Inc., Minneapolis, MN) to an internal temperature of ca. 75°C. Prepared samples were then analyzed by headspace purging of volatile compounds. In each case just prior to initiating headspace purging, ethyl heptanoate (30 μg in 200 μL ethyl ether; internal standard) was added to the inside wall of the flask. Headspace volatiles were collected on Tenax GC (60–80 mesh, ENKA N.V., Holland) as described by Olafsdottir et al. (1985) by passing a stream of purified nitrogen (80 mL/min for 15 hr; 21°C) over the samples. The nitrogen stream was first bubbled through distilled water in a separate chamber, and then over the sample to minimize evaporative water losses. Volatiles were subsequently eluted into concentrate tubes (Laboratory Research Co., Los Angeles, CA) from the Tenax GC traps with 1 mL redistilled ethyl ether (Fisher Scientific, Fairlawn, NJ), and were concentrated under a slow stream of nitrogen at room temperature (21°C) to approximately 10 μL.

Concentrates of volatile compounds in ethyl ether were then analyzed using capillary column gas chromatography in conjunction with mass spectrometry using a Carbowax 20M (60 m × 0.25 mm i.d.) fused silica capillary column (J & W Scientific, Inc., Rancho Cordova, CA) operated with helium carrier gas. A program rate of 50°C (5 min) to 140°C at 6°C/min followed by a rate of 10°C/min from 140°C to 220°C was employed.

Compound identifications

Identification of 2-butenal, hexanal, 2-octenal and the 2,4-decadienal isomers was based on computer matching of the full mass spectra of these compounds published in "EPA/NIH Masa Spectral Data Base" (Heller and Milne, 1975, 1980) with those from the isolated compounds in the model systems, as well as coincidence for retention indices (I_E, Van den Dool and Kratz, 1963).

Other selected compounds were tentatively identified by their mass spectral fragmentation pattern and Fourier transform-infrared (FT-IR) absorption spectra. FT-IR spectroscopy was performed on the isolated compounds using a Hewlett Packard 5890 series gas chromatograph (Hewlett Packard Co., Palo Alto, CA) coupled to a Nicolet FT-IR spectrometer (Nicolet Instrument Corp., Madison, WI). These compounds were first cold trapped (dry ice) in capillary tubes as they eluted from a Carbowax 20M packed glass column (3 m × 2 mm i.d.), and then washed with hexane (200 μL ea) from the tubes before concentrating to 30 μL. Components of these concentrates were then separated for coupled FT-IR analysis on a DB-1 (30 m × 0.31 mm i.d.) fused silica capillary column (J & W Scientific, Inc., Rancho Cordova, CA) operated with helium carrier gas, and programmed at a rate of 50°C to 200°C at 8°C/min.

All quantitative data were obtained with a Spectra-Physics computing integrator (SP 4200). Data are reported as the percent of 2,4-decadienal converted to the compound of interest on a mole basis.

RESULTS & DISCUSSION

Retro-aldol degradation of 2,4-decadienal in aqueous model systems

Degradation of 2,4-decadienal (2*t*, 4*t* and 2*t*, 4*c* mixture) in water held at pH 7.5 under nitrogen and air atmospheres for time intervals to 120 hr yielded 2-octenal and hexanal along with two additional components that were designated as compounds 1 and 2 (Fig. 1). In both air- and nitrogen-blanketed model systems, hexanal was found to accumulate to a significantly greater extent than 2-octenal. Hexanal and 2-octenal are predicted degradation products from 2,4-decadienal by both classic autoxidation (Matthews et al., 1971; Schieberle and Grosch, 1981) and alpha/beta double-bond hydration, retro-aldol condensation reactions (Fig. 2). In addition, small amounts

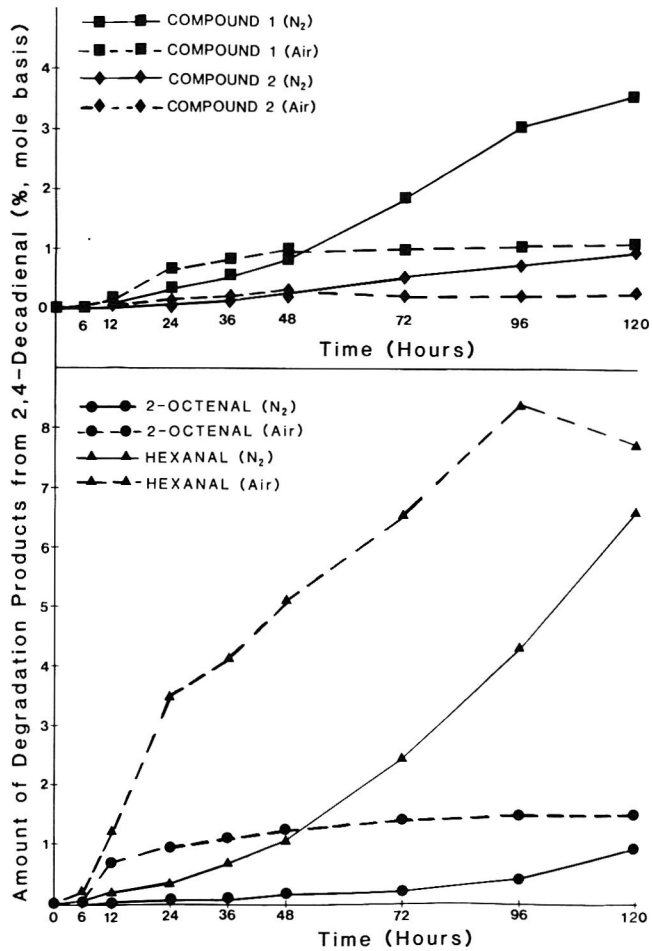

Fig. 1—Effect of headspace (air vs nitrogen) on the degradation of 2,4-decadienal to form 2-octenal, hexanal and two significantly abundant components designated compounds 1 and 2.

of the intermediate degradation product, 3-hydroxy-4-decenal, [I_E = 16.53, Carbowax 20M; 43(100), 41(95), 55(78), 39(44), 69(41), 42(37), 54(34), 110(28), 84(28), 81(26), 96(17), 152 M-18 (17), 123(15), 170 M+ (4); CI-MS: molecular weight = 170 daltons] were observed in the water-mediated degradation products of 2,4-decadienal (Fig. 2).

The reaction scheme in Fig. 2 shows two possible nonoxidative pathways (A_1 and B_1) which involve some degree of degradation of 2,4-decadienal via double-bond hydration, retro-aldol condensation reaction. In pathway A_1, 2,4-decadienal undergoes alpha/beta double bond hydration to form 3-hydroxy-4-decenal. Retro-aldol degradation of 3-hydroxy-4-decenal later yields 2-octenal and ethanal. Subsequently, 2-octenal undergoes a similar series of reactions to yield hexanal and another molecule of ethanal. In pathway B_1, 2,4-decadienal would undergo delta/epsilon double-bond hydration to form 5-hydroxy-2-decenal, which would require the cleavage of the 4-5 bond to yield hexanal and 2-butenal. 2-Butenal could then undergo retro-aldol condensation to yield two ethanal molecules.

2-Butenal was found only as a minor decomposition product in the aqueous model system stored under an air atmosphere, and the model system stored under a nitrogen atmosphere was devoid of any measurable 2-butenal throughout the experimental trials (120 hr). Hexanal and 2-octenal should be formed non-oxidatively only through alpha/beta double-bond hydration, retro-aldol degradations when 2,4-decadienal is held in an aqueous environment under a nitrogen atmosphere (Fig. 1). Therefore, the results of the experiments seem to indicate that 2-butenal arises from 2,4-decadienal through an autoxidative mechanism, even though it theoretically could also be formed

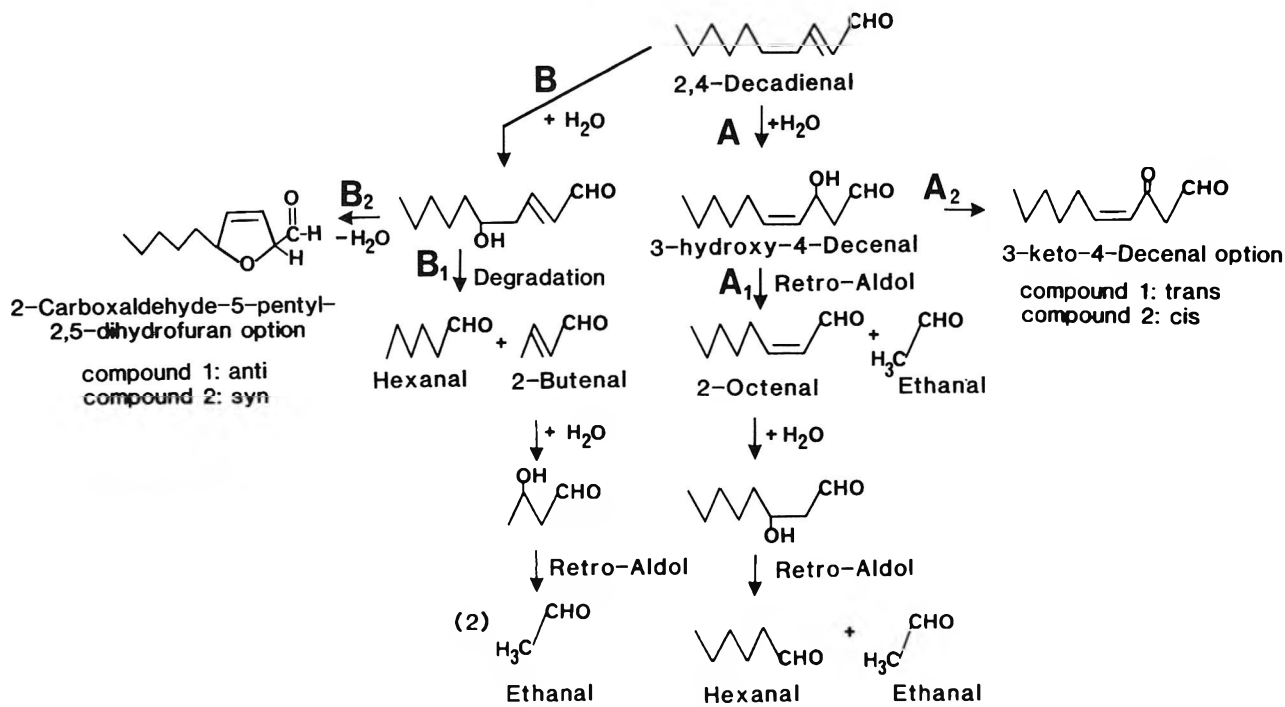

Fig. 2.—Proposed degradation of 2,4-decadienal by non-oxidative water-mediated reaction. Evidence for the existence of pathway B, was not observed.

by either water-mediated degradation of 2,4-decadienal (Fig. 2), autoxidation of 2.4-decadienal (Matthews et al., 1971; Michalski and Hammond, 1972; Schieberle and Grosch, 1981) or aldol condensation and dehydration of two ethanal molecules. The accumulation of 2-octenal and hexanal (Fig. 1) in the aqueous model system held under nitrogen, and the lack of 2-butenal occurring in this anaerobic environment, supports an interpretation that 2,4-decadienal undergoes water-mediated degradation only via pathway A_1.

The formation of substantially higher concentrations of hexanal and 2-octenal from 2,4-decadienal held under an atmosphere of air, compared to when 2,4-decadienal was held under an atmosphere of nitrogen, was expected because of autoxidation contributions (Fig. 1). After 96 hr the sample held under air showed a decline in the amount of hexanal present which reflected secondary reactions involving oxidation to acid and polymerizations (Streitwieser and Heathcock, 1981), both of which were noted in later analyses.

Compounds 1 and 2 (Fig. 1) which also became more abundant with time possessed identical mass spectral fragmentation patterns, and were tentatively identified as either 3-keto-*t*4-decenal and 3-keto-*c*4-decenal or the *anti* and *syn* isomers of 2-carboxaldehyde-5-pentyl-2,5-dihydrofuran. The mass spectral fragmentation pattern for both of these compounds was 68(100), 39(86), 41(81), 43(75), 55(68), 69(59), 42(57), 40(56), 57(53), 56(48), 53(28), 84(25), 83(24), 139 M-29 (8), 152(1), 168 M+ (1); CI-MS: molecular weight = 168. The structure for either of these two pairs of isomers appear to satisfy the mass spectrometric data was well as theoretical means of formation (Fig 2: Pathways A_2 and B_2).

When compounds 1 and 2 were analyzed by GC-coupled FT-IR, only weak IR spectra were obtained. However, evidence for a carbonyl functional group was found in a C=O stretch absorption band at 1711 cm^{-1}. A weak absorption band at 1640-1660 cm^{-1} was indicative of C=C stretch absorption, and the only other absorption bands occurred at 2930-2970 and 2860-2880 cm^{-1} which were apparently for C-H stretches. The two isomers were not resolved from one another when GC separations were attempted on a non-polar, fused silica capillary column (DB-1), but were resolved on a polar Carbowax 20M column with retention indices (I_E; Van den Dool and Kratz, 1963) for compounds 1 and 2 of 13.33 and 13.17, respectively. These observations indicate that some geometry differs between the molecules which affects the overall polarity of each.

Odor assessments for compounds 1 and 2 during elution from packed GC column separations revealed that both exhibited an odor quality similar to that of 2,4-decadienal, but the aroma of each contained less pleasant notes that were reminiscent of old fried, stale-like aromas. Both compounds 1 and 2 were found abundantly in the aqueous model system (pH 7.5) stored under nitrogen (Fig. 1) which establishes that their formation does not directly involve atmospheric oxygen. However, compounds 1 and 2 were not formed to any appreciable extent in an aqueous model system adjusted to pH 2.8 (data not shown), and degradation products other than those noted at pH 7.5 were not detected.

2-Carboxyaldehyde-5-pentyl-2,5-dihydrofuran could be formed, and it would likely be derived from a ring closure of 5-hydroxy-2-decenal which theoretically could form as a result of delta/epsilon hydration of 2,4-decadienal (Fig. 2; Pathway B_2). Thus, the two peaks could be rationalized by the presence of *anti* and *syn* isomers formed at about equal concentrations at the time of ring closure. However, evidence for the occurrence of the 5-hydroxy-2-decenal precursor was not observed in the spectral data.

When only GC-purified *t*2, *t*4-decadienal was used rather than a mixture of *t*2, *t*4-and *t*2, *c*4-decadienal in retro-aldol-related degradation experiments, both compounds 1 and 2 were still formed (Fig. 3). If the *cis* and *trans* 3-ketodecenals account for compounds 1 and 2, this would seem to imply that the delta/epsilon double-bond of 2,4-alkadienals (Fig. 1) provides a source of mobile electrons and/or some degree of conjugational stability to a resonance stabilized transitional intermediate. Such an intermediate structure could direct a hydroxyl-oxidation at carbon-3 of 3-hydroxy-4-decenal. Overall this would require the transitional double bond to assume either a *cis* or *trans* conformation when the keto group was formed. However, a mechanism for this type of conversion does not appear to be available in the literature.

Heating aqueous model systems (pH 7.5) containing 2,4-decadienal held under a nitrogen atmosphere caused an en-

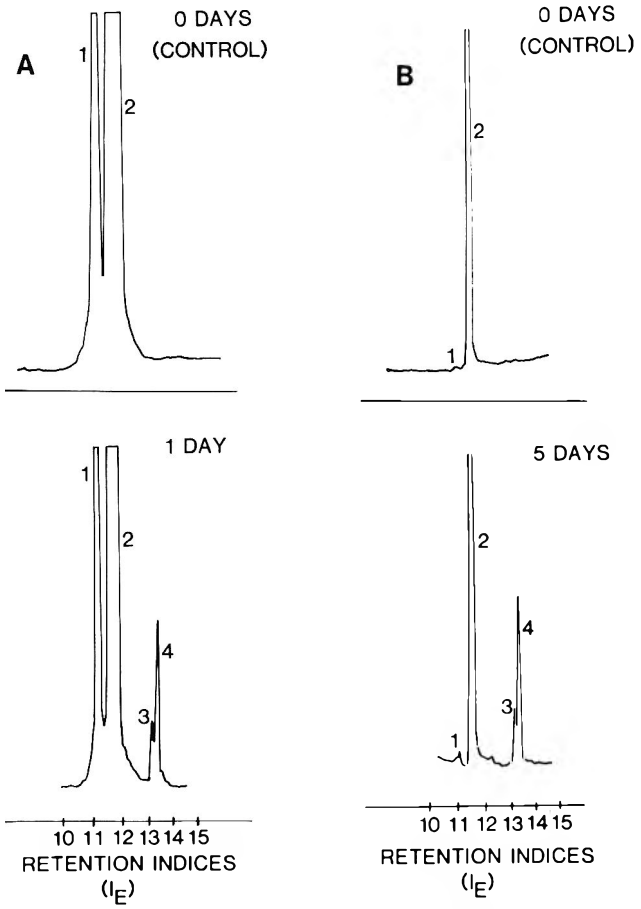

Fig. 3—Selected region from a Carbowax 20M packed column gas chromatographic separation of hexane-extracted components formed during a water-mediated degradation of (A) t2, c4-decadienal (peak 1) and t2, t4-decadienal (peak 2) and (B) nearly pure t2, t4-decadienal (peak 2) model systems. Peak 3 = compound 2 and peak 4 = compound 1.

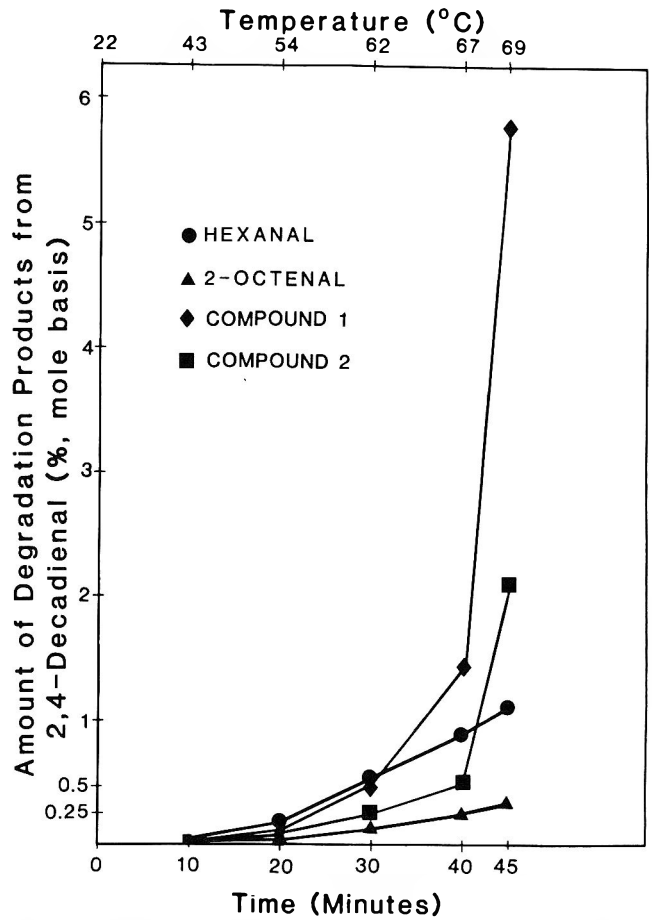

Fig. 4—Effect of temperature on the degradation of 2,4-decadienal to form 2-octenal, hexanal and two significantly abundant components designated compounds 1 and 2 in models systems under a nitrogen atmosphere.

hanced formation of 2-octenal, hexanal and compounds 1 and 2 (Fig. 4). Similar enhanced rates of double-bond hydration, retro-aldol condensations of *t*2, *c*6-nonadienal were observed earlier when aqueous model systems of this 2-alkenal were subjected to elevated temperatures (Josephson and Lindsay, 1987). However, in the earlier study substances analogous to compounds 1 and 2 were not observed, and only 3-hydroxy-*c*6-nonenal was found. This suggests that a unique degradation mechanism for the conjugated 2,4-alkadienals occurs which gives rise to additional products compared to 2-alkenals.

Retro-aldol-related degradations in the staling of foods

Since 2,4-decadienal is one of the more abundant carbonyls derived through autoxidation of n-6 fatty acids (Nawar et al., 1978; Frankel, 1980), it has been qualitatively observed in many cooked meats, including chicken and beef (Watanabe and Sato, 1971; Liebich et al., 1972; Harkes and Begemann, 1974). However, high temperature aqueous isolation procedures, including steam distillation, create conditions where 2,4-decadienal would be susceptible to nonoxidative, water-mediated degradation. As a result, the lack of occurrence of 2,4-decadienal in fried chicken volatiles isolated by nitrogen purging of water slurries of meat held at an elevated temperature (Tang et al., 1983) probably can be attributed to artifactual retro-aldol-related degradations during heating.

Headspace analysis of volatiles in freshly deep-fat fried chicken pieces (Fig. 5) and pollack fillets revealed that the concentrations of the 2,4-decadienals were higher in fish than in chicken both soon after frying and after 3 days of refrigerated storage (Table 1). However, in both foods the concentrations of 2,4-decadienal isomers were notably reduced after the refrigerated holding. Levels of 2-octenal increased substantially during refrigerated storage in both fish and chicken indicating an anticipated build-up of a degradation product of 2,4-decadienal. A major difference between samples was observed for hexanal contents of both freshly fried and refrigerated chicken and fish. For chicken, an initially large hexanal content tripled during storage indicating that oxidation of n-6 fatty acids occurred extensively in the meat. However, the concentration of hexanal in fish declined indicating that oxidation of n-6 fatty acids in fish occurred to a much lesser extent than in chicken. This could reflect a lower concentration of precursor n-6 fatty acids in fish than in chicken or more likely that heme-catalyzed lipid oxidation was much more pronounced in the chicken than in the fish (Ke and Ackman, 1976; Pearson and Gray, 1983; Kanner et al., 1986). Compounds 1 and 2 were not positively identified in isolates from the Tenax GC porous polymer headspace entrainment procedure because of their low volatility, but tentative mass spectral evidence was obtained for their presence in samples held 3 days at 6°C.

Although the products of 2,4-decadienal degradations by retro-aldol related reactions cannot be segregated from those resulting from classic secondary oxidations in the fried foods, the data (Table 1) support a view that some staling flavors arising during refrigerated storage can probably be attributed to retro-aldol related reactions. Aroma alterations were also observed in aqueous 2,4-decadienal model systems (pH 7.5) which initially exhibited rich, fresh-fried aroma that gave way to distinct stale-type aromas as the retro-aldol related degra-

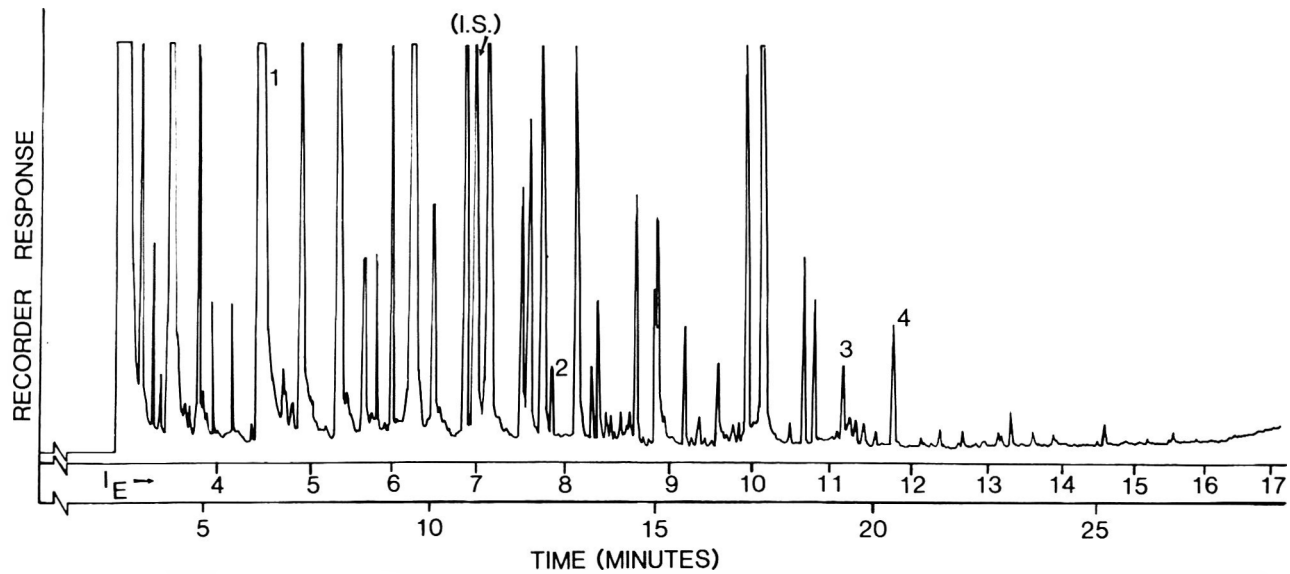

Fig. 5—Carbowax 20M fused silica capillary gas chromatographic separation of headspace volatiles from freshly deep-fat fried and battered chicken. 1 = hexanal, 2 = 2-octenal, 3 = t2, c4-decadienal, 4 = t2, t4-decadienal, (I.S.) = Internal Standard; ethyl heptanoate.

Table 1—Concentrations of selected volatile aldehydes in fresh and refrigerated deep-fat fried chicken and fish

	Chicken		Fish	
Compound	Fresh	3 days	Fresh	3 days
	μg kg (ppb)			
*t*2, *c*4-Decadienal	4	1	14	3
*t*2, *t*4-Decadienal	5	1	24	7
2-Octenal	3	44	5	26
Hexanal	798	2769	95	81

dations progressed.

Water-mediated degradation of unsaturated aldehydes likely also affect quality and stability of other chicken flavors, such as stewed chicken. A number of unsaturated long-chain aldehydes have been reported as important characterizing contributors to stewed chicken flavor (Harkes and Begemann, 1974), and these aldehydes arise from oxidations of arachidonic and linoleic acids. However, some of the aldehydes, including *t*2, *c*5-undecadienal and *c*4-decenal, possess unusual positions of unsaturation that are not readily rationalized from arachidonic and linoleic acids by classic oxidation mechanisms. In these instances, retro-aldol related degradations of primary lipid oxidation products can readily account for these aldehydes. For example, *t*2, *c*5-undecadienal can arise from *t*2, *c*4, *c*7-tridecatrienal through an alpha/beta double-bond hydration, retro-aldol degradation reaction sequence. *t*2, *c*5-Undecadienal could then serve as a precursor to *c*3-nonenal through similar reactions, and this compound was also identified in stewed chicken. Formation of *c*4-decenal by retro-aldol related conversions of *t*2,*c*6-dodecadienal has been confirmed in our laboratory (data not shown). The primary precursor products, *t*2, *c*4, *c*7-tridecatrienal and *t*2, *c*6-dodecadienal, were both reported in stewed chicken by Harkes and Begemann (1974), and are readily formed from the oxidation of arachidonic and linoleic acids, respectively.

In summary, data have been presented for the degradation of 2,4-decadienal in aqueous model systems held at neutral pH which extends knowledge of degradations of unsaturated aldehydes through alpha/beta double-bond hydration and retro-aldol condensation reactions. Additionally, evidence was obtained for novel hydration-induced compounds that are not products of retro-aldol reactions of 2,4-decadienal in aqueous systems. These products and reactions appear to be influential in altering fresh-like flavors to those perceived as stale in some foods, but they may also be responsible for the development of characterizing flavors in other foods.

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Frozen Storage Stability of Modified Pollock Theragra chalcogramma) Blocks Containing 15% or 30% Minced Pollock

J. K. BABBITT, K. D. REPPOND, AND A. HARDY

ABSTRACT

Pollock were removed from ice or refrigerated seawater after 2, 4 and 6 days and processed into fillet blocks or fillet blocks containing 15% or 30% minced flesh. Although all the samples were acceptable after 12 months, the addition of minced flesh and storage at −18°C affected texture and flavor. This was particularly evident for blocks containing 30% minced flesh, and results suggest the addition of minced flesh should be limited to 15%. Changes in dimethylamine and expressed thaw drip may explain the changes in sensory attributes since there were small changes in the control blocks held at −34°C.

INTRODUCTION

IN 1984, domestic processors of fishery products purchased over 300 million pounds of fillet and minced blocks mainly using cod and other members of the gadoid family such as Walleye pollock, *Theragra chalcogramma* (Thompson, 1985). Over 99% of these blocks were imported. Part of the difficulty facing domestic production of pollock blocks is minimizing production costs to compete with imported blocks. High speed filleting and skinning machines are available but even the best machines will produce a significant number of fillets with bones, patches of skin and other defects. An alternative to the labor intensive effort of manually removing the defects from these fillets is the use of mechanical flesh separators to produce a boneless, skinless mince that could be mixed with the fillets to form a modified block. Although minced flesh deteriorates much faster during frozen storage than intact fillets (Tokunaga, 1964; Babbitt et al., 1972), Licciardello et al. (1979) found that blocks of Atlantic cod (*Gadus morhua*) containing up to 30% mince were not distinguishable from standard fillet blocks during 60 weeks storage at -18°C. Using pollock that had been previously frozen, Babbitt et al. (1984) demonstrated that an acceptable block can be produced using chopped fillets with 20% mince but blocks with 50% mince were not acceptable. The purpose of this study was to determine the effects of incorporating minced fresh into blocks of fillets from pollock held in ice or refrigerated seawater (RSW).

MATERIALS & METHODS

Materials

Trawl caught pollock (1500 kg) were delivered to the laboratory within 2 hr after the fish were caught on the evening of August 9, 1984. Upon delivery, the pollock were either heavily iced in an insulated chest or placed in refrigerated seawater (RSW). The RSU system (3.5% NaCl; weight ratio—(2 fish:1 brine) was maintained at 0°C to −0.5°C during the experiment. The following morning, 250 kg of iced pollock were filleted. The fillets were skinned, rinsed, drained, and pin-bones removed using a V-cut. Minced fresh was prepared by passing the pin-bone trimmings through a Bibun mincer having 8mm drum orifices. The boneless fillets were sliced into 7.5 cm chunks to facilitate packing the blocks and mixed by hand with appropriate amounts of minced flesh to form modified blocks containing 0, 15, or 30% mince. These blocks (zero-time samples) were frozen overnight at −40°C in a Dole plate freezer, removed and overwrapped in 2 mil polybags, placed into 25 kg master cartons and then held at −18°C throughout the experiment. Several 8.4 kg blocks of fillets were also prepared and stored at −34°C during the experiment to serve as controls. At 2, 4 and 6 days of holding, 150 kg pollock were removed from the ice and RSW. Modified blocks containing 0, 15% and 30% minced flesh were prepared from fish from each system and treated in the same manner as the zero-time samples. Approximately 1 kg of fillets from the various treatments were frozen and held at −34°C for chemical analysis. These samples were analyzed within 2 wk of frozen storage.

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Chemical analyses

Samples for chemical analysis were tempered overnight at 4°C and ground through a 6 mm plate while partially frozen. Analysis for trimethylamine was performed by the method of Bullard and Collins (1980). Analyses for dimethylamine (Dowden, 1938) and trimethylamine oxide (Dyer et al., 1952) were made on the same 5% trichloroacetic acid (TCA) extract. At subsequent periods of frozen storage, the TCA extract was diluted with additional 5% TCA in the analysis for dimethylamine. Moisture and salt were determined by AOAC (1980) methods. Portions (2x2x7cm) cut from the blocks were used for the determination of expressed thaw drip by a centrifugal method described by Miyauchi (1962).

Sensory analyses

Breaded portions (12x45x77mm) were deep-fat fried from the frozen state at 177°C for 5 min. All portions were served hot to 10 panelists trained testing portions with excellent and poor quality. For the sensory evaluations, portions from fillet blocks held at -34°C were used as a control to relate the various handling procedures to the changes in sensory attributes during frozen storage at -18°C. Sensory evaluations were conducted after 30, 90, 220 and 375 days of frozen storage. At each evaluation, the labelled control sample was served as a reference. The control was evaluated first, then samples containing 0, 15 and 30% minced flesh were examined in that order yet coded so that panelists did not know the history of the samples. Panelists evaluated color, flavor, texture and juiciness on a 7-point scale with the control sample assigned a value of 4 for each attribute. A score greater than 4 indicated that the panelist thought the sample was whiter, had better flavor, had a tougher texture (more resistance to chewing) or was more moist than the control. A score less than 4 indicated that the panelist thought the sample was darker, had a poorer flavor, was softer or drier than the control. The panelists also judged the desirability of all samples including the control on a 9-point hedonic scale as follows: 9, very desirable; 5, neither like/dislike; 1, very undesirable. The scores of the panelists were subjected to analysis of variance, ANOVA and Duncan's muitiple range procedure (Sokal and Rohlf, 1969; Bowman and Cahill, 1975).

RESULTS & DISCUSSION

Yield

The pollock used in this study were in excellent condition when received and uniform in size averaging 768 ± 22 g. Recoveries of edible flesh were increased from 25% to 36% by incorporation of minced V-cut trimmings into modified pollock blocks.

Chemical changes

Changes in moisture, salt, trimethylamine (TMA), dimethylamine (DMA) and trimethylamine oxide (TMAO) of pollock held in ice and refrigerated seawater (RSW) are shown in Table 1. Salt remained largely unchanged for pollock in ice but increased steadily for fish held in RSW. TMA remained relatively unchanged to 4 days in ice and then rapidly increased. The increase in TMA was more rapid in fish held in RSW than that held in ice. Similar results have been reported by Nelson (1982) and Reppond et al. (1985). Although TMA was significantly correlated to undesirable flavor scores in other work (Kramer and Nordin, 1979; Reppond et al., 1985), the correlation was not significant in this experiment.

DMA of pollock held in ice or RSW remained relatively unchanged to 4 days but was higher at 6 days (Table 1). In a previous experiment (Reppond et al., 1985), DMA increased linearly with time of holding for fish in ice or RSW. Both TMA and DMA are formed from TMAO, and TMAO content decreased slightly during the 6 days of fresh holding.

During frozen storage, the formation of DMA is accompaned by equimolar amounts of formaldehyde (Tokunaga, 1964). The reaction of formaldehyde with muscle proteins is believed to result in a toughening of the flesh of the fish (Tokunaga, 1965; Castell et al., 1973). Changes in DMA of all the modified blocks were similar and Fig. 1 depicts the changes in DMA of modified blocks prepared from freshly caught (zero-time) pollock during frozen storage at −18°C. No changes in DMA were observed in the corresponding fillet blocks held at −34°C.

More DMA was formed in the blocks with minced flesh than in the fillet blocks during the freezing process but the subsequent accumulation of DMA during frozen storage at −18°C was similar in all the modified blocks. Although the results are not shown, the changes in initial expressed thaw drip values of 3.2–5.7 mL/20g to 7.3–7.4 mL/20g at 375 days were similar to DMA and were significantly correlated with DMA content in pollock from either the RSW (correlation coefficient, $r=0.836$, $P<0.01$) or iced ($r=0.874$, $P<0.01$) systems. Addition of 30% mince to the fillet block increased thaw drip values by 32% at 30 days of frozen storage at −18°C but was the same as the fillet block at 375 days.

Sensory attributes

All the sensory attributes of the portion from blocks prepared from freshly caught pollock or from pollock held for 2, 4 and 6 days in ice or RSW changed in a similar manner during frozen storage. Thus, only the results of the desirability of modified blocks held at −18°C prepared from freshly caught (zero-time) pollock (Fig. 2) and desirability and sensory attributes of modified blocks held at −18°C prepared frompollock held in ice and RSW for 6 days (Fig. 3-6) will be discussed.

The desirability of the control (fillet block held at −34°C) changed very little after one year of frozen storage (Fig. 2)

Table 1—Moisture, salt, trimethylamine (TMA), dimethylamine (DMA) and trimethylamine oxide (TMAO) content[a] of pollock fillets held in ice or refrigerated seawater (RSW)

Time of holding (Days)	Moisture %	Nacl %	TMA	DMA	TMAO
			----------MgN/100g----------		
ICE					
0	82.25	0.10	0.20	0.83	61.2
2	82.19	0.10	0.26	0.98	67.0
4	83.13	0.12	0.27	0.91	61.4
6	82.85	0.15	0.70	1.48	59.1
RSW					
2	81.73	0.27	0.23	1.04	62.1
4	81.41	0.47	1.24	1.09	60.7
6	82.01	0.60	4.28	2.20	55.1

[a] Mean of duplicate samples.

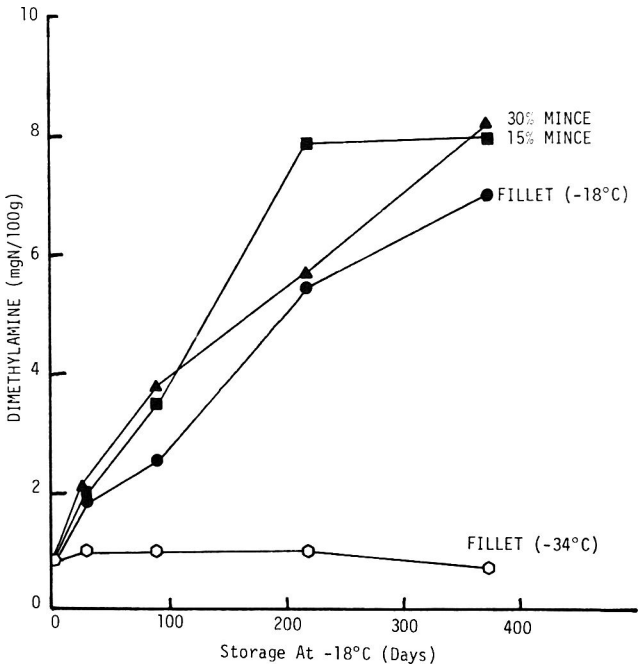

Fig. 1—Changes in dimethylamine during storage at −18°C of modified blocks prepared from freshly caught pollock.

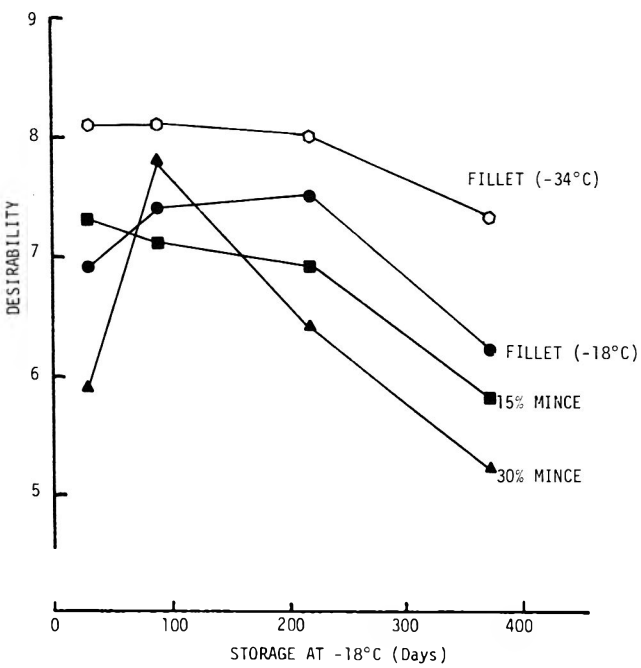

Fig. 2—Changes in desirability during storage at −18°C of modified blocks prepared from freshly caught pollock.

and this coupled with no change in DMA (Fig. 1) indicated that pollock flesh is very stable at low temperatures. Differences were detected in the modified blocks prepared from freshly caught pollock held at −18°C but these differences were small and not statistically ($P>0.05$) different (Fig. 2). Only the block containing 30% mince after 1 yr of frozen storage was statistically ($P<0.05$) different in desirability from the fillet block held at −34°C. The desirability value of 5.2 for the 30% mince treatment would still, however, be considered to be acceptable.

Flavor scores generally decreased as the holding time of pollock in ice and RSW increased (Reppond et al., 1987) and the flavor scores of all samples decreased during frozen storage (Fig. 3) . At 6 days, the accumulation of salt (Table 1) in the

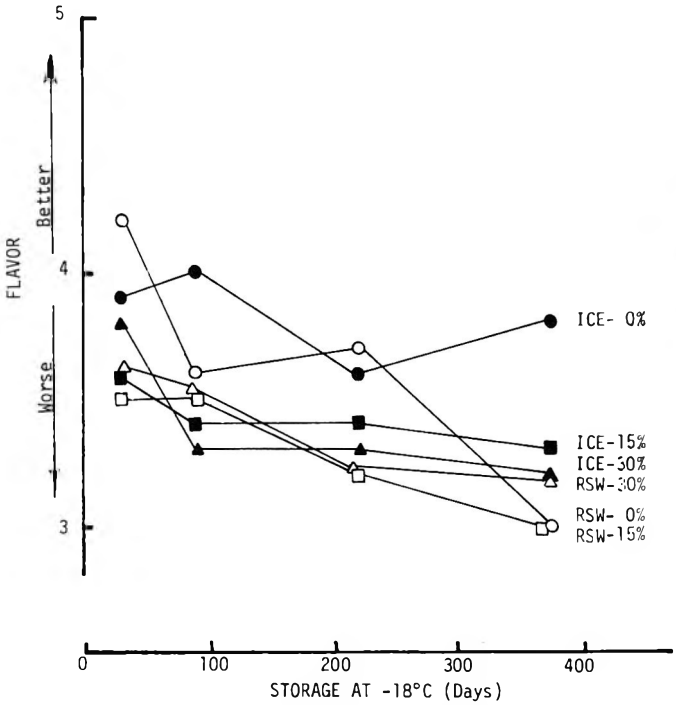

Fig. 3—Changes in flavor during frozen storage at -18°C of modified blocks containing 0, 15%, or 30% mince prepared from pollock held in ice or refrigerated seawater (RSW) for 6 days.

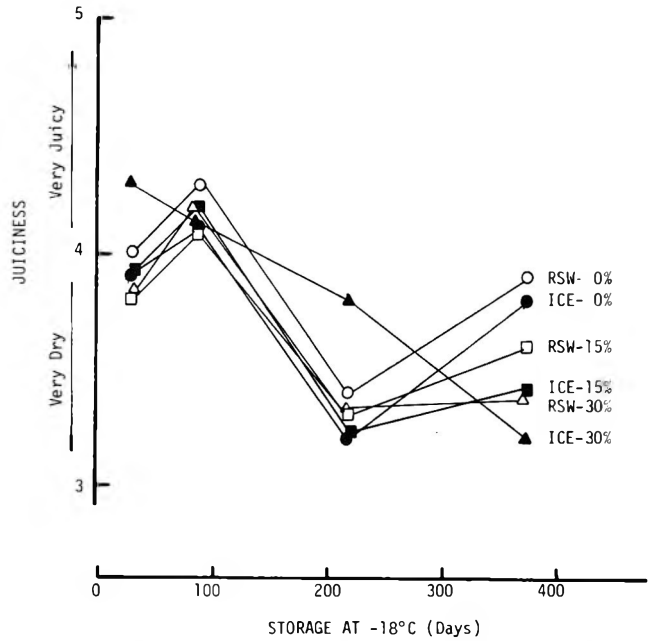

Fig. 5—Changes in juiciness during frozen storage at −18°C of modified blocks containing 0, 15%, or 30% mince prepared from pollock held in ice or refrigerated seawater (RSW) for 6 days.

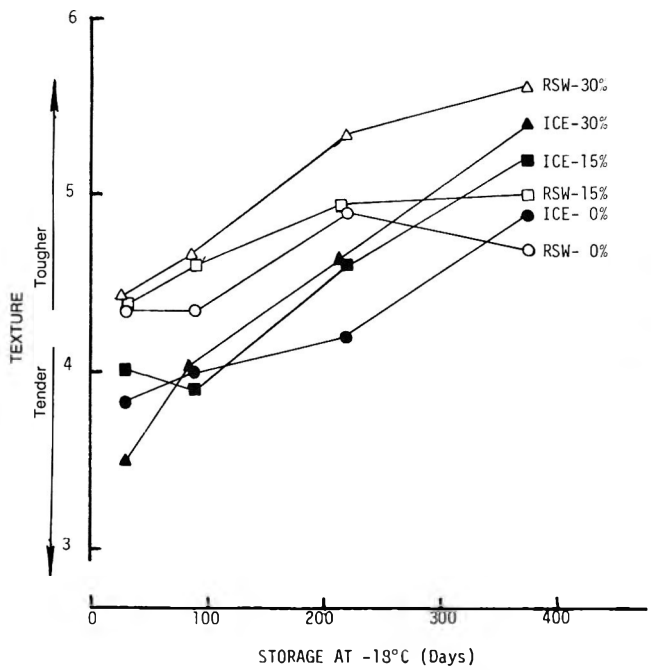

Fig. 4—Changes in texture during frozen storage at −18°C of modified blocks containing 0, 15%, or 30% mince prepared from pollock held in ice or refrigerated seawater (RSW) for 6 days.

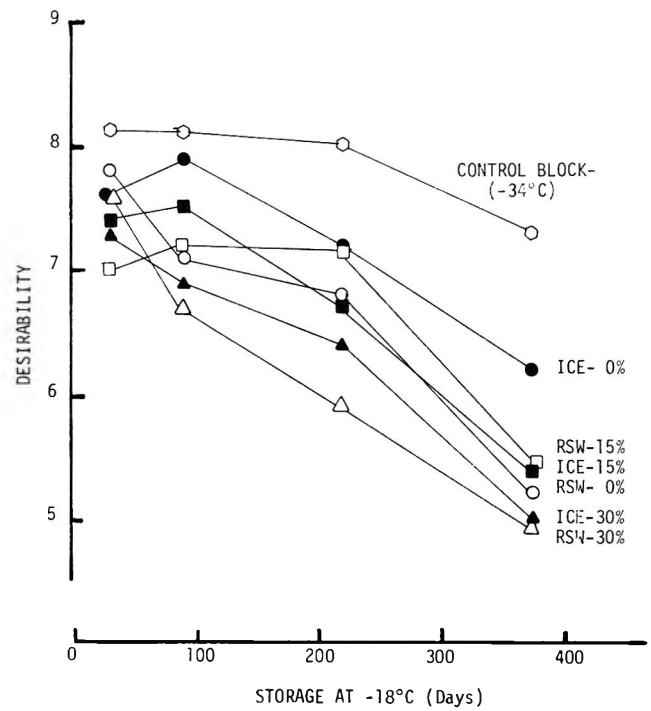

Fig. 6—Changes in desirability during frozen storage held at −18°C of modified blocks containing 0, 15%, or 30% mince prepared from pollock held in ice or refrigerated seawater (RSW) for 6 days.

pollock from the RSW system may explain the lower scores as most panelists detected the presence of salt.

Texture scores (Fig. 4) increased and juiciness scores (Fig. 5) decreased during frozen storage. The addition of minced flesh resulted in increased texture scores (toughness) and lowered juiciness scores for portions from fish held in ice but the changes were not statistically significant ($P>0.05$). For RSW samples, these changes were more pronounced. The significant correlation of flavor and texture scores with DMA content suggested that perhaps DMA may be a good indicator for monitoring undesirable changes that occur during frozen storage of pollock.

In general, desirability decreased as the time of holding in ice and RSW or as the frozen storage time at −18°C increased (Fig. 6). Addition of minced flesh tended to lower desirability scores and RSW samples tended to have slightly lower scores than iced samples. However, nearly all samples retained high desirability scores through 220 days at −18°C. Only the 6-day RSW sample with 30% mince at 375 days of frozen storage was rated (4.9) as only moderately acceptable. In addition,

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Detection of Microsomal Phospholipase Activity in Myotomal Tissue of Atlantic Cod (*Gadus morhua*)

PARVEEN CHAWLA and RICHARD F. ABLETT

ABSTRACT

Incubation of microsomes prepared from myotomal tissue of Atlantic cod yielded detectable phospholipase activity following incubation with 1,2-dipalmitoyl-9,10-[^{3}H]-phosphatidylcholine over a period of 24 hr. No significant incidence of nonenzymatic hydrolysis was found to accompany the determined phospholipase activity. Phospholipid hydrolysis increased linearly in the presence of increasing levels of microsomal protein (0–4 mg). Optimal phospholipase enzyme activity was determined following incubation at 20°C and maximal activity was determined in the presence of 0.1 nmole unlabelled lecithin substrate. The myotomal microsome preparation of Atlantic cod proved to be a suitable model for direct evaluation of phospholipase and perhaps might be useful in evaluation of frozen storage impact on the resultant integrity of the enzyme system.

INTRODUCTION

DURING FROZEN STORAGE, especially at marginal temperatures, fish muscle exhibits deteriorative changes in texture and flavor characteristics. These changes can be of great commercial importance, for they determine not only the storage life of the frozen seafood, but also the acceptance of the product by the consumer. The problems of deterioration vary among species but members of the lean-fleshed Gadoid family, including Atlantic cod (*Gadus morhua*), are particularly prone to problems associated with toughened texture. Among several pathways, it is recognized that deterioration of muscle texture during frozen storage can be attributed in part, to protein denaturative events in which myofibrillar proteins have been shown to undergo crosslinking interactions with accumulated free fatty acids (FFA). The evidence indicates FFA are derived from endogenous phopholipase activity which continues to hydrolyze phospholipid despite frozen storage temperatures (Sikorski et al., 1976).

The possibility of a relationship between the events associated with protein denaturation and lipid hydrolysis in frozen cod fillets was first postulated by Dyer and Fraser (1959). It was shown that the formation of FFA preceded loss of extractability of myofibrillar proteins. The mechanism underlying 'lipid-protein interactive' texture changes is now known to include attachment of FFA to appropriate binding sites on hydrophobic, polar or ionized fragments of polypeptide chains. In turn, this contributes to a decreased solubility of structural proteins (Sikorski et al., 1976).

Previous studies have established FFA accumulation in gadoid myotomal tissue to be attributed almost exclusively to endogenous phospholipase activity (Olley et al., 1962). The presence of these enzymes among different fish species has been recently reviewed and the evidence indicates the catalytic hydrolysis of phospholipids to be principally under the control of phospholipases A_1 and A_2 (Shewfelt, 1981). Although the impact of the phospholipase system on frozen storage deteriorative changes in cod muscle is recognized, it has never been demonstrated through direct determination methods.

Several previous studies among species which include winter flounder (Shewfelt et al., 1981) and rainbow trout (Neas and Hazel, 1984) have established the microsomal fraction of myotomal tissue to yield detectable phospholipase activity. Thus, it is anticipated this fraction would also provide a suitable model to directly investigate the activity of phospholipase in Atlantic cod muscle. A recent study established an isolation procedure for microsomes prepared from this tissue (Ablett et al., 1986). Therefore, the present study was undertaken to define the presence or absence of determinable phospholipase activity in a microsomal fraction prepared from fresh myotmal tissue of Atlantic cod. A primary objective was to assess the microsome model as the basis for further studies which would evaluate the impact of frozen storage on the integrity of the enzyme in Atlantic cod.

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METHODS & MATERIALS

Animals

Live Atlantic cod (*Gadus morhua*) weighing between 500–1500g were obtained from the marine facilities of the Federal Department of Fisheries and Oceans, Scotia Fundy Region Laboratory, Halifax. All fish were maintained in 2m diameter circular tanks with constant flowing seawater (10 L/min) and fed *ad libitum* with a diet consisting of chopped herring. Immediately prior to experimentation, the fish were transported live to the Canadian Institute of Fisheries Technology, Halifax in plastic tanks containing 10L sea water.

Isolation of microsomes

All fish were killed by a cranial blow and immediately bled by severance of the caudal vein. Subsequently, caudal flank epaxial musculature was exposed and samples removed onto ice by excision with a surgical blade. Fresh tissue samples were used immediately following excision.

Microsomes were prepared from myotomal tissue as previously described (Ablett et al., 1986). Samples (45g) of tissue were macerated with a safety blade in the presence of 3 mL 0.21M KCl; 5.0 mM histidine buffer (pH 7.3). Subsequently, the tissue was homogenized in the same buffer at a ratio of 1:4 (tissue:buffer, w/w) in an ice-cold beaker for 60 sec using a Brinkman Polytron tissue homogenizer. The homogenate was then centrifuged at 21,400 x *g* for 30 min in a Beckman SW 27 rotor. The supernatant was then further centrifuged at 105,000 x *g* for a period of 60 min to recover the crude microsomal fraction in the pellet. The pellet was suspended in 0.6M KCl; 5 mM histidine buffer (pH 7.3) to solubilize residual myofibrillar proteins. The final microsomal fraction was obtained by centrifugation at 105,000 x *g* for 60 min. The supernatent was discarded and the sediment resuspended in 10 mL 0.12M KCl; 5 mM histidine (pH 7.3) using three passes of a Potter Elvehjem tissue homogenizer.

Protein was determined according to the method of Lowry et al. (1951), and the suspended microsomal fraction as a source of phospholipase was further used to measure the activity of the enzyme system. Freshly prepared microsomes were retained at 4°C for a maximal period of 4 days prior to experimentation.

Determination of phospholipase activity

Phospholipase activity was determined according to the radiolytic method of Neas and Hazel (1985a). By this method, the recovery of

intact radiolabelled phosphatidylcholine (PC) and free fatty acid (FFA) was determined following incubation of 1,2-dipalmitoyl-9,10-[^{3}H]-phosphatidylcholine (New England Nuclear, Montreal, PQ) in the presence of the microsomal suspension. The assay medium was prepared by placing 0.1 microcurie of 1,2-dipalmitoyl-9,10-[^{3}H]-phosphatidylcholine in a test tube. Organic solvents were removed under nitrogen and following addition of 0.9 ml assay medium (0.1M Tris-acetate, 8 mM $CaCl_2$, 0.1% Triton X-100; pH 8.0 at 20°C), the mixture was sonicated for 2 min in a bath sonicator. All assays were initiated by the addition of an aliquot of microsomal protein (2.0 mg) in the appropriate resuspension buffer and incubated over a period of 0, 8 and 24 hr at 20°C. Assays were terminated by the addition of 2.5 mL methanol and 12.5 mL $CHCl_3$; samples were stored overnight at 4°C. Subsequently, 1.25 mL $CHCl_3$ and 1.25 mL H_2O were added and the samples centrifuged for 30 min at 2,500 x *g* (Bligh and Dyer, 1959). The lipid layer was removed and dried under nitrogen, washed twice with methanol and twice with $CHCl_3$ and dried under nitrogen. Lipid samples were dissolved in 100 µL $CHCl_3$:methanol (2:1 v/v) and spotted on silica gel TLC plates along with standards of phosphatidylcholine (PC), lysophosphatidylcholine (LPC) and fatty acid. Thin layer plates were developed in a solvent system of $CHCl_3$/methanol/acetic acid/H_2O (25:15:4:2 v/v) and lipid spots visualized under ultraviolet light following spraying with 2,7-dichlorofluorescein (0.2% by weight in 90% ethanol). Spots corresponding to PC and fatty acid were scraped directly into liquid scintillation vials and 0.5 mL Beckman Tissue Solubilizer was added. The mixture was incubated for 4 hr at 40°C, then 5 mL nonaqueous scintillation cocktail containing acetic acid (7 mL/L) was added. Counts per minute (CPM) were measured on a Beckman Model LS 1801 liquid scintillation counter and converted to disintegrations per minute (DPM) using an external standard and quench calibration curve.

Establishment of enzymatic and nonenzymatic hydrolysis

To determine the presence or absence of nonenzymatic hydrolysis, phospholipase activity was measured in microsomal fractions isolated from fresh myotomal tissue heated at 100°C for 5 min to induce thermal denaturation of enzyme activity. All assays were conducted in duplicate over an incubation period of 0, 8 and 24 hr at 20°C.

Incubation time and temperature

An investigation was conducted to evaluate the influence of incubation time and temperature on detectable phospholipase activity measured as described above. In this experiment, fresh microsomes and labelled PC substrate were incubated in duplicate at 4°C, 20°C, and 36°C for periods of 2, 4, 8, and 24 hr, respectively.

Effect of microsomal protein level on phospholipase activity

To investigate the influence of increasing amounts of protein, and hence enzyme availability, phospholipase activity was measured in the presence of 0, 1, 2 and 4 mg microsomal protein. All assays were conducted using the radiolytic method as described above, following incubation in duplicate for 0, 8, and 24 hr at 20°C. Corresponding blanks were run with similar levels of microsomal protein heated for 5 min at 100°C.

Effect of substrate concentration on phospholipase activity

To evaluate the influence of enhanced substrate availability, phospholipase activity was measured in the presence of 0.00175 nmoles radiolabelled PC with the addition of 0–10 nmoles additional unlabelled egg lecithin (Sigma Chemical Co., St. Louis, MO). All assays were conducted in duplicate over an incubation period of 24 hr at 20°C.

Statistics

Where appropriate, the differences between means were evaluated by "t-test" and "paired comparison t-test" (Steel and Torrie, 1980). Differences between mean values with $p<0.05$ were considered statistically significant.

RESULTS & DISCUSSION

THE RESULTS of the present study demonstrated the presence of an active phospholipase enzyme system in fresh myotomal microsomes of Atlantic cod. Figure 1 shows an overall decline ($p<0.01$) of labelled PC substrate over a 24 hr incubation period in comparison with corresponding boiled blank samples, which demonstrated no change ($p>0.05$) over the same period. A commensurate rise ($p<0.01$) in free fatty acids was observed over the 24 hr incubation period with no change ($p>0.05$) in the corresponding boiled blanks (Fig. 2). These data provided reasonable assurance that the integrity of both the microsome preparation and the assay medium was maintained throughout the period of incubation. The absence of non-enzymatic hydrolysis indicated that no particular problems associated with chemical degradation of the radiolabelled PC substrate were encountered under the conditions of the assay.

Figures 3 and 4 show that as the period and temperature of incubation were increased, there was a corresondingly greater

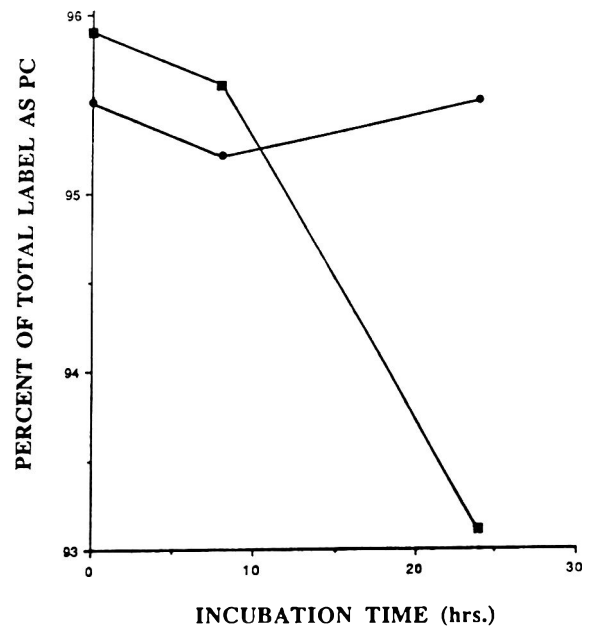

Fig. 1—Effect of phospholipase on dipalmitoyl-9,10-[^{3}H]-phosphatidylcholine substrate in (■) fresh and (●) boiled samples of myotomal microsomes.

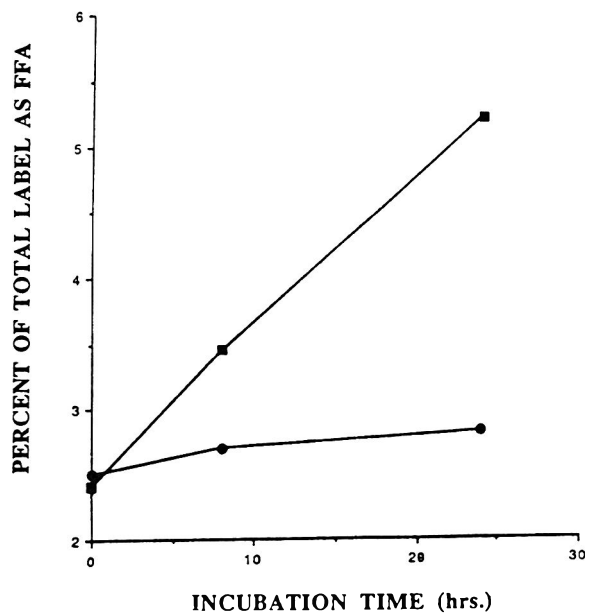

Fig. 2—Effect of phospholipase on FFA accumulation using dipalmitoyl-9,10-[^{3}H]-phosphatidylcholine substrate in (■) fresh, and (●) boiled samples of myotomal microsomes.

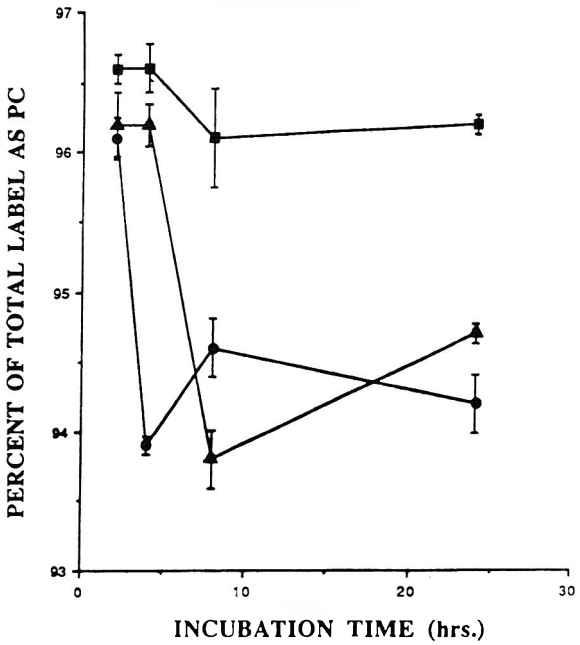

Fig. 3—Effect of incubation time on PC decline using dipalmitoyl-9,10-[³H]-phosphatidylcholine substrate in the presence of myotomal microsomes at 4° (■), 20° (●), and 36°C (▲).

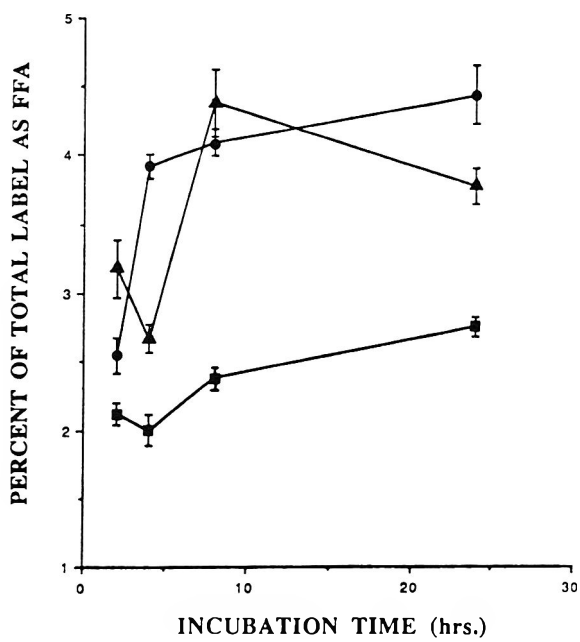

Fig. 4—Effect of incubation time on FFA accumulation using dipalmitoyl-9, 10-[³H]-phosphatidylcholine substrate in the presence of myotomal microsomes at 4° (■), 20° (●), and 36°C (▲).

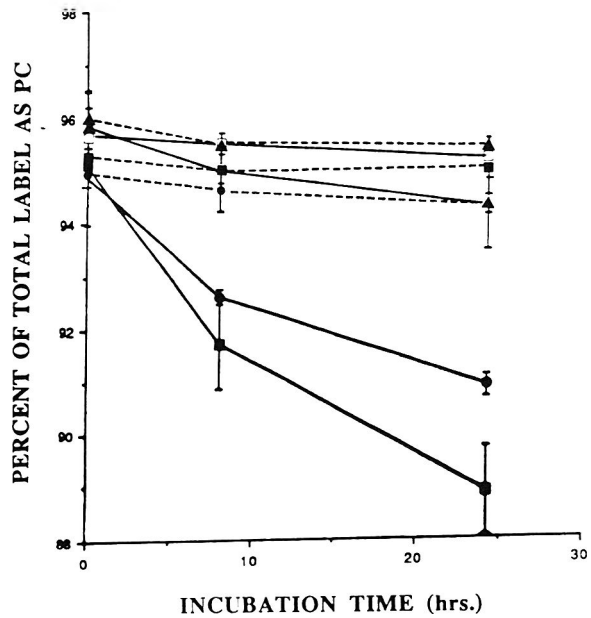

Fig. 5—Decline of PC using dipalmitoyl-9,10-[³H]-phosphatidylcholine substrate in the presence of 0 (□), 1 (▲), 2 (●), and 4 mg (■) microsomal protein and its corresponding blank samples.

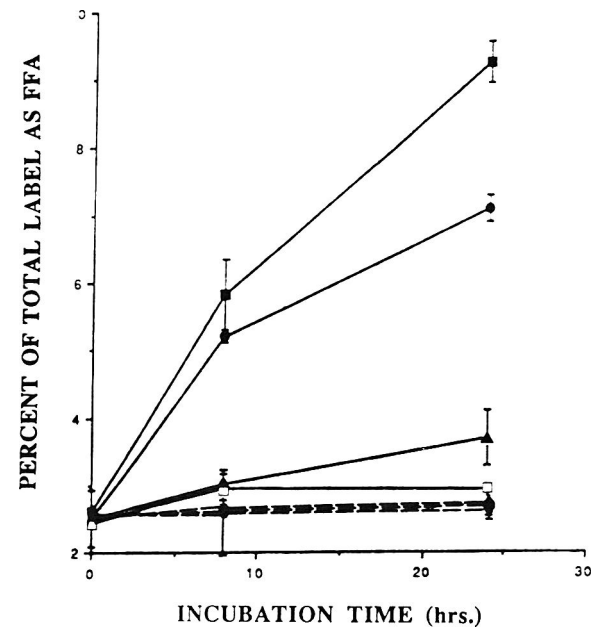

Fig. 6—Accumulation of FFA using dipalmitoyl-9,10-[³H]-phosphatidylcholine substrate in the presence of 0 (□), 1 ▲), 2 (●), and 4 mg (■) microsomal protein and its corresponding blank samples

decline of PC and increase of FFA production. In all instances, the rise in FFA between 0 and 24 hr was significant at 4°C ($p<0.05$), 20°C ($p<0.05$) and 37°C ($p<0.05$). Under the present conditions of the assay, maximal hydrolytic activity was determined following incubation at 20°C (Fig. 4) and this was consistent with the findings of previous authors investigating hepatic microsomal phospholipase in rainbow trout (Neas and Hazel, 1985 a,b).

Following the addition of variable amounts of microsomal protein, it was observed that as the amount of protein was increased from 0 to 4 mg/ml, a greater decline ($p<0.05$) of PC (Fig. 5) and corresponding increase of FFA (Fig. 6) occurred, thereby showing enhanced phospholipase activity. The slope of percent increase of FFA was found to be 0.048 ($p<0.05$), 0.780 ($p<0.01$) and 0.268 ($p<0.01$) per hr following addition of 1,2 and 4 mg microsomal protein, respectively. Correspondingly, the slope of percent increase of FFA for the boiled blank samples was 0.007, 0.001 and 0.004 per hr and this was considered to be negligible. A positive correlation (0.97) was determined between change in slope of percent increase of FFA per hr and increase in protein concentration (Fig. 6). In accordance with similar findings by neas and Hazel (1985[a]), these data strongly support the enzymatic nature of the determined phospholipid hydrolysis in the present study.

Table 1 shows the results obtained following the addition of variable amounts of unlabelled egg lecithin substrate in the presence of a standard quantity (0.00175 nmoles) of labelled

Table 1—Phospholipase activity using dipalmitoyl-9,10-[^{3}H]-phosphatidylcholine substrate in the presence of unlabelled lecithin

Unlabelled lecithin (nmoles)	Percentage of total label	
	PC	FFA
0.00	93.6 ± 0.1[a]	5.6 ± 0.0[a]
0.01	93.0 ± 0.1[a]	6.0 ± 0.2[a]
0.10	91.1 ± 0.2[b]	7.6 ± 0.4[b]
1.00	93.0 ± 0.1[a]	5.9 ± 0.1[a]
10.00	93.8 ± 0.1[a]	5.5 ± 0.0[a]

[a,b] Values in columns with the same subscript are not different ($p>0.05$). Values represent the mean ± standard deviation of triplicate assays expressed as percentages of total label recovered.

PC. Over an incubation period of 24 hr, greatest PC hydrolysis ($p<0.05$) with a commensurate rise in FFA ($p<0.05$) was observed in the presence of 0.1 nmoles unlabelled lecithin substrate. Hydrolytic activity was reduced ($p<0.05$) when unlabelled lecithin was present in quantities in excess ($\geq$ 10 nmoles) or less ($\leq$ 0.00175 nmoles) than 0.1 nmoles.

As evidenced by the sensitivity of phospholipase to enhanced protein loading (Fig. 5 and 6), the enzyme system was obviously responsive to the present conditions of the assay. However, several questions remain as to the efficiency of conditions for detection of the identified enzyme system. For example, it was felt that the overall detectable activity, expressed as a percent of total label hydrolysis, was relatively low under the present assay method. Possibly some limitations associated with substrate specificity might account for the limited levels of determined activity. Although beyond the scope of the present study, it is suspected the endogenous microsomal phopholipase may eventually prove more responsive in the presence of more substrate specific SN-1 and SN-2 acyl moieties. Previous evidence in hepatic microsomes of rainbow trout, reveal substantial phospholipase activity was detected following incubation with 1-acyl-2-[^{3}H]-oleoyl phosphatidylcholine (Neas and Hazel, 1985[a]). As with mammalian phospholipases (Newkirk and Waite, 1971; Waldman et al., 1984), the specificity of the enzyme system among fish species appears most responsive to the presence of long chain polyunsaturated-SN-2-acyl moieties (Shewfelt, 1981). In this preliminary study, no attempt was made to separate the observed phospholipid hydrolysis as specific phospholipases A_1 or A_2 activity. The use of 1,2-dipalmitoyl-9,10-phosphatidylcholine as the substrate probe precluded clear dissemination of hydrolysis attributed to each specific enzyme. An investigation to separate the specific components of detectable phospholipase A_1 and A_2 activity and the optimization of substrate specificities is presently underway in our laboratory.

Some questions also remain as to the suitability of the microsome preparation as an optimal source of phospholipae activity in cod muscle. In an early study, phospholipase activity was demonstrated in the tissue homogenate derived from skeletal muscle (Bilinski and Jonas, 1966a) and lateral line muscle of rainbow trout (Jonas and Bilinski, 1967). Specifically, these authors determined that greater enzyme activity was present in the lateral line dark muscle compared with skeletal muscle tissue. Moreover, it was reported the activity of phospholipase in the microsomal fraction was four to six times higher than that of a comparable mitochondrial suspension (Bilinski and Jonas, 1966b). More recently, phospholipase enzymes have been detected in myotomal tissue of flounder (Shewfelt et al., 1981) and Atlantic pollock (Audley et al., 1978). In isolation and characterization studies, it has been determined that phospholipase of Atlantic pollock showed optimal activity in a temperature range of 37–42°C and in the alkaline pH range 8.5–9.0 (Audley et al., 1978). The molecular weight was established to be 13,700 daltons which was comparable to that reported among mammalian studies. Calcium was found to stimulate the enzyme whereas other divalent ions (Fe^{2+}, Mg^{2+}) and the chelating agent, ethylenediaminetetraacetic acid (EDTA) inhibited phospholipase activity. Attempts to clarify and establish an optimal assay medium for the phospholipase system of Atlantic cod muscle are presently under further investigation.

In a future study, it is planned to use the present microsome model to probe the determinable activity of phospholipase following storage of intact myotomal tissue under several subzero temperature regimes. Using this approach, it is anticipated the physicochemical impact of the frozen state environment on the resulting detectable activity of phospholipase can be directly evaluated.

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Ms received 12/15/86; revised 2/27/87; accepted 2/27/87.

This work was supported by an Operating grant (RFA) from the Natural Science and Engineering Research Council, Canada. The authors thank Ms. Roberta Norton-McKay for preparation of the manuscript.

A Comparison of Dogfish and Bovine Chymotrypsins in Relation to Protein Hydrolysis

MALLUR RAMAKRISHNA, HERBERT O. HULTIN, and MOKHTAR T. ATALLAH

ABSTRACT

An extracellular protease, chymotrypsin, from a low temperature-adapted poikilotherm (dogfish) was compared with the bovine enzyme against several protein substrates to determine potential advantages in the use of an enzyme adapted to functioning in a low temperature environment. In general, the dogfish enzyme showed a higher rate of reaction and hydrolyzed more peptide bonds than did the bovine enzyme whether the substrates were soluble or insoluble proteins. Dogfish chymotrypsin was slow to produce clotting of milk, but once formed the clot had good stability. The bovine enzyme was more efficient in incorporating methionine into soy protein in a plastein-type reaction.

INTRODUCTION

MANY AREAS of our planet are exposed to low temperatures, and organisms living in these regions have found different ways to adjust to these conditions. Although many organisms become less active at low temperatures, there are large numbers which function well under these conditions. A primary example of these are animals from the ocean where 95% of the environment has a temperature below 5°C throughout the year. For organisms to successfully survive under these conditions it is clear that their enzymes must be capable of functioning well at low temperatures. There have been many strategies by which enzymes from organisms adapted to living at low temperatures (hereafter referred to as low temperature enzymes) have adapted to these conditions (Hultin, 1980). They include changes in Michaelis constant, molecular activity, activation energy, cold stability, sensitivity to modulators and specificity. Most of the the studies related to the technique by which low temperature enzymes have adapted to their environment have dealt with intracellular enzymes, principally those concerning carbohydrate metabolism. Limited substrate concentrations, limited space and limited water content of the intracellular space are all factors which probably affect how the enzyme might adapt but should not be factors for extracellular digestive, hydrolytic enzymes.

There has been only limited work dealing with low temperature proteases. Wiggs (1974) observed with partially purified preparations that the overall apparent K_M for hemoglobin for a crude protease preparation was higher for fish caught in the summer than for those caught in the winter indicating an adaptation by the fish to make the enzymes more efficient in the wintertime. Ooshiro (1968) showed that mackerel pyloric caecal proteases hydrolyzed 70% of the peptide bonds in casein compared to 15% by bovine chymotrypsin. Simpson and Haard (1984a, b) found that a purified cod trypsin had higher activity, higher optimal pH and a lower activation energy than the comparable bovine enzyme although the fish enzyme was less stable than the bovine. They suggested that this might be caused by the low number of cysteine residues (8) in the molecule making the cysteine maximal number of disulfide bonds it was capable of forming (4) less than the 6 found in bovine trypsin.

Recently, dogfish and bovine chymotrypsins were shown to differ in several activation parameters (Racicot, 1984). These differences are expressed primarily as compensatory enthalpy/entropy changes since overall free energy changes were comparable in the reactions catalyzed by the two enzymes. These thermodynamic parameters, however, are expressed as relatively small effects in the action of the two enzymes on several small, low molecular weight artificial substrates. The purpose of the work described here was to extend these studies to more natural substrates of chymotrypsin, proteins, in making comparisons between the dogfish and bovine enzymes. Chymotrypsin was chosen for these studies since it was desired to work with a well characterized enzyme.

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MATERIALS & METHODS

Materials

Bovine chymotrypsin type II, N-benzoyl-L-tyrosine ethyl ester (BTEE), bovine serum albumin (BSA) prepared from Fraction V Albumin, and collagen type I, insoluble from bovine achilles tendon, were purchased from Sigma Chemical Co., St. Louis, MO. The casein used was ANRC reference protein (Humko Sheffield Chemical, Krafto Corp., Madison, WI). Soy Protein Isolate-Ardex R was a gift from Archer Daniels Midland Co., Decatur, IL and corn gluten meal was a gift from Corn Products, Argo, IL. All other chemicals were the purest available commercially.

Isolation of dogfish chymotrypsin

Dogfish pancreases were a gift of North Atlantic Products, Inc. of Rockland, ME. The pancreases were obtained from one- to two-day postmortem iced spiny dogfish (*Squalus acanthias*) and were transported on dry ice to the laboratory in Gloucester, MA and stored at −80°C until used. The purification procedure was that of Prahl and Neurath (1966) with modifications described by us (Ramakrishna et al., 1987). The purified preparation showed a single band on SDS-polyacrylamide gel electrophoresis.

Standard assay for chymotrypsin activity

A standard procedure was carried out so that comparable concentrations of the two enzymes could be added in the various experiments. Hydrolysis of BTEE was followed at 256 nm in a Perkin-Elmer Model 556 spectrophotometer coupled to a Hewlett-Packard Model 7045A X-Y recorder. Temperature was controlled at 23°C with a Lotemptrol 154 refrigerated/heated bath (Precision Scientific, Chicago, IL). After allowing substrate and buffer to come to temperature separately, 1.5 mL of 1.07 mM BTEE in 50% methanol (v/v) and 1.4 mL of 0.1M Tris buffer, pH 7.8, were mixed. The reaction was then started with the addition of 0.1 mL chymotrypsin solution. In the reference cuvette, the enzyme solution was replaced with buffer. The increase in absorbance at 256 nm was followed for 2 to 5 min, and the slope of the initial linear portion of the curve was used to determine activity. An extinction coefficient (ϵ) equal to 964 $cm^{-1}M^{-1}$ was used. Protein concentrations of the enzyme solutions were determined by the method of Lowry et al. (1951). A unit of activity was defined as the amount of enzyme that hydrolyzed 1 μmol of substrate per min at 25°C.

Protein hydrolysis by chymotrypsin

Proteins were dissolved or suspended in 100 mM sodium borate buffer at pH 8. Enzyme was added and incubation was carried out as indicated below. At various periods of time, 1.0 mL aliquots from the reaction mixture were pipetted into tubes containing 0.2 mL 20% trichloroacetic acid to stop the enzymic reaction and precipitate the protein. The suspension was neutralized with KOH, the volume made to 5.0 mL, and then centrifuged to remove the precipitated protein and trichloroacetate. From the supernatant fraction, a 0.1 ml aliquot was taken and amino nitrogen was determined by the ninhydrin procedure (Rosen, 1957). Substrate concentrations, total volumes and units of enzyme added were comparable between the enzymes for each experiment. Details are noted in the Results and Discussion section.

The insoluble protein fraction of fish muscle was prepared as follows: 35g red hake (*Urophycis chuss*) were homogenized in 50 mL 0.15M KCl for 2 min in a blender and centrifuged in a tabletop centrifuge (Clinical Centrifuge, International Equipment Co., Needham Heights, MA) at maximum speed. The sediment was extracted 5 times with 0.15 M KCl solution to remove soluble proteins. The washed insoluble fraction was suspended in 100 mM borate buffer, pH 8, at a concentration of 8.3 mg protein per mL.

The theoretical percentage hydrolysis of protein that took place in each experiment was estimated by determining the total amount of protein used (Lowry et al. 1951), estimating the total number of amino acid residues in that amount of protein by assuming an average molecular weight for each residue of 132, and comparing this with the number of moles of amino groups that were liberated as determined by the ninhydrin reaction. Leucine was used as the standard for this determination.

The effect of dogfish and bovine chymotrypsins on milk clotting was determined by adding 7.5 units enzyme to 5 mL whole pasteurized milk purchased at a local store, incubating for a given period of time, and then measuring the time necessary for the milk to drain from a standardized 1 mL pipette.

Chymotrypsin catalysis of the plastein reaction

The following were mixed together in a paste: 1.16g of soy protein isolate, 100 mg $NaHCO_3$, 30 mg Na_2CO_3, 14.1 mg L-methionine ethyl ester and 35 units enzyme in a total of 3.75 mL water. Samples were taken at 0 and 60 hr after incubation at 37°C in an evacuated desiccator. Five gram samples were mixed with 20 mL ethanol and centrifuged. The precipitate was washed twice with 80% ethanol, dried and powdered. Fifty milligrams precipitate were mixed with 0.5 mL water and 1.0 mL 6N HCl in sealed tubes and heated at 110°C for 16 hr. The acid was removed by repeated evaporation, and the sample was made up to 5 mL. This sample was analyzed for amino acids by the procedure of Moore and Stein (1963).

RESULTS & DISCUSSION

THE PREPARATION PROCEDURE used in this work for producing dogfish chymotrypsin A was essentially that of Prahl and Neurath (1966) with some modifications that primarily take into account modern developments in separation technology (Ramakrishna et al., 1987). DEAE-Sephadex and CM-Sephadex were substituted for the cellulose powders originally used and the final step involved an affinity chromatographic column of CH-Sepharose-4B-D-tryptophan methyl ester, a ligand with a high specificity for chymotrypsin. The preparation of the chymotrypsin A appeared as a single band on SDS-polyacrylamide gel electrophoresis. In addition, the preparation of dogfish chymotrypsin A had similar responses to the inhibitors indole, indole propionic acid, β-naphthyl acetate and β-naphthyl propionate against two substrates, p-nitrophenyl acetate and BTEE, as did the bovine enzyme (Ramakrishna et al., 1987). Based on these characteristics of the enzyme and the essentially identical free energies of activation of the dogfish and bovine chymotrypsins against a series of p-nitrophenyl esters (Racicot, 1984), it is concluded that this preparation represents a true chymotrypsin and is most likely not contaminated with the other digestive proteases.

To carry out the experiments described in this report, the amount of the two enzymes added in each reaction mixture was adjusted to be equal by determining the activity of the enzymes against BTEE at pH 7.8 in a medium containing 25% methanol. It was later found, however, that the activity observed with this substrate for the two enzymes was dependent on both the polar solvent which was used to help dissolve the substrate as well as its concentration. As had been found with two other small molecular weight substrates by Prahl and Neurath (1966), dogfish chymotrypsin A was relatively more sensitive to methanol than was bovine chymotrypsin. Thus, we realized we did not have an accurate measure of the comparable activities of the two preparations. A superior way of determine the active enzyme concentration of serine proteases is to titrate the active site. We had been unsuccessful in using this technique originally because the published procedure using cinnamoyl imidazole (Schonbaum et al., 1961) did not give reasonable results with the fish enzyme. This was probably caused by the fact that there was significant hydrolysis of this compound by the fish enzyme. This problem was eventually solved by performing the active site titration with p-nitrophenyl acetate (Racicot, 1984). It was determined that matching activities against BTEE resulted in using three times as much active bovine chymotrypsin as dogfish chymotrypsin A.

The greater activity of the dogfish chymotrypsin compared to the bovine enzyme vs a 1% solution of bovine serum albumin (BSA) is shown in Fig. 1. In addition to its greater rate of catalysis, the fish enzyme was still causing the reaction to proceed at 4 hr while it appeared that the bovine chymotrypsin-catalyzed reaction was over at approximately 2 hr. On the basis of active enzyme, fish chymotrypsin was almost five times as active against BSA as was the bovine enzyme. The leveling off of the reaction catalyzed by the bovine enzyme would presumably not be dependent on the amount of the enzyme used. The theoretical % hydrolysis at the end of 4 hr was 4.2 for the bovine enzyme and 11.5 for the fish.

Dogfish chymotrypsin had a greater activity towards soy protein isolate at three temperatures (Fig. 2.) This was true during the initial phase of the reaction and in the second stage, after the initial rapid rate. Whereas the rate with the bovine enzyme slowed down significantly after an initial rapid burst, this decrease was less pronounced with the fish enzyme. The theoretical hydrolysis with bovine chymotrypsin after 20 hr at 35°C was 5.2%, while it was 18.2% under the same conditions with the fish enzyme.

With casein as substrate at 37°C, dogfish chymotrypsin again showed greater activity than did the bovine enzyme (Fig. 3). It appeared that the fish enzyme hydrolyzed casein in a series of at least four steps, each one characterized by a lag phase

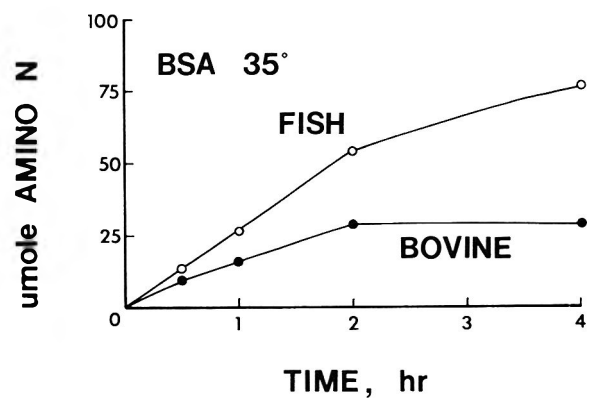

Fig. 1. Hydrolysis of bovine serum albumin (BSA) by dogfish and bovine chymotrypsins. The concentration of BSA was 1% and 15.6 units of enzyme were used in a total volume of 12 mL at 35°C. The ordinate shows total number of umole amino groups produced in the total volume. Percent theoretical hydrolysis was 11.5% for the reaction catalyzed by dogfish chymotrypsin and 4.2% for that catalyzed by bovine chymotrypsin after 4 hr.

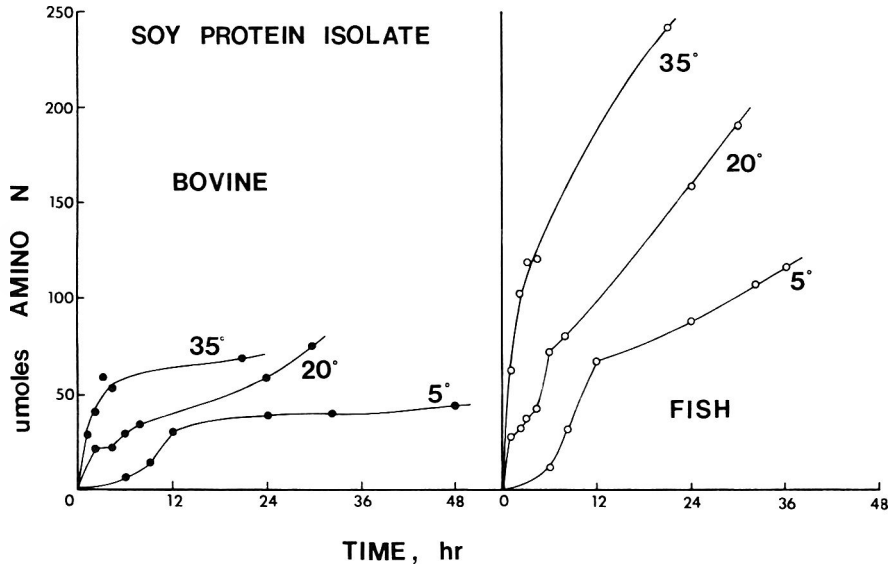

Fig. 2. Hydrolysis of soy protein isolate by dogfish and bovine chymotrypsins at three different temperatures. A 2% suspension of soy protein isolate was used with 31.2 units of enzyme in a total volume of 12 mL. The ordinate shows total number of umole amino groups produced in the total volume. Percent theoretical hydrolysis after 20 hr at 35°C was 5.2% with the bovine enzyme and 18.2% with dogfish chymotrypsin.

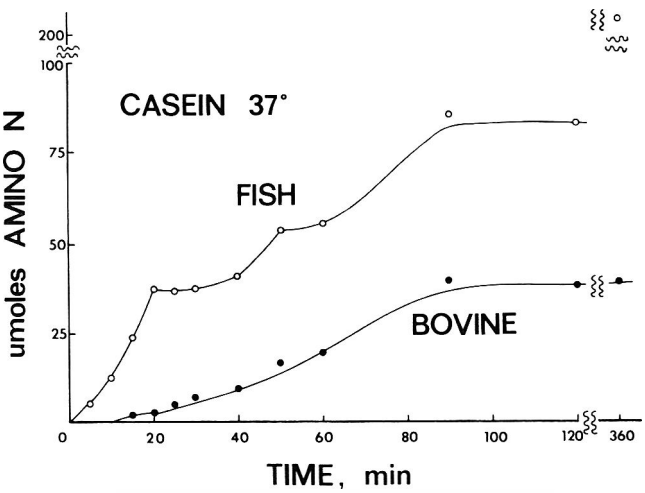

Fig. 3. Hydrolysis of casein by dogfish and bovine chymotrypsins. The concentration of casein was 1% and there were 15.6 units of chymotrypsin in a total volume of 15 mL at 37°C. The ordinate shows total number of umole amino groups produced in the total volume. Percent theoretical hydrolysis at the end of 6 hr was 3.8% with the bovine enzyme and 20.5% with the fish enzyme.

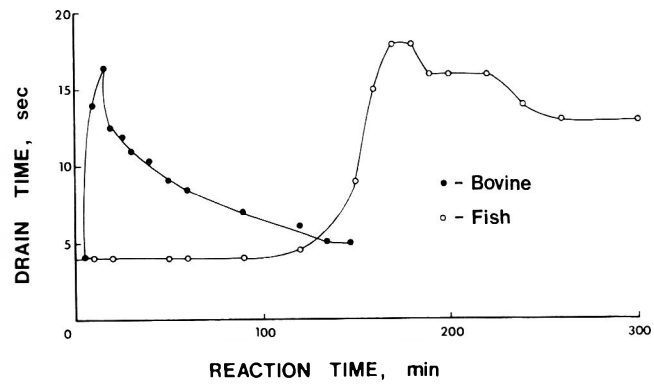

Fig. 4. Milk clotting activity of dogfish and bovine chymotrypsins. To 5 mL milk were added 7.5 units of either dogfish or bovine chymotrypsin. The time required for the milk to drain from a narrow, standardized 1 mL pipette was measured.

followed by a more rapid rate of activity. It is of interest that the first "step" catalyzed by the fish enzyme was quantitatively almost exactly equal to the one step observed with the bovine enzyme in terms of total substrate hydrolyzed. The reaction catalyzed by the fish enzyme reached this plateau much more rapidly than did the reaction catalyzed by the bovine protease, i.e., 20 min vs about 90 min. The data could be explained by a process whereby the fish enzyme attacked a certain number of exposed bonds and then caused more unfolding of the protein exposing additional bonds which were attacked, through four stages. The theoretical hydrolysis at the end of 6 hr was 3.8% for the reaction catalyzed by the bovine enzyme and 20.5% for that catalyzed by the fish enzyme.

When the action of the two enzymes on the rate of milk-clotting at 23°C was compared (Fig. 4), unusual behavior was observed with the fish enzyme. The time course of the reaction with bovine chyomtryspin was what has been typically observed with this and related enzymes (Dalgleish, 1982). There is a very rapid clotting of the milk followed by a quick breakdown of the clot. This is likely due to hydrolysis of the κ-casein at a point near the sensitive amino acid residues followed by non-specific hydrolysis leading to dissolution of the clot. The formation of the clot with the fish enzyme took a much longer time, i.e., close to 3 hr, but produced a relatively stable clot.

Yoshinaka et al. (1978) reported collagenolytic activity in various digestive organs of 19 species of teleosts and suggested that the enzyme activity originates from the pancreas. It has also been reported that there is a tryspin-like enzyme in the fiddler crab (*Uca pugilator*) that has collagenolytic activity (Welgus et al., 1982; Grant et al.,1983; Welgus and Grant, 1983). Both bovine and fish chymotryspin have activity against insoluble bovine tendon collagen with the fish enzyme displaying greater activity than the bovine enzyme (Fig. 5). There was an apparent levelling off of the bovine enzyme after approximately 4 hr and a theoretical hydrolysis of 2.9%. With the fish enzyme, the reaction was still proceeding after at least 8 hr with 6.2% of the bonds hydrolyzed at that time.

Since the chymotryspin from dogfish would be used to digest the proteins of fish ingested by the shark, the relative activities of dogfish and bovine chymotryspin on an insoluble fraction prepared from fish muscle at both 37°C (Fig. 6) and 4°C (Fig. 7) were examined. Somewhat unusual behavior was observed in that initially the bovine enzyme produced more hydrolysis than the fish enzyme; however, there was almost

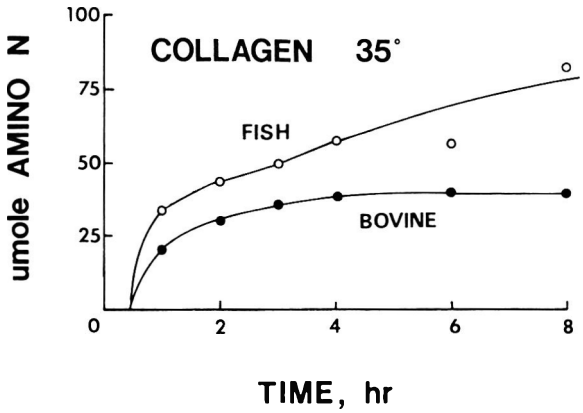

Fig. 5. Hydrolysis of insoluble collagen by dogfish and bovine chymotrypsins. A 2% suspension of collagen in a volume of 15 mL was used to which were added 15.6 units of enzyme at 35°C. The ordinate shows total number of umole amino groups produced in the total volume. After 8 hr incubation, the theoretical hydrolysis was 2.9% for the bovine enzyme and 6.2% for the fish.

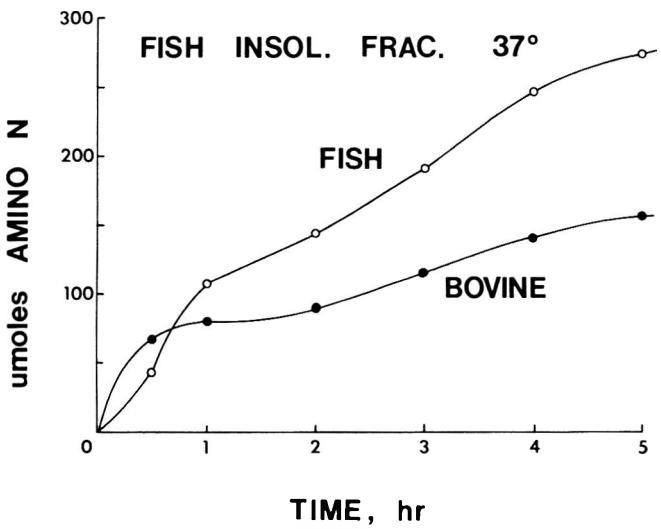

Fig. 6. Hydrolysis of an insoluble fraction of fish muscle by dogfish and bovine chymotrypsins at 37°C. To a suspension containing 8.3 mg protein per mL in a total volume of 15 mL trypsin were added 15.6 units of either dogfish or bovine chymotrypsin at 37°C. The ordinate shows total number of umole amino groups produced in the total volume.

three times as much active bovine enzyme present as active fish enzyme. Later the amount of substrate hydrolyzed by the fish enzyme was greater than that by the bovine enzyme. After 5 hr at 37°C, the reaction catalyzed by both enzymes was still proceeding. The rates of both enzymes at 4°C were much lower than they were at 37°C. Based on extent of hydrolysis, it can be seen that the reaction rate patterns were very similar at the two temperatures. What occurred at 4°C over a 24 hr period was similar to what occurred at 37°C in less than 1 hr qualitatively and quantitatively.

Corn gluten meal, which is a by-product of the corn oil processing industry, is a granular, intractable material with very little solubility in water. Bovine chymotryspin had very little effect on this substrate over a 48 hr incubation period (Table 1) producing only 0.5% theoretical hydrolysis in that time period. On the other hand, under comparable conditions, the fish enzyme produced 9.3% theoretical hydrolysis. The reaction with the fish enzyme was completed by 40 hr.

Incubating 1.16g of soy protein isolate with 14.1 mg of the

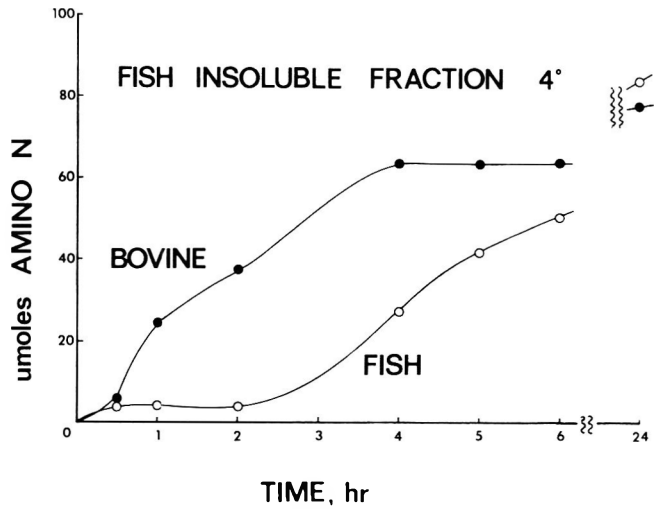

Fig. 7. Hydrolysis of an insoluble fraction of fish muscle by dogfish and bovine chymotrypsins at 4°C. Other conditions were as described in Fig. 6

Table 1—Hydrolysis of corn gluten meal[a] by dogfish and bovine chymotrypsins[b]

Time, hr	Total μmoles of amino nitrogen released	
	Dogfish	Bovine
3	5	0
4	13	0
16	84	7
25	93	7
40	126	7
48	124	7

[a] 2% corn gluten meal was used with 15.6 units of chymotrypsin in a total volume of 12 mL at 35°C.

[b] % theoretical hydrolysis was 9.3% for the reaction catalyzed by dogfish chymotrypsin and 0.5% for that catalyzed by the bovine enzyme.

ethyl ester of L-methionine and 35 units of chymotryspin for 60 hr at 37°C resulted in a net incorporation of 170 nmol of methionine per mg protein in the presence of the fish enzyme and 240 nmol of methionine per mg protein with bovine chymotryspin. The soy protein isolate had an initial methionine concentration of 50 nmol per mg protein. Thus, both chymotryspins were capable of incorporating the methionine ester into the soy protein isolate, but the bovine enzyme caused greater incorporation than the fish enzyme. Both soluble and immobilized chymotryspins have been used for plastein production (Tanimoto et al., 1972; Yamashita et al., 1976; Pallavicini et al., 1983). Noar and Shipe (1984) showed that methionine incorporation into soy protein involved primarily noncovalent bonding of the methionone to the apolar regions of the protein although there was also some evidence for covalent binding.

It is clear from the above results that dogfish chymotryspin has very different properties than the bovine enzyme against a variety of protein substrates. In other work (Racicot, 1984) using p-nitrophenyl esters of varying size and hydrophobicity, the association and acylation and deacylation rate constants were determined for the two enzymes as well as the corresponding thermodynamic activation parameters. The results showed that although the free energies of activation for both enzymic reactions were similar, individual parameters such as activation enthalpy and activation entropy varied considerably, indicating different reaction mechanisms. In addition, the absolute values of the association constants and the acylation and deacylation rate constants were different for the two chymotryspins, and the extent of the difference depended on the substrate used. In general, association constants were greater for the dogfish than the bovine enzyme with opposite temperature

dependencies observed for the two enzymes with large hydrophobic substrates. Bovine chymotryspin had greater acylation rate constants than did dogfish chymotryspin, while the fish enzyme had larger deacylation rate constants than the bovine.

In another study, (Ramakrishna et al., 1987) it was shown that the two enzymes had similar kinetic properties with respect to BTEE and a series of n-fatty acid esters of p-nitrophenol. The only major difference was that the molecular activity (k_{cat}) for the dogfish enzyme was 1.5 to 3-times greater than that of the bovine enzyme, the exact number depending on substrate and assay conditions. The generally greater rate of proteolysis observed with the fish enzyme in the present study could not have been predicted from the results of the work with small molecular weight substrates. This greater rate with the fish enzyme was observed even when the the activities of the two enzymes were adjusted to equal values with BTEE as substrate, a situation where, in fact, there was three times as much active bovine enzyme as fish. Obviously, reaction rates with small molecular weight substrates do not provide a good relative assessment of how the enzymes will act on protein substrates.

In addition to the greater rate of reaction, the dogfish chymotrypsin in most cases hydrolyzed a greater number of bonds in the protein molecules. It is not clear whether this broader specificity is due to the activity of the enzyme on bonds other than those next to the hydrophobic residues usually attacked by this enzyme, i.e., tyrosine, phenylalanine and tryptophan, or whether the enzyme is able to reach other aromatic residues due to a greater flexibility. It has been suggested that the greater catalytic activity of some low temperatures enzymes is due to a more flexible structure of the enzyme (Low and Somero, 1974). Kato et al. (1985) have suggested that flexibility of proteins may be detected by their susceptibility to proteases. Since the same substrates were used in our study for both enzymes, it may be that the flexibility resides in the enzyme itself, and the greater extent of hydrolysis of soy protein isolate, casein, insoluble collagen and corn gluten meal may be a reflection of the greater flexibility of the dogfish chymotrypspin compared to the bovine enzyme. Prahl and Neurath (1966) also found that dogfish chymotrypsin had a broader specificity than bovine chymotryspin towards glucagon. By examination of the "fingerprints" of the digested glucagon, they found that the fewest number of peptides were obtained with bovine chymotrypsin A and the largest number with dogfish chymotrypsin A. Bovine chymotrypsin B was intermediate between the two. Kalac (1978) presented evidence that anionic chmotrypsins isolated from the pyloric caeca of herring and capelin had greater relative activities against BTEE than casein compared to bovine α-chymotrypsin, a finding opposite to what we have observed in this work with dogfish cationic chymotrypsin A.

There are potentially useful applications of proteolytic enzymes from low temperature-adapted organisms (Haard et al., 1982). Superior functioning at low temperatures, different specificities, higher reaction rates, decreased bacterial contamination and an ability to easily deactivate the enzyme are cogent reasons why these catalysts should be examined. This study points out the inadequacy of extrapolation of data from small molecular weight artificial substrates to proteins in determining the usefulness of some of these enzymes.

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Ms received 6/19/86; revised 1/17/87; accepted 2/3/87.

This work was supported in part by the Massachusetts Agricultural Experiment Station, by the Graduate School of the University of Massachusetts at Amherst and by National Science Foundation Grant CPE-7910816. We thank North Atlantic Products, Inc. (Rockland, ME) for the dogfish pancreases and for their generous assistance. We are grateful to Bruce Krasin for the amino acid analyses. We thank Marcia M. Stevens for editorial assistance.

Characteristics of an alkaline proteinase and exopeptidase from shrimp (*Penaeus indicus*) muscle

S. N. DOKE and V. NINJOOR

ABSTRACT

Activities of an alkaline proteinase and an exopeptidase were detected in the muscle of shrimp. Both enzymes could be extracted with unbuffered 0.5% KCl. The shrimp muscle alkaline proteinase, optimally active at pH 8.0 and 60°C, was partially purified and characterized. The heat stable enzyme had an apparent molecular weight of 250 KDa. Protein substrates such as casein, azocasein, azocoll and hemoglobin were hydrolyzed by the enzyme while it showed no action on bovine serum albumin and the synthetic substrates of proteinases. Active site directed inhibition experiments suggested that the enzyme was a metal-dependent serine proteinase. The shrimp exopeptidase cleaving amino acid naphthylamides at pH 6.8 and 40°C exhibited the characteristics of an aminopeptidase because of its susceptibility to bestatin, puromycin and a metal chelator.

INTRODUCTION

TISSUE PROTEINASES have been implicated as adversely affecting the quality of stored muscle foods due to the sustained action of endopeptidases and exopeptidases that are involved in the complete breakdown of tissue proteins (Goll et al., 1983). In fish skeletal muscle, several of these proteinases such as cathepsin D (Doke et al., 1980), neutral proteinase (Makinodan et al., 1983), cathepsin B (Chen and Zall, 1986), alkaline proteinase (Makinodan et al., 1983) and some peptidases (Osnes and Mohr, 1985a) have been identified and characterized. Among these, alkaline proteinase seemed to mediate changes in the muscle texture when processed at 50°-70°C (Makinodan et al., 1985) apparently due to its heat stability.

Although alkaline proteinase has been purified from the muscle of fish varieties such as Antarctic krill (Osnes and Mohr, 1985b), Atlantic croaker (Lin and Lanier, 1980), white croaker (Busconi et al., 1984) and carp (Iwata et al., 1973), information on the enzyme from crustacean species is scarce. Also, there are conflicting reports on the nature of the enzyme. While some investigators (Lin and Lanier, 1980; Busconi et al., 1984) suggested that the enzyme is sulfhydryl dependent, others showed it to be either a metallo (Kozlovskaya and Elyakova, 1975) or a serine proteinase (Kozlovskaya and Elyakova, 1975; Busconi et al., 1984). Differences also seemed to exist between mammalian and invertebrate enzyme (Eguchi and Kuriyama, 1983; Dahlmann and Reinauer, 1978).

The objectives of this study were to establish the presence of alkaline proteinase in shrimp muscle and describe its isolation, partial purification and characterization. Some data on an exopeptidase of shrimp muscle were also determined.

MATERIALS & METHODS

Extraction of alkaline proteinase and an exopeptidase from shrimp muscle

Fresh shrimps (*Penaeus indicus*) from a commercial source were brought to the laboratory in iced condition. After removal of shell and veins, the muscle was collected and cleaned in running water.

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A homogenate (10% w/v) was prepared in different extraction media (Table 1) by blending the finely minced muscle for 2 min in a Sorvall omnimixer with the rheostat setting at 3. After standing for 1 hr, the homogenate was centrifuged at 12,100x*g* for 30 min in a SS-34 rotor of Sorvall RC-2 refrigerated centrifuge. The supernatant was dialyzed against 20 volumes of the respective media for 18 hr and clarified by centrifuging at 12,100x*g* for 20 min and the precipitate discarded. The supernatant served as the source of enzymes. All steps of enzyme preparation and purification were carried out at 0–4°C.

Partial purification of alkaline proteinase

Extraction of alkaline proteinase. Since 0.5% KCl extracted maximum enzyme activity (Table 2), the muscle tissue (100g) was scissor-minced and homogenized in 0.5% KCl solution in a Waring Blendor for 2 min. The homogenate was centrifuged at 12,100x*g* for 20 min. The sediment was resuspended by homogenization in the above solution and centrifuged. The combined supernatants from both centrifugations served as the source of enzyme.

Acetone fractionation. Cold acetone was added with stirring to the above supernatant to give 80% saturation. The precipitated protein collected by centrifugation at 12,100x*g* for 20 min was dissolved in 50 mM phosphate buffer pH 7.2 and dialysed against the same buffer with two changes.

Ion exchange chromatography on DEAE-Sephacel

The dialyzed enzyme was applied to a DEAE-Sephacel column (1.5 x 16 cm) previously equilibrated with 50 mM phosphate buffer, pH 7.2. After elution of the unbound proteins by washing with the buffer, the column was developed with a linear gradient of 0–1.0M NaCl in 50 mM phosphate buffer pH 7.2. Fractions of 3.5 mL were collected at a flow rate of 25 mL/hr. The active fractions were pooled and concentrated by ultrafiltration over YM-10 membrane in an Amicon Diaflow ultrafiltration unit (Amicon Instruments, Lexington, MA).

Gel filtration on Sephadex G-200

The concentrated enzyme was applied to a Sephadex G-200 column (2.4 x 93 cm) previously equilibrated with 50 mM phosphate buffer, pH 7.2 containing 0.15M NaCl and eluted with the same buffer in 3.0 mL fractions at a flow rate of 18 mL/hr. The active fractions were pooled and concentrated to about 4.0 mL.

Determination of molecular weight

The molecular weight of alkaline proteinase was estimated by gel filtration with a Sepharose 6B column (4x87 cm) employing thyroglobulin (Mr-669,000), ferritin (Mr = 440,000), catalase (Mr = 232,000) and aldolase (Mr = 168,000) as standard proteins. Blue dextran was employed to measure the void volume. The flow rate was 18 mL/hr and 4.5 mL fractions were collected. Samples were eluted with 50 mM sodium phosphate buffer, pH 7.2 containing 150 mM NaCl.

Polyacrylamide gel electrophoresis (PAGE)

The procedure of Brewer and Ashworth (1969) was employed for electrophoresis at pH 8.3. The protein bands were stained with coomassie brilliant blue G and destained with 7% acetic acid in 5% methanol. For the location of enzyme activity the unstained gels were cut into equal segments and homogenized manually in 1.0 mL, 50 mM borate buffer, pH 8.0. The enzyme activity was determined in these homogenates.

Table 1—Comparative study of different extraction media for alkaline proteinase and exopeptidase activities from shrimp muscle[a]

Medium of extraction	Protein (mg/mL)	Specific activity: Alkaline proteinase (units/mg)	Specific activity: Exopeptidase (units/mg) Arginine-N Nap hydrolase	Specific activity: Exopeptidase (units/mg) Leucyl-N Nap hydrolase
0.5% KCl	3.13	642	146.8	116.2
0.2M KCl (pH 7.2)	3.11	366	116.8	111.4
0.25M Sucrose + 1 mM EDTA	3.80	348	34.8	9.6
0.6M KCl + 10 mM EDTA (pH 7.2)	4.10	240	10.2	4.8
0.05M Tris-HCl (pH 8.0)	3.70	210	34.2	10.8
0.05M Tris-HCl (pH 9.0)	4.70	234	27.0	9.6
0.05M Tris-HCl + 0.2M KCl (pH 9.0)	5.70	108	—	—
0.05M Tris-HCl + 0.2M KCl (pH 7.2)	3.70	204	—	—
0.05M Phosphate buffer + 0.2M KCl (pH 7.2)	5.42	138	—	—
0.05M Borate buffer + 0.2M KCl (pH 8.0)	4.28	198	—	—

[a] The alkaline proteinase activity was measured as nanomoles of tyrosine liberated per hour and the exopeptidase activity was expressed as nanomoles of 2-naphthylamine liberated per hour. Each value represents an average of three determinations.

Table 2—Purification of alkaline proteinase from shrimp muscle

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification ratio (fold)
Muscle homogenate	14136	2.2×10^6	156	100	—
Supernatant (12,100 × *g*)	4125	2.2×10^6	533	100	3.4
Acetone fraction	666	7.3×10^5	1096	33	7.0
DEAE-Sephacel chromatography	108	4.7×10^5	4352	21	28.0
Sephadex G-200 gel filtration	4	3.3×10^4	8250	1.5	53.0

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Gel electrophoresis was performed in the presence of SDS on 12% slab gels (Laemmli, 1970) at pH 8.3. Briefly, the method consisted of treating the enzyme in Tris-HCl buffer, pH 6.8 with 2% sodium dodecyl sulfate, 2% mercaptoethanol and 15% glycerol and heating this mixture (260 μL approx.) at 100°C for 2 min. A portion of this and molecular weight standards were subjected to electrophoresis at 20 mA for 4 hr. The gel was stained by coomassie blue and destained by 7% acetic acid in 5% methanol.

Assay of enzyme activity

Proteolytic activity was determined using casein (Makinodan et al., 1985) or azocasein (Ninjoor and Srivastava, 1985) as substrates. The caseinolytic activity was assayed in a total volume of 1.0 mL that contained 5% casein solution prepared in 50 mM $Na_2B_4O_7$ buffer, pH 8.0 and an aliquot of the enzyme. After incubating at 60°C for 30 min, the reaction was terminated by the addition of 1.0 mL 5% trichloroacetic acid (TCA). The control tubes received the substrate after stopping the reaction. An aliquot from the TCA filtrate was analyzed for proteolytic end products (Miller, 1959). The unit of activity was nmoles tyrosine released/hr.

The effect of pH on the enzyme activity was examined by assaying at different pH values. Casein dissolved in 50 mM buffers of acetate (pH 4.0 and 5.0), phosphate (pH 6.0 and 7.0), borate (pH 8.0 and 9.0), and carbonate (pH 10.0, 11.0 and 12.0) was used. Assays were performed at 45°, 50°, 55°, 60°, 65°, and 70°C to determine the optimum temperature of the enzyme at pH 8.0.

When azocasein was used as the substrate, the reaction mixture contained 2% azocasein, 50 mM borate buffer, pH 8.0 and an aliquot of the enzyme in a total volume of 1.0 mL. The assay mixture was incubated at 60°C for 30 min and the reaction was stopped by the addition of 1.0 mL 5% TCA. Appropriate controls were maintained. The release of soluble dye was monitored at 366 nm. The unit of activity was change in A_{366} times 10^2/hr.

Activity towards synthetic substrates

Substrate concentration of α-N-benzoyl DL-arginine β-naphthylamide (bz-arg-NNap), L-leucyl- β-naphthylamide (leu-NNap) and L-arginine β-naphthylamide (arg-NNap) was 5 mM and that of cbz-arginine arginine β-naphthylamide (Z-arg-arg-NNap) was 1 mM. Endopeptidase activity was measured by reacting the enzyme (0.01–1 mg) with the preincubated substrate (either bz-arg-NNap or Z-arg-arg-NNap) that contained 1 mM EDTA and 2 mM cysteine. While assaying exopeptidase, the enzyme was reacted with either arg-NNap or leu-NNap at pH 6.8 in a total volume of 1.0 mL. Cysteine and EDTA were omitted in the reaction mixture. The incubation was at 40°C for 10-60 min and mersalyl reagent was used to terminate the reaction and to couple the liberated β-naphthylamine with Fast Garnet GBC (Barrett and Kirschke, 1981). The intensity of red color was measured at 520 nm. The unit of activity was nmoles β-naphthylamine released/min.

Action on other protein substrates

The activity on azocoll, hemoglobin and bovine serum albumin was measured at pH 8.0 according to Ninjoor and Srivastava (1985). Protein was determined by Miller's (1959) method using bovine serum albumin as standard.

Materials

The reagents and chemicals required for electrophoresis and column chromatography were procured from Eastman Kodak (Rochester, NY) and Pharmacia Fine Chemicals Inc. (Uppsala, Sweden), respectively. Bz-arg-NNap, arg-NNap, leu-NNap, benzoyl arginine ethyl ester, bnezoyl tyrosine ethyl ester, azocasein, azocoll, hemoglobin, phenylmethylsulfonyl fluoride (PMSF), N-α-p-tosyl-L-lysine chloromethyl ketone-HCl (TLCK), p-chloromercuribenzoic acid (p-CMB), N-ethyl maleimide (NEM), o-phenanthroline, chymostatin, leupeptin and bestatin were the products of Sigma Chemical Co. (St. Louis, MO); EP-459 was a gift from Dr. K. Hanada, Taisho Pharmaceutical Co. (Saitama, Japan). N-Cbz-arginine arginine β-naphthylamide (Z-arg-arg-NNap) was purchased from Bachem Fine Chemicals (Torrence, CA).

RESULTS

Extractibility of enzymes in different media

The degree of extraction of both the enzymes, i.e., alkaline proteinase and exopeptidase (arg-NNap hydrolase) and protein varied depending upon the media of extraction used. The data included in Table 1 show that while increased yield of protein could be achieved by extracting with 50 mM Tris-HCl buffer, pH 9.0 and 50 mM phosphate buffer, pH 7.2 in the presence of KCl, greater solubilization of the enzymes could be attained by homogenizing in unbuffered 0.5% KCl. Therefore, this medium was selected as the ideal extracting solution to solubilize the enzymes from the muscle of shrimp. Increase in the concentration of KCl as in 0.6M resulted in the gelation of the homogenate.

Partial purification of alkaline proteinase

The enzyme was purified 53-fold with 1.5% recovery by steps included in Table 2. There was a sharp fall in the recovery by acetone precipitation. Efforts to increase the yield at this stage by varying the concentration of acetone did not succeed; neither did ammonium sulfate fractionation. In fact, the latter treatment markedly decreased the specific activity of the enzyme. Acidification and heat treatment were the other two steps which failed to increase yield. The exopeptidase was also concentrated by acetone precipitation. When the acetone fraction was subjected to ion exchange chromatography, both alkaline proteinase and exopeptidase were found to be adsorbed to the resin (Fig. 1), and no activity was traceable in the unbound protein. Application of salt gradient resolved alkaline proteinase and exopeptidase efficiently. The exopeptidase was the first to appear at 0.1–0.2M NaCl concentration. The second protein peak corresponded exclusively to alkaline proteinase which eluted at 0.2–0.35M NaCl and remained well separated from the exopeptidase activity. Further increase in NaCl concentration resulted in another peak of exopeptidase activity. Thus, the results of Fig. 1 indicated that alkaline proteinase could be separated from the contaminating arg-NNap hydrolase activity. It should be noted that none of the protein fractions separated on DEAE-Sephacel hydrolyzed bz-arg-NNap or Z-arg-arg-NNap showing the complete absence of cathepsin B type activity. Fractions 82–112 which contained the major peak of alkaline proteinase on DEAE-Sephacel were subjected to molecular sieving on Sephadex G-200 column as shown in Fig. 2. The enzyme eluted immediately after void volume as a single peak that coincided with the protein peak. This preparation, after concentration, was subjected to PAGE and SDS-PAGE. The enzyme gave a sharp band on PAGE with two minor bands suggesting possible non-homogeneity (Fig. 3). When the gel segments were analyzed for enzyme activity, the major band exhibited the entire proteinase activity.

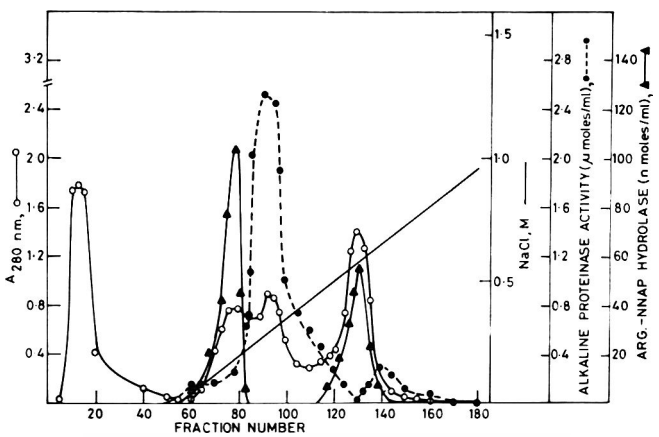

Fig. 1—DEAE-Sephacel column chromatography of alkaline proteinase from shrimp muscle. The sample applied on the column (1.5 x 16 cm) was eluted with 50 mM phosphate buffer pH 7.2 in 3.5 mL fractions at a flow rate of 25 mL/hr. The bound protein was eluted by applying a gradient of NaCl (0–1.0 M) in the buffer.

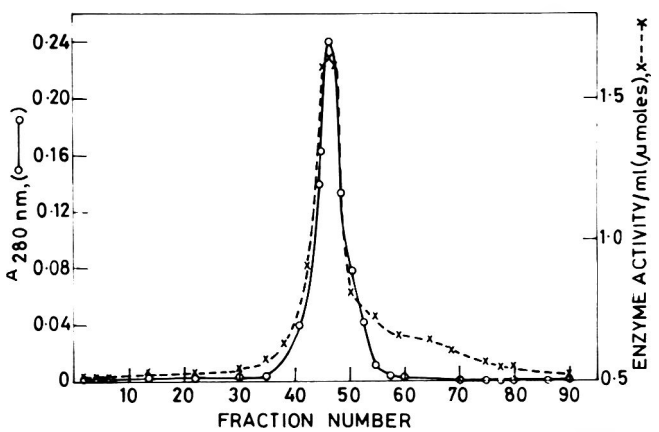

Fig. 2—Elution profile of alkaline proteinase on a Sephadex G-200 column (2.5 x 93 cm). The sample was eluted with 50 mM phosphate buffer, pH 7.2 containing 150 mM NaCl in 3.0 mL fractions at a flow rate of 18.0 mL/hr.

Molecular weight

Molecular exclusion chromatography on Sepharose-6B indicated that the molecular weight of alkaline proteinase is 250,000 (Fig. 4). However, on SDS-PAGE, the major band corresponded to 96,000 with 2 minor bands (data not shown).

pH and temperature optima of alkaline proteinase

As shown in Fig. 5 the caseinolytic activity of the enzyme was evaluated at pH 4.0 to 12.0 and the optimal activity of the enzyme was found at pH 8.0. The enzyme displayed maximum activity at 60°C.

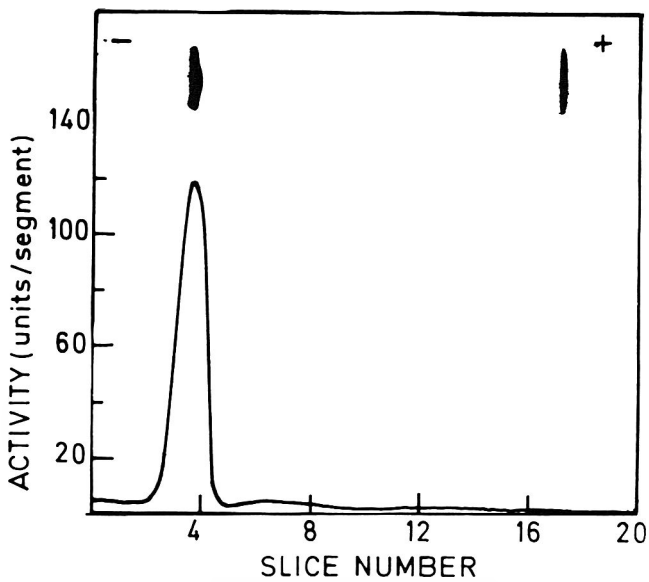

Fig. 3—PAGE pattern of partially purified alkaline proteinase. The enzyme activity of unstained gel segments was determined and plotted in the lower panel.

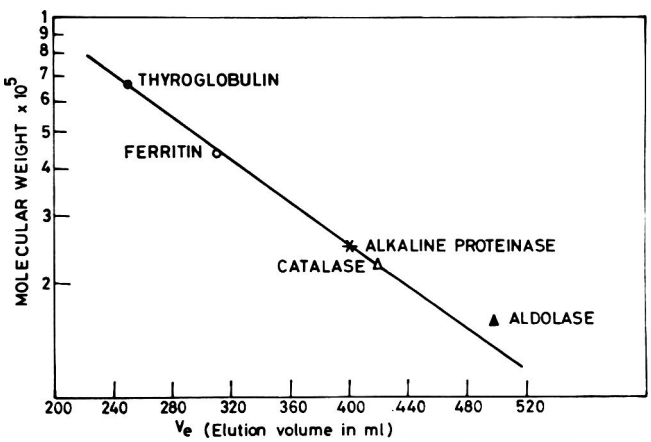

Fig. 4—Determination of molecular weight of shrimp muscle alkaline proteinase by gel filtration on Sepharose-6B. The column (4 x 87 cm) was equilibrated and eluted by 50 mM phosphate buffer pH 7.2 containing 150 mM NaCl. The flow rate was 18 mL/hr and the fraction size 4.5 mL. The standard proteins used for calibration were thyroglobulin, 669 KDa; ferritin, 440 KDa; catalase, 232 KDa; and aldolase, 168 KDa. The molecular weight was determined from the linear semilogarithmic plot.

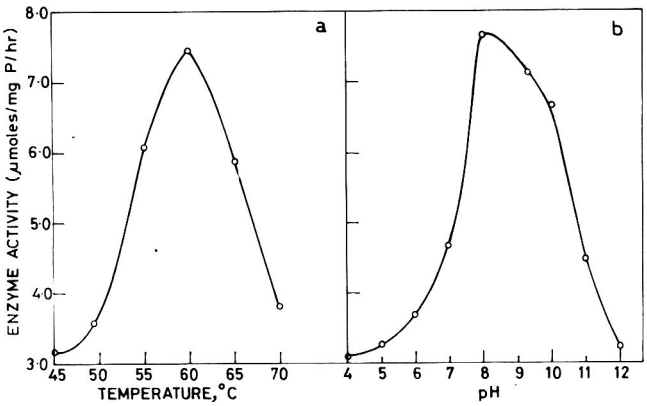

Fig. 5—Effect of temperature and pH on the activity of alkaline proteinase from shrimp muscle: (a) Effect of temperature at pH 8.0; (b) pH curve at 60°C.

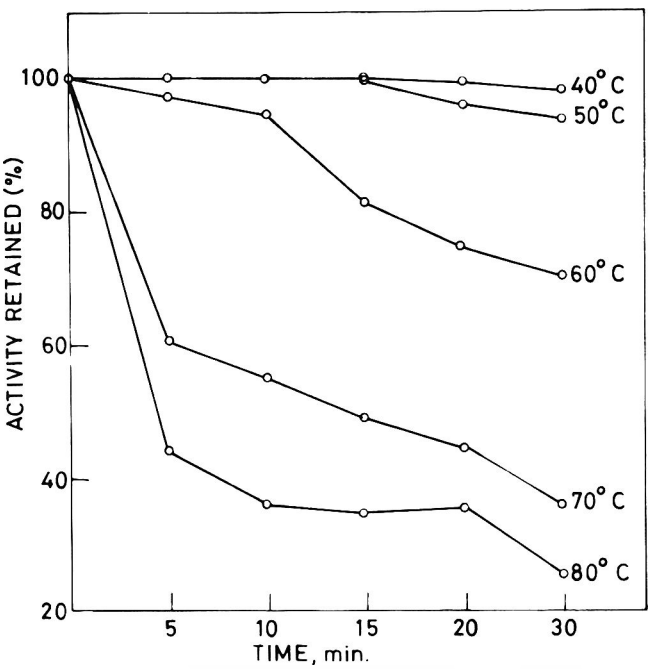

Fig. 6—Influence of heat on the stability of alkaline proteinase. The enzyme was incubated for different intervals of time at stated temperatures following which the residual activity was measured.

Thermal stability of alkaline proteinase

The influence of various temperatures on the caseinolytic activities of alkaline proteinase is illustrated in Fig. 6. The enzyme was stable at both 40°C and 50°C for 30 min. When exposed to 60°C, there was hardly any loss in activity during 10 min, while after 30 min. 30% of the activity was abolished. Further increase in temperatures resulted in the rapid loss of activity as in the case of exposure at 70° and 80°C. However, even after thermal treatment at 70° and 80°C for 30 min, the enzyme retained 30 and 25% activity, respectively.

Action of the enzyme on different protein substrates

Alkaline proteinase was able to hydrolyze intact proteins and the data on its action on casein, azocasein, hemoglobin, bovine serum albumin and azocoll are included in Table 3. Excepting bovine serum albumin all other protein substrates could be hydrolyzed by alkaline proteinase although the degree of hydrolysis varied from protein to protein. Among the proteins tested, casein seemed to be the ideal substrate for alkaline proteinase.

Table 3—Action of alkaline proteinase on different protein substrates

Substrate[a]	Specific activity (units mg protein)
Casein	668.20 ± 123.54
Azocasein	35.88 ± 7.99
Hemoglobin	30.94 ± 3.70
Azocoll	32.80 ± 3.13
Bovine serum albumin	0

[a] The concentration of casein was 5% while those of azocasein, bovine serum albumin and hemoglobin were 2% in 50 mM sodium borate buffer pH 8.0. In the case of azocoll 10 mg substrate suspension was made in 3.0 mL of the buffer. The units were as described in the text. The values are the average of three experiments ± S.D.

Table 4—Effect of proteinase inhibitors on alkaline proteinase activity of shrimp muscle[a]

Effector compound	Final conc (mM)	Activity retained (%)
Control	—	100
p-chloromercuribenzoic acid	2	93
N-ethyl maleimide	2	96
EP-459	2	97
Iodoacetamide	2	100
Leupeptin	10^{-6}	90
Chymostatin	0.1 μg/mL	97
Phenylmethylsulfonyl fluoride	2	60
Soybean trypsin inhibitor	0.2 μg	60
Soybean trypsin inhibitor	0.4 μg	41
N-α-p-tosyl-L-lysine chloromethyl ketone	2	102
8-Hydroxyquinoline	2	170
o-Phenanthroline	2	41
$MnCl_2$	2	17
$ZnCl_2$	2	61
$CoCl_2$	2	0
$CaCl_2$	2	100
EDTA	0.1	87
EDTA	1.0	68
EDTA	5.0	35

[a] The enzyme preparation was preincubated with the inhibitors at 37 C for 30 min and the residual activity estimated. Appropriate controls were included with the test samples. The values are means of two determinations in replicate.

Hydrolysis of synthetic substrates

Among the synthetic substrates tested the enzyme failed to cleave benzoyl tyrosine ethyl ester, a substrate of chymotrypsin and benzoyl arginine ethylester, a substrate of trypsin. However, a specific activity of 0.13 was obtained with bz-arg-NNap at pH 8.5 in the presence of Ca^{2+} (50 mM) indicating a trace of trypsin-type activity. Cathepsin B type activity was absent since both bz-arg-NNap and Z-arg-arg-NNap were not hydrolyzed by the enzyme at pH 6.0. Similarly, the amino peptidase substrates viz arg-NNap and leu-NNap were not cleaved by alkaline proteinase.

Effect of inhibitors

The effect of several known proteinase inhibitors and metal ions on the activity of alkaline proteinase is included in Table 4. Cysteine proteinase inhibitors such as pCMB, iodoacetamide, NEM and EP-459 did not cause enzyme inhibition. Among metallo proteinase inhibitors, 8-hydroxy quinoline stimulated the activity while o-phenanthraline caused more than 50% inhibition at 2 mM concentration. EDTA at 10 mM and above inhibited the activity completely. Serine proteinase inhibitors such as PMSF and STI also displayed inhibitory activity towards alkaline proteinase. Chymostatin, leupeptin and TLCK did not affect the enzyme while cations such as Co^{2+}, Mn^{2+} and Zn^{2+} exerted inhibitory activity. Ca^{2+}, however, failed to show any influence on alkaline proteinase.

Exopeptidase from shrimp muscle

An exopeptidase hydrolyzing both arg-NNap and leu-NNap was found in the shrimp muscle homogenate. This enzyme was concentrated by acetone treatment and some of its properties were investigated. The enzyme hydrolyzed both arg-NNap and leu-NNap maximally at pH 6.8 and 40°C (results not shown). Unlike cathepsin B and H the enzyme was not dependent on cysteine or EDTA for its maximum activity and was stable at 60°C for 30 min.

Table 5—Effect of different inhibitors on the activity of exopeptidase from shrimp muscle[a]

Effector compound	Final conc (mM)	Activity retained (%)
Control	—	100
Iodoacetamide	2	100
N-ethyl maleimide	1	85
EP-459	1	90
Chloromercuribenzoic acid	1	81
Chloromercuribenzoic acid	10	46
Leupeptin	10^{-3}	100
Antipain	10^{-3}	100
Chymostatin	1 μg mL	100
Puromycin	1	19
Bestatin	1	28
Phenylmethylsulfonyl fluoride	1	88
Soybean trypsin inhibitor	1	90
Pepstatin	10^{-2}	95
$ZnCl_2$	1	100
$MnCl_2$	1	18
$CoCl_2$	1	72

[a] All inhibitors were preincubated with the partially purified enzyme at 37°C for 30 min and assayed for residual activity. Appropriate controls were also included. The values are means of two determinations.

Table 6—Effect of EDTA and cysteine on exopeptidase from shrimp muscle[a]

Addition	Final conc (mM)	Activity retained (%)
EDTA	0	100
	1	34
	2	34
	5	27
	10	27
Cysteine	1 to 5	100
	10	93
EDTA + cysteine	1 + 1	100
	5 + 5	83
	10 + 10	26

[a] The values are the averages of duplicate experiments.

Effect of inhibitors on the exopeptidase activity

Data on the influence of several proteinase inhibitors on shrimp exopeptidase are included in Table 5. Among cysteine proteinase inhibitors iodoacetamide, NEM and EP-459 hardly displayed any influence on the enzyme activity. p-CMB on the other hand was inhibitory at high concentration (10 mM). An aspartic proteinase inhibitor like pepstatin and serine proteinase inhibitors PMSF and STI also failed to exert any action on the enzyme. Similarly, cathepsin B specific inhibitors like leupeptin and antipain and chymotrypsin inhibitor chymostatin did not suppress exopeptidase activity. However, puromycin and bestatin proved to be potent inhibitors. Among cations tested only Mn^{2+} proved highly inhibitory while Ca^{2+} showed moderate inhibition.

The data on the action of EDTA and cysteine on the exopeptidase are summarized in Table 6. The enzyme was inhibited by EDTA and addition of cysteine could retard this inhibitory influence although cysteine alone was not able to stimulate the activity. However, it is interesting to note that the inhibition caused by higher concentration of EDTA could not be reversed by cysteine.

DISCUSSION

THE PRESENCE of alkaline proteinase capable of hydrolyzing casein at 60–65°C has been documented in the skeletal muscle of four species of fresh water fish and twenty-one species of marine fish (Iwata et al., 1974). The results of the present study reveal that the muscle of a crustacean, shrimp, contains an endopeptidase active at alkaline pH and an exopeptidase capable of hydrolysing N-blocked amino acid derivatives. The endo- and exopeptidase activities were apparently distributed both in myofibrillar and sarcoplasmic protein fractions of the shrimp muscle since the enzymes could be extracted in low salt solutions. The data included in Table 1 suggest that low salt concentrations (0.5% KCl) offer the best yield of enzyme and therefore, confirm the observation of Siebert (1973) and Bonete et al. (1984).

Alkaline proteinase purified from shrimp muscle in the present study despite showing apparent homogeniety in gel permeation chromatography (Fig. 2) on Sephadex G-200 and PAGE, appeared far from homogeneous as revealed by SDS-PAGE. Also, the yield of enzyme was low. None of the conventional procedures such as acidification, heat treatment, and ammonium sulfate fractionation was successful in isolating the enzyme. A marked loss in both yield and specific activity was noted with these steps. Therefore, acetone precipitation was used and found superior to others in attaining 33% yield of the enzyme. Anion exchange chromatography separated alkaline proteinase from the contaminating exopeptidase and gel filtration over Sephadex G-200 gave partially pure enzyme. Existence of an inhibitor for alkaline proteinase in fish skeletal muscle has been reported (Busconi et al., 1984). The enzyme may be bound to the endogeneous inhibitor firmly during the purification steps and hence results in apparent loss in specific activity and yield. This may also explain the additional bands obtained during SDS-PAGE, wherein the inhibitor is perhaps dissociated from the enzyme during the adverse conditions of denaturation.

The molecular weight of shrimp alkaline proteinase determined by gel filtration over Sephadex was 250,000 and by SDS-PAGE, 96,000 indicating that the enzyme was a dimer containing apparently identical subunits of 96,000. The rest was attributable to the tightly bound nonenzymic protein (inhibitors). The molecular mass of alkaline proteinase from the muscle of white croaker has been found to be 132,000 for protease I and 1,363,000 for protease II by Busconi et al. (1984), while it was estimated to be 86,000 for the enzyme from Atlantic croaker (Lin and Lanier, 1980). Similarly, Iwata et al. (1973) demonstrated the molecular weight of carp alkaline proteinase as 780,000. In constrast, molecular mass of alkaline proteinase from mammalian sources seemed to vary from 30,800 (Dahlmann and Reinauer, 1978) to 120,000 (Dahlmann et al., 1981).

Shrimp muscle alkaline proteinase resembles alkaline proteinases of other fish varieties in terms of heat stability (Iwata et al., 1973; Lin and Lanier, 1980; Deng, 1981; Makinodan et al., 1985). In fact, carp muscle alkaline proteinase has been shown to be inactive below 55°C and to require conformational changes brought about by treatments like heat exposure, addition of urea or gamma irradiation to exhibit its maximal activity (Makinodan and Ikeda, 1977). The shrimp enzyme had an optimum temperature of 60°C and remained partially active even after exposure to 80°C. Apart from exogenous substrates alkaline proteinase is capable of degrading myofibrillar proteins (Lin and Lanier, 1980; Folco et al., 1984) and, therefore, is implicated in the textural degradation of minced fish meat (Deng 1981; Lanier et al., 1981; Makinodan et al., 1985). Although myofibrillar proteins were not used as substrates in the present study, shrimp muscle alkaline proteinase seemed to be capable of degrading all protein substrates with the exception of bovine serum albumin. It was pointed out by Lin and Lanier (1980) that among the various substrates tested, alkaline proteinase of Atlantic croaker was inactive towards hemoglobin and azocoll. It seems, therefore, that alkaline proteinases from different sources exhibit differences in their substrate specificity. The results of the present study also indicated that shrimp alkaline proteinase was devoid of any action towards the synthetic substrates of cathepsin B and aminopeptidase. There was no action on arg-NNap following the addition of cysteine showing the absence of cathepsin H-type activity. The enzyme failed to hydrolyze the substrates of chymotrypsin and trypsin while it cleaved bz-arg-NNap in the presence of 50 mM Ca^{2+}, though insignificantly, showing the presence of some trypsin-type activity. It is noteworthy that the muscle homogenate of shrimp is devoid of bz-arg-NNap or Z-arg-arg-NNap hydrolysing activity indicative of absence of cathepsin

B which confirms earlier observations (Eitenmiller, 1974, Sherekar et al., 1986).

The response of shrimp alkaline proteinase towards various inhibitors suggested that the enzyme was a metal dependent serine proteinase, since serine proteinase inhibitors, PMSF and STI and the metallocnzyme inactivator EDTA suppressed the activity. Inhibition by two types of proteinase inhibitors seems to be typical for alkaline proteinase isolated from vertebrate and invertebrate species. While Busconi et al. (1984) isolated two alkaline proteinases from white croaker, one of which was trypsin-like and the other a cysteine proteinase. Kozlovskaya and Elyakova (1975) demonstrated that star fish alkaline proteinases contained serine and metallo proteinases. An alkaline proteinase from the digestive tract of silk worm has been shown to be a serine proteinase having a cysteine residue (Eguchi and Kuriyama, 1983) while that from fish has been demonstrated to be either a cysteine (Makinodan et al., 1985) or serine (Iwata et al., 1974) proteinase. In contrast, many workers who isolated and characterized alkaline proteinases from mammalian sources have documented evidence to suggest that the enzyme is either a trypsin-like or chymotrypsin-like serine proteinase (Kido et al., 1985). The mammalian muscle alkaline proteinase, especially that from the rat, seems to have originated from mast cells (Woodbury and Neurath, 1981). However, fish muscle under normal conditions is devoid of mast cells (Busconi et al., 1984) and, therefore, the enzyme may be localized in the myogen.

Apart from alkaline proteinase, occurrence of several endopeptidases such as cathepsins B and D (Sherekar et al., 1986, Chen and Zall, 1986), neutral proteinase (Makinodan et al., 1983) and calpain (Makinodan et al., 1985) has been reported in fish muscle. While these enzymes apparently hydrolyze the polypeptide chain randomly, action of amino and carboxypeptidase is necessary to complete the proteolytic breakdown; and the occurrence of such enzymes has been reported in fish (Osnes and Mohr, 1985a). The preliminary results of the present study indicate that shrimp muscle contains a peptidase capable of hydrolysing arg-NNap and leu-NNap equally efficiently. Like alkaline proteinase this enzyme also displays maximum solubilization in 0.5% KCl (Table 1). The enzyme active at 40°C and pH 6.8 showed stability at 60° (results not shown). Naphthylamides of arginine and leucine are generally used as substrates of cathepsin H, and endoaminopeptidase (Barrett and Kirschke, 1981) belonging to cysteine proteinase group. However, the exopeptidase of shrimp is neither activated by sulphydryl reagents like cysteine nor inhibited by potential cysteine proteinase inhibitors such as iodoacetamide, NEM, EP-459 and p-CMB (Table 4). High concentration of p-CMB (10 mM) suppressed the enzyme activity by 50% which could be attributed to the effect of heavy metal on the enzyme. The inhibition profile of the enzyme also suggested that serine proteinase inhibitors like PMSF and STI and an aspartic proteinase inhibitor pepstatin were devoid of any influence. However, puromycin and bestatin, known competetive inhibitors of aminopeptidases (Stratford and Lee, 1986), markedly decrease the activity of shrimp exopeptidases. Bestatin has been shown to inhibit leucine aminopeptidase as well as amino peptidase N and B (Suda et al., 1976) while puromycin has been shown to be inhibitory to aminopeptidases N and B (McDonald and Schwabe, 1977) but not leucine aminopeptidase (McDonald et al., 1964). Since the shrimp enzyme is inhibited both by puromycin and bestatin it could be classed as an aminopeptidase B (arginine aminopeptidase). Aminopeptidases are metalloenzymes and EDTA blocks their activity (Vo et al., 1983, Mantle et al. 1985). The shrimp muscle aminopeptidase loses its activity by the addition of EDTA which could be restored by cysteine. However, the restoration in enzyme activity occurred only at low concentrations and the inhibitory effect of EDTA persisted at higher concentrations irrespective of added cysteine. Among the metal ions, Mn^{2+} and Co^{2+} have been found to be activators for leucine and alanine aminopeptidases, respectively (Stratford and Lee, 1986). In the case of shrimp aminopeptidase, however, both these metal ions seemed to block the activity, Mn^{2+} showing higher inhibition than Co^{2+} which is in good agreement with the properties of rat arginine aminopeptidase (Vanha-Perttula and Jauhiainen, 1983). The available results seemed to suggest that shrimp muscle aminopeptidase was different from leucine aminopeptidase. Further work on the characterization of the enzyme is necessary to prove this point.

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Polyunsaturated Fatty Acids and Fat in Fish Flesh for Selecting Species for Health Benefits

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ABSTRACT

An awareness of the protective roll of n-3 polyunsaturated fatty acids against development of cardiovascular disease has prompted the promotion of fish consumption. It is assumed that those fish rich in polyunsaturated fat and particularly those having high ratios of n-3 to n-6 polyunsaturated fatty acids would confer the most benefit. The total fat and fatty acid compositional data are reported herein for 41 different species of fish that are mostly found in the coastal waters of the Eastern United States.

INTRODUCTION

RECENT EPIDEMIOLOGICAL STUDIES have linked the dietary intake of n-3 polyunsaturated fatty acids (PUFA) in Greenland Eskimos to their low incidence of coronary heart disease (Dyerberg and Bang, 1979; Dyerberg et al., 1978). The most important of the n-3 PUFA seems to be eicosapentaenoic acid (EPA) which has an anti-aggregatory potency that results from competitive inhibition of the synthesis of thrombotic eicosanoid thromboxane (Budowski, 1981; Bronsgeest-Schoute et al., 1981) or alternatively results from increased production of prostacyclins which are anti-aggregatory agents (Goodnight et al., 1981).

Dietary trials aimed at reducing the risk of cardiovascular diseases have emphasized the importance of ingesting marine oil and fish products that are rich in n-3 PUFA and poor in n-6 PUFA (Singer et al., 1983; Herold and Kinsella, 1986). The beneficial effects have been attributed to an increased ratio of n-3 to n-6 PUFA in blood lipids and cell membrane lipids.

The fat of most common fish is 8% to 12% in EPA and 10% to 20% in docosahexaenoic acid (DHA). Fats of more exotic seafoods, such as scallops, oysters, and red caviar contain more than 20% of either EPA or DHA (Hepburn et al., 1986). The fat content and fatty acid composition of other fish are less well known and they may be an important nutritional source. It is the purpose of this study to give a brief survey of the fat content and fatty acid composition of commercially available fresh table fish.

MATERIALS & METHODS

ALL FISH were purchased from a fish market in the Atlanta area. Triplicate samples were taken from fresh fish fillets by removing a 5–6g piece of flesh from the center of the fillet. Whenever possible, the fillet was taken from the middle portion of the fish. Aliquots were weighed accurately, cut into small pieces and immediately homogenized in 20 mL chloroform/methanol (1:1, V/V) according to the method of Bligh and Dyer (1969). To prevent oxidation, t-butyl hydroquinone was added to all samples during processing.

The resulting lipid fraction was weighed and a fraction removed for analysis. Lipid samples were converted to their fatty acid methyl esters by heating the lipids in a mixture of 3% H_2SO_4 in methanol at 80°C in a nitrogen atmosphere for 1 hr. Analysis of methyl esters was performed by gas-liquid chromatography on 1.5 m columns (2 mm i.d.) packed with SP2310 on Chromosorb W AW (Supelco, Inc., Bellefonte, PA). The chromatographic conditions were as follows: injection port temperature 240°C, flame ionization detector 260°C, and oven temperature 205°C. The carrier gas used was nitrogen set at a flow rate of 20 mL/min.

Samples were also routinely injected onto 4.8m (2 mm i.d.) columns packed with 15% OV 275 on Chromosorb P AW-DMCS (Supelco, Inc.) under the following conditions: injection port temperature 260°C, flame ionization detector 280°C, and oven temperature 245°C. The carrier gas used was nitrogen set at a flow rate of 25 mL/min.

Retention times and peak areas were computed automatically by a computing integrator (Laboratory Data Control, Riviera Beach, FL). Standard methyl esters (Nu-Check-Prep, Elyssian, MN and The Hormel Institute, Austin, MN) were routinely chromatographed to establish the identity of the fatty acid methyl esters of fish samples and to determine the response of the detector to different methyl esters. Quantitative response factors were determined and used in the calculations of the data. There were no differential losses of PUFA.

Total fatty acid methyl esters were separated according to their number of double bonds by thin layer chromatography, on preparative plates (500 μ) of Silicagel H impregnated with 10% $AgNO_3$. Chromatograms were developed twice with hexane-ethyl ether-acetic acid (94:4:2, V/V). The plates were sprayed with 2% dichlorofluorescein solution, and the spots were visualized under UV-light. Sample spots corresponding to fatty acid methyl ester standards of 0,1,2 and 3 double bonds respectively were obtained.

RESULTS & DISCUSSION

THE OIL CONTENT in the flesh of the 41 different species of fish studied is listed in Table 1. The oil content (in weight percent) varies widely among species, from 0.4% for grouper and bream to 21.5% for eel. Similar findings have been previously noted (Stansby, 1969; Stansby, 1973; Exler and Weihrauch, 1976). A difference in oil content has also been observed between spring and fall fish; fall fish generally contain increased fat in the muscle (Dyer et al., 1977; Knipprath and Meade, 1965). The data in Table 1 are for spring fish and are consistent with results reported by Exler et al. (1975).

The fatty acid composition of flesh fish oils is shown in

Table 1—Oil content in flesh of fresh fish[a]

Fish	Total oil (wt %)	Fish	Total oil (wt %)
Sea bass	2.9	Pompano	4.0
Black bass	4.3	Porgy	3.1
Bluefish	6.8	Pink salmon	4.2
Bream	0.4	Coho salmon	7.2
Butterfish	2.3	Sardines	9.7
Channel catfish	9.8	Shark	0.9
Carp	4.5	Smelt	4.2
Atlantic cod	1.1	Pink snapper	0.8
Pacific cod	0.9	Red snapper	1.2
Eel	21.5	White snapper	1.0
Flounder	1.5	Sole	1.8
Grouper	0.4	Spots	2.3
Haddock	1.1	Swordfish	2.6
Halibut	3.1	Tilapia	1.6
Herring	11.2	Rainbow trout	1.8
Common jack	1.9	Ocean trout	1.4
Mackerel	7.2	Tuna	7.5
Mullet	5.6	Susi tuna	2.3
Boston perch	3.8	Whiting	2.0
Ocean perch	3.7	Small whiting	2.7
Pike	0.9		

[a] Mean of three samples

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Table 2 for the 41 different species studied. The values are given as weight percent of total fatty acid methyl esters. Fatty acids are designated by number of carbon atoms: number of double bonds. In PUFA with methylene-interrupted sequence of double bonds, the digit after n states the number of carbon atoms from the methyl end of the acyl chain to the nearest double bond. Common names are not indicated because fatty acids in fish are primarily unsaturated and they are mixtures of several isomers, that is, the double bonds are located in different positions. Only those fatty acids that were consistently detected at the level of 0.5% or more of the total are given. Table 2 also gives the amount of PUFA of n-3 and n-6 families. This amount is the sum of all PUFA belonging to the same family: 18:3, 18:4, 20:3, 20:5, 22:5, 22:6 of the n-3 series and 18:2, 20:2, 22:4, 22:5 of the n-6 series, respectively. The ratio of n-3 over n-6 is given for comparative purposes.

With regard to n-6 PUFA, species such as bluefish, bream, butterfish, flounder, grouper, common jack, perch, pike, pompano, shark, swordfish and tilapia contained more than 10% of total fatty acids. The major n-6 fatty acid present was 20:4. Other fatty acids of the n-6 series present included 18:2, 22:4 and 22:5.

With regard to n-3 PUFA, cod, haddock, macherel, sardines, smelt, ocean trout, and whiting all contained more than 40% n-3 PUFA. Channel catfish, carp and eel were lowest in percentage of n-3 PUFA; all had less than 13%.

The ratio of n-3/n-6 PUFA was nearly one for bream, butterfish, catfish, carp, pike, shark and tilapia. As expected, however, n-3/n-6 PUFA ratio for most species was high; it exceeded 10 for sea bass, cod, haddock, herring, sardines and smelt.

Although it is generally recognized that PUFA composition may vary among species of fish, in selecting fish for general diets in the past, little attention was paid to the PUFA composition of different species. All fish were considered of similar nutritional value, and selection was made chiefly on the basis of availability, freshness, flavor, etc. When fish is intended, however, as a means of improving health, both the fat content and the content of PUFA must be considered. This usually means selecting species after consideration is given to the n-3 and n-6 PUFA content.

Fish oils rich in n-3 PUFA have been proposed as antithrombotic dietary supplements (Budowski, 1981; Goodnight et al. 1982). Oils rich in n-3 would provide substrates for the synthesis of platelet antiaggregatory factor such as thromboxane and prostacyclin of the 3-series. On the other hand, there is ample evidence suggesting that prostacyclions of the 2-series which are derived from 20:4 n-6 have a very important role to play in the chemistry of hemostasis and thrombosis (Needleman et al, 1979). The use of fish or fish oils rich in both n-3 and n-6 PUFA may provide a more balanced dietary approach especially when the potentially deleterious effect of fish oil feeding is considered, i.e., increased bleeding times and thromboycytopenia (Goodnight, et al., 1981; 1982).

It is hoped that the present study along with others of similar nature (Sohn et al., 1961; Shimma and Taguchi, 1964; Gruger, 1964; Ackman, 1980; Hepburn et al., 1986) may provide valuable information in selecting fish and fish oils for nutritional studies. Much could be learned by conducting comparative studies in which several fish and marine oils from different species are utilized in the same subjects.

Table 2—Fatty acid composition of fish (wt %)[a]

	Fatty acid																						Ratio
Fish	14:0	15:0	16:0	16:1	18:0	18:1	18:2 n-6	18:3 n-6	20:1	18:4 n-3	20:2 n-6	22:0	20:3 n-3	20:4 n-6	22:1	20:5 n-3	24:1	22:4 n-6	22:5 n-6	22:5 n-3	22:6 n-3	Total n-3	Total n-6	n-3/ n-6
Sea bass	2.0	0.8	17.4	6.5	4.1	22.5	0.8		3.0					2.2		8.5	1.9			1.7	23.5	33.7	3.0	11.1
Black bass	2.4	1.9	12.2	8.9	9.8	16.5	2.2		2.8	1.5				3.2		10.0	1.2	0.5	0.8	2.0	24.0	37.5	6.7	5.6
Bluefish	2.8	1.8	9.9	6.5	8.0	12.2	4.5		4.0	1.2				4.7		8.5		2.3	1.1	3.9	22.4	36.0	12.6	2.9
Bream	1.3	1.2	16.2	2.8	10.8	12.0	1.6		2.5		0.7		1.2	13.8		9.3		4.1	2.3	6.2	12.8	29.5	22.5	1.3
Butterfish	3.3	1.8	17.8	6.6	4.3	23.1	2.3	3.1	4.4		2.9		0.9	5.8	0.6	5.1		3.1	2.7	2.7	10.4	22.2	16.8	1.3
Channel catfish	1.6		20.6	4.1	6.3	31.0	6.8		3.4		2.1			2.4	0.7	4.8				2.0	10.6	17.4	11.3	1.5
Carp	1.2		17.8	11.6	5.9	32.4	13.7	0.7						5.2		7.1					3.9	11.7	18.9	0.6
Atlantic cod	0.5		22.1	2.1	4.8	9.5	1.2		2.0		0.8			1.5	2.1	16.3				0.7	36.1	53.1	3.5	15.2
Pacific cod	1.0		17.5	3.8	5.2	13.2	1.8		1.6		0.7			1.2	1.4	15.8				2.0	28.1	45.9	3.7	12.4
Eel	5.5		18.9	13.1	1.3	39.3	4.6		2.5						2.3	2.2				4.3	5.8	12.3	4.6	2.7
Flounder	3.2		17.9	5.9	7.1	9.9	1.8	1.9		3.2				7.1		9.7	3.3	3.4	2.3	5.9	16.8	37.5	14.6	2.6
Grouper	1.1	1.1	17.8	4.7	10.0	11.4	0.7		0.8	1.4				5.5		2.3		4.4	3.7	2.0	30.0	35.7	14.3	2.5
Haddock	1.2		21.7	4.8	4.7	13.8	2.1		3.3	3.4				2.0		15.1				1.2	24.6	44.3	4.1	10.8
Halibut	2.8	0.5	16.2	10.1	3.6	24.2	0.9		9.2	4.3				2.6		12.3	4.0			3.1	8.6	28.3	3.5	8.1
Herring	5.1		16.5	8.9	2.6	18.1	1.8	0.8	7.1	2.9					4.8	9.2	4.5			1.8	8.9	23.6	1.8	13.1
Common jack	2.3	2.0	23.7	3.2	16.5	11.8	0.6		1.1	2.2			0.5	7.4		4.3		1.6	1.2	4.1	15.3	26.4	10.8	2.4
Mackerel	2.8	1.2	17.5	6.0	5.8	7.8	1.9		4.1	2.4			0.7	6.9	0.7	11.2	2.4		0.8	4.1	22.8	41.2	9.6	4.3
Mullet	4.1	3.4	11.8	9.4	10.3	12.1	5.7		1.6	1.2	1.1		3.5	5.8		11.2	0.8	0.7	0.7	5.7	7.7	29.3	14.0	2.1
Boston perch	4.2	2.1	12.0	8.5	9.1	14.3	4.5		10.1	3.0				5.8	1.6	10.6	0.6		2.2	3.3	10.9	27.8	12.5	2.2
Ocean perch	4.6		13.1	8.2	3.6	18.9	1.5		8.2	1.9				6.2		8.8			2.0	1.8	12.0	24.5	9.7	2.5
Pike	3.0	1.4	6.6	5.7	3.6	6.8	3.1		13.6	4.6				20.0	1.8	7.5	2.1			2.5	15.2	29.8	23.1	1.3
Pompano	3.2	2.5	14.4	9.1	16.0	9.7	1.4		3.4	0.6				6.6		4.2		1.3	1.8	5.1	19.8	29.7	11.1	2.7
Porgy	4.2	1.1	13.4	10.2	7.8	17.4	1.9		3.6	1.4				3.8		10.9			0.6	5.5	14.6	32.4	6.3	5.1
Pink salmon	3.3		13.0	5.2	3.0	14.0	2.0	1.6	3.5	4.2	2.0			2.8	1.3	11.0	1.3			3.1	20.0	39.9	6.8	5.9
Coho salmon	3.7	0.5	10.2	6.7	4.7	18.6	1.2	0.6	9.1	2.6				2.5		13.8	3.4	0.5	0.8	3.2	15.9	36.1	5.0	7.2
Sardines	3.4	1.3	14.5	7.0	4.9	15.4	1.4		4.2	3.8				0.9	1.3	17.3		0.8	0.6	2.5	25.8	43.4	3.7	11.7
Shark	2.9	2.5	14.7	1.7	14.5	14.7	1.4		0.8					13.1		2.2		6.1	3.0	4.5	17.7	24.4	23.6	1.0
Smelt	3.5	1.5	9.4	8.6	3.5	12.2	2.5		4.6	3.3		2.5		1.0		14.7				1.5	21.8	41.3	3.5	11.8
Pink snapper	3.2	1.3	13.9	8.8	8.5	18.7	2.6		4.1		1.7		1.0	4.1		7.8				1.8	21.3	31.9	8.4	3.8
Red snapper	2.5	1.5	14.2	4.4	11.2	17.8	1.2		1.3					3.6		5.7				3.5	23.7	32.9	4.8	6.9
White snapper	2.0	2.0	21.7	8.4	14.5	19.6	1.8		1.1					5.1		6.5					16.3	22.8	6.9	3.3
Sole	4.9	0.7	16.5	15.3	3.6	12.2	1.4	1.7	4.8	1.7				4.9		16.4				8.3	7.6	35.7	6.3	5.7
Spots	4.0	2.7	10.5	10.3	8.8	14.1	2.4		7.9		1.5	0.9	1.3	4.3	1.8	11.5		2.6	1.1	4.9	9.0	26.7	11.9	2.2
Swordfish	3.5	1.9	8.0	8.6	7.7	20.0	1.5		5.8	0.9				5.4	0.9	4.4	2.6	1.1	1.6	5.8	18.6	29.7	9.6	3.1
Tilapia	4.8	1.3	15.4	11.7	9.0	20.0	2.2		4.1	1.6			0.6	6.3		1.9		2.3	2.3	5.0	11.6	20.7	13.1	1.6
Rainbow trout	4.7		11.4	8.2	7.3	17.4	12.3	3.5		2.1				1.4	0.9	5.1				2.6	16.8	30.1	13.7	2.2
Ocean trout	1.7		19.1	3.3	8.0	9.9	1.3		1.6					4.1		6.7	0.8		1.7	2.0	39.3	48.0	7.1	6.8
Tuna	3.1		9.5	7.5	7.9	17.5	1.8		2.6	1.2				4.1	0.7	7.5	1.8		2.1	2.5	26.4	37.6	8.0	4.7
Susi tuna	3.1	0.8	14.6	7.3	8.2	19.8	0.6		1.8	0.8				3.7		5.8	1.5		2.2	1.6	27.7	35.9	6.5	5.5
Whiting	2.0	0.5	9.1	3.3	7.9	15.4	1.9	0.7		3.1				3.6		13.2		3.6	2.0	3.7	23.9	44.6	11.1	4.0
Small whiting	4.2	1.3	10.6	5.1	6.2	11.2	3.2	0.6		5.1				3.3		21.3		1.0	1.1	2.2	23.5	52.7	8.6	6.1

[a] Mean of three samples

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Ms received 12/18/86; revised 2/17/87; accepted 3/4/87.

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Presented at the 46th Annual Meeting of the Institute of Food Technologists, Dallas, TX, June, 1986.

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Ms received 10/20/86; revised 2/5/87; accepted 3/23/87.

Functionality and Microbial Stability of Ultrapasteurized, Aseptically Packaged Refrigerated Whole Egg

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ABSTRACT

Raw, liquid whole egg was ultrapasteurized and aseptically packaged to extend the refrigerated shelf-life and maintain functional quality. Nine processes ranged from 63.7–72.2°C for 2.7 to 192.2 sec and resulted in shelf-lives ranging from 4 to >24 weeks at 4°C. Egg pasteurized by an essentially conventional process (26.2 sec, 63.7°C), aseptically packaged and stored at 4°C spoiled in 4–8 weeks. Overall, little or no change was observed in the chemical (protein and solids contents, soluble protein, pH) and functional properties (performance in cakes and custards) of the ultrapasteurized, aseptically packaged, 4°C refrigerated egg compared to raw egg. Egg of acceptable quality may be produced by this process with a 3–6 months shelf-life at 4°C.

INTRODUCTION

THE EGG PRODUCTS INSPECTION ACT of 1970 (FDA, 1971) led to regulations requiring that all egg products be rendered free from *Salmonella* by the application of appropriate pasteurization processes (Cotterill, 1977). Since the implementation of those regulations in 1971, there have been no reported instances of *Salmonella* food poisoning attributable to commercially prepared egg products (Anonymous, 1986). Thus, a relatively mild heat process, for example 60°C for 3.5 min for whole egg, and proper handling practices eliminates *Salmonella* from egg products.

The heat processes used to pasteurize egg products in the United States and other countries (Cunningham, 1977) minimally constitute 9-D treatments with respect to *Salmonella* inactivation; vegetative and sporeforming microorganisms capable of causing post-pasteurization spoilage may survive. Furthermore, spoilage microorganisms may be introduced into the product after processing. The survival of spoilage microflora and their effect on shelf-life of pasteurized, refrigerated eggs are addressed by York and Dawson (1973), who observed shelf-lives of 12 days at 2°C or 5 days at 9°C. To date, there have been no developments which would allow more severe heat treatments to further reduce the numbers of potential spoilage microorganisms without damaging the egg quality. From a practical standpoint, avoiding post-pasteurization spoilage has required: (1) prompt use (<14 days) of liquid refrigerated egg; (2) addition of 10% salt or acidification (pH 5) in conjunction with prompt use of refrigerated egg; (3) dehydration (spray drying, pan drying or freeze drying); or (4) frozen storage. Of the above, freezing has been the primary means to preserve eggs. More than 320 million pounds of eggs are frozen annually in the U.S.

Although providing a long shelf-life, inconveniences result and undesirable changes occur in whole egg due to freezing and thawing. Gelation of egg magma, increase in viscosity, color change and separation of liquids and solids are observed in thawed, frozen whole egg (Cotterill, 1977). Functionality of egg products (Forsythe, 1970) also is impaired by frozen storage (Ijichi et al., 1970; Pearce and Lavers, 1949). Storage and transportation of frozen products are cost intensive compared to refrigerated products. Additionally, one must foresee in advance the product demand to allow time for thawing. The thawed product must be consumed rapidly; therefore, there is an increased chance of waste if large frozen egg containers are involved.

More severe heat treatments (ultrapasteurization) coupled with aseptic packaging have been suggested as a means of gaining extended refrigerated shelf-life of liquid egg and avoiding the costs and disadvantages associated with frozen eggs. Essary et al. (1983) reported significant shelf-life extensions for various egg products pasteurized with a nonconventional heat exchanger and aseptically packaged in sterile glass jars in an ethylene oxide-flushed glove box. Hamid-Samimi et al. (1984) determined the effects of elevated temperatures (55–75°C) on the viscosity and denaturation of whole egg proteins as the basis for developing design criteria for production of ultrapasteurized, extended shelf-life liquid egg (Hamid-Samimi and Swartzel, 1985a, b).

The objective of this study was to determine the potential of thermal processing and aseptic packaging for producing long shelf-life, refrigerated whole egg which would have good functional performance by applying the design criteria developed by Hamid-Samimi and Swartzel (1985a, b) and evaluating the microbiological stability and functional performance of the egg.

MATERIALS & METHODS

ASEPTICALLY PACKAGED liquid whole egg, pasteurized by nine test conditions, was evaluated during storage and compared to raw and commercially pasteurized (minimum process of 60°C for 3.5 min) egg samples.

Raw egg product

Raw, liquid whole egg was obtained from a local breaking plant (Gold Kist, Durham, NC) by manually filling 13.6 kg (30 lb) metal cans with egg from a holding tank agitated to blend ca. 13,600 kg egg. The egg was kept refrigerated before transporting 13.2 km in an insulated truck to the laboratory where it was held overnight at 4°C or allowed to stand at room temperature (20°C) for up to 12 hr prior to processing.

Thermal processes

The process trials were designed to be equal to or more severe than minimum conditions defined by the 9-D line for *Salmonella* inactivation in whole egg (USDA, 1969) and within predetermined limits of denaturation of egg protein defined as percent soluble protein loss (Hamid-Samimi et al., 1984).

The thermal processing system consisted of, in order, a plate-type heat exchanger for preheat, a timing pump, a heating section, holding tubes (various modifications of the No-Bac Unitherm model XLV processing unit, Cherry Burrell Corp., Cedar Rapids, IA, to achieve different holding times) an aseptic homogenizing pump operated at 10.34 mPa, a cooling section and an aseptic packaging system (model AB3-250, Tetra Pak Inc., Dallas, TX). The flow rate was 2.89×10^{-4} m^3/sec (275 GPH) with heating, holding and cooling pipe diameters of 12.45 mm i.d. stainless steel.

Before each process the system was sterilized with hot water to achieve a discharge temperature of at least 121°C for 30 min. Approximately 1 hr prior to each process, 408.2 kg raw egg were transferred from the metal cans to a 568 L vessel (Creamery Package MFG Co., Chicago, IL) and stirred at ca. 30 rpm at room temperature. The processing time was approximately 20 min; therefore, the last liter of

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Table 1—Microbiological methods[a] for analyzing aseptically packaged liquid whole egg.

Organisms/Test	Medium	Reference
Aerobic Colony Count (35°C, 2 d)	Plate count agar[b]	Busta et al. (1984)
Salmonella	Lactose broth[b]	Andrews et al. (1984)
	Selenite cystine broth[b]	
	Tetrathionate broth[b]	
	Bismuth sulfite agar[b]	
	Xylose lysine deoxycholate agar[b]	
	Hektoen enteric agar[b]	
	Triple sugar iron agar[b]	
	Lysine iron agar[c]	
Coliforms	Lauryl sulfate tryptose broth[c]	Mehlman et al. (1984)
	Brilliant green bile broth[b]	
S. aureus	Baird-Parker agar[b]	Bennett (1984)
	Brain heart infusion broth[b]	
	Coagulase plasma (rabbit) with EDTA[b]	
B. cereus	Mannitol yolk polymyxin agar	Harmon (1984)
	KG agar	
Enterococci	KF streptococcus agar[b]	Deibel and Hartman (1976)
Yeast and mold	Acidified potato dextrose agar[b]	Koburger and Marth (1984)
Psychrotrophic microorganisms (7°C, 7d)	Plate count agar[b]	Gilliland et al. (1984)
Spores of facultative and/or anaerobic mesophilic microorganisms (35°C, 2d)	Fluid thioglycollate broth[b]	d, e
Spores of aerobic mesophilic microorganisms (35°C, 2d)	Plate count agar[b]	e, f
Campylobacter[g]	Brucella broth with Roman/Doyle Supplement[h] and 7% lysed horse blood[h]	Doyle and Roman (1982)
	Campy BAP agar	Blaser et al. (1979)

[a] All dilutions were made using 0.1% peptone water.
[b] Difco Laboratories, Detroit, MI.
[c] BBL, Baltimore, MD.
[d] A three-tube most probable number procedure using freshly prepared fluid thioglycollate broth overlaid with sterile mineral oil was used.
[e] Spores were selected by heating diluted samples 15 min at 80°C prior to enumerating.
[f] Spores were enumerated by the aerobic colony count procedure.
[g] Evaluated for egg from processes 1, 2, 3, 4, and 5 only.
[h] Oxoid U.S.A., Inc., Columbia, MD.

Table 2—Times and temperatures of the processes, with equivalent times and temperatures, heating values and theoretical soluble protein yield.

Process number	Holding Time (sec.)	Holding Temp (°C)	Equivalent[a] Time (sec.)	Equivalent[a] Temp (°C)	Total thermal effect[b] ($G \times 10^{42}$)	Theoretical %SP[c]
1	26.2	63.7	30.4	63.1	6.104	99.48
2	92.0	63.8	97.5	63.3	20.75	98.26
3	192.2	65.3	192.4	65.1	72.75	94.03
4	9.2	67.8	15.7	66.5	8.370	99.29
5	56.9	68.2	63.0	67.5	44.58	96.30
6	123.0	68.3	125.5	67.9	99.43	91.90
6.1	123.0	68.9	129.0	68.4	117.6	90.50
7	2.7	71.5	9.5	68.5	8.911	99.25
8	30.1	72.2	36.5	71.5	78.94	93.54
9	56.7	72.0	62.5	71.8	146.8	88.30

[a] Equivalent times and temperatures include the heating experienced in the heat exchanger in addition to the holding tube (Swartzel, 1986).
[b] G-values are determined based on the activation energy (Ea) for soluble protein loss (Hamid-Samimi and Swartzel, 1985b); $G = (te/2.303) \times \exp(-Ea/RTe)$. Where te = equivalent time (sec); Te = equivalent temperature (K) and R = gas constant (Swartzel, 1986).
[c] Percent soluble protein (SP) was calculated as $\% SP = \exp(-2.303 \times \exp(88.8) \times G) \times 100$ (Hamid-Samimi and Swartzel, 1985b).

egg was processed no later than 80 min after removing from the cooler. During processing, product and heating media temperatures were recorded for evaluation of the thermal effect of the heating section and to determine the total thermal effect (G) (Swartzel, 1986).

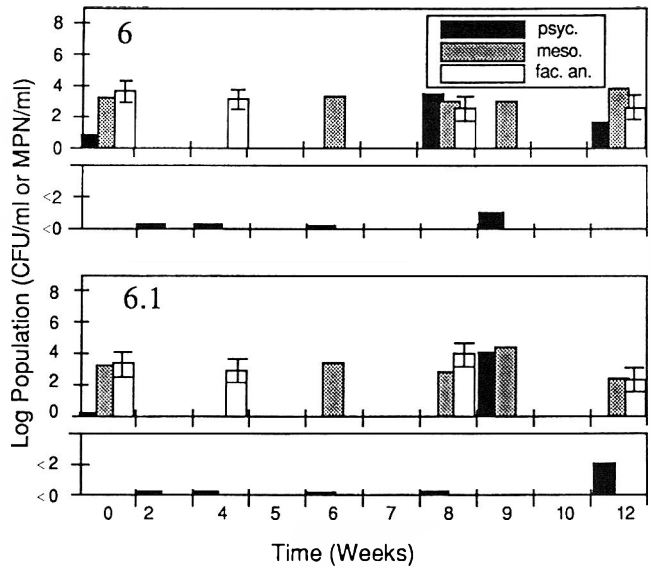

Fig. 1—Comparison of microbiological populations in egg from process 6 and 6.1. Subgraphs show populations which were below detectable limits. Black, grey and white bars indicate psychrotrophic, mesophilic, and facultatively anaerobic or anaerobic mesophilic populations, respectively. Mesophilic and psychtrotrophic microbial populations are reported as colony forming units (CFU)/mL while the facultative or anaerobic populations are reported as the most probable number (MPN)/ml with 95% confidence intervals indicated.

Temperatures were measured using type T thermocouples and recorded on an electronic data logger (model 9302, Monitor Labs., Inc., San Diego, CA). Thermocouples were scanned every 30 sec for all trials except for the first trial where scan intervals were 1 min. Thermocouple locations in the system are detailed by Swartzel and Jones (1985). Average readings from temperature probes at the beginning and end of the holding tube were used with the heating time-temperature curve to calculate equivalent point times and temperatures (Swartzel, 1986). Equivalent point times and temperatures were used to give the best overall thermal description of the heating process. The equivalent point is not dependent on individual constituent kinetics. The activation energy for soluble protein loss was used to calculate G (Swartzel, 1986).

After heating, the temperature was reduced to <10°C in 17.3 to 26 sec, except when prevented by technical difficulties. In those instances, the packaged egg was exposed to −20°C for 20 min prior to refrigeration. The packaging material was a low oxygen permeable laminate of polyethylene, paper and aluminum foil formed into 250-mL containers. Each package was dated and coded so that egg processed in the first 7 min, the second 7 min, and the remaining time was identified. The processed and packaged egg was held overnight at 4°C prior to sampling and storing at 4° or 10°C.

Tests were conducted at nine sets of process conditions, designated 1 through 9. Two days after process 6, a replicate trial (6.1) was performed on the same lot of raw egg. Egg for processes 6.1 to 9 was held at room temperature for not more than 12 hr to achieve a higher initial microbial load and simulate abused product and chilled to 4°C before processing. The 10 processes were performed in numerical order over a 7 month period of time.

Sampling

Samples of raw egg for physical, chemical and functional testing were aseptically removed from the mixing vat ca. 30 min prior to processing and held overnight at 4°C for evaluation with the pasteurized egg. Microbiological samples were removed at the same time and analyzed within 1 hr. Pasteurized samples were obtained the day after processing and at selected times during storage. Sampling of stored egg continued as long as the egg was sensorily sound, as determined by one experienced panelist. The criteria for that judgement were: (1) that the color was normal, i.e., within the expected range of color observed for eggs immediately post-processing; and (2) that there were no objectionable aromas. Because the procedure typically involved sampling at four week intervals, the shelf-life of the product

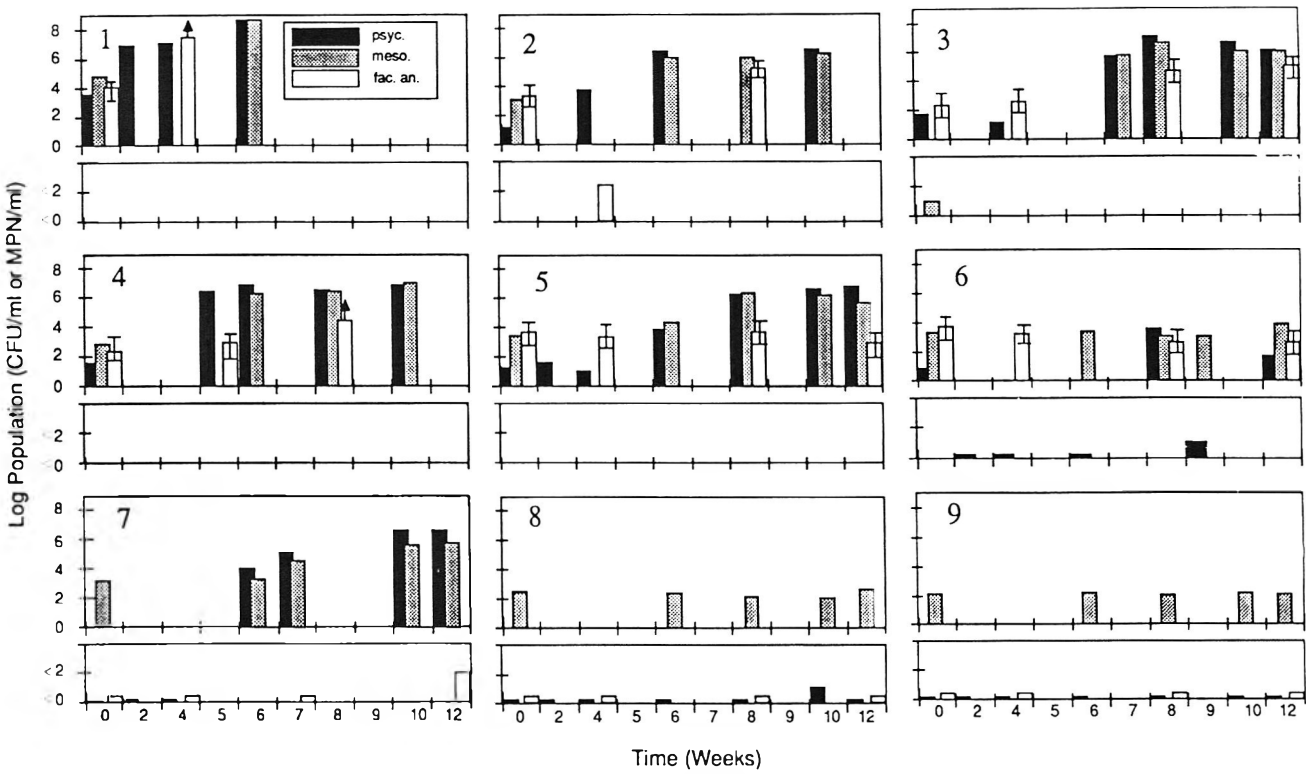

Fig. 2—Numbers of psychrotrophic, mesophilic, and facultative or anaerobic microorganisms/ml of ultrapasteurized, aseptically packaged egg at selected times during 4°C storage. Subgraphs show populations which were below detectable limits. Symbols, CFU, MPN and confidence intervals are as indicated for Fig. 1. Arrows indicate populations greater than those indicated.

Table 3—Microbiological results for raw, processed, and stored liquid whole egg and 4°C shelf-life

	Initial results			Results near end of storage time	
Process	ACC[a] (log_{10} CFU/mL)[b]	Decrease in ACC[c]	Shelf life (wk)	Sampling time (wk)	PCC[d] (log_{10} CFU/mL)
1	6.26	1.6	4-8	6	8.51
2	6.32	3.2	8-12	11	6.52
3	4.23	>3.2	12-16	12	6.08
4	4.82	2.0	8-12	10	6.78
5	5.88	2.4	18-20	16	7.86
6	6.48	2.2	18-24	20	5.67
6.1	7.41	3.2	>24	20	5.95
7	6.23	3.1	12-18	12	6.95
8	7.60	5.1	20-24	20	5.85
9	6.28	4.2	18-20[e]	16	4.49

[a] Aerobic colony count.
[b] Population in raw egg.
[c] Logg reduction of numbers of mesophiles after processing.
[d] Psychrotrophic colony count.
[e] Refrigeration system failed resulting in loss of temperature control in the 20th week of storage.

Table 4—Thermal effects on α-amylase activity, protein content, % soluble protein and viscosity of ultrapasteurized and commercially pasteurized egg compared to raw egg controls

Process no.	Viscosity ratio[c]	α-Amylase (% Relative activity)[a]		Micro-Kjeldahl ratio[b]		% Soluble protein[e]
		UP[d]	Coml	UP	Coml	
1	0.69	93.8	45.5	0.923	1.000	92.7
2	0.71	5.6	ND[f]	0.950	ND	102.3
3	0.79	5.2	ND	0.970	ND	101.3
4	0.80	93.5	21.2	0.997	0.973	101.1
5	0.97	4.6	ND	0.994	ND	100.3
6	0.96	4.7	ND	1.006	ND	100.9
6.1	1.04	3.5	ND	0.987	ND	103.3
7	0.94	6.8	ND	1.013	ND	104.5
8	1.46	6.3	ND	1.021	ND	100.4
9	2.73	6.1	ND	1.003	ND	100.7

[a] Percent relative activity is based on the changes in $A_{585\ nm}$ in the α-amylase assay due to processing. Activity is inversely proportional to absorbance values. Thus, % relative activity = [(A due to commercially pasteurized or ultrapasteurized egg/A due to control raw egg from the same lot)$^{-1}$] 100. Absorbance values due to α-amylase in the raw egg controls ranged from 0.029-0.063.
[b] Calculated as % protein in commercially pasteurized or ultrapasteurized egg % protein in control, raw egg from the same lot. Protein contents of raw egg controls ranged from 10.19 to 13.10%.
[c] Viscosity of ultrapasteurized egg control, raw egg (from the same lot). The viscosity of raw egg ranged from 0.012-0.0129 Pa·sec with a mean of 0.0115 Pa·sec.
[d] UP = ultrapasteurized.
[e] ($A_{280\ nm}$ of ultrapasteurized egg $A_{280\ nm}$ of control, raw egg from the same lot)100 (Hamid-Samimi et al., 1984).
[f] ND = Not Done.

was reported as a range of time in weeks representing the time of sampling when the product was acceptable and the time when the product was judged unacceptable. Egg which was bright yellow, had an off-aroma or a pH value below 7 was judged to be spoiled. Analyses were made on the combined contents of three of four individual packages representing the first, second, and third portions of each process run. When four packages were required, two packages from the second portion of the process were used. Sediment on the bottom of the cartons was scraped out and mixed into the egg by magnetic stirring.

Microbiological evaluation

An aerobic colony count was determined on each lot of raw egg. Processed egg was analyzed the day after processing to determine numbers of mesophilic and psychrotrophic microorganisms, coliforms, *Staphylococcus aureus*, *Bacillus cereus*, enterococci, yeasts and molds, anaerobic or facultative anaerobic mesophilic microorganisms and spores of anaerobic or facultative and aerobic mesophilic microorganisms. Additionally, the presence or absence of *Salmonella* sp. and *Campylobacter* sp. was determined. Procedures and media used are detailed in Table 1. Numbers of selected groups of microorganisms were determined at ca. 4 week intervals for ca. 12 weeks, depending upon when sensory spoilage was detected. Packages of egg were immersed in a freshly prepared sodium hypochlorite (ca. 200 mg/L) solution for ≥15 min and aseptically opened using a sterile surgical blade. Contents of the three cartons

Table 5—Functional properties of cakes and custards made from ultra-pasteurized, raw and commercially pasteurized egg

Variable	Treatment	Means[a]	n[a]	C.V.[a]
	Cakes			
Cake Height (mm)	Raw	65.1	10	1.66
	UP	61.7	10	5.26
	Commercial	64.8	2	0.11
Maximum Force[b] (N)	Raw	10.02	10	17.3
	UP	10.49	10	10.5
	Commercial	9.73	2	2.5
Relaxation[c] Parameter	Raw	0.394	10	3.24
	UP	0.410	10	3.34
	Commercial	0.402	2	0.17
Springiness[d]	Raw	0.83	10	1.94
	UP	0.83	10	2.47
	Commercial	0.82	2	1.32
Water Activity	Raw	0.873	10	2.53
	UP	0.875	10	2.37
	Commercial	0.892	1	—
	Custards			
Penetration Force (N)	Raw	0.82	10	8.20
	UP	0.70	10	8.56
	Commercial	0.79	2	3.69
Custard Height (mm)	Raw	35.7	10	1.66
	UP	35.6	10	5.26
	Commercial	34.6	2	0.11
%Sag	Raw	7.2	10	16
	UP	7.1	10	13
	Commercial	4.6	2	59

[a] Means and coefficients of variation (C.V.) include data from egg of all process trials. The number of trials or raw egg samples for which egg function was evaluated is indicated by n. For each n, 3 measurements taken from each of 2 cakes or 5 measurements taken from each of 2 custards have been used to calculate the mean for each individual trial.

[b] Force required to deform the cake sample by 26 mm.

[c] Asymptotic relaxation value as defined by Peleg and Normand (1983).

[d] Ratio of the second peak force to the first peak force.

Table 6—Functional properties of cakes and custards made from ultra-pasteurized egg stored at 4°C.

Variable[b]	Storage	Means[a]	n[a]	C.V.[a]
	Cakes			
Cake Height (mm)	4	61	10	6.02
	8	64	9	2.06
	12	64	7	2.7
	18	63	2	3.0
	24	65	2	0.22
Maximum Force (N)	4	9.78	10	21.3
	8	10.37	9	20.5
	12	11.29	7	18.2
	18	11.42	2	13.6
	24	10.53	2	12.9
Relaxation Parameter	4	0.3937	10	3.7
	8	0.3876	9	3.9
	12	0.3846	7	3.0
	18	0.3788	2	3.1
	24	0.3745	2	1.5
Springiness	4	0.829	10	1.7
	8	0.834	9	2.0
	12	0.834	7	0.9
	18	0.831	2	0.3
	24	0.815	2	4.9
Water activity	4	0.860	10	4.2
	8	0.884	9	1.1
	12	0.879	7	2.0
	18	0.872	1	—
	24	0.879	2	0.4
	Custards			
Penetration Force (N)	4	0.68	10	12.7
	8	0.70	9	9.1
	12	0.75	7	20.3
	18	0.94	2	12.6
	24	0.76	2	10.5
Custard Height (mm)	4	35	10	2.4
	8	35	9	1.6
	12	35	7	1.2
	18	36	2	0.4
	24	35	2	1.0
%Sag	4	7.1	10	38.1
	8	5.9	9	20.5
	12	6.5	7	19.5
	18	7.2	2	15.9
	24	6.6	2	36.0

[a] Means and coefficients of variations (C.V.) initially include data for egg from all process trials. The numbers of observations (n) decrease with storage time since egg from certain processes spoiled and was not included. See Table 5 for further explanation of n.

[b] See Table 5 for definitions of variables.

were aseptically mixed together, and an 11-mL sample was withdrawn for analyses.

Chemical assays

The pH was determined with a Fisher Accumet pH meter (model 600) equipped with calomel reference and glass indicating electrodes. Solids were determined from the averages of triplicate 2-mL samples dried in disposable aluminum pans in a forced draft oven for 24 hr. The micro-Kjeldahl procedure (AOAC, 1985) was used to determine protein. Percent soluble protein was determined as measure of protein denaturation (Hamid-Samimi et al., 1984). Alpha-amylase activity was determined as described by Shrimpton and Monsey (1962).

Viscosity measurements

A Brookfield Synchro-Lectric viscometer with a UL adaptor and 20-mL cup and bob assembly was used to determine viscosity at 4°, 7°, and 20°C using 20-mL samples (Model LVT, Brookfield Engineering Laboratories, Inc., Stoughton, MA). Shear rates of 0.734, 1.835, 3.671, 7.342, 12.68 and 36.71 sec^{-1} were used. Three readings at each shear-rate and temperature were averaged for viscosity calculations.

Sponge cakes

True sponge cakes were made according to the method of Gorman and Ball (1986) by using half of the ingredient amounts suggested and omitting vanilla. Two separate batches of batter were prepared for each treatment and 340g were baked in rectangular metal pans (21.5 × 11.5 × 6.5 cm, i.d.) at 191°C for 25 min. Cakes were inverted onto a wire rack and held at room temperature overnight. Heights were determined from the mean of four measurements along the center line of the long axis of the cake taken 1 cm and ca 5.5 cm from each edge. Cakes were kept in plastic bags for rheological evaluations within 24 hr.

Textural properties of the cakes were evaluated using an Instron Universal Testing Machine (Model 1122, Instron Engineering Corp., Canton, MA) controlled by a micro-computer (Apple Computer Co., Inc., Cupertino, CA). A plate was connected to a 2000-g load cell and positioned at the surface of the 50 ± 2 mm cube of cake (cut from the center of the cake trimmed free of surface skin). Cross head and chart speeds were 200 mm/min and 100 mm/min, respectively. Three samples from each cake were subjected to a compression-relaxation cycle consisting of a 26 mm compression, 20 sec relaxation, 26 mm decompression (removal of force), 20 sec relaxation, 26 mm compression and 26 mm decompression. The peak forces and relaxation curves were used in analyses.

Water activities at ca 20°C of the cake samples were determined by averaging the readings from three Durotherm (Lufft, West Germany) water activity meters placed in the plastic bags. Each meter was calibrated daily with a barium chloride solution (a_w 0.90 at 20°C).

Custards

Duplicate flan-type custards were made by mixing 190-mL evaporated milk, 196-g sweetened condensed milk, 125-g whole egg, 50-g water, and 1-teaspoon vanilla in a Waring Blendor at low speed for 1 min. The mixtures (360g) were weighed into rectangular metal pans (14.3 × 8.0 × 5.6 cm, i.d.) which had been sprayed with Pam (Boyle-Midway, Inc., New York, NY). Pans were placed in a tray containing a 2.5 cm depth of water, baked at 191°C for 40 min, and stored at 10°C overnight prior to determining the custard height from the mean of three measurements made with calipers along the center line of the long axis of the custard in the pan. Custards were removed from the pans, allowed to stand 45 min at room temperature, and the height was again determined. Percent sag was calculated according to Gardner et al. (1982). Custards were stored in plastic bags at 10°C for rheological evaluation using the Instron Universal Testing Machine within 24 hr.

A 10.5-mm diameter rod with a semi-spherical tip was used to obtain penetration force for each custard. Cross head speed was 100 mm/min and all other parameters were the same as used in the cake evaluations. Five values were averaged to obtain the maximal force at failure for each custard.

Statistical evaluation

This study was designed as a feasibility study. Economic limitations and the nature of the project permitted or warranted only one replica (except process 6) of each process. Additionally, eggs used for the 10 processes came from 9 different lots. Thus, statistical design was compromised by raw product heterogeneity and lack of process replications. To eliminate lot-to-lot variability, a raw sample from each lot of egg was used as a reference for processed egg from the same lot. However, raw product spoiled quickly during storage and could not serve as a reference throughout the experiment. Therefore, we assumed, and the data indicated, that differences between the lots of egg were insignificant. Hence, generally the mean data for ultrapasteurized egg were compared to commercially pasteurized or raw egg. This allowed assessment of the general effect of ultrapasteurization rather than specific, incremental changes due to particular processes. This approach was consistent with the objective of determining the feasibility of this processing concept but did not allow assessment of effects specific to a particular process.

RESULTS & DISCUSSION

Thermal processes

Holding tube temperatures and mean residence times and equivalent point times and temperatures (Swartzel, 1986 are presented in Table 2. The equivalent point time and temperature included the thermal contribution of the heat exchanger as well as the holding tube and was always less than the holding tube temperature. Processes 3, 9, 6.1, 6, and 8 had the highest G values, thus are considered the most severe processes. Theoretical percent soluble protein (based on the total thermal effect, G) also is shown in Table 2.

Microbiological properties of processed eggs

Table 3 presents aerobic colony counts and the log reductions in aerobic colony counts (log population in raw egg less the log population in ultrapasteurized egg measured the day after processing) for egg from each of the processes. The raw egg was microbiologically diverse, with initial populations of aerobic mesophilic microorganisms ranging from 1.6×10^4 to 4×10^7 colony forming units (CFU)/mL. For the first six processes, raw egg was continuously refrigerated until mixing for pasteurization. The egg was generally of high quality and the numbers of microorganisms present were frequently low ($<10^5$ CFU/mL). Since this study was intended to explore the feasibility of this process, the raw egg for processes 6.1 to 9 was held at room temperature from 4 to 12 hr to simulate abused egg and consequently lower the microbiological quality. The additional treatment of egg for process 6.1 (4 hr at room temperature plus 2 days at 4°C) resulted in an initial mesophilic population approximately ten-fold (1 log) greater than in egg for process 6.

Heat treatments reduced the aerobic colony counts by 1.6 to 5.1 log cycles. The decrease in mesophilic population of eggs for process 6.1 was one log cycle greater than process 6, off-setting the increased initial numbers, so that the numbers of mesophilic microorganisms in the freshly processed product from processes 6 and 6.1 were essentially identical. This suggested that the population of microorganisms which proliferated most rapidly during abuse of the raw egg was relatively heat sensitive. Indeed, the numbers of mesophilic, psychrotrophic and facultative or anaerobic mesophilic microorganisms were essentially identical in freshly processed (0 weeks storage) egg from processes 6 and 6.1 and did not differ substantially during storage (Fig. 1).

The reduction of mesophilic populations was >3 log cycles for egg from processes 2, 3, 6.1, 7, 8, and 9, with trials 8 and 9 resulting in the largest (>5 and >4 logs, respectively) reductions (Table 3). The total thermal effect (Table 2) suggests that processes 3, 6, 6.1, 8, and 9 should have resulted in the most inactivation, which compares favorably with the observed reduction in mesophilic populations. Only relative comparisons of the log reduction in mesophilic populations can be made since this value will depend upon the heat sensitivity of the microbial flora present which may vary from lot-to-lot and due to storage prior to processing. Hence, perfect correlation of relative process severity and reduction in numbers of the heterogeneous microbial flora cannot be expected.

The approximate shelf-life of egg from each trial is presented in Table 3. Figure 2 illustrates the populations of psychrotrophic, mesophilic and facultative or anaerobic microorganisms present at selected times during storage up to 12 weeks at 4°C. The data in Fig. 2 indicate that each of these populations may increase in number during storage at 4°C. The increase in numbers of mesophiles probably reflected the psychrotrophic population increase. Egg pasteurized at a time-temperature combination which was essentially a standard pasteurization process (process 1) had the largest population of each of these groups of microorganisms initially, and populations rapidly exceeded 10^7 CFU or most probable number (MPN)/mL. Although a relationship could not be established between spoilage and the number of any single group of microorganisms examined, egg from processes 6, 6.1, 8, and 9 generally had both the lowest initial populations and the least development of significant numbers of microorganisms during storage at 4°C (Table 3 and Fig. 2). Egg from processes 8 and 9, which did not spoil earlier than 18 weeks, had neither detectable psychrotrophic nor facultative or anaerobic populations through 10 or 12 weeks of storage and mesophilic populations never exceeded 10^3 CFU/mL throughout this storage period. These data are in agreement with data of York and Dawson (1973) from thawed refrigerated pasteurized egg and indicate that a unique microorganism or group of microorganisms is not responsible for spoilage. Undoubtedly, spoilage is dependent on the initial microbial population in raw egg, the types of organisms and times and temperatures of processing and storage.

Psychtrotrophic populations were monitored throughout the 4°C shelf-life of the egg. The psychrotrophic populations at the sampling time nearest but not exceeding the end of the 4°C shelf-life are presented in Table 3 and ranged from 3.1×10^4 to 3.2×10^8 CFU/mL. The metabolic activities of the particular spoilage microorganisms will influence the numbers of microorganisms required for spoilage to be detected. A variety of microorganisms were isolated from the processed egg immediately after processing and during storage at 4°C for several weeks. Identification and characteristics of these isolates are reported by Foegeding and Stanley (1987).

The processed egg was *Salmonella*-negative (evaluated for all processes) and *Campylobacter*-negative (evaluated for egg from processes 1-5) (<1/25 mL and <1/10 mL, respectively). For each trial and at each sampling time populations of *S. aureus*, *B. cereus*, coliforms, enterococci, yeasts and molds, spores of aerobic mesophilic microorganisms and spores of anaerobic or facultative anaerobic mesophilic microorganisms were below the detection limits except in the following instances. Egg from process 8 contained 5.0 MPN anaerobic or facultative anaerobic mesophilic microorganisms/ml immediately after processing. Egg from processes 2 and 4 contained 2.5 CFU and 2.0 CFU enterococci/mL immediately after processing; these populations remained constant or increased slightly during storage. Egg from process 5 contained 5.5 CFU yeast/mL immediately after processing and this population decreased below detectable limits during storage. Egg from process 2 contained 1.8×10^1 CFU *B. cereus*/mL immediately after processing and remained essentially constant during storage.

The shelf-life of ultrapasteurized egg stored at 10°C never

exceeded 4 weeks, highlighting the importance of a low storage temperature. Extensive microbiological and functional studies are not reported for egg stored at 10°C because of the unacceptably short shelf-life.

Physical and chemical properties of processed egg

Ultrapasteurized egg, commercially pasteurized egg and raw control egg did not differ overall in solids. Average solids were 24.5, 24.6, and 24.8% for ultrapasteurized, commercially pasteurized and control egg, respectively. The average pH values of egg from all processes were 7.5, 7.1, and 7.5 for ultrapasteurized, commercially pasteurized and control egg. The thermal processes had essentially no effect on protein or soluble protein (Table 4). Ranges of the micro-Kjeldahl ratios and percent soluble protein of processed relative to control egg were 0.923–1.021 and 92.7–104.5%, respectively. The apparent increase in the amount of protein in processed egg (>1 micro-Kjeldahl ratio or >100% soluble protein) was probably due to the shearing effect of the system (homogenizing valve, etc) which might disperse aggregated egg components. Since processing had a minimal effect on solids and protein, pH values, and the percent soluble protein contents of liquid whole egg, observed differences in rhelogical and functional properties of the egg must result from the effect of processing on the egg components rather than a gross composition change.

Viscosity of protein solutions frequently is used as an indicator of a heating effect on, or denaturation of, proteins. Table 4 indicates the effect of the thermal processes on egg viscosity at one temperature (20°C) and one shear rate (7.342 sec^{-1}). Data from other conditions followed a similar pattern, thus are not reported. Processes with lower G values (1, 2, 4, and 7) had little impact on viscosity. For these processes, the heating and homogenization effects appeared to balance each other so that the net effect was essentially the same viscosity as the raw egg controls (viscosity ratio ca. 1.0). With the exception of process 3, processes with higher G values (6, 6.1, 8, and 9) and process 5 appeared to show some thermal effect on viscosity. Thus, the heating effect was great relative to the homogenization effect resulting in increased viscosity of the processed egg relative to the raw egg controls (viscosity ratio 1.46 or 2.73). Alpha-amylase activity assays (Table 4) indicate that the enzyme was inactivated by mild heat processes which essentially did not affect viscosity. The lack of alpha-amylase heat stability is in agreement with results reported by others (Cunningham, 1977).

The percent soluble protein and and proximate analysis data indicated that the processes did not result in extensive damage to the egg proteins although the viscosity data suggested some denaturation occurred. Electropherograms obtained from the heated egg (not shown) were similar to those published by Woodward and Cotterill (1983), indicating that there were heat effects on individual proteins. The theoretical percent soluble protein values presented in Table 2 predicted a more severe effect of processing on soluble protein than was observed (Table 4). The theoretical values were based on studies conducted with small amounts of egg (1 mL) heated in the cup of a Brookfield viscometer with various shear rates applied to simulate egg under the dynamic conditions of conventional heat exchangers (Hamid-Samimi et al., 1984). This model system may have over estimated the processing effect on egg protein and resulted in a successful, but conservative, process design for this study. Alternatively, thermally initiated aggregation that may have occurred during the holding period may have been dispersed by homogenization and the mechanical effects of the pumps, resulting in a positive physical effect on the heated protein. Dispersion of aggregates or other factors favoring solution or formation of stable sols in the solubility test would partially explain the difference in actual and model system estimates of percent soluble protein. Further investigations on the effect of homogenization on functionality of long shelf-life liquid whole egg are needed.

Function of processed eggs

The functional properties of the processed eggs agreed with the physical and chemical results; data presented in Table 5 for freshly ultrapasteurized egg indicated that the thermal process had little effect on the performance of egg in cakes and custards relative to raw or commercially pasteurized egg. Cake height (indicator of the leavening ability), force to deform, relaxation parameter (Peleg and Normand, 1983), and springiness (indicators of textural properties) were similar for all eggs examined. The water activities of the cakes were similar indicating that experimental handling procedures were uniform, thus should not have affected the textural evaluations. The custard properties indicated that all evaluated egg had good ability to form gels. The relatively low percent sag suggested the gels had excellent integrity, yet penetration forces suggested the gels were tender. Although weep was not measured, little syneresis was noted even after holding the custards overnight. Limited sensory evaluation of the processed eggs indicated that the thermal processes did not affect flavor or aroma in scrambled eggs. The USDA (1984) palatability scores were 7 to 8 (data not presented), with 8 being the best possible score.

Refrigerated storage effect on chemical and functional properties of processed egg

As long as the processed egg was not obviously spoiled, as indicated by sensory evaluations, storage at 4°C maintained the chemical and functional properties of the processed egg for up to 24 weeks for some samples (Table 6). Means and coefficients of variation suggested rheological properties of cakes and custards, cake and custard heights, cake water activities and % sag of custards were not significantly affected by time of refrigerated storage at 4°C. Functional properties of egg stored at 10°C also were maintained throughout the shelf-life (data not presented).

The stored egg did not change in solids or pH (data not presented). Visual observations revealed little sedimentation during storage. A thin layer of a fine, light-yellow, granular-like material was observed on the bottom of cartons after 4 weeks of refrigerated storage and, although not quantified, did not appear to increase during storage beyond 4 weeks. Martinez and Maurer (1975) observed that homogenized egg held for ≤ 1 week under refrigeration did not separate as quickly as non-homogenized eggs held under the same conditions. The homogenization process applied in this study probably helped to avoid extensive separation and sedimentation of the egg during storage.

CONCLUSIONS

OVERALL, little or no change was observed in the chemical and functional properties of the ultrapasteurized, aseptically packaged, 4°C refrigerated egg compared to raw egg. This indicated that a product with superior quality compared to frozen egg could be produced. Egg stored at 10°C spoiled 2–8 times faster than when stored at 4°C, highlighting the significance of an acceptable, low storage temperature to the total process. The types and numbers of microorganisms causing spoilage varied reflecting the heterogeneous microflora of the raw product. The egg industry may benefit practically and economically from such a product, although studies must be conducted to determine the economy of a given process. Prior to application, optimization of the process and storage conditions must be considered.

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Ms received 11/1/86: revised 2/7/87; accepted 2/27/87.

Paper Number 10750 of the Journal Series of North Carolina Agricultural Research Service, Raleigh, NC 27695-7601.

The use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Research Service of the products named, nor criticism of similar ones not mentioned.

Financial assistance from the Southeastern Poultry and Egg Association and the North Carolina Board of Science and Technology is gratefully acknowledged. We thank J. McGuire, N. Stanley, and S. Winn for technical assistance.

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Ms received 10/22/86: revised 2/10/87; accepted 2/27/87.

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

Growth and Inactivation of Microorganisms Isolated from Ultrapasteurized Egg

P.M. FOEGEDING and N. W. STANLEY

ABSTRACT

Microorganisms which survived and grew at 4° or 10°C in ultrapasteurized, aseptically packaged liquid whole egg were isolated and identified. Growth at 4° and 10°C, and inactivation from 50 to 70°C, of selected isolates were evaluated in ultrapasteurized egg. *Bacillus circulans* and a *Pseudomonas* species grew well at 4°C, with generation times of 1.0 and 0.3 days, respectively. *Enterococcus faecalis* and *B. circulans* were the most heat resistant isolates studied. Because *B. circulans* grew at 4°C and was relatively heat resistant it would be a useful test organism for many applications while *E. faecalis* would be useful since it was heat resistant and would grow rapidly when the storage temperature is less than ideal.

INTRODUCTION

IN THE UNITED STATES, liquid whole egg is pasteurized at a minimum of 60°C for 3.5 min. Other countries have slightly different pasteurization requirements. Pasteurization is designed to produce a salmonellae-negative product and prolongs the shelf life of the egg. Kraft et al. (1967) reported pasteurization extended the shelf life of liquid egg yolk by 4 days at 10°C and 20 days at 1.7°C. York and Dawson (1973) reported that the shelf life of pasteurized (61°C, 3.5 min) liquid whole egg was 12 and 5 days when stored at 2° and 9°C, respectively. Because the refrigerated shelf life is relatively short, the pasteurized product is typically frozen. Essary et al. (1983) suggested an increased thermal treatment followed by aseptic processing to produce pasteurized liquid whole egg with a long refrigerated shelf life. In a pilot-plant feasibility study, Ball et al. (1987) verified that ultrapasteurization and aseptic processing produced liquid whole egg with good functional properties and a refrigerated shelf life of more than four months.

Ball et al. (1987) observed that several types of spoilage of ultrapasteurized egg eventually occurred, depending on the particular microorganisms present in the raw product which survived the thermal process and proliferated. Several reports detail the types of microorganisms which contaminate egg shells and raw liquid whole egg (Board et al., 1964; Mayes and Takeballi, 1983; Wrinkle et al., 1950). Debris on the egg shell is largely responsible for the microorganisms in raw egg products. *Micrococcus, Enterococcus* (formerly *Streptococcus* sp.; Moore et al., 1985), *Bacillus, Escherichia, Pseudomonas, Flavobacterium* spp. and others frequently are identified on or in raw eggs. Despite the initial diversity, the microorganisms present in spoiled eggs have remained relatively constant over time and geographical location (Board, 1977). Thus, intrinsic egg properties largely dictate the types of spoilage microorganisms which develop.

Pasteurization or ultrapasteurization select for the more heat-resistant spoilage organisms. Shafi et al. (1970) analyzed nine pasteurized, frozen whole egg samples from five plants and reported averages between 6 x 10^6 and 6.3 x 10^2 colony forming units (CFU)/g of mesophilic aerobic miroorganisms, Gram-positive cocci, coliforms, *Pseudomonas* (fluorescent-type) sp., anaerobic or faculatively anaerobic microorganisms and psychrotrophic microorganisms. Payne et al. (1979) reported coryneform bacteria related to *Microbacterium lacticum*, many unidentified cocci or coccobacilli and *Bacillus* sp. typically survived pasteurization (65°C, 3 min) in egg melange. The most common and most heat resistant isolates were the coryneform types. Two such isolates had $D_{80°C}$-values of ca. 10 and 7 min in 100 mM potassium phosphate buffer, pH 7.1, grew well in laboratory media at 10°C, but grew poorly or did not grow at 5°C. Using four strains of *Streptococcus faecium* and four strains of *S. faecalis*, Payne and Gooch (1980) found from 0.001% to 94.6% of the initial populations survived 3 min at 65°C in egg. For four of the eight strains, cultures grown in egg rather than laboratory medium prior to pasteurization were more heat resistant. However, two strains were equally resistant and two were less resistant if prepared in egg.

One objective of this study was to identify microorganisms which survived ultrapasteurization of egg and to evaluate the ability of these organisms to grow in liquid whole egg at 4° and 10°C. A second objective was to evaluate the resistance of the isolates when heated in egg at or near pasteurization or ultrapasteurization temperatures. These data will expand our knowledge of potential spoilage microflora for ultrapasteurized egg and provide information for selecting indicator organisms to be used in process design and evaluation or for evaluating quality of raw egg intended for ultrapasteurization.

MATERIALS & METHODS

Test microorganisms

Microorganisms were isolated from liquid whole egg which had been ultrapasteurized as detailed previously (Ball et al., 1987). Ultrapasteurization times and temperatures ranged from 2.7 to 192.2 sec and 63.7° to 72.2°C. The microorganisms were selected immediately after ultrapasteurization or during storage of the ultrapasteurized egg at 4° or 10°C. The twenty-eight isolates were selected preferentially from egg which had received the more severe heat treatments tested and from egg stored at 4°C or from psychrotrophic colony counts of egg stored at 10°C. Gram stains, cellular and colony morphology and origin were recorded for each isolate. From these isolates, four representative microorganisms were selected for further study. Additionally, *Bacillus cereus* T was selected for comparative studies as a reference strain. The identity, origin and tests used for classification of these four isolates and *B. cereus* T are presented in Table 1. Cultures were maintained on TSA slants at 4°C. Spores of *B. cereus* T and the *B. cereus* isolate also were prepared and maintained at 4°C as detailed previously (Foegeding et al., 1986).

Medium for growth and inactivation studies

To evaluate growth and inactivation of these isolates in liquid whole egg, a relatively clean, or ideally sterile, product was needed since (1) growth of the surviving microflora would occur with few competitors, and (2) a system was needed to detect the test isolates amongst competitors. Therefore, ultrapasteurized egg was selected for the growth and inactivation studies. Growth was evaluated in a 50:50 v/v mixture of egg processed at 72.2°C for 30.1 sec and at 72.0°C for 56.7 sec (processes 8 and 9 reported by Ball et al., 1987) and frozen at −40°C in 250-mL laminated paper packages with low oxygen permeability for 10–12 wk immediately after processing. The aerobic mesophilic and psychrotrophic populations of egg from these processes were each

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Table 1—Sources and identification scheme of test and reference microorganisms studied

Microorganisms	Origin[a] and remarks	Number of trials from which similar isolates were recovered	Tests used for identification
Bacillus cereus	92sec/63.8°C; 0 wk storage	*B. cereus* from 2 process trials.	No acid from mannitol aerobically, lecithinase +, ground glass-appearing colonies on Mannitol Yolk Polymyxin agar (35°C, 24 hr); Gram +, catalase +, oxidase −, sporeforming rods[c]
B. cereus T	NCSU culture collection[a]	NA[b]	NA
Bacillus circulans	192sec/65.3°C; 8 wk storage at 4°C	11 Gram + sporeformers (not all *B. circulans*) from 7 process trials.	Oxidase −, catalase +, Gram + sporeforming rod; spores were elliptical and located centrally to terminally; acid without gas from glucose aerobically, acetoin not produced, no growth at 55°C, starch hydrolysed, casein not decomposed, growth on Sabourand agar[d,e]
Pseudomonas sp.	56.9sec/68.2°C; 8 wk storage at 4°C	3 *Pseudomonas* sp. from 3 process trials.	Yellow colonies on PCA at 7°C, Gram −, catalase +, oxidase + rods, oxidative in OF glucose[f]
Enterococcus faecalis (formerly *Streptococcus faecalis*)	123sec/68.3°C; 4 wk storage at 10°C	*Enterococcus* sp. from 8 process trials (all from 10°C stored egg).	Small white colonies on PCA, catalase −, oxidase −, Gram + cocci in short chains; small red colonies on KF agar; grew in BHI at 10°C and 45°C, with 7.5% NaCl (37°C), arabinose −[g,h]

[a] Process temperature/time and weeks of storage at indicated temperature of ultrapasteurized egg from which the microorganism was isolated and/or other appropriate information.
[b] Not applicable.
[c] Harmon and Goepfert (1984).
[d] Gordon et al. (1973).
[e] Gibson and Gordon (1974).
[f] Douderoff and Palleroni (1974).
[g] Diebel and Seeley (1974).
[h] Moore et al. (1985).

Table 2—Types of microorganisms isolated from ultrapasteurized egg

Microbial type	Number isolated
Gram + cocci	8
Gram + rods	
sporeforming	11
nonsporeforming	4
Gram − rods	5

$<10^{\circ}$ CFU/mL. Five cartons of egg from each process were thawed overnight in the refrigerator, aseptically opened (Ball et al., 1987) and mixed together, distributed in 125-mL aliquots in polyethylene bottles and frozen at −20°C for growth studies.

The egg for inactivation studies was similarly ultrapasteurized at 72°C for 90 sec. Cartons (250 mL, laminated paper) were refrigerated overnight, aseptically opened and mixed together, and 10- and 50-mL volumes were aseptically dispensed into polyethylene bottles and frozen at −20°C until needed (≤ 6 mo). Prior to use, egg for growth or inactivation studies was thawed overnight at 4°C.

Growth studies

Each isolate was grown individually in Trypticase Soy Broth (TSB; BBL, Baltimore, MD) to provide an inoculum for the egg. The TSB cultures were incubated at 35°C for 24 ± 2 hr except the *Pseudomonas* species which was incubated at room temperature (ca. 21.5°C) for 24 ± 2 hr.

The TSB populations were estimated by direct microscopic count and appropriate dilutions were made in 0.1% peptone (Difco Laboratories, Detroit, MI) water for inoculation of a thawed 125-mL volume of egg to achieve ca. 10^2-10^3 CFU/mL of egg with ≤1 mL of inoculum. The inoculated egg was stirred with a sterile magnetic stir bar at a medium speed for 5 min at room temperature. Three-mL aliquots of the inoculated egg were dispensed into sterile test tubes, overlayed with 1 mL of sterile mineral oil to minimize free oxygen exchange, and the tubes were split into two groups for incubation at 4°C and 10°C.

Initially and at selected time intervals one tube of egg was diluted in 0.1% peptone water, and the population was enumerated by the spread plate procedure on Trypticase Soy Agar (TSA; BBL) after 48 hr incubation at the temperature specified above for growth in TSB, except the *B. cereus* cultures which were incubated 24 hr. Populations also were enumerated on a selective and differential medium where appropriate. Selective and differential media employed were crystal violet tetrazolium agar (incubated at ca. 21.5°C, 2 days) for the *Pseudomonas* sp. (Gilliland et al., 1984), KF Streptococcus agar (incubated at 35°C, 2 days) for *E. faecalis* (Deibel and Hartman, 1984), and mannitol yolk polymyxin agar (incubated at 35°C, 24 hr) for *B. cereus* (Harmon and Goepfert, 1984). *B. circulans* was enumerated on TSA only and colony and microscopic cell morphology were used to verify that the counts were the test microorganisms and not an organism present due to the egg microflora. Counts obtained from the selective and differential media were always essentially identical to TSA counts, indicating the test isolate was enumerated by TSA without interference from the egg microflora. This was verified by colony and microscopic cell morphology. Therefore, populations reported herein represent those enumerated using TSA.

Inactivation studies

Isolates were grown individually in TSB as detailed above to obtain an active culture for inactivation trials. Spores of *B. cereus* T and the *B. cereus* isolate were used to inoculate TSB, the culture was heat shocked at 70°C for 15 min, and incubated 24 hr at 35°C.

Except for the *B. circulans* culture for which a 1-mL inoculum was used, 0.1 mL of each TSB culture was used to inoculate 10 mL thawed egg and the inoculated egg was incubated 18-24 hr at the same temperature used for the TSB culture. A 50-mL volume of egg was mixed and 4-mL aliquots were dispensed into 16 x 150 mm test tubes. Each 4-mL tube of egg was inoculated with 1 mL of the selected egg culture diluted 100-fold in 0.1% peptone water. Thus, the population was ca. 10^6 CFU/mL egg for each inactivation study.

Inactivation temperatures ranged from 50° to 70°C. Inoculated egg was placed in the water bath, held to allow the egg to reach the inactivation temperature, and 0-time and subsequent samples were removed. Heated samples were immediately placed in ice water to cool. The cooled egg was promptly diluted with 0.1% peptone water and survivors were enumerated using TSA spread plates as detailed above.

A type K thermocouple connected to a digital thermometer (Model 871, Omega Engineering Inc., Stamford, CT) was placed in one tube of egg inoculated with peptone water to monitor temperature throughout each inactivation trial. The come-up time was approximately 2 min and populations declined ≤0.5 log cycle during this period.

RESULTS

THE TYPES of microorganisms isolated from the egg and frequency of isolation are detailed in Table 2. The data pre-

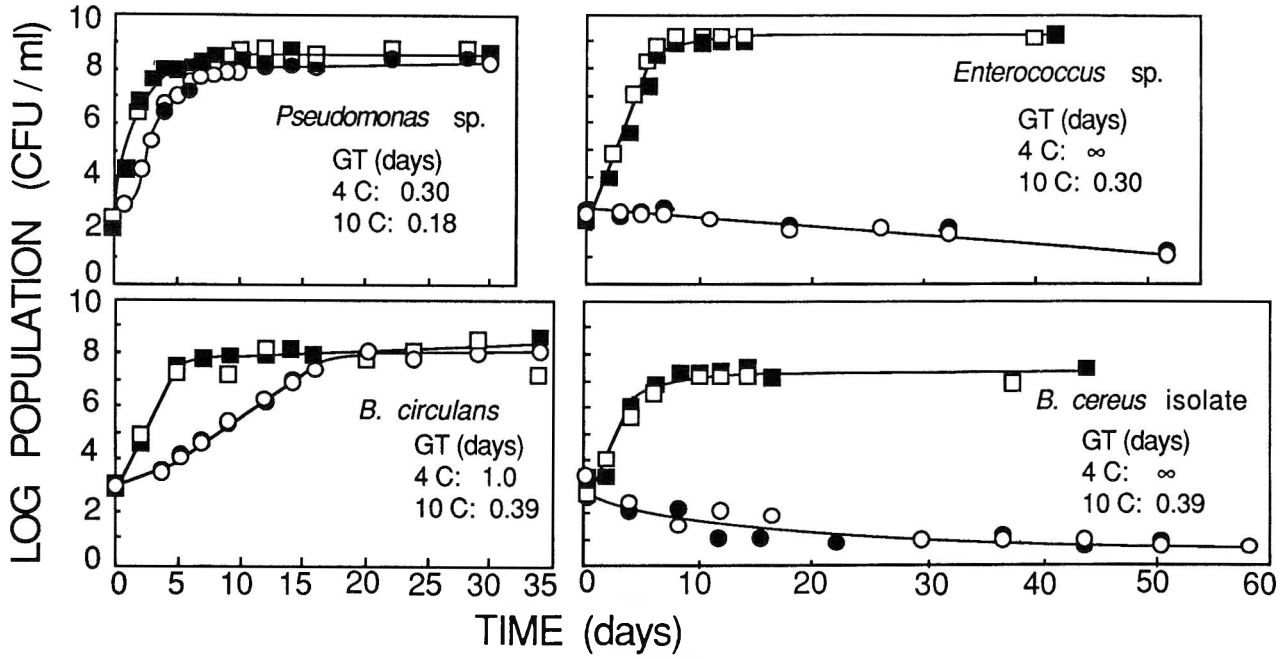

Fig. 1—Growth in ultrapasteurized egg at 4°C (○, ●) and 10°C (□, ■) of Pseudomonas *sp.,* Enterococcus *sp.,* B. circulans *and* B. cereus *isolated from ultrapasteurized egg. Open and closed symbols represent duplicate trials. Generation times (GT) in days are reported at each temperature (GT infinitely long are indicated by ∞).*

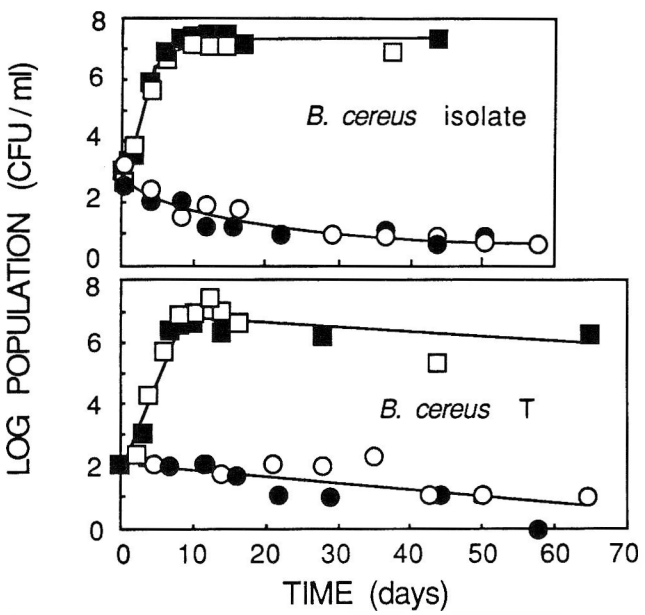

Fig. 2—Growth in ultrapasteurized egg at 4°C (○, ●) and 10°C (□, ■) of B. cereus *T and a* B. cereus *strain isolated from ultrapasteurized egg. Open and closed symbols represent duplicate trials.*

sented in Table 2 should not be used to infer population profiles of the egg; this information has been presented by Ball et al. (1987). The four isolates selected for further studies (Table 1) were chosen to represent the various types of organisms present, except a Gram-positive nonsporeforming rod was not included since preliminary studies using two of these isolates indicated growth was extremely slow and sporadic in egg.

Figure 1 shows growth curves for each of the four ultrapasteurized egg isolates monitored at 4° and 10°C in ultrapasteurized egg. Of the four microorganisms from egg, only the *Pseudomonas* sp. and *B. circulans* grew in egg at 4°C, with generation times of 0.3 days and 1.0 days, respectively. Numbers of *Enterococcus* sp. and *B. cereus* slowly declined at 4°C. Generation times at 10°C were 0.21, 0.33, 0.34 and 0.48 days for *Pseudomonas* sp., *B. circulans*, *Enterococcus* sp. and *B. cereus*, respectively. Maximum populations reached during the stationary growth phase at 10°C ranged from ca. 3.0 x 10^7 to ca. 1.5 x 10^9 CFU/mL for *B. cereus* and the *Enterococcus* sp., respectively. Figure 2 compares growth of the *B. cereus* isolate to *B. cereus* T. These two organisms grew similarly at 10°C and neither culture grew at 4°C.

Survival curves in egg are presented in Fig. 3 for each of the four isolates from egg. The background population in the egg used for these inactivation studies was ca. 5 x 10^2 CFU/mL ($\log_{10}$ = 2.70). This background microflora survived the ultrapasteurization process (72°C, 90 sec) and, therefore, was relatively heat resistant. The survival curves representing higher thermal inactivation temperatures for each test microorganism tailed at ca. 1.7 to 3.3 $\log_{10}$, reflecting primarily or in part the microflora of the egg, as confirmed by colony morphology.

Decimal reduction time curves were constructed (Fig. 4) from D-values (time required for a 90% reduction in the population) determined from the initial portions of the survival curves (Fig. 3), prior to the appearance of tails. The z_D-values (change in temperature resulting in a 10-fold change in the D-value) of the *Enterococcus* sp., *Pseudomonas* sp. and *B. circulans* were each 6.3°C. The z_D-value for the *B. cereus* egg isolate was 4.6°C. The responses to temperature of each of the egg isolates indicated that the relative sensitivities of the egg isolates to temperature were essentially the same throughout the inactivation range studied (ca 50–70°C). Inactivation kinetics of vegetative cells of the reference *B. cereus* T culture and *B. cereus* egg isolate differed (Fig. 5 and 6). The z_D-values in egg were 3.1°C and 4.6°C for *B. cereus* T and the egg *B. cereus* isolate, respectively.

DISCUSSION

Ultrapasteurized, refrigerated egg ultimately will spoil due to growth of psychrotrophic microorganisms. The organisms causing spoilage are dependent on (1) the microflora of the raw product, (2) the process time and temperature and microorganisms surviving the process, and (3) the storage environ-

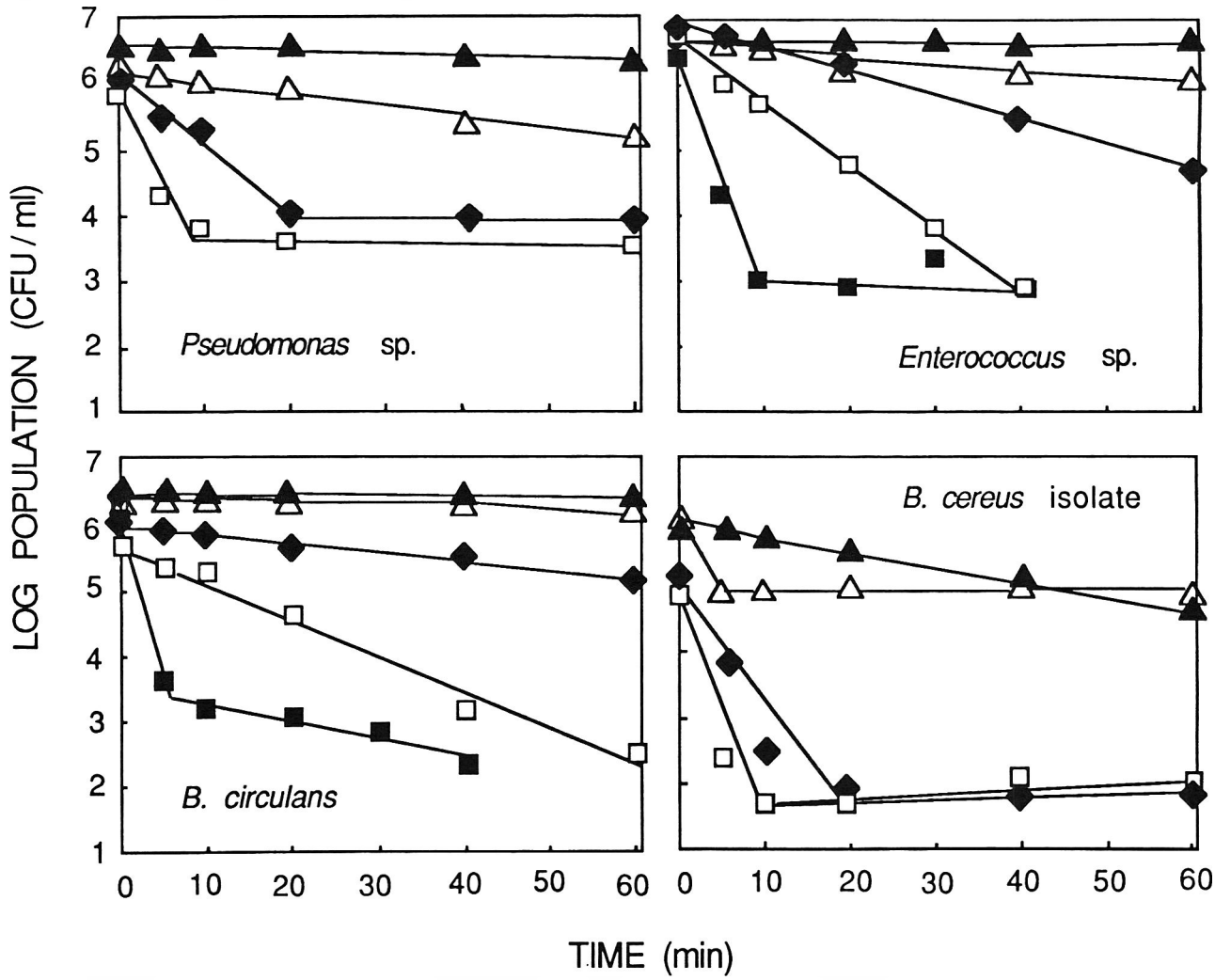

Fig. 3—Survival curves for four microorganisms isolated from ultrapasteurized egg. Inactivation was in ultrapasteurized egg. Pseudomonas *sp. was inactivated at 49.5°C (▲), 52.5°C (△), 56.8°C (♦), 61.1°C (□).* Enterococcus *sp. was inactivated at 52.6°C (▲), 57.1°C (△), 60.5°C (♦), 65°C (□), and 69.0°C (■).* B. circulans *was inactivated at 49.5°C (▲), 54.0°C (△), 58.0°C (♦), 61.6°C (□), and 65.9°C (■).* B. cereus *was inactivated at 50.6°C (▲), 54.6°C (△), 52°C (♦), and 56°C (□). Data represent resistance of vegetative cells.*

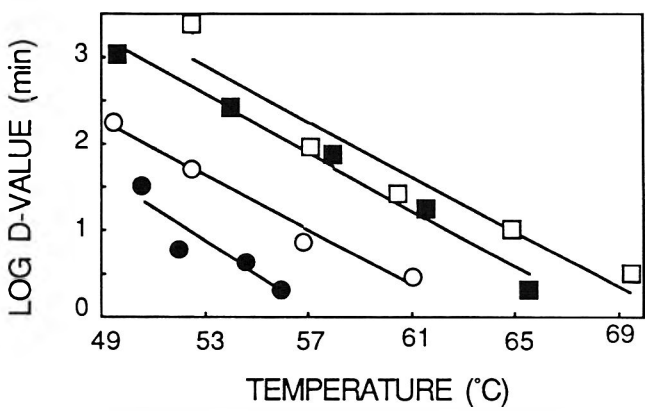

Fig. 4—Decimal reduction time curves for Enterococcus *sp. (□),* B. circulans *(■),* Pseudomonas *sp. (○), and* B. cereus *(●) isolated from and inactivated in ultrapasteurized egg. Data represent resistance of vegetative cells.*

ment. The storage environment includes nutritional quality of the egg (relatively constant), aerobioses of the product (relatively constant for a given package) and temperature. Commercially, storage temperature will vary among batches of processed egg and during storage of any given lot or package of egg. Ideally, refrigeration should be a low, constant temperature. Realistically, the storage temperature will fluctuate and exposure to temperatures ranging from 4-10°C is expected.

The microorganisms which originally were isolated from egg stored at 4°C were able to grow well at 4°C, with generation times for the *Pseudomonas* sp. and *B. circulans* ≤1 day. Using the generation times determined in this study and assuming spoilage of the product occurred when the populations reached 10^7 CFU/mL, if one *B. circulans* cell was present per 250 mL of the freshly ultrapasteurized egg, then the product could spoil in 34 days assuming no injury or prolonged lag time. Using the same assumptions for the *Pseudomonas* sp., spoilage could occur in 9 days. The carton size used in a previous feasibility study was 250 mL (Ball et al., 1987). Egg from which these *B. circulans* and *Pseudomonas* isolates were recovered took much longer times (>12 wks and >18 wk, respectively) to spoil than these predictions suggested. This must primarily reflect the injured nature of the cells surviving the process as well as competition for growth factors and antagonism among the few various types of microorganisms surviving the process. The growth curves presented in this study resulted from an inoculum of 10^2 to 2 x 10^3 healthy cells/mL which had been grown in, and therefore, metabolically adapted to, ultrapasteurized egg from the same lot. Hence, these spoilage predictions reflect worst case conditions for ultrapasteurized egg stored constantly at 4°C.

Each test isolate grew well at 10°C, with generation times of <10 hr. This was expected since isolates were recovered

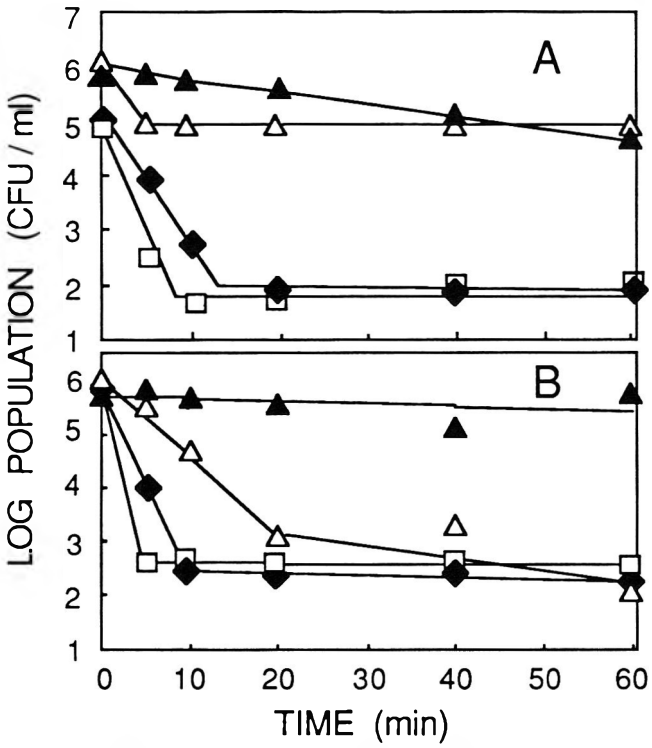

Fig. 5—Survival curves for B. cereus *isolated from egg (A) and* B. cereus *T (B). Inactivation was in ultrapasteurized egg. The* B. cereus *egg isolate was inactivated at temperatures detailed in Fig. 3.* B. cereus T was inactivated at 49.3°C (▲), 52.4°C (△), *54.3°C (♦), and 56.2°C (□). Data represent resistance of vegetative cells.*

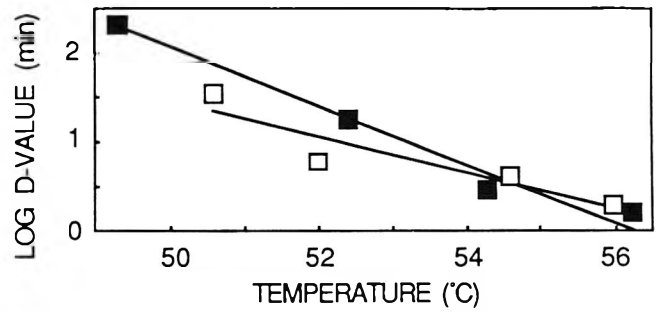

Fig. 6—Decimal reduction time curves for the B. cereus *isolated from egg (□) and* B. cereus *T (■). Data represent resistance of vegetative cells.*

or similar species were known to have developed in egg stored at 10°C. A 250 mL carton of egg initially containing one healthy cell would spoil within 12 days. Under these conditions, spoilage by the *Pseudomonas* species would occur in ca. 6 days. These fast spoilage rates are not realistic, even as a worst-case scenario, since constant storage at 10°C would not be anticipated. However, comparing predictions based upon the 4° and 10°C growth curves indicated that spoilage would be expected to occur at least 1.5 to 3 times faster if egg were stored at the higher temperature. This is essentially what was observed in a prior study (Ball et al., 1987) where spoilage occurred 2–8 times faster at 10°C than at 4°C. The predicted increased rates of spoilage were considered minimal since damaged cells most likely would recover more rapidly at 10°C resulting in faster population increases than these predictors based on growth alone would estimate. Realistically, refrigerated storage temperatures will fluctuate. It is well established that growth rates change logarithmically with temperature, hence even short time periods at higher storage temperatures within the growth range can significantly shorten the time to spoilage.

Heat resistances evaluated at constant temperatures, by a batch process, in egg indicated the *Enterococcus* sp. was the most resistant, followed by the *B. circulans, Pseudomonas* sp. and *B. cereus* egg isolate. This relationship held true throughout the temperature range studied (49–70°C), assuming the decimal reduction time curves could be extrapolated through this range. The four egg isolates were similar or identical in their responsiveness to temperature in the range studied. The *B. cereus* T reference strain was the most responsive to temperature fluctuation, as reflected by the small z_D-value (3.1°C) for this strain. Ultrapasteurization processes select for survival of organisms with moderate to high heat resistance above ca. 63°C. Thus, isolates such as the *B. cereus* T reference strain with small predicted D-values at or above 63°C would not be expected to survive. The difference in resistances of *B. cereus* T and the egg *B. cereus* isolate highlights the importance of appropriate strain selection for process or product evaluation. Use of strains isolated from ultrapasteurized egg will provide the requisite minimal heat resistance which would be realistic to consider.

The least heat resistant egg isolate of those studied was the *B. cereus* isolate. Extrapolation of the decimal reduction time curve suggested the D-value at 63°C would be 3.5 sec, an admittedly gross estimate. Nonetheless, for this organism to survive ultrapasteurization the population in the raw egg would need to be relatively high or spores must be present. Laboratory-prepared spores of the *B. cereus* egg isolate, *B. circulans* or *B. cereus* T were not inactivated (data not presented) throughout the temperature range used in ultrapasteurization of liquid whole egg (up to 72.2°C). Aerobic, mesophilic spore counts on egg from each of the ten trials reported by Ball et al. (1987) were <10/mL for eight of the processes and 5 and 10/mL, estimated, for two processes. These populations were in freshly processed egg (processed and held overnight at 4°C) and are expected to reflect the minimal spore population in raw egg since bacterial spores would not be killed at ≤72.2°C in egg and germination probably would not occur to a significant extent at the low storage temperature but may occur during the cooling period. Raw egg from the same plant obtained and evaluated at a later date contained 5.5×10^1 CFU aerobic mesophilic spores/mL.

The most heat resistant isolate throughout the temperature range studied was the *Enterococcus* sp. (formerly *Streptococcus faecalis*). Payne and Gooch (1980) also reported heat resistant fecal streptococci survived pasteurization in egg. However, several isolates studied by Payne and Gooch (1980) were more heat resistant than observed for the *Enterococcus* sp. reported here. Payne et al. (1979) recovered heat resistant coryneform bacteria yet these types of organisms were not recovered from ultrapasteurized egg.

From the available data, one useful indicator of the quality of raw egg intended for ultrapasteurization would be an aerobic mesophilic or psychrotrophic spore count. This count could definitely preclude processing of unsuitable product and may be high enough to trigger concern when the numbers of other commonly used indicator microorganisms are low. For process development or optimization using inoculated product, suggested test microorganisms would be *B. circulans* or the *Enterococcus* sp. The former is a concern even in the best storage and distribution environment since it can grow at 4°C. However, an ideal environment is unlikely and exposure to 10°C is probable, hence *Enterococcus* sp. control is necessary because of the frequent occurrence of this genus in raw egg products and the relative heat resistance of enterococci.

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Formation of Cholesterol-5,6-Epoxides during Spray-drying of Egg Yolk

JEFFREY N. MORGAN and DAVID J. ARMSTRONG

ABSTRACT

An indirect heated spray dryer with an evaporative capacity of up to 7 kg H_2O/hr was used to identify factors responsible for formation of cholesterol-5,6-epoxides (CEs) in spray-dried egg yolk. CEs were not formed under various operating conditions without addition of H_2O_2 to liquid yolk. At 10% H_2O_2, 90° and 150°C outlet temperatures, concentrations of CEs were 27 and 37 ppm, respectively. At 5% H_2O_2, 90° and 150°C outlet temperatures, concentrations of CEs were undetected and 16 ppm, respectively. Antioxidants appeared to lower CEs. Small amounts of CEs were formed in products dried in the presence of low concentrations of nitrogen oxide gases (NO_x). CEs increased greatly during storage of products dried from liquid yolk containing H_2O_2.

INTRODUCTION

CHOLESTEROL CAN UNDERGO spontaneous autoxidation in the presence of air, light and/or various other prooxidants. In an excellent monograph on cholesterol autoxidation, Smith (1981) has documented numerous cholesterol oxidation products.

Recently, cholesterol oxidation products have received considerable attention in light of their various reported biological activities associated with human disease. Cholesterol-5α,6α-epoxide (α-CE) has received much of this attention because of studies which have associated it with atherogenesis (Imai et al, 1976; Gray et al., 1971) and carcinogenesis (Bischoff, 1969; Black and Lo, 1971; Black and Chan, 1976). Other toxic effects attributed to α-CE include inhibition of endogenous cholesterol biosynthesis via inhibition of HMG-CoA reductase (Kandutsch and Chen, 1978) and association with DNA (Parson and Goss, 1978; Blackburn et al., 1979).

The reported toxicological properties of cholesterol oxidation products have led to growing concern about their occurrence in foods. A comprehensive review by Finocchiaro and Richardson (1983) has documented the occurrence of various cholesterol oxidation products in numerous foodstuffs. Of these, egg yolk has been studied extensively because of high cholesterol and because dehydrated egg yolks are a common ingredient in many popular foodstuffs including baby foods, bakery products, cake mixes, pancake mixes, salad dressings, noodles and military rations. Chicoye et al. (1968) identified, but did not quantitate, five oxidation products in spray-dried egg yolk irradiated with both sunlight and fluorescent light. Tsai et al. (1980) developed a method for quantitative estimation of cholesterol-5,6-epoxides (CEs) in eggs. Using this method, Tsai and Hudson (1985) quantitated CEs in commercially spray-dried egg products. Other recent studies have also reported quantitative data on CEs and other oxidation products in dried egg or egg-containing products (Missler et al., 1985; Fischer et al., 1985; Sugino et al., 1986). However, these studies have been primarily method development work in which commercial products were subjected to some form of oxidative stress to generate measurable levels of oxidation products.

To date no systematic study of the effects of various processing parameters on formation of CEs in spray-dried egg yolk has been reported. The present study was designed to investigate factors responsible for formation of CEs in spray-dried egg yolk powder and to manipulate these factors to the extent necessary to generate CEs at levels equivalent to maximum levels found in commercial products. Factors investigated include inlet and outlet air temperatures, solids content of the liquid egg yolk, addition of prooxidants and antioxidants to the liquid egg yolk, addition of prooxidants to the drying air and the effects of storage.

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MATERIALS & METHODS

Preparation of liquid egg yolk

Batches of liquid egg yolk were prepared from fresh Grade A large eggs purchased locally. Yolks were separated from albumen, blended at low speed for 15 sec and stored under refrigeration (4°C) until dried.

Hydrogen peroxide, 50%, was added directly or in combination with distilled water in appropriate amounts to achieve the desired concentration in liquid egg yolk. Distilled water was used to dilute liquid egg yolk for drying studies at low solids concentrations.

Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) were added from stock solutions in ethanol. Stock solutions were prepared in appropriate concentrations so that 1 mL stock solution added to 100 mL liquid yolk produced the desired final concentration. Propyl gallate was prepared in water solution and added in a manner identical to that for BHA and BHT.

Spray dryer operation

Spray-drying studies were conducted using a Niro Atomizer Mobile Minor laboratory spray dryer (Niro Atomizer, Columbia, MD) equipped with an electric air heater (indirect heating), a 24-vaned centrifugal atomizer wheel and a peristaltic pump for pumping liquid egg yolk to the atomizer. Air flow through the drying system and air pressure to the atomizer were maintained at constant levels for all studies. Inlet temperature was adjusted by using dryer heater controls, while outlet temperature was adjusted by altering flow rate of the liquid egg yolk. Thermocouples used to monitor temperatures at the spray dryer inlet and outlet were calibrated against a known EMF. The dryer was equipped with air-driven vibrators to minimize sticking of egg yolk powder to chamber or duct walls. Only the powder that exited the dryer was collected for analysis.

Determination of CEs

CEs were quantitated using minor modifications of the method of Tsai et al. (1980).

Extraction of lipids

Dry yolk (0.5g) was blended with 100 mL chloroform/methanol (2:1, v/v), and the mixture was filtered into an evaporation flask. The residue was washed with a total of 25 mL solvent. The combined filtrate was dried under vacuum with a rotary evaporator and redissolved in 5 mL hexane.

Enrichment of cholesterol oxides

The lipid extract was applied to a Sep-Pak silica cartridge (Waters Associates, Milford, MA) which had been previously wetted with 2 mL hexane. The flask was rinsed with 5 mL hexane, which was also applied to the Sep-Pak. The Sep-Pak was eluted with 10 mL hexane/ethyl ether (95:5, v/v), 30 mL hexane/ethyl ether (90:10, v/v) and 50 mL hexane/ethyl ether (50:30, v/v). The hexane/ethyl ether (50:30, v/v) eluate, containing all recoverable CEs, was dried under a nitrogen stream and redissolved in 25 μL chloroform in a 1 mL conical vial.

Quantitation of CEs by gas-liquid chromatography (GLC)

A Perkin-Elmer Sigma 115 gas chromatograph with flame ionization detection was used. A nickel column (2.1 mm i.d. x 2m) packed with 3% OV-17 on 100-120 mesh Gas-Chrom Q was used. Carrier gas, helium, was delivered at a flow rate of 30 mL/min. Oven temperature was programmed from 240° to 280°C at a rate of 5°C/min and isothermal at 280°C to completion. Injection port and detector temperatures were 270 and 320°C, respectively.

One μL chlorofrom solution was injected into the gas chromatograph. The peak appearing within ±4% of standard α-CE (Sigma Chemical Co., St. Louis, MO) was integrated. Peak area was converted to quantity of CEs by the external standard method. No attempts were made to differentiate α- and α-isomers of cholesterol-5,6-epoxide. Lipids were extracted from each egg yolk powder in duplicate and each extract was analyzed in duplicate by GLC.

Efficiency of recovery of CEs from egg yolk powder and the coefficient of variation (CV) for the analytical method were determined by analyzing freeze-dried egg yolk spiked with 50 ppm α-CE. Lipids were extracted in triplicate form three freeze-dried yolk portions each containing 50 ppm α-CE, and each extract was analyzed in triplicate by GLC. The mean recovery of CEs for the nine analyses was 104% with a CV of 16%. The limit of determination for this method was estimated as 2.5 ppm.

A coefficient of variation which included both the spray-drying process and the analytical method was also calculated. Four liquid egg yolk test samples containing 10% H_2O_2 were dried at 300°C inlet temperature and 150°C outlet temperature. Lipids were extracted from each dried powder in duplicate and each extract analyzed by GLC for CEs in duplicate. The mean concentration of CEs was 44 ppm with a CV of 24%.

Analytical methods

Total cholesterol was determined using a method and test kit supplied by Boehringer-Mannheim Biochemicals (Indianapolis, IN). Total solids were determined using AOAC (1984) vacuum oven methods. NO_x ($NO + NO_2$) gases in the spray dryer exhaust were measured using Draeger tubes (Leichnitz, 1985) placed in the exhaust air duct. Residual H_2O_2 in dried egg powder was determined using a H_2O_2 test kit (Chemetrics, Calverton, VA) after rehydration of dried yolk.

RESULTS & DISCUSSION

Indirect heating

CEs were not found in egg yolk powder spray-dried with indirect heating at inlet temperatures from 150° to 400°C, outlet temperatures from 75° to 150°C and solids concentrations from 11.3 to 45% in the liquid egg yolk. Low concentrations (3-5 ppm) of CEs were reported in 3 of 10 commercial yolk powders dried with indirect heating (Tsai and Hudson, 1985).

Prooxidants

Since indirect heating alone was not sufficient to promote formation of CEs, prooxidants were added to the liquid egg yolk to create oxidizing conditions. The effects of addition of prooxidants to liquid egg yolk were studied using H_2O_2 as a prooxidant. H_2O_2 was chosen because its action favors formation of CEs (Smith, 1981) and because it is sometimes used in enzymatic desugarization of liquid egg products before drying. Thus, H_2O_2-induced oxidation may be a potential problem in commercial products. While liquid egg yolk in commercial practice is not routinely desugared before drying, some specialty applications require glucose removal. Industrial glucose removal practices remain proprietary; however, generalizations can be made. Enzymatic glucose removal systems use a glucose oxidase-catalase enzyme system and H_2O_2. Catalase catalyzes breakdown of H_2O_2, yielding O_2 required in the glucose oxidase-catalyzed conversion of glucose to gluconic acid. H_2O_2 may be added batchwise or continuously, but concentrations in the liquid yolk do not exceed 0.5%. The reaction is carried out for the period of time needed to reduce glucose to suitable levels. This reaction time depends on several factors including enzyme activity, glucose concentration and liquid yolk temperature. However, conditions conducive to removing glucose in a period of 2.5 to 3 hr are recommended.

Addition of low concentrations of H_2O_2 to liquid egg yolks did not produce CEs in the dried powder, making it necessary to use high H_2O_2 concentrations to generate CEs in amounts found in commercial products. A concentration of 5% H_2O_2 in the liquid egg yolk resulted in formation of CEs (Table 1). CEs were produced at 9 ppm at an outlet temperature of 150°C, but no CEs were detected at an outlet temperature of 90°C. A concentration of 10% H_2O_2 in the liquid egg yolk produced CEs at 37 ppm at an outlet temperature of 150°C and 27 ppm at an outlet temperature of 90°C (Table 2). Concentrations of CEs in products dried from liquid egg yolk containing 10% H_2O_2 were in the same range as maximum levels found in most commercial spray-dried yolk (Tsai and Hudson, 1985).

Table 1—Effect of 5% H_2O_2 in liquid egg yolk[a] on formation of cholesterol-5,6-epoxides in egg yolk powder spray-dried with indirect heating

Inlet temp (°C)	Outlet temp (°C)	Cholesterol-5,6-epoxides[b] ppm, Solids basis	mg/g Cholesterol
200	150	8	0.3
250	150	6	0.2
300	150	16	0.5
350	150	7	0.2
		mean[c] 9[d]	0.3[e]
150	90	ND[f]	0.1
250	90	ND	0.1
		mean[g] ND[d]	0.1[e]
Liquid egg yolk			ND

[a] Solids of liquid egg yolk ranged from 36 to 38%.
[b] Mean of results from two portions analyzed in duplicate.
[c] Mean of all products dried at 150°C outlet temperature.
[d] Means significantly different ($p < 0.05$) using Student's t-test. Assumes no effect of inlet temperature.
[e] Means significantly different ($p < 0.05$) using Student's t-test. Assumes no effect of inlet temperature.
[f] ND = not detected.
[g] Mean of all products dried at 90°C outlet temperature.

Table 2—Effect of 10% H_2O_2 in liquid egg yolk[a] on formation of cholesterol-5,6-epoxides in egg yolk powder spray-dried with indirect heating

Inlet temp (°C)	Outlet temp (°C)	Cholesterol-5,6-epoxides[b] ppm, Solids basis	mg/g Cholesterol
200	150	26	1
250	150	42	2
300	150	40	2
325	150	29	1
350	150	48	2
		mean[c] 37[d]	2[e]
150	90	19	1
175	90	19	1
200	90	35	1
225	90	28	1
250	90	33	1
		mean[f] 27[d]	1[e]
Liquid egg yolk		ND[g]	

[a] Solids of liquid egg yolk ranged from 36 to 38%.
[b] Mean of results from two portions analyzed in duplicate.
[c] Mean of all products dried at 150°C outlet temperature.
[d] Means significantly different ($p < 0.1$) using Student's t-test. Assumes no effect of inlet temperature.
[e] Means significantly different ($p < 0.025$) using Student's t-test. Assumes no effect of inlet temperature.
[f] Mean of all products dried at 90°C outlet temperature.
[g] ND = not detected after 4 hr at ambient temperature.

Varying inlet temperature while keeping outlet temperature constant had little effect on formation of CEs in products dried from liquid egg yolk containing either 5 or 10% H_2O_2 (Tables 1 and 2, respectively). This suggested that inlet temperature was not a key factor in cholesterol oxidation during the spray-drying process. Lack of significant effects of inlet temperature at constant outlet temperature was not surprising since the actual temperature of dry egg powder would not be expected to exceed that of the outlet air. Differences in outlet air temperature more accurately reflect actual differences in powder temperature than do differences in inlet temperature. Thus, concentrations of CEs might be expected to be more closely related to variations in outlet temperature. Results of the present study supported this claim. In fact, when data from test samples prepared from liquid yolks containing 5% H_2O_2 were subjected to statistical treatment using the Student's t-test, differences in CEs in products processed at 90° and 150°C outlet temperatures were significant ($p < 0.05$). For liquid egg yolk containing 10% H_2O_2, CEs in products dried at 90° and 150°C differed ($p < 0.10$). No correlation between outlet temperature and CEs in commercial products was observed (Tsai and Hudson, 1985). This is probably due to the low and relatively narrow outlet temperature range (49–82°C) used in the commercial drying processes reported.

The reasons why such high concentrations of H_2O_2 are required to generate CEs are not clear, but several possibilities exist. One possible explanation concerns evaporation or decomposition of H_2O_2. Much of the H_2O_2 may evaporate or decompose very early in the drying process, resulting in an effective H_2O_2 concentration that is considerably less than the initial H_2O_2 concentration of 5 or 10% in the liquid egg yolk. Secondly, in this study only formation of CE was monitored. While H_2O_2-induced cholesterol oxidation favors formation of CEs (Smith, 1981), other egg yolk components may be preferentially oxidized. A third possibility is that the relatively slow rate at which cholesterol oxidation occurs necessitates use of high H_2O_2 concentrations to generate CEs in the relatively short time involved in the drying process. Naber and Biggert (1985) reported that 100 hr at 100°C in air was required to promote a fourfold increase, up to 200 ppm, in some oxidized cholesterol derivatives in dried egg yolk. Thus, it is not surprising that an extremely high H_2O_2 concentration (10%) was required to generate CEs at 37 ppm during a drying process of only about 20 min, including time exposed to hot drying air in the powder collection jar, used in this study.

While reasons for the high H_2O_2 requirements were not clear, results of indirect heating studies with and without H_2O_2 in liquid egg yolk suggested that cholesterol did not autoxidize to a measurable extent during the spray-drying process in the absence of prooxidants. This conclusion supports the hypothesis that initiators of cholesterol oxidation, probably nitrogen oxides (NO_x) in the flue gases in direct-fired commercial systems, must be present during spray-drying to form significant amounts of CEs (Tsai and Hudson, 1985).

In an attempt to generate NO_x gases in an indirect heating system, nitrous oxide (N_2O) was introduced into the spray dryer at the electric heater. At temperatures above 300°C, N_2O dissociates into a mixture of oxidizing nitrogen oxide gases (NO_x). Thus, N_2O can be used to produce NO_x gases in an indirect heating system, thereby avoiding direct use of highly toxic NO or NO_2 gases. However, NO_x in the exhaust air, as measured with Draeger tubes, was only 3 ppm when N_2O was added at 1000 ppm and the dryer was operated at 360°C inlet temperature, and they were only 5 ppm when N_2O was added at 10,000 ppm. Thus, this method was not efficient in generating high concentrations of NO_x. Nevertheless, CEs were formed at concentrations up to 7 ppm when N_2O was present in the drying atmosphere. These concentrations were in the same range as 50% of commercial products dried with direct heating systems (Tsai and Hudson, 1985). No effects were observed with various N_2O concentrations, probably since NO_x concentrations did not differ to an appreciable extent at the N_2O concentrations used. Nevertheless, these results lend further support to the hypothesis that initiators of cholesterol oxidation must be present during spray-drying to generate significant amounts of CEs.

Antioxidants

Effects of antioxidants added to the liquid egg yolk on formation of CEs in egg yolk powder are shown in Table 3. Propyl gallate at 67 ppm reduced CEs in the control from 40 to 18 ppm. Increasing propyl gallate to 200 ppm had no further effect on reduction of CEs. BHA and BHT at 67 ppm had little effect on concentrations of CEs. However, at 200 ppm, BHA and BHT reduced CEs to 26 and 22 ppm, respectively. Although there appeared to be a trend toward reduced CEs in test samples dried in the presence of antioxidants, the observed differences in CEs were probably not statistically significant because of the relatively high CV that was inherent in the spray-drying process and analytical method.

Storage

Results presented in Table 4 suggest that storage plays a key role in oxidation of cholesterol in egg yolk powders. Concentrations of CEs in the powder dried from liquid egg yolk containing no prooxidants increased from undeterminable initial concentrations to 20 ppm after 9 months of storage. CEs in a product dried from liquid egg yolk containing 5% H_2O_2 increased 7.8-fold to 122 ppm during 4 months of storage. No further increases were observed during an additional 3 months storage. This result was typical for products dried from liquid egg yolk containing 5% H_2O_2.

Products prepared from yolks containing 10% H_2O_2 showed more widely varying results of storage. CEs increases ranged from 3- to 54-fold (result in Table 4 is for extreme case) after 4 months of storage. These concentrations increased only slightly after an additional 3 months storage. Such varying results might possibly be attributed to varying amounts of residual H_2O_2 present in the dried powders. The product which showed the largest increase was dried under the mildest temperature con-

Table 3—Effects of antioxidants in liquid egg yolk[a] on formation of cholesterol-5,6-epoxides in egg yolk powder spray-dried[b] with indirect heating

Antioxidant	Antioxidant conc (ppm, yolk solids)	Cholesterol-5,6-epoxides[c] ppm, Solids basis	mg/g Cholesterol
None	—	40	2
Propyl gallate	67	18	1
	200	21	1
BHA	67	33	1
	200	26	1
BHT	67	35	1
	200	22	1

[a] Liquid egg yolk contained 10% H_2O_2 and 36% solids.
[b] Inlet temperature = 300°C; outlet temperature = 150°C.
[c] Mean of results from two portions analyzed in duplicate.

Table 4—Effect of storage[a] on concentrations of cholesterol-5,6-epoxides in spray-dried egg yolk

Treatment	Cholesterol-5,6-epoxides[b] (ppm) Initial	4 months	7 months
None	0	6	20
5% H_2O_2	16	123	123
10% H_2O_2	19	1030	1320
10% H_2O_2 + 67 ppm BHT	35	182	223
N_2O	7	8	—

[a] All test samples stored in glass jars with polyethylene caps at ambient temperature in the dark.
[b] Data are for individual test samples in each group and show most extreme storage effect.

ditions (150°C inlet; 90°C outlet) and thus might be expected to contain relatively higher residual peroxide than those dried under more severe conditions. In fact, residual peroxide concentrations were 0.27, 0.41 and 0.78% in egg yolk powders dried at inlet/outlet temperature combinations of 350°/150°C, 200°/150°C and 150°/90°C, respectively. Thus, preliminary results suggested that residual peroxide concentrations were dependent on drying conditions and influenced formation of CEs during storage. These results suggested that while CEs might be formed during spray-drying, some formation of CEs might occur after drying but while the powder still remained exposed to hot drying air. This conclusion should be tested further.

CEs in products dried from liquid egg yolk containing 10% H_2O_2 and antioxidants increased to the same degree as in most of those containing only 10% H_2O_2. Thus, antioxidants at the concentrations tested did not appear to be effective in reducing oxidation of cholesterol at the concentrations of H_2O_2 present during storage of dried egg yolk powders.

CEs in products dried in the presence of N_2O did not increase significantly during storage for 4 months. These results paralleled results for test samples dried from liquid egg yolk containing no additives and suggested that prooxidants must be present in the dried egg yolk powder (residual H_2O_2) to promote significant further oxidation during storage.

Increases in CEs in stored dried egg yolks have previously been observed (Tsai and Hudson, 1985). Similar increases were also observed with whole egg powders (Tsai and Hudson, 1985; Sugino et al., 1986) and military issue powdered egg mixes (Missler et al., 1985).

SUMMARY

THIS STUDY DEMONSTRATED that, contrary to what might be expected, the presence of prooxidants during spray-drying was necessary to promote formation of measurable concentrations of CEs in egg yolk powders. However, the data did not establish whether formation of CEs occurred during the few seconds that particles were retained in the drying chamber or during the period (up to 20 min) that the dried powder was exposed to hot drying air while being collected in the powder collection jar. Nevertheless, outlet temperature was the only spray dryer operating parameter that exerted any effect on CEs with an indirect heating spray dryer. Further increases in CEs were observed during storage and this pointed to storage as an important factor in determining CEs concentration.

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Ms received 11/1/86; revised 2/5/87; accepted 2/5/87.

Paper Number 10772 of the Journal Series of North Carolina Agricultural Research Service, Raleigh, North Carolina 27695-7601.

This research was supported by the Southeastern Poultry and Egg Association, the NC Board of Science and Technology and project No. 3814 of the NC Agricultural Research Service.

The assistance of H.R. Ball, Jr. in securing egg and H. Shyu in preparation of figures is gratefully acknowledged.

The use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Research Service of the products named, nor criticism of similar ones not mentioned.

Nutritional, Sensory and Microbiological Qualities of Labneh Made from Goat Milk and Cow Milk

D. R. RAO, A. ALHAJALI, and C. B. CHAWAN

ABSTRACT

Labneh, a concentrated yogurt product routinely consumed in the Middle East, was prepared from cow milk and goat milk by a procedure normally used in the Middle East. Goat labneh was significantly higher in ash than cow labneh. Protein, fat and total solids were the same. Storage for six months had no significant influence on the chemical composition. Levels of Ca, P and K were higher in goat labneh. Goat labneh protein had significantly higher levels of aspartic acid, threonine, proline, valine and phenylalanine than cow labneh. On the other hand, cow Labneh protein had significantly higher levels of histidine and arginine. Total aerobic counts, lactic acid bacterial counts and counts of yeast and molds decreased by 2 to 4 $\log_{10}$ numbers during 6 months of storage in both labnehs. Storage of goat labneh, but not cow labneh, for six months significantly improved the sensory qualities.

INTRODUCTION

ALL MILK and milk products consumed by humans in the Middle Eastern countries were estimated at 7 to 7.5 million metric tons in 1975 (Haddad, 1983). Per capita daily consumption of milk and milk products in the Middle Eastern countries varies from almost 0 to 0.3L (McDowell, 1977). Thus, dairy products contribute significant amount of nutrients to the populations in the Middle Eastern countries. Leben-type (yogurt) products are consumed during the hot summer with bread as the major part of the mid-day meal whereas labneh-type (concentrated yogurt) products are consumed with bread as a major part of breakfast and supper.

Nutritionally, labneh-type products provide energy, protein and significant amounts of calcium, phosphorus and vitamins. Because of low-moisture and high-salt, labneh-type products have good shelf life and lend themselves for use in the winter (Tamime and Robinson, 1978; Hafez and Hamad, 1984). Furthermore, these products have historically formed part of the dietary pattern of the Middle Eastern countries. Therefore, there are no social problems involved with their use. Other advantages include relatively simple equipment and skill requirements to make these products. In the Middle Eastern population the homemade concentrated yogurt (Labneh) submerged by vegetable oil is highly appreciated and consumed with bread all year-around.

Several articles pertaining to general procedures of yogurt preparation from goat milk have been published (Aggarwal, 1974; Duitschaever, 1978; Loewenstein et al., 1980). However, the nutritional, sensory and microbiological qualities of yogurt products such as labneh from goat milk are not available in literature. Therefore, the objective of this study was to determine the nutritional, sensory and microbiological properties of concentrated yogurt product - labneh, made from goat milk and compare it with labneh made from cow milk.

MATERIALS & METHODS

Milk

Homogenized and pasteurized cow milk was obtained from a local market in Alabama. Pasteurized goat milk was obtained from a local farm in Alabama.

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Starter culture

Fifty milliliters cow milk or goat milk were boiled for 3 min and cooled to 45°C. A mixture of yogurt culture (activated culture consisting of 1 mL each of *Lactobacillus bulgaricus*, ATCC-11842 and *Streptococcus thermophilus*, strain from University of Nebraska) was used to inoculate the milks. The inoculated milks were incubated at 45°C for 12 hrs and refrigerated for no more than 24 hr before use.

Labneh preparation

Labneh was made by a procedure normally used in the Middle East. The milk was boiled for 3 min, cooled to 45°C and inoculated with 2% starter culture. The milk was incubated for 12 hr at 45°C. This fermented milk was subsequently held in a refrigerator at 4–5°C for 12 hr. The coagula were held at room temperature (21°C) for 7 days in hanging cloth bags to drain the wheys. The resulting creamy, cheese-like product was mixed by hand and salt was added at 2% level. The curds were shaped into balls of about 3 cm diameter (labneh). The balls were submerged in vegetable oil in glass jars and stored for 6 months at room temperature.

Analytical methods

Proximate analyses on labneh were according to procedure outlined in AOAC (1980). Fresh samples were analyzed for pH and acidity and aliquots of samples were frozen until further analyses. Total nitrogen concentration and nitrogen in protein-free filtrates were determined by micro-Kjeldahl method (AOAC, 1980). A factor of 6.38 was used to calculate protein.

Levels of K, Ca, and Zn were determined using the methods described in AOAC (1980) with an atomic absorption spectrophotometer (Perkin Elmer Model 460). Phosphorus, chlorine and sodium were determined by AOAC (1980) procedures.

Amino acids were analyzed on the Dionex Model D-502 (Dionex Corporation, Sunnyvale, CA) amino acid analyzer. One gram of each sample was hydrolyzed for the determination of total amino acids with

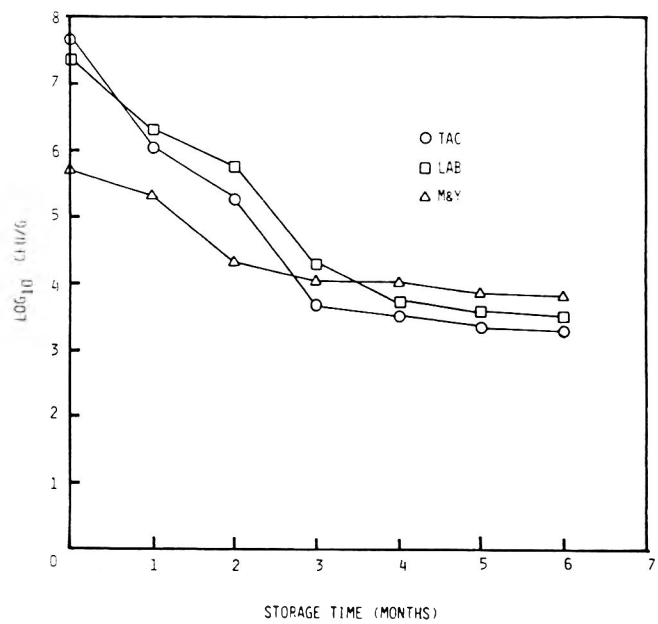

Fig. 1—Total aerobic (TAC), lactic acid bacterial (LAB), and mold and yeast (M&Y) counts in goat labneh.

Table 1—Effect of storage on chemical composition of labneh

Species	Storage time (months)	Protein	Nonprotein nitrogen	Fat	Ash	Total solids	Acidity (%)	pH
		g/100 g sample						
COW	0	19.22 (0.64)	1.90[a] (0.07)	29.58 (2.28)	4.79[a] (0.12)	53.59[a] (2.17)	2.19 (0.10)	4.37 (0.18)
	6	19.15 (0.56)	1.98 (0.06)	30.97 (2.10)	4.76[a] (0.08)	54.88[ab] (3.10)	2.22 (0.13)	4.33 (0.15)
GOAT	0	20.24 (0.54)	2.14[ab] (0.08)	31.04 (1.76)	5.06[b] (0.03)	56.34[ab] (2.09)	2.25 (0.13)	4.24 (0.20)
	6	20.46 (0.58)	2.25[b] (0.10)	32.30 (1.55)	5.10[b] (0.06)	57.76[b] (1.80)	2.19 (0.15)	4.31 (0.22)

[a,b] Mean values in the same column bearing different superscripts are significantly different ($P < 0.05$) by Duncan's New Multiple Range Test (Steel and Torrie, 1980) (Comparisons for fat and total solids within storage time). Each value is a mean of four replications (No. in parenthesis is standard error of mean).

Table 2—Mineral composition of fresh labneh

Mineral	Cow labneh	Goat labneh
	mg/100 g	
Na	1950[a] (110)	1820[b] (130)
K	55[a] (4)	85[b] (7)
Ca	110[a] (6)	140[b] (5)
P	286[a] (20)	382[b] (25)
Zn	1.63[a]	1.40[b] (0.05)
Cl	2658 (200)	2880 (180)

[a,b] Means in the same row bearing different superscripts are significantly different ($P < 0.05$) by Duncan's New Multiple Range Test (Steel and Torrie, 1980). Each value is a mean of four replications. (No. in parenthesis is standard error of mean).

20 mL 6N HCl for 24 hr at 110°C in capped tubes under nitrogen. Prepared samples in amounts of 20μL were injected into the amino acid analyzer. For preparation of protein free filtrates, the samples were treated with 10% trichloracetic acid (TCA) (10g sample + 10 mL 10% TCA). The precipitated protein was removed by centrifugation at 10,000 × g for 20 min to recover the clear supernatant. Nonprotein nitrogen (NPN) in the supernatant was determined by micro-Kjeldahl method. TCA supernatants were hydrolyzed by 6N HCl for free amino acids as described above.

Stored samples were analyzed at monthly intervals according to Standard methods for the Examination of Dairy Products (APHA, 1967) for total aerobic counts (TAC), lactic acid bacterial (LAB) counts, and mold and yeast (M&Y) counts. For TAC, pour plates of plate count agar were incubated at 32°C for 2 days. For LAB counts, pour plates of Elliker agar containing 0.2% bromcresol purple were incubated at 35°C for 3 days. Colonies surrounded by yellow zones were counted as LAB. For M&Y, pour plates of Potato Dextrose agar (acidified to pH 3.5 with sterile 10% tartaric acid) were incubated at 22°C for 5 days.

Samples stored for 0 days and 6 months were tested for flavor, mouth feel, and overall acceptability by 9 untrained panelists using a 5-point hedonic scale (Peryam and Pilgram, 1957). Samples were coded and served to each panelist on the same day. Analysis of variance was used for statistical evaluation of the data, and mean separation was performed using the Duncan Multiple Range Test (Steel and Torrie, 1980) when significant ($P < 0.05$) main effects were observed.

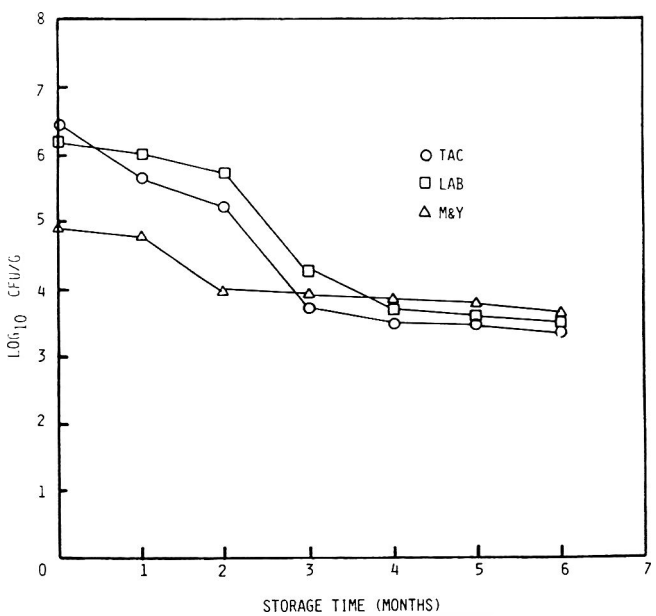

Fig. 2—Total aerobic (TAC), lactic acid bacterial (LAB), and mold and yeast (M&Y) counts in cow labneh.

RESULTS & DISCUSSION

Fresh labneh compositions

Goat labneh was similar to cow labneh in gross composition (Table 1). However, goat labneh had higher ($P < 0.05$) ash (5.06% vs 4.79%) than cow labneh. Goat and cow labnehs in this study had higher protein, fat and total solids than in cow labneh reported by Rosenthal et al. (1980). The possible reasons for different results obtained here may be due to breed of animal used and the lengths of draining whey from yogurt.

Effects of storage on labneh compositions

During the six-month storage period, there were no significant changes in chemical composition in both labneh types (Table 1). Nonsignificant increase in fat was probably due to preserving the products in vegetable oil. Nonprotein nitrogen (NPN) increased but was not significant after 6-month period in both labneh types. The nearly constant pH during the six-month period of storage of both labnehs probably indicates a cessation of bacterial activity.

Mineral composition

Levels of Ca, P, and K were higher in goat labneh (Table 2) than in cow labneh. Both species apparently are excellent sources of K, Ca, and P. The high level of these minerals was due to concentration effect of products even though there were losses of some minerals in whey during the draining periods. The sodium chloride levels of both labneh types were very high as expected because 2% sodium chloride was added to bring the products to desirable taste. However, the Na level was high and labneh could probably be prepared with much lower levels of added salts without sacrificing the keeping quality.

Amino acid composition

Amino acid composition of labneh (mg/g labneh) is shown in Table 3. Total levels aspartic acid, threonine, proline, valine and phenylalanine were significantly higher in goat labneh than in cow labneh. However, histidine and arginine were lower in goat

Table 3—Amino acid composition of fresh labneh

Product	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	His	Lys	Arg	Total
								Total Amino Acids (mg g Labneh)									
Cow labneh	14.95[a] (0.92)	6.69[a] (0.42)	11.38 (0.76)	44.03 (3.50)	17.00[a] (0.35)	3.70 (0.23)	6.94 (0.40)	12.56[a] (0.86)	5.37 (0.33)	9.94 (0.62)	19.49 (1.25)	10.23 (0.65)	4.48[a] (0.38)	5.42[a] (0.33)	15.78 (0.78)	6.86[a] (0.29)	94.82
Goat labneh	18.10[b] (1.50)	6.91[b] (0.50)	10.89 (0.80)	40.86 (4.20)	24.12[b] (0.55)	3.96 (0.30)	6.75 (0.45)	21.23[b] (0.90)	6.03 (0.25)	10.88 (0.70)	21.38 (2.10)	8.58 (0.80)	5.49[b] (0.65)	3.98[b] (0.25)	16.32 (0.75)	4.26[b] (0.20)	209.74
								Free Amino Acids (mg/100g)									
Cow labneh	1.78 (0.11)	1.13 (0.09)	1.50[a] (0.10)	2.22[a] (0.15)	1.39 (0.11)	0.41[a] (0.04)	0.73 (0.07)	0.72[a] (0.06)	0.30[a] (0.007)	1.07[a] (0.08)	0.94 (0.06)	0.07[a] (0.004)	0.27[a] (0.02)	0.36[a] (0.03)	0.91 (0.06)	0.47 (0.03)	14.25
Goat labneh	1.68 (0.18)	1.08 (0.10)	1.14[b] (0.08)	3.04[b] (0.15)	1.41 (0.10)	1.41[b] (0.09)	0.79 (0.09)	1.20[b] (0.10)	0.11[b] (0.01)	0.72[b] (0.06)	1.04 (0.07)	0.17[b] (0.03)	0.08[b] (0.003)	0.51[b] (0.04)	0.96 (0.07)	0.55 (0.04)	15.96

[a,b] Means in the same column bearing different superscripts are significantly different ($P < 0.05$) by Duncan's New Multiple Range Test (Steel and Torrie, 1980). Each value is a mean of four replications. (No. in parenthesis is standard error of mean).

Table 4—Sensory scores of labneh[a]

Qualities	Products: Cow Labneh (Fresh)	Goat Labneh (Fresh)	Cow Labneh Stored for 6 months	Goat Labneh Stored for 6 months
Taste Flavor	3.75[b]	3.51[bc]	3.17[c]	4.26[d]
	(0.23)[2]	(0.19)	(0.46)	(0.15)
Mouthfeel	3.46[b]	3.49[b]	3.51[b]	4.12[d]
	(0.40)	(0.24)	(0.37)	(0.25)
Overall acceptability	3.62[b]	3.47[b]	3.35[b]	4.11[d]
	(0.37)	(0.04)	(0.38)	(0.10)

[a] Scored on 1-5 hedonic scale where 1 = dislike definitely and 5 = like definitely

[b,c,d] Means in the same row bearing different superscripts are significantly different ($P < 0.05$) by Duncan's New Multiple Range Test (Steel and Torrie, 1980). Each value is a mean of four replications. (No. in parenthesis is standard error of mean).

labneh when compared to cow labneh. Levels of these amino acids also showed similar pattern in goat and cow milk (Chawan, 1987). Arginine and histidine are essential for children and in this regard goat labneh may be inferior to cow labneh.

There were no significant differences in free amino acids between either type of labneh (Table 3). However, the individual amino acids including glutamic acid, glycine, valine, tyrosine and histidine were higher in goat labneh; serine, methionine, isoleucine, phenylalanine were higher in cow labneh.

Microbial counts of labneh during storage

The microbial counts of labneh before and through storage are presented in Fig. 1 and 2. In general, any difference obtained in microbial counts of labneh can be attributed to the variety of the milks since the same starter culture of similar microbial quality was used for production of both labneh types.

The total aerobic counts (TAC) in both labneh types decreased from about 10^7 colony forming units (CFU)/g before storage to slightly more than 10^3 CFU/g after six months storage. Lactic acid bacterial (LAB) counts also followed the same trend. TAC and LAB counts remained fairly constant after 4 months storage. Almost similar counts of TAC and LAB indicated that most of bacteria present were lactic acid bacteria. It is known that lactic cultures are commonly used to improve the shelf life of various food products because of the metabolic products such as lactic acid, propionic acid, diacetyl and antibiotic-like substances produced by these organisms. These end products have a profound inhibitory effect on variety of gram-negative food spoilage bacteria (Babel, 1976; Pulusani et al., 1979; Rao et al., 1981). Moreover, the anaerobic conditions of preserving labneh with vegetable oil together with low pH and high acidity probably had significant inhibition of TAC and LAB during storage.

Mold and yeast counts in both labneh types decreased from 10^5 CFU/g before storage to 10^4 CFU/g after six months storage. The early three months storage seemed to have significant effects in decreasing mold and yeast counts. This again was due to anaerobic condition of preserving labneh with vegetable oil. Mold and yeast counts were higher than that of TAC and LAB counts after four months storage. This may be due to the fact that molds and yeast have the ability to survive at low pH (high acidity).

Sensory evaluation of labneh

Fresh labneh and labneh after storage for six months were evaluated for flavor, mouthfeel and overall acceptability. The sensory scores are shown in Table 4. There were no significant differences between fresh cow labneh and fresh goat labneh for the sensory parameters tested. Flavor, but not mouthfeel and overall acceptability, decreased ($P < 0.05$) in cow labneh after storage. In contrast, storage of goat labneh for 6 months improved ($P < 0.05$) all the sensory qualities. Thus, it appeared that storage improved the quality of goat labneh whereas storage had minimal or no effect on cow labneh quality. Apparently, these differences in keeping qualities are attributable to the source of milk.

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Ms received 9/26/86; revised 2/6/87; accepted 3/3/87.

Fractionation of Anhydrous Milk Fat by Superficial Carbon Dioxide

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ABSTRACT

Milk fat was fractionated with supercritical CO_2 (SC-CO_2) into 8 fractions at temperatures of 50° and 70°C, over a pressure range of 100–350 bar. Two fractions (L1 and 2) were liquid, 3 fractions (I1-3) were semi-solid and 3 fractions (S1-3) were solid at 20°C. The peak melting temperature progressively increased (9.7° to 38.3°C) from fraction L1 to S3. The concentration of short chain (C24-C34) triglycerides decreased from fraction L1 to S3 while that of long chain (C42-C54) triglycerides increased gradually. The medium chain triglycerides were more concentrated in fractions L2 and I1-3. The proportion of short (C4-C8) and medium (C10-C12) chain fatty acids decreased and that of long (C14-C18) chain fatty acids increased gradually from fraction L1-S3. The weight average molecular weights and geometric mean-carbon number of milk fat fractions were in the range from 625.6 to 805.0 and 34.2 to 47.6, respectively, in comparison to 729.3 and 41.0, respectively, for native milk fat, suggesting SC-CO_2 effected a fair degree of molecular weight separation.

INTRODUCTION

MILK FAT has been used traditionally for the most part as butter, being the most important product in the dairy industry. Milk fat is a mixture of triglycerides of a range of molecular weights and degree of unsaturation, exhibiting a broad and variable melting range (Mulder and Walstra, 1974). The pleasing flavor of milk fat is among its most important advantages. However, its unique physical characteristics, especially its melting properties and plastic range do not suit it to a number of food-fat applications. Furthermore, the nature of feed and plan of nutrition of the cow affect the fatty acid composition and hence, milk fat characteristics (Jenness and Patton, 1959). Economic fractionation of milk fat into oil and plastic fat fractions which differ markedly from one another in chemical composition and physical characteristics will facilitate an increased utilization of milk fat in many food applications, such as confectionary and bakery products, and in creating convenient (e.g., frigo-spreadable) and dietetic (e.g., cholesterol-reduced or short and medium chain-triglyceride enriched) butter types. Differences in molecular weight, melting temperature (molecular weight and entropy of fusion), volatility and intermolecular interaction energy of constituent triglycerides, provide the physical property basis for separation of milk fat triglycerides.

There has been a growing interest in supercritical gas extraction, over the past few years. Liquid-like densities of dense gases result in liquid like solvent powers. This property and faster mass transport characteristics relative to liquids due to low dense gas viscosity make dense fluids attractive extraction agents. Substances can be selectively dissolved by changing the density of the gas. Dense gas extraction involves the phenomena of distillation and extraction simultaneously (Zosel, 1978); enhancement of vapor pressure, ideal solubility and phase separation play a role. A mixture of compounds differing in physical properties, i.e., molecular weight, volatility, entropy of fusion and intermolecular interaction energy, such as milk fat triglycerides, can be fractionated with a variation in the solvent power of the dense gas. Among the potential gases, carbon dioxide is attractive as a fractionating agent (Biernoth and Merk, 1985), being relatively a poor solvent for non-polar substances compared to hydrocarbons such as propane due to its molecular volume (Webster, 1953; Brunner and Peter, 1981; Allada, 1984). Besides, CO_2 does not react chemically with food constituents, even in supercritical state (Weder, 1984). It is neither flammable nor toxic and is available in large quantities at relatively low cost; its use does not pose the problem of processing residues (Hubert and Vitzthum, 1980; Randall, 1982). Many workers (Stahl et al., 1980; Stahl and Quirin, 1982; Friedrich et al., 1982, 1984; King, 1983; List et al., 1984; Bulley and Fattori, 1984; Eggers, 1985) have investigated CO_2 as a solvent for the extraction and fractionation of oils from oil seeds; Kaufmann et al. (1982) have obtained two fractions of milk fat by supercritical CO_2 (SC-CO_2) extraction.

The purpose of this study was to determine the feasibility of fractionation of milk fat by SC-CO_2 into a range of fractions, differing from one another in molecular weight and physicochemical characteristics.

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MATERIALS & METHODS

Anhydrous milk fat preparation

Commercial butteroil (Agropur Cooperative Agro-Alimentaire, Granby, Quebec) was used after removal of protein residues by centrifugation of melted milk fat at 55°C for 20 min at 250 × *g*.

Supercritical extraction procedure

Extraction of milk fat triglycerides was carried out with SC–CO_2 at temperatures of 50° and 70°C, over a pressure range of 100–350 bar in a flow system as shown in Fig. 1. The system consists of a double-end diaphragm compressor (Superpressure, Inc., Silver Spring, MD), a temperature controlled extraction vessel (2 cm i.d. × 30 cm long standard high pressure tubing), a heated micrometering valve (flow regulating and pressure reducing), two glass U-tubes (Kimax, Inc.) in series, an in-line flow meter (Fischer-Porter, Inc.) and a flow totalizer (Singer, Inc., Philadelphia, PA). The inlet and outlet of the extractor were packed with glass wool, and 6.05g milk fat was placed into the reactor vessel. The extractor was sealed and carbon dioxide (commercial grade, Airco, Inc.) was supplied at about 87 bar pressure and 40°C to the suction side of

Table 1—Yield and physical appearance of various milk fat fractions obtained by supercritical CO_2 extraction of anhydrous milk fat

Fractions	State at room temperature	Weight (g)	Yield (%)
Milk fat (feed)	Solid	6.05	—
L1	Liquid	0.25	4.1
L2	Liquid	0.48	8.1
LF[a]	Liquid	0.73	12.2
I1	Semi-solid	0.57	9.4
I2	Semi-solid	0.80	13.1
I3	Semi-solid	0.95	15.7
IF[b]	Semi-solid	2.32	38.2
S1	Solid	0.65	10.7
S2	Solid	1.05	17.3
S3	Solid	1.30	21.5
SF[c]	Solid	3.00	49.5

[a] Total liquid fraction.
[b] Total intermediate fraction.
[c] Total solid fraction.

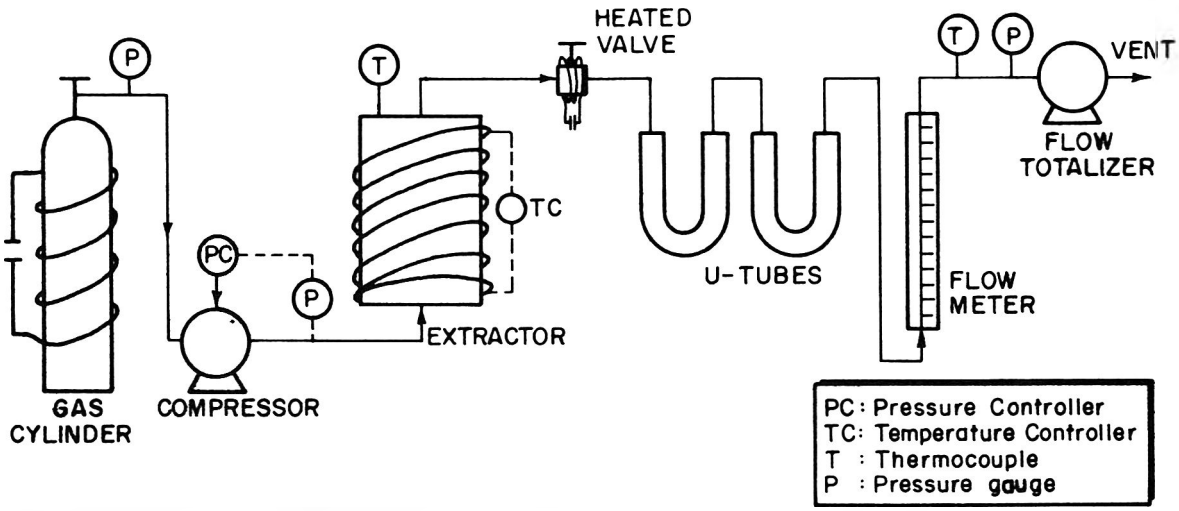

Fig. 1—Schematic diagram of supercritical fluid extraction system.

Table 2—Triglyceride composition of milk fat and milk fat fractions (weight %)[a]

Acyl carbon number	Milk fat	Fractions							
		L1	L2	I1	I2	I3	S1	S2	S3
C24	0.5	2.9	1.6	1.1	0.8	0.6	0.3	0.1	tr[b]
C26	0.3	3.7	2.0	0.7	0.2	0.3	0.3	tr	tr
C28	0.8	6.8	3.8	2.0	1.2	0.5	tr	tr	tr
C30	1.5	8.9	6.1	4.2	2.3	1.1	0.2	0.1	tr
C32	3.0	11.3	10.1	7.9	4.8	3.0	1.1	0.4	0.2
C34	6.5	16.3	11.1	14.9	12.0	8.0	4.4	2.0	0.5
C36	10.9	19.0	22.7	22.4	20.3	15.1	10.9	6.5	1.4
C38	14.0	13.7	19.2	20.3	20.0	20.3	18.0	12.4	3.5
C40	10.5	6.8	10.5	11.7	14.2	16.9	16.6	12.7	4.9
C42	7.0	2.8	4.6	5.5	7.7	9.2	11.0	10.6	5.2
C44	6.8	1.4	2.8	3.2	5.1	6.9	9.1	10.4	7.7
C46	7.0	0.9	1.7	2.2	4.5	5.7	8.1	11.2	10.9
C48	8.3	0.4	1.3	1.6	3.1	4.8	7.9	11.1	15.9
C50	10.3	0.8	0.8	1.1	2.2	4.0	7.3	12.0	22.1
C52	9.0	1.7	0.8	0.8	1.1	2.6	3.3	8.7	18.0
C54	3.6	2.6	0.9	0.4	0.5	1.0	1.5	1.8	9.7

[a] The analysis of each sample was performed in duplicate.
[b] Traces.

the compressor and was compressed to the required pressure. The high pressure gas passing downstream of the compressor was heated in a tube preheater to the required temperature prior to entering the temperature controlled extraction vessel.

The SC–CO_2, containing dissolved solute, was passed through the heated micrometering valve and expanded to ambient pressure. The dissolved solute precipitated in the first U-tube in the system. The second U-tube was employed to insure trapping of all the solute. Both tubes were packed with glass wool at the outlet. A gas flow rate of about 10 SLM was maintained. The mass of the collected solute was quantified by gravimetry. The milk fat fractions were later analysed for their melting temperature profile and triglyceride and fatty acid composition. Five fractions (L1 and 2, I1-3) were obtained by sequentially increasing the pressure of CO_2 in the pressure range from 100 to 250 bar at 50°C and 3 fractions (S1-3) were obtained at 70°C in the pressure range from 250 to 350 bar.

Differential scanning calorimetry

Melting curves were performed on a Dupont model 990 Thermal analyser (DuPont Instruments, Toronto, Ontario) by the method of Timms (1980).

Determination of triglyceride composition by gas liquid chromatography (GLC)

The native anhydrous milk fat and its fractions were analysed for their triglyceride composition (acyl carbon number) on a gas chromatograph equipped with a flame ionization detector (HP5890, Hewlett Packard, Avondale, PA) using the cold on-column injection technique (Freeman, 1981; Traitler and Prévot, 1981). The separation was carried out in a fused silica capillary column (15 m × 0.25 mm i.d.) coated with SE-54 durabond (0.5 μm) (J and W Scientific, Inc., Rancho Cordova, CA). Triglycerides from C-24 to C-54 were identified from the retention times of a standard mixture of triglycerides (Applied Science Laboratories, Inc., State College, PA). The fat samples were diluted (1:100) in n-heptane (Matheson Coleman and Bell, East Rutherford, NJ) and an aliquot of 0.5 μL was injected onto the column. The temperature program was held isothermally at 90°C for 2 min, with subsequent programming of the column temperature at a rate of 10°C/min to 350°C and a final isothermal hold at 350°C for 10 min. The carrier gas (hydrogen) was maintained at a constant pressure head of 1.5 bar.

Determination of fatty acid composition by GLC

Fatty acids from the original milk fat and its fractions, were converted into their methyl esters by the method of Luddy et al. (1968). The separation of the fatty acid esters was carried out in a fused silica capillary column (30 m × 0.25 mm i.d.) coated with OV-225 Durabond (0.25 μm) (J and W Scientific Inc., Rancho Cordova, CA). The same gas chromatograph with hydrogen as carrier gas was used as for triglyceride analyses. The samples were injected by the split technique (30:1). The temperature program was, 60°C for 2 min, 10°C/min until 190°C and held at 190°C for 8 min. Relative response factors were determined by analysis of a standard mixture of fatty acid methyl esters having a composition which was quite similar to an average butterfat sample (Applied Science Laboratories, Inc., State College, PA).

RESULTS & DISCUSSION

EIGHT FRACTIONS of anhydrous milk fat were obtained by SC–CO_2 extraction, and their yields are presented in Table 1.

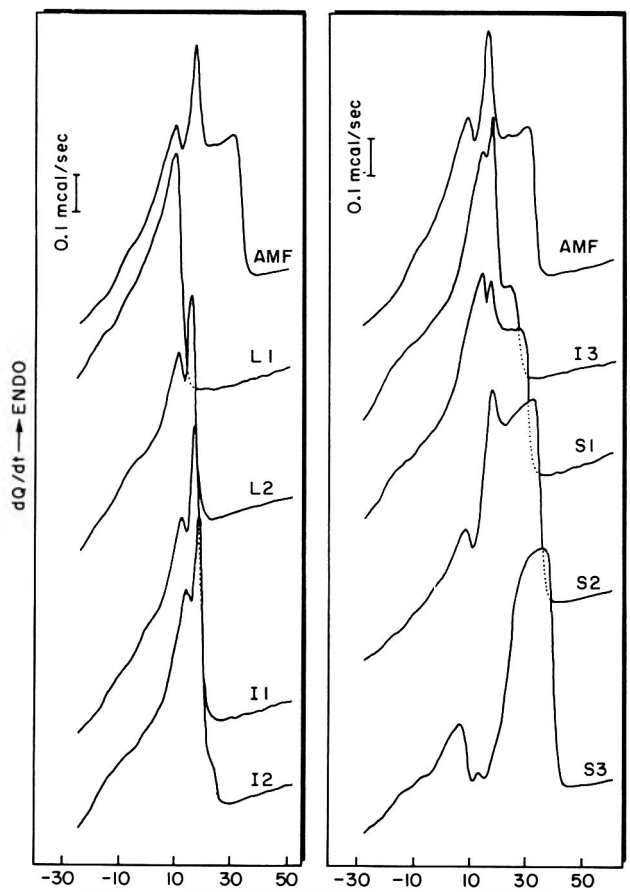

Fig. 2.—Melting curves of anhydrous milk fat (AMF) and milk fat fractions (L1-2, I1-3, and S1-3) obtained by differential scanning calorimetry.

Fractions L1 and L2 were liquid at room temperature (20°C), fractions I1-3 were of semi-solid consistency and fractions S1-3 were solid.

Melting point profile

The thermograms show differences between fractions and native milk fat in their melting point distribution (Fig. 2). Three major melting zones of milk fat, liquid (low temperature melting), intermediate and solid (high temperature melting), were repartitioned gradually in different proportions in milk fat fractions going from L1 to S3. Fraction L1 was entirely composed of liquid portion of the milk fat with a peak melting point at 9.7°C. Fractions L2, I1 and I2 were composed of liquid and intermediate portions while fractions I3 and S1 were composed of liquid, intermediate and solid portions of the milk fat but in varying proportions. Fractions S2 and S3 greatly increased in the solid portion of the milk fat with peak melting point in the range of 33–38°C. Fraction S3, however, was composed of a small liquid portion but the intermediate melting portion was almost absent.

Triglyceride composition

The triglyceride composition (acyl carbon number) of various fractions obtained by SC–CO_2 extraction is presented in Table 2. The concentration of short chain triglycerides (C24-C34) gradually decreased from fraction L1 to S3 (Fig. 3). Fractions L2 and I1-3 were enriched in medium chain triglycerides. Fractions S2 and S3, compared to milk fat, were low in both short and medium chain triglycerides. The concentration of long chain triglycerides increased from fraction L1 to S3 and they were highly concentrated in fractions S2 and S3.

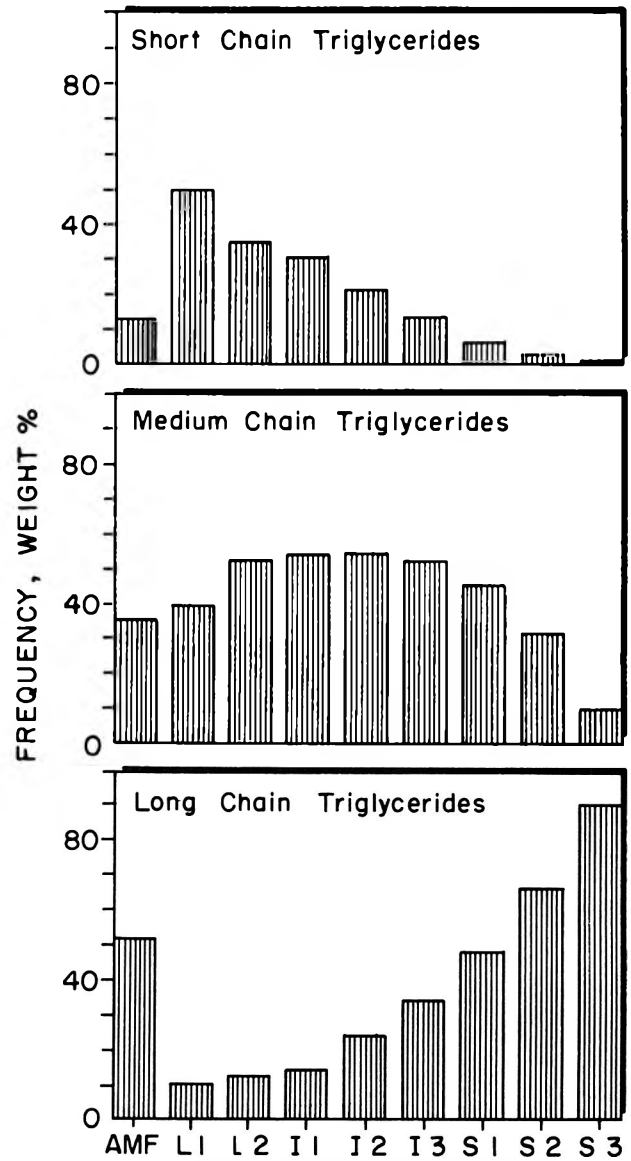

Fig. 3—Distribution of short, medium and long chain triglycerides in anhydrous milk fat (AMF) and milk fat fractions (L1-2, I1-3, and S1-3).

These trends are similar to those observed in fractions obtained by short-path distillation (SPD) (Arul et al., 1987a) and melt crystallization (MC) (Makhlouf et al., 1987) processes. However, the proportion of short chain triglycerides in the liquid fractions or medium chain triglycerides in the intermediate fractions or long chain triglycerides in the solid fractions, obtained by SC–CO_2 extraction, are intermediate between SPD and MC distributions (SPD > SC–CO_2 > MC) (Table 3). The composition of various SC–CO_2 fractions, in short, medium and long chain triglycerides is different from that of native milk fat (Table 2). Fraction L1 was essentially composed of short and medium chain triglycerides while fraction S3 was mainly composed of long chain triglycerides.

Fatty acid composition

Short chain (C4-C8) and medium chain (C10-C12) fatty acids gradually decrease from fraction L1 to S3 and this is reversed for long chain (C14-C18) fatty acids (Table 4 and Fig. 4). These trends are also similar to those observed in SPD and MC processes for the fractionation of milk fat (Arul et al., 1987a; Makhlouf et al., 1987). The proportion of short and

Table 3—Distribution of short, medium and long chain triglycerides in milk fat and its fractions obtained by SC–CO_2, SPD and MC (weight %)

Triglyceride Group	Milk fat	Liquid fraction			Intermediate fraction			Solid fraction		
		SC–CO_2	SPD[a]	MC[b]	SC–CO_2	SPD[a]	MC[b]	SC–CO_2	SPD[a]	MC[b]
Short chain C24 – C34	12.1	40.0	45.8	17.4	20.4	19.3	14.2	2.7	1.4	11.5
Medium chain C36 – C40	35.7	47.9	42.3	37.1	53.7	60.4	39.1	25.2	19.3	28.8
Long chain C42 – C54	52.2	12.2	12.0	45.5	26.0	20.5	46.7	72.3	79.4	59.7

[a] Arul et al. (1987a).
[b] Makhlouf et al. (1987).

Table 4—Fatty acid composition of milk fat and milk fat fractions (mole %)

Fatty acid	Milk fat	Fractions							
		L1	L2	I1	I2	I3	S1	S2	S3
C4:0	11.8	26.4	27.4	20.7	17.6	12.5	12.3	6.0	9.3
C6:0	6.8	12.7	13.4	11.4	10.2	8.3	9.1	7.0	1.8
C8:0	2.3	5.6	4.9	3.7	3.6	3.2	3.1	3.3	0.0
C10:0	5.1	7.6	6.7	5.4	5.5	5.3	4.8	6.3	2.7
C12:0	4.4	5.4	4.9	5.0	4.6	4.1	3.7	4.8	2.9
C14:0	12.6	12.6	12.3	13.8	13.6	13.0	12.1	13.4	10.6
C14:1	1.5	tr[a]	tr	tr	1.3	1.4	tr	1.5	1.4
C15:0	1.0	tr	tr	tr	0.8	1.0	tr	tr	tr
C16:0	26.0	19.2	21.5	26.8	26.9	27.2	31.1	30.4	29.3
C16:1	1.2	tr	tr	tr	tr	1.2	0.0	0.0	1.4
C18:0	8.5	1.4	1.9	4.5	5.2	7.2	7.4	9.0	12.2
C18:1	17.8	5.6	7.1	8.9	10.6	14.6	16.5	18.4	27.2
C18:2	1.0	3.4	tr	tr	tr	0.8	tr	tr	1.1

[a] Traces.

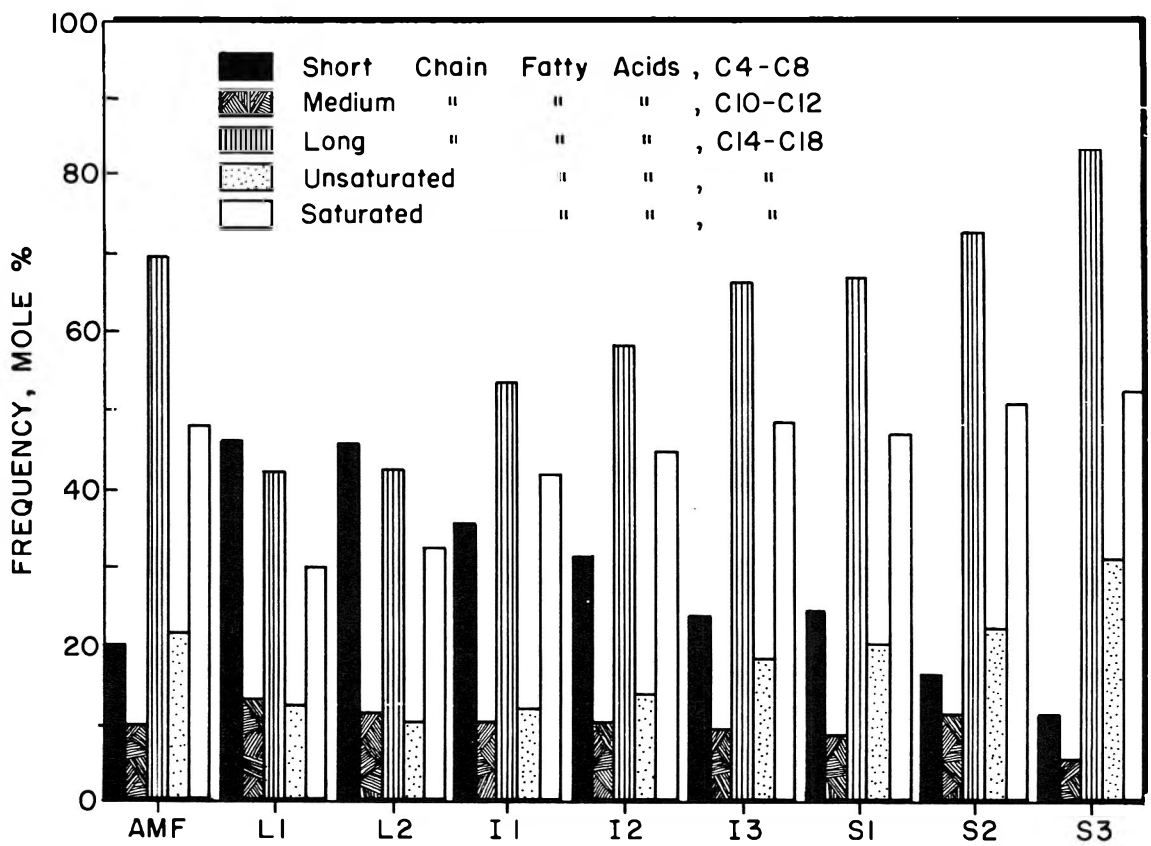

Fig. 4.—Distribution of fatty acid groups in anhydrous milk fat (AMF) and milk fat fractions (L1-2, I1-3, and S1-3).

medium chain fatty acids in all SC–CO_2 fractions was higher than those of SPD and MC fractions (SC–CO_2 > SPD > MC) in the liquid and intermediate fractions and SC–CO_2 > MC > SPD in the solid fractions. The concentration of long chain fatty acids in the liquid and intermediate fractions of SC–CO_2 extraction was lower than those of SPD and MC (SC–CO_2 < SPD < MC) and, in the solid fractions, it was in the order SC–CO_2 < MC < SPD. However, the concentration of unsaturated fatty acids in the liquid and intermediate fractions was similar to SPD but lower than that of MC and was intermediate between SPD and MC (SPD > SC–CO_2 > MC) in the solid fractions (Table 5).

Table 5—Distribution of short, medium and long chain fatty acids in milk fat and its fractions obtained by SC–CO_2, SPD and MC (mole %)

Fatty acid group	Milk fat	Liquid fraction			Intermediate fraction			Solid fraction		
		SC–CO_2	SPD[a]	MC[b]	SC–CO_2	SPD[a]	MC[b]	SC–CO_2	SPD[a]	MC[b]
Short chain C4 – C8	16.9	45.3	26.0	20.1	29.3	16.9	17.3	15.9	4.5	12.6
Medium chain C10 – C12	8.2	12.1	12.8	8.7	9.9	8.8	7.7	8.1	5.4	6.2
Long chain C14 – C18	74.7	42.5	61.2	71.2	60.8	74.3	75.0	75.8	90.1	81.2
Unsaturated	24.0	10.9	13.5	30.9	15.2	19.3	25.8	25.4	33.1	22.5
Saturated	50.7	31.6	47.7	40.3	45.6	55.0	49.2	50.4	57.0	58.7

[a] Arul et al. (1987a).
[b] Makhlouf et al. (1987).

Table 6—Average triglyceride size of milk fat and milk fat fractions

	Wt avg mol wt	Geometric mean carbon number
Native milk fat	729.3	41.0
Short chain triglycerides	584.0	32.5
Fraction L1	625.6	34.2
Fraction L2	643.7	35.4
Total liquid fraction	637.0	34.8
Medium chain triglycerides	666.8	37.2
Fraction I1	661.1	36.0
Fraction I2	682.3	37.0
Fraction I3	702.5	38.2
Total intermediate fraction	683.4	37.2
Long chain triglycerides	806.3	46.9
Fraction S1	720.3	39.7
Fraction S2	750.8	42.9
Fraction S3	805.0	47.6
Total solid fraction	765.7	47.6

Solubility phenomena and milk fat properties

At low densities of the gas, the short chain triglycerides are dissolved into the supercritical fluid phase. As the pressure (density) of the gas is increased at a constant temperature, intermediate and higher molecular weight triglycerides commence migration into the mobile phase at a threshold value of density or pressure. Consequently, there is a distinctive level of compression at which solubility of a species is observed. Dense gas extraction involves the phenomena of distillation and extraction simultaneously (Zosel, 1978). Distillation involves the vapor pressure enhancement (Diepen and Scheffer, 1948; Robin and Vodar, 1953; Webster, 1953) due to applied pressure (Poynting effect) (Webster, 1953; Prausnitz, 1969) and supercritical gas solubility in the oil phase under pressure (Webster, 1953; McHugh and Yogan, 1984). Extraction involves ideal solubility (Bailey, 1950; Morrison and Boyd, 1973; Krukonis and Kurnik, 1985) and phase separation (Hildebrand and Scott, 1950; Rowlinson and Richardson, 1959; Vetere, 1979; Schneider, 1980; King, 1983).

Milk fat is a mixture of triglycerides with a range of molecular weights from 470 to 890, and with a degree of packing within the crystal (triglycerides composed of fatty acids of uneven length and unsaturated fatty acids with cis-configuration) as well as variation in nature (dispersion and polar) and magnitude of intermolecular or cohesive energies. Variation in size and packing regularity of crystal structure (entropy) lead to a wide variation in melting points (-30°C to 40°C) for milk fat triglycerides. Variation in molecular weight and unsaturation lead to differences in volatility of triglycerides (Perry et al., 1949). In a homologous series, i.e., of similar nature of intermolecular forces, the cohesive energy is a function of the molecular size. Volatility of triglycerides, therefore, decreases with their molecular weight. Variations in the nature and magnitude of cohesive energy density (δ^2 = cohesive energy/molar volume, δ is termed solubility parameter) of triglycerides would lead to differences in solute-solvent interactions of the triglycerides (regular solution theory, Hildebrand and Scott, 1950). Solution process, like the vaporization process, causes mutual displacement of solute molecules. From regular solution arguments, it follows that the solute-solvent mixing is best achieved when the nature and cohesive energy densities of the solute and solvent molecules are matched, i.e., heat of mixing or interchange energy density, $(\delta_{solute} - \delta_{solvent})^2$ is zero when the solubility would be maximum. The regular solution Scatchard-Hildebrand model (Prausnitz, 1969) also indicates that with increasing molar volume (size) of the solute molecules of similar cohesive energy densities, the solubility would decrease, i.e., a large number of solvent molecules would be required to cluster around or saturate a larger molecule than for a smaller one to effect the dissolution process. The variation among the tryglycerides in the molecular packing of crystals would lead to differences in their ideal solubility (i.e., solubility due to close or loose molecular packing of crystals) (Morrison and Boyd, 1973). The principle of fractionation by extraction lies in the difference in the size, enthalpic (intermolecular interaction) and entropic (molecular packing of crystals) characteristics of the solute molecules as well as the solvent effect.

Controlling principles in milk fat fractionation

The general tendency in SC–CO_2 extraction, SPD and MC processes is that the concentration of short chain triglycerides and fatty acids decreases from liquid to solid fractions and vice versa for long chain triglycerides and fatty acids. This would indicate that the molecular size has a primary influence in the molecular separation of milk fat triglycerides. However, the differences in the proportion of various groups of triglycerides or fatty acids in liquid, intermediate and solid fractions obtained by these processes, would suggest that other principles which may or may not depend on the molecular size, should also be operative in the separation of triglycerides (Arul et al., 1987b).

The proportion of short, medium and long chain triglycerides in liquid, intermediate and solid fractions, respectively, obtained by SC–CO_2 extraction, was close to those obtained by SPD. Furthermore, the trend of increasing concentration of unsaturated fatty acids from liquid to solid fractions was common for both SC–CO_2 and SPD and SPD processes. These similarities between SC–CO_2 extraction and SPD processes support the fact that the enhancement of vapor pressure of triglycerides due to applied pressure (Poynting effect) and supercritical gas solubility in the oil phase weakening its intermolecular force play a role in SC–CO_2 extraction. Relatively high volatile short chain triglycerides are thus eluted at a lower gas pressure. The presence of liquid portion in fraction S3 (Fig. 2), though it is limiting in short chain triglycerides (Fig. 3), is possibly due to the presence of triglycerides with high entropy of fusion.

On the other hand, the proportion of short, medium and long chain triglycerides in liquid, intermediate and solid fractions of the SC–CO_2 extraction was intermediate between the SPD and MC. Furthermore, the proportion of long chain and unsaturated fatty acids in the solid fraction was somewhat sim-

ilar to that of MC process. This would suggest that the ideal solubility principle may also be involved in SC–CO_2 extraction of milk fat triglycerides. The data of Krukonis and Kurnik (1985) on the solubility of o-, m-, p-hydroxybenzoic acid in SC–CO_2 illustrate this principle.

The fatty acid composition of the SC–CO_2 milk fat fractions is different from those of SPD and MC processes, although their melting temperature profiles are essentially the same as those of SPD and MC processes (Arul et al., 1987a; Makhlouf et al., 1987). This indicated that the separation of triglycerides by SC–CO_2 extraction involved the additional principle of solute-solvent interaction or affinity leading to miscibility of triglycerides with the mobile CO_2 phase. The cohesive energy density (δ^2) of SC–CO_2 increases with pressure (Allada, 1984). As the cohesive energy density of SC–CO_2 approaches that of triglycerides (Arul et al., 1987b), the affinity of the gas molecules to the triglycerides would increase and would be maximum when $\delta_{solute} = \delta_{gas}$, as postulated by the regular solution theory. The clustering of gas molecules around triglyceride molecule to effect the dissolution process is driven by their affinity for the solute (solute-solvent interaction), and the number of gas molecules which can cluster around the solute molecule is dictated by the size of the solute molecule. Low gas concentration (density) is adequate to saturate a small molecule and hence short chain triglycerides of low cohesive energy density are thus dissolved at low pressure. Subsequent increases in gas pressure increase the gas concentration as well as its cohesive energy density and hence short chain triglycerides of high cohesive energy density shall commence migration into the fluid phase. Separation of higher molecular weight species is effected with further increases in gas pressure, by the interplay of affinity and size of the triglycerides.

Size separation efficiency

The weight average molecular weights (WAMW) and geometric mean carbon number (GMCN) of various fractions and groups of triglycerides are presented in Table 6. GMCN was obtained from the square root-normal distribution plot on a probability scale. There was a gradual increase in WAMW as well as GMCN with increasing pressure or density of the gas at a constant temperature. This suggested that SC–CO_2 effected a fair degree of molecular weight separation; nevertheless, there was a certain overlapping of molecular weights in all the fractions as SC–CO_2 extraction involved more than one principle of separation.

SUMMARY

DENSE CO_2 EXTRACTION selectively dissolved components of a mixture as its solvent power was manipulated by the change of pressure and temperature. It offered an excellent opportunity for obtaining milk fat fractions with distinctive differences in chemical and physical properties which could satisfy the requirements of many food applications. This also presented an opportunity in developing convenient (e.g., frigospreadable butter) as well as dietetic butter rich in short and medium chain triglycerides for dyspeptic disorders (Bour, 1980). Semi-quantitative analysis of cholesterol (results not shown) indicated that fractions L1 and L2 were enriched while fractions S2 and S3 were lower in cholesterol content than the native butterfat. The cholesterol content in fractions 11-S1 was comparable to that of milk fat.

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Ms received 7/18/86; revised 10/31/86; accepted 3/17/87.

This work was supported by the Canadian Dairy Commmission, contract no. OSU81-00410. The authors gratefully acknowledge Dr. V.J. Krukonis, Phasex Corporation, 360 Merrimac St., Lawrence, MA 01843 for carrying out the fractionation of milk fat; Mr. C. Gosselin for his technical assistance and Dr. R.N. Giroux, Agropur Cooperative Agro-Alimentaire, Granby, Quenbec, J2G 7G2 for his encouragement during the course of this investigation. The support for this publication by the Conseil de Recherche et Service du Québec (CRSAQ) is gratefully acknolwedged.

Reduction of Beta-Lactoglobulin Content of Cheese Whey by Polyphosphate Precipitation

S.A. AL-MASHIKH and S. NAKAI

ABSTRACT

When cheddar cheese whey was treated under the optimized conditions, i.e., 1.33 mg/mL sodium hexametaphosphate (SHMP) at pH 4.07 and 22°C for 1 hr, more than 80% of β-lactoglobulin was removed by precipitation. In the supernatant, almost all of the immunoglobulins and the major portion of α-lactalbumin were retained, as indicated by SDS gel electrophoresis. Immunochemical assays showed that approximately 90% of immunoglobulin G activity was found in the supernatant. By dialysis against water for 48 hr, 72.2% and 45.3% phosphorous was removed from the supernatant and precipitate, respectively.

INTRODUCTION

BETA-LACTOGLOBULIN has been regarded as a major allergen for bottle-fed infants (Lebenthal, 1975; Wharton, 1981; Moneret-Voutrin et al., 1982; Pahud et al., 1985). The fact that human milk contains only a trace of this protein (Liberatori and Napolitano, 1980; Friend et al., 1983) justifies the elimination of β-lactoglobulin from infant formulae.

Based on immunological reactions, human milk contains a trace of β-lactoglobulin (Brignon et al., 1985). However, it is rich in immunoglobulins (Igs), lactoferrin (Lf), lysozyme (Ly), leucocytes, lactoperoxidase, the bifidus factor and interferon (Packard, 1982; Friend et al., 1983) as compared to cow's milk, the basic replacer of human milk. Fortifying cow's milk or whey protein based formula with such biologically important proteins would improve the therapeutic value and the resistance of infants against gastrointestinal diseases. Although the benefits of feeding infants with nonimmunized Ig have not yet been clearly established as they have been for feeding hyperimmunized Ig (Hilpert et al., 1974/1975; Ballabriga, 1982; Ebina et al., 1984, 1985), some uses may be warranted in collaboration with other antimicrobial components in cow's milk as discussed by Packard (1982). Oral administration of human colostrum to high risk, low birth weight infants is reported to significantly reduce the rate of infection (Narayanan et al., 1983).

Recently, a ferric chloride method was established for selectively precipitating β-lactoglobulin from bovine whey (Kuwata et al., 1985; Kaneko et al., 1985). However, because of the potential loss of the antimicrobial activity of lactoferrin when it is saturated with iron, nonferric methods have been duly investigated. Polyphosphates have been extensively used as additives in food processing. Gordon (1945) used polyphosphates to precipitate proteins from cheese whey.

The objective of this paper was to establish a new method for eliminating β-lactoglobulin from cheddar cheese whey by using food grade polyphosphate.

MATERIALS & METHODS

CHEDDAR CHEESE WHEY was obtained from Dairyland Foods (Burnaby, B.C.). The whey was centrifuged at 5000 x *g* for 10 min to remove the residual casein curd. Sodium hexametaphosphate (SHMP, purified grade) was purchased from Fisher Scientific Company (Fairlawn, NJ); β-lactoglobulin, α-lactalbumin and bovine immunoglobulin (Ig) were purchased from Chemalog (South Plainfield, NJ); Ig was also purchased from Sigma Chemical Company (St. Louis, MO). Rabbit anti-bovine whey protein antiserum was a gift from Mead Johnson & Co., (Evansville, IN).

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Optimization procedure

The mapping super simplex optimization (MSO) of Nakai et al. (1984) was used to find the most suitable conditions for the polyphosphate treatment of cheese whey which would give the maximum separation efficiency of Igs and a minimum amount of β-lactoglobulin in the supernatant. An IBM PC computer was used for computation for the MSO and centroid mapping optimization (CMO) by the method of Aishima and Nakai (1986). The experimental conditions (factors) used in MSO and CMO were within the following ranges: pH 4.0-4.5, SHMP concentration 1.0-1.4 mg/mL. All experiments were carried out at room temperature (22°C).

Surface plot

Contour and 3-dimensional surface plots were obtained using the UBC Surface Visualization Routines program (Mair, 1982), on an Amdahl 470 V/8 computer. The 3-dimensional plot was rotated and tilted for the best view of the surface: (a) The "about" angle was the angle of turn, in degrees, of rotation about the z-axis, measured clockwise from the positive x-axis; (b) the "above" angle was the angle of tilt, in degrees, of rotation about the y-axis, measured above the xy plane. In this work, x = pH, y = SHMP and z = SE (separation efficiency).

SHMP treatment of cheese whey

An aliquot of 10% sodium hexametaphosphate solution was added to 25 mL pH adjusted cheese whey while maintaining the pH by dropwise addition of 3N NaOH or 3N HCl. The mixture was held for 1 hr, then centrifuged at 10,000 x *g* for 15 min. The precipitate was dispersed in 5 mL 0.5M Tris-HCl buffer, pH 6.8 and made up to 25 mL after further pH adjustment to 6.8, whereas, the supernatant was neutralized to pH 6.8 with 3N NaOH. The samples were dialyzed against distilled water for 48 hr and then freeze dried (Fig. 1).

Dodecyl sulfate-polyacrylamide gel electrophoresis

The method of Laemmli (1970) was used after some modifications as reported by Kaneko et al. (1985). Polyacrylamide gel electrophoresis in the presence of 0.2% sodium dodecyl sulfate (SDS-PAGE) was performed in a slab type vertical gel system (3% and 10% for stacking and separating gel, respectively) using the Atto SJ 1060 SDH Electrophoresis unit (Atto Co., Tokyo, Japan).

Evaluation of separation efficiency

Peak areas of whey proteins on the electrophoretograms were analyzed using a Kontes fiber optic scanner (Model K-494800, Kontes Scientific Instruments, Vineland, NJ) together with a Varicord variable response recorder (Model 42 B, Photovolt Corp, New York). Separation efficiency (SE) was expressed as the "Igs to β-Lg ratio" calculated from peak area of Igs (PA_{Igs}) and β-Lg ($PA_{\beta\text{-}Lg}$) on the densitometric patterns;

$$SE = PA_{Igs} / (PA_{Igs} + PA_{\beta\text{-}Lg})$$

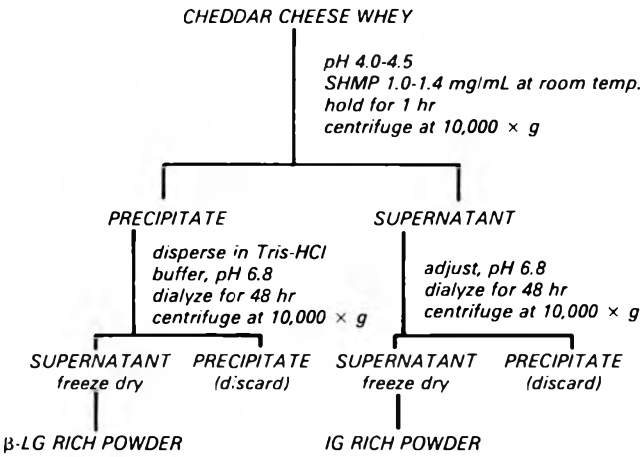

Fig. 1—Flow diagram of the procedure for elimination of β-lactoglobulin from cheddar cheese whey with SHMP.

PA_{Igs} was estimated by multiplying the heavy chain peak area by a coefficient of 1.4 because the determination of light chain peak area was difficult due to overlapping with other minor proteins. The coefficient 1.4 was derived from analysis of IgG standard.

For quantitative analysis, the variation of staining and destaining conditions during electrophoresis was standardized using an internal standard of ovalbumin (ICN Pharmaceutical Inc., Cleveland, OH.). Ten microliters 0.1% ovalbumin solution, treated with SDS and 2-mercaptoethanol (similar to the treatment of sample), was added to each whey sample solution and analyzed simultaneously. The ovalbumin peak area measured for every run was compared with the peak areas measured for a series of standard ovalbumin. The ratio of ovalbumin values, thus obtained, was used as a correction factor.

Immunochemical analysis

Immunochemical analysis and immunodiffusion were carried out according to the methods of Williams and Chase (1971). Nine milliliters 1% agarose in 0.05 M barbital buffer, pH 8.6, was gelatinized over Gelbond film (0.02 × 7.5 × 10cm, FMC Corporation, Marine Colloid Division, Bioproducts, Rockland, ME). A 2.5 μL sample (10 times concentrated) was applied to a punched sample well with a diameter of 2mm and electrophoresis was performed at room temperature (22°C) for 60 min with a constant voltage of 60 volts. Fifty microliters antibody were added to the trough and diffusion was performed overnight at 5°C.

Immunochemical quantitation of IgG was carried out by radial immunodiffusion (R.I.D.) with R.I.D. Kit (Miles Laboratories Inc., Elkhart, IN). Whey samples were dialyzed against 0.01M phosphate buffer, pH 7.2, for two days and freeze-dried. The powders were dissolved in 0.05M barbital buffer, pH 8.6, to give a concentration 10 times that of the original whey for both supernatant and precipitate.

Phosphorus determination

The phosphorus distribution of the fractions was determined according to the method of Morrison (1964).

RESULTS & DISCUSSION

Optimum conditions for separation of Igs and β-Lg

Mapping simplex optimization with two factors led to performing three experiments called "initial simplex." After obtaining the response values which were in our case the separation efficiency (SE) of immunoglobulins, the values were reported back to the computer to obtain new vertices (experimental conditions) as a form of the repetitive sequences of centroid, reflection and curve-fit. After performing nine vertices for the MSO, mapping was done by plotting the response values obtained against the factor levels. A crude approximation of the response surface appeared to direct search for higher SE towards acidic conditions (pH 4.0–4.2); therefore, the range for lower and upper limit of pH was narrowed down to 4.0–4.2. In a similar manner, the general trend of SHMP for higher SE was toward the higher polyphosphate concentrations (1.2–1.4 mg/mL); therefore, the range of concentration of SHMP was reduced to 1.3–1.4 mg/mL.

With the new lower and upper limits for the pH and SHMP, CMO program was applied. After entering the new ranges, a new initial simplex (three vertices) was created. The experiments were carried out and the response values were reported. Upon significant improvement in the response values, simultaneous shift was implemented after six experiments in the centroid search (Fig. 2A, 2B). With the present best values of 4.07 and 1.31, the target values (T) of 4.03 and 1.36 were set for pH and SHMP, respectively. However, because of failure to achieve further improvement in response values, experimentation was discontinued.

Examining the best response value (Vertex 15, Figs. 2A and 2B), it was found that pH 4.07 and SHMP 1.33 mg/mL would give about 80% elimination of β-lactoglobulin from cheddar cheese whey into the precipitate, with almost complete recovery of immunoglobulins in the supernatant. The major portion of α-lactalbumin was found in the supernatant as indicated by SDS-PAGE (Fig. 3). However, most of the bovine serum albumin was precipitated along with β-lactoglobulin, with only a small amount remaining in the supernatant. Recovery of Igs in the supernatant is evident immunochemically as shown in Fig. 4. Supernatants showed a long, outer precipitating line corresponding to the standard IgG arc, while precipitates showed no such precipitating line. The inner precipitating lines (by sample application well) might represent lactoferrin, transferrin and IgM. Serum albumin and β-lactoglobulin arcs are indicated by the inner and the outer precipitating lines, respectively, formed toward the anode, far from the application well. The response of antibovine whey protein antiserum toward α-lactalbumin was rather weak (not shown), probably due to its low molecular weight and consequently lower antigenicity. Approximately 90% of Igs in the supernatant was determined to be IgG by means of R.I.D. (not shown).

Graphic illustration of pH, SHMP and SE relationship

Contour and 3-dimensional surface plots were generated by computer to aid in visualization of the relationship between pH, SHMP and SE of immunoglobulins in cheddar cheese whey. Figures 5A and 5B show that, in general, combinations of low pH and high SHMP concentration result in good separation efficiency. Separation efficiency was improved by decreasing pH values below 4.25 and by increasing SHMP concentration above 1.2 mg/mL.

The contour plot (Fig. 5A) shows three humps with SE of 0.508, 0.577 and 0.302, which can be seen also in Fig. 5B. Multiple peaks might have been caused by the absence of data points between SE 0.508 and the other two peaks with SE 0.302 and 0.577; in other words, more experiments under the conditions with closer intervals between pH 4.2 and 4.08 might be required for obtaining smoother surfaces.

Elimination of phosphorus

Table 1 shows the phosphorus distribution in the supernatant and the precipitate. 72.2% and 45.3% of the total phosphorus have been removed from the supernatant and the precipitate, respectively, by dialysis against distilled water for 48 hr. Facile removal of polyphosphate-phosphorus from whey preparations by dialysis might indicate weak binding of phosphorus with whey proteins. Since no precipitation of whey proteins by SHMP was observed at pH values higher than 5, it was assumed that ionic interaction might be involved in the interaction between SHMP and proteins. At pH lower than the isoelectric point, positively charged groups (basic amino acid residues) in protein molecules might interact with each other via SHMP and cause the aggregation of the proteins. By increasing the pH

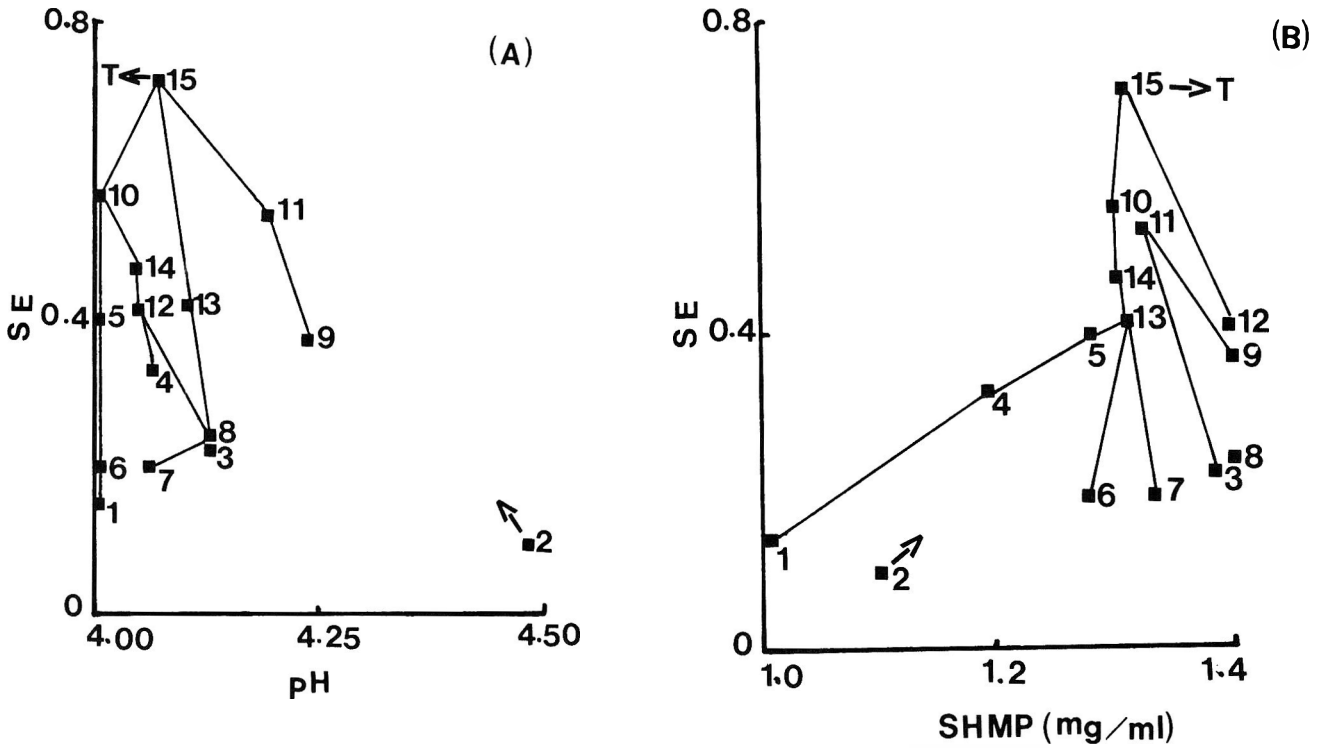

Fig. 2—Approximate response surface patterns for (A) pH and (B) SHMP concentration obtained by mapping accumulated data from simplex optimization (Vertices 1-9) and centroid optimization (Vertices 10-15). T, target values of pH and SHMP.

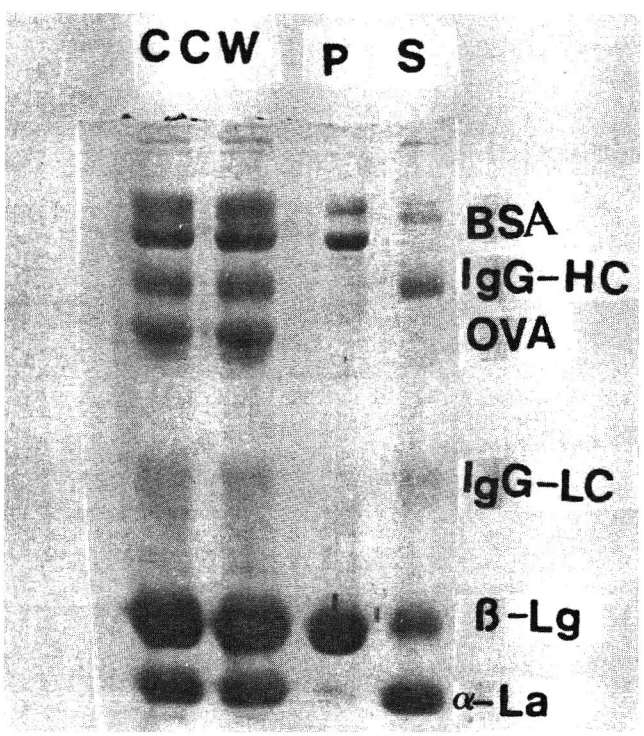

Fig. 3—SDS-PAGE of supernatant (S) and precipitate (P) obtained after treatment with 1.31 mg/mL SHMP at pH 4.07. CCW, cheddar cheese whey; α-La, α-lactalbumin; β-Lg, β-lactoglobulin; IgG-HC, immunoglobulin G heavy chain; IgG-LC, immunoglobulin G light chain; BSA, bovine serum albumin; OVA, ovalbumin.

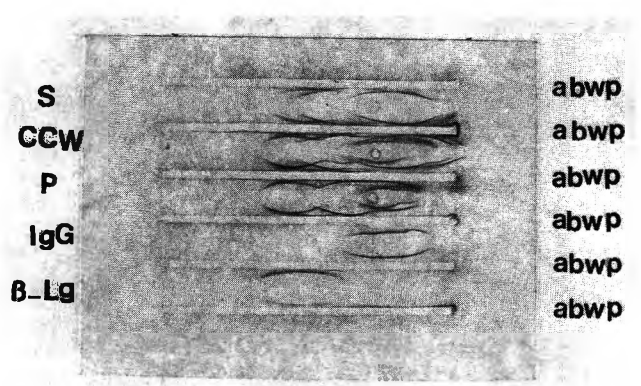

Fig. 4—Immunoelectrophoretic pattern of cheddar cheese whey. CCW, cheddar cheese whey; S, supernatant; P, precipitate; IgG, immunoglobulin G; β-Lg, β-lactoglobulin; abwp, antibovine whey proteins.

above the isoelectric point and increasing net negative charges, this interaction might be disrupted and the free SHMP could be removed by a simple dialysis process, thereby separating a whey protein fraction containing phosphorus within the level recommended in infant formula (1.56%).

Proposal of new infant formula

Table 2 compares the composition of the new infant formula to that of human and cow's milk and the current commercial SMA (whey-based) formula. In the commercial SMA formula, the ratio of casein/whey proteins (79/21) of cow's milk has been changed to 40/60 in order to mimic the ratio found in human milk. However, simple adjustment of casein/whey protein ratio does not minimize the compositional differences between cow and human milk proteins, i.e. higher contents of α-lactalbumin, lactoferrin, immunoglobulins and lysozyme in human milk as compared to cow's milk.

By eliminating β-Lg completely with full retention of other whey proteins, the whey protein composition of the new β-

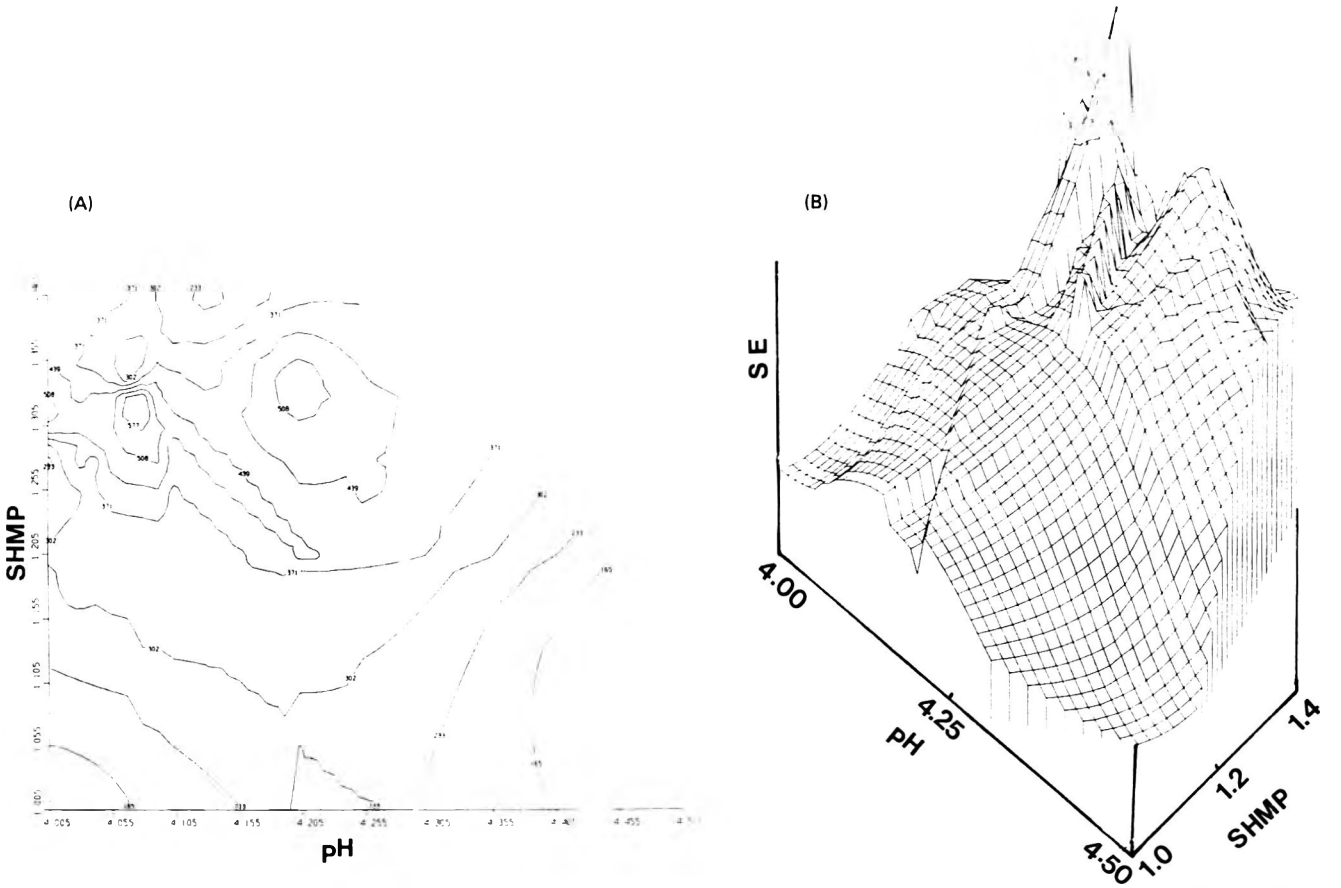

Fig. 5—Contour (A) and 3-dimensional (B) surface plots of relationship between pH, SHMP and separation efficiency (SE) of cheese whey treatment. ("about" angle = 60° and "above" angle = 35° for 3-dimensional plot).

Table 1—Phosphorus (P) distribution in supernatant and precipitate obtained by SHMP treatment

Fraction	mg P/100 mL	mg P/100 mL after dialysis	% Removed by dialysis
Supernatant	65.0	18.1	72.2
Precipitate	16.0	8.75	45.3
Cheese whey	50.0	8.6	82.8

Table 2—Protein composition of human and cow's milks and whey-based and proposed β-lactoglobulin (β-Lg)-free infant formula

Protein	Human[a] Total %	Cow Total %	Whey-based formula Total %	β-Lg free formula Total %
Total	100	100	100	100
Caseins	35.0	79.0[a]	40.0[c]	40.0
Total whey	65.0	21.0[a]	60.0[c]	60.0
α-lactalbumin	17.0	2.8[b]	8.0	17.2[d]
β-lactoglobulin	--	11.2[b]	32.0	--
immunoglobulins	11.0	2.3	6.6[d]	14.1[d]
serum albumin	6.0	1.8	5.1[d]	10.9[d]
lactoferrins	17.0	1.7[b]	4.9[d]	10.5[d]
lysozyme	6.0	--	--	--
others	8.0	1.2[b]	3.4[d]	7.3[d]

[a] Gurr (1981)
[b] Calculated from electrophoretic scanning of cheddar cheese whey
[c] Friend et al. (1983)
[d] Whey protein composition is calculated based on our data (b)

Lg-free formula can be seen in Table 2. In this proposed formula, immunoglobulins and lactoferrin are much closer in their quantities to their counterpart in human milk. In addition, lysozyme separated from egg white can be incorporated in this new infant formula as proposed by Friend et al. (1983), to improve the therapeutic value of infant formula. Thus, the β-Lg-reduced supernatant can be incorporated into infant formulae, and since it is rich in immunoglobulins, it can be an additional benefit to infant feeding.

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Calcium Chelation and Other Pretreatments for Flux Improvement in Ultrafiltration of Cottage Cheese Whey

J. PATOCKA and P. JELEN

ABSTRACT

Cottage cheese whey was treated to minimize effects of calcium on membrane fouling during ultrafiltration in a stirred Amicon cell. The treatments used were stepwise pH adjustment from 4.5 to 1.5; chelation of calcium with EDTA or citric acid: calcium chelation followed by pH adjustment to 2.5; and calcium replacement with sodium by ion exchange. All treatments resulting in elimination of free calcium improved the flux. Highest flux increase (53%) in the 8-hr processing runs was for citric acid (1.25 meq/one meq Ca) after pH adjustment to 2.5. Addition of $CaCl_2$ decreased the flux. Confirmatory experiments with DDS-Lab 20 equipment showed 25% flux increase after treatment with either EDTA (one meq/one meq Ca) or citric acid (1.25 meq/one meq Ca) with pH adjusted to 2.5.

INTRODUCTION

ULTRAFILTRATION (UF) has become an accepted industrial process of separation and purification. One of the successful commercial applications is the UF of cheese whey. The major limiting factor in UF is the decline of flux with time due to membrane fouling, a condition in which a membrane undergoes plugging and coating by some components in the feed stream in such a way that its output (flux) is reduced (Eykamp, 1978; Merin, 1979). Lee and Merson (1976) reported that in UF of cottage cheese whey, membrane fouling can be caused by several factors, including inorganic ions (P and Ca) which may be deposited on the membrane. Muller and Harper (1979) demonstrated that various protein-membrane, calcium-membrane and phosphate-membrane interactions are involved in the membrane fouling phenomenon. There is a tendency in the literature to confuse fouling and concentration polarization. Both lead to lowering of the flux but from the definition of concentration polarization (Michaels et al., 1971; Cheryan, 1977) it can be seen that the latter is a reversible process which is independent of time, while fouling is often irreversible.

Most of the calcium in whey is present in the form of salts, mainly as calcium phosphates (Hill, 1986). Part of the total calcium is bound to major whey protein fractions, β-lactoglobulin (Zittle et al., 1957) and α-lactalbumin (Hiraoka et al., 1980; Kronman et al., 1981; Bernal and Jelen, 1984). Cottage cheese whey contains almost twice as much calcium as the cheddar, swiss and other types of rennet wheys (Merin, 1979). The whey UF flux improvement is possible by decreasing pH to solubilize the calcium phosphate (Kuo and Cheryan, 1983), but this does not exclude an interaction of calcium with the membrane (Merin, 1979). The sequestration of calcium by chelating agents can eliminate this possibility, but removing calcium from the calcium-protein complexes can supposedly destabilize the proteins with the following increase in fouling due to higher deposits of proteins on the membrane (Lee and Merson, 1976). The exact effect of calcium on the flux in ultrafiltration of cheese wheys is still poorly understood.

The objective of this study was to investigate effects of several pretreatments of cottage cheese whey aimed at maximizing the UF flux by manipulating the content and/or availability of calcium. The pretreatments studied included (a) pH adjustment, b) calcium sequestration by EDTA or citric acid, (c) calcium sequestration followed by pH adjustment, (d) calcium replacement with sodium by ion exchange, and (e) addition of calcium chloride as a control measure.

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MATERIALS & METHODS

Materials

Cottage cheese whey (pH 4.5-4.6) was obtained from local dairy plants, cooled to 7°C, and sedimented casein fines were removed. City water used for flushing and cleaning the UF membrane and other tasks in this work was deionized by passing through a reverse-osmosis (RO) purification system (Milli-Q Purification System, Millipore Corp., Bedford, MA). The whey pH was adjusted with 1N HCl as needed. Calcium chelation was accomplished with disodium ethylenediamine tetraacetic acid (EDTA; Fisher Scientific, Fair Lawn, NJ) or citric acid (J.T. Baker Chem. Co., Phillipsburg, NJ). Calcium replacement with sodium was achieved by ion exchange on the Bio-Rex 70 resin (Bio-Rad Laboratories, Richmond, CA) used in the sodium form, capacity 10 meq/g. To further study the effects of free calcium ions on ultrafiltration flux, 0.013M $CaCl_2$ (J.T. Baker Chem. Co., Phillipsburg, NJ) was added to the control whey in one series of experiments.

Equipment

Most of the ultrafiltration experiments were carried out on an Amicon 8400 stirred cell ultrafiltration unit (Amicon Corp., Lexington, MA) using PM-10 flat sheet membranes with diameter of 76 mm, MW cut-off 10,000 daltons, area 41.8 cm^2. The unit was pressurized to 3.5 bar with nitrogen.

Selected confirmatory experiments were also carried out on a DDS 20-Lab ultrafiltration module (De Danske Sukkerfabrikker, Nakskov, Denmark) containing 10 sandwiches of GR 61-PP membranes with MW cut-off 20,000 daltons; overall filtration area was 0.36 m^2. The pH was measured by an Orion Model 601A pH meter (Orion Research Inc., Cambridge, MA). The calcium and phosphorus contents were determined in duplicate for each experiment with atomic emission spectrophotometer AtomScan 2000 (Jarrell-Ash Div., Fisher Sci. Co., Waltham, MA).

Experimental procedures

In each experiment with the Amicon system, approximately one liter treated whey was ultrafiltered in an 8 hr run. The permeate was collected in a tared beaker to measure the flux and returned to the feeding tank. All runs were carried out at a constant pressure of 3.5 bar at room temperature (range 21–23°C). At the end of each experiment the feed and permeate were analyzed for calcium and phosphorus content by atomic emission. The constant level of liquid in the stirred unit (the feed side) was controlled manually using the Amicon Concentration/Dialysis Selector.

The replacement of calcium with sodium by ion exchange followed the method described by Ennis et al. (1981). Cottage cheese whey was passed through a downflow column filled with the Bio-Rex resin in sodium cycle. Initially, the resin was wetted with RO-treated deionized water. The content of calcium was determined before and after passing the whey through the column. Because of the increase of pH from 4.5 to 8.0, the pH was re-adjusted to the original value of 4.5 with 1N HCl.

Before each run the UF membrane was pretreated by running RO-

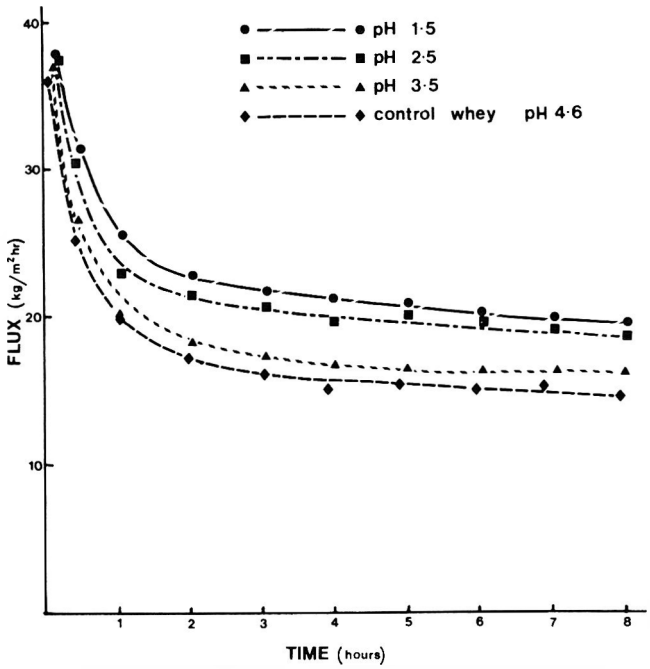

Fig. 1—Effect of pH on flux in ultrafiltration of cottage cheese whey.

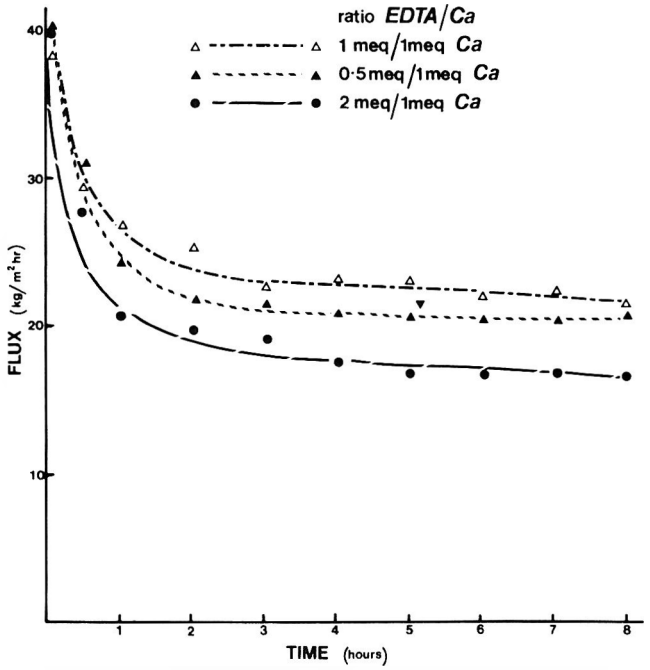

Fig. 2—Effect of calcium sequestration by EDTA on ultrafiltration of cottage cheese whey.

deionized water through the system until a constant flux was obtained. After finishing a run the membrane was cleaned by a standard cleaning procedure (Chong et al., 1985) with 1.2M NaOH and 0.01M EDTA to ensure removal of proteins and other deposits. Finally, the UF unit and all feed lines were rinsed with deionized water to remove the cleaning solution.

In the experiments carried out on the DDS-UF module, a 25L batch of cottage cheese whey was recirculated from a 50L feeding tank by a high pressure piston pump (model 12-18/50, Rannie, Coppenhagen). The retentate was continuously recycled to the feeding tank. The permeate was returned to the feeding tank in specific time intervals necessary for determination of the flux rate. Experiments were performed at the standard temperature of 21 ± 1°C and constant transmembrane pressure of 3.5 bar.

All ultrafiltration runs were replicated three times with different batches of whey. Correlation coefficients for all flux progress curves were determined using a routine Univ. of Alberta computer program.

RESULTS & DISCUSSION

THE pH ADJUSTMENT seems to be the simplest way to minimize membrane fouling in the ultrafiltration of cottage cheese whey. It has been observed that acidification of whey enhanced permeation rates (Hayes et al., 1974; Lee and Merson, 1976; Bresslau and Kilcullen, 1977; Cheryan and Merin, 1980). The change in pH is considered to affect the formation of the membrane deposit rather than the composition of the whey (Merin, 1979).

The flux changes observed in our experiments for pH conditions between 4.5 (untreated whey) and 1.5 are shown in Fig. 1. The variability of the replicate data points summarized in this and subsequent figures was small (absolute mean error ± std dev of all data was 5.6 ± 4.2, n = 414) and the range of correlation coefficients of the third degree best fit polynomials for all curves reported was 0.94-0.98. With pH adjustment to 2.5 or 1.5 the flux was 20% and 24% higher, resp., than in the original whey; however, at pH 1.5 an objectionable change in the odor of the whey was observed, thus making this treatment questionable for industrial practice. The same defect was noted by Kuo and Cheryan (1983). Kuipers and Meggle (1975) pointed out the possibility of proteolysis at this low pH.

Contents of Ca and P in the feed and the permeate are summarized in Table 1. The differences observed indicate little retention of calcium on the feeding side or on the membrane. There was a slightly diminishing difference in the retention of both calcium and phosphorus with decreasing pH. The solubility of whey salts, particularly calcium phosphates, was apparently enhanced by the low pH (Lehninger, 1982) and the permeation of both Ca and P ions through the membrane increased (Kuo and Cheryan, 1983).

Table 1—Content of calcium and phosphorus in cottage cheese whey ultrafiltered at different pH conditions

	% Calcium		%	% Phosphorus		%
pH	Feed	Permeate	Retention	Feed	Permeate	Retention
4.5	0.119	0.101	15	0.088	0.066	25
3.5	0.121	0.103	15	0.086	0.072	16
2.5	0.118	0.106	10	0.083	0.080	4
1.5	0.120	0.110	8	0.085	0.082	3.5

Several investigators (Hayes et al., 1974; Lee and Merson, 1976) have suggested that calcium, present in the acid whey mainly in the ionic state, may be sequestrated by the addition of chelating agents. When calcium chelation was carried out by the EDTA or citric acid additions, increases in flux were observed (Fig. 2 and 3). The EDTA was added directly to the whey and dissolved in various equimolar ratios matched to the calcium content. The lowest increase in the flux rate—25% above the control whey—was recorded for the use of excess EDTA (2 meq/one meq Ca). The highest increase in the flux was obtained by adding EDTA in ratio 1 meq/1 meq Ca. Slightly lower flux was observed when using 0.5 meqEDTA/1 meq Ca. The calcium content in the permeate compared to the calcium level of incoming whey (feed) is shown in Table 2. The decrease in the difference between feed and permeate calcium is obvious when using excess EDTA, while the amount of retained phosphorus did not change appreciably. This may indicate that the role of phosphorus in UF flux losses may be minimal.

Similar effects, including the increase in flux, were seen after addition of citric acid (Fig. 3). The highest improvement in the flux rate—38% above the control whey—corresponded to the addition of citric acid in the ratio 1.25 meq/1 meq Ca. With excess citric acid the flux rose only 20%, while for the

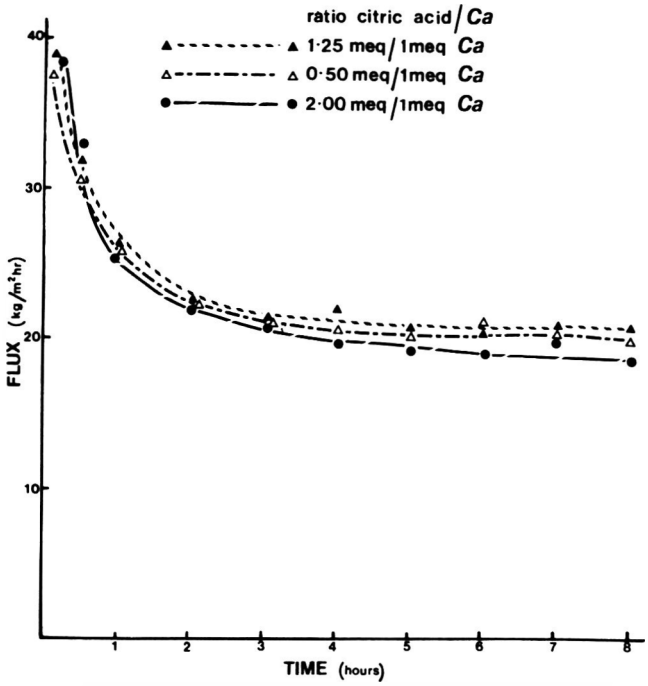

Fig. 3—Effect of calcium sequestration by citric acid on ultrafiltration of cottage cheese whey.

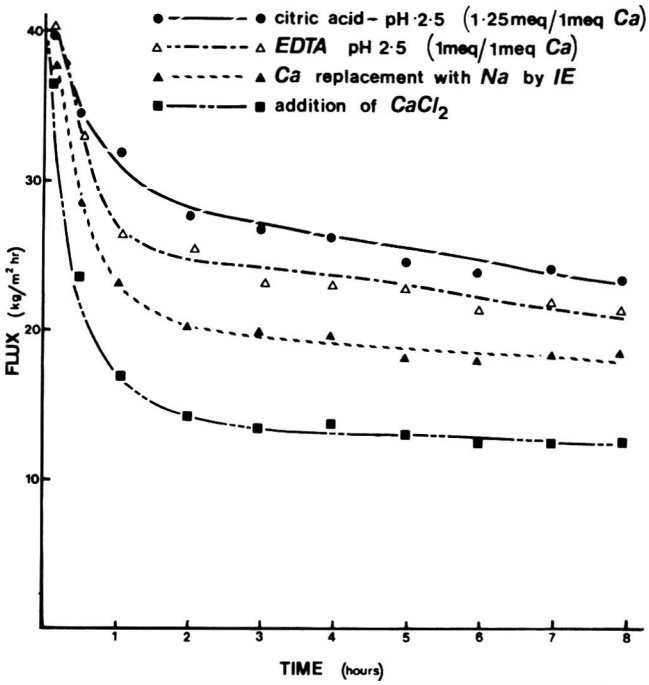

Fig. 4—Comparison of flux change for various treatments of cottage cheese whey.

Table 2—Content of calcium and phosphorus in UF cottage cheese whey with addition of EDTA (pH 4.0)

Ratio EDTA/Ca	% Calcium		% Retention	% Phosphorus		% Retention
	Feed	Permeate		Feed	Permeate	
0.5	0.123	0.107	13	0.088	0.069	22
1	0.122	0.110	10	0.086	0.072	16
2	0.119	0.117	2	0.088	0.070	20

Table 3—Calcium and phosphorus in UF cottage cheese whey with addition of citric acid (pH 3.5)

Ratio Citric acid/Ca	% Calcium		% Retention	% Phosphorus		% Retention
	Feed	Permeate		Feed	Permeate	
0.50	0.121	0.110	9	0.082	0.069	16
1.25	0.124	0.114	8	0.084	0.069	18
2.00	0.118	0.112	5	0.082	0.070	15

0.50 meq/1 meq Ca ratio the increase in flux was 34%. Deposits on the membrane had similar appearance as in the case of EDTA treatment. Calcium and phosphorus contents and their retention (Table 3) showed a pattern generally similar to the values recorded for the EDTA pretreatments.

In the next series of experiments, the chelation of calcium was followed by pH adjustment to 2.5 (Fig. 4). For the chosen amount of EDTA (1 meq/1 meq Ca) no further improvement in the flux was observed in comparison with whey treated only by EDTA without subsequent acidification to pH 2.5. The addition of EDTA alone changed the whey pH to 4.0 When citric acid was used as a chelating agent (1.25 meq/1 meq Ca), the subsequent lowering of pH to 2.5 resulted in the largest flux increase—53% above the control whey. The values of calcium and phosphorus contents for both experiments are shown in Table 4. The differences between the feed and permeate were small. It appears that most of the calcium was chelated and transferred through the membrane together with the phosphorus solubilized as a result of the low pH. Due to calcium complexing, one cannot expect the availability of free Ca ions for membrane-calcium interactions typically observed in UF of whey (Muller and Harper, 1979; Nisbet et al., 1981).

The reason for replacing calcium with sodium instead of complete demineralization was to avoid major changes in the ionic strength of whey. This treatment again resulted in improvement of the flux (Fig. 4); however, the increase was smaller than that found for the use of a chelating agent combined with pH adjustment.

Table 4—Calcium and phosphorus in ultrafiltered cottage cheese whey after Ca sequestration and pH adjustment to 2.5

Chelating agent	% Calcium		% Retention	% Phosphorus		% Retention
	Feed	Permeate		Feed	Permeate	
EDTA	0.120	0.114	5	0.084	0.080	5
Citric acid	0.121	0.113	6	0.088	0.082	7

Lee and Merson (1976) observed improvement in the flux rate with increasing ionic strength of whey by addition of $CaCl_2$ which resulted in better dispersion of the proteins in the whey. In an attempt to confirm their observations (40% flux increase for 0.005M, and 72% increase for 0.2M $CaCl_2$ concentrations), 150 mL 0.1M $CaCl_2$ was added to 1L whey to obtain 0.013M $CaCl_2$ concentration. This caused the pH of whey to drop to 3.97; the pH was subsequently adjusted to 2.5 with 1N HCl. The expected improvement in the flux rate was not observed; on the contrary, the flux was 20% lower than that of the control whey (Fig. 4). However, this result is in agreement with the trends observed in other experiments reported herein, as the increased amount of free calcium resulted in lower flux.

Changes in flux for cottage cheese whey ultrafiltered on the DDS UF unit are shown in Fig. 5. With the use of similar treatments which gave the highest improvement in ultrafiltration rate on the Amicon UF system, the flux was also enhanced. The improvement was less dramatic than that observed with the stirred cell, possibly due to the different engineering characteristics - and thus different hydrodynamic conditions - of the plate-and-frame system. The increase in flux, about 25% above the control whey, was almost identical for the use of EDTA (1 meq/1 meq Ca) or citric acid (1.25 meq/1 meq Ca) with the pH of whey adjusted to 2.5.

CONCLUSIONS

ALL PRETREATMENTS of cottage cheese whey resulting in the elimination of free calcium appeared to increase the UF

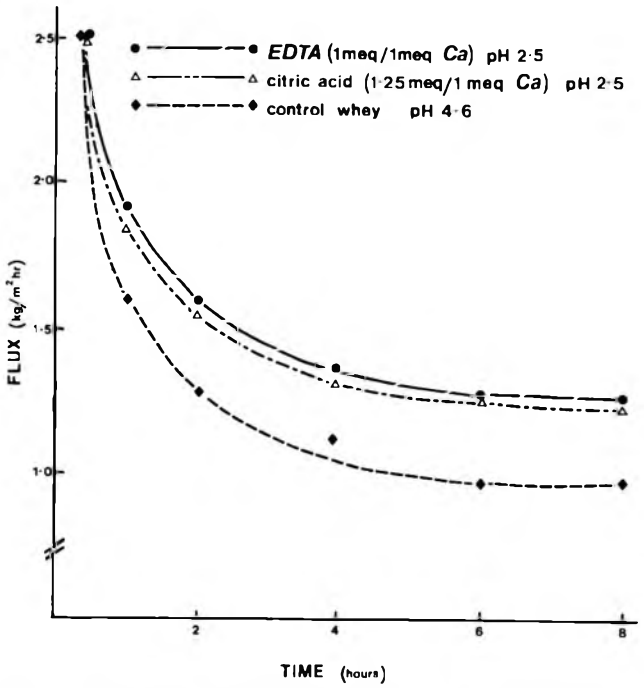

Fig. 5—Changes in flux for pretreated cottage cheese whey ultrafiltration on DDS-Lab 20 UF module (P_T = 3.5 bar).

flux in comparison to the untreated whey. Lowering of the pH to the 2–3 range improved both flux and calcium permeation rate. Binding of calcium in chelated complexes seemed to be the best way to minimize the availability of calcium for the presumed calcium-membrane interactions. The amount of used chelating agent which was equivalent to free calcium in the cottage cheese whey resulted in the highest flux improvement. Chelating agents may be preferred to demineralization particularly for a greater flexibility in degree of calcium sequestration.

This study confirmed previous evidence of calcium effects during ultrafiltration of acid whey. It may be expected that most of the treatments used for flux improvement could be utilized in industrial design of ultrafiltration processes for cottage cheese whey. For commercial applications the use of citric acid seems to be more suitable than that of EDTA. The treatment is simple and inexpensive, and there are no perceived legal complications so that it could be readily used by the whey processing industry. With manipulation of operating parameters it may be possible to improve the flux rate about 50% above that of whey ultrafiltered without any pretreatment.

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Sincere thanks are expressed to Mr. R. Currie and other staff from the O.S. Longman Food Laboratory, Alberta Agriculture, for assistance with the atomic absorption analyses of Ca and P.

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Protein Quality of Cheddar Cheese Compared with Casein and Fabricated Cheese in the Rat

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ABSTRACT

The protein quality of freeze-dried cheddar cheese, spray-dried cheddar cheese, freeze-dried fabricated cheddar cheese (with casein as the main protein source), and sodium caseinate was evaluated in rats using the protein efficiency ratio (PER) assay (AOAC procedures) in two feeding experiments with casein as the control. Biological evaluation of the products showed that PER values for freeze-dried cheddar cheese were significantly higher than casein (3.7 vs 2.5). Freeze-dried cheddar also had a PER value significantly higher than spray-dried cheddar cheese (3.7 vs 3.0). Freeze-dried fabricated cheddar cheese and sodium caseinate had PER values not significantly different from the casein control.

INTRODUCTION

MANY NEW DAIRY FOOD SUBSTITUTES made in semblance of traditional dairy foods have recently been introduced to the market. The use of imitation and substitute cheeses, particularly by the pizza industry, has been increasing rapidly in the last few years from 2% of the total U.S. cheese production in 1978 to 5% by 1980 (Graf, 1981). Since milk and milk-based products constitute a valuable source of dietary protein and other nutrients, the Food and Drug Administration (FDA) has proposed nutritional equivalency standards for dairy product substitutes (Federal Register, 1978). Included in the proposed regulations are protein quality standards based on the protein efficiency ratio (PER) assay in rats. According to the proposed regulations issued in September 1978, "If the cheese or cheese product substitute is made with milk protein or protein of vegetable origin, the PER must be at least 100% that of casein."

This standard for "nutritional equivalency" assumes that the PER of traditional cheese is not greater than casein. Although cheese proteins are mainly represented by calcium paracaseinate (Kosikowski, 1977), this protein is degraded considerably during ripening of some cheeses. Studies investigating the nutritional significance of the ripening process have been circulating. Beadles et al. (1933) found the digestibility of protein from cheese to be 1–2% less than the protein from milk curd. However, they also found that the capacity of the cheese to promote weight gain in young rats was greater than that of milk curd. Randoin and Causeret (1956) reported that cheese proteins were more digestible than proteins of skim milk powder as tested in the rat. Using the protozoan *Tetrahymena pyriformis W*, Gregori et al. (1973) reported that Grana, Crescenza, and a processed cheese had relative protein values of 200, 151, and 150% those of the casein control. The amino acid composition and available lysine content of a variety of cheeses were determined by Vuyst et al. (1973) who reported that the biological value of the cheese proteins were all high, being 91–97% that of milk proteins.

In contrast, Korolczuk et al. (1977) found that the proteolytic changes during the ripening of Gouda and Tilsit types of cheeses did not result in significant differences in nutritive value when compared to casein plus fresh milk fat. They reported that they found no changes in Net Protein Utilization (NPU) and PER during ripening and storage (remaining at 75% and 2.8%, respectively).

Nutritional quality differences among cheese varieties have also been noted. A range of NPU values for a number of cheeses were reported by Siudak and Poznanski (1971) including 56 for Edam and Camembert, 60 for Tilsit, 65 for Roquefort, and 67 for Mysliwski cheese. From the viewpoint of free amino acid content, Toma and Grisaru (1971) reported that cheddar-type cheeses are nutritionally superior to cows' milk Kachkaval cheeses.

Our earlier work (Kotula et al., 1983) showed that a cheddar cheese and a fabricated cheddar cheese were not nutritionally equivalent for the rat in terms of their vitamin/mineral composition. Little information, however, is available on the protein quality of fabricated cheese. Although a fabricated cheese may be formulated from protein(s) having a PER equal to 100% of casein; processing conditions, nutrient interactions and other factors may affect protein digestibility and bioavailability. Thus, one of our objectives was to test the protein quality of a commercially manufactured fabricated cheese that met the protein quality standards for nutritional equivalency. Since the literature contains conflicting information on the protein quality of ripened cheese, another objective was to determine the protein quality of a commonly consumed ripened cheese (cheddar cheese) using standard AOAC (1980) PER procedures.

MATERIALS & METHODS

Samples

A spray-dried pasteurized, process cheddar cheese (ingredients listed in Table 1) was supplied by Kraft, Inc. (Glenview, IL). Fresh, mild cheddar cheese (Table 1) was obtained from a local supermarket. A fabricated cheese (L.D. Schreiber Cheese, Co., Inc.) providing protein primarily derived from casein was purchased from a local supplier. Sodium caseinate was purchased from Nutritional Biochemicals Corp. (Cleveland, OH). Since the mild cheddar cheese and fabricated cheese were obtained in the hydrated state, removal of moisture was

Table 1—Proximate analysis of protein sources

	Moisture %	Protein %	Crude fat %	Ash %	NaCl %
Cheeztang[a]	4	34	59	9	5
Cheddar cheese-mild					
Fresh[b]	37	20–24	35	4	1.7
Freeze-dried	2–3	31–37	54	6	3
Fabricated cheddar cheese					
Fresh	48	22	20	6	2
Freeze-dried	2-3	40-42	38	11	4
Sodium caseinate	8	86	—	2	(1.3)*

[a] Ingredients (from label): Cheddar cheese, cream, salt, disodium phosphate, lactic acid.

[b] Ingredients (from label): Pasteurized milk, salt, rennet, annatto (vegetable color).

[c] Ingredients (from label): Water (43%), calcium caseinate (24%), partially hydrogenated soybean oil (20%), natural flavor (4%), sodium aluminum phosphate (2%), whey (2%), skim milk (2%), salt (2%), and less than 1% of lactic acid, sodium citrate, trisodium phosphate, artificial flavor, sorbic acid, and artificial color.

* 1.3% sodium

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required prior to addition to the diets. Samples were grated using a Hobart Food Cutter, Model 84-141 (Hobart Mfg. Co., Troy, OH) and then freeze-dried in a Model 10-MR-TR shelf unit (Virtis, Gardiner, NY). Products were placed in 30 × 44 × 4 cm stainless steel trays (2 kg/tray × 3 trays) and frozen at −40°C shelf temperature. With a vacuum of 5 mm to 50 μm Hg and a final shelf temperature of 25°C, the product was dried in about 72 hr. The freeze-dried samples were then grated in a blender to a size that would pass through a 10 mesh US sieve.

Proximate analyses

Proximate analyses for moisture, Kjeldahl nitrogen, crude fat, and ash were determined on all samples by standard AOAC (1980) methods (Table 1). The approximate sodium chloride content of the test products was obtained from the respective manufacturers.

Rat feeding experiments

For both experiments, male Sprague-Dawley rats of 45–55g body weights, 21 days old, were individually housed in stainless steel wire-bottomed hanging cages and maintained in a 23°C environment with a 12 hr light-dark cycle. In experiment I, animals were acclimated for 4 days on the casein (Vitamin Free Test Casein, Teklad Test Diets, Madison WI) control diet. In Experiment II, animals were acclimated for 5 days on the ANRC casein (Sheffield Products, Memphis, TN) control diet. The rats were then randomly allocated to the treatment groups on the basis of body weight and weight gained during the acclimation period. There were no significant differences in mean group weights on day zero. Both experiments were of 28 days duration with food and deionized water supplied *ad libitum*.

Experiment I

Three different products were tested in this experiment: spray-dried processed cheddar cheese, freeze-dried mild cheddar cheese, and freeze-dried fabricated cheddar cheese. As indicated in Table 1, the natural and fabricated cheeses tested had a higher content of sodium chloride. Even when fed at levels providing 10% protein, these products provided more sodium chloride than that used in the AOAC casein control diet (Table 2). Dori (1976) has shown that sodium chloride in diets at levels ranging from 1–3% can reduce the rat's food intake by 10–15%. Thus, to avoid increasing the sodium chloride content of diets containing the test products, we modified the AOAC PER mineral mix so that it did not contain sodium chloride. This mineral mix was used in all the diets at the levels shown in Table 2. A level of 0.7% sodium chloride was then added to the casein control diet (the level of AOAC procedures). The sodium chloride in the test products, however, varied according to the nature and amount of product used. To determine the effects, if any, feeding more than 0.7% sodium chloride would have on the PER, we added an additional casein control diet containing 2.8% sodium chloride, or four times the level in the AOAC casein control diet.

Experiment II

In this second experiment, we again conducted a PER test on freeze-dried cheddar cheese and freeze-dried fabricated cheddar cheese. Since some fabricated cheeses are made from sodium caseinate, sodium caseinate was also included in this trial. Due to the product's high ash content, we noted that adherence to the AOAC diet formulation guidelines in Experiment I resulted in diets possibly deficient in some minerals such as copper and manganese. To ensure nutritional adequacy of the trace elements, a 5% level of the AOAC mineral mix was used in all diets (Table 2). However, as in Experiment I, sodium chloride was again added only to the casein control and sodium caseinate diets (at the AOAC level of 0.7%). In addition, in the second experiment 1% cellulose was included in all diets. AOAC procedures allow for use of either sucrose or cornstarch in the PER diets. Although the use of some cornstarch resulted in a diet of better consistency, cornstarch does contain small quantities of protein, moisture, and other impurities. For this reason we limited the amount of cornstarch to 20% of the variable carbohydrate component. The protein and moisture content of the cheddar cheese used in Experiment II differed slightly from that used in Experiment I. Thus, different levels of freeze-dried cheddar cheese were used in the two experiments to obtain diets that provided 10% protein.

Blood chemistry and organ weight determinations

Rats were anesthetized with gaseous CO_2 and blood samples collected into heparinized syringes via heart puncture. Death occurred by exsanguination. Hematocrit values were determined by using a micro-hematocrit centrifuge (Adams Autocrit Centrifuge CT 2095, by Clay-Adams, Inc., New York, NY) on individual heparinized blood samples in capillary tubes. The hemoglobin levels were determined by the cyanomethemoglobin method (Cannon, 1958).

In addition to the above, in Experiment II the liver, kidney, spleen, testes, and heart were removed, blotted, wrapped individually in aluminum foil, refrigerated and then weighed on a Mettler H34 analytical balance (Mettler Instrument Corp., Hightstown, N.J.) within a few hours of the autopsy.

Statistical analyses

Adjusted PER was calculated with the PER of the casein group set at 2.5. The PER values of the other test groups were then calculated as percentages of the casein group value.

Statistical differences among means of all parameters were determined by one-way analysis of variance followed by Tukey's Honestly Significant Test Difference (0.05 level of significance). Data were analyzed with a standard SPSS (Statistical Packages for Social Sciences) Program (Nie et al., 1975).

RESULTS & DISCUSSION

THE PER, adjusted PER values, and PER as a percent of casein for the first and second experiments are shown in Tables

Table 2—Percent diet composition for test groups

	Experiment I					Experiment II			
	1 Casein[a] control 0.7% NaCl	2 Casein[a] control 2.8% NaCl	3 Real cheddar cheese (Freeze-dried)	4 Fabricated cheddar cheese (Freeze-dried)	5 Real cheddar cheese (Spray-dried)	1 ANRC casein control	2 Real cheddar cheese (Freeze-dried)	3 Fabricated cheddar cheese (Freeze-dried)	4 Sodium caseinate
Ingredients (%)									
Sample	11.3	11.3	32.1	24.1	29.5	10.9	26.4	24.8	11.6
Cottonseed oil	17.3	17.3	0	8.3	0	14.1	0	4.9	14.2
Mineral mix[b] minus NaCl	4.9	4.9	3.1	2.3	2.5	5.0	5.0	5.0	5.0
NaCl added	0.7	2.8	0	0	0	0.7	0	0	0.7
present[c]	–	–	1.0	1.0	1.6	–	0.8	1.0	–
Vitamin mix[b]	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Cellulose	0	0	0	0	0	1.0	1.0	1.0	1.0
Water distilled	4.2	4.2	4.5	4.6	3.9	4.5	4.6	4.6	4.1
Sucrose powdered	29.8	28.8	29.2	29.4	31.1	49.4	48.8	46.2	49.8
Cornstarch	29.8	28.8	29.2	29.4	31.1	12.4	12.2	11.6	12.4

[a] Vitamin Free Test Casein, Teklad Test Diets, Madison, WI

[b] Salt mixture and vitamin mixture prepared according to AOAC procedures (1980).

[c] Amount of NaCl provided by the cheese or fabricated cheese.

Table 3—PER of test proteins and the effect of NaCl levels (Experiment I)

Dietary treatment	PER	Adjusted PER[d]	PER as a % of casein
1. Casein (0.7% NaCl)	2.57 ± 0.29[a]	2.50 ± 0.28[a]	100%
2. Casein (2.8% NaCl)	2.74 ± 0.39[a,b]	2.67 ± 0.38[a,b]	107%
3. Cheddar cheese (freeze-dried)	3.76 ± 0.19[c]	3.66 ± 0.18[c]	146%
4. Fabricated cheddar cheese (freeze-dried)	2.92 ± 0.21[b]	2.84 ± 0.21[b]	114%
5. Real cheddar cheese (spray-dried)	3.09 ± 0.22[b]	3.01 ± 0.22[b]	120%

[a-c] Mean ± SD. Different superscripts within a column denote significant differences ($P<0.05$)
[d] PER values adjusted to casein at 2.5 PER.

Table 4—PER of test proteins and the effect of sodium caseinate (Experiment II)

Dietary treatment	PER	Adjusted PER[c]	PER as a % of casein
1. ANRC Casein	2.70 ± 0.24[a]	2.50 ± 0.23[a]	100%
2. Cheddar cheese (freeze-dried)	3.03 ± 0.12[b]	2.81 ± 0.11[b]	112%
3. Fabricated cheddar cheese (freeze-dried)	2.79 ± 0.41[a,b]	2.59 ± 0.38[a,b]	104%
4. Sodium caseinate	2.66 ± 0.23[a]	2.47 ± 0.21[a]	99%

[a,b] Mean ± SD. Different superscripts within a column denote significant differences ($p<0.05$)
[c] PER values adjusted to casein at 2.5 PER

3 and 4. In Experiment I the spray-dried cheddar cheese had a significantly lower PER value than the freeze-dried cheddar cheese. This finding of reduced protein quality of spray-dried cheddar cheese is in contrast to the results of Deodhar and Duggal (1981). They reported that the heat treatment (160°C) used during preparation of a cheese spread powder did not have any deleterious effects on protein quality. In contrast, Hackler et al. (1965) reported that the spray-drying of soybean milk protein at temperatures above 160°C resulted in a temperature dependent decrease of PER values which were inferior to the values of analogous freeze-dried products. Although the testing of cheeses was not performed, a similar effect on protein quality would be expected.

The inferior PER of the spray-dried vs freeze-dried cheddar does not appear to be due to sodium chloride toxicity. When fed at the 10% protein level, spray-dried cheddar cheese provided 1.6% sodium chloride, or over twice the 0.7% sodium chloride level in the casein control diet. However, our data show that even a diet containing 2.8% sodium chloride (group two), or four times the sodium chloride in group one, did not adversely affect the PER value.

Controlling for ash as outlined in the AOAC procedures resulted in the spray-dried cheddar cheese diet containing only 2.5% of the mineral mix. Except for copper and manganese, a 2.5% level of the mineral mix along with the minerals in the ash from the cheese probably provided sufficient levels of the other minerals.

In both experiments, freeze-dried cheddar cheese had PER values significantly ($p < 0.05$) higher than the casein controls. Our PER values for cheddar cheese are comparable to Deodhar's et al. (1981) nonadjusted "PER" value of 3.78 for processed cheddar cheese. Deodhar et al., however, used no casein controls and their animals received the test diet for only 10 days. In contrast, Steinke et al. (1980) using standard AOAC procedures reported a low PER of 2.16 for freeze-dried processed cheese. Their cheese had been autoclaved at 108°C for 30 min prior to lyophilization which would possibly cause some protein denaturation.

One reason for the higher PER of cheddar cheese vs casein may be proteolytic changes that occurred during ripening of the curd. During the progressive proteolysis in cheese processing, the paracasein and some of the minor proteins are gradually converted to simpler nitrogenous compounds, namely proteoses, peptones, amino acids, and ammonia. Since amino acids can be absorbed more rapidly from small peptides than the equivalent amino acids (Adibi and Phillips, 1968; Matthew et al., 1968) it is possible that the peptides in cheese enhance apparent protein availability. Silk et al., (1975) have compared the absorption of an amino acid mixture simulating casein and a hydrolysate of casein prepared by papain treatment followed by use of hog kidney peptidases. They reported that a number of amino acids were absorbed more rapidly than the enzymatic hydrolysate. Thus, dietary proteins that have similar amino acid composition may not necessarily be similar in their protein quality as measured biologically.

In both trials, fabricated cheddar cheese had PER values that were not significantly different than casein. Considering that whey proteins have been shown to have a higher nutritive value than casein (Lange and Potgieter, 1979), it is notable that the fabricated cheese did not support higher PER values than the reference protein. The soluble proteins albumin and globulin provided by the 2% whey and 2% skim milk contained in the fabricated cheese apparently were inadequate to improve significantly the protein quality above that of the casein alone.

The PER of sodium caseinate was not significantly different from the PER value of ANRC casein. The treatment of casein with sodium induces a number of changes including formation of crosslinked amino acids such as lysinoalanine and racemization of amino acid residues (Friedman, 1977; Friedman et al., 1981; Masters and Friedman, 1980). Sodium caseinate and other alkali-treated caseins are used in formulating fabricated cheeses. Although sodium caseinate alone may not have an inferior PER as compared to casein, processed foods containing alkali-treated casein are expected to contain significantly higher amounts of D-amino acids. Consequently, the resulting lower relative levels of L-amino acids could cause a decrease in the protein quality of that food (Friedman et al., 1981).

Very few differences were noted in hemoglobin-hematocrit analyses and organ/carcass ratios (Table 5). All hemoglobin and hematocrit levels were within normal limits. Although in Experiment I, the casein control group containing 2.8% sodium chloride had a mean hemoglobin value which was statistically lower than those of other groups, this would not have biological significance.

In Experiment II, differences were noted in spleen/carcass and kidney carcass ratios. The smallest spleen/carcass ratios were found in the group receiving sodium caseinate. The smallest kidney/carcass ratios were found in the group receiving freeze-dried cheddar cheese. The reasons for these differences and their significance need further study.

Although the actual PER numbers obtained in Experiments I and II were somewhat different, the ranking of the proteins tested was the same for both experiments. The numerical differences in the PER values between Experiments I and II could be attributed to the small differences in protocol.

CONCLUSIONS

THIS STUDY provides evidence that the protein of the cheddar cheese tested is of a significantly higher quality than cas-

Table 5—Hemoglobin and organ/carcass ratios from Experiments I and II

Dietary Treatment	Experiment I	Experiment II	
	Hemoglobin g 100 mL	Spleen Carcass × 100	Kidney Carcass × 100
Casein (0.7% NaCl)	13.7 ± 0.6[a]	0.345 ± 0.041[a,b]	1.36 ± 0.08[6]
Casein (2.8% NaCl)	12.9 ± 0.5[b]	—	—
Cheddar cheese (freeze-dried)	13.0 ± 0.7[a]	0.372 ± 0.052[b]	1.25 ± 0.08[a]
Fabricated cheddar cheese (freeze-dried)	13.4 ± 0.5[a]	0.339 ± 0.025[a,b]	1.29 ± 0.09[a,b]
Cheddar cheese (spray-dried)	13.2 ± 0.7[a]	—	—
Sodium caseinate	—	0.320 ± 0.033[a]	1.39 ± 0.10[b]

[a,b] Mean ± SD. Different superscripts within a column denote significant difference ($p < 0.05$).

ein. In both experiments, the traditional cheddar cheese was found to have significantly higher PER values than the casein control. The fabricated cheese values, however, were statistically comparable to those of the casein group. Therefore, although the fabricated cheese tested met the FDA's protein quality standards, the fabricated cheese provided a protein quality that was neither better than casein nor comparable to the traditional cheddar cheese. Consequently, the traditional and fabricated cheese did not provide proteins of equivalent nutritional value and, thus, were not "nutritionally equivalent." The nutritional implications to humans of the use of such products certainly deserve consideration. Much more biological testing of fabricated compared with real foods is required to help elucidate the nutritional differences that exist in these products.

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Ms received 12/1/86; revised 3/13/87; accepted 3/20/87.

This research was partially supported by the National Dairy Council, Rosemont, IL 60018. We thank Fran Powers for typing this manuscript.

Elongational Viscosity Measurements of Melting American Process Cheese

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ABSTRACT

The elongational viscosity of melting process American cheese of two commercial brands was determined by compression tests at various temperatures in the range 36–62°C. The elongational viscosity determined at low strain rates (0.01–0.04 sec^{-1}) had little or no rate dependency in one brand and a moderate dependency in the other. Plots of log viscosity vs reciprocal of the absolute temperature indicated that both Arrhenius' and the WLF models failed to accurately account for the cheese melts temperature dependency and that the differences between the types of cheeses could be expressed in whether the plots had upward or downward curvature. Comparison with the Schreiber meltability index demonstrated that although the latter was associated with viscosity, it could not account for different flow rates at different temperatures.

INTRODUCTION

"MELTABILITY" is one of the most important physical properties of process cheese, cheese analogs and various natural cheeses, notably mozzarella. It permits their use in a variety of popular foods such as toasted sandwiches, pizzas and other Italian foods. Despite many efforts to study the phenomenon of cheese meltability, the currently used methods for its evaluation are basically empirical in nature. The most popular among these are the Schreiber and Arnott tests (Kosikowsky, 1977). They are based on measuring the change in the diameter or height of a cylindrical specimen after being exposed to elevated temperature in an oven. It has recently been demonstrated (Park et al., 1984) that the results of the Schreiber and Arnott tests do not correlate. This was not totally unexpected since the flow of cheese, under such circumstances, can hardly be characterized by a single empirical parameter. Conventional instrumental methods to determine the viscosity of melted cheese are also of limited practicality because of slip and viscoelastic effects (Smith et al., 1980). A way to overcome these difficulties is to use squeezing flow rheometry based on compression of a specimen between two parallel plates (Casiraghi et al., 1985). The main advantage of this method, as has recently been demonstrated in peanut butter (Campanella and Peleg, 1987), is that the presence of slip, as a result of self-lubrication, is not only acknowledged but is actually a prerequisite for a proper test.

Because the deformation rates experienced by a melting cheese in a sandwich or on a pizza are very low, the compression test can be performed at similar low rates by either adjusting the compression rate or the magnitude of the imposed load (if the test is of a "creep" type). Under these conditions viscoelastic effects are considerably reduced and have insignificant influence on the results. The objective of this work was to demonstrate the applicability of the compressional testing methodology to melted American process cheese and to evaluate the effect of temperature on the melt's rheological properties.

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THEORY

THE KINEMATICS of elongational flow with the geometry shown in Fig. 1 can be described by the following equations (Chatraei et al., 1981)

$$V_z = \dot{\epsilon}_T H(t) \tag{1}$$

$$V_r = -\dot{\epsilon}_T \frac{r}{2} \tag{2}$$

$$V_\theta = 0 \tag{3}$$

where V_z, V_r and V_θ are the normal (vertical), radial and angular velocity components, respectively, H(t) the momentary specimen height at time t, and r the radial distance.

The momentary strain rate, $\dot{\epsilon}_T$, is defined as:

$$\dot{\epsilon}_T = -\frac{1}{H(t)} \frac{dH(t)}{dt} \tag{4}$$

In a Universal Testing Machine operated in the constant displacement rate mode (constant crosshead speed V_z), the momentary strain rate is given by:

$$\dot{\epsilon}_T = \frac{V_z}{H_o - V_z t} \tag{5}$$

where H_o is the initial height of the undeformed specimen

The average normal stress difference $T_{rr} - T_{zz}$ can be calculated as the momentary compressive force F(t) divided by the cross-sectional area of the deformed part of the specimen or in our case, by the plates cross-sectional area as seen in Fig. 1, i.e.,

$$T_{zz} - T_{rr} = \frac{F(t)}{\pi R^2} \tag{6}$$

where R is the plate's radius.

Elongational flow is regulated by a material function known as elongational viscosity. The latter is calculated by the expression:

$$\mu_b = \frac{T_{rr} - T_{zz}}{\dot{\epsilon}_b} \tag{7}$$

where ϵ_b is the biaxial extensional strain rate which is defined by (Chatraei et al., 1981):

$$\dot{\epsilon}_b = \frac{1}{2} \dot{\epsilon}_T \tag{8}$$

Incorporating Eq. (5), (6) and (8) into Eq. (7) gives the relationship

$$\mu_b = \frac{2F(t)(H_o - V_z t)}{\pi R^2 V_z} \tag{9}$$

from which μ_b can be calculated directly.

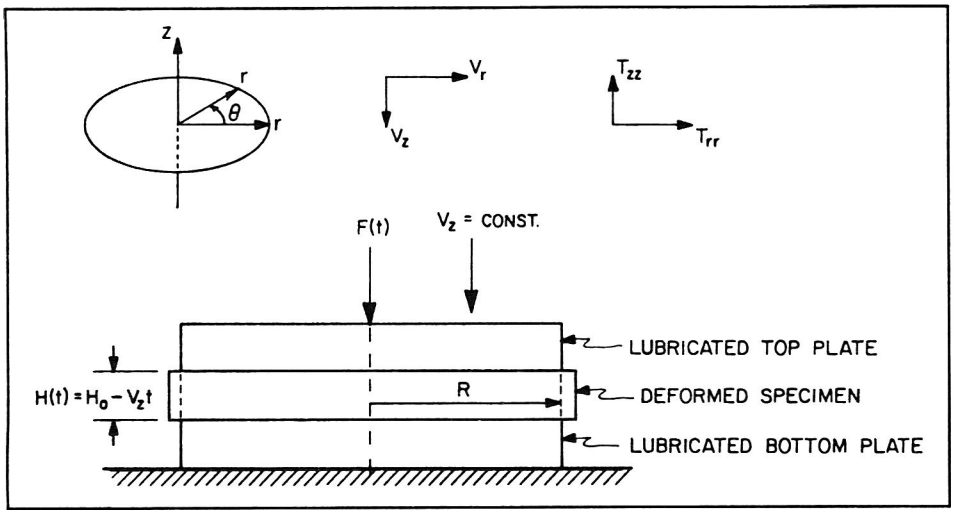

Fig . 1—Schematic view of the test array to determine elongational viscosity and the system's cylindrical coordinates. z, r and θ are the coordinates, F(t) the momentary force at time t, V_z and V_r the normal (vertical) and radial velocity components respectively, T_{zz} and T_{rr} are the normal and radial stresses respectively, H_o the initial specimen height, and H(t) its momentary height at time t.

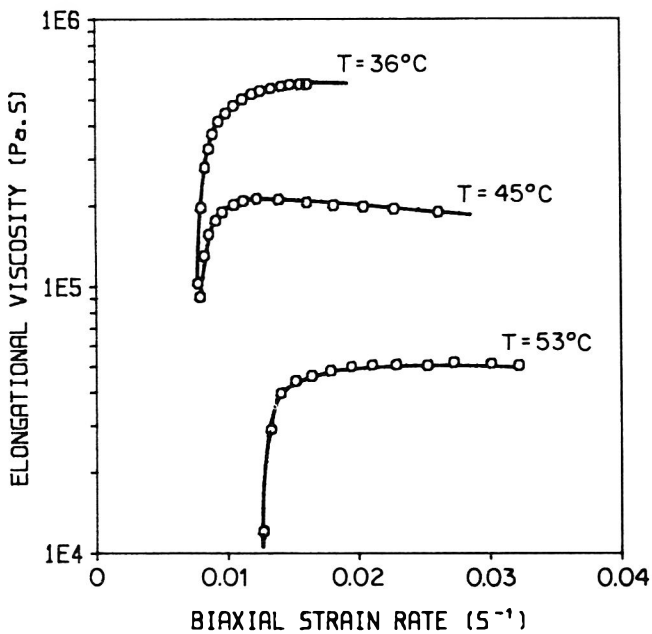

Fig. 2—Elongational viscosity (μ_b) vs strain rate (ϵ_b) of process American cheese at various temperatures — National brand.

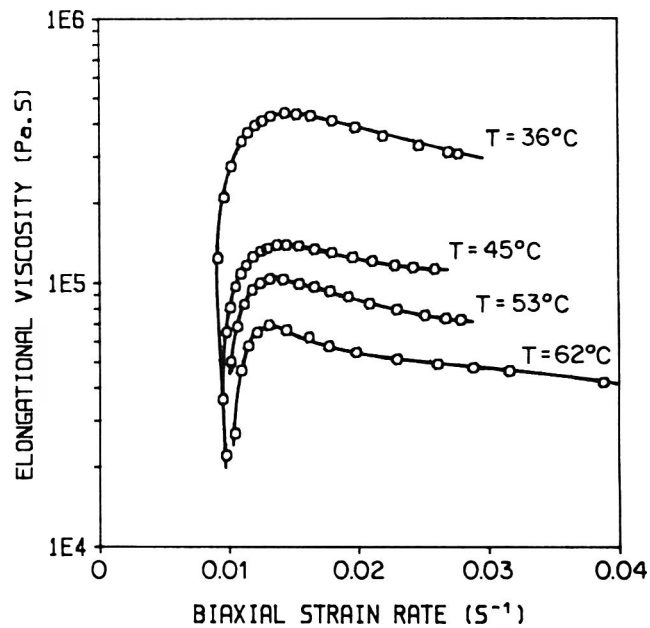

Fig. 3—Elongational viscosity (μ_b) vs strain rate (ϵ_b) of process American cheese at various temperatures — Supermarket chain.

MATERIAL & METHODS

PROCESSED American cheese, a national brand and a supermarket chain brand, were purchased at a local supermarket. They were left to equilibrate at the laboratory ambient temperature (22–24°C) for 24 hr prior to testing.

Cheese samples were mounted on an Instron Universal Testing Machine model TM equipped with a temperature controlled chamber (model 3111). The samples were placed between two parallel teflon coated platens 31.8 mm in diameter lubricated with silicone oil (Dow Corning 500 Cst). The samples were heated and maintained at the desired temperature for at least 11 min before the test was started. This was the minimum time necessary to ensure equilibrium with the chamber temperature as indicated by thermocouples inserted into cheese specimens placed in the chamber for this purpose. The compression tests were performed at a crosshead speed of 1 cm.min $^{-1}$ and each test was repeated four times with a fresh sample.

RESULTS & DISCUSSION

ELONGATIONAL VISCOSITY vs biaxial strain rate relationships of the two process cheese types are presented in Fig. 2 and 3. The figures show that after a short period of a transient flow regime, the viscosity reached a maximum value which either remained stable or slightly declined with the rate increase. Casiraghi et al. (1985) reported similar plots for cheese spread at room temperature. They are different in the sense that in the cheese spread the decrease following the peak viscosity was much more pronounced and approached the same asymptotic slope over a fairly wide range of rates. In comparison with the cheese spread at room temperature, the melting process cheese can be considered as having a practically constant elongational viscosity at the rates tested.

Plots of the elongational vs the reciprocal of the absolute temperature show that the Arrhenius relationship, which would have been manifested in a straight line, was not applicable to the cheese melts under the reported test conditions (Fig. 4 and 5). If the curvature is ignored and the data are subjected to linear regression, the resulting activation energy would be on the order of 28 and 17 Kcal/mole for the national and supermarket brands, respectively. It is interesting to note that while the two types of cheese had elongational viscosities that were roughly on the same order of magnitude, the curvature of the plots was concave downward in one case (the natioanl brand) and concave upward in the other (supermarket chain brand).

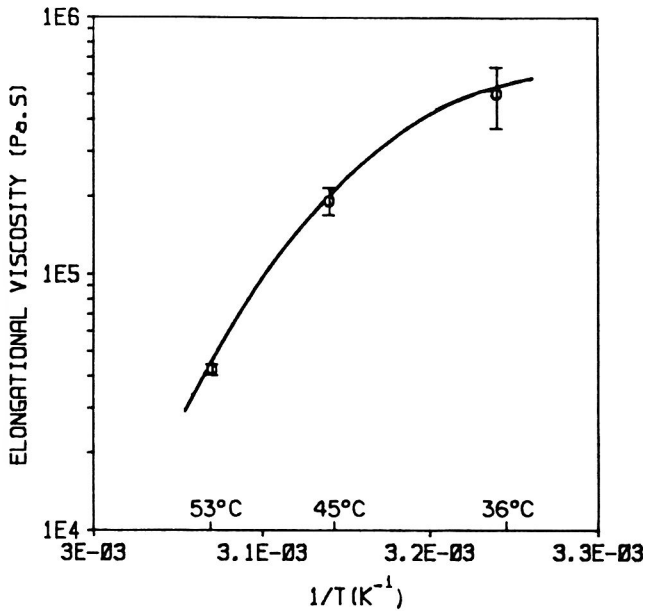

Fig. 4—Effect of temperature on the elongational viscosity of process American cheese at 0.015 sec⁻¹ biaxial strain rate. National brand.

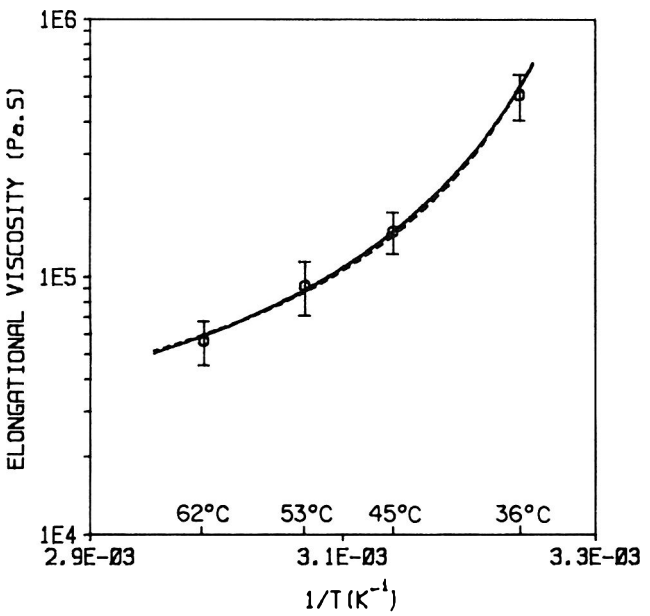

Fig. 5—Effect of temperature on the elongational viscosity of process American cheese at 0.015 sec⁻¹ biaxial strain rate. Supermarket chain brand. Note the clearly concave down slope of the curve. The solid line represents the fit of the WLF model with $T_o = 36°C$ and dashed line with $T_o = 45°C$.

This of course would have a profound effect on the behavior of the cheese in any real life situations where the cheese temperatures vary and on the results of an empirical meltability test performed under arbitrary time-temperature conditions. Applying the Schreiber test to the two cheese brands (5 min at 232°C) resulted in a meltability index of 5.3 ± 0.73 for the national brand and 1.4 ± 0.68 for the supermarket brand. Since the measured spread in a Schreiber test (Park et al., 1984) is affected by the total history of the specimen, it is not possible to distinguish between early and late flow regimes (let alone separate heat transfer rates) that are the result of the actual temperature history. Thus, it is very probable that when the meltability of two cheeses is compared by such a test, differences will indeed be found, but their magnitude will be totally inadequate to predict the two cheeses behavior under different time-temperature conditions. This is clearly evident in our case and it appears that the higher observed "meltability" of the national brand is primarily a result of its lower viscosity at higher temperatures (i.e., T>60°C) (Fig. 4 and 5), an effect which is also strengthened by improving the heat transfer rates and the temperature distribution within the sample.

A concave upward plot of log viscosity *vs* the reciprocal of the absolute temperature (Fig. 5) has been observed in various polymer melts. A model that describes this behavior is known as the WLF equation after Williams, Landel and Ferry (Williams et al., 1955) or

$$\log \frac{\mu_b(T)}{\mu_b(T_o)} = \frac{-C_1 (T - T_o)}{C_2 + (T - T_o)} \qquad (10)$$

where T_o is a reference temperature and C_1 and C_2 constants

Application of this model to the supermarket chain cheese showed a very good fit (Fig. 5) irrespective of the reference temperature. The difficulty with this model, however, is that the relative magnitude of C_1 and C_2 changes significantly, although not drastically, with the selection of the reference temperature. For example, for $T_o = 36°C$, $C_1 = 1.7$, and $C_2 = 21K$ while for $T_o = 45°C$, $C_1 = 1.1$ and $C_2 = 27K$ with no change in the goodness of fit.

The curvature of the Arrhenius plot and the failure of the WLF model to account for the flow behavior of both cheeses was not surprising. Since the phenomenon involved structural and phase changes, it could hardly be treated by models that were primarily developed for homogeneous or simple systems. It appears, therefore, that if a proper account of melting cheese flowability is required, there is no other way but to apply comprehensive rheological analysis. This can be done using the same or a similar procedure that is described in this work extended to a larger temperature range. The main advantage of applying lubricated squeezing flow rheometry to cheese melts is in the relative simplicity and low cost of the equipment. The elimination of a major source of artifacts, namely, slip, is an added benefit that does not require instrumental or procedural refinement.

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Ms received 1/15/87; revised 2/26/87; accepted 2/26/87.

Contribution of the Massachusetts Agricultural Experiment Station at Amherst. The authors express their gratitude to Mr. Richard J. Grant for the graphical aid.

Ascospore Heat Resistance and Control Measures for *Talaromyces flavus* Isolated From Fruit Juice Concentrate

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ABSTRACT

Talaromyces flavus were isolated from fruit juice concentrates in two unrelated spoilage incidences of packaged reconstituted fruit juice. The population of survivors in heated fruit concentrate was 0.6 colony forming units (CFU) per gram. The ellipsoidal ascospores diameters were 4.5 × 6 μm. Potassium sorbate and sodium benzoate prevented outgrowth of *T. flavus*, the concentration required was lower when the pH of the growth medium was 3.5 instead of 5.4. Conidia were sensitive to 70% ethanol, but ascospores were resistant. Ascospores required heat activation before maximum outgrowth occurred, the time for activation was less with higher activation temperature. Thermal death rate curves were nonlogarithmic but approached logarithmic death rate at higher temperatures. A calculated thermal destruction rate, using the exponential formula, for 1 $\log_{10}$ was 2–7 min at 90°C depending upon the strain; and the approximate z-value was 10.3°C.

INTRODUCTION

HEAT RESISTANT MOLDS occur sporadically and are a continuing problem to the food industry. They were first reported by Olliver and Smith (1933) with their classification of the mold *Byssochlamys fulva*. Several molds are capable of withstanding the normal thermal processes given to fruit products and, thus, occasionally cause spoilage of processed fruit products. Reports of heat resistant molds have been summarized by Splittstoesser and King (1984). Ten species of heat resistant molds from seven genera were listed. The most frequently reported heat resistant mold was *B. fulva*. Less frequently reported were *Neosartorya fischeri* and *Talaromyces flavus*.

T. flavus is the perfect stage of *Penicillium dangeardii* (*P. vermiculatum*). Growth of the *T. flavus* reported here was alleged to have caused spoilage in packaged fruit juice. *T. flavus* has been previously isolated from spoiled apple juice in South Africa by Van der Spuy et al. (1975), who reported that ascospores and cleistothecia were heat resistant but not conidia or mycelial fragments. The objectives of this study were to isolate *T. flavus* from fruit concentrate, characterize its heat resistance, and describe methods to control its growth.

METHODS

T. FLAVUS CULTURES NRRL 13535 and NRRL 13536 were isolated from fruit juice concentrate. The juice concentrate was heated (70°C, 60 min) to eliminate heat sensitive organisms and to heat activate spores to germinate before plating. Cultures NRRL 13537 and NRRL 13538 were isolated from spoiled fruit drink. It was submitted for isolation of the spoilage mold, classification and determination of heat resistance. The cultures have been deposited at the ARS Culture Collection Northern Regional Research Lab (NRRL) for maintenance. Cultures FRR 1976, FRR 2098, and FRR 2386 were obtained as reference cultures from Dr. A.D. Hocking, Curator, Culture Collection, CSIRO, Division of Food Research North Ryde, NSW, Australia. Malt extract agar (MEA) (Pitt, 1979) was selected as the medium for outgrowth after comparisons with potato dextrose agar (PDA).

Ascospores were produced by growing cells on 25 mL malt extract broth for 30 days at 25°C in 20 mL Erlenmeyer flasks or 100 mL in fernbach flasks. Ascospores were prepared by first blending the flask contents in a Waring Blendor for 2 min, washing three times in peptone water (lg/L) with centrifuging action to remove debris. Then cleistothecia and asci were broken by passing twice through a French pressure cell at not more than 8000 PSIG (Michener and King, 1974). To disrupt aggregates of ascospores, cells were mixed in a Potter-Elvehjem tissue grinder immediately before testing heat resistance. Spores were counted microscopically with a Levy-Hauser (A.H. Thomas Co., Philadelphia, PA) counting chamber. Ascospore size was measured microscopically at 500 magnifications on 25 ascospores by each of two observers.

Heat resistance was determined by heating ascospores in 1.6 mL of 16° Brix glucose-tartrate (5 mg/mL) heating medium at pH 5.0, in thermal death time tubes in a water bath controlled to within 0.1°C. The artificial heating medium was used so these data could be compared with previous work on heat resistant molds (King et al., 1979; Bayne and Michener, 1979). Viable survivors were counted after plating on MEA containing 7.5 mg/L rose bengal to restrict radial colony growth (Splittstoesser et al., 1970). Counts were recorded periodically after up to 7 days incubation at 25°C. Preliminary experiments showed continued incubation did not result in late outgrowth, but overgrowth and crowding became a problem.

The effect of sodium benzoate and potassium sorbate on growth of *T. flavus* was measured at 100 μg/mL intervals of the salts from 0 to 600 μg/mL in acidified PDA and on MEA. MEA was prepared and autoclaved, and, after cooling, appropriate amounts of sodium benzoate or potassium sorbate were added to the MEA and mixed before pouring plates.

PDA with appropriate amounts of either sorbate or benzoate were autoclaved and then acidified to pH 3.5 with sterile tartaric acid before pouring plates. Duplicate plates were then stab inoculated with the *T. flavus* cultures at three equally spaced spots per plate and incubated upright in plastic sacks at 25°C. Colony diameters were recorded periodically for up to 14 days, as was the presence or lack of growth. The influence of temperature on growth rate was similarly determined by measuring radial colony growth on MEA at daily intervals.

RESULTS

Cultures

Cultures NRRL 13535 and NRRL 13536 were isolated from concentrated pineapple juice. Cultures NRRL 13537 and NRRL 13538 were isolated from spoiled fruit drink made with concentrated pineapple juice. These four cultures were similar and consistant with the taxonomic descriptions for the genus and species of *Talaromyces flavus* (Pitt, 1979; Domsch et al., 1980; Stolk and Samson, 1972) for both the anamorphic and teleomorphic stages. Colonies typically developed on MEA after about 3 days of incubation at 25°C and appeared as a *Penicillium* colony with Greenish-Grey color. After about a week of

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incubation the gymnothecia dominated and the colony appeared rough and bright yellow with the Petri plate reverse showing a reddish color under the colony. Measurement of 25 or more ascospores gave average maximum diameter of 6.3μm and standard deviation of 0.58um for culture NRRL 13535, 6μm and 0.41um for NRRL 13536, 5.9um and 0.34um for NRRL 13537 and 5.9um and 0.37um for NRRL 13538. These dimensions agree with those reported for *T. flavus* var. *macrosporus* (Stolk and Samson, 1972). The population of viable *T. flavus* in pineapple juice concentrate, thought to be the source of this spoilage mold in reconstituted juice, was 0.6 colony forming unit per gm. of heated concentrate.

Colony counting

Rose bengal added to fungal medium limits the radial diameter of mold colonies and causes a more defined colony edge, thus making colony counting more accurate (Splittstoesser et al., 1970). Tests were run with 0, 5, 7.5, 10 and 15 ug/ml rose bengal to determine its influence on colony counts of heated *T. flavus* ascospores. Colonies of *T. flavus* had a more defined colony perimeter and were slightly smaller with than without rose bengal in MEA. No statistically significant difference in count was found over the range of 0 to 15 μg/ml using the least significant difference test (Bender et al., 1982). Similarily, there was no statistical difference shown when the number of colonies on MEA with 7.5 μg rose bengal was compared with MEA using the paired t-test. Average $\log_{10}$ colony counts on MEA were 7.00 with standard deviation of 0.223. With 7.5 μg/mL added rose bengal the log 10 colony counts and the standard deviation were 7.02 and 0.324, respectively. We thus used 7.5 μg/mL rose bengal in MEA as an aid in counting colonies.

Heat activation

T. flavus ascospores are subject to heat activation (Katan, 1985). Heat activation is evidenced by an increase in viable count after mild heating (Fig. 1) whereas longer heating results in fewer viable cells. The time to reach maximum viable population for cultures NRRL 13535, NRRL 13536, and NRRL 13537 was 12-15 min at 80°C, 6–8 min at 85°C, and 1–2 min at 90°C. The maximum viable count after heat activation was 0.4 $\log_{10}$ below the microscopic count of about $\log_{10}$ 6.0. Thus, the majority of ascospores are viable and subject to heat activation.

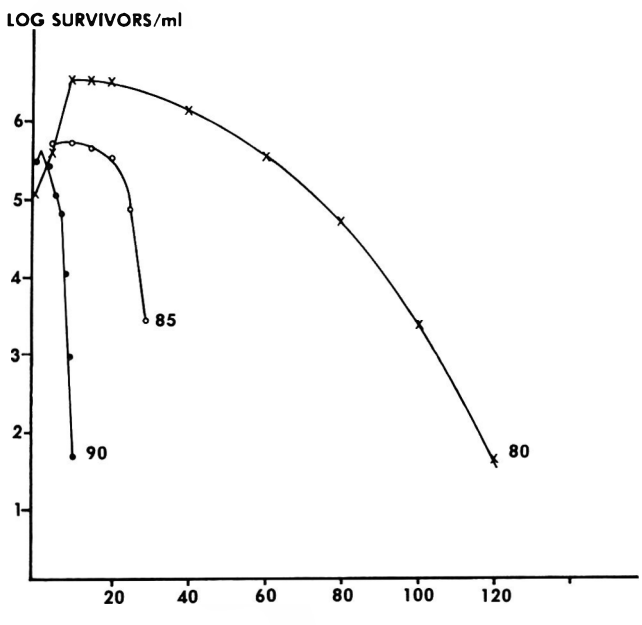

Fig. 1—Thermal death rate curves for T. flavus *NRRL 13535 heated at 80°, 85°, and 90°C.*

Temperature and growth rate

The six cultures were tested for maximum radial growth rate by daily measuring colonies growing on MEA at temperatures from 15° to 40°C. The maximum growth rate was from 30° to 35°C with some strain variations (Fig. 2). A steep decline in growth rate from 35° to 40°C indicates that 40°C is near the maximum. The lower growth limit was below 15°C and above 5°C, where no growth was observed. Strains were fairly uniform in regard to growth rate, those strains that had the highest growth rate at one temperature were generally highest at all temperatures and vice versa. FRR 2098 and FRR 2386 grow nearly twice as fast as the slowest growing culture, FRR 1976.

Sorbate and benzoate effect on growth

Preservatives can be used to control growth of *T. flavus* (Table 1) depending upon the pH and concentration. *T. flavus* grew only on control plates of acidified PDA (pH 3.5) without benzoate or sorbate, addition of either compound at concentrations of 100 μg/mL or greater prevented growth. The added antifungal compounds were less effective at controlling growth of *T. flavus* in MEA (pH 5.4) than in acidified PDA. Growth of NRRL 13536 and FRR 1976 occurred on MEA at 300 μg/mL sorbate and below after 10 days. After 35 days there was growth at 400 μg/mL with FRR 1976. Cultures NRRL 13535 and NRRL 13537 had growth on MEA at 0, 100 and 200 μg/mL but not at higher concentrations. MEA plates with added benzoate had growth at all concentrations tested, the highest being 600 μg/mL. The different response of growth to similar concentrations of the two antifungal compounds on PDA or MEA was due to the differences of pH in the plates or to the

Table 1—Lowest level of preservative (μg/mL) that prevents growth of T. flavus *on agar plates after 35 days incubation*[a]

	PDA pH 3.5		MEA pH 5.4	
Culture No.	Benzoate	Sorbate	Benzoate	Sorbate
FRR 1976	100	100	>600	500
NRRL 13535	100	100	>600	300
NRRL 13536	100	100	>600	400
NRRL 13537	100	100	>600	300

[a] Concentrations tested—0, 100, 200, 300, 400, 500, 600 μg mL.

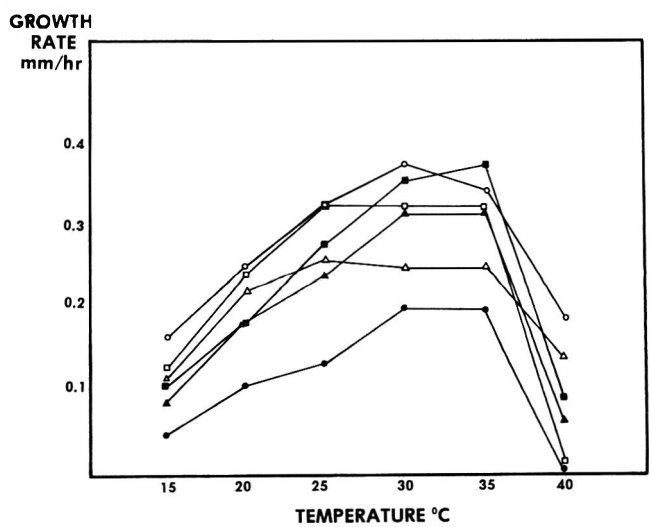

Fig. 2—Radial growth rate curves (mm/hr) for T. flavus *cultures: ○ FRR 2098, ■ FRR 2386, □ NRRL 13536, ▲ NRRL 13537, △ NRRL 13535, and ● FRR 1976 at different temperatures.*

different composition of the media. Growth on control plates with no preservative indicated that the major difference was due to the pH of the medium with added preservative. Sorbate was more effective at the pH of MEA than benzoate. At the lower pH both compounds were effective. The four strains displayed similar growth on either media alone or media containing either of the two antifungal compounds. Both sodium benzoate and potassium sorbate were effective at controlling growth at concentrations above 100 μg/mL at pH 3.5. At pH 5.4 sodium benzoate did not control growth below 600 μg/mL while potassium sorbate did at 300-400 μg/mL.

Resistance of ascospores to ethanol

A preparation of ascospores, mycelial fragments and conidia in 70% ethanol had a decrease in viable count in 10 min from $\log_{10}$ 6.3 to $\log_{10}$ 5.1: then, no further count reduction of 360 min. This is interpreted as a loss of viability of conidia and mycelium sensitive to alcohol and a surviving population of ascospores stable to 70% ethanol.

Resistance of ascospores to heat

Figure 1 shows nonlogarithmic thermal death rate curves for *T. flavus* NRRL 13535 ascospores heated at 90°, 85°, and 80°C. These curves are characterized by an initial rise in viable count caused by heat activation, then a plateau and finally a decline. The decline approaches linearity at the highest heating temperature in contrast with the lower temperatures. Using the formula $(\log N_o - \log N)^a = kt + C$, these curves can be converted into terms for calculating thermal process times (Alderton and Snell, 1970; King et al., 1979). The highest count after heat activation was used as the term No. Table 2 lists the calculated values and shows times calculated from these values to destroy a 3 $\log_{10}$ ascospore population. Table 2 also lists the value l/k derived from the formula $l/k = t/(\log N_o - \log N)^a$ (assuming $C = 0$) and is similar to $D = t/\log n_o - \log N$ in function (King et al., 1979). D is defined in logarithmic death rate calculations as the time at a temperature for a 1 $\log_{10}$ decrease in survivors. The different death rate times for the four *T. flavus* cultures tested indicated different heat resistance for the strains and for different spore preparations of the same strain. The NRRL 13535 preparation with the highest heat resistance (Table 2) was grown in fernback flasks, whereas the other was grown in 250m/L erlenmeyer flasks. Ascospores of FRR 1976, FRR 2098 and FRR 2386 were also tested for heat resistance. None of these three had heat resistance that exceeded 1–2 min at 90°C with an inoculum greater than $\log_{10}$ 4.48.

DISCUSSION

TALAROMYCES FLAVUS was separated into two varieties by Stolk and Samson (1972) on the basis of the ascospore size. *T. flavus* var *flavus* was described as having ascospores 3.5–5 × 2.5–3.2 μm while *T. flavus* var. *macrosporus* has ascospores 5.0–6.5 × 3.5–5.2 μm, the larger spored variety coming from warmer areas (Pitt and Hocking, 1985). Cultures isolated from pineapple concentrate would have come from a warm climate and thus should be the larger spored variety of *T. flavus* if they did not come from processing contamination. According to Pitt and Hocking (1985) isolates with small ascospores have not been reported from spoiled products and must be assumed to have lower heat resistance. A contamination rate of 0.6 CFU per gm is about the same as that reported for *Byssochlamys fulva*, 0.1 per gm (Splittstoesser et al., 1971).

Because of the nonlogarithmic death rate of these molds (and some other microorganisms), calculation of death rate requires using a formula with an exponent as shown by Alderton and Snell (1970) and expanded by King et al. (1979). Firstenberg-Eden et al. (1980) examined the calculations used here for heat resistance and compared them with calculations based upon the assumption of a flat shoulder in which no death occurred followed by logarithmic inactivation. They concluded that the two methods were in agreement. However, they preferred to use their assumption as the basis for calculation because it gives results that could be compared with published D and z values.

Rearrangement of the formula used by Alderton and Snell (1970) can give two different values useful for calculation of thermal process times. Here we have used the calculation of l/k to determine a value corresponding to D in logarithmic death rate kinetics (King et al., 1979). Table 2 shows l/k values of 2–8 min for *T. flavus* at 90°C. A value equivalent to z for these molds was estimated to be 10.3 from a plot of l/k versus temperature. Rearrangement of the primary formula also allows calculation of time to destroy a given number of ascospores (Table 2). This is shown in Table 2 by the time to inactivate 3 $\log_{10}$ ascospores. Beuchat (1986) has reported D values for *T. flavus* at 88°C from 4.1 to 22.3 min and at 91°C from 2.1 to 5.4 min based upon the linear portion of the inactivation curves. z values for these organisms in different fruit products were from 5.2° to 10.8°C. Bayne and Michener (1979) studied heat resistance of *Byssochlamys fulva* and reported that 8 of the 25 cultures studied were heat resistant. The calculated 3 $\log_{10}$ inactivation time for their cultures of *B. fulva* ranged form 1.2 to 46 min at 90°C. The *T. flavus* cultures studied here using the same techniques had 3 log inactivation times of 5 to 12 min at 90°C. Thus, these *T. flavus* cultures were possibly less heat resistant than the maximum heat resistant *B. fulva* cultures reported by Bayne and Michener (1979). Yet they are quite resistant by comparison with other molds and certainly resistant enough to cause spoilage after normal processing of fruit juice. The thermal resistant of *Neosartorya (Aspergillus) fischeri* has been reported by Splittstoesser and Splittstoesser (1977) and Beuchat (1986).

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Table 2—Thermal resistance values for T. flavus *cultures*

Culture No.	Heating temp (°C)	a[a]	k[b]	C	Minutes[c] 1/k	Minutes[c] 3 log
NRRL 13535	80	0.4053	0.01549	0.05552	63	97
	85	0.2020	0.03977	−0.04023	22	32
	90	0.2934	0.1511	−0.05232	7	9
NRRL 13535	80	0.3239	0.007736	0.1819	113	116
	85	0.4188	0.64842	−0.04236	20	34
	90	0.2534	0.2496	0.06275	4	5
NRRL 13536	80	0.7258	0.01049	0.2617	96	236
	85	0.3060	0.03985	−0.02408	26	36
	90	0.5499	0.3145	−0.06918	3	6
NRRL 13537	90	0.4150	0.3010	−0.06149	2	5
NRRL 13538	90	0.3781	0.1319	−0.0111	8	12

[a] a—The reciprocal slope of the plot of log (log No − log N) against log time. This is a rearrangement of the basic formula $(\log No - \log N)^a = kt + C$.
[b] k—Values were determined from the slope of the linearized plot using the previously determined a-value in the basic formula and C is the intercept.
[c] Time for decrease in 1 k value by 1 $\log_{10}$, and calculated time for 3 $\log_{10}$ drop in viable count.

Identification of Some Phenolic Compounds in Bilberry Juice *Vaccinium myrtillus*

M. AZAR, E. VERETTE, and S. BRUN

ABSTRACT

The phenolic compounds of the ethyl acetate extractable fraction of bilberry juice—*Vaccinium myrtillus*—were separated and collected by semi-preparative HPLC. Three flavonoids [quercetin-3-rhamnoside (quercitrin), quercetin-3-galactoside (hypersoide), quercetin-3-glucoside (isoquercitrin)], six phenolic acids [caffeic, chlorogenic, paracoumaric, ferulic, syringic, hydroxybenzoic derivative] previously reported, and other phenolic acids [gallic, protocatechic, *p*-hydroxybenzoic, *m*-hydroxybenzoic, vanillic, *m*-coumaric, *o*-coumaric] were detected by analytical RP-HPLC, TLC procedures, acid and alkaline hydrolyses and spectral characteristics examination. Some of these compounds have not yet been reported for bilberries. The absence of flavan-3-ols and their weakly condensed derivatives, which have already been identified in this fruit, would be attributed to the maturity stages of bilberries and to the strong reactivity of monomeric and dimeric flavan-3-ols.

INTRODUCTION

FRIEDRICH AND SCHONERT (1973) identified two phenolic acids in the fruit of *Vaccinium myrtillus*: caffeic and chlorogenic acids; four flavonol glycosides: quercitrin, isoquercitrin, hyperoside and astragalin (kaempferol-3-glucoside); and finally two flavan-3-ols: (+)-catechin and (−)-epicatechin by TLC. Pogorzelski (1976) identified three phenolic acids in the fermented juice of bilberry: caffeic, chlorogenic and *p*-coumaric acids by TLC.

Brenneissen and Steinegger (1981a) found six phenolic acids: chlorogenic, caffeic, *p*-coumaric, ferulic, syringic, a hydroxybenzoic acid derivative, and three flavonol glycosides: hyperoside, isoquercitrin and quercitrin in the bilberry fruit by HPLC. the occurrence of procyanidines B_1, B_2, B_3, B_4 and monomeric flavan-3-ols: (+)-catechin and (−)-epicatechin, was also confirmed by these authors.

This study grew out of a program initiated to determine phenolic compounds of bilberry juice—*Vaccinium myrtillus*—used in medicine for its pharmacological properties. The phenolic compounds extracted from bilberry juice after fermentation constitute the active principle of a french drug used for its vascular properties. In the present work especially phenolic acids and flavonols which had not been systematically and thoroughly studied until now were investigated. Several techniques were jointly applied to separate, isolate, identify and confirm the presence of some phenolic compounds in the juice of wild bilberry harvested in Poland.

MATERIALS & METHODS

THE FRESH JUICE of bilberry was prepared in the laboratory of a pharmaceutical industry in France, from wild bilberries harvested in Poland and transported frozen. After thawing, bilberries were crushed at 25°C and after pressing, juice was stored and transported to our laboratory at −20°C.

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Extraction

The bilberry juice (pH = 3.5) (50 mL) was extracted several times (6 × 50mL) with ethyl acetate after the addition of sodium bisulfite (Wulf and Nagel, 1980). The sodium bisulfite discolors anthocyanin monomers occurring in the juice and keeps them in the aqueous phase. The ethyl acetate was dried over anhydrous Na_2SO_4 and then concentrated in a rotary evaporator under vacuum at 30°C. The concentrate was taken up in methanol and stored at 0°C under nitrogen.

Thin layer chromatography (TLC) of phenolic compounds

TLC was applied to the extract comparatively to standard phenolic compounds. The two dimensional TLC of phenolic acids was carried out on commercial 0.1 mm cellulose layers (Ref. 5730, Merck Darmstadt, R.F.A.) using the solvent system described by Andary (1975): Solvent I: acetic acid-water (2:98, v/v); Solvent II: benzene-acetic acid-water (60:22:1.2, v/v). the phenolic acids were located by spraying chromatograms first with the diazotized *p*-nitroaniline and then with sodium bicarbonate 15% (v/v) in water (Andary, 1975). Solvent III: *n*-butanol-pyridine-water (14:3:3, v/v) was used for chromatographical separation of chlorogenic acid (Steck, 1976).

Flavonols were chromatographed in parallel on silica gel plates (Ref. 5626 Merck Darmstadt, R.F.A.) with solvent IV: ethyl acetate-ethyl methyl ketone-formic acid-water (5:3:1:1:, v/v) and on cellulose plates as suggested by Mabry and al. (1970) using solvent V: tert-butanol-acetic acid-water (3:1:1, v/v) in the first dimension and solvent VI: acetic acid-water (15:85, v/v) in the second dimension. They were detected with 5% (w/v) $AlCl_3$ solution in MeOH or with Neu (1957) reagent (1% (w/v) diphenyl-boric acid-ethanolamine solution in MeOH).

HPLC/UV spectoscopy of phenolic compounds

A Varian 5000 Liquid Chromatograph equipped with a variable wavelength Varian U.V.-100 detector was used. Two columns were used for reversed phase analysis preceded by a 25 × 4 mm Lichrosorb RP 18/Hibar-Lichrocart (Merck) precolumn. A 250 x 4 mm Hibar stainless steel column packed with 5 μm Lichrosorb RP 18 (Merck)

Table 1—Detection of phenolic acids on TLC plates[a]

Compounds	a	b
gallic acid	fl. blue	yellow-brown
protocatechic acid	d. mauve	mauve-purple
caffeic acid	fl. blue	beige
chlorogenic acid	fl. blue	yellow
ferulic acid	fl. blue-purple	blue-green
p-hydroxybenzoic acid	d. mauve	pink
m-hydroxybenzoic acid	mauve purple	d. pink
p-coumaric acid	d. mauve	blue-grey
m-coumaric acid	fl. blue-purple	d. pink
o-couramic acid	fl. blue-yellow	mauve-purple
vanillic acid	invisible	d. purple
syringic acid	d. mauve	d. blue-grey

[a] fl: fluorescent; d: dark; (a) under UV light; (b) after spraying first with diazotized *p*-nitroaniline and then with sodium bicarbonate solution.

Table 2—Detection of flavonol glycosides[a]

Compounds	a	b	c	d
Hyperoside	mauve brown	yellow	yellow	orange yellow
Isoquercitrin	mauve brown	yellow	yellow	orange yellow
quercitrin	mauve brown	yellow	yellow	orange yellow

[a] under UV light in absence of NH_3; (b) under UV light in presence of NH_3; (c) after spraying $AlCl_3$ reagent; (d) after spraying Neu reagent.

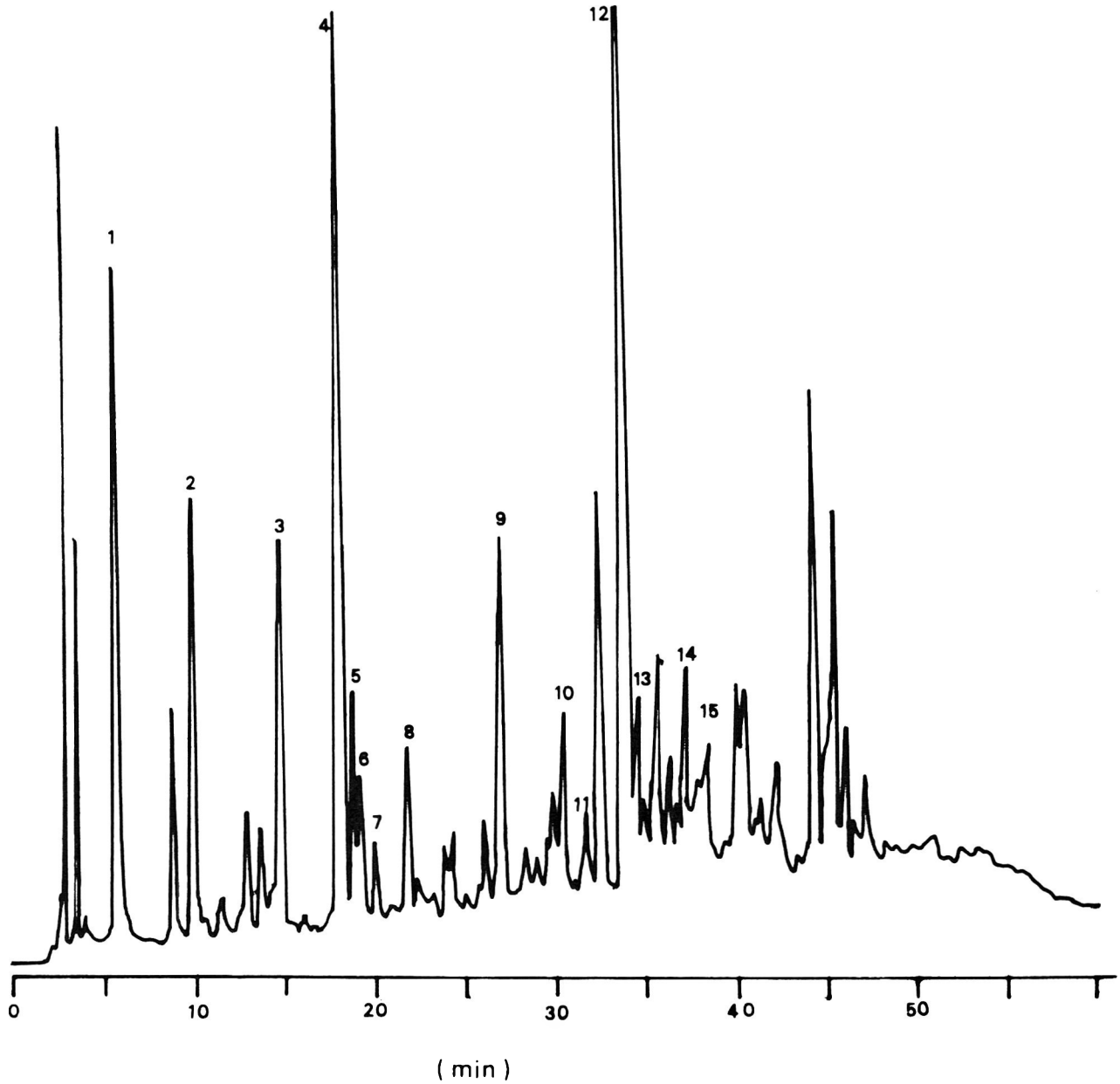

Fig. 1—Analytical HPLC separation of phenolic compounds of the ethyl acetate extract of bilberry juice; for conditions see Experimental. Compounds identified by peak numbers are given in Table 3.

was used for analytical separations and a 250 × 10 mm Hibar stainless steel column packed with 7 μm Lichrosorb RP 18 for semi-preparative chromatographical separations. Linear gradient elution was carried out using solvent I and solvent VII (acetic acid-acetonitrile-water: 2:30:68, v/v) from 7% to 100% VII in I in 50 min and holding at 100 % VII for 10 min. The other parameters used were: flow rate: 1 mL/min, 10 μL sample loop for analytical HPLC and 3 mL/min, 100 μL sample loop for semi-preparative HPLC. In both HPLC separations the column temperature was set at 30°C and UV detector at 280 nm. A Perkin Elmer LC 85 detector was used to record spectra between 200 and 380 nm of separated compounds, according to their elution sequence from the HPLC column.

Acid and alkaline hydrolyses

The flavonol monoglycosides occurring in the ethyl acetate extract were hydrolyzed by heating at 100°C in 2 N HCl-ethanol (1:1 v/v), as described by Harborne (1965), in stoppered capillary tubes. A mild acid hydrolysis in 0.1N HCl at 100°C was used to break the C-C bonds between flavan-3-ol units. Samples were taken at suitable time intervals (0, 10, 20, 30 min) and chromatographed to identify the released compounds (aglycone, sugars, flavan-3-ols).

Alkaline hydrolysis was carried out at room temperature (20°C) in 1N NaOH under nitrogen for 1hr and stopped by acidification with 4 N HCl to pH 3.0. Phenolic acids were then extracted by ether and identified by TLC as described previously. The aqueous phase was chromatographed to identify the quinic acid released from chlorogenic acid.

Identification of hydrolysis moieties

Sugars and aglycone moieties of flavonol glycosides were identified by TLC on cellulose plates in the presence of standard compounds. Over run chromatography of sugars was accomplished with the solvent system: *n*-butanol-pyridine-water (6:3:1, v/v). Sugars were located on TLC plates by spraying with the following solution: 5 mL *n*-butanol, 5 mL ethanol, 5 mL water, 0.5 mL aniline, 0.5g tartaric acid and heating for 10 min in an oven at 105°C. Chromatography of the aglycone (quercetin) was carried out in BAW: n-butanol-acetic acid-water (4 : 1 : 5, upper phase v/v). Detection was carried out as mentioned before.

The quinic acid released after alkaline hydrolysis was identified by TLC on cellulose plates in the following solvent system: n-propanol-acetic acid-water (80:2:18, v/v) and revealed with green bromocresol reagent (Bounias, 1983); it was also identified by HPLC as described by Goiffon et al. (1985).

Table 3—UV spectral characteristics (λ_{max}); retention times (t_R) and capacity factors (K') of phenolic compounds identified in the ethyl acetate extract by HPLC

Peak number	Compound	t_R (min)	K'	λ_{max}[a] (nm)	
1	gallic acid	5.62	1.25	272	
2	protocatechic acid	9.87	2.95	260	295
3	p-hydroxybenzoic acid	14.73	4.90	260	
4	m-hydroxybenzoic acid	17.95	6.19	290	
5	chlorogenic acid	18.77	6.52	295[b]	325
6	vanillic acid	19.20	6.69	260	295
7	caffeic acid	19.98	7.01	295[b]	325
8	syringic acid	21.79	7.73	280	
9	p-coumaric acid	26.91	9.78	300[b]	310
10	ferulic acid	26.92	10.99	295[b]	325
11	hyperoside	31.10	11.46	256	356
12	m-coumaric acid	33.74	12.52	276	312[b]
13	isoquercitrin	34.48	12.82	256	356
14	o-coumaric acid	36.79	13.74	274	322[b]
15	quercitrin	37.91	14.19	256	356

[a] Measured in HPLC eluent.
[b] Shoulder.

Chromatography of flavan-3-ols and their weakly condensed forms was carried out on silica gel plates using the solvent system : toluene-acetone-formic acid (3:3:1, v/v); the reagent sprayed for visualization was 5% vanillin in ethanol (w/v) mixed with concentrated HCl in the ratio 4:1 (v/v) just prior to use.

RESULTS & DISCUSSION

THE ETHYL ACETATE extractable fraction of bilberry juice contained phenolic acids, some flavonoids and flavan-3-ols in their monomeric and weakly condensed form. The two dimensional TLC (solvent I and II) of the extract separated free phenolic acids according to the nature of substituents on the phenolic ring. The combined phenolic acids were separated according to Steck (1976) using solvent III which allowed the separation of glucose esters ($0.55 < R_f < 0.75$) from glycosides and quinic esters ($0.1 < R_f < 0.3$) of phenolic acids. The detection techniques helped to characterize the acids mentioned in Table 1.

The two-chromatographical systems applied to the ethyl acetate extract and standard flavonols using solvent IV for monodimensional chromatography and solvent V and VI for two-dimensional chromatography; all detection techniques identified three flavonoids (Table 2). Unidentified spots observed on TLC plate were attributed to aglycones after examination of their position on the two-dimensional TLC according Mabry et al. (1970).

The R.P.-HPLC method (Reminiac and Goiffon, 1982) slightly modified for our purpose, separates the phenolic compounds of the ethyl acetate extract (Fig.1). Some peaks of this extract were identified by several procedures: (1) comparison of retention times between unknown peaks and standard phenolic compounds; (2) co-chromatography of the extract and standard phenolic compounds; (3) examination of peak intensity variations at different wavelengths and comparison with chromatograms of standard phenolic compounds recorded using the same conditions. Thus at 260 nm peaks corresponding to gallic, protocatechic, hydroxybenzoic, syringic and vanillic acids, at 320 nm peaks corresponding to cinnamic acids, and at 360 nm peaks corresponding to flavonols were characterized.

All phenolic compounds are detected at 280 nm so this wavelength was selected for the analysis. UV spectra of substances were recorded according to their elution sequence from the HPLC column after appropriate detection. Spectral characteristics of phenolic compounds of the ethyl acetate extract as well as their retention times and capacity factors are illustrated in Table 3.

To confirm the identity of phenolic acids and flavonols, semi-preparative HPLC was applied to collect the corresponding fractions, which were chromatographed on TLC plates in the presence of reference compounds with solvent II and solvent IV. By this way, phenolic compounds mentioned in Table 1 were confirmed.

The acid and alkaline hydrolyses were carried out to study the different combined forms of phenolic compounds in the ethyl acetate extract. Acid hydrolysis released quercetin and three sugars : glucose, galactose and rhamnose which were all identified by TLC. These results confirmed the presence of three monoglycosides of quercetin. Mild acid hydrolysis for release of monomeric flavan-3-ols from weakly polymeric forms did not allow the identification of flavan-3-ols by TLC. However, alkaline hydrolysis of the fraction relative to chlorogenic acid, collected by semi-preparative HPLC, released caffeic and quinic acids which were confirmed by means of HPLC and TLC.

Analysis of the fresh bilberry juice resulted in the identification of the following phenolic acids: gallic, protocatechic, *p*-hydroxybenzoic, *m*-hydroxybenzoic, vanillic, chlorogenic, caffeic, syringic, *p*-coumaric, ferulic, *m*-coumaric, *o*-coumaric and three glycosides of quercetin: quercetin-3 rhamnoside (quercitrin), quercetin-3 glucoside (isoquercitrin), quercetin-3 galactoside (hyperoside). The presence of these compounds was confirmed by different analytical methods.

Work relating to phenolic acids in bilberry fruits previously mentioned (Brenneissen and Steinegger, 1981a) revealed the presence of chlorogenic, a hydroxybenzoic derivative, caffeic, *p*-coumaric, ferulic and syringic acids. Our study confirmed the presence of these acids and led to the identification of six other phenolic acids. As for flavonols, the identification of quercitrin, isoquercitrin and hyperoside is in agreement with the results of Friedrich and Schönert (1973) and Brenneissen and Steinegger (1981a, 1981b).

The presence of monomeric or polymeric flavan 3-ols in ethyl acetate was not ascertained by applying several procedures. The absence of flavan-3-ols would be attributed to the maturity stages of bilberries and to the strong reactivity of monomeric and dimeric flavan-3-ols (Brenneissen and Steinegger, 1981a, 1981b). All these results are reported (Azar, 1986).

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Ms received 10/23/85; revised 5/8/87; accepted 5/11/87.

Measurement of Enzymatic Browning at Cut Surfaces and in Juice of Raw Apple and Pear Fruits

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ABSTRACT

Reflectance procedures were developed to measure the extent of enzymatic browning at cut surfaces and in the raw juice of apple and pear fruits. Reflectance L and a measurements, made at transversely cut surfaces of plugs bored from fruit halves, were linear or bilinear with log time and related to the extent of browning in six apple cultivars. With apple and pear juices, tristimulus values changed linearly with time in samples undergoing browning. Differences between initial and final tristimulus values were better indices of browning than the slopes of time curves. The suitability of these procedures for evaluating the effectiveness of browning inhibitors was demonstrated with SO_2 and ascorbic acid treatments.

INTRODUCTION

BROWNING of raw fruits and vegetables due to mechanical injury during postharvest handling and processing is an important cause of quality and value loss in affected commodities. This reaction results from the polyphenol oxidase (PPO)-catalyzed oxidation of phenolic compounds to *o*-quinones which subsequently polymerize to form dark-colored pigments (Joslyn and Ponting, 1951; Mayer and Harel, 1979; Vamos-Vigyazo, 1981). Enzymatic browning in unblanched, cut fruits and vegetables may be controlled by the application of sulfur dioxide (Joslyn and Braverman, 1954), ascorbic acid (Bauernfeind and Pinkert, 1970), and various other antioxidants, chelating agents, salts, enzymes and enzyme inhibitors (Vamos-Vigyazo, 1981). In recent years, however, concern over adverse health effects from sulfite, the most effective browning inhibitor, has stimulated a search for alternative antibrowning compounds (Taylor and Bush, 1983; Labell, 1983; Andres, 1985).

To evaluate the effectiveness of experimental treatments in controlling enzymatic browning and to compare them with conventional treatments, accurate measurements of the extent of browning are required. Spectrophotometric procedures, usually entailing absorbance measurements at 420 nm, have been used to measure brown pigments in clarified juices (Toribio et al., 1984) and in vegetable extracts (Hendel et al., 1955). However, such procedures are slow relative to the rate of enzymatic browning in macerated fruits and vegetables and are not applicable to the evaluation of browning at cut surfaces.

Tristimulus reflectance colorimetry (usually the measurement of Rd or Hunter L values) has been used to follow the extent of enzymatic browning in juices (Smith and Cline, 1984) and apple slices (Ponting et al., 1972). Published information on sample preparation and presentation for colorimetry, the sensitivity of different tristimulus color scales in responding to enzymatic browning and the kinetics of such changes is limited. While reflectance methods are rapid and nondestructive, preliminary studies have shown them to be limited in accuracy and precision with heterogeneous samples or samples that were subject to physical changes during the time of measurement (Sapers, 1985). The objective of this study was to develop accurate and precise, nondestructive tristimulus reflectance procedures, that could be applied to the cut surfaces or juice of raw fruits, yielding data that could be correlated with visual and spectrophotometric assessments of browning and used to determine the effectiveness of treatments to control enzymatic browning.

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MATERIALS & METHODS

Browning at cut surfaces

Apple and pear samples representing common cultivars were obtained from local food stores during the fall and winter of 1985–86 and stored briefly at 4°C until needed. One hour prior to use, fruits were removed from the refrigerator and equilibrated to room temperature (ca 20°C). Each apple or pear was cut in half along the stem axis, and the halves were positioned in a Petri dish, cut side down, under an electric cork borer (Sargent-Welch, Skokie, IL) so that uniform plugs could be bored perpendicular to the cut surface, on either side of the point of greatest thickness, with a 22 mm stainless steel cutting tube. At the start of an experiment, a transverse cut was made in the plug, at least 1 cm from the skin end (to exclude the effects of bruising), exposing fresh surface. An arrow was cut at the opposite end of the plug to mark its orientation during reflectance measurements.

Colorimetry was performed with a Gardner XL-23 Tristimulus Colorimeter (Pacific Scientific, Silver Spring, MD), operated with large diameter illumination and with a 19 mm opening aperture plate. The instrument was standardized against a white tile (Y = 84.60, X = 82.21, Z = 97.64) before each measurement. The transversely cut surface of a plug was centered over the aperture, oriented so that the arrow cut in the opposite end pointed away from the colorimeter operator. Values of the tristimulus coordinates in the L, a, b and Y, X, Z systems were recorded at 1, 10, 30, 60, 90, 120, 150, 180, 240, 300, 360, and 420 min. Between measurements, plugs were held in covered glass crystallizing dishes to minimize dehydration at the cut surface. The tristimulus coordinates were plotted against log time, and the slopes of linear portions of these curves were obtained by linear regression.

Browning in juice

Juice samples were prepared from individual apples or pears with an Acme Supreme Model 6001 Juicerator (Acme Juicer Manufacturing Co., Lemoyne, PA), lined with Whatman No. 1 filter paper. A 25 or 50 mL aliquot of thoroughly mixed juice was pipetted into a cylindrical clear glass optical cell (57.1 mm i.d.) to a depth of about 10 or 20 mm for colorimetry. Care was taken to exclude foam from the cell. Colorimetry was performed as described above but with the optical cell placed in a 50 mm diameter support ring in place of the 19 mm aperture plate. Tristimulus values were recorded at 1, 2, 3, 4, 6, 8, 10, 15, 20, 30, 40, 50, 60, 75 and 90 min and were plotted against time rather than log time.

To permit the direct comparison of reflectance and spectrophotometric data for browning apple juice, 10 mL aliquots of a 100-125 mL juice sample (mixed slowly with a magnetic stirrer) were taken for spectrophotometry at 15 min intervals, when reflectance measurements also were made. The aliquots were clarified by a modification of the method of Meydav et al. (1977) entailing rapid mixing with an equal volume of 95% ethanol and 0.3g Celite Analytical Filter Aid (Fisher Scientific, Pittsburgh, PA) followed by filtration through Whatman No. 50 paper under suction. The absorbance of the filtrates was determined at 420 nm with a Perkin-Elmer Model 552 UV-visible spectrophotometer (Perkin-Elmer, Oak Brook, IL)

Evaluation of browning inhibitors

To determine the suitability of the tristimulus reflectance procedure for evaluating browning inhibitors applied to cut surfaces, Red Delicious and Stayman Winesap apple plugs (2 per half, taken on either side of the core axis) were cut in half, yielding 4 pairs of plug halves per fruit, each pair having a common cut surface. Treatments were applied to one plug half from each pair, the other half serving as a control, so that 4 levels of a treatment and corresponding untreated controls could be compared, using only 1 apple. Treatments consisted of 90 sec dips in freshly prepared 0.01%, 0.02%, 0.04% or 0.08% $NaHSO_3$ solutions or in freshly prepared 1% citric acid monohydrate solutions containing 0.4%, 0.8%, 1.6% or 3.2% ascorbic acid. After dipping, the plugs were drained, blotted dry with absorbent tissue and then held for 7 hr at 20°C during which time tristimulus reflectance measurements were made at intervals.

The suitability of the reflectance procedure for evaluating browning inhibitors in juice was tested with Golden Delicious and Granny Smith apples. The freshly prepared juice from 2 apples was briefly stirred to assure uniformity and then divided into 5 25-mL portions, rapidly dispensed by burette into optical cells containing 1 mL H_2O (the control), 1 mL 0.406% sodium bisulfite (100 ppm SO_2 in the juice), 0.75 mL H_2O + 0.25 mL 0.1% ascorbic acid (AA) (10 ppm), 0.50 mL H_2O + 0.50 mL 0.1% AA (20 ppm), and 1.0 mL 0.1% AA (40 ppm). Reflectance L- and a-values for controls and treated juices were measured at intervals during 1½ hr at 20°C. To compensate for sedimentation in the optical cells, the samples were briefly stirred prior to each reading.

RESULTS & DISCUSSION

Measurement of browning at cut surfaces

Preliminary observations of the cut surfaces of apple and pear plugs indicated that enzymatic browning occurred gradually over several hours with unblemished tissue. Little or no variation in the degree of browning could be seen along the length of the bore hole remaining in the fruit half after removal of a plug, indicating that the exact location of the transverse cut probably was not critical. Tristimulus reflectance measurements made at the cut surface of fruit plugs yielded values which were linear or occasionally bilinear when plotted against log time and appeared to be related to the extent of browning (Fig. 1). The logarithmic relationships were unexpected and may be a consequence of the gradual depletion of PPO substrates in the free juice adhering to disrupted cell layers at the cut surface. Initial flat regions or deviations from linearity in the reflectance curves may be indicative of the depletion of endogenous browning inhibitors such as ascorbic acid prior to the onset of browning (Ponting and Joslyn, 1948), or of changes in the albedo of the cut surface due to physical processes such as the evaporation of free juice.

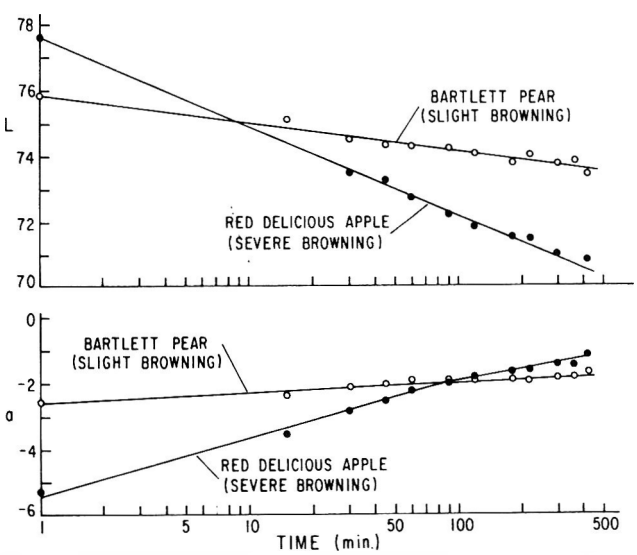

Fig. 1—Reflectance L- and a-values at cut surfaces of apple and pear plugs held at 20°C.

Since treatments to inhibit enzymatic browning might be evaluated by comparing one or several plugs taken from opposite halves of a single fruit (each plug receiving a different treatment level), the similarity of reflectance vs time relationships for multiple plugs taken from individual Red Delicious and Stayman apples was examined (Table 1). Regression slopes for the linear portion of reflectance vs log time curves as well as differences between the final and initial L- or a-values (Δ values) for the entire curve generally were similar for different plugs obtained from the same fruit. Since some variation in slopes or Δ values was obtained within individual apples (see Red Delicious I B-1, for example), the procedure was modified so that a treated plug could be compared with a control representing the same plug. This was done by using half-plugs, one half receiving the treatment and the same surface on the other half serving as its control. Variation in cut apple surfaces could be related to the location of plugs and transverse cuts relative to the core, with vascular bundles showing up as streaks or spots, depending on the orientation of the surface. Such heterogeneity largely could be eliminated by boring plugs on either side of the core axis at the widest part of the apple.

To determine which of the tristimulus coordinates gave the most accurate indication of browning at cut surfaces, Hunter L, a, b and CIE Y, X, Z values were measured for plugs taken from 4 fruits for each of 6 apple cultivars (Cortland, Granny Smith, Idared, McIntosh, Red Delicious and Stayman) over a 3 hr period. This study was not extended to pear fruits since the cultivars examined in preliminary studies (Anjou, Bartlett, Bosc, Red Bartlett and Seckel) browned too slowly to be useful in evaluating browning inhibitors. Values of Y, X, Z and L for the browning apple surfaces decreased with time, while values of a and b increased. In all cases, a linear relationship was seen between the tristimulus coordinate and the logarithm of time, correlation coefficients for the regression usually exceeding 0.9. However, changes in X, Z and b appeared to be unrelated to the extent of browning. Ponting et al. (1972) reported that total reflectance values (Rd = Y) correlated better with browning in apple slices than did a- or b-values (determined with the Rd coordinate system). Bolin et al. (1964) was able to use the L-value ($L = 10^{1/2}$ to determine the effectiveness of SO_2 in

Table 1—Reflectance characteristics of cut surfaces of plugs from opposite sides of Red Delicious and Stayman apples undergoing browning at 20°C

				L		a	
Cultivar	Fruit	Side	Plug	Slope[a]	ΔL[b]	Slope[a]	Δa[b]
Red Delicious	I	A	1	−2.7	−5.2	1.7	3.2
			2	−2.9	−5.6	1.8	3.5
		B	1	−2.1	−4.2	1.3	2.6
			2	−2.9	−5.9	1.7	3.4
	Mean ± S.D.[c]			−2.6 ± 0.4	−5.2 ± 0.7	1.6 ± 0.2	3.2 ± 0.4
	II	A	1	−2.1	−4.3	1.4	2.8
			2	−1.5	−2.8	1.0	1.8
		B	1	−2.0	−4.0	1.3	2.8
			2	−2.4	−4.7	1.6	3.3
	Mean ± S.D.			−2.0 ± 0.4	−4.0 ± 0.8	1.3 ± 0.2	2.7 ± 0.6
Stayman	I	A	1	−3.9	−7.7	2.4	4.9
			2	−3.2	−6.5	1.9	4.3
		B	1	−4.0	−8.1	2.4	5.0
			2	−3.6	−7.2	2.2	4.8
	Mean ± S.D.			−3.7 ± 0.4	−7.4 ± 0.7	2.2 ± 0.2	4.8 ± 0.3
	II	A	1	−2.8	−5.5	1.6	3.2
			2	−3.7	−7.4	2.3	4.5
		B	1	−3.3	−6.4	2.0	4.0
			2	−3.8	−7.8	2.3	4.6
	Mean ± S.D.			−3.4 ± 0.4	−6.8 ± 1.0	2.0 ± 0.3	4.1 ± 0.6

[a] Linear portion of L or a vs log time curve (at least 5 data points); correlation coefficients for regression > 0.98.

[b] Difference between 180 min and 1 min values.

[c] Standard deviation.

inhibiting browning in apple wedges. Since the most characteristic manifestation of enzymatic browning is sample darkening (changes in hue being commodity-dependent), a negative correlation between browning and the Y- (or L-) value, which is defined as the luminosity or lightness function (Clydesdale, 1978), would be expected.

The absolute values of the reflectance measurements could not be used to compare different samples undergoing browning because of apple-to-apple variability in natural pigmentation. Therefore, the slopes of the reflectance curves (change in L- or a-value per log cycle time) as well as the differences between final and initial values of L or a (ΔL or Δa) were compared, the latter approach being applicable to curves with bilinear or nonlinear regions for which a single slope value would be meaningless (Table 2). The apples compared in this study varied greatly in degree of browning, both between and within cultivars. Such variability probably results from differences in PPO activity, polyphenol content and/or ascorbic acid content (Vamos-Vigyazo, 1981). The extent of apple-to-apple variability within samples would mitigate against the use of more than one apple to carry out comparisons of multilevel treatments to control browning. Among the cultivars compared, Idared and Granny Smith tended to brown less while Stayman browned more; Red Delicious, Cortland and McIntosh were intermediate in browning. Slopes and Δ values for L and a both appeared to be related to the extent of browning, changes in L being larger and consequently more sensitive than changes in a. Because of occasional inconsistencies between L and a, perhaps due to changes in sample albedo, both ΔL and Δa values should be used to determine the extent of browning in apple plugs. Measurements should be made at times appropriate to the samples and treatments.

Evaluation of browning inhibitors applied to cut surfaces

To demonstrate the applicability of the reflectance procedure to cut apple surfaces treated with browning inhibitors, untreated cut surfaces of Red Delicious and Stayman plug halves, which undergo severe browning, were compared with surfaces of the corresponding plug halves that had been dipped in solutions containing different concentrations of sulfite or ascorbic acid. The extent to which these treatments inhibited browning was expressed on a percent basis, i.e., the percent difference between the control and treatment ΔL or Δa values after a specified storage time t:

$$\% \text{ Inhibition} = \frac{\Delta L \text{ control} - \Delta L \text{ treatment}}{\Delta \text{ control}} \times 100$$

where ΔL (or Δa) is the difference between the L- (or a-value) at time t and the value at 1 min. Positive values of the percent inhibition between 0 and 100 would indicate that the treatment is effective as a browning inhibitor to the extent calculated. Values greater than 100%, if significant, would indicate sample bleaching by the treatment, while negative values would indicate that the treatment promoted rather than inhibited browning. The inhibition data clearly showed that the reflectance procedure could detect the differing degrees of browning inhibition obtained in plugs treated with different levels of sulfite or ascorbic acid and then stored for different periods of time (Table 3). Inhibition data for Red Delicious apples (not shown) were similar to those obtained with Stayman apples.

Measurement of browning in apple and pear juice

Raw juice might represent a more useful system than the cut surface of plugs for the comparison of multilevel treatments to inhibit browning since it would be homogeneous and more easily manipulated. However, preliminary experiments with a number of apple cultivars (Jonathan, McIntosh, Red Delicious, Rome, Stayman and Winesap) and pear cultivars (Bartlett, Red Bartlett and Seckel) indicated that browning in the freshly pre-

Table 2—Measurement of browning at cut surface of apple plugs held at 20°C by tristimulus reflectance colorimetry

Cultivar	Apple	Extent of browning (180 min)[a]	L - value		a - value	
			Slope (r)[b]	ΔL[c]	Slope (r)[b]	Δa[c]
Idared	1	V. sl.	−0.7 (−0.85)	−1.5	0.8 (0.95)	0.8
	4	V. sl.	−2.8 (−0.99)	−3.4	1.4 (0.99)	1.9
	2	Sl.	−2.8 (−0.99)	−3.0	2.8 (0.99)	2.5
	3	Sl.	−3.4 (−0.99)	−3.9	2.0 (0.99)	2.4
	Mean ± S.D.[d]		−2.4 ± 1.2	−3.0 ± 1.0	1.8 ± 0.8	1.9 ± 0.8
Granny Smith	1	None	−1.9 (−0.93)	−0.6	1.2 (0.98)	1.1
	3	V. sl.	−1.8 (−0.99)	−2.4	1.1 (0.99)	1.4
	4	Sl.	−2.7 (−0.96)	−2.9	1.9 (0.99)	1.6
	2	Mod.	−3.9 (−0.97)	−5.9	2.7 (0.99)	4.0
	Mean ± S.D.		−2.6 ± 1.0	−3.0 ± 2.2	1.7 ± 0.7	2.0 ± 1.3
Red Delicious	2	Mod.	−2.0 (−0.98)	−4.3	1.4 (0.98)	2.8
	4	Mod.	−2.1 (−0.98)	−4.9	1.8 (0.99)	3.4
	1	Sev.	−2.6 (−0.99)	−6.0	1.5 (0.99)	3.6
	3	Sev.	−3.2 (−0.99)	−5.9	2.0 (0.99)	3.8
	Mean ± S.D.		−2.5 ± 0.6	−5.3 ± 0.8	1.7 ± 0.3	3.4 ± 0.4
Cortland	2	Sl.	−3.7 (−0.99)	−5.6	1.0 (0.99)	1.4
	3	Mod.	−5.1 (−0.99)	−7.7	2.1 (0.99)	3.0
	1	Sev.	−4.2 (−0.97)	−8.8	1.3 (0.99)	2.8
	4	Sev.	−6.2 (−0.99)	−9.1	2.7 (0.99)	4.3
	Mean ± S.D.		−4.8 ± 1.1	−7.8 ± 1.6	1.8 ± 0.8	2.9 ± 1.2
McIntosh	2	Mod.	−3.2 (−0.97)	−6.4	2.2 (0.99)	3.4
	3	Mod.-Sev.	−3.2 (−0.99)	−7.0	2.6 (0.99)	3.7
	1	Sev.	−4.6 (−0.99)	−6.4	1.9 (0.99)	4.1
	4	Sev.	−4.5 (−0.99)	−7.5	2.1 (0.99)	3.9
	Mean ± S.D.		−3.9 ± 0.8	−6.8 ± 0.5	2.2 ± 0.3	3.8 ± 0.3
Stayman	3	Mod.-Sev.	−2.5 (−0.99)	−5.2	2.2 (0.99)	4.0
	1	Sev.	−4.4 (−0.99)	−8.1	2.8 (0.99)	5.9
	2	Sev.	−4.6 (−0.99)	−8.0	3.1 (0.99)	5.6
	4	Sev.	−5.1 (−0.99)	−9.6	3.4 (0.99)	6.1
	Mean ± S.D.		−4.2 ± 1.1	−7.7 ± 1.8	2.9 ± 0.5	5.4 ± 1.0

[a] V. sl. = very slight; Sl. = slight; Mod. = moderate; Sev. = severe.
[b] Slope and correlation coefficient for linear portion of L or a vs log time curve (at least 5 data points).
[c] Difference between 180 min and 1 min values.
[d] Standard deviation.

Table 3—Evaluation of treatments to inhibit browning of cut surfaces of Stayman apple plugs by reflectance tristimulus colorimetry

Browning inhibitor	Treatment level (%)	Percent inhibition[c] Calculated from ΔL 2 hr	4 hr	6 hr	Calculated from Δa 2 hr	4 hr	6 hr
$NaHSO_3$[a]	0.01	75	69	69	69	54	48
	0.02	97	94	92	91	84	81
	0.04	96	96	80	97	89	74
	0.08	101	99	94	101	95	89
Ascorbic acid[a,b]	0.4	52	24	12	46	15	9
	0.8	102	91	79	100	87	75
	1.6	104	102	100	100	97	93
	3.2	102	102	101	103	100	97

[a] 90 sec dip.

[b] All solutions contain 1% citric acid.

[c] (Δcontrol − Δtreatment) × 100 ÷ Δ control; Δ values are differences between L or a values at indicated storage time and values at 1 min.

pared juice occurred too rapidly to permit sample treatment and evaluation. Juices prepared from Cortland, Golden Delicious, Granny Smith and Idared apples or from Anjou and Bosc pears browned more gradually.

Reflectance measurements, made with these slower browning cultivars, indicated an inverse relationship between browning and Z- and L- (or Y) values; a-values increased in the browning juices (Fig. 2). Plots of the tristimulus coordinates vs time were linear or bilinear over 1 hr with some nonlinearity during the first 5 or 10 min, possibly due in part to the development of turbidity or dissipation of air bubbles. Reflectance measurements usually were not extended beyond 1-2 hr because of interference due to sedimentation.

The occurrence of initial nonlinearity or bilinearity precluded the use of slopes of tristimulus coordinate vs time curves as indices of browning. No clear advantage could be seen in choosing one tristimulus coordinate over another to monitor juices undergoing browning. Johnson et al. (1976) have noted that the Z coordinate will respond to browning since brown pigments absorb at 400 nm, near the maximum for the z-function of the CIE standard observer. While the Z-value for pear juice did decrease in browning samples, it also decreased by a similar amount in some freshly prepared juices that did not yet develop brown color. Apparently, Z responds to physical changes such as the development of turbidity or the dissipation of air bubbles as well as to browning.

To establish the validity of the juice system without recourse to visual observations of browning, which are limited in accuracy and dependent on fruit-to-fruit variability in browning rates, reflectance L and a data for browning apple juice samples were compared with spectrophotometric measurements, i.e., the absorbance of clarified juice at 420 nm (A_{420}) (Table 4). The A_{420} values increased linearly with time (sometimes after an initial lag) while the reflectance a-values also increased and the L-values decreased. The A_{420} and reflectance data were highly correlated. Slopes and intercepts were considerably more variable for the a vs A_{420} relationship than for the L vs A_{420} relationship, indicating that the a-value was influenced by some characteristic of juice besides the extent of browning. Consequently, the use of L (or Y) rather than the a-value to monitor browning in the juice system is recommended. In a recent study of browning in apple juice, Smith and Cline (1984) obtained a good correlation between the Hunter L-value and visual rankings of the samples. Correlations between visual rankings and values of a, b, a/L, a/b and $\cot^{-1}$ a/ b were not significant.

It is apparent from the data in Table 4 that juices from individual apples of the same cultivar varied greatly in the extent of browning. Therefore, comparisons of multilevel treatments to inhibit browning should be carried out with the juice from one fruit (or the pooled juice from several fruits), apportioned among the treatments and control.

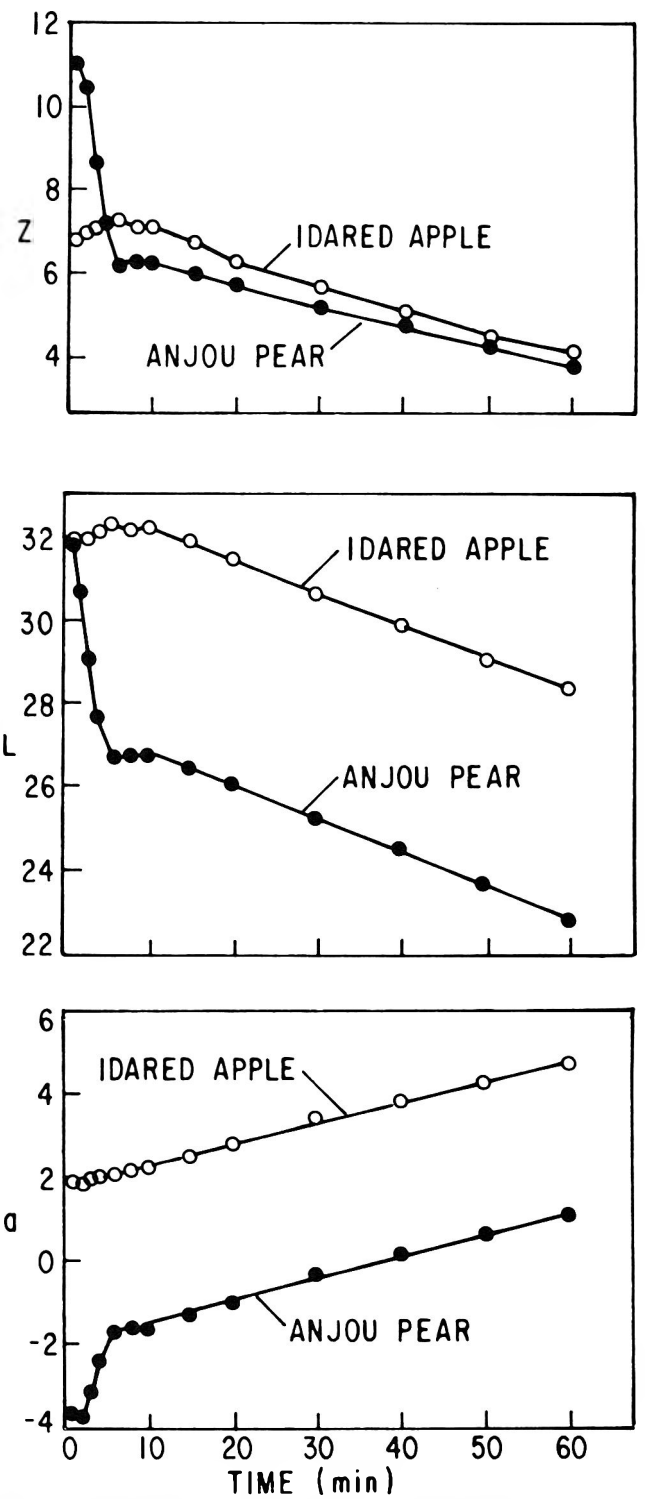

Fig. 2—Reflectance Z-, L- and a-values for apple and pear juice held at 20°C.

Evaluation of browning inhibitors in the juice system

Comparisons of tristimulus reflectance data for untreated apple juice samples with the same juice containing SO_2 or ascorbic acid illustrate the use of the juice system to evaluate browning inhibitors (Table 5). These results were expressed as percent inhibition values, calculated in the same way as for cut surface browning. However, because more time was required to prepare and stabilize samples in the juice system, the initial L- and a-values used as the basis for Δ values were read at 1.5 min for Golden Delicious and at 5 min for Granny Smith

Table 4 – Correlation between reflectance and spectrophotometric measurements of browning in apple juice

Cultivar	Trial	Extent of browning at 30 min: Visual[a]	× L[b]	× a[b]	A_{420}	L vs A_{420}[c]: Slope	Intercept	Correlation coeff.	a vs A_{420}[c]: Slope	Intercept	Correlation coeff.
Cortland	1	Mod	−6.8	2.6	0.168	−37.9	34.8	−0.96	18.5	0.5	0.99
	2	Mod-sev	−7.2	1.1	0.252	−26.2	33.2	−0.97	8.2	4.0	0.94
	3	Mod-sev	−6.9	4.9	0.238	−36.0	36.4	−0.97	15.4	1.7	0.95
	4	Mod-sev	−6.9	3.1	0.287	−21.2	34.1	−0.99	14.1	1.8	0.99
	C.V.[d]		2.5	53.6	21.1	26.2	4.0	–	30.7	72.1	–
Granny Smith	1	Sl	−3.7	1.9	0.109	−33.2	28.2	−0.98	23.4	−3.4	0.99
	2	None	−1.0	0.2	0.028	−33.9	24.8	−0.96	16.1	−1.6	0.97
	3	Mod-sev	−8.2	7.8	0.197	−33.8	33.2	−0.94	18.2	−1.7	0.93
	4	Sl	−0.3	0.3	0.036	−39.9	26.9	−0.99	20.0	−2.7	0.99
	C.V.		108.5	140.6	85.0	8.9	12.7	–	15.9	37.8	–
Idared	1	Mod	−6.3	5.3	0.175	−44.3	37.1	−0.98	34.8	2.9	0.97
	2	Mod-sev	−3.6	−1.2	0.184	−28.9	32.4	−0.99	3.4	5.2	0.99
	3	Mod	−4.3	2.7	0.168	−29.1	29.8	−0.99	17.7	0.1	0.98
	4	Mod	−3.4	−0.3	0.151	−28.0	32.5	−0.98	2.3	7.5	0.96
	C.V.[d]		30.1	182.4	8.2	24.0	9.2	–	104.6	189.0	–

[a] Sl = slight, Mod = moderate, Sev = severe.
[b] Difference between 30 min and 1 min values of L or a.
[c] Slopes, intercepts and correlation coefficients determined by linear regression based on 5 data points per trial.
[d] Coefficient of variation (%).

Table 5—Use of tristimulus colorimetry to evaluate the effectiveness of browning inhibitors in raw apple juice held at 20°C

Cultivar	Treatment[a]	% Inhibition[b]: Calculated from ΔL: 30 min	60 min	90 min	Calculated from Δa: 30 min	60 min	90 min
Golden Delicious	10 ppm AA	6	5	6	14	10	8
	20 ppm AA	23	18	15	19	15	11
	40 ppm AA	36	23	20	35	23	19
	100 ppm SO_2	90	100	104	105	105	105
Granny Smith	10 ppm AA	63	38	35	48	26	30
	20 ppm AA	85	57	43	76	57	44
	40 ppm AA	96	73	50	76	60	45
	100 ppm SO_2	67	92	96	70	90	91

[a] AA = ascorbic acid.
[b] (Δ control - Δ treatment) · 100 ÷ Δ control; Δ values are difference between 30, 60 or 90 min values and initial values (1.5 min for Golden Delicious and 5 min for Granny Smith).

(which took longer to give stable reflectance values) rather than at 1 min, as with the cut surface procedure. No browning was observed in these samples when the initial tristimulus measurements were made. Under the conditions of this experiment, both Granny Smith and Golden Delicious juices underwent severe browning over the course of 30-60 min at 20°C. Color changes were paralleled by decreasing L-values and increasing a-values. Browning in juices of both varieties was almost completely inhibited by the addition of 100 ppm SO_2, resulting in little or no change in L or a. Ascorbic acid at concentrations of 10-40 ppm was less effective in inhibiting browning with Golden Delicious juice than with Granny Smith juice, the percent inhibition increasing with increasing ascorbic acid concentration and decreasing with time.

It is important when using the juice system to employ cultivars that undergo sufficient browning to reveal differences between treatment levels but not so much that all treatments are ineffective. Among the cultivars compared in this study, Granny Smith and Golden Delicious not only meet these criteria but are also widely available for most of the year. Experiments designed to evaluate browning inhibitors should include both an untreated control and a sample treated with sufficient SO_2 to completely inhibit browning. Inclusion of the former will provide a basis for determining the extent to which an experimental treatment inhibits browning, i.e., the percent inhibition. Inclusion of the latter will permit the correction of sample reflectance values for changes unrelated to browning, i.e., the dissipation of air bubbles and development of turbidity during the first few minutes after juice preparation.

CONCLUSIONS

ENZYMATIC BROWNING at cut surfaces of plugs from apple and pear fruits can be monitored by measuring changes in reflectance L and a values. This technique may be used with fruits that are subject to severe browning such as Stayman or Red Delicious apples to evaluate the effectiveness of new browning inhibitors. Because of fruit-to-fruit variability in the extent of enzymatic browning, multilevel treatments with browning inhibitors should be compared using several plugs from the same fruit, half of each plug serving as a control for the treatment applied to the other half. Browning in raw apple juice can be monitored by measuring reflectance L values. If the juice system is used to evaluate the effectiveness of browning inhibitors, a fruit that browns slowly such as Granny Smith apple should be employed.

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Optimization of Apple Juice Production by Single Pass Metallic Membrane Ultrafiltration

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ABSTRACT

Apple puree was treated with various amounts of commercial liquefaction enzyme (0% - 0.066%) for 2 hr at 50°C. To produce clarified juice purees were pumped through a metallic oxide membrane ultrafiltration system, consisting of 3.27 ft^2 (0.304 m^2) of membrane coated internally onto a 1-1/4 inch (i.d.) x 10 ft (3.12 cm x 3.04 m) sintered stainless steel tubing. All enzyme treatments reduced viscosity 70–80% within 1 hr but steady state flux increased with higher concentrations of enzyme. Alcohol insoluble solids (AIS) and total pectins of the apple puree after enzyme treatment decreased as enzyme concentration increased to 0.044%. A large scale system, 1-1/4 inch (i.d.) x 120 ft (3.12 cm x 36.48 m), was operated at 86% juice yield with minimal pressure drops as predicted by a mathematical model.

INTRODUCTION

ULTRAFILTRATION has been used successfully in the clarification of pressed or prefiltered apple juice and other pressed fruit juices (Kirk et al., 1983). Fouling of membranes increases as feed concentration increases, as seen in the permeate fluxes of hollow fiber of thin channel polymeric membrane systems (Breslau and Kilcullen, 1977). Conventional ultrafiltration systems can only be applied to the clarification of pressed or prefiltered juices. However, Thomas et al. (1986) recently described a single pass, metallic membrane ultrafiltration system for producing clarified apple juice directly from apple puree. In that study, the apple puree was treated with both cellulase and pectinase to reduce viscosity, and 5/8 inch (1.56 cm) diameter tubes were used at a pressure drop of 700 psi (4830 kPa) to achieve an 85% yield of apple juice. However, a steady retenate flow could not be maintained at high juice yields. It was suggested that larger diameter tubes should not be as drastically affected by high concentrate viscosity at high juice yields, and high yields could possibly be achieved with lower pressure drops. This study was designed to optimize viscosity reduction of the puree and membrane flux by utilizing a commercial liquefaction enzyme preparation and to evaluate pressure drop at high juice yields with a predictive mathematical model.

MATERIALS & METHODS

RED DELICIOUS APPLES were obtained from a local juicing operation, Carolina Products (Greer, SC). Whole apples were comminuted in a Fitz mill with a 65 mil screen. To reduce viscosity and total pectin, commercial liquefaction enzyme preparations were used. Both Novo Pectinex Ultra-SP-L (Novo Laboratories, Inc., Wilton, CT) and Rohapect 7016 (Rohm Tech, Inc., Malden, MA) were used in this study at concentrations ranging from 0 to 0.066% based on total weight of apple puree. Typically, the treatment was for 2 hr at the recommended optimum temperature of 50°C with constant stirring, except in experiments where optimal treatment time was being determined. Relative viscosity measurements were made with a Brookfield Synchroelectric viscometer at regular intervals during the treatment period.

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The ultrafiltration system used in the optimization of the enzyme treatment consisted of a 1-1/4 inch (i.d.) x 10 ft (3.12 cm x 3.04 m) sintered stainless steel tubing with a food-grade metallic oxide membrane (CI-201-1) formed-in-place within the matrix of the sintered stainless steel tube. The CI-201-1 membranes were manufactured by CARRE, Inc. (Seneca, SC). All enzyme-treated samples were ultrafiltered at 300 psi (2070 kPa) inlet pressure and 50°C. The concentrate flow was controlled by a ball valve, which also served as a pressure control valve.

The ultrafiltration system used in the determination of juice yields consisted of a two-stage single pass. The first stage was 1-1/4 inch (i.d.) x 80 ft (3.12 cm x 24.32 m) of sintered stainless steel tubing followed by a second stage consisting of 1-1/4 inch (i.d.) x 40 ft (3.12 cm x 12.16 m) sintered stainless steel tubing. Both stages contained the CI-201-1 membrane. The concentrate from the first stage was collected and then pumped through the second stage. In both stages the concentrate flow was controlled by a variable speed gear pump, which also served as a pressure control valve. Inlet pressure was controlled by adjusting flow from the pumps with a manually operated by-pass valve (Thomas et al., 1986).

The pumping system for both units consisted of a centrifugal suction booster pump feeding a diaphragm pressurizing pump. Permeate flux measurements were made by the stopwatch and graduated cylinder method and reported as gallons/ft^2 membrane/day (GFD). (1 GFD is equal to 1.698 L/m^2/hr).

Alcohol insoluble solids (AIS) were prepared from enzyme treated and untreated apple puree for each enzyme concentration according to Shewfelt (1965). The samples were dried overnight in a forced-air oven at 50°C and weighed to determine the reduction of AIS by enzyme treatment. Samples were then ground in a Wiley mill with a 60 mesh screen for analysis of total pectin.

Each ground AIS sample was analyzed in triplicate for total pectin by mixing 7.5 mg with 5 mL cold concentrated sulfuric acid in tubes held in an ice water bath until the AIS was well suspended (Ahmed and Labavitch, 1977). Then 1.25 mL deionized water was added dropwise as the mixture was stirred. After 5 min another 1.25 mL deionized water was added dropwise. The AIS was digested for 1 hr in the ice water bath, and aliquots were assayed for uronic acids using the m-hydroxydiphenol method of Kintner and Van Buren (1982).

All graphs were prepared using a cubic regression program, which gave the best fit curve within a 95% confidence limit (SAS, 1985).

RESULTS & DISCUSSION

THE RELATIVE VISCOSITY of apple puree was reduced by 70–80% for all enzyme treatments within approximately 40 min (Fig. 1). Thus, even small amounts of liquefaction enzyme effectively reduced the viscosity of apple puree, but there was a notable difference in the flux achieved with the various enzyme treatments. Steady state flux was observed to increase as the concentrations of enzyme were increased up to 0.044% (Fig. 2), indicating that viscosity reduction could not be used as an indicator of achievable flux.

To determine the possible cause of improved flux with increased enzyme treatment, AIS and total pectin were analyzed. AIS were reduced as enzyme concentration was increased up to 0.044% (Fig. 3). Also, reduction of total pectin was maximal at 0.044% concentration of enzyme (Fig. 4). These results correlate very well with the effect of enzyme concentration on maximum permeate flux, which was achieved with an enzyme concentration of 0.044% (Fig. 2). Kirk et al. (1983) proposed that pectins in fruit juice form a compressible gel layer which

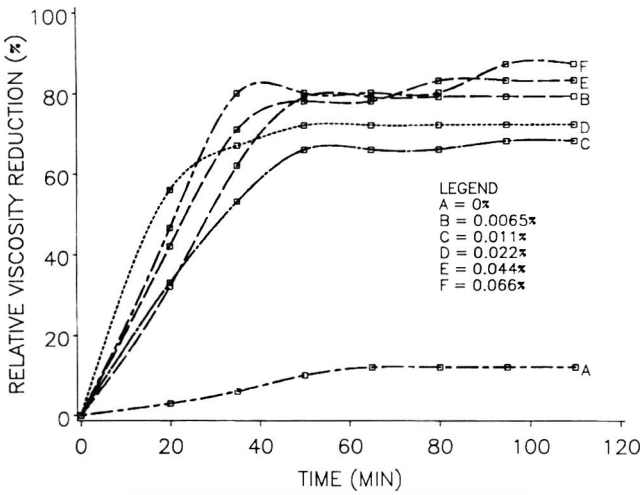

Fig. 1—Relative viscosity reduction of apple puree with increasing amounts of liquefaction enzyme (Novo Ultra-SP-L) at 50°C and constant stirring.

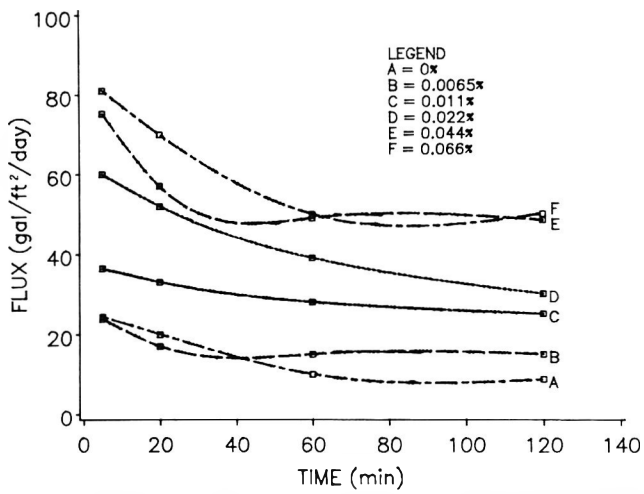

Fig. 2—Effect of enzyme concentration (Novo Ultra-SP-L) on steady state flux of ultrafiltered apple puree. 1 GFD is equal to 1.698 L/m²/hr.

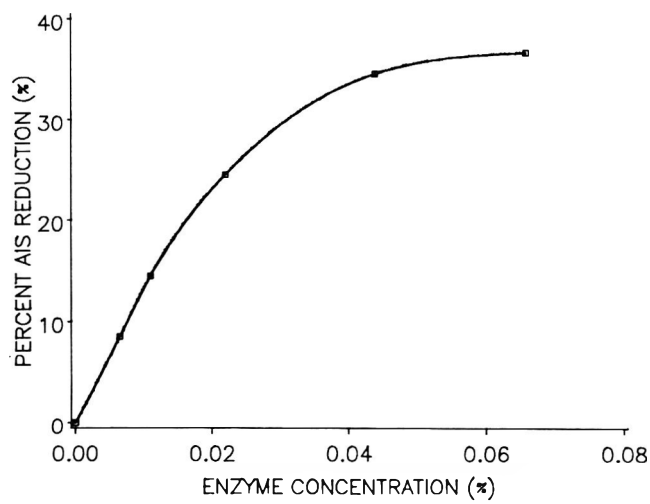

Fig. 3—Percent AIS reduction in apple purees with increasing concentrations of liquefaction enzyme (Novo Ultra-SP-L).

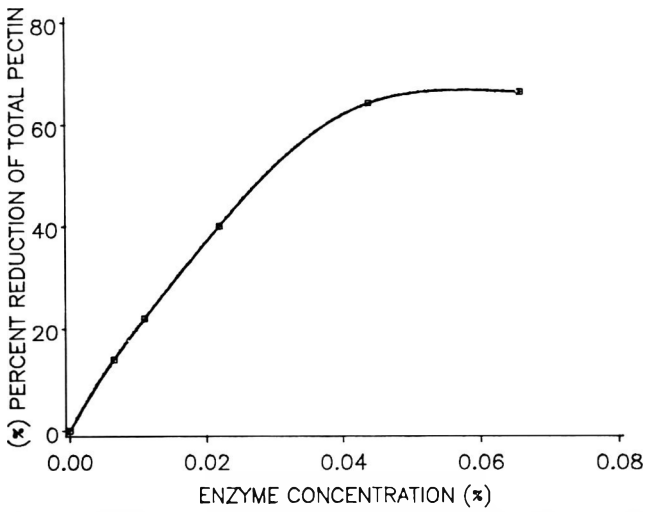

Fig. 4—Percent reduction of total pectin in apple puree with increasing concentrations of liquefaction enzyme (Novo Ultra-SP-L).

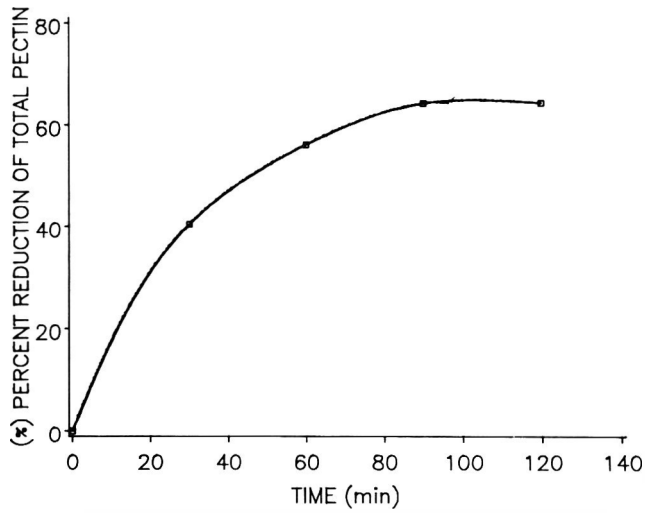

Fig. 5—Optimum time for reduction of total pectin in apple purees using 0.044% liquefaction enzyme. (Novo Ultra-SP-L and Rohapect 7016 gave nearly identical results).

could lead to severe fouling of ultrafiltration membranes. It would appear that permeate flux is directly dependent on the amount of total pectin present in the apple purees. At an enzyme concentration of 0.044%, maximum reduction of pectin was achieved in 60–80 min (Fig. 5). Both commercial enzyme preparations, Novo Ultra - SP-L and Rohapect 7016, gave nearly identical results. Therefore, treatment of apple puree with 0.044% liquefaction enzyme for 1 hr at 50°C was considered optimal to obtain the highest possible permeate flux, as well as maximum reduction of viscosity.

The optimization of any membrane process typically involves the achievement of maximum permeate flux for maximum performance per unit surface area. In this process where high solids, viscous material is ultrafiltered in a single pass operation, maximum reduction of viscosity is also important. As higher yields are achieved in a single pass operation the viscosity of the concentrate increases dramatically and can cause severe pressure drops and operational difficulty (Thomas et al., 1986). The amount of liquefactin enzyme necessary to achieve maximum viscosity reduction was considerably less than that necessary to achieve maximum permeate flux since

the total pectin content must be reduced significantly to prevent membrane fouling.

It was desirable to estimate the degree of importance of viscosity reduction on the operation of the extraction system. The flow is modeled as a non-Newtonian power-law fluid and membrane flux as a linear function of transmembrane pressure. The solvent is juice while the solute a mixture of suspended cell debris and macromolecules. The solute causes the puree to be quite viscous and of shear-thinning nature. It was possibly thixotropic but no systematic observations were recorded. The membrane extraction is modeled as being linear in volume flux versus applied pressure, but in reality the flux is near a limting value established by gel formation. Such gel formation (Blatt et al., 1970; Porter, 1972) has been observed in a number of situations and probably is due, in the case of apple puree, to fragments of pectin.

A volume flow of puree, represented by F, flowing in a tube at an axial location, x, of diameter, D, and a volume flux, J, is being extracted. If the density of juice and puree are the same the decrease in flow is according to:

$$dF = -J\pi D dx \tag{1}$$

The flux is modeled by $AP = J$, where A is a membrane permeance assumed to be constant, and P is the driving pressure.

Apple puree has viscous properties which conform reasonably well to an Ostwald power-law model:

$$\tau = B^n \dot{\theta}^n \tag{2}$$

Here τ is the fluid shear stress, B^n is a pseudo viscosity, and $\dot{\theta}$ is the strain rate oriented appropriately to the shear stress. The exponent n depends on concentration, and has an average value of about 0.3. The pseudo viscosity depends on temperature, enzyme treatment, and solute concentration but not on the motion of the fluid. In most reasonable situations the flow of apple puree will be laminar in nature and the pressure drop will obey an equation (Metzner and Reed, 1955):

$$\frac{dP}{dx} = -\frac{4B^n}{D^{1-n}}\left(\frac{6n+2}{n}\right)^n \bar{u}^n \tag{3}$$

where $\bar{u}$ is the average velocity $4F/\pi D^2$. As juice is extracted, the concentration of solute rises and results in an increase in viscosity and pressure drop. The concentration C is related to its initial value, C_o, and the initial flow, F_o, as:

$$\frac{C_o}{C} = \frac{F}{F^o} \tag{4}$$

The pseudo viscosity is related to the concentration as:

$$B^n = B_o{}^n f\left(\frac{C}{C_o}\right) \tag{5}$$

The function f is frequently modeled as exponentially dependent on concentration.

Elimination of dx from Eq. (1) and (3) results in:

$$B_o^n f u^n dF = \frac{A\pi D^{2+n}}{4}\left(\frac{n}{6n+2}\right)^n P dP$$

Using the relation between velocity and flow and Eq. (4) to eliminate F in favor of concentration results in:

$$\frac{B_o^n f d\left(\frac{C}{C_o}\right)}{(C/C_o)^{n-2}} = -\frac{\pi^{1-n}}{4^{1-n}}\left(\frac{n}{6n+2}\right)^n \frac{D^{2-3n} A P dP}{F_o{}^{1-n}} \tag{6}$$

The left side of this equation depends on the way concentration affects the viscous behavior, on the degree of shear thinning (through n) and on the combined effect of the enzyme preparation on B_o^n, f, and n. The enzyme preparation also affects the value of membrane permeability A but with some independence of the effect on B_o^n.

The designer may choose the value of tube diameter, D, the flow, F_o, and within limits, operating pressure. Because of the gel forming tendencies of the solute there is a maximum value for flux and, hence, inlet pressure. The left hand side of Eq. (6) may be integrated and evaluated at limits for C_e/C_o of 5 or 10, corresponding to juice extraction fractions of 80% or 90%. Then, within a constant, Eq (6) integrates to:

$$-P_e{}^2 + P_o{}^2 = \text{CONST}\,\frac{F_o{}^{1+n}}{A} D^{-2-3n} \tag{7}$$

The constant depends on the pseudo viscosity and concentration effect, f. The designer must limit P_o, and P_e must be positive. The relation between D and F_o becomes:

$$F_o \propto D^{\frac{2+3n}{1+n}} A^{\frac{1}{1+n}} \tag{8}$$

As the diameter increases, the entering flow increases more rapidly. With $n = 0.3$, $F_o \propto D^{2.23}$. Lower permeability systems permit more flow also because the limit in flux occurs at higher pressure and hence permits more pressure drop. The pressure drop becomes a real design factor in high viscosity fluid situations. Perhaps this is best illustrated in writing Eq. (7) in terms of an average flux, $<J>$, and the pressure drop:

$$2<J>(P_o - P_e) = \text{CONST}\quad F_o{}^{1-n} D^{-2-3n}$$

The pressure limit is really a limit on J, hence $<J>$, and $P_o - P_e$ can increase as the permeability decreases.

The most important single result of this analysis in that the design flow in a channel is proportional to the 2 to 2.5 power of tube diameter, other things being equal. Reduction of viscosity in the flow channel is expected to yield greater design flow rates by decreasing the pressure drop. Membranes with lower permeability actually allow greater yield though at the expense of higher pressure operation. As can be seen most clearly through Eq. (6), the viscosity (as B_o^n) has a reciprocal effect on the flow which may be processed. In other terms, the viscosity remains linearly proportional to the system pressure drop.

To illustrate these concepts produced by the model, a 120 ft (36.48 m) single-pass system was constructed of 1-1/4 inch (3.12 cm) diameter tubes. The system was operated in two stages using apple puree enzymatically treated under the optimal conditions described above. The first stage pass was operated at approximately 60% yield. The concentrate was collected and pumped through the second stage to achieve additional recovery of juice. This allowed for a separate analysis of the pressure drop at the end of a normal one stage pass, where the effects of concentrate viscosity are greatest. The first stage pressure drop at 60% recovery was only 50 psi (345 pKa), and in the second stage, pressure drops were higher and increased as overall recovery increased (Table 1). There was large experimental variation in the second stage pressure drop due to

Table 1—Juice recovery versus pressure drop in a two-stage single pass system using 1-1/4 inch (3.12 cm) diameter tubes.

Run	Overall recovery (5)[a]	Pressur e drop 1st stage	Pressure drop 2nd stage
1	75	50psi (345 kPa)	100-200 psi (690-1380 kPa)
2	86	50 psi (345 kPa)	200-300 psi (1380-2070 kPa)

[a] First stage recovery was 60%. Overall recovery represents yield from both stages collectively. Inlet pressure was 300 psi (2070 kPa) for first stage and 400 psi (2760 kPa) for second stage.

the sensitivity of pressure drop to higher concentrate viscosity. Second stage pressure drop constantly fluctuated within the ranges shown in Table 1. However, these pressure drops were approximately 500 psi (3450 kPa) lower than the pressure drops reported with 5/8 inch (1.56 cm) diameter tubes at the same yield values (Thomas et al. 1986), indicating the importance of tube diameter on flow characteristics. The aforementioned model using equivalent average flux for both sizes predicts a reduction in pressure drop from 700 psi (4830 kPa) in 5/8 inch (1.56 cm) tubes to a value of 288 to 120 psi (1987 to 828 kPa) for the larger, 1-1/4 inch (3.12 cm), tubes at the same recovery of 86%, depending on the value of n used (0.3 and 1, respectively). The total pressure drop (sum of both stages in Table 1) was observed to be 250 to 350 psi (1725 to 2415 kPa) and, allowing for errors due to observation, was reasonably substantiated.

Mathematical modeling indicated that good yields can still be achieved with lower permeability membranes as long as viscosity is reduced and the tube diameter is large. This collectively minimizes the pressure drop, although operating pressure will still be higher with lower permeability membranes than with higher permeability membranes. Therefore, reduced fouling is still preferred for ease of operation, as well as for the economic consideration of production with the least possible membrane surface area. Also, it should be considered that an enzyme preparation be developed specifically for the purpose of providing greater activity for reducing membrane fouling in this process rather than primarily for the reduction of viscosity. This would significantly reduce the amount of enzyme needed in the process.

CONCLUSION

THIS STUDY demonstrated that single pass ultrafiltration of apple juice could be achieved with good flux and yield and with minimal pressure drop with the proper enzymatic treatment of the puree and operation with larger diameter tubes. It appears likely that even larger tubes could be utilized for greater volume handling with nearly the same space requirements and with less control requirements. The optimum diameter for tubes does not appear to be a subject completely related to fluid behavior. Other factors of economics associated with space and waste in start up/shut down probably will dominate the selection of tube diameter.

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Ms received 8/18/86; revised 2/13/87; accepted 3/5/87.

Presented at the 46th Annual Meeting of the Institute of Food Technologists, Dallas, TX, June, 1986.

The authors acknowledge the advice and consultations with Dr. H. G. Spencer, Dept. of Chemistry, Dr. Susan Barefoot, Dept. of Food Science, and Dr. C. A. Brandon of CARRE, Inc. Mr. S. B. Brandon and D. N. Mahony of CARRE, Inc. provided assistance in test operation, data evaluation, and evaluation of potential system control requirements.

T. FLAVUS FROM FRUIT JUICE CONC. . .From page 1254

The undissociated form of acid preservatives is the effective form. The pK value for benzoic acid is 4.19 and for sorbic acid is 4.76. At pH 3.5 the amount of undissociated benzoic acid is 83% and for sorbic acid 95%. At pH 5.4 (the pH of MEA) the respective percent of the two undissociated acids are 6 and 19. Thus, at the pH of MEA neither preservative is as effective as at the lower pH of PDA. Because of the higher pK of sorbic acid, it is more effective at the pH of MEA than is benzoic acid.

T. flavus was resistant to ethanol inactivation and to heat and probably to other chemical or physical methods of control except for filtration. The relatively large ascospore size makes it possible to filter them if suitable to the product (King et al., 1969). Prevention of outgrowth by antifungal compounds such as benzoate or sorbate is another method of control.

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pH Dependence of Complex Formation Between Condensed Tannins and Proteins

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ABSTRACT

The interaction between condensed grape tannins and some proteins was studied turbidimetrically. Tannins precipitated protein efficiently at pH values up to the isoelectric point of the individual protein. At slightly higher pH values there was a sharp decrease in complex formation. This "critical pH" was usually observed within approximately 0.5 pH unit of the isoelectric point. A close correlation ($r = 0.96$) was obtained between the isoelectric point and the critical pH of proteins. The effects of pH and ionic strength on turbidity formation by tannins and bovine serum albumin showed that an increasing salt concentration tended to increase turbidity at pH 3, but it had no significant effect at pH 4. In contrast at pH 5 high salt concentration tended to decrease turbidity. Employing an ultrafiltration technique, the binding behavior of interacting tannin and protein was described by a typical Brunauer type II adsorption curve.

INTRODUCTION

TANNIN-PROTEIN INTERACTION phenomena have long been believed to be largely responsible for the formation of hazes in natural beverages (White, 1957), astringency of unripe fruits and various natural beverages (Bate-Smith, 1954, 1973) impaired nutritional quality of some cereals (Butler and Price, 1980) and inactivation of digestive enzymes (Tamir and Alumot, 1969; Milic et al., 1972; Oh and Hoff, 1986).

Binding between tannins and proteins has been largely attributed to multiple hydrogen bond formation between the hydroxyl groups of tannins and the carbonyl groups of the protein peptides (Gustavson, 1954; Cannon, 1955; Loomis and Battaile, 1966; Van Sumere et al., 1975). However, more recent evidence suggests that hydrophobic interactions could be the more important force in the formation of tannin-protein complexes (Oh et al., 1980; Hagerman and Butler, 1980). On the other hand, tannin-protein interaction as a physical process remains poorly understood (McManus et al., 1981). A further understanding of the interaction between tannins and proteins is of considerable practical importance for effective control and removal of hazes in natural beverages such as beer, wine and fruit juice.

It has been shown previously (Oh et al., 1985) that release of proteins from immobilized tannins occurred at pH values close to the isoelectric points of the proteins. In the present study the effect of pH on complex formation between grape tannins (condensed tannins) and a number of proteins in free solution was investigated. It was of particular interest to observe whether differences in the pH effect between individual proteins could be associated with some other characteristics of the proteins. Binding behavior of tannin to bovine serum albumin (BSA) in the formation of soluble complexes was also investigated using an ultrafiltration technique.

MATERIALS & METHODS

Materials

Characterization of the grape tannin preparation used in this study has been previously reported (Oh and Hoff, 1979). It consists of a mixture of procyanidins ranging in apparent molecular weight from 900 to 3000. Protein and enzyme sources were as follows: BSA (Fraction V), ovalbumin, and β-globulin were purchased from Sigma Chemical Co. (St. Louis, MO.). Hemoglobin was obtained from Worthington Biochemical Corporation (Freehold, NJ.), β-lactoglobulin, from ICN Nutritional Biochemicals (Cleveland, OH) and gelatin, from J.T. Baker Chemical Co. (Phillipsburg, NJ), α-amylase from porcine pancreas (2X cryst.), alkaline phosphatase from porcine intestinal mucosa and trypins from procine pancreas (cryst.) were all obtained from Sigma Chemical Co.

Turbidimetry

Proteins, other than enzymes, were dissolved in various buffers (0.1M) at a concentration of 1 mg/mL unless otherwise specified. The following buffers were used at the indicated pH ranges: pH 3.0–7.4, citrate-phosphate; pH 7.5–9.4, barbital; pH 9.5–10.5, sodium carbonate-bicarbonate. Enzymes were dissolved in 0.01M NaCl and mixed with buffer to give a final buffer molarity of 0.05M and a final enzyme concentration of 0.5 mg/mL. Turbidimetric determinations were performed according to Armstrong (1976) as described previously (Oh et al., 1980). Tannin solution (100 μL, 15 mg/mL H_2O) was injected into the sample cuvette (3 mL) and mixed. The resulting turbidity was determined as the absorbance at 450 nm 5 min after mixing at which time it was fully developed. The "critical pH" was defined as the pH at which a marked change in turbidity was observed. For the study of effects of pH, ionic strength and protein concentration on the tannin-protein complex formation, the above tannin solution was injected into a cuvette containing 3 mL BSA solutions at appropriate concentrations, pH values and ionic strengths. Ionic strength was adjusted by adding appropriate amounts of NaCl to 0.05 citrate-phosphate buffers.

Ultrafiltration of tannin-BSA mixtures

Tannins and BSA were dissolved separately in 0.1M phosphate buffer, pH 6.0 both at 1 mg/mL and mixed at various ratios (v/v) of tannin to BSA ranging from 1:7 to 1:0.33. Each mixture (4 mL) was placed in a Centriflo membrane (Type CF-25, m.w. cutoff: 25,000, Amicon Corp., Danvers, MA.) and centrifuged for 2 min at 1,000 x g. Control tannin solutions were run in the same manner in the absence of BSA. Tannin in the ultrafiltrate (0.5 mL) was determined using the Folin-Ciocalteau method as modified by Singleton and Rossi (1965). A standard curve was prepared using catechin as a standard.

RESULTS & DISCUSSION

PRECIPITATION OF PROTEINS in the presence of tannins can reasonably be expected to be a two-step reaction, whereby the first step involves the binding of the tannin molecules to the protein, and the second step is an aggregation of the initial tannin-protein complexes into larger units, which then precipitate. The extent to which the second step takes place should thus depend on the ability of the cross-linking tannin-protein bonds in overcoming repulsive electrostatic forces that might exist between the protein molecules. Such repulsive forces are presumably at a minimum at the isoelectric point of the protein, which in part explains the generally observed minimum solubility of a protein at this point. It should, according to this reasoning, be expected that precipitation of proteins by tannins will be particularly pronounced in the vicinity of the isoelectric point. Hagerman and Butler (1978) reported that maximum amounts of tannin were precipitated within one pH unit of the isoelectric point of the protein. The tendency towards aggregation and formation of precipitable complexes will become

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less evident as the protein gains either positive charges at pH values lower than the isoelectric point or negative charges at higher pH values. Minor conformational changes resulting from pH or ionic strength perturbations would seem likely to affect the number and type of binding sites available to the tannin, and thereby modulate the basic theme of minimum ionic repulsion as the dominating factor in the flocculation.

Few systematic studies on the pH dependence of tannin-protein interactions involving several proteins have been published. The behavior of a system consisting of proteins and sorghum tannins (Hagerman and Butler, 1978) is in general accord with expectations. The observations by Armstrong (1976) and Oh et al. (1985) that proteins trapped by immobilized condensed tannins were released when the pH was raised above their respective isoelectric points also tends to support the concept described above.

In the present work a series of enzymes and other proteins were allowed to interact with condensed tannins at pH values from 3 to 10 (Fig. 1) and the resulting turbidity measured (see Methods). The extent of turbidity was taken to be proportional to the extent of tannin-protein complex formation. Preliminary experiments had shown that turbidity was proportional to tannin concentration. It was in general found that beyond a certain pH, specific for each protein, the degree of complex formation abruptly decreased (Fig. 1). This pH, here referred to as the "critical pH," was usually observed within 0.5 pH unit of the isoelectric point of individual proteins and digestive enzymes. Gustavson (1956) found that condensed tannins were bound to polypeptides at any pH below 7-8, while the binding decreased sharply above pH 8. The present work indicated that this was generally true with the exception of trypsin, which formed complexes with tannin up to pH 9.9. Similarly, Hagerman and Butler (1978) also observed that lysozyme with an isoelectric point of 11.0 bound to sorghum tannin up to that pH.

Loomis and Battaile (1966) speculated that the binding of condensed tannins with proteins involved phenolic hydroxyl groups of tannins. The sharp drop in the formation of tannin-protein complexes was attributed by these workers to the ionization of phenolic groups (phenolate formation) at pH values greater than 9, making fewer hydroxyl groups available for hydrogen bonding with the peptide linkages of protein. However, most critical pH values observed in this study were far below the average pKa (9.9) of tannins. Since condensed tannins do not contain negatively charged groups at pH values below the pKa of the phenolic groups, they cannot contribute to electrostatic repulsion in the vicinity of the majority of the critical pHs reported here. The sharp drop in complex formation at the critical pH can be more reasonably related to a decrease in the over-all electrostatic charge of the protein in the vicinity of the isoelectric point (Tanford, 1961). Hagerman and Butler (1981) reported that when the protein conformation has the more open conformation, protein binds tannin more strongly. In contrast to hydrolyzable tannins, charged groups are absent in the condensed tannins (Haslam, 1966). The effect is therefore more likely attributable to a minor conformational perturbation of the protein rather than an effect of pH on the tannins, thereby, affecting the number and types of binding sites available to tannins (Oh et al., 1985). A close correlation ($r = 0.96$) was obtained between isoelectric point and the critical pH of proteins (Fig. 2). The overall results obtained from tannin-protein interactions in free solution parallel those obtained from immobilized tannins (Oh et al., 1985).

To see whether the critical pH values (Fig. 1) are valid under different reaction conditions for a specific protein, BSA, the effects of varying pH, ionic strength and protein concentration on the extent of tannin-protein complex formation were investigated. The results showed that, at pH 3, the extent of complex formation (turbidity) increased at high ionic strength (Fig. 3). However, at this pH the salt effect decreased with an increase in protein concentration. It is reasoned that high ionic strength at relatively low protein concentrations effectively shielded the

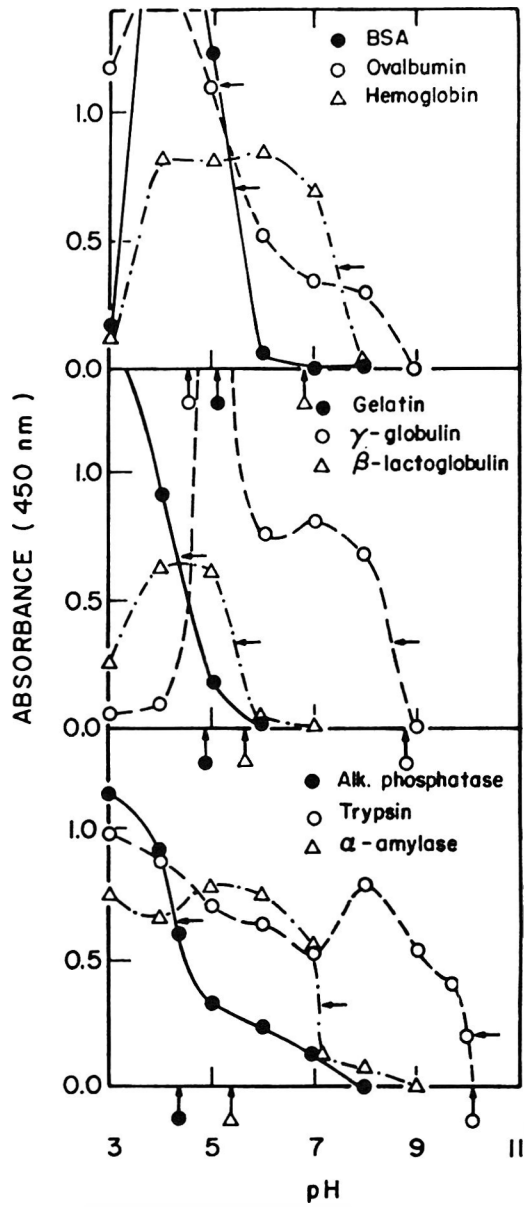

Fig. 1—pH dependence of complex formation between tannins and proteins and digestive enzymes. The extent of complex formation was determined turbidimetrically by monitoring change in absorbance at 450 nm for 5 min after injection of tannin into the protein solution. (See Materials & Methods for details.) Horizontal arrows indicate the critical pH of each protein. Vertical arrows on x-axis indicate the pI of the proteins.

electrostatic repulsion between protein molecules, thereby, increasing other interacting forces involved in tannin-protein complex formation. Previously, hydrophobic forces were shown to be importantly involved in tannin-protein complex formation (Oh et al., 1980). The effectiveness of the protein charge shielding by salt progressively decreased with increasing protein concentration. At pH 4.0 where maximum tannin-protein interaction was observed, ionic strength had no significant effect on the turbidity regardless of the protein concentration, although the extent of turbidity increased somewhat with higher protein concentrations. Maximal interaction between condensed tannins and BSA occurred at pH 4 (Fig. 1; McManus et al., 1981). In contrast, at pH 5.0 which is in the vicinity of the observed critical pH of BSA (Fig. 1), increasing salt concentration tended to decrease the turbidity with the effect becoming more pronounced with an increase in protein concentration. At pH 6 very little tannin-protein complex formation occurred regardless of ionic strength and protein con-

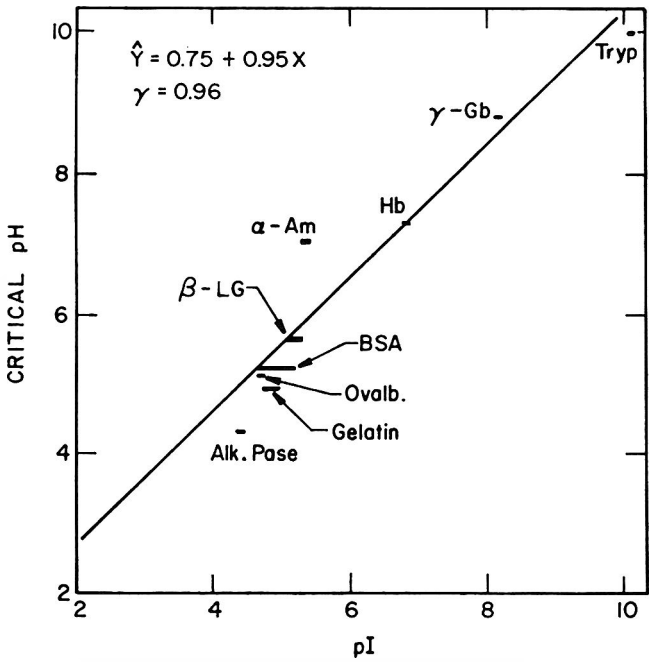

Fig. 2—Correlation between isoelectric points and critical pH values of proteins and digestive enzymes. Tryp, trypsin; β-Gb, β-globulin; Hb, hemoglobin; α-Am, α-amylase; β-LG, β-lactoglobulin; BSA, bovine serum albumin; Oval, ovalbumin; Alk. Pase, alkaline phosphatase.

centration. The results demonstrated that the extent of the interaction of condensed grape tannins with BSA depended on the relative amounts of tannins and proteins, as well as on pH and salt concentration. The results also suggest that the critical pH values observed in Fig. 1 are valid under different reaction conditions, although the extent of tannin-protein complex formation can be influenced by various factors such as pH, ionic strength and relative concentration of tannins and proteins as well as their absolute concentrations (Calderon et al., 1968). The non-stoichiometric increase in turbidity with increased protein concentration (Fig. 3) could be in part due to formation of soluble tannin-protein complexes.

Formation of soluble complexes between tannic acid and gelatin was first reported by Calderon et al. (1968) and their existence was further demonstrated by gel filtration (Van Buren and Robinson, 1969). Whether condensed tannins also form soluble complexes with BSA was investigated by means of an ultrafiltration technique.

When the amount of bound tannin was plotted against that of free tannin, a typical Brunauer Type II adsorption curve (Adamson, 1960) was obtained (Fig. 4). The initial portion of the curve at low levels of free tannin, in analogy with gas-solid or solute-solid adsorption phenomena, is likely to be represented by the attachment of tannin molecules to binding sites on the protein surface to form a monolayer of adsorbate. But the analogy with gas-solid adsorption cannot be applied to the curve at higher levels of free tannin since a capillary pore condensation process is clearly excluded. It seems more likely that in this portion of the curve the protein conformation becomes increasingly perturbed by the attached tannin, a process which results in exposure of additional binding sites from the interior of the protein molecules. This interpretation is supported by our observations of an increased digestibility of BSA by trypsin when tannins are present (Oh and Hoff, 1986).

Determination of binding sites or binding capacity and of binding constants (affinity between tannin and protein) would be useful in characterizing tannin-protein interactions (Van Buren and Robinson, 1969). These two parameters can be conveniently estimated by treating the results obtained from ultrafiltration with the Langmuir isotherm equation $T_f/T_b = 1/b.n +$

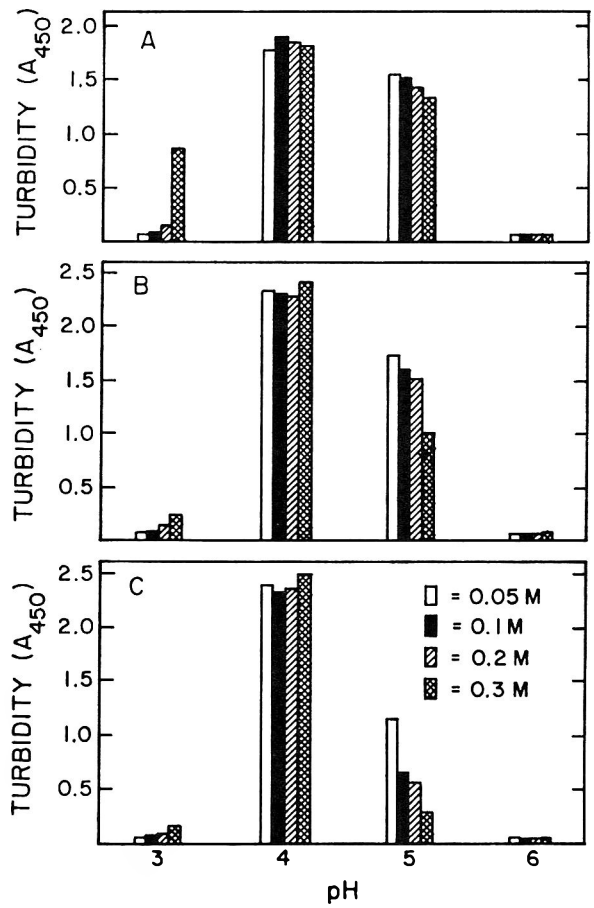

Fig. 3—Effects of variation in pH, ionic strength and protein concentration on complex formation between tannins and BSA. (A) BSA concentration: 1 mg/mL. (b) BSA concentration: 2 mg/mL. (C) BSA concentration: 3 mg/mL. Tannin solution (100 μL, 15 mg/mL H_2O) was injected into a cuvette containing 3 mL of BSA solution and mixed. (See Materials & Methods for details.)

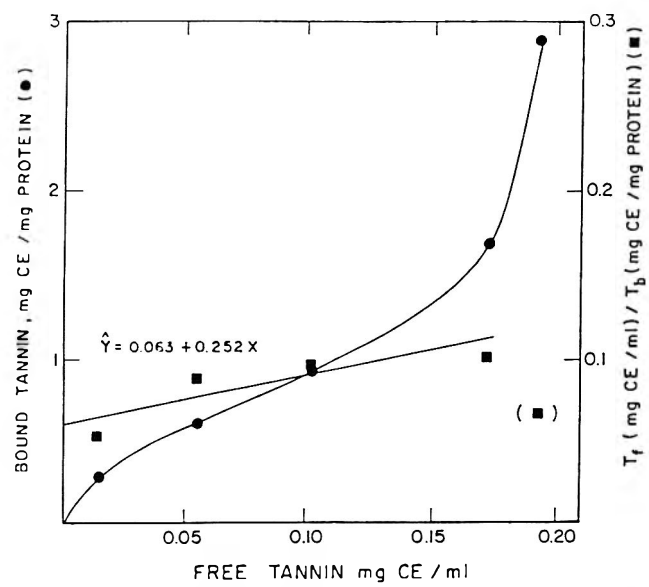

Fig. 4—BET adsorption isotherm of tannin on BSA at pH 6. Details are described in text. T_f: free tannin, T_b: bound tannin, CE: catechin equivalent. The data point indicated in parenthesis was excluded from computation of the equation.

T_f/n, where T_f = free tannin, T_b = bound tannin, n = binding capacity, and b = association constant. A plot of T_f/T_b vs T_f

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Ethylene Production in Staling Beer

PATRICIA A. LYNCH and C. W. SEO

ABSTRACT

Levels of ethylene in the headspace of three brands of staling lager beers which were allowed to stale at 40°C increased with storage time. TBA values and browning of staling beers also increased with storage time and coincided with increase in ethylene production. Results indicated that measurement of ethylene in the headspace of beer might be useful to follow beer staling.

INTRODUCTION

The development of stale flavor in beer during storage has been a major problem in the brewing industry. Studies indicated that stale flavor was attributed to aldehyde formation in beer (Hasimoto and Kuroiwa, 1975; Hasimoto and Eshima, 1977; Chang et al., 1970; Meilgaard, 1972; Visser and Lindsay, 1971). Schmitt and Hoff (1979) reported that acetaldehyde concentration in beer was correlated with mean panel scores for stale flavor. Aldehydes are also good precursors of hydrocarbons in free radical generating systems. Lieberman and Kunishi (1967) showed that propanal was a good precursor of ethylene in the copper-ascorbate catalyzed model system. Seo (1976) also demonstrated that methane and ethane were produced from ethanal and pentane from hexanal in the Fe (II)-EDTA and hydrogen peroxide catalyzing system. From these facts, it was assumed that the short-chain hydrocarbons such as methane, ethylene and ethane would be formed in staling beer.

Grigsby and Palamand (1976) reported that TBA (thiobarbituric acid) values were correlated with the extent of beer staling. Zenz (1973) indicated that 2-methyl-2-butene was a typical selective and sensitive indicator of light-spoilage of beer and isoprene production was an index of both light and heat-spoilage of beer.

The purpose of this study was to evaluate ethylene production in relation to browning and TBA values in beer stored at 40°C.

MATERIALS & METHODS

Preparation of samples

Three popular brands of lager beer were obtained from the local market. The beers were chilled overnight in a 0°C bath. The following morning the beers were carefully measured under sterile conditions with a 50 mL graduated cylinder and gently poured into 100 mL standard serum bottles. The bottles were sealed with silicone rubber septa which were covered with the inert self-adhesive teflon tape. The sample container system was adapted from Seo and Joel (1980). The samples were then stored in an incubator at 40°C.

Gas chromatography

At the time of analysis, samples were removed from the incubator. A 4-mL sample of the headspace gas was taken from each bottle by a gas-tight syringe and injected into the gas chromatograph equipped with a flame ionization detector. The gas chromatograph (Hewlett-Packard Model 5830A, Palo Alto, CA) was operated under the following conditions: column, 182.9 cm (6 ft) aluminum, 0.32 cm (1/8 in.) diam; column packing materials, 60/80 activated alumina (Coast Engineering Laboratory, Redondo Beach, CA); or Chromosorb 102, 60/80 (Supelco, Inc., Bellefonte, PA); carrier gas, nitrogen at flow rate of 30 mL/min, hydrogen, 30 mL/min and air, 240 mL/min; both injection port and detector port temperature, 200°C; chart speed, 1.27 cm (0.5 in.)/ min.

The activated alumina column used in this study is known to absorb irreversibly all polar oxygen containing compounds allowing nonpolar compounds such as hydrocarbons to pass (List et al., 1965). However, the Chromosorb 102 column is recommended for the analysis of permanent gases, alcohols and other oxygen containing compounds. The chromosorb 102 column was used to support the data obtained from the activated alumina column.

The Finnigan 3300 gas chromatograph-mass spectrometer (GC-MS) equipped with the Incos datasystem was used to identify ethylene obtained from the beer samples. The gas chromatographic conditions were the same as described for the 5830A gas chromatograph and the transfer line/jet separator was run at room temperature. The MS mode was electron impact (EI) at 70 eV. In order to increase sensitivity a selected ion monitoring (SIM) technique was employed. The m/z 26 ($C_2H_2^+$) was monitored which is fairly unique to ethylene.

Brown pigment

The nonenzymatic browning reaction was followed by measuring the absorbance of the product at the same time headspace gases were taken. The samples were filtered through Millipore 0.45 micron filters. The absorbance was measured at 430 nm by a Bausch and Lomb 210A spectrophotometer. The results were expressed in absorbance of sample. Absorbance data were based on three sample analyses.

pH Analysis

The pH was measured on each sample cooled to 20°C by a Fisher Accument 230A pH meter.

TBA test

For the TBA test, duplicate beer samples were mixed with 5 mL 0.02M 2-thiobarbituric acid solution and heated in a water bath for 35 min. The absorbance was measured at 535 nm by a Bausch and Lomb 210A recording spectrophotometer. This method was adapted from the procedures of Grigsby and Palamand (1976) and Tarladgis et al. (1960).

Statistical analysis

Analysis of variance and Duncan's multiple range test were used to analyze the data for significant differences between means (Steel and Torrie, 1980).

RESULTS & DISCUSSION

ETHYLENE was the major short-chain hydrocarbon produced from beer stored at 40°C. The ethylene production increased signficantly with increased storage times, rising rapidly initially and leveling off thereafter (Fig. 1).

Of the three brands studied, Brand A consistently gave the highest amount of ethylene followed by Brand B and Brand C, respectively. The means of ethylene production and browning for the three brands over the entire storage time were compared statistically. The ethylene production and browning were significantly different between brands (Table. 1) Ethylene was also indentified by the retention time data obtained by GC with

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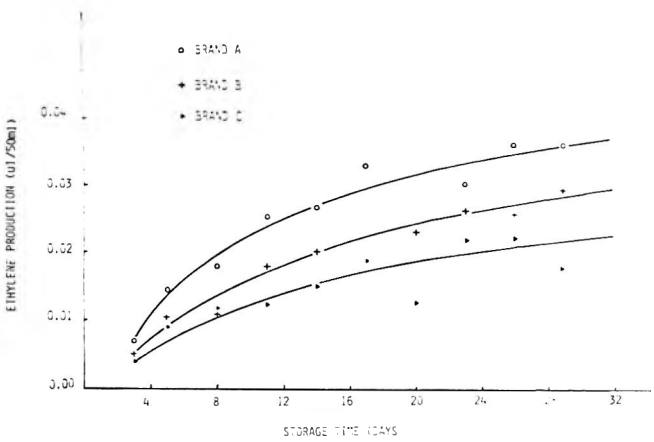

Fig. 1—Ethylene production in staling beer during storage.

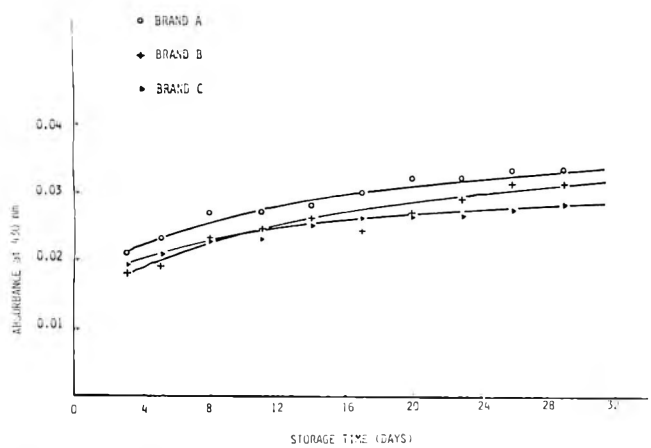

Fig. 3.—Browning of staling beer during storage.

Table 1—Mean ethylene production and browning for each brand of beer

Brand	Ethylene production (µL/50 mL)	Browning (Absorbence at 430 nm)
A	0.026[a]	0.286[a]
B	0.017[b]	0.253[b]
C	0.015[c]	0.246[c]

[a,c] Means with different superscripts are significantly different ($P<0.01$)

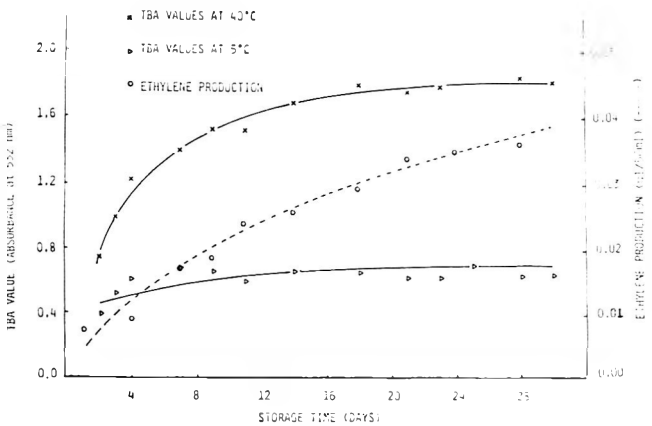

Fig. 2—TBA value (absorbance at 532 nm) of beer samples stored at 40°C and 5°C and ethylene production in staling beer during storage.

the Supelco Chromosorb 102 column. Regarding the identity of ethylene, the GC-MS data of the m/z 26 chromatogram further confirmed the presence of ethylene in the beer headspace gas. Another set of beer samples from a different batch (Brand A) was stored at 40°C, and ethylene determination and TBA tests were conducted at the same time. The TBA values increased with increased storage time and with increased ethylene production from the beer (Fig. 2). The beer samples stored at 5°C showed much lower TBA values than the abused beer at 40°C and also showed no significant increase in the TBA values. Apparently, storing beer at 40°C caused the staling. As cited in the introduction, Grigsby and Palamand (1976) reported that TBA values were correlated with the extent of beer staling.

Browning formation increased as the storage time increased. Brand A gave the highest level of browning throughout the storage period (Fig. 3). In the later stage of storage, the browning production was parallel to ethylene production. The brand that gave the highest amount of ethylene also had the most browning and the brand which yielded lowest ethylene gave the least browning. Color development is known to be a manifestation of beer oxidation (Grigsby et al., 1974). After 8 days of storage, color development paralleled ethylene production. Ethylene production in the later stage of beer oxidation might be related to color development. However, Grigsby et al. (1974) indicated that it was an indication of only a part of the entire staling process.

Regarding the origin of ethylene in staling beer, three pathways were speculated based on data published by several workers: (1) Ethylene could originate from the breakdown of aldehydes which have been reported as good precursors for hydrocarbon formation. As cited earlier, Lieberman and Kunishi (1967) demonstrated that propanal, a breakdown product of peroxidized linolenate, was a very effective precursor of ethylene in the copper-ascorbate catalyzed model system. Seo (1976) also reported that propanal produced ethylene in a model system catalyzed by Fe (II)-EDTA. Aldehydes could be formed by many different reactions. Hashimoto and Kuroiwa (1975) presented evidence that volatile aldehydes in bottled beer came from oxidation of higher alcohols by melanoidins, oxidative degradation of isohumulones, Strecker degredation of amino acids and autoxidation unsaturated fatty acids. (2) Ethylene could be formed from the oxidative degradation of polyunsaturated fatty acids. Ethylene was the major hydrocarbon from the oxidation of linolenic acid catalyzed by cupric-ascorbic acid system (Lieberman and Mapson, 1964). (3) Amino acids might be the precursors of ethylene. Methionine could be broken down to ethylene in the presence of a free radical generating system. Lieberman et al. (1965) reported that ethylene was formed from methionine degradation catalyzed by copper and ascorbate in a phosphate buffer. Ethylene was derived from the $—^{4}CH_2—^{3}CH_2—$carbon skeleton of methionine. Visser and Lindsay (1971) reported the presence of methional in staling beer which was possibly produced from methionine through Strecker degradation. Methional, a sulfur containing aldehyde, was identified as a compound which could contribute to the overall flavor deterioration of beer. All three types of reactions may occur simultaneously in beer, but it is difficult to speculate which of the three reactions is more predominant.

The pH of each brand was different. Brand A was 4.11, 4.50 for Brand B and 4.15 for Brand C, respectively. There was no change in pH during storage. Grigsby et al. (1974) reported that by raising the pH, the rate of formation of certain staling products by esterification and by dehydration which were indicative of certain aspects of staling was diminished. In this study no relationship between ethylene formation or color development and pH of the beer was indicated.

Aldehyde determination has proven to be a reliable method in following beer staling (Schmitt and Hoff, 1979). However, aldehydes may undergo secondary reactions such as carbonyl-amine type or polymerization and disappear during storage through these reactions. Ethylene is more stable and is easily determined. It was concluded from these facts that ethylene

determination might be a useful technique to follow beer staling.

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Ms received 11/3/84; revised 12/26/85; accepted 8/11/87.

Acknowledgement is given to the assistance of Dr. Fields C. Gunsett, Associate professor in Animal Science for the statistical treatment of data.

Presented at the 44th Annual Meeting of the Institute of Food Technologists, Anaheim, CA, June 10-13, 1984. This study was supported by CSRS Research Grant NCX-027-5-80-170-1 and NCX-068-5-84-170-1 from the US Dept. of Agriculture.

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should, in theory, give a straight line of slope 1/n and intercept, 1/b·n which can be used to estimate n (slope) and b (intercept at $T_f = 0$). The present study (Fig. 4) gave a value of 4.3 mg C.E./mg BSA for n, and 3.7 (mg/mL)$^{-1}$ for b. The rather low value for the association constant suggested that the association of condensed grape tannins with BSA was relatively weak in soluble complexes.

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Ms received 12/15/86; revised 3/14/87; accepted 3/30/87.

Journal paper no. 10,962 of the Purdue Agricultural Experiment Station.

Analysis of Judge Performance in Wine-Quality Evaluations

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ABSTRACT

The use of correlation coefficients, F-statistics and LSDs was described for measuring judge performance in terms of agreement, reliability, discrimination, stability, and variability. The technique was applied to a number of wine-quality evaluation experiments. It was shown that a single analysis of variance of all judges' scores in an experiment will often be inappropriate. Further, it was demonstrated that judges' performances varied over time. It was, therefore, recommended that each judge's performance be monitored continually and that when judges were unreliable and nondiscriminating, they would be ignored in drawing conclusions from the experiment. The analysis for wine differences should be based on a separate analysis of each set of homogeneous judges as determined from the measures for agreement, reliability, and variability.

INTRODUCTION

DIFFERENCES IN QUALITY between wines, foods and perfumes are often evaluated by sensory means whereby a score is assigned to a sample of a product presented to several "experts." In the wine industry this method is widely used for evaluating wines. When such data are analyzed statistically, the whole set of scores is subjected to an analysis of variance that will include the examination of product differences, judge differences and product-judge interactions. Such analyses rely on a number of assumptions, the most important being homogeneity of variance and correlations (Box, 1954; Huynh and Feldt, 1980) and absence of certain interactions. This implies that the judges exhibit a high degree of homogeneity in their scoring. However, it is well known that individuals vary, for many reasons, in their evaluations of food and beverages (Amerine et al., 1965). Such variations occur irrespective of whether the evaluations are based on ranks (Weiling and Schoeffling, 1976), composite scores (Ough and Winton, 1976) or score cards incorporating graphic scales (Kwan and Kowalski, 1980).

To overcome these variations emphasis has been placed on screening and training judges prior to their participation in taste panels (Amerine et al., 1965; Amerine and Roessler, 1976). These authors point out that the proposed screening methods are not completely satisfactory since (1) a judge's performance varies in time and with the type and quality of the product, (2) tests of taste acuity, often used to screen judges, do not necessarily correlate with subsequent more complex evaluations of quality sensations, and (3) judge selection tests are usually impractical and impolitic. Whether or not training and screening are employed, the individuals in a panel for a particular tasting will almost certainly display some heterogeneity in their evaluations. This makes it desirable to monitor judges' performances *in each sensory evaluation*.

Previous authors (Hopkins, 1946; Overman and Li, 1948; Moser et al., 1950; Giradot et al., 1952; Bennett et al., 1956; ASTM, 1968; Cross et al., 1978; Hovenden et al., 1979; Malek et al., 1986) have utilised standard deviations (SD), correlations (r) and F-statistics (F) calculated separately for each judge to measure judge performance in a single sensory evaluation experiment. Generally, they have calculated only one such statistic. This has the disadvantage of measuring only a single aspect of judge performance. In order to improve the conclusions drawn from sensory experiments, a method of comprehensively measuring judge performance by using novel and traditional statistical methods based on the statistics SD, r and F was proposed in this study. This method was used to analyse the data from several wine evaluation experiments and show that each statistic measured a different aspect of judge performance. Also, this study was designed to demonstrate that these methods can be used to check assumptions to be met for analyses of variance for mean differences and that the analysis of variance appropriate for a particular experiment will depend on the results of these checks.

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MATERIALS & METHODS

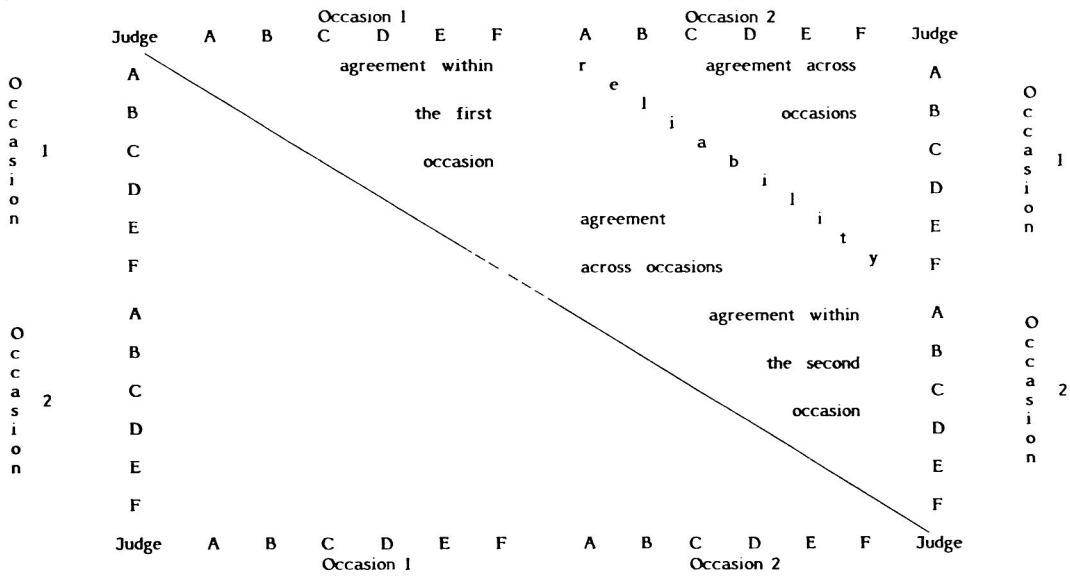
Experiment 1

Baker and Amerine (1953) describe an experiment in which 13 Cabernet Sauvignon and 17 Riesling wines were scored by five judges who were "highly trained" and "had extensive acquaintance with both Cabernet and Riesling wines." The wines were commercial Californian wines that "covered a cross section of the best and worst wines of these types." The judges were told the type of wine and asked to score the wine using score sheets based on scoring 10 aspects of a wine and summing these to a form a single composite score, the maximum value of which is 100. The judge had 4 or 5 wines to score at a session; however, the allocation of wines to sessions is not reported by Baker and Amerine (1953) and so is ignored here.

Experiments 2-5

A series of four previously published experiments concerned with the effects of viticultural treatments on wine quality were to be tested. There were gaps of one year between each of the first three experiments and two years between the third and fourth. In each experiment, viticultural treatments were applied to plots; the grapes of each plot were made into a separate lot of wine by small-scale winemaking techniques (Antcliff and Kerridge, 1976). The wines were then evaluated by at least six experienced wine-makers who used the judging system used for Australian Wine Shows (Rankine, 1974), with which they were familiar. In this system, a single composite score is assigned to each wine within the range 0 to 20 (the maximum being composed of three points for color, seven for aroma and ten for taste characteristics). Every sensory experiment was a *two-occasion* experiment, i.e. all the wines were presented to the judges on each of two occasions, the second occasion differing from the first only in the order of presentation of the wines. Each occasion consisted of several *sessions*, at each of which 8 or 12 wines were presented for simultaneous evaluation. Wines were assigned to sessions so that all treatments were equally replicated at a session.

Experiment 2—In this experiment (May, 1977), four lots of Shiraz grapes were obtained by dividing one row of vines into four parts and harvesting each part by a different method. After crushing, each lot was divided into three portions and the resultant twelve lots of must were made into wine. Two bottles from each of the three winemaking replicates of each of the four field treatments, 24 bottles in all, were used for sensory evaluation. Six judges were asked to evaluate the



wine in each bottle twice on the same day (occasions one and two). Within each occasion, two sessions were held: in each, one of two bottles of each of the field-treatment × winemaking-replicate combinations was presented.

Experiment 3—In this experiment (May, 1977), Shiraz grapes were harvested from 8 plots arranged in the field in two blocks of four. Four harvesting treatments had been randomly assigned to the plots in each block. Each of the eight lots of grapes were crushed and divided into six portions and each portion was made into wine. Sensory evaluation of the wine was performed by six judges. At a first occasion, the 48 winemaking replicates were evaluated in six sessions and, at each session, eight glasses were presented that contained the wines from one winemaking replicate of each field replicate of each treatment. This process was repeated with the same 48 wines at the second occasion on the day after the first.

Experiment 4—In this experiment (May et al., 1976), Crouchen grapes were harvested from 16 plots, four plots coming from each of four blocks. Four trellising-pruning treatments had been randomly assigned to the plots in each block. Each of the 16 lots of grapes were crushed and divided into three portions and each portion made into wine. Sensory evaluation of the wine was performed by six judges. On an occasion the 48 winemaking replicates were evaluated in six sessions; at each session eight glasses were presented that contained wine from one winemaking replicate of the same two blocks for all treatments. This process was repeated with the same 48 wines on a second occasion on the day after the first.

Experiment 5—In this experiment (Hale and Brien, 1978), Shiraz grapes were harvested from 12 half-plots, there being two half-plots in the two plots from each of three blocks. To avoid irrelevant complications, the treatments applied in this experiment will be ignored. Each of the 12 lots of grapes were crushed and divided into two portions and each portion made into wine. Sensory evaluation of the wine was performed by eight judges. The 24 winemaking replicates were evaluated in six sessions which were held over one day; at each session eight glasses of wine were presented that contained the eight wines from a block. The eight wines were presented first in clear glasses and at the succeeding session in black glasses to eliminate the effect of color on the evaluation of the wines.

Statistical analysis

The performance of judges was assessed in terms of: (a) agreement, the consistency in scoring between judges; (b) reliability, the ability of the judge to reproduce score differences between samples; (c) discrimination, the ability of the judge to separate wines of differing quality; (d) stability, the lack of change in the overall level of scoring of a judge from one occasion to the next; and (e) variability, the spread in the scores assigned by a judge in making multiple evaluations of sets of wine.

Measuring agreement and reliability—These are measured using correlation matrices calculated from the scores of several judges (multijudge correlation matrices) or the scores of several judges on several occasions [multioccasion-multijudge correlation matrices (Campbell and Fiske, 1959). Their general form is given in Table 1. Clearly, multioccasion-multijudge correlation matrices consist of three classes of correlations, viz. Class 1: BETWEEN JUDGES WITHIN OCCASIONS – correlations between two judges' scores for the same set of wines given on the same occasion (= AGREEMENT); Class 2: BETWEEN OCCASIONS WITHIN JUDGES – correlations between a judge's scores on one occasion and those for the same set of wines on subsequent occasion(s) (= RELIABILITY); Class 3: BETWEEN OCCASIONS AND JUDGES – correlations between two judges' scores for the same set of wines given on different occasions (= ACROSS OCCASION AGREEMENT).

It is desirable that the elements of multioccasion-multijudge correlation matrices exhibit patterns similar to those already stipulated by Campbell and Fiske (1959). Firstly, the reliability correlations (class 2) should, in each case, be significantly greater than zero and sufficiently large if the judge is to be considered reliable. Secondly, the scores of a particular judge should result in significantly greater reliability correlations than of agreement (class 1) or across occasion agreement (class 3) correlations. That is, judges should correlate more highly with themselves than with others. Thirdly, the magnitude of the agreement and across occasion agreement correlations between the same two judges should be approximately equal as this implies independence of the occasions involved. Finally, the agreement and across occasion agreement correlations should not differ significantly and should be sufficiently large for the judges to be considered in agreement. This last condition also specifies the pattern desirable for multijudge correlation matrices.

The methods of Brien et al. (1984) and Brien et al. (1986) are used for analysing these matrices to test for the equality of certain of their elements; the methods consist of analysis of variance performed on the well-known Fisher z-transforms $\{z = 0.5 \ln[(1+r)/(1-r)]\}$ of the elements of a correlation matrix.

Measuring discrimination, stability, and variability—These are measured using statistics obtained for each judge from the analysis of variance of his wine scores. The analysis of variance for an individual judge is derived from the experimental structure as described by Brien (1983). The formulae for the experimental structures reflect the design of the experiment which will consist of production and sensory evaluation phases. The first tier derives from the sensory evaluation phase and the remaining tiers from the production phase. Thus, the first tier is determined by the procedure of the evaluation taking into account that the wines were evaluated on two occasions or in two types of glass, at several sessions within each occasion or type and in several glasses within each session. The second tier, and often a third tier, are determined by the procedures employed in the production phase. So, they take into account the treatments applied and the field, winemaking and bottle replication incorporated in the experiment. The formulae for the experimental structures for experiments 2-5 are given in Table 2A. Tiers 2 and 3 for these experiments reflect the fact that in the production phase of most of these experiments, blocks were split into plots and treatments assigned to plots; several lots of wine

Table 2—Analyses of variance for a judge in experiments 2–5: (A) Experimental structure, (B) Analysis of variance tables, and (C) Formulae for calculating F-statistics for discrimination and stability from the mean squares

EXPERIMENT 2

A) Tier Structure formulae (no. levels in {})

1 Occasions {2}/Sessions {2}/Glasses {12}
2 (Treatments {4}/Winereps {3}/Bottles {2})*Occasions
3

B)

	SOURCE	DF
a	Occasions	1
	Occasions.Sessions	2
b_1	Treatments.Winereps.Bottles	1
b_2	Treatments.Winereps.Bottles.Occasions	1
	Occasions.Sessions.Glasses	44
c_1	Treatments	3
c_2	Treatments.Winereps	8
c_3	Treatments.Winereps.Bottles	11
c_4	Treatments.Occasions	3
c_5	Treatments.Winereps.Occasions	8
c_6	Treatments.Winereps.Bottles.Occasions	11
	TOTAL	47

(C) $F_{Discrimination} = (c_2 + c_2 + c_3)/(c_4 + c_5 + c_6)$
$F_{Stability} = (a + c_6)/(b_2 + c_4)$

EXPERIMENT 3
Structure formulae (no. levels in {})

Occasions {2}/Sessions {6}/Glasses {8}
Blocks {2}/Plots{4}/
Winereps{6})*Occasions
Treatments {4} * Occasions

SOURCE		DF
a	Occasions	1
	Occasions.Sessions	10
b_1	Blocks.Plots.Winereps	5
b_2	Blocks.Plots.Winereps.Occasions	5
	Occasions.Sessions.Glasses	84
c_1	Blocks	1
	Blocks.Plots	6
d_1	Treatments	3
d_2	Residual	3
c_2	Blocks.Plots.Winereps	35
c_2	Blocks.Occasions	1
	Blocks.Plots.Occasions	6
e_1	Treatments.Occasions	3
e_2	Residual	3
c_4	Blocks.Plots.Winereps.Occasions	35
	TOTAL	95

$F_{Discrimination} = (c_1 + d_1 + d_2 + c_2)/(c_3 + e_1 + e_2 + c_4)$
$F_{Stability} = (a + c_4 + e_2)/(b_2 + c_3 + e_1)$

EXPERIMENT 4

A) Tier Structure formulae (no. levels in {})

1 Occasions {2}/(Sessions{6},Glasses{8})
2 (Blocks{4}/Plots{4}/Winereps{3}/*Occasions
3 Treatments{4}*Occasions

B)

	SOURCE	DF
a	Occasions	1
	Occasions.Sessions.Glasses	94
b_1	Blocks	1
	Blocks.Plots	12
c_1	Treatments	3
c_2	Residual	9
b_2	Blocks.Plots.Winereps	32
b_3	Blocks.Occasions	3
	Blocks.Plots.Occasions	12
d_1	Treatments.Occasions	3
d_2	Residual	9
b_4	Blocks.Plots.Winereps.Occasions	32
	TOTAL	95

(C) $F_{Discrimination} = (b_1 + c_1 + c_2 + b_2)/(b_3 + d_1 + d_2 + b_4)$
$F_{stability} = (a + d_1)/(b_3 + d_1)$

EXPERIMENT 5
Structure formulae (no. levels in {})

Glasstype{2}/Sessions{3}/Glasses{8}
(Blocks{3}/Plots{2}/Halfpots{2}/Winereps{2})*Glasstype

SOURCE		DF
a	Glasstype	1
	Glasstype.Session	4
b_1	Blocks	2
b_2	Glasstype.Blocks	2
	Glasstype.Session.Glasses	42
c_1	Blocks.Plots	3
c_2	Block.Plots.Halfplots	6
c_3	Blocks.Plots.Halfplots.Winereps	12
c_4	Blocks.Plots.Glasstype	3
c_5	Blocks.Plots.Halfplots.Glasstype	6
c_6	Blocks.Plots.Halfplots.Winereps.Glasstype	12
TOTAL		47

$F_{Discrimination} = (b_1 + c_1 + c_2 + c_3)/(b_2 + c_4 + c_5 + c_6)$
$F_{stability}$ = none available

were made from the fruit of each plot. The skeleton analysis of variance tables for experiments 2-5, derived from their experimental structures, are given in Table 2B.

From the table for the analysis of variance, variability is assessed by the LSD for the difference between any two wine scores and is calculated from the last mean square in each table, $t \times (\text{mean square} \times 2)^{1/2}$. Discrimination is assessed by the F-statistic appropriate for testing the combined sources of variation derived from the production phase (Table 2C) while stability is assessed by the F-statistic for testing Occasion differences (Table 2C). As both of these F-statistics are ratios of sums of mean squares they are only approximately distributed as F values and the degrees of freedom have to be calculated using Satterthwaite's (1946) approximation. Also, it needs to be borne in mind that the otherwise useful statistic describing discrimination may be misleading if any of the mean squares in its denominator is significant (see Table 2C).

RESULTS

Agreement in Experiment 1

The multijudge correlation matrix, the elements of which are the correlations between the scores of pairs of judges, for experiment 1 is given in Table 3; the Fisher z-transform matrices and the analyses performed on them are also presented in Table 3.

The analysis of the results for the Riesling evaluation indicates that the scores were correlated to a similar extent for all pairs of judges, this correlation being about 0.58 (calculated as the inverse-z-transform of the mean of the z-transforms). There appeared to be some agreement between the judges in scoring the wines.

This was not the case for the Cabernet wines. Here, the analysis of the scores (Table 3) indicates that the correlations between two judges' scores vary with the pair of judges involved. There was poor agreement between some judges and better agreement between others, e.g., judges 1 and 2 were poorly correlated, but judges 1 and 4 were well correlated.

Agreement and reliability in Experiment 2

The multioccasion-multijudge correlation matrix and z-transforms for experiment 2 are reproduced in Table 4 and the results of the analysis in Table 5. The analysis indicated that the correlations were extremely heterogeneous: the Occasions·Judges Interaction line was highly significant ($p<0.001$) both in the "Agreement Within Occasions" and "Across Occasion Agreement" sections and the Judges line was highly significant ($p<0.001$) in the "Reliability" section. Thus, the judges differed in their reliability. Further, agreement between

Table 3—Multijudge correlation matrix (above the diagonal) and Fisher z-transform matrix (below the diagonal) and analyses of the z-transforms for experiment 1

Correlation and Fisher z-transform matrices

	Cabernet					Riesling				
Judges	1	2	3	4	5	1	2	3	4	5
1		0.07	0.55	0.90	0.34		0.54	0.75	0.58	0.70
2	0.07		0.37	0.34	0.24	0.60		0.52	0.46	0.51
3	0.62	0.39		0.50	0.12	0.97	0.58		0.44	0.70
4	1.48	0.35	0.55		0.43	0.66	0.50	0.48		0.54
5	0.35	0.25	0.12	0.45		0.86	0.56	0.86	0.61	
Variance	41.3	25.6	22.4	47.8	30.6	25.9	218.5	20.9	20.4	37.3
			υ = 11					υ = 15		

(υ = degrees of freedom of correlation matrix)

Analyses of Fisher z-transforms

		Cabernet			Riesling		
SOURCE	DF	SSQ	DIV	χ^2	SSQ	DIV	χ^2
Mean	1	2.15	0.100	21.55***	4.46	0.071	62.39***
Judges	4	0.85	0.098	8.62^{NS}	0.21	0.064	3.32^{NS}
Judges Interaction	5	0.58	0.049	11.87*	0.04	0.028	1.53^{NS}
Corrected Total	9	1.42	–	20.49	0.26	–	4.86

DF = Degrees of Freedom; SSQ - Sums of Squares; DIV- Divisor.

Table 4—Multioccasion-multijudge correlation (above the diagonal) and Fisher z-transform (below the diagonal) matrices for experiment 2

(all values × 100)

		Occasion 1						Occasion 2							
	Judge	A	B	C	F	G	I	A	B	C	F	G	I	Judge	
Occasion 1	A		79	67	63	57	0	74	52	67	58	78	−20	A	Occasion 1
	B	108		69	61	50	−9	68	66	78	64	73	−37	B	
	C	81	85		78	32	20	39	73	91	72	81	−25	C	
	F	75	70	103		40	29	61	60	83	88	71	−2	F	
	G	65	55	33	43		44	57	42	41	47	39	30	G	
	I	0	−9	21	30	47		0	10	22	34	−6	77	I	
Occasion 2	A	96	82	41	71	65	0		38	49	64	55	−6	A	Occasion 2
	B	57	80	94	69	44	10	40		74	63	62	−14	B	
	C	82	104	155	119	43	22	54	96		84	79	−24	C	
	F	66	75	90	139	50	36	76	75	121		58	12	F	
	G	105	92	113	90	42	−6	62	73	108	67		−40	G	
	I	−21	−39	−25	−2	31	102	−6	−14	−24	12	−42		I	
	Variance	80	281	391	257	72	204	105	293	365	242	99	154		
	Judge	A	B	C	F	G	I	A	B	C	F	G	I	Judge	
		Occasion 1						Occasion 2							

(υ = 22 = degrees of freedom of observed correlations)

judges depended on the pair of judges and the occasion involved.

In the hope of obtaining a more homogeneous set of judges, the correlation matrix was examined in detail. This revealed that Judge 1 disagreed with the other judges as all his agreement correlations were low, but he was reasonably self-consistent as his reliability correlation was 0.77. Judge G was unreliable (r = 0.39), and agreed only rarely with other judges (with C and F on the second occasion r = 0.79 and r = 0.58, respectively). In an analysis of the correlations of the remaining judges A, B, C, and F only (Table 5), the significant lines are Occasions-Judges (O.J) in the "Agreement Within Occasions" section, Judges (J) in the "Reliability" section, and Mean (M) in the section "Across Occasion Agreement." This indicated that the agreement of a judge with other judges on one occasion varied according to the judge and the occasion involved. The parameter estimates, which were essentially means calculated from the z-transforms, in Table 5B revealed (a) from O.J, that judge A and B tended to agree less with the other two judges on the second occasion than on the first, (b) from J, that the reliability of the four judges differed and that, in particular, that of Judge B was lower than those of judges C and F, and (c) from M, that across-occasion agreement was nonzero. It would appear that the only genuinely homogeneous subset comprised judges C and F who were both reliable and in agreement.

Individual performance in Experiments 2–5

The statistics summarizing individual performances of judges for experiments 2–5 are presented in Table 6. In experiment 2 all the F-statistics for discrimination, except that for judge G, were highly significant ($p<0.001$), judges C and F being clearly superior in their separation of the wines. All judges, except judge B, appeared to show similar variability as evidenced by the similar LSD values. Judge G exhibited central tendency as indicated by a low discrimination and a low LSD. All judges scored at the same overall level on the two occasions.

In experiment 3 reliability was generally lower and variability higher than in experiment 2. The higher variability might be due to lower reliability and/or to the use of a larger scoring range. Judges C and H had similar reliability but differed in their variability; judge C exhibited central tendency whereas

Table 5—Analysis of Fisher z-transforms for experiment 2 for all judges (A,B,C,F,G,I) and four of the judges (A,B,C,F).

(A) **Analysis of Variance**

Source	6 Judges DF	6 Judges x^2	4 of 6 Judges DF	4 of 6 Judges x^2
Agreement Within Occasions				
Mean	1	158.0***	1	169.0***
Judges	5	50.4***	3	4.0$^{n.s.}$
Judges Interaction	9	11.0$^{n.s.}$	2	5.1$^{n.s.}$
Occasions	1	0.9$^{n.s.}$	1	0.7$^{n.s.}$
Occasions.Judges	5	12.9*	3	9.6*
Occasions.Judges Interaction	9	43.4***	2	4.0$^{n.s.}$
Corrected Total	29	118.6	11	23.4
Reliability				
Mean	1	131.7***	1	116.1***
Judges	5	20.6***	3	10.9*
Corrected Total	5	20.6	3	10.9
Across Occasion Agreement				
Mean	1	170.0***	1	158.0***
Judges	5	53.6***	3	5.5$^{n.s.}$
Judges Interaction	9	12.1$^{n.s.}$	2	4.8$^{n.s.}$
Occasions.Judges	5	10.7$^{n.s.}$	3	3.2$^{n.s.}$
Occasions.Judges Interaction	10	42.5***	3	4.4$^{n.s.}$
Corrected Total	29	118.9	11	17.9

(B) **Table of Fisher z-transform parameter estimates for significant lines in analysis of 4 out of the 6 judges**

Agreement Within Occasions

Occasion.Judges (O.J)

		Judges A	B	C	F
Occasion	1	0.45	0.45	0.47	0.37
	2	0.08	0.28	0.58	0.58

Reliability

Judges (J)

A	B	C	F
0.96	0.80	1.55	1.39

Across Occasion Agreement

Mean (M) 0.79

judge H scored haphazardly. In spite of the higher variability and lower reliability, four of the six judges were able to discriminate between wines, although not to the same extent as judges C and F in experiment 2. Judge D shifted his level of scoring from one occasion to the next, his mean score for the first occasion being 17.1 and for the second occasion 16.1.

In experiment 4 also, reliability was generally lower than in experiment 2 but higher than in experiment 3. Overall, variability was highest in this experiment and discrimination lowest. The lower discrimination suggested that the wines in this experiment were more similar than in the other experiments.

In experiment 5 reliability and discrimination were high and variability low. It was not possible to test stability because of confounding.

DISCUSSION

Judge performance

The method of assessing judge performance outlined above could be described as a post-evaluation screening procedure in that assessment of individual, rather than combined, performance became an integral part of the sensory experiment. Of course, the same statistical analysis could also be performed on the results of a suitably designed pre-evaluation screening trial. In both situations a number of points need to be borne in mind when interpreting the results of such analyses.

Reliability

The method of measuring reliability used here is called the test-retest method in the psychological literature. Its purpose here was to measure an individual's ability to reproduce relative score differences between products. As Carmines and Zeller (1979) point out, it is intuitively appealing but has serious limitations. In sensory evaluation experiments, the material may change between evaluations and this would invalidate the measurement of reliability. Secondly, reliability will be limited by changes in a judge's concept of quality and in his or her utilization of the score card. Such changes could result from discussions amongst judges between sessions or from learning during the experiment. They are more likely to occur in experiments covering a considerable time span. A third point is that overestimation of reliability occurs when the re-evaluation is not independent of the initial evaluation; for instance, when the judge recognizes a particular sample at the later occasion and remembers his original evaluation of it. Close spacing of the evaluations will increase the likelihood of this occurring, but blind presentation will assist in avoiding it. Finally, the measure of a judge's reliability will tend to be low when evaluating products that differ only slightly.

The use of correlation coefficients for measuring reliability is not common. One example is its use by Hovenden *et al.* (1979) to measure both combined and individual judge repeatability in rating steak samples. However, their association of repetitions with the weeks in which they were taken offends against the condition required for intra-class correlations that repetitions of different samples must not be related. Thus reliability would have been better measured by the more usual (product-moment) correlation coefficients. Appropriate use of intra-class correlation coefficients can be made in experiments with a replicated composite complete-incomplete block design (Cornell and Knapp, 1974). In this instance, repetitions occur within as well as between sessions and their order of presentation is randomized for each session. The intraclass correla-

Table 6—Individual performances of 13 judges in scoring wines in experiments 2-5.

	Experiment 2				Experiment 3				Experiment 4				Experiment 5			
Judge	Reliability	Variability	Discrimination	Stability	Reliability	Variability	Discrimination	Stability	Reliability	Variability	Discrimination	Stability	Reliability	Varability	Discrimination	Stability
A	0.74	1.4	10.4***	3.4^{ns}	0.59	3.2	12.8***	1.2^{ns}	0.76	3.6	9.0***	5.9^{ns}	0.95	1.9	243.00***	–
B	0.66	3.2	12.5***	0.2^{ns}	0.40	3.1	16.4***	0.4^{ns}	0.16	5.3	1.7^{nsa}	3.9^{ns}				
C	0.91	1.4	41.3***	2.0^{ns}	0.42	2.6	2.2^{ns}	0.8^{ns}	0.58	5.6	6.6***	0.8^{ns}				
D					0.35	3.7	19.0***	7.3*	0.40	5.2	3.4**	1.0^{ns}	0.80	2.9	17.9*^{a}	
E					0.56	2.7	11.2***	0.8^{ns}	0.70	5.4	6.1**^{a}	0.6^{ns}				
F	0.88	1.6	32.6***	2.1^{ns}					0.63	4.4	4.9***^{a}	0.6^{ns}	1.00	–	–	–
G	0.39	1.5	2.1^{ns}	0.7^{ns}									0.58	3.4	9.2*^{a}	–
H					0.40	4.1	2.9^{ns}	4.0^{ns}								
I	0.77	1.8	16.0***	9.8^{ns}												
J													0.56	3.4	16.9***	–
K													0.61	3.8	11.8**	–
L													0.59	3.2	7.6*	–
M													0.85	2.2	34.8***	–

[a] The mean squares in the denominators of these Discrimination F-values are not homogeneous and so they may be misleading.

tion coefficient will measure the correlation between repetitions occurring in the same session, ignoring the order of presentation.

Agreement

The condition that correlations between the scores of pairs of judges be significantly different from zero and sufficiently large will be met if there is *construct validity* (Carmines and Zeller, 1979); that is, if the judges have the same concept of product quality and use the score system in a similar manner. However, the fulfilling of this condition does not guarantee agreement as it will be met if the judges assign similar scores to quite different characters. Construct validity is a desirable prerequisite for a set of judges. Its absence makes conclusions about product differences judge-specific.

The use of agreement as the sole criterion for selecting judges for a small panel is inappropriate as one cannot determine from it alone whose concept of quality is acceptable. In large panels it seems reasonable to exclude judges showing poor agreement.

A related approach is to measure the agreement between a judge's scores and the overall mean scores for the products. One could correlate in turn the scores of each judge with the product means (Hopkins, 1946; Moser *et al.*, 1950; Malek *et al.*, 1986) or coefficients based on the mean of differences from judge means (Asselin *et al.*, 1983). As such an approach requires that individual judges have minimal effect on the consensus opinion of the products it is most satisfactory for large panels of judges who are nearly all in agreement (Finlay and Wilkinson, 1963). In contrast, erroneous assessment of judge agreement is likely to occur with a small panel of heterogeneous judges.

Variability

It is evident from the examples presented in Table 6 that a judge's variability in evaluating the same samples more than once depends on (1) his reliability, and (2) his use of the scoring scale. Thus, variability does not appear to be an appropriate measure on its own to gauge reproducibility, despite such an approach being advocated (Overman and Li, 1948; Bennett *et al.*, 1956; Liming, 1966). For example in experiment 4, a comparison of variabilities of judges whose reliabilities were similar indicated that the increased variability was due to the use of a greater spread in scoring rather than to reduced reliability. This was not surprising since this was the only experiment in which the judges were asked to "ensure that any discernible differences in quality characteristics were reflected in the scores and to use only full points for scoring" (May *et al.*, 1976) rather than the usual half points. The judges apparently responded to this request by amplifying their scoring range, hence the greater spread in scoring.

However, the joint use of the measures of reliability and variability was useful for determining how the scoring scale was used. Likely factors that might influence the use of the scoring scale were the experience of the judges and the spread in quality of the products presented. Less experienced judges tended to use a narrower range that might lead to central tendency, while even experienced judges might feel tempted to amplify their scoring range for a set of products which were similar.

Discrimination

Clearly, discrimination and reliability are related: an unreliable judge will have difficulty discriminating and a judge who is unable to discriminate is likely to be unreliable. However, this can only be taken as a broad guideline as these two aspects of performance are complementary. Even moderately reliable judges may be able to discriminate between distinctly different products. Such judges may also discriminate when the set of products to be evaluated involves sufficient replication in the sensory evaluation phase or of the growing, processing, or storage operations that constitute the production phase of the experiment. Experiments 2-5 were of this kind, with the wines being comprised of several treatments each of which had been replicated in the field (blocks) and during the winemaking process. In experiment 3 the judges exhibited poor reliability in their duplicate evaluations of a wine, but several of them nevertheless found quality differences between the wines from different plots because six wines from each were evaluated. In experiments involving a *simple set of products* which did not involve the kind of replication discussed above, such as experiment 1, moderately reliable judges were not likely to discriminate unless each wine was evaluated many times.

Stability

In experiments in which treatments were balanced with respect to occasions, stability (as defined above) was important only when it was desired to establish the absolute equality level of the products. Instability was not of concern in those experiments in which only a relative measure of product quality was required as it affected all product means equally and thus did not change the differences between the product means.

Implications for analysis for product differences

Product differences are usually tested in a combined analysis of variance of all judges' scores (Amerine and Roessler, 1976). As mentioned above, the assumptions underlying such analyses require that the judges display a certain degree of homogeneity in their performances.

For example, a two-way analysis of variance would be used to test for wine differences in experiment 1 (Amerine and Roessler, 1976). The first assumption underlying this analysis was the absence of judge-wine interaction; in other words, each judge established the same differences between the wines, except for random variation. Secondly, so that the standard error of the differences between wine means was the same for all pairs of wine means, it was assumed that all observations had the same variance and all pairs of observations showed the same correlation (of course, the wines had to be equally replicated also). Other assumptions included normality of the observations which was not crucial as the analysis was robust to departures from it and independence of the observations that could be ensured only by organizing the evaluations appropriately.

It can be shown that, under the first two assumptions, the elements of the multijudge correlation matrix are expected to be equal. Thus, in experiment 1, the multijudge correlation matrix computed from the scores for Riesling wines exhibited the appropriate pattern for performing the two-way analysis of variance in contrast to the matrix from those for the Cabernet wines where it indicated that a combined analysis of the judges scores should not be performed.

Brien (1980) has reported the results of the analysis for judge agreement and reliability used in this paper for experiments 2–5 and it is evident that the judges are heterogeneous in their performance. Further, the judges have varied in their performance over the lengthy period during which these experiments were conducted (Table 6).

Given this situation, which agrees with that reported generally in the literature on sensory evaluation, the routine application of the combined analysis is inappropriate and post-evaluation screening of judges becomes required. Hence it is recommended that, like Weiling and Schoeffling (1976), one should initially analyze the scores assigned in the experiment by each judge separately to contrast the judges' individual performances. The results of unreliable, nondiscriminating judges should be discarded as they do not provide any useful information; if retained, they may obscure the results of more re-

liable judges. Combined analyses of variance should be performed subsequently for each subset of judges exhibiting agreement and similar reliability and variability; individual analyses of variance (Table 2) should be performed for heterogeneous judges.

Unfortunately, separate analyses are not possible for simple-set experiments, such as experiment 1. Consequently, there is no valid analysis of variance when there is a lack of agreement between the judges. However, the scores of each judge can be analyzed separately for nonsimple experiments which may involve the evaluation of at least some of the products more than once, or the products can be divided into sets that contain replicates from different field locations, from separate processing batches or from different containers. This replication will have to be sufficient to ensure that the statistics used to measure judge performance have adequate degrees of freedom (as a guide, a minimum of 10 for correlation coefficients) to enable meaningful measures of judge performance to be obtained. The examples presented in Table 6 are ones in which separate analyses are possible, although the results reported for experiment 2 indicate that a combined analysis of variance performed on the scores of judges C and F only would be appropriate. Ewart et al. (1985) provide a further example of the application of the proposed method of analysis. When the results of the analysis indicate the judges are heterogeneous, such as in these examples, one is left with the unsatisfactory, judge-specific conclusion that the perceived product differences vary between judges; the results must report the product differences established by each group of homogeneous judges as overall differences do not reflect adequately the evaluation of the products by the judges.

Single attribute scores

The experiments analyzed here involved the use of a single composite score purportedly reflecting wine quality. Since quality is an inherently multidimensional construct there is obviously scope for considerable variation in a composite score and this is supported by the results reported here. However, sensory experiments are increasingly being based on the assessment of single attributes, each such attribute being subjected to a separate analysis of variance. There is less room for variation when only a single attribute is involved, although the usual factors affecting the physiological and psychological responses of judges (e.g. inherited sensitivity, environment, motivation, experience, training and so on) will still operate. Nonetheless, one would expect judges to be more homogenous in their evaluation of single attributes. Whether they will be sufficiently homogeneous to allow combined analysis of all judges scores is a matter of conjecture at this stage. Results presented by Kwan and Kowalski (1980) indicate that they may not be and those presented by Malek *et al.* (1986) indicate that they are not when beer is evaluated. It would be beneficial to apply the methods outlined in this paper to such data from panels of judges suitably trained in single attribute evaluation of wine; they would have to be applied to each attribute separately.

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Ms received 5/8/86; revised 11/17/86; accepted 2/4/87.

The authors thank Mr. P.R. Clingeleffer and Mr. G.H. Kerridge who prepared the wine and Messrs. D. Armstrong, J. Brown, A. Hoey, A. Hood, P. John, G. Kerridge, N. Lindsay, R. Moody, G. Prass, M. Tummel, B. Tyson, C. Weeks, and P. Weste who were the judges (experiments 2-5). They are also grateful to the referees for many helpful comments.

Adjustment of Nonlinear Models for Lignin and Its Degradation Products during the Aging of Armagnac

J.-L. PUECH and B. GOFFINET

ABSTRACT

Changes in lignin and in its degradation products, namely aromatic acids and aldehydes, were monitored in Armagnac brandies stored in old or new barrels. The concentration of these substances was always higher in the brandies aged in new barrels than in those aged in older barrels. Among the various mathematical models proposed, the parabolic function always showed the least residual error. A parameter n that might characterize the age of a barrel was defined.

INTRODUCTION

WHEN IT LEAVES THE STILL, Armagnac is colorless and fiery. The latter feature abates during aging in oak barrels, and a smooth product, pleasant on the palate and rich in bouquet and flavor is finally obtained as a result of the extraction of certain substances from the wood by the brandy during maturation. The cellulose is not broken down by the alcohol, while the hemicelluloses are slightly degraded into pentoses (arabinose, xylose) and hexoses (fructose, galactose and glucose) (Reazin, 1981; Nykanen et al., 1984). On the other hand, 55% of the lignin and its degradation products, particularly the aromatic aldehydes, give smoothness, bouquet and character. Approximately 4% of the wood lignin, called "sol-lignin," is solubilized in the brandy (Puech, 1984). This compound is extracted by hydroalcoholysis at room temperature (approximately 18°C); this is similar to hydrolysis and causes the formation of benzoic and cinnamic aldehydes. Oxidation of these compounds then produces aromatic acids. All the constituents derived from "sol-lignin" form the brandy ligno-complex.

There has been no publication to date concerning an experimental study on the changes in these substances during aging. These compounds have only been investigated in spirits of different origin or age such as Armagnac, Cognac, rum, whisky or brandy (Bricout, 1971; Egorov and Borisova, 1957; Guymon and Crowell, 1968; Hardy, 1969; Lehtonen, 1983; Puech et al., 1984; Skurikhin and Efimov, 1972).

The first phase of brandy aging is the extraction of wood phenolics. This is achieved by placing the brandy into new barrels. In the second phase, oxidation is obtained by transferring the brandy into older barrels. In order to assess the differences in wood storage quality, two series of experiments were carried out on Armagnac brandy aged in new or old barrels. The lignin and its degradation products aromatic aldehydes (vanillin, syringadehyde, coniferaldehyde, sinapaldehyde) and aromatic acids (vanillic and syringic acids), were analyzed in this study because of their beneficial effect on the sensory properties of brandies. The data thus collected were processed using non linear models to observe the behavior of the substances extracted from brandies during maturation.

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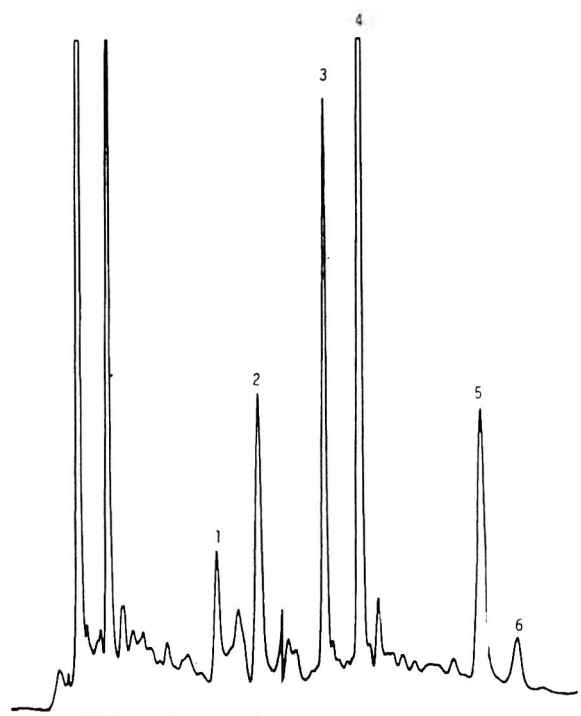

Fig. 1—Aromatic aldehydes and acids in Armagnac.

MATERIALS & METHODS

IN 1968, four barrels which had contained Armagnac for about 10 years, and which were thus considered as being old were filled again with Armagnac and stored in a warehouse at the Cave Cooperative in Nogaro. Temperature ranged from 4°C in winter to 20°C in summer. Samples were taken annually, each sample collected being replaced by an equal amount of brandy of similar age stored in one of these barrels.

In 1970, further tests were carried out in the same Cave Cooperative following the same procedure, except that the brandy was stored in new barrels. This warehouse had a minimum winter temperature of 6°C and a maximum summer temperature of 24°C. The two series of trials were continued until 1981.

Quantitative determination of the ligno-complex of Armagnac brandies

The methoxyl groups were separated by boiling in the presence of hydriodic acid, leading to the formation of alkyl iodides. These compounds were carried away by a flow of CO_2 and trapped in toluene as described by Deibner et al. (1976). Quantitative determination was made by gas chromatography to separate the methyl and ethyl iodides (Puech et al., 1978).

Aromatic acids and aldehydes

The monomers derived from lignin and present in the Armagnac brandies were identified after purification by HPLC. The aromatic acids and aldehydes were extracted from each 50-mL sample with 250

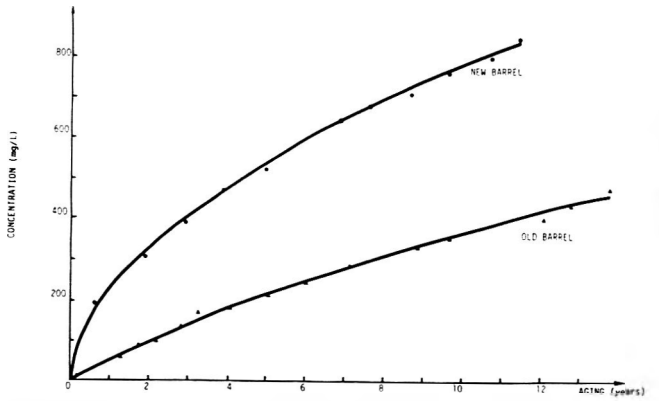

Fig. 2—Mean values of experimental points observed for evolution of ligno-complex firstly for new barrels and secondly for old barrels, and the theoretical parabolic curves adjusted using these values.

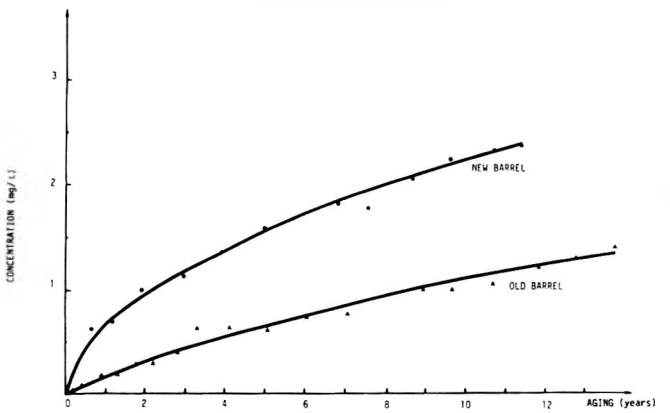

Fig. 3—Mean values of experimental points observed for evolution of vanillin firstly for new barrels and secondly for old barrels, and the theoretical parabolic curves adjusted using these values.

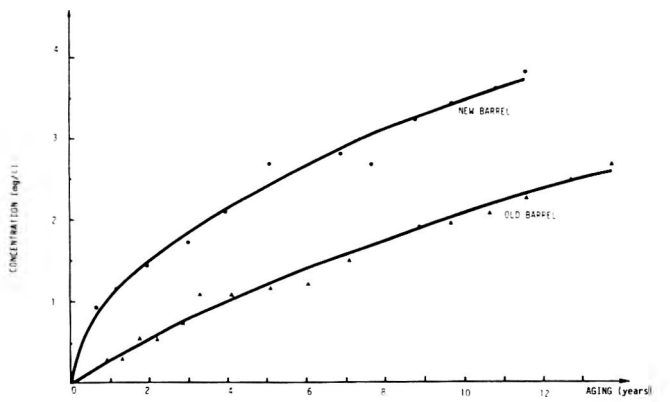

Fig. 4—Mean values of experimental points observed for evolution of syringaldehyde firstly for new barrels and secondly for old barrels, and the theoretical parabolic curves adjusted using these values.

mL ethyl ether by means of a Soxhlet-Handorf extractor for 3 hr at 50°C. The ether extract was evaporated and rediluted in a 12% acetonitrile-water mixture acidified with 1% acetic acid. Extracts were filtered through a 0.45 μm Millipore membrane and adjusted to 5 mL. From 10 to 100 μL of the extract, depending on the acid and aldehyde content of the water, were injected into an HPLC chromatograph. The experiments were conducted using a Waters ALC 200 chromatograph equipped with a M 660 solvent programmer with 6000 A pumps, a U6K injector and a M440 detector. Integration was carried out with a Data module M730 integrator, on the same channel at 280 nm for

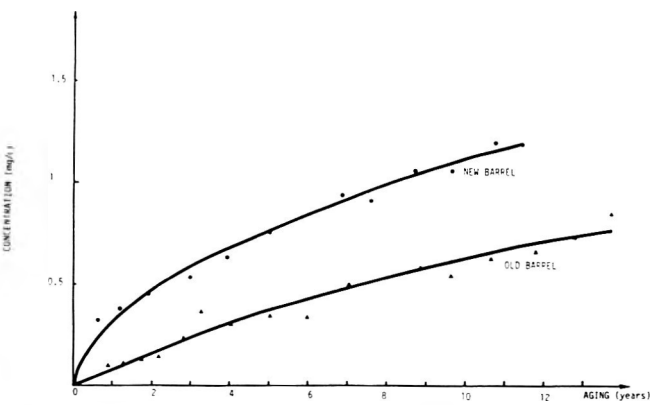

Fig. 5—Mean values of experimental points observed for evolution of vanillic acid firstly for new barrels and secondly for old barrels, and the theoretical parabolic curves adjusted using these values.

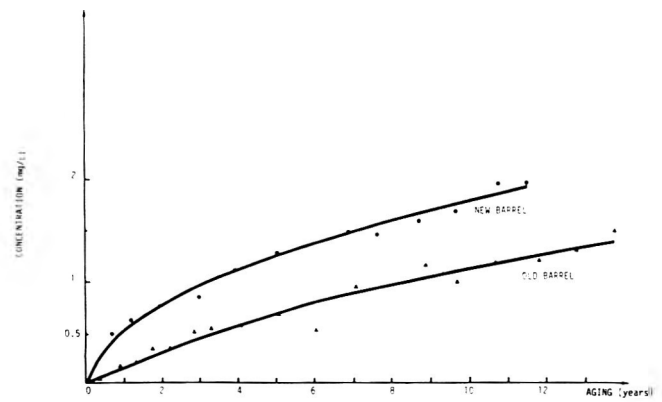

Fig. 6—Mean values of experimental points observed for evolution of syringic acid firstly for new barrels and secondly for old barrels, and the theoretical parabolic curves adjusted using these values.

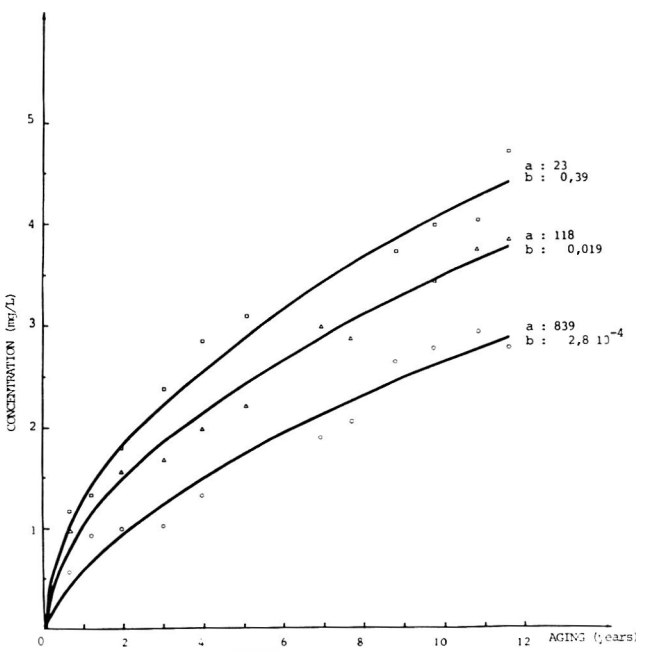

Fig. 7—Experimental points and the theoretical curves with parameters a and b corresponding to model 7 in the case of syringaldehyde from new barrels.

Table 1—Estimation of $\hat{m}$, $\hat{n}$ and confidence interval at 5% for parameters m and n for several compounds in old barrels

Barrels	Ligno-complex	Vanillin	Syringaldehyde	Vanillic acid	Syringic acid
A	$\hat{m}$ = 336 ± 11	$\hat{m}$ = 0.94 ± 0.03	$\hat{m}$ = 1.79 ± 0.07	$\hat{m}$ = 0.63 ± 0.04	$\hat{m}$ = 1.19 ± 0.07
	$\hat{n}$ = 0.40 ± 0.03	$\hat{n}$ = 0.38 ± 0.02	$\hat{n}$ = 0.35 ± 0.03	$\hat{n}$ = 0.36 ± 0.05	$\hat{n}$ = 0.38 ± 0.05
B	$\hat{m}$ = 262 ± 6	$\hat{m}$ = 0.50 ± 0.04	$\hat{m}$ = 1.24 ± 0.06	$\hat{m}$ = 0.41 ± 0.02	$\hat{m}$ = 0.75 ± 0.04
	$\hat{n}$ = 0.37 ± 0.02	$\hat{n}$ = 0.34 ± 0.05	$\hat{n}$ = 0.35 ± 0.04	$\hat{n}$ = 0.35 ± 0.04	$\hat{n}$ = 0.38 ± 0.04
C	$\hat{m}$ = 346 ± 6	$\hat{m}$ = 1.39 ± 0.08	$\hat{m}$ = 2.16 ± 0.11	$\hat{m}$ = 0.52 ± 0.03	$\hat{m}$ = 0.97 ± 0.06
	$\hat{n}$ = 0.44 ± 0.02	$\hat{n}$ = 0.49 ± 0.06	$\hat{n}$ = 0.49 ± 0.06	$\hat{n}$ = 0.53 ± 0.06	$\hat{n}$ = 0.55 ± 0.06

Table 2—Estimation of $\hat{m}$, $\hat{n}$ and confidence interval at 5% for parameters m and n for several compounds in new barrels

Barrels	Ligno-complex	Vanillin	Syringaldehyde	Vanillic acid	Syringic acid
E	$\hat{m}$ = 642 ± 12	$\hat{m}$ = 1.59 ± 0.07	$\hat{m}$ = 2.38 ± 0.14	$\hat{m}$ = 0.82 ± 0.04	$\hat{m}$ = 1.39 ± 0.07
	$\hat{n}$ = 0.55 ± 0.02	$\hat{n}$ = 0.54 ± 0.04	$\hat{n}$ = 0.50 ± 0.07	$\hat{n}$ = 0.56 ± 0.04	$\hat{n}$ = 0.55 ± 0.05
F	$\hat{m}$ = 752 ± 14	$\hat{m}$ = 2.04 ± 0.08	$\hat{m}$ = 3.18 ± 0.1	$\hat{m}$ = 1.12 ± 0.05	$\hat{m}$ = 1.77 ± 0.09
	$\hat{n}$ = 0.51 ± 0.02	$\hat{n}$ = 0.57 ± 0.03	$\hat{n}$ = 0.55 ± 0.03	$\hat{n}$ = 0.54 ± 0.04	$\hat{n}$ = 0.54 ± 0.05
G	$\hat{m}$ = 702 ± 18	$\hat{m}$ = 2.4 ± 0.09	$\hat{m}$ = 3.74 ± 0.18	$\hat{m}$ = 1.06 ± 0.05	$\hat{m}$ = 1.65 ± 0.08
	$\hat{n}$ = 0.57 ± 0.02	$\hat{n}$ = 0.57 ± 0.02	$\hat{n}$ = 0.57 ± 0.04	$\hat{n}$ = 0.56 ± 0.04	$\hat{n}$ = 0.57 ± 0.04

the phenolic acids and at 313 nm for the aromatic aldehydes. HPLC determination was carried out using a column (4 x 250 mm) packed with micro-Bondapak C18, 5μm (Waters Associates, Milford, MA). A gradient elution with two solvents was used: solvent A consisted of H_2O acidified with 1% acetic acid (Merck) and solvent B was acetonitrile (Rathburn, Walkerburn, Scotland) acidified with 1% acetic acid. Eluants were filtered through a Millipore membrane (0.45 μm) and degassed in an ultrasonic bath. The flow rate was 0.9 mL/min. The initial condition was 12% solvent B. After 9 min a gradient was introduced. B was increased to 20% in 3 min and maintained at 20% for 25 min. A chromatogram obtained on Armagnac is shown in Fig. 1.

RESULTS & DISCUSSION

SYSTEMATIC ANALYSIS for lignin and its degradation products was carried out for each barrel and each annual sampling. Nonlinear regression analysis was applied to the experimental data obtained per barrel, and to the mean values of new barrels and those of older barrels. The following seven models were used: (1) $y = a + bx$ (linear); (2) $y = a\,e^{bx}$ (exponential); (3) $y = a\,x^b$ (power); (4) $y = a + b/x$ (hyperbolic); (5) $y = 1/(a + bx)$ (hyperbolic); (6) $y = x/(ax + b)$ (hyperbolic); (7) $y = a(\sqrt{1 + bx} - 1)$ (parabola); where y represents the amount of a compound in a barrel or the mean amount of a compound for three barrels of the same series.

The first 6 models are derived from a study conducted by Baldwin and Andreasen (1974) on whisky. This study was then applied by Puech et al. (1985) to the mean experimental data obtained for dry extract, lignin and tannin in Armagnac brandies. Model 7 was added because of the parabolic appearance of the curves representing the quantities of each compound in Armagnac.

Figures 2, 3, 4, 5 and 6 show the theoretical curves representing the mean values for old and new barrels. The model corresponds to Eq (7) for reasons given below. All concentrations were higher in new than in old barrels, e.g. for 10 years of wood storage, the amounts of ligno-complex, vanillin, syringaldehyde, vanillic acid and syringic acid were 2.15, 2.02, 1.68, 1.76, 1.58 times as high in new barrels as in old barrels. These figures do not emphasize the variations that occur between the barrels in the same series. For example, Fig. 7 shows the experimental points and theoretical curves corresponding to model 7 in the case of syringaldehyde from new barrels, together with the values of the corresponding parameters. Within each series of barrels, each barrel was an individual unit; therefore, the "behavior" of each unit in addition to the differences between series was studied.

With respect to the lignin and its degradation products in the series of new barrels, in most cases model 7 exhibited the least residual variance or a value slightly higher than the best model proposed. Figure 7 illustrates the quality of the adjustment using model 7. Thus, the differences between the observed values and the model-values followed a pattern that justified models with only two parameters. In the older barrels, model 7 often showed the least residual variance, but the qualities of models 3 and 6 were very similar.

This work made it possible to characterize the lignin and its degradation products as well as the new and old barrel series by parameters corresponding to model 7. The very strong correlation (0.9 on average) between the estimators of parameters a and b of model 7 induced considerable confidence intervals for the estimate of these parameters. Estimates $\hat{m}$ and $\hat{n}$ of the values $m = f(3000)$ and $n = f(1000)/f(3000)$ where $f(x) = a(\sqrt{1 + bx} - 1)$, are, therefore, listed in Tables 1 and 2. Parameter m represents the prediction given by the 3000-day model and n the ratio of the 1000-day prediction to the 3000-day prediction. The two values m and n associated with the form defined by function f(x) adequately define the curve; m and n characterize curve 7 in the same way as a and b do, but they also display a smaller confidence interval.

Little variation was found between the values of parameter n for the various constituents in a same barrel, whether old or new. This parameter can, therefore, be considered as characteristic of the barrel. The close values of n for the three new barrels suggested that this parameter might characterize the age of a barrel. This work has made possible an approach to determine the quality of barrel wood. However, further work on spirits aged in new wood and then transferred to old wood would lead to a more complete understanding of the question.

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Postharvest Use of Sucrose Polyesters for Extending the Shelf-life of Stored 'Golden Delicious' Apples

S. R. DRAKE, J. K. FELLMAN, and J. W. NELSON

ABSTRACT

The post-storage uses of a sucrose-fatty acid polyester (SPE) formulation, commercial fruit wax and a combination of both were investigated on 'Golden Delicious' apples after fruit were removed from both controlled atmosphere (CA) and refrigerated storage. Quality parameters measured included color (skin and internal), firmness, soluble solids and titratable acidity. Physiological parameters measured were carbon dioxide and ethylene concentrations both evolved and internal. Refrigerated stored apples, treated with SPE, displayed retarded color development, higher acid and greater firmness values compared to controls. CA stored, treated apples possessed similar attributes but no firmness retention. Ethylene evolution was reduced in SPE-coated apples. In comparison to control apples, an internal accumulation of carbon dioxide and a reduction in ethylene was observed.

INTRODUCTION

QUALITY PRESERVATION is a major consideration in modern fresh fruit marketing systems. Predominant methods used to preserve fresh fruit during handling and subsequent marketing include controlled atmosphere (CA) or modified atmosphere techniques in conjunction with refrigerated storage. Modified atmospheres have been used to supplement temperature control and extend the postharvest shelf-life of fruits by reducing respiration and delaying senescence.

A recent approach has been the use of a coating that is edible and semi-permeable to CO_2 and O_2. Lowings and Cutts (1982) reported that an edible coating mixture composed of sucrose fatty acid esters, sodium carboxymethylcellulose and mono- and diglycerides would produce a semi-permeable modified atmosphere within fresh fruit after application. Sucrose polyesters (SPE) have been tested extensively on bananas (Banks, 1983, 1984a, 1984b); changes in the internal concentration of O_2, CO_2 and C_2H_4 and delay of ripening have been noted. In a study with pears, Meheriuk and Lau (1985) reported fruit were firmer, had greater acid concentration and greener skin when coated with SPE prior to storage, but the fruit failed to ripen properly. Chen (1986) reported increased firmness and titratable acidity with reduced C_2H_4 production of 'Bartlett' and 'Bosc' pears after treatment with SPE, but no apparent benefit to 'd'Anjou' pears treated with SPE was evident. Elson et al. (1985) found that SPE applied to apples suppressed CO_2 and C_2H_4 production in several cultivars and quality retention was evident in pears after storage with a coating of SPE.

Chu (1985) determined that a post-storage application of SPE reduced the softening of low oxygen stored 'McIntosh' and CA stored 'Delicious' apples during a 21-day shelf-life period but did not influence the firmness of CA stored 'McIntosh' apples. Working with 'Cox's Orange Pippin', Smith and Stow (1984) reported SPE applied after storage reduced yellow color, loss of firmness and increased internal CO_2 levels during 21 days of a simulated marketing period. Banks (1985) reported changes in the internal atmosphere of apples after SPE application.

Semperfresh™ is an improved formulation of earlier SPE products. The major difference is improved dispersion due to incorporation of a higher proportion of short chain unsaturated fatty acid esters. This study was conducted to determine the influence of Semperfresh™ on the quality of refrigerated and CA stored 'Golden Delicious' apples upon removal from storage.

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MATERIALS & METHODS

THIS STUDY was conducted using size 113 Fancy 'Golden Delicious' apples from commercial refrigerated and CA storage depending on the date of the experiment. This methodology more closely matches industry practices. The refrigerated apples were removed from storage in October and divided into nine lots of 20 kg each. Three untreated lots were used as the control; three lots were treated with SPE (Semprefresh™ obtained from Inotek International Inc., Painesville, OH); and three lots were treated with the SPE plus apple wax (Pennwalt™ #221 obtained from Decco Tiltbelt, Monrovia, CA), all consisting of three replications each. After treatment the apples from refrigerated storage were placed back in refrigerated (0°C) storage.

The CA apples were removed from storage in January and divided into twelve lots of 40 kg each. There were three replications of each treatment (control, SPE, apple wax, SPE plus apple wax). After treatment, the apples from CA were placed in refrigerated (0°) storage.

All the apples in this study were run through a model commercial packing line at the USDA lab in Wenatchee, WA. Apples treated with SPE were dipped in a 1% (w/v) concentration and passed through a wax dryer operating at 49°C with a dwell time of 2 min. Apples were waxed on the same packing line. The wax was applied with a traveling sprayer at the rate of 4 mL/min and also dried at 49°C. Apples were treated with a combination of SPE and wax as described for those treatments alone (e.g., dipped in sucrose ester and subsequently sprayed with wax).

Apples from refrigerated storage were treated and stored for 60 days, removed from storage, and quality determined after 0 and 7 days at ambient temperature (20°C). Apples from CA storage were treated, stored for 30 or 60 days, removed from storage and quality determined after 4 and 9 days at ambient temperature.

Skin color of the apples was determined with an Agtron Model E-5W reflectance spectrophotometer. An LC Model EP1 pressure tester (Lake City Technical Products, Kelowna, B.C.), was used to determine firmness, and values reported in Newtons. Internal color (green and blue) of the fruit was measured by cutting the apple in half equatorially and placing the exposed surface (blossom end up) in the sample cup of an Agtron Model 500-A reflectance spectrophotometer (Magnuson, Reno NV). Instrument span was set using discs 30 and 63 in the blue mode, 63 and 97 in the green mode. Soluble solids of the fruit was determined with an Abbe-type refractometer with a sucrose scale calibrated at 20°C. Titratable acidity and pH were determined with a Radiometer titrator, Model TTT85 (Radiometer, Copenhagen). Acids were titrated to pH 8.2 with 0.1N NaOH and expressed as percent malic acid.

Apple respiration (carbon dioxide generation) and ethylene evolution were measured by placing approximately 4 kg samples for each treatment and replication in plexiglass chambers through which ethylene-free compressed air was passed. Gas samples from the chambers were introduced into a Perkin-Elmer Model 3720 GC/FID via a 0.25 mL Valco gas sampling loop. Carbon dioxide was converted to methane (for FID quantitation) using a ruthenium H_2 catalyst positioned at the end of a (2 m × 0.3 cm) Porapak Q column. Instrument calibration

was achieved using Matheson certified standards. Evolved samples were taken 24, 48 and 72 hr after placement in the chambers at ambient temperature. Internal gases were obtained by direct syringe needle penetration into the cavity of the apple after 72 hr ambient storage. Evolved gas determinations are expressed in µl/hr/kg of apples; internal gas content is expressed as mL/L or ppm per apple. A multiple range comparison procedure (Waller and Duncan, 1969, 1972) was used to analyze all traits according to SAS (1985).

RESULTS & DISCUSSION

THE APPLICATION of SPE to 'Golden Delicious' apples treated and stored 60 days at 0°C significantly maintained the keeping quality of the apples (Table 1). Even before the 7 days ripening period, the skin color of apples treated with SPE was greener than either the control or the SPE + wax treatment. After 7 days at ambient temperature, the control apples lost more of their green skin color than either of the two treated apple samples. The more rapid change in color suggested a much reduced shelf life for the control apples; whereas the lack of color change suggested increased shelf life for the treated apples. Initially, the SPE-treated apples or the fruit treated with a combination of SPE + wax were firmer than the control, but after 7 days ripening, the apples treated with a combination of SPE + wax were the firmest. In fact, the SPE treated apples lost firmness at a much more rapid rate than did the control apples.

After 4 days ripening, no differences in internal green color were noted regardless of the coating treatment. After 9 days ripening, the only significant difference in internal green color was due to SPE + wax treatment (Table 2), resulting in a darker green color to persist suggesting an inhibition of ripening.

Internal Agtron blue color, an inverse indicator of yellow color, was significantly decreased (lower value = darker blue) from the control at 9 days ripening by SPE or SPE plus wax treatments. This difference suggested an inhibition of yellow color development associated with treatment with SPE type coatings (Table 2).

Internal blue color was darker for the SPE and SPE + wax-treated than the control apples (Table 1). After 7 days ripening, the control and SPE-treated apples were similar in color, but more yellow than the SPE + wax-treated apples. Prior to ripening, SPE-treated apples had 13% higher acid than control fruit. But, when combined with wax, SPE-treated apples did not have any more acid than the control apples before or after 7 days ripening. After 7 days ripening, the control apples lost 22% of the existing acid, whereas the SPE-treated apples with or without wax had lost only 14% acid. This retention of acid was a strong indication of apples that were not ripening as rapidly as control fruit.

Effects of the coating treatments on skin color, firmness and acid on apples from CA storage were similar (Table 3). Skin color of the SPE-treated apples was greener with significantly less yellow development than the control or other treatments. Apples waxed or treated with SPE + wax developed skin color at the same rate as the control. SPE and SPE + wax did not significantly slow the loss of firmness of CA fruit. Acidity of SPE-treated apples without wax was much greater than either the control or the apples with wax only. Apples with SPE + wax retained high acid concentrations significantly different from apples with wax only, but not different from the control.

The development of a yellow skin color and a corresponding loss in titratable acids was significantly reduced for apples from both refrigerated and CA storage after treatment with SPE. As previously reported (Elson et al., 1985) retention of firmness with the use of SPE was evident for the fruit from refrigerated storage.

There was a significant interaction between coating treatments and ripening time for internal color (Table 2). After 4 days ripening, coatings did not influence internal green color. After 9 days ripening internal green color was significantly darker for SPE + wax than control only, and the data suggested that either SPE or wax applied singularly to fruit might result in a darker internal green color. A darker internal green color after a ripening period was also evident for apples treated and placed only in refrigerated storage (Table 1). There were only small differences in internal blue color between treatments during the first 4 days of ripening (Table 2). After 9 days of ripening, the internal blue color of the control and wax treated apples were similar with little change from the 4 days color readings. Both the SPE-treated apples and the SPE + wax-treated apples displayed a 7–12% enhancement of internal blue color. A large increase in internal blue color was also evident for the SPE-treated apples stored only in refrigeration.

The use of SPE coating on 'Golden Delicious' apples reduced ethylene production (Table 4). Reduced ethylene production was evident in apples taken from CA storage and then refrigerated for 60 days. Even though there was no difference in CO_2 production after 30 days of refrigerated storage, there was a marked slowing of C_2H_4 production for apples coated with SPE or SPE plus wax. After 60 days those apples coated with SPE or SPE plus wax continued to show reduced C_2H_4 production. Apples coated with wax only were not different from the control apples in the production of CO_2 and C_2H_4.

Table 1—Quality attributes of refrigerated 'Golden Delicious' apples coated with SPE or SPE plus wax and stored 60 days at 0°C

Ripening time	Treatment	Skin color (Agtron) (E-5W)	Firmness (newtons)	Internal Agtron color		Titratable acidity (% malic)
				Green	Blue	
0 days	Control	55.4a[z]	51.4b	81.8b	42.0c	0.37b
	SPE	53.8b	54.3a	84.8a	48.7a	0.42a
	SPE + wax	55.7a	54.2a	82.8ab	45.2b	0.35b
7 days	Control	59.1a	47.4a	83.1a	42.6a	0.29b
	SPE	54.8c	48.5a	81.3a	42.3a	0.36a
	SPE + wax	56.8b	52.0b	79.3b	39.8b	0.30b

[z] Means in a column within ripening time followed by different letters are significantly different ($P < 0.05$).

Table 2—Internal color of CA 'Golden Delicious' apples coated with SPE, wax or SPE plus wax as influenced by the interaction of treatment and ripening time

Ripening time	Treatment	Internal Agtron color	
		Green	Blue[y]
4 days	Control	51.9 ns	62.5a[z]
	SPE	51.7	60.2ab
	Wax	52.2	59.5b
	SPE + wax	50.1	58.5b
9 days	Control	50.7a	60.3a
	SPE	46.3ab	52.9b
	Wax	48.2ab	57.4a
	SPE + wax	45.3b	54.8b

[z] Melans in a column within ripening time followed by different letters are significantly different ($P < 0.05$).

[y] The absence of blue (higher value) is a measure of yellow color. In every case a lower value indicates a darker color.

Table 3—Quality attributes of 'Golden Delicious' apples influenced by coating with SPE, wax or SPE plus wax

Treatment	Skin color (Agtron) (E-5W)	Firmness (newtons)	Titratable acidity (% malic)
Control[z]	51.6a[y]	56.2ab	0.32bc
SPE	50.9b	55.2b	0.34a
Wax	51.7a	55.5b	0.31c
SPE + wax	51.7a	57.6a	0.33ab

[z] Apples were removed from CA storage, coated and stored for 60 days at 3°C.

[y] Means in a column followed by different letters are significantly different ($P < 0.05$).

Table 4—Evolved carbon dioxide and ethylene values of 'Golden Delicious' apples removed from CA storage, treated with coatings and held for 30 or 60 days in refrigerated air[z]

Treatment	Carbon dioxide (mL kg^{-1} hr^{-1})		Ethylene (μL kg^{-1} hr^{-1})	
	30 days	60 days	30 days	60 days
Control	9.4[y]	13.9	142.0ab	163.7ab
SPE	9.1	7.9	97.7b	82.7b
Wax	11.1	16.2	170.0a	200.0a
SPE + wax	10.6	10.3	97.7b	73.3b

[z] Values represent emissions 72 hr after removal from cold storage.
[y] Means in a column followed by different letters are significantly different ($P < 0.05$). The absence of a letter indicates statistical nonsignificance.

Table 5—Internal carbon dioxide and ethylene values of refrigerated 'Golden Delicious' apples treated with SPE or SPE plus wax[z]

Treatment	Carbon dioxide (mL/L)	Ethylene (ppm)
Control (n = 13)	19.16a[y]	303.2a
SPE + wax (n = 12)	34.97b	184.3b
SPE (n = 12)	35.06b	191.4b

[z] Samples were drawn 72 hr after removal from cold storage.
[y] Means in a column followed by different letters are significantly different ($P < 0.05$). Samples size is in parentheses following treatment description.

To determine if the reduced evolution of CO_2 and C_2H_4 were solely due to restricted gas diffusion across these coatings, internal gases were measured. Apples coated with SPE or SPE + wax demonstrated a 183% increase in CO_2 and 39% decrease in C_2H_4 internal concentrations (Table 5). This increase suggested a dual effect of SPE coatings in that restriction of CO_2 diffusion caused secondary physiological changes in the ripening process. In a recent review Kader (1986) listed the processes affected by elevated CO_2 in fruits and vegetables. High CO_2 concentrations reduced respiration rates, prevented or delayed responses to ethylene and reduced losses in acidity. Smith and Stow (1984) suggested that the effects of SPE coatings were different from effects of elevated CO_2 alone, because an 8% CO_2 storage environment could not duplicate the effects of coating with SPE. In our study, quality parameters influenced by SPE coatings could, by inference, also be attributed to increased internal CO_2 levels.

Regardless of the mechanisms involved, the use of SPE coatings delayed 'Golden Delicious' apple ripening. Apples treated after refrigerated storage displayed slower skin color development and slower firmness and acid loss in comparison with controls. Apples treated after CA storage displayed similar attributes, but no difference in firmness was evident. Apples that were wax-coated had similar, if not identical, quality attributes in comparison with control apples. In most instances, apples coated with the SPE + wax showed quality attributes similar to the singular use of SPE coating. The combination of SPE + wax appeared no more beneficial than the use of SPE alone. Possible benefits that could be derived from using the mixture would be enhanced quality attributes coupled with a desirable fruit finish.

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Water Stress and Quality Decline during Storage of Tropical Leafy Vegetables

HAMID LAZAN, ZAINON MOHD. ALI, AI'ANI MOHD., and FALEHATUN NAHAR

ABSTRACT

The effect of water stress (excessive water loss) on postharvest quality during storage of tropical leafy vegetables *Brassica juncea* L. and *Amaranthus caudatus* L. at ambient (24°/28°C) and low temperature (2°/4°C) regimes has been investigated. Exposed, nonsealed *B. juncea* and *A. caudatus* leaves lost water rapidly during storage at either temperature regime resulting in accelerated decrease in leaf turgidity (wilting), chlorophyll, ascorbic acid and soluble protein content, and an increase in amino acids, in the stressed leaves. Alleviation of water stress by sealing the leaves with thin plastic film restricted chlorophyll, soluble protein and ascorbic acid loss, particularly when the leaves were cooled, and maintained their turgidity. Both leafy vegetables appeared tolerant to chilling injury.

INTRODUCTION

LEAFY VEGETABLES are ranked among the most perishable post-harvest products (Coursey, 1983; Salunkhe and Desai, 1984). This commodity is particularly prone to mechanical injury during handling, and loses water easily due to its high surface area to volume ratio (Burton, 1982). Excessive water loss leads to the development of water stress in the leaf. Consequently, leaf senescence and the attendant decline in post-harvest quality will be accelerated, as is the case with fruits and other vegetables (Ezell and Wilcox, 1959; Grierson and Wardowski, 1978; Thomas and Stoddart, 1980; Ben-Yehoshua et al., 1983; Kailasapathy and Koneshan, 1986). The purpose of this study was to investigate the effect of excessive water loss and storage temperature on changes in physical appearance as well as in water, ascorbic acid, chlorophyll, soluble protein and amino acids content of tropical leafy vegetables, *Amaranthus caudatus* (L.) and *Brassica juncea* (L.).

MATERIALS & METHODS

MATURE *Brassica juncea* (L.) and *Amaranthus caudatus* (L.) leaves of uniform color and size were obtained from the local market. The leaves were washed thoroughly with running water, and divided into two groups: individual leaves in one group were sealed by wrapping the entire leaf lamina with thin plastic film (low density polyethylene); the other group of nonsealed leaves served as controls. Unless stated otherwise, the leaves were stored at either ambient temperature (range 24–28°C) under low light condition (50–100 μmol m^{-2} sec^{-1} for 10–12 hr daily), or at low temperature (range 2–4°C) in the dark for a six-day experimental period.

Each day, leaf tissues, 2 × 40 cm^2, were cut from each side of the midrib for analysis of water, amino acid, soluble protein, ascorbic acid and chlorophyll content. Leaf midribs, which represent the major vascular tissues of the leaves, were left intact.

Soluble protein was extracted in 0.01M sodium phosphate buffer, pH 7.5, in a ratio of 10:1 (mL/g tissue fresh weight). After centrifugation (27000 × *g* for 30 min at 4°C), the protein in the supernatant fraction was precipitated with an equal volume of 10% trichloroacetic acid (TCA). The precipitate was recovered by centrifugation, redissolved in 0.1M NaOH and the protein content estimated by the method of Lowry et al. (1951) using bovine serum albumen as a standard.

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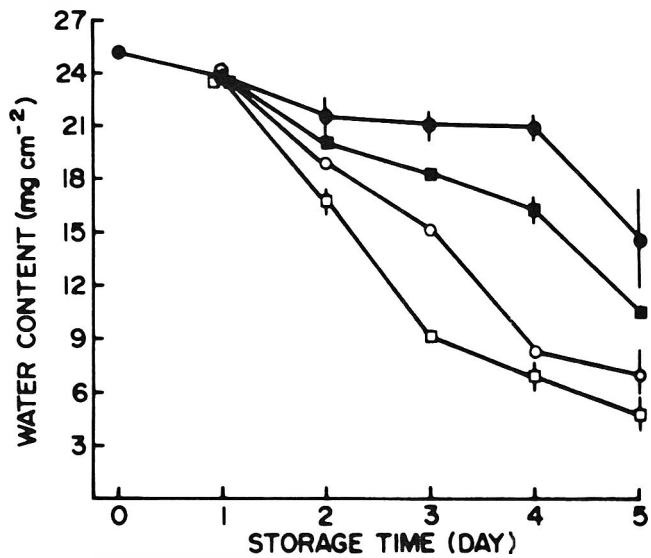

Fig. 1—Changes in water content of exposed (□,○) and wrapped in plastic (■,●) B. juncea leaves during storage at either 24–28°C (square) or 2–4°C (circles). Bars indicate standard error of means of three leaves.

The amino acid content in the supernatant, measured as α-amino nitrogen, was determined by the modified procedure of Moore and Stein (1954) using leucine as a standard. Chlorophyll in the residue fraction was extracted quantitatively in cold 80% acetone, and its amount estimated using the equation of Arnon (1949).

Ascorbic acid in *A. caudatus* was extracted in 0.4% oxalic acid, and its amount estimated by the procedure of Pearson (1970). Ascorbic acid in *B. juncea* was extracted in 3% metaphosphoric acid-acetic acid solution, and its content determined by the method of AOAC (1984). Amount of water in a given tissue was estimated as the difference between fresh and dry weight. Leaves were dried in an oven at 65°C for 24 hr.

Since leaves lost weight during storage, all results were thus expressed as amount per unit leaf area.

RESULTS & DISCUSSION

WATER CONTENT of exposed *B. juncea* and *Amaranthus caudatus* leaves decreased rapidly during storage for six days at both temperature regimes (Fig. 1 and 2). Rate of water loss was faster at ambient (24°/28°C) than at low temperature (2°/4°C). Sealing the leaves with thin plastic film effectively retarded evaporative water loss, thus enabling the leaves to maintain high water content during storage.

Leaf appearance and ascorbic acid content

Water deficit developed rapidly in the exposed leaves; the stressed leaves wilted, and later shrivelled due to excessive turgor loss. Leaf yellowing, as characterized by decrease in chlorophyll content, was also accelerated by water stress (Fig. 3; Table 1). Storage temperature seemed to have no effect on the rate of yellowing of *A. caudatus* leaves (Table 1). How-

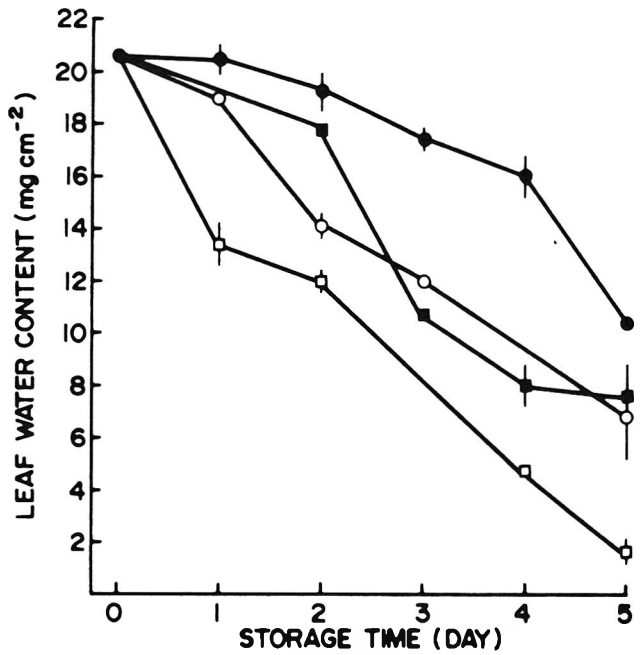

Fig. 2—Changes in water content of exposed (□,○) and wrapped in plastic (■,●) A. caudatus leaves during storage at either 24–28°C (squares) or 2–4°C (circles). Bars indicate standard error of means of three leaves.

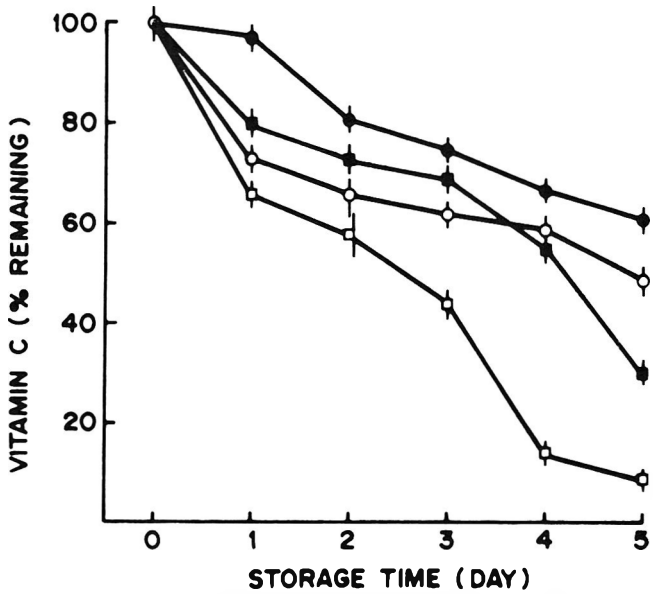

Fig. 4—Loss of ascorbic acid (vitamin C) during storage of exposed (□,○) and wrapped in plastic (■,●) B. juncea leaves at either 24–28°C (squares) or 2–4°C (circles). Initial content of vitamin C at day 0 of storage was 18.04 ± 1.08 μg cm⁻². Bars indicate standard error of means of three leaves.

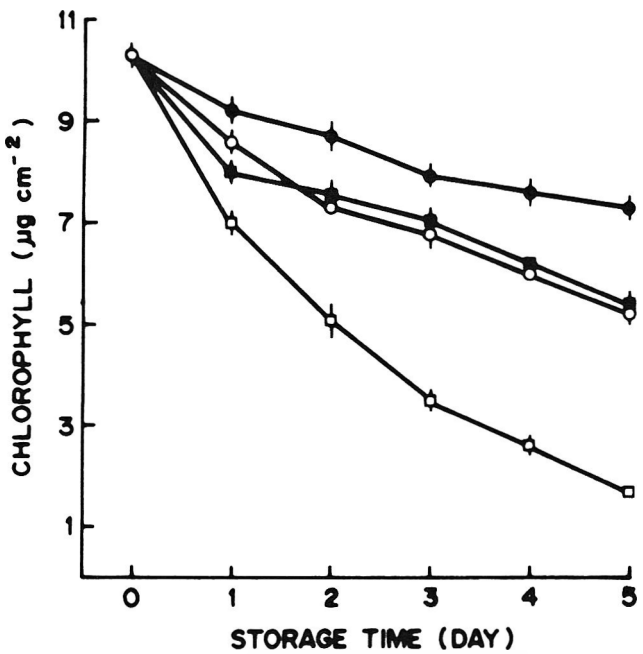

Fig. 3—Loss of chlorophyll during storage of exposed (□,○) and wrapped in plastic (■,●) B. juncea leaves at either 24–28°C (squares) or 2–4°C (circles). Bars indicate standard error of means of three leaves.

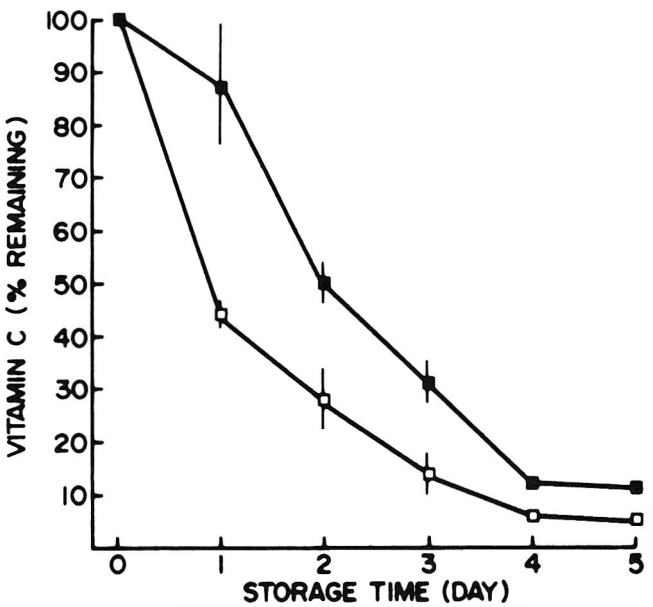

Fig. 5—Loss of ascorbic acid (vitamin C) during storage of exposed (□) and wrapped in plastic (■) A. caudatus leaves at 24–28°C. Initial content of vitamin C at day 0 of storage was 271.75 ± 10.18 ng cm⁻². Bars indicate standard error of means of three leaves.

ever, in *B. juncea* (Fig. 3), low temperature reduced the decrease in chlorophyll content during storage, and the effect was more pronounced in exposed than in sealed leaves.

Apart from leaf yellowing, water stress also accelerated the decrease in ascorbic acid content during storage of both leafy vegetables (Fig. 4 and 5). This ascorbic acid loss was markedly retarded when water loss from the leaves was restricted, particularly when the leaves were cooled (Fig. 4). Compared with fruits and other vegetables, ascorbic acid content in leafy vegetables is generally not stable, partly because of the tendency for the leaves to lose water rapidly (Fafunso and Bassir, 1976; Burton, 1982), and the decrease in ascorbic acid upon leaf

Table 1—Chlorophyll content (μg cm⁻²) of exposed and sealed A. caudatus *L. leaves during storage at either ambient or low temperature regime*[a]

Storage time (days)	Ambient (24–28°C)		Low Temp (2-4°C)	
	Sealed	Exposed	Sealed	Exposed
0	20.87 ± 1.43	20.87 ± 1.43	19.57 ± 2.00	19.57 ± 2.00
1	20.27 ± 1.35	18.47 ± 2.28	18.07 ± 1.53	18.80 ± 0.55
2	17.47 ± 1.10	16.13 ± 0.58	16.07 ± 0.32	15.20 ± 0.23
3	15.93 ± 0.97	14.70 ± 1.00	14.70 ± 1.03	11.93 ± 1.11
4	14.10 ± 1.56	12.83 ± 0.35	13.13 ± 0.23	9.97 ± 0.29
5	12.93 ± 0.77	9.50 ± 0.50	12.60 ± 1.69	7.07 ± 3.05

[a] Values are means of three leaves ± standard errors.

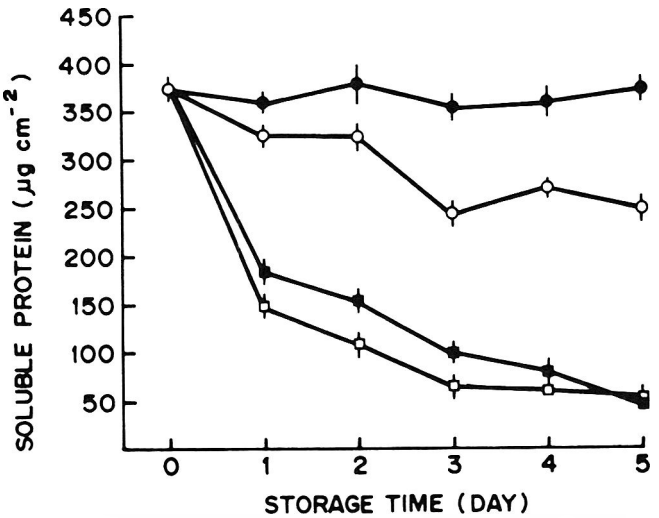

Fig. 6—Loss of soluble protein during storage of exposed (□,○) and wrapped in plastic (■,●) B. juncea leaves at either 24–28°C (squares) or 2–4°C (circles). Bars indicate standard error of means of three leaves.

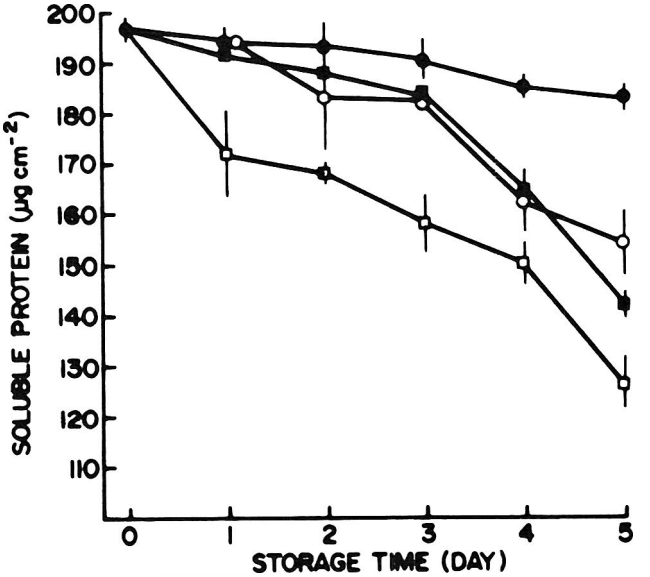

Fig. 7—Loss of soluble protein during storage of exposed (□,○) and wrapped in plastic (■,●) A. caudatus leaves at either 24–28°C (squares) or 2–4°C (circles). Bars indicate standard error of means of three leaves.

Table 2—Changes in soluble protein content (μg cm⁻²) of B. juncea *L. leaves kept either in the dark or exposed to ambient light condition*

Storage time (days)	Dark storage[a]	Ambient light condition[a]
0	278.54 ± 5.56	278.54 ± 5.56
1	260.87 ± 50.59	248.54 ± 13.49
2	258.01 ± 68.28	251.36 ± 8.42
3	243.01 ± 32.74	226.27 ± 42.75
4	222.63 ± 10.00	210.19 ± 3.03
5	199.96 ± 8.73	184.20 ± 10.00

[a] Storage temperature was 24–28°C, and ambient light condition was 50–100 μmol m^{-2} sec^{-1} for 10–12 hr daily. Leaf wilting was alleviated by immersing the petiolar base of the leaves in water. Values are means of three leaves ± standard errors.

Table 3—Soluble protein and α-amino nitrogen content (μg cm⁻²) during storage of exposed and sealed B. Juncea *leaves at 24–28°C*[a]

Storage time (days)	Sealed		Exposed	
	Sol. protein	α-Amino N	Sol. protein	α-Amino N
0	176.23 ± 25.09	31.72 ± 1.13	176.23 ± 25.09	31.72 ± 1.13
1	158.61 ± 25.41	56.33 ± 7.49	126.72 ± 22.11	47.27 ± 1.28
2	136.92 ± 31.28	62.31 ± 3.88	47.50 ± 6.75	77.95 ± 2.87
3	113.33 ± 9.32	43.39 ± 6.55	38.30 ± 0.32	88.30 ± 9.43
4	111.25 ± 7.39	27.07 ± 3.20	22.07 ± 2.99	92.88 ± 11.26
5	113.71 ± 11.00	29.02 ± 4.55	14.70 ± 2.69	91.95 ± 7.90

[a] Values are means of four leaves ± standard errors

wilting can sometimes be retarded by storage at low temperature (Ezell and Wilcox, 1959; Kailasapathy and Koneshan, 1986). The rapid loss of ascorbic acid in water-stressed leaves may be related to increased oxidation because of the reported increase in activity of ascorbic acid oxidase and other relevant oxidising enzymes in the stressed leaves (Todd, 1972).

Soluble protein and amino acid content

Soluble protein content of both leafy vegetables generally decreased during storage (Table 2; Fig. 6 and 7). Storing the leaves in the dark at ambient temperature (Table 2) failed to promote soluble protein decrease in *B. juncea* although darkness can promote protein degradation and leaf senescence in many plant species (Thimann, 1980). Water stress, too, may promote degradation as well as inhibit synthesis of proteins (Hsiao, 1973; Bewley, 1981), thus explaining the accelerated decrease in soluble protein content in the stressed leaves (Table 3; Fig. 6 and 7). Low storage temperature assisted in the preservation of soluble protein content in both stressed and nonstressed leaves respectively (Fig. 6 and 7). The fact that low temperature did not promote soluble protein and chlorophyll decrease suggests that both leafy vegetables are tolerant to chilling injury even though many tropical fruits and vegetables are susceptible to such injury (Wade, 1979; Graham, 1983; Kailasapathy and Koneshan 1986).

Amino acids released from protein hydrolysis would accumulate in the leaf's lamina (Table 3), a typical observation for detached-leaf systems (Lazan et al., 1983). Accumulation of amino acids in the sealed, nonstressed *B. juncea* leaves was only transitory; the amino acid content later decreased during storage. In the stressed leaves, amino acids continue to accumulate over the experimental period probably indicating a permanently impaired basipetal translocation of nitrogenous products out of the mesophyll cells into and through the leaf vascular tissues (Lazan et al., 1983). In both stressed and nonstressed leaves, the accumulated amino acids will be easily lost via leakage during cooking as the result of membrane damage.

Quality maintenance: water loss vs storage temperature

A combination of excessive water loss and higher storage temperature was highly detrimental to storage quality of *B. juncea* and *A. caudatus*. In *Brassica*, protein loss (Fig. 6) was affected more by high temperature than by water stress. In addition, the retarding effect of low storage temperature on chlorophyll and ascorbic acid loss from water-stressed *B. juncea* leaves (Fig. 3 and 4) was comparable with that of sealing treatment of leaves stored at ambient temperature. Likewise, similar observation was found with respect to soluble protein loss in *A. caudatus* leaves (Fig. 7). Thus, low storage temperature was relatively more effective in preserving postharvest quality of both leafy vegetables than alleviation of water stress. Relatively similar results were also reported by Ezell and Wilcox (1959; 1962) with other *Brassica* species. In the present study, greater extension of shelf-life can be achieved if the chilled leaves maintained their turgidity. Sealed *B. juncea* leaves, for instance, retained their green color and fresh appearance up to 35 days in the cold.

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Influence of Water Stress on Cold-Induced Sweetening in Leafy Vegetable, *Brassica juncea L.*

HAMID LAZAN, ZAINON MOHD. ALI, AI'ANI MOHD., and GIM BEE ONG

ABSTRACT

The occurrence of low temperature sweetening in *Brassica juncea* L., an apparently chilling-tolerant leafy vegetables, has been investigated in relation to the effect of water stress and postharvest age (i.e. fresh harvest vs previously stored samples). The magnitude of cold-induced sweetening in *B. juncea* leaves was estimated to vary from 10–145% relative to the corresponding nonchilled samples. Previously stored leaves showed greater tendency to accumulate sugars but was apparently less responsive to cold-induced sweetening than freshly harvested leaves. Water stress synergistically enhanced this low temperature sweetening response: markedly greater and/or faster accumulation of sugars occurred in leaves that were both chilled and stressed. The majority of the total sugar increase was attributable to non-reducing sugars.

INTRODUCTION

LOW TEMPERATURE SWEETENING, a phenomenon characterized by accumulation of sugars at low temperature, has been reported to occur in a wide variety of plants (Levitt, 1980; ap Rees et al., 1981). In some plants, cold-induced sweetening is believed to confer resistance against chilling injury (Heber and Santarius 1976; Santarius and Milde, 1977; Steponkus, 1984). However, ability to accumulate solutes rapidly is also a feature for many water-stress tolerant plants (Hanson and Hitz, 1982; Handa et al., 1983; Morgan, 1984). Interestingly, some aspects of chilling injury are reported to be identical with injury attributed to water stress (Heber and Santarius, 1976; Fukushima et al., 1977). Tropical leafy vegetables are particularly prone to excessive water loss during storage (Lazan et al., 1987). Many of them, too, are susceptible to chilling injury, although some, like *Brassica juncea* L. and *Amaranthus caudatus* L., appeared to be tolerant to such injury (Wade, 1979; Kalisapathy and Koneshan, 1986; Lazan et al., 1987). The purpose of the present study is to investigate low temperature sweetening in *B. juncea* and the effect of water stress on this response.

MATERIALS & METHODS

FRESH *Brassica juncea* leaves were obtained directly from farmers where experiments began within 2–4 hr of harvesting. Previously stored *Brassica* leaves of unknown postharvest age were purchased from the market. Following purchase, the leaves were selected for uniformity of size and color and treated as described previously (Lazan et al., 1987). Individual leaves were sealed with thin polyethylene film and stored either in the dark at low temperature 2–4°C) or under low light (50–100 μmol m^{-2} sec^{-1}) at ambient temperature (24–28°C). Corresponding set of stressed leaves for the respective storage temperature were also prepared. The stressed leaves were not sealed and, thus, would incur excessive water loss during storage for six days. Leaves were sampled at daily intervals for analysis of water and sugar content.

Leaf segments of known area (2 × 40 cm^2) were extracted three times with hot 80% ethanol. The combined ethanolic extracts were evaporated *in vacuo* at 35°C and the solutes redissolved in distilled water. After passing through a cellulose acetate filter (0.4μ), total sugars in the clear aqueous extract was determined by phenol-sulfuric acid method (Dubois et al., 1956) and reducing sugars by the procedure of Nelson (1944). Nonreducing sugar was estimated as the difference between total and reducing sugars.

Leaf water content (mg cm^{-2}) was determined as described previously (Lazan et al., 1987). Relative water content (RWC) of the leaf was determined using the following equation:

$$RWC = \frac{\text{Fresh wt} - \text{Oven dry wt}}{\text{Turgid wt} - \text{Oven dry wt}} \times 100$$

Following fresh weight determination, the leaf was immersed in distilled water at 24°C for 6 hr and its turgid weight recorded. Leaf drying was in an oven at 65°C for 24 hr. All results are presented as mean values of three leaves ± standard errors.

RESULTS & DISCUSSION

Changes in leaf water status during storage

Leaf water status can be expressed in a variety of ways such as RWC (Fig. 1) and also as water content per unit area, WC (Fig. 2) . Both RWC and WC are effective indicators of changes in leaf water status during storage of *B. juncea* leaves for six days.

Water status of stressed (nonsealed) leaves decreased rapidly during storage particularly those kept at ambient temperature (Fig. 1 and 2). In contrast, water status of nonstressed (sealed) leaves kept at low temperature was maintained at a steady level of about 94% RWC. Those nonstressed leaves kept at ambient temperature, however, incurred some water loss following storage for one day. Thereafter, their water status was maintained at about 78% RWC. Water stress would accelerate premature senescence of leaves as characterized by rapid degradation of chlorophyll and proteins (Thimann, 1980; Thomas and Stod-

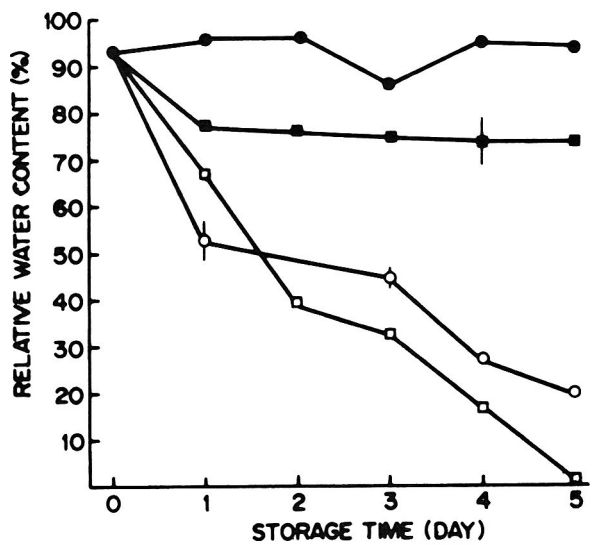

Fig. 1—Changes in relative water content (RWC) of freshly harvested stressed (□,○) and nonstressed (■,●) leaves of B. juncea during storage at either 24—28°C (squares) or 2–4°C (circles).

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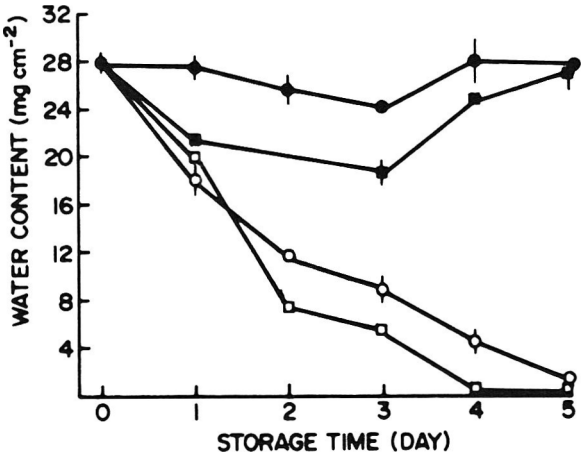

Fig. 2—Changes in water content (WC) of freshly harvested stressed (□,○) and nonstressed (■,●) leaves of B. juncea during storage at either 24–28°C (squares) or 2–4°C (circles).

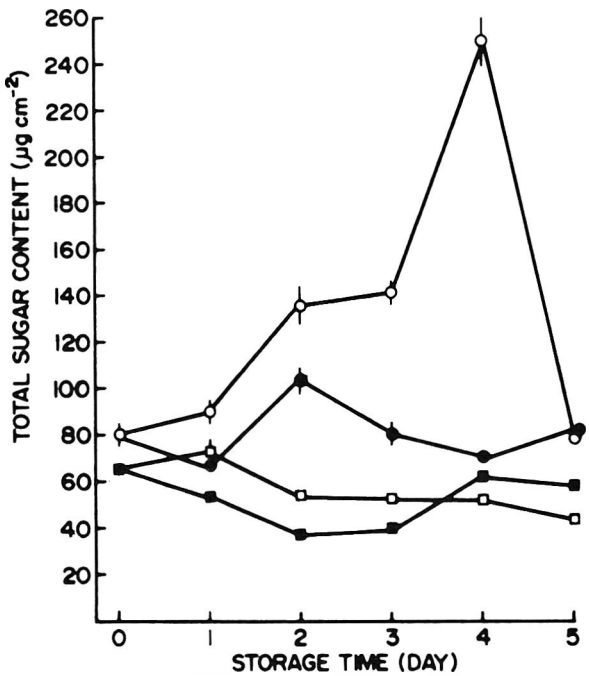

Fig. 3—Changes in total sugar content of freshly harvested stressed (□,○) and nonstressed (■,●) leaves of B. juncea during storage at either 24–28°C (squares) or 2–4°C (circles).

dart, 1980). However, alleviation of water stress by sealing *B. juncea* leaves with polyethylene film retarded not only leaf yellowing and protein degradation but also the decrease in ascorbic acid content during storage (Lazan et al., 1987). Nevertheless, evidence with some chilling-tolerant leafy vegetables indicated that low storage temperature was apparently more effective in preserving postharvest quality than the maintenance of high water status (Ezell and Wilcox, 1959; 1962; Kailasapathy and Konoshan, 1986; Lazan et al., 1987).

Changes in sugar levels during storage

Changes in total sugar levels, and its reducing and nonreducing sugar components during storage of freshly harvested *B. juncea* leaves are shown in Fig. 3 and 4. Total sugar level in nonstressed leaves kept at ambient temperature intially decreased during storage. The sugar level later increased and tended to stabilize at an initial day 0 level of about 60 μg cm $^{-2}$ (Fig. 3). Exposed leaves, despite losing water rapidly, also did

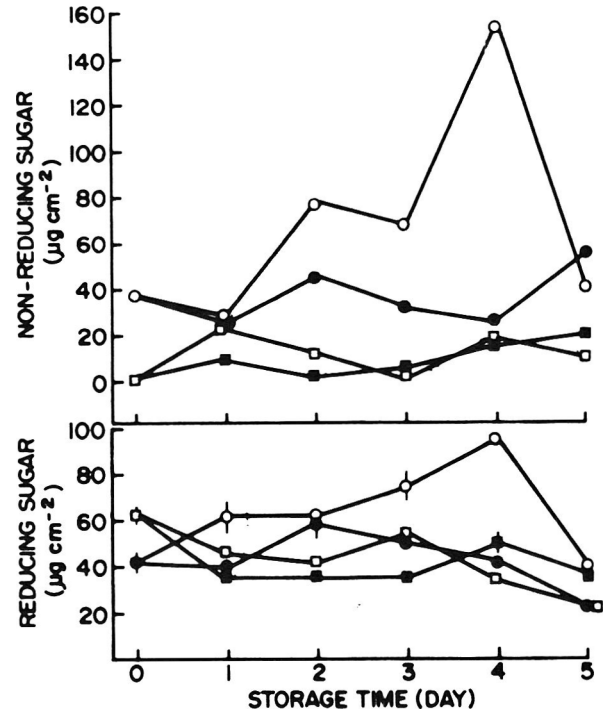

Fig. 4—Changes in reducing and non-reducing sugar level of freshly harvested stressed (□,○) and nonstressed (■,●) leaves of B. juncea during storage at either 24–28°C (squares) or 2–4°C (circles).

not accumulate sugars as commonly observed in water-stressed tissues (Munns et al., 1979; Aspinall, 1986; Barlow, 1986) probably indicating an unimpeded sugar transport into and through the leaf's vascular tissues (Lazan et al., 1983). The sugar content of the exposed leaves, instead, decreased during storage. The stressed leaves, however, maintained higher total sugar levels than the corresponding non-stressed leaves during the first four days of storage. In contrast, substantial accumulation of sugars occurred in stressed leaves kept at low temperature (Fig. 3). Total sugar also accumulated, albiet temporarily, in the corresponding non-stressed leaves but the accumulation was not as extensive at that observed in the stressed leaves.

The extent of cold-induced sugar accumulation at any given time during storage of nonstressed leaves can be estimated as the difference in amounts of sugar between leaves kept at ambient and those kept at low temperature (Fig. 3 and 5; Table 1). From Table 1 it appeared that low temperature sweetening also occurred in previously stored leaves but the magnitude of the sweetening response differed from that of the freshly harvested samples. In the previously stored leaves, the maximum observed cold-induced-sweetening response was about 27.5% compared with 145% in the freshly harvested samples. Under local conditions, it is not uncommon for the previously stored leaves to experience repeated incipient wilting and rewetting during storage and handling. Such conditions may probably be conducive to build up of sugars in the leaves as reflected by the continued increase in total sugar content during storage of the previously stored leaves irrespective of experimental treatments (Fig. 5). Although the previously stored leaves appeared to have greater tendency to accumulate sugars (Fig. 5 vs Fig. 3), they however, were found to be relatively less responsive to cold-induced sweetening than the freshly harvested samples (Table 1). Nonetheless, it is conceivable that leaf age at harvest and time of day when the leaf was harvested may also influence the extent of low temperature sweetening response in *B. juncea* because both factors may determine the amount of starch present in the leaves prior to harvesting (see discussion below). The amount of sugar accumulated at low temperature was gen-

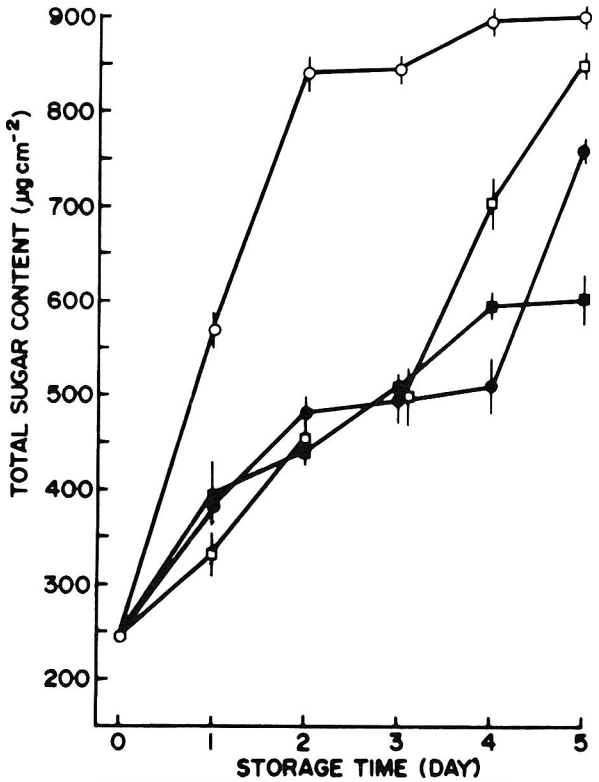

Fig. 5—Changes in total sugar content of previously stored stressed (□,○) and nonstressed (■,●) leaves of B. juncea during storage at either 24–28°C (squares) or 2–4°C (circles).

Table 1—Cold-induced sugar accumulation in freshly harvest and previously stored non-stressed leaves of B. juncea

Storage time (Day)	Freshly harvested[a]	Previously stored
	%	
0	0	0
1	11.0[b]	(s.d)[d]
2	144.8	9.3
3	64.3	(s.d.)
4	(18.7)[c]	(16.6)
5	13.6	27.6

[a] Corrected for differences in sugar content at day 0 between leaves stored at 24–28 C and at 2–4°C.

[b] Numbers indicate differences in amounts of sugar content between leaves kept at 2–4 C and those kept at 24–28°C.

[c] Values in brackets ( ) indicate sugar content in leaves kept at 24–28°C was higher than those kept at 2–4°C.

[d] s.d. small difference; not exceeding 3.5%.

erally reported to be small; it varies from 40% to 900% of initial value depending on plant type, varietal difference and growth conditions prior to harvest (ap Rees et al., 1981; and references cited therein). In *B. juncea* the observed cold-induced sweetening response in fresh and stored samples varied from 10% to 145% (Table 1).

Consistent with results reported earlier (Fig. 3), water stress also modified the observed low temperature sweetening response in previously stored *B. juncea* leaves (Fig. 5). Both factors, water stress and low temperature, acted synergistically. Markedly faster and/or greater accumulation of total sugar occurred in leaves that experienced simultaneous wilting and chilling. This response was observed even in leaves that failed to accumulate sugars when stressed at ambient temperature (Fig. 3 vs Fig. 5). In both chilled and non-chilled leaves, the majority of the sweetening effect was attributable to non-reducing sugars (Fig. 4 and 6). Likewise, non-reducing sugars was also the principal contributor to total sugar increase in *A. caudatus* (data unpublished). However, unlike *B. juncea*, cold-induced sweetening response was not observed in *A. caudatus*;

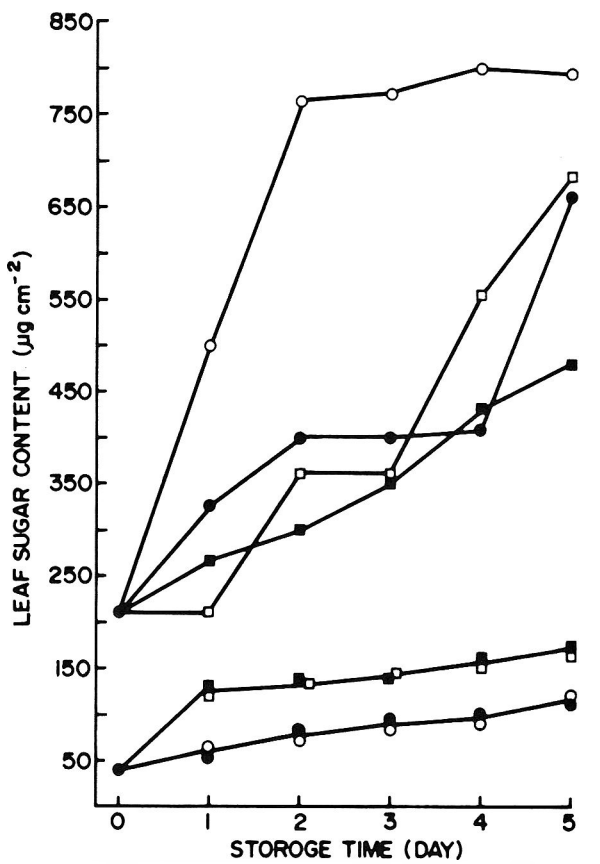

Fig. 6—Changes in reducing (bottom graph) and nonreducing (top graph) sugar level of previously stored stressed (□,○) and nonstressed (■,●) leaves of B. juncea during storage at either 24–28°C (squares) or 2–4°C (circles).

the leaves appeared to be more responsive to water stress than to low storage temperature.

Sugar accumulation: probable carbohydrate source and significance

Some of the physiological consequences that may arise following leaf detachment are promotion of leaf senescence and also, in some cases, cellular respiration (Thimann, 1980; Solomos, 1983). In addition, basipetal transport of substances out of the leaf would also be restricted and this may have consequence on the rate of accumulation of sugars and other solutes within the detached leaf (Lazan et al., 1983). Since leaf midrib was not included in the analysis of sugar, thus, net accumulation of sugar in the lamina of *B. juncea* leaves at any given time during storage will be jointly influenced by the rate of sugar production and its export into and through the leaf's midrib, and also on the rate of respiration. Water stress and storage temperature may modify the relative influences of these factors on sugar accumulation. Low temperature may restrict export into the vascular tissue while water stress may affect hydrolysis of polymeric carbohydrates, and these may probably account for the observed synergistic effect of low temperature and water stress on sugar accumulation in *B. juncea* leaves. However, the inhibitory effect of low temperature on translocation in plants is usually not permanent (Moorby, 1981) while sugar production in the detached leaves may eventually be limited by lack of substrate. Thus, the decrease in sugar content later during storage of leaves in the cold may probably be attributed primarily to respiratory loss, and partly also to recovery of sugar export into and through the vascular tissues.

In any detached leaf system, sugars that accumulate within the leaf must be produced exclusively *in situ*. In the case of *B. juncea* leaves kept at ambient temperature, contribution of

current photosynthesis to sugar accumulation may be expected to be small, partly because of the relatively low light conditions and partly due to the effect of leaf aging. In the case of leaves kept at low temperature and in the dark, sugar production would ultimately be derived from hydrolysis of starch stored in the chloroplast, and probably, also, from degradation of relatively more resistant structural (i.e. cell wall) polysaccharides. Our more recent data (unpublished) indicated that the decrease in starch content during storage of *B. juncea* could account only partially for the observed cold-induced increase in sugar content in water-stressed leaves. Cell wall polyuronide content, on the other hand, increased during storage, thus, discounting its likely involvement as a possible and additional carbohydrate source for sugar accumulation in the cold. In potatoes kept in the cold, the accumulated sugar, which was predominantly sucrose, was derived solely at the expense of starch (Isherwood, 1976; ap Rees et al., 1981). In many plants, sucrose or its derivatives, viz. raffinose and stachyose, were the principal sugars (i.e. nonreducing) involved in low temperature sweetening (Levitt, 1980; ap Rees et al., 1981).

Accumulation of sugars and other nontoxic solutes at low temperature may have adaptive value because of the ability of these solutes to preserve membrane stability against chilling injury (Heber and Santarius, 1976; Santarius and Milde, 1977; Steponkus, 1984). Water stress tolerant plants also adjust osmotically by accumulating solutes rapidly and extensively when stressed (Hanson and Hitz, 1982; Morgan, 1984; Aspinall, 1986). The stimulation of cold-induced sugar accumulation in *B. juncea* by water stress may be indicative of its adaptive response to stressful situations. Thus, the ability of *B. juncea* leaves to tolerate chilling injury (Lazan et al., 1987) was perhaps a natural consequence of such adaptation.

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Quality of Fungicide Treated and Individually Shrink-Wrapped Tomatoes

GREG J. HULBERT and SANTI R. BHOWMIK

ABSTRACT

Fungicide-treated mature green and pink tomatoes were individually shrink-wrapped with plastic films and stored at 13°C and 18°C. Shrink-wrapped pink tomatoes lost less water than unwrapped tomatoes, but there were no significant shelf-life or quality differences. The shelf-life of mature green tomatoes at 18°C, however, was increased 10 days by shrink-wrapping. Color development of wrapped mature green tomatoes at 18°C was delayed. Wrapped fruit at both temperatures were firmer than the controls. Titratable acidities and soluble solids of wrapped fruit were lower than those of the unwrapped fruit at 13°C, while no significant changes were observed in citric acid or monosaccharide concentrations. The differences in titratable acidities and soluble solids in the wrapped and unwrapped fruit stored at 18°C were not significant.

INTRODUCTION

THE WORLD FOOD SHORTAGE and the consumer's increasing desire for high-quality and nutritional foods, has created a demand for improved methods of storage of fresh fruits and vegetables. Because of increased consumption of fresh produce, a longer market season would be desirable for both domestic and export markets. This is especially true of tomatoes which rank number one in the contribution of vitamins and minerals to the U.S.A. diet (Rick, 1978). Although tomato fruits have a per capita consumption rate of 25.5 kg/yr (Rick, 1978), surveys show that consumers are unhappy about the quality of most fresh tomatoes found in the supermarket. In one study (Handy and Pfaft, 1975), tomatoes received the highest dissatisfaction rating of the 31 food products that were rated. Complaints were made about the ripeness, texture, taste and appearance of supermarket tomatoes. Today, criticism of the dull-orange, gas-ripened fruits sold during the winter months, is especially high. An ideal storage method would increase the shelf-life of tomatoes without causing significant loss of quality.

Currently, tomatoes are preserved by storage in environmental chambers at low temperatures, high humidities and/or modified atmospheres. Although these methods are beneficial in many ways, they also have several drawbacks. Low temperatures may prevent tomatoes from developing a full red color (Pantastico et al., 1975). They also tend to increase the acid content (Kader et al., 1978) and decrease the sugar content (Buescher, 1975) of the fruits. Even brief refrigeration may cause a loss of the characteristic "tomato-like" flavor and leave them tasting bitter or bland (Rick, 1978). In addition, the high concentration of CO_2 in controlled or modified atmospheres may lead to tissue damage, increased rotting and/or off-flavor production (Parsons et al., 1970).

Recently, various researchers (Ben-Yehoshua, 1983; Bhowmik and Sebris, 1984; Hulbert, 1986) have used the individual shrink-wrapping technique for prolonging the shelf-life of fresh produce. This technique involves the use of a heat-shrinkable polymeric film that is wrapped around an individual fruit or vegetable to create a second protective layer. Individual packaging of fruits in this manner has many advantages. Ripening is delayed due to the altered microatmosphere that is created around the fruit, and the film acts as a very good barrier to water so that almost no moisture loss occurs. It also prevents the spread of rot from one fruit to another, improves sanitation during handling by consumers' and facilitates the pricing and labeling of individual fruit.

Early studies using this method were applied mainly to citrus fruits such as grapefruit, oranges and lemons. Because the application of shrink-wrapping to citrus fruits was successful in these studies, fruit-producing companies have begun to proceed with commercial development. By September 1985, almost 500,000 cartons of shrink-wrapped citrus had been sent to the market (Idol, 1985).

However, the individual shrink-wrapping method has not been extensively applied to tomatoes. Ben-Yehoshua et al., (1979) found an extension in the shelf-life of tomatoes that were wrapped at the breaker stage due to reduced weight loss, delayed ripening and a greater firmness retention. Similar results were obtained for mature green fruits that were wrapped immediately after or immediately before treatment with ethylene (Risse et al., 1984, 1985). However, in both these studies, the decay rate was approximately the same for the wrapped and unwrapped fruits. There was an increase in the quality of the tomatoes due to shrink-wrapping but no real extension in the shelf-life. The objective of this study was to determine the effects of individual shrink-wrapping on the quality and shelf-life of pink and mature green tomatoes that were wrapped after being dipped in a fungicide solution.

MATERIALS & METHODS

Fruit handling

Flora-Dade cultivar tomatoes were harvested from the horticultural farm at Clemson University (Clemson, SC). Tomatoes were picked at mature green and pink stage at random and selected by visual examination and feel. Fruits were washed with water and dipped in a 0.708% solution of Dithane M-22 (manganese ethylene bisdithiocarbamate) with zinc fungicide for 90 sec. Wet surfaces of the fruits were dried by allowing them to stand in air circulated by a fan at ambient temperature (24°C). Tomatoes at each maturity level were divided into two groups. The control group was not wrapped, and the other group of tomatoes was individually wrapped in shrink-film with an impulse heat sealer (Weldetron Corporation, Newark, NJ, Model 6100-S) and a shrink tunnel (Bestronic Mini Shrink Tunnel, Model MT14-8, Beseler Corporation, Florham Park, NJ). Both wrapped and unwrapped fruits were stored at 13°C or 18°C and at 85–90% relative humidity. Mature green tomatoes were wrapped with 60-gauge Cryovac D-950 shrink-film, water vapor transmission rate, (WVTR) = 0.88g/24 hr, m^2, atm at 38°C and 0% RH; O_2 transmission rate = 7119 mL (at standard temperature and pressure)/24 hr, m^2, atm at 23°C and 0% RH). Water vapor transmission rate and oxygen transmission rate were determined at Cryovac Division, W.R. Grace Company (Duncan, SC) using PERMATRAN-W1 and OX-TRAN-100 instruments, respectively (Modern Controls, Inc., Minneapolis, MN). Eighty wrapped and eighty unwrapped tomatoes were stored at each temperature level.

Sample tomatoes were tested weekly to determine changes in weight, pH, titratable acidity, % soluble solids, color and firmness. On alternating weeks, samples were frozen for subsequent analyses of sugar

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and citric acid. Fruits were also visually examined twice a week for microbiological decay or physical deterioration as indicated by formation of dark spot or fungal growth. Spoiled fruits were immediately discarded.

Pink tomatoes were wrapped with 60-gauge Cryovac D-950 shrink-film in one study and 60-gauge Cryovac D-955 shrink-film (WVTR = 0.80 g 24 hr. m^2. atm at 38°C; O_2 transmission rate = 6000 mL/24 hr. m^2, atm at 23°C and 0% RH) in the other. In both studies, 40 wrapped and 40 unwrapped pink tomatoes were stored side-by-side at each storage temperature. They were subjected to the same analyses as the mature green fruits except that sugar and citric acid were not determined.

Color and firmness

Four tomatoes from each of the four test groups were randomly chosen for weekly analyses. Color was measured externally with a Hunter Color Difference Meter (Model D25-2, Hunter Associates, Ralston, VA) and the L.a $_L$ and b_L color values were recorded for each fruit. These values were computed as the average of four measurements taken along the equator of the fruit using a 14mm diameter orifice.

After determining the color, the firmness of the same tomatoes was measured with an ASCO firmness meter (Model FM-3C, Agricultural Specialty Company, Hyattsville, MD). This particular firmness meter was designed especially for use with tomatoes based on investigation of Garett et al. (1960). These researchers reported excellent agreement between firmness index determined by this apparatus and firmness ranking of a sensory panel for tomatoes. The tomato was placed in the gripping chain of the firmness meter with the calyx end facing the operator. The counterweight brake was then released so that the counterweight pulled the chain firmly around the fruit. After resetting the brake, a 500g weight was released for 10 sec causing the chain to tighten even more firmly around the fruit. The relative compression of the tomato under that force was recorded directly from the instrument as a dimensionless number, termed the firmness index. As in color analyses, four measurements were performed on each tomato. Fruits were rotated a quarter of a turn after each measurement.

The four tomatoes in each group were combined into one sample for determination of pH, titratable acidity and % soluble solids. After removal of the cores, the tomatoes were pureed in a Sunbeam Food Processor (Model 14-11, Sunbeam Corp., Chicago, IL).

Soluble solids and titratable acidity

The % s.s was determined by using a drop of the blended sample in a refractometer (ABBE Refractometer, Model 10450, American Optical Corp., Buffalo, NY). For titratable acidity, 50g distilled water were added to 10g puree and blended with an Osterizer (Oster Corporation, Milwaukee, WI) pulse-matic blender. The blended sample was then titrated with standard 0.1N NaOH solution to a pH 8.0 using a PH ION meter (Fisher Accumet, Model 230A, Fisher Scientific Company, Springfield, NJ). The acidity as expressed in terms of milliequivalent (meq) of normal sodium hydroxide solution per 10g tomato.

Sugar and citric acid

The sugar and citric acid analyses were performed by a method adapted from Buescher (1975). Glucose, fructose and citric acid derivatives were prepared by adding a mixture of hexamethyldisilazane, trimethylchlorosilane and pyridine in 3:1:9 portions to the acid and sugar fractions of the samples (Johnson and Carroll, 1973). A solution containing 1meq of citric 10g, 1% fructose and 1% glucose in distilled water was used as a reference solution to check the accuracy of this method before running the experimental analysis. Sugars and citric acid were finally determined using a gas chromatograph (Model ME-220, Microtek, Tracor Analytical Instruments, Austin, TX) containing a 305cm × 3mm stainless steel column packed with 80/100 mesh 3% SE-52 Chromosorb WHP (Supelco, Inc., Bellefonte, PA). The chromatograph had a flame ionization detector and an automatic digital integrator (Model CRS-204, Infotronics, Austin, TX).

Statistical analysis

A factorial treatment design was employed to analyze the firmness and color difference data. The experimental design was a split plot with treatments as the whole plot factor, and days of storage as the sub-plot factor. An analysis of variance (SAS, 1982) was used to determine the magnitude of error. In a significant treatment effect ($P<0.05$) was detected, a least significant difference test ($\alpha = 0.05$) was used to find treatments that were different. The statistical analysis of the % s.s. and titratable acidity were conducted in the same manner except that the experimental design was a randomized block design with days as blocks.

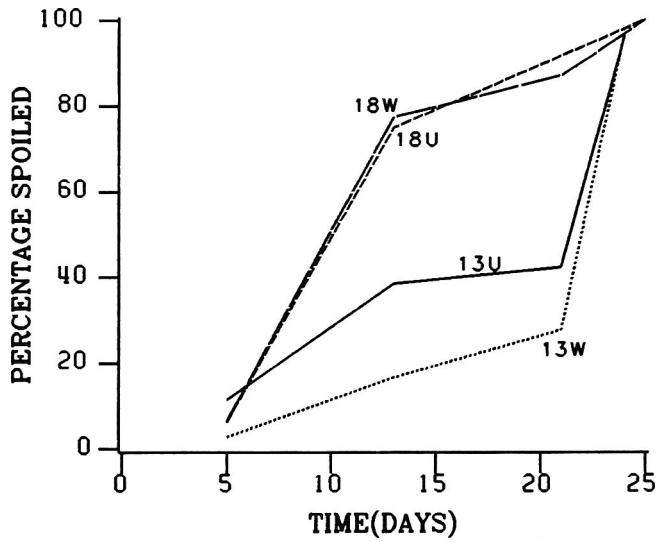

Fig. 1.—Spoilage rate of pink tomatoes wrapped in Cryovac D-950 shrink-film. The symbols 13U and 13W represent the unwrapped and wrapped fruits stored at 13°C and the symbols 18U and 18W represent the unwrapped and wrapped fruits stored at 18°C.

Table 1—Weight loss[a] of pink tomatoes wrapped in Cryovac D-950 shrink film

	Weight loss (%/day)	
	18°C	13°C
Unwrapped	0.38	0.14
Wrapped	0.03	0.01
Unwrapped Wrapped (Ratio)	11.3	12.3

[a] Weight loss is reported as single value because linear correlation coefficients (r -0.99) were obtained in least square analysis.

RESULTS & DISCUSSION

THERE WAS NO SUBSTANTIAL CHANGE in the shelf-life of pink tomatoes due to shrink-wrapping as indicated in Fig. 1.spoilage rates of wrapped and control fruits at 18°C were almost identical due to the fact that fruits at the pink stage of maturity had already reached the climacteric respiration period, and respiration rates were decreasing. Because the films had only a moderate oxygen barrier, the oxygen supply was not sufficiently depleted to further reduce fruit respiration rates. Thus, senescence of wrapped fruits was not significantly retarded. The slight increase in shelf-life of wrapped fruits at 13°C was probably due to increased water retention. However, a very low percentage of the pink tomatoes lasted longer than 25 days. No significant quality differences were observed over this short storage period.

Weight losses of pink fruits during storage are shown in Table 1. At both temperatures shrink-wrapping reduced the weight loss by more than a factor of 10, whereas storage at the lower temperatures decreased the weight loss threefold due to temperature effects on vapor pressure difference. Therefore, the advantage of shrink-wrapping pink tomatoes was greater water retention, resulting in reduced shrinkage and shriveling of the fruits.

The shelf-life of mature green tomatoes stored at 18°C was increased 10 days due to shrink-wrapping. This difference was

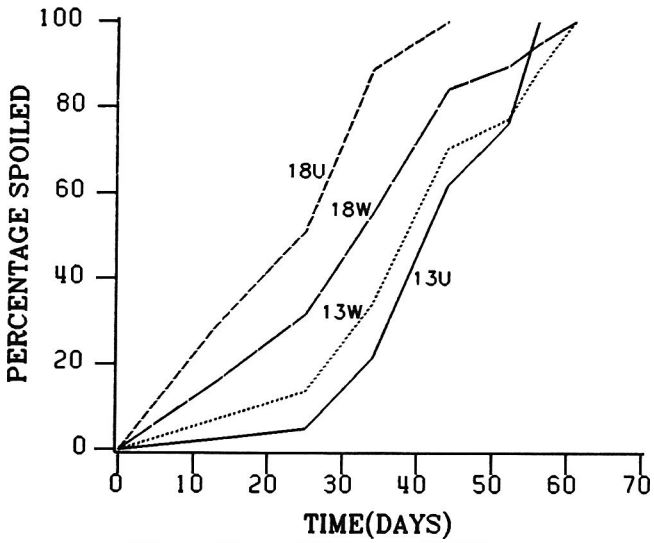

Fig. 2.—Spoilage rate of mature green tomatoes. The symbols 13U and 13W represent the unwrapped and wrapped fruits stored at 13°C and the symbols 18U and 18W represent the unwrapped and wrapped fruits stored at 18°C.

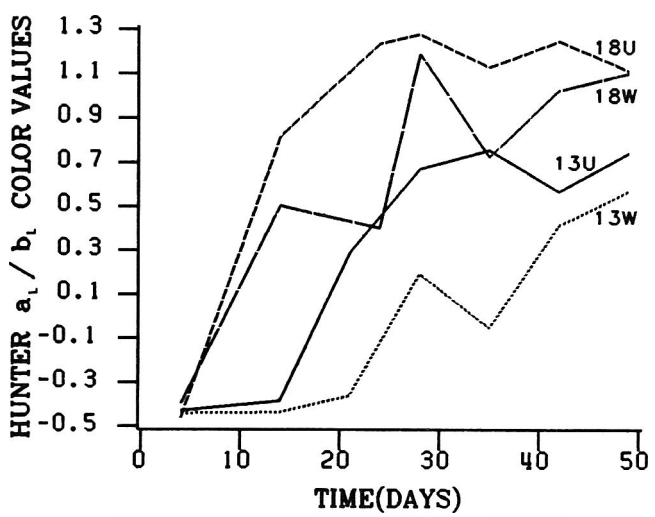

Fig. 3.—Color development of mature green tomatoes. The symbols 13U and 13W represent the unwrapped and wrapped fruits stored at 13°C and the symbols 18U and 18W represent the unwrapped and wrapped fruits stored at 18°C.

Table 2—Weight loss[a] of mature green tomatoes wrapped in Cryovac D-950 shrink film

	Weight loss (% day)	
	18°C	13°C
Unwrapped	0.25	0.16
Wrapped	0.03	0.01
Unwrapped Wrapped (Ratio)	7.9	11.0

[a] Weight loss is reported as single value because linear correlation coefficients ($r > 0.99$) were obtained in least square analysis.

Table 3—pH values of mature green tomatoes[a]

Days of storage	pH of tomatoes in test groups			
	13U	13W	18U	18W
5	4.10	4.04	4.01	4.20
14	3.96	4.04	3.79	3.94
23	4.18	4.34	4.07	4.10
28	4.02	4.14	4.07	4.18
35	4.13	4.26	3.90	4.18
42	4.17	4.35	4.14	4.11
49	4.08	4.21	4.08	4.10

[a] The symbols 13U and 13W represent the unwrapped and wrapped fruits stored at 13°C and the symbols 18U and 18W represent the unwrapped and wrapped fruits stored at 18°C.

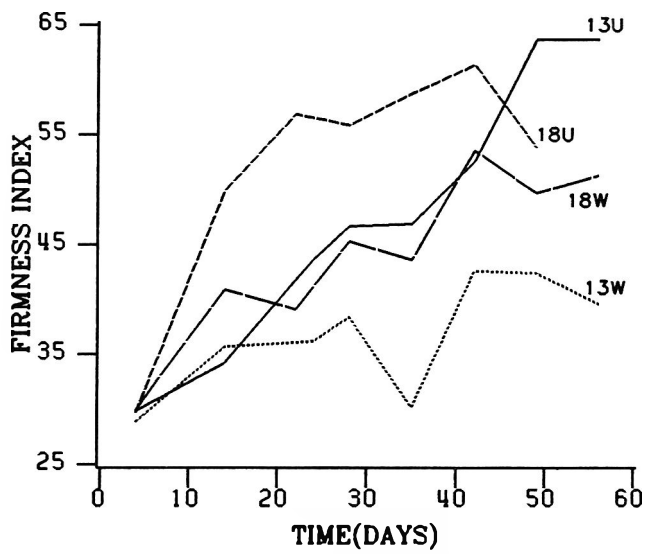

Fig. 4.—Firmness index of mature green tomatoes. The symbols 13U and 13W represent the unwrapped and wrapped fruits stored at 13°C and the symbols 18U and 18W represent the unwrapped and wrapped fruits stored at 18°C. Firmness is inversely proportional to firmness index.

significant at the 95% confidence level [t-test (LSD)], but these fruits still spoiled at a greater rate than tomatoes stored at 13°C. Shrink-wrapping prolonged the shelf-life of mature green fruits at 18°C by decreasing the respiration rates. The fruits were wrapped before they reached the climacteric stage, and therefore, the rapid rise in respiration that occurred during this ripening stage was inhibited due to the barrier properties of the film. Low temperatures decreased spoilage rates even further because they lowered both the microbiological activity and respiration rates. However, the effects of shrink-wrapping and low temperature storage were not additive. Wrapped fruits stored at 13°C spoiled as quickly as controls. Figure 2 indicates that shrink-wrapping can be used to reduce spoilage of mature green tomatoes at 18°C.

Figure 3 shows that the color development of mature green fruits was delayed by shrink-wrapping. However, at 18°C, wrapped and control fruits eventually developed a full-red color, whereas at 13°C both groups developed only a dull-orange color. The dull-orange color is highly undesirable to consumers, and thus, shrink-wrapping had a definite advantage over low temperature storage as far as color development was concerned.

Changes in firmness of the mature green tomatoes are shown in Fig. 4. At both 13°C and 18°C, wrapped tomatoes were considerably more firm than controls ($P<0.05$).

The primary reason for the retention of firmness of shrink-wrapped tomatoes was the decrease in water loss. Decrease in water loss is expected to be associated with significant reduction in the movement of water molecules (water stress) from the cell structure of the tomato flesh. The unbound water, together with the bound water remaining in the cells, maintains the firmness of the fruits. Disruption of the cell structure matrix due to movement of water molecules was also reduced.

Table 2 shows the weight loss of mature green tomatoes. Shrink-wrapping reduced the weight loss of fruits at 18°C by a factor 7.9 and the weight loss of fruits at 13°C by a factor of 11.0.

Figure 5 shows the titratable acidity of the mature green

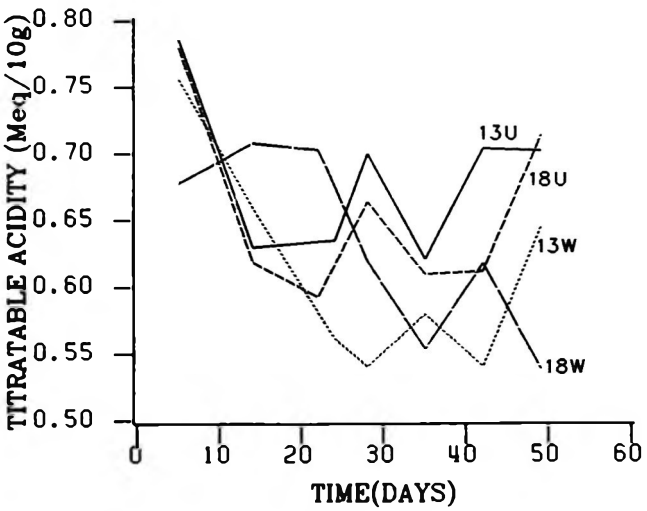

Fig. 5.—Titratable acidity of mature green tomatoes. The symbols 13U and 13W represent the unwrapped and wrapped fruits stored at 13°C and the symbols 18U and 18W represent the unwrapped and wrapped fruits stored at 18°C.

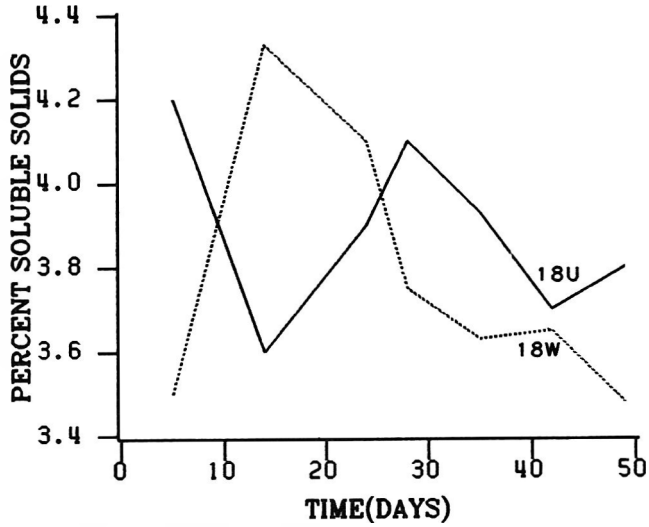

Fig. 6.—Percent soluble solids of mature green tomatoes held at 13°C. The symbols 13U and 13W represent the unwrapped and wrapped fruits stored at 13°C.

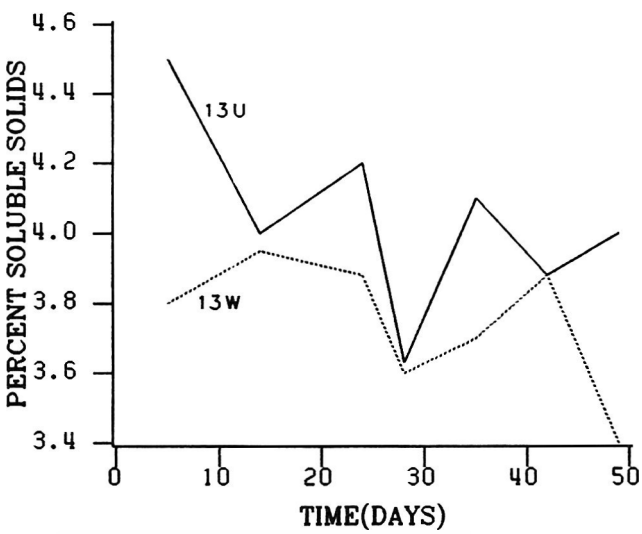

Fig. 7.—Percent soluble solids of mature green tomatoes held at 18°C. The symbols 18U and 18W represent the unwrapped and wrapped fruits stored at 18°C.

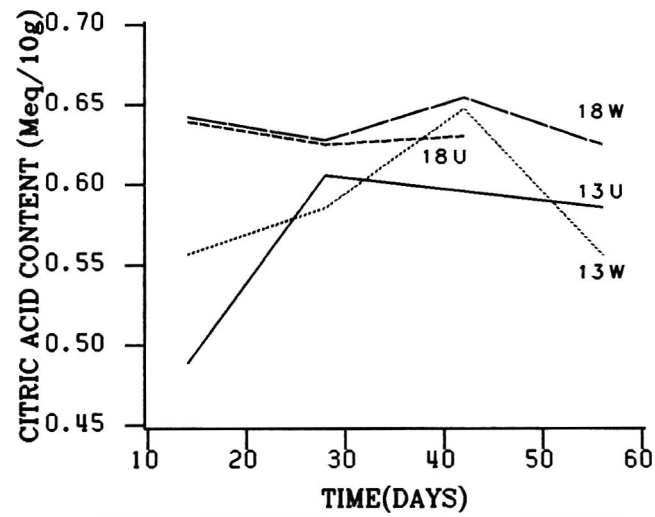

Fig. 8.—Citric acid concentration of mature green tomatoes. The symbols 13U and 13W represent the unwrapped and wrapped fruits stored at 13°C and the symbols 18U and 18W represent the unwrapped and wrapped fruit stored at 18°C.

tomatoes. There was considerable fluctuation in the data, but the general trend was a decrease in the titratable acidity with time. This trend was more pronounced for wrapped fruits than for unwrapped fruits. The lower titratable acidity of wrapped fruits was probably due to the fact that they retained more water than the controls. The difference in titratable acidity between wrapped and unwrapped tomatoes at 13°C was significant ($P<0.05$), but the difference between unwrapped and wrapped tomatoes at 18°C was negligible.

The results of the percent soluble solids study are shown in Fig. 6 and 7. Again, the general trend was a decrease in the % soluble solids with time. Because percent soluble solids acts as a rough index of the amount of sugar in tomatoes, it would be expected that these values would increase rapidly during the ripening of tomatoes and then decrease. Wrapped fruits stored at 18°C followed this pattern. Unwrapped fruits at 13°C and 18°C had, however, reached the point of decreasing % soluble solids during the first 2 wk of storage. The ripening process of wrapped tomatoes stored at 13°C was inhibited to the degree that maximum percent soluble solids remained less than 4.0 during the entire storage period. However, no differences in test groups were statistically significant ($P<0.05$).

The pH of the mature green tomatoes (Table 3) ranged from 3.79–4.35. However, none of the four test groups exhibited a considerable change in pH as most of the values fell in the 4.00–4.20 range. The pH of wrapped fruits was consistently higher than that of unwrapped fruits. This is compatible with the fact that the wrapped fruits had a lower titratable acidity than the controls stored at 13°C.

The results of the citric acid, fructose, and glucose analyses of mature green tomatoes are shown in Fig. 8, 9, and 10, respectively. Only three data points were obtained for the unwrapped fruits stored at 18°C because the fruits were spoiled before collecting the fourth sample. The values obtained for the citric acid content were approximately equal to those of titratable acidity measurements because citric acid that existed in the salt form (citrate) was not accounted for in the titratable acidity measurement (Ryugo, 1965; Fernandez-Flores et al., 1970). During the early part of the experiment the citric acid concentrations tended to be slightly lower than the values for

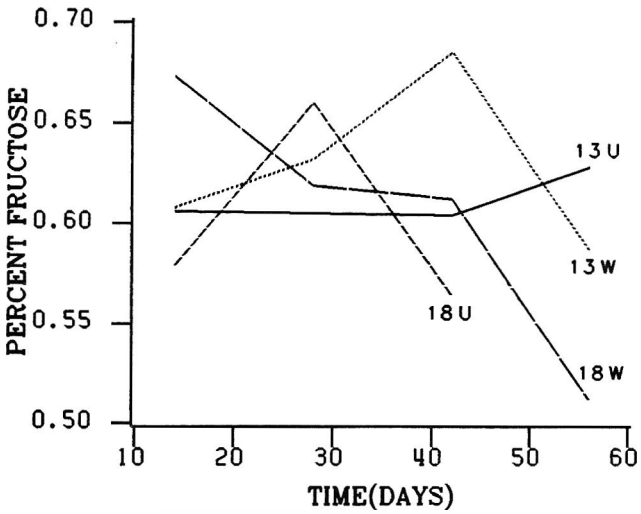

Fig. 9.—Fructose concentration of mature green tomatoes. The symbols 13U and 13W represent the unwrapped and wrapped fruits stored at 13°C and the symbols 18U and 18W represent the unwrapped and wrapped fruits stored at 18°C.

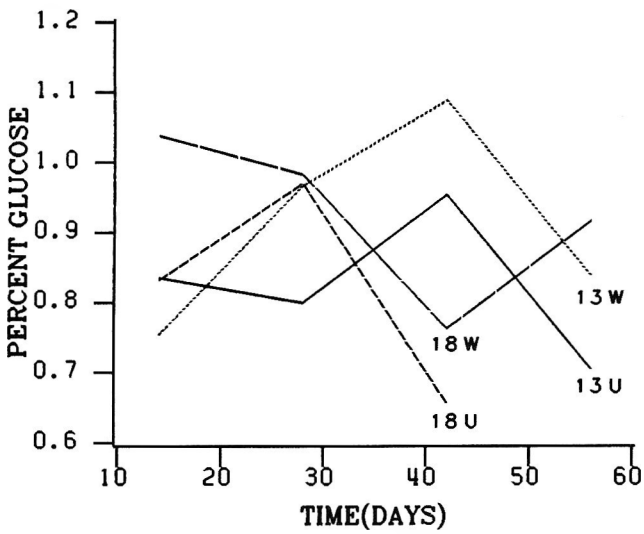

Fig. 10.—Glucose concentration of mature green tomatoes. The symbols 13U and 13W represent the unwrapped and wrapped fruits stored at 13°C and the symbols 18U and 18W represent the unwrapped and wrapped fruits stored at 18°C.

titratable acidity indicating that only a small amount of citrate was present.

Citric acid of both unwrapped and wrapped fruits stored at 18°C had already reached their maximum after 14 days of storage and remained relatively constant for the remainder of the storage period. Citric acid of the fruits stored at 13°C increased to a maximum and then decreased slightly. These findings are in agreement with other research which shows that the citric acid of tomatoes increases until ripeness and then either falls (Villarreal et al., 1960) or shows no further significant change (Davies, 1966). Low temperature storage had a significant effect ($P<0.05$) on decreasing the citric acid, but shrink-wrapping did not.

Fructose ranged from 0.5–0.7%, and glucose from 0.65–1.1%. Thus, total sugar, which is approximately equal to the sum of the fructose and glucose, was on the average about 1.5%. This is lower than the 2–3% sugar content that is characteristic of most tomatoes (Gould, 1974) but is partially due to the fact that tomato fruits that are harvested mature green tend to have lower sugar than those that are vine-ripened (Rick, 1978). In addition, tomato monosaccharides as low as 1.1% have been reported to occur even during normal ripening on the planet (Goodenough and Thomas, 1980).

Several of the test groups showed a rapid rise in the fructose and glucose concentrations followed by a rapid decline. The pyramid-shaped curves that were obtained are almost identical to those reported by Goodenough et al. (1982). The monosaccharides are produced by polysaccharide degradation but are utilized as substrates for respiration (Goodenough and Thomas, 1980). Thus, their concentrations increase until all the storage carbohydrates are utilized, and then they decrease.

Changes in monosaccharides did not correspond very well to the changes in the percent soluble solids. Wrapped fruits had a higher concentration of both fructose and glucose but lower soluble solids. There are two main reasons for these discrepancies: first the monosaccharides were lower than for most tomato fruits and only represented about 40% of the soluble solids. Therefore, changes in the sugar did not appreciably alter the total soluble solids; and secondly, differences in the soluble solids were not significant ($P<0.05$).

Conclusion

Individual shrink-wrapping extended shelf-life of fungicide-treated mature green tomatoes stored at 18°C and at 85–90% RH by 10 days. The only beneficial effect of shrink-wrapping the pink tomato was retention of moisture and firmness of the fruits without any appreciable extension of shelf-life. The short-term rentention of firmness of the wrapped tomatoes stored at 18°C was as good as the unwrapped fruits stored at 13°C, and long-term retention was better. The higher moisture retention in the wrapped fruits lowered the concentration of other constituents as detected in the titratable acidity and percent soluble solids. However, there was no lowering of fructose, glucose or citric acid content due to shrink-wrapping. Therefore, it was found that individual shrink-wrapping with polyolefin film could be used both to increase the shelf-life and improve the quality of fungicide-treated mature green tomatoes.

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Effect of Feathery Mottle Virus Infection on Sweet Potato Sensory Properties

W. M. WALTER JR. and J. W. MOYER

ABSTRACT

Four sets of sweet potato clones obtained by meristem tip culture were used to investigate the effect of feathery mottle virus (FMV) infection on the appearance, sensory quality and chemical composition of the storage roots. This study, encompassing four generations, indicated that FMV infection had a slight effect on carbohydrate metabolism but no effect on the polyphenoloxidase-phenol system. Flavor and texture profile analyses indicated that for one clone, FMV-infected roots were more desirable when baked than healthy roots. Considering the study as a whole, FMV infection did not appear to adversely affect sweet potato sensory properties. Clones produced from the same parent by meristem tip culture might have different compositional and sensory properties.

INTRODUCTION

SWEET POTATO VIRUS DISEASES (SVD) have been reported in many parts of the world (Terry, 1982) and have in some instances been shown to affect yields (Hahn, 1979) and chemical composition (Chung et al., 1981). The names given to SVD are indicative of the symptoms produced on the foliage, in the roots, or on general plant morphology, and as a result, the identity of many of these diseases is not well established.

In the United States, most commercially grown sweet potatoes are infected with feathery mottle virus (FMV). The virus name describes the major observed symptom which is an irregular chlorotic lesion associated with the leaf veins of the plant foliage. Although selected strains cause symptoms in roots of certain cultivars, it is not known what effect the virus infection has on yields and quality as a result of altering normal metabolism in symptomless roots.

With improvement in methods to eliminate viral infection and to index (monitor) plants for viral infection (Ebenshade and Moyer, 1982), it has become possible to obtain sufficient plant material to measure the effect of FMV on sweet potato quality. This study was conducted to determine the effect of FMV on the biochemical and chemical composition of sweet potato roots and to relate these changes to root sensory properties.

MATERIALS & METHODS

Sweet potatoes

Four sets of healthy and virus-infected clones obtained from a common parent ('Jewel') by a meristem tip culture procedure (Moyer and Collins, 1982) were field-grown using recommended horticultural practices for North Carolina. Each clone originated from a different meristem. The virus-infected clones were obtained by graft-inoculating rooted cuttings of each healthy clone with FMV-injected scions of *Ipomoea setosa*. The original clones were determined to be healthy or virus-infected by repeated graft-indexing to *I. setosa*. Sprouts were transplanted in June and were harvested in October, approximately 120 days after transplanting. This study was conducted for four generations of plants, beginning in 1981 and ending with the 1984 crop. In 1983 insufficient sprouts were produced due to poor bedding conditions and, thus, the third generation was not included. After harvest, the roots were cured for 1 wk at 30°C and 85% relative humidity (RH) followed by storage at 13°C and 85% RH for 60 days.

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Compositional analyses

After storage, each clone was divided into two lots. One lot was analyzed immediately; the second lot was baked prior to sensory evaluation and compositional analysis. Roots which were baked were prepared by thoroughly washing the periderm with water followed by air drying. The skin was pierced several times with a fork and the roots wrapped individually in aluminum foil. The roots were put on a tray and placed in a preheated, gas-fired convection oven set at 195–200°C. The roots were baked at that temperature for 90 min. After removal from the oven, the baked roots were cooled. The interior flesh was removed and passed through a collander (2 mm hole diameter). The collandered material was used for subsequent analyses and sensory evaluation. Moisture and alcohol-insoluble solids were measured on both raw and baked tissues (Walter, 1987). For those roots which were not baked, the peel was removed, the roots were quartered along the long axis, and a representative portion of the tissue was grated. The grated material served as the analytical samples.

Sugars. Ten-gram samples of tissue were blended with 50 mL 95% ethanol and 8 mL H_2O for 1 min. The mixture was quantitatively transferred to a 100 mL volumetric flask and held for 1 wk at room temperature. Individual sugars were measured on derivitized samples using gas chromatography (Walter and Hoover, 1984). The sugar content of both raw and baked material was measured.

Phenolics. Weighed tissue samples (ca. 10g) were homogenized with 95% ethanol, diluted to a final volume of 100 mL (80% ethanol) and allowed to equilibrate. A 4 mL aliquot was removed and evaporated to dryness under a stream of nitrogen followed by evacuation at ca. 1 mm Hg vacuum until constant weight was attained. The sample was removed and 1 mL ethanol containing 40 μg of coumarin (internal standard) was added. The mixture was sonicated, allowed to stand for 1 hr and filtered through a 0.2 μm filter. From 4–7 μL were injected into an HPLC for each analysis. The HPLC system was similar to that described earlier (Walter and Giesbrecht, 1982) except a Waters model 660 solvent programmer replaced the two-chambered gradient maker. The gradient program began with 16% methanol in 0.033 M phosphate buffer (pH 3.3) and ended with 40% methanol in the same buffer. Curve 6 on the programmer was used; the gradient change occurred during the first 15 min of the 27-min run. Phenols were detected at 334 nm with a Varian Varichrome variable wavelength detector. Quantitation was accomplished by electronic integration of peak areas.

Polyphenoloxidase and darkening. Polyphenyloxidase (PPO) activities and darkening values were measured as previously described (Walter and Purcell, 1980). Phenolics, PPO activity and darkening values were measured on raw sweet potatoes only.

Sensory analysis

Flavor and texture profiles were established by a panel trained in both flavor (Cairncross and Sjostrom, 1950) and texture (Civille and Szczesniak, 1973) profiling. The panel consisted of 6 or 7 individuals for each session. Scores for flavor and texture notes were based on a descriptive intensity scale that was converted to a 1 to 14 numerical scale for statistical analyses. A score of 1 = not detectable and a score of 14 = extremely intense. A single lot of canned sweet potatoes was used as the standard for treatment comparisons for both years.

Table 1—Composition of healthy and virus-infected sweet potato clones (raw)[a,b]

Clone	% Moisture[c]	% Alcohol-insoluble solids[c]	Polyphenol-oxidase activity[d]	Chloro-genic acid[e]	Isochloro-genic acid[e]	% Total sugars[c]	% Fructose	% Glucose[c]	% Sucrose[c]	% Darken-ing[f]
J-49										
Infected	74.74	17.70	44.69	5.30	8.13	6.78	0.96	1.21	4.60	0.143
Healthy	74.86	18.32	55.59	4.45	6.46	6.93	1.05	1.31	4.57	0.169
N[a]	13	11	13	13	13	9	9	9	9	13
J-574										
Infected	74.64	17.41	56.67	6.31	10.31	7.07	1.07	1.37	4.63	0.203
Healthy	74.02	16.72	49.84	6.88	9.37	6.94	1.00	1.28	4.55	0.189
N[g]	13	11	13	13	13	9	9	9	9	13
J-88										
Infected	76.14	15.36	54.37	4.39	7.97	6.05	0.97	1.13	3.95	0.213
Healthy	75.05	17.30	47.88	5.27	9.31	5.58	0.95	1.07	3.56	0.212
N[g]	13	11	13	13	13	9	9	9	9	13
J-299										
Infected	74.95	16.69	37.44	4.57	6.65	5.65	0.98	1.17	3.51	0.193
Healthy	74.55	17.01	44.87	4.36	7.35	5.45	1.11	1.32	3.60	0.233
N[g]	13	11	13	13	13	9	9	9	9	13

[a] Combined data for three generations.
[b] No statistically significant differences ($P \leq 0.05$) observed between infected and healthy samples from the same clone for each of the compositional parameters.
[c] % of fresh weight.
[d] Change in absorbance per min per g tissue at 450 nm.
[e] mg/100 g tissue.
[f] Absorbance of tissue homogenate at 420 nm.
[g] No. of observations.

Table 2—Composition of healthy and virus-infected sweet potato clones (baked)[a]

Clones	% Moisture	% Alcohol-insoluble solids	% Total sugars	% Fructose	% Glucose	% Sucrose	% Maltose
J-49							
Infected	71.89	9.58	15.19	1.07	1.29	3.86	8.96
Healthy	71.98	10.04	15.64	1.11	1.25	3.84	8.88
N[b]	9	9	9	9	9	9	9
J-574							
Infected	70.55	10.26	14.74	0.92	1.08	3.57	9.16
Healthy	71.66	9.94	15.42	1.04	1.33	3.94	9.19
N[b]	9	9	9	9	9	9	9
J-88							
Infected	73.54	7.88	13.54	1.11	1.21	3.78	7.25
Healthy	73.84	7.83	13.24	1.19	1.29	3.55	7.21
N[b]	9	9	9	9	9	9	9
J-299							
Infected	72.30	8.77	14.13	1.09*[c]	1.27**[c]	3.54	8.22
Healthy	71.44	8.73	15.71	1.28	1.48	3.97	8.98
N[b]	9	9	9	9	9	9	9

[a] All values in % of cooked weight (collandered material). Combined data for three generations.
[b] No. of observations.
[c] Means are different at $P \leq 0.05$ level (*) or $P \leq 0.01$ level (**).

Table 3—Composition of the second and fourth generations of healthy and virus-infected sweet potato clones (baked)[a]

Clone	Compositional component	Treatment	Mean
J-49	% Alcohol-insoluble solids	Infected	9.54
		Healthy	10.25
J-574	% Sucrose	Infected	3.45
		Healthy	4.14
J-299	% Fructose	Infected	1.12
		Healthy	1.37
	% Glucose	Infected	1.23
		Healthy	1.53

[a] Within each clone and compositional component, means for healthy and infected are different at $P \leq 0.05$.

The number of flavor and texture profile descriptive notes developed by Hamann et al. (1980) were reduced to eliminate interdependent notes (Syarief et al., 1985a, b). Flavor notes were: (1) sweet basic—sweet perceived on the taste buds of the tongue; (2) starch—resembling the typical flavor of white potato, an awareness of potato starch; (3) caramel—cooked sugar flavor. In addition, the panels detected an off-flavor note in some clones which was described as cold damage. The texture notes were: (1) first bite moistness—degree to which sample feels moist in mouth; (2) mastication gumminess—amount of energy required to disintegrate the sample for swallowing; (3) residual ease of swallow—effort required to swallow sample.

Sensory evaluations were performed from 10:00 a.m. to 11:00 a.m. At each session, the panelists evaluated four coded samples. Each set of four samples consisted of two clones, one clone of each set was virus-infected, and the other was healthy. Individual scores and the consensus score for each character note were subjected to statistical analysis.

Statistical analysis

The SAS PROC TTEST procedure (SAS, 1982) was performed to determine the significance between infected and healthy tissue. Significant differences were accepted at the 5% level of probability.

RESULTS & DISCUSSION

Compositional analyses

Comparison of the composition of healthy and virus-infected, raw sweet potatoes gave no indication that the infection had affected any of the parameters measured over the four generations in this study (Table 1). The parameters selected for analysis represent aspects of sweet potato quality. The moisture content, alcohol-insoluble solids and sugar content have an impact on the sensory properties, sweetness and mouthfeel. The PPO, chlorogenic acid, isochlorogenic acid and degree of darkening influence the visual appeal of sweet

Table 4—Flavor and texture scores for healthy and virus-infected, baked sweet potato clones[a,b]

Clone	Flavor notes			Texture notes			
	Sweet basic	Starch	Caramel	First-bite moistness	Mastication gumminess	Ease of swallow	Cold hurt
J-49							
Infected	6.40**	2.00	4.2**	9.07	5.93*	11.40**	1.00**
Healthy	4.73	2.33	3.0	8.53	7.33	10.20	2.14
N	15	15	15	15	15	15	14
J-574							
Infected	5.73*	2.33	4.00**	8.07	7.73	9.53	2.08**
Healthy	4.40	2.47	2.67	8.27	7.47	10.07	1.00
N	15	15	15	15	15	15	13
J-88							
Infected	5.14	2.29	3.00	8.50	7.71	10.57	1.57
Healthy	4.28	2.43	2.57	8.43	7.00	10.92	1.64
N	14	14	14	14	14	14	14
J-299							
Infected	5.07	2.57	2.71	7.86**	7.29*	10.50*	1.71
Healthy	5.07	2.79	3.07	6.50	8.93	9.29	1.36
N	14	14	14	14	14	14	14

[a] Data for two generations.
[b] Means for healthy and infected are different; * at P≤ 0.05; ** at P≤0.01.

Table 5—Compositional data for raw sweet potato clones[a]

Clone	Dry matter[b]	Alcohol-insoluble solids[b]	PPO[c]	Chlorogenic acid[d]	Isochlorogenic acid[d]	Darkening[e]	Fructose[b]	Glucose[b]	Sucrose[b]	Total sugar[b]
J-49	25.20^{A}	18.01^{A}	50.14^{A}	4.87^{B}	7.29^{B}	0.156^{B}	1.01^{A}	1.26^{AB}	4.59^{A}	6.85^{A}
J-574	25.67^{A}	17.06^{AB}	53.25^{A}	6.59^{A}	9.84^{A}	0.196^{AB}	1.03^{A}	1.33^{A}	4.59^{A}	6.95^{A}
J-88	24.41^{B}	16.34^{B}	51.12^{A}	4.84^{B}	8.64^{AB}	0.213^{A}	0.96^{A}	1.10^{B}	3.75^{B}	5.81^{B}
J-299	25.26^{A}	16.85^{B}	41.15^{B}	4.47^{B}	7.00^{B}	0.213^{A}	1.04^{A}	1.26^{AB}	3.55^{B}	5.85^{B}

[a] Clones produced from a common parent (cv. 'Jewel') by meristem tip culture. Means in the same column with the same superscript are not different (P≤0.05).
[b] Percent of raw weight.
[c] Change in absorbance per min per g tissue at 450 nm.
[d] mg 100 g of tissue.
[e] Absorbance of tissue homogenate at 420 nm.

Table 6—Compositional data for baked sweet potato clones[a,b,c]

Clone	Dry matter	Alcohol-insoluble solids	Fructose	Glucose	Sucrose	Maltose	Total sugar
J-49	28.06^{B}	9.81^{A}	1.09^{A}	1.27^{B}	3.85^{A}	8.92^{A}	15.13^{A}
J-574	28.90^{B}	10.09^{A}	0.98^{B}	1.20^{B}	3.75^{A}	9.18^{A}	15.11^{A}
J-88	26.31^{A}	7.85^{C}	1.15^{A}	1.25^{S}	3.76^{A}	7.23^{B}	13.39^{B}
J-299	28.13^{B}	8.75^{B}	1.18^{A}	1.38^{A}	3.67^{A}	8.60^{A}	14.83^{A}
LSD[d]	1.02	0.43	0.10	0.10	0.30	0.85	1.03

[a] Percent of baked weight.
[b] Clones produced from a single parent ('Jewel') by meristem tip culture.
[c] Means in the same column with the same superscript are not different (P≤0.05).
[d] Least significant difference (P≤0.05).

potatoes. These parameters are related to the phenolic-PPO system which causes a brown discoloration of the flesh. The enzyme (PPO) and the substrates (chlorogenic acid and its isomers) have been implicated in discoloration of various fruits and vegetables (Walter and Purcell, 1980; Weaver and Charley, 1974; Mondy et al., 1967).

Since it is possible that the viral infections worsen with each succeeding generation, the composition within clones for the last two generations was separated and statistically analyzed. The only statistically significant difference was observed for PPO activity in clone J-299. For PPO the infected clone had a PPO activity of 20.6 units, while the healthy clone had an activity of 35.4 units (P≤0.01). Thus, the infected clone appeared to be less susceptible to enzymatic discoloration due to PPO than did the healthy clone.

When a sweet potato is baked, endogenous amylolytic enzymes hydrolyze the starch into sugars (mainly maltose) and thus cause a sweet taste and the mouthfeel sensation to become softer and smoother than the uncooked root. If viral infection interfered with the starch hydrolysis, quality would be impaired. The data showed this did not occur. The only statistically significant differences for three generations between healthy and virus-infected clones were in fructose and glucose concentrations (Table 2). For both sugars the healthy tissue had a slightly high concentration than the infected tissue. However, the differences were so slight as to be nondetectable sensorially. Analysis of data from the second and fourth generations showed that three of the clones had statistically different amounts of several of the components. In cultivar J-49 the healthy tissue contained more alcohol-insoluble solids, while in clones J-574 and J-299 healthy tissue had higher levels of sucrose and fructose plus glucose, respectively (Table 3). This indicated that the virus infection might have had some effect on the carbohydrate metabolism.

Sensory analysis

In the United States, the preferred sweet potato has a deep orange color, tastes sweet and has a smooth, creamy consis-

tency when masticated. This smooth, creamy texture is commonly referred to as "moistness." In a comparison of two treatments such as were used in this study, the following would be a desirable change. For flavor notes, the preferred sweet potato would have a higher sweet basic score, a lower starch score and a higher caramel score. For texture notes, the preferred sample would have a higher first-bite moistness score, a lower mastication gumminess score and a higher ease of swallow score.

The data indicate (Table 4) that for cultivars J-49 and J-574, the infected roots have more desirable flavor notes, sweet basic and caramel. The starch flavor note scores were not different. Among the texture note scores for J-49, infected roots had a higher ease of swallow score than did healthy roots and a lower mastication gumminess score. All of these scores are consistent, with J-49 infected being more desirable sensorially than J-49 healthy. The only other clone showing different scores for texture notes was J-299. Again, infected clone scores were consistent with a higher quality baked root than healthy clone scores. Healthy and infected texture note scores for clone J-574 were not different. For the remaining clone, J-88, no differences were detected in either flavor or texture note scores between healthy and infected roots.

The off-flavor detected (cold hurt) by the panel was found both years in infected roots from clone J-574, indicating that under some environmental conditions the virus might cause an off-flavor in this clone. In clone J-49, the off-flavor was noted in healthy roots for 1 year only and, thus, was probably due to an environmental effect only. Although detected in the remaining clones, there was no statistically significant difference in scores between healthy and virus infected tissues.

The data obtained in this study indicated that feathery mottle virus infection might cause subtle changes in the metabolic pathway affecting carbohydrates. There was no evidence to indicate that the phenol-PPO system was affected by virus infection. Sensory studies suggested that for one clone, J-49, infected roots were more desirable than healthy roots. Considering the study as a whole, FMV infection did not appear to adversely affect sweet potato quality.

Although this virus has little effect on the sensory qualities of the clones of 'Jewel' sweet potato, the observed between-clone variability for compositional and sensory properties (Tables 5 and 6) is consistent with variation in flesh and skin color observed between meristem tip culture-derived clones of 'Jewel' (Moyer and Collins, 1983). Thus, it might be necessary to evaluate cultivars following meristem tip culture to select clones for propagation with properties equal to or better than the original clone and to avoid inadvertent selection of a less desirable clone.

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The authors thank Mr. Gaillard, Director of the Cave Cooperative in Nogaro for his assistance in this study and Mrs. H. Mondiès for technical assistance.

Double Direct Shear Test for Potato Texture

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ABSTRACT

A double direct shear device was developed to examine shear strength parameters of mealy and waxy potatoes. Cohesion and coefficients of friction were measured at peak and ultimate shear with two machine speeds. Coefficients of friction of potato types did not differ. However, mealy potatoes displayed greater cohesion than waxy potatoes and a greater difference between peak and ultimate cohesion values. Stem ends of mealy potatoes were the most firm. Shear strength was related to strain rate.

INTRODUCTION

TEXTURE is often considered the most important sensory quality in cooked potatoes (Warren and Woodman, 1974; Harada and Paulus, 1986); however, the study of texture is confusing because of differences in definitions, variations in potatoes, and lack of accepted objective measures.

Though the term "mealy" has most often been used to describe cooked potato texture, the combination of physical forces could be described more accurately, according to Warren and Woodman (1974), as "viscosity." Reeve (1977) listed a variety of applicable terms, including "thick," "cottony," "floury," and "fluffy,". Linehan and Hughes (1969) related mealiness to a combination of intercellular adhesion and dryness, with a mealy potato exhibiting cell separation and dry mouthfeel. "Grainy" was the term used most frequently to describe potato texture by one taste panel; their sensory scores did not correlate with any Instron Texture Profile Analysis parameters except the product of adhesiveness and cohesiveness (Leung et al., 1983).

Objective testing of this important sensory characteristic has often been made without sensory analyses, assuming because of data in existing literature that the higher specific gravity and/or more mealy potatoes have greater strength (Sterling and Bettelheim, 1955; Sharma et al., 1959; Lujan and Smith, 1964; Ross and Porter, 1969; Iritani et al., 1971; Iritani and Weller, 1974; Nonaka, 1980; Nonaka and Timm, 1983). Nevertheless, Warren and Woodman (1974) pointed out that if cell separation were directly related to mealiness, the mealy potatoes should exhibit lower strength than nonmealy varieties. In fact when potatoes were whipped in a mixer, mealy potatoes offered less "shear" resistance than waxy (Unrau and Nylund, 1957).

The variety of terminology, conditions of testing, and instrumentation used has added to the difficulty of developing a standardized method for examining potato texture. The term "shear force" has been used when dull blades passed through strips confined in a slotted cell (Ross and Porter, 1969; Iritani et al., 1971), when a plunger passed through an unconfined slice positioned over a hole (Harada and Paulus, 1986), when raw unconfined cubes were compressed with a larger flat plunger, and when mashed potatoes confined in a cup were compressed with a smaller flat plunger (Lujan and Smith, 1964). "Shear strength" described the readings when a guillotine-type apparatus passed through unconfined rectangular strips (Sterling and Bettelheim, 1955). Unrau and Nylund (1957) measured "resistance to shear" as wattage required to whip potatoes in a mixer. Nonaka (1980) reported "compressive force" required to plunge a double layer of cooked discs, smaller in diameter than a kitchen ricer, through side and bottom holes of the ricer. Hughes et al. (1975), using a Wolodkewitsch tenderometer, discussed changes in intercellular adhesion as measured by "compressive strength" of discs. Unconfined samples compressed between flat plates were evaluated by Texture Profile Analysis (TPA) parameters (Loh and Breene, 1981, 1982; Leung et al., 1983). A penetrometer plunger measured "penetration resistance" (Sharma et al., 1959). However, by utilizing pure shear action as described by physicists and engineers, interpretations basic to the properties of the material tested as measured by shear could be employed (Bourne, 1982).

The objective of the present study was to modify a direct shear instrument commonly used in engineering applications the shearing stress developed along two parallel planes (as a section of the test material under a vertical, normal force was moved horizontally at a constant rate) could be measured, in order to calculate the coefficient of friction as well as the cohesion.

MATERIALS & METHODS

THE MACHINE USED (Fig. 1) was a modification of a direct shear machine (Lambe, 1951). Modifications were made in the design of a smaller shear box to fit a typical size potato sample and in the design of double direct shear faces to permit increased sensitivity (Fig. 2).

Sixty-three red potatoes and 77 Russet potatoes were purchased from a local grocery store (cultivars unknown) to represent waxy and mealy characteristics, respectively. Bud and stem halves were separated. Cylindrical samples, 4 cm in length and 4 cm in diam were cut perpendicular to the long axes of the raw potato halves. Only one sample could be obtained from a half potato. Samples were steamed for 25 minutes and heat penetration of all samples appeared similar. Steaming appeared to result in more even cooking than boiling. From the cooked potato cylinders, cylindrical subsamples were cut 3 cm in diam by 3 cm in length to eliminate any sloughed tissue (though little occurred during steaming). Subsamples were placed in the shear box while still hot, and shear was carried out within 5 minutes of cooking.

Shear testing was done using a variety of arbitrarily selected normal forces, 7.75, 38.07, 137.41, 201.64, 266.57, 309.30, 330.92 or 373.64, g/cm^2, in order to evaluate the potatoes according to the Mohr-Coulomb theory (Harr, 1977). The center cross section of the potato sample, 1.5 cm thick, was pulled horizontally from between the top and bottom of the sample (Fig. 3) at a rate of either 2.5 mm/min (80 samples) or 15.0 mm/min (60 samples). The shear stress (g/cm^2), measured with a proving ring, was a read at each 0.05 cm of horizontal deformation up to a maximum of 0.50 cm. Peak shear stress was defined as the highest stress attained, and ultimate shear stress was defined as the stress at 0.50 cm deformation, representing the stress attained at large deformations.

An analysis of variance model was utilized to determine the significance of the data.

RESULTS & DISCUSSION

THE MOHR-COULOMB THEORY has been used successfully to describe the shear strength of particulates (Harr, 1977), and was selected to analyze the data in this study.

Mohr's theory states that, on any plane, the shearing stress,

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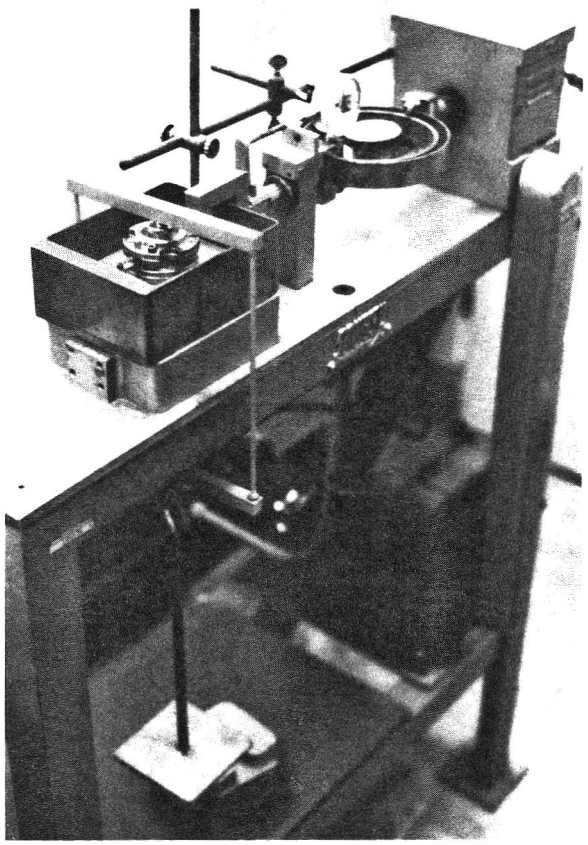

Fig. 1 Double direct shear machine.

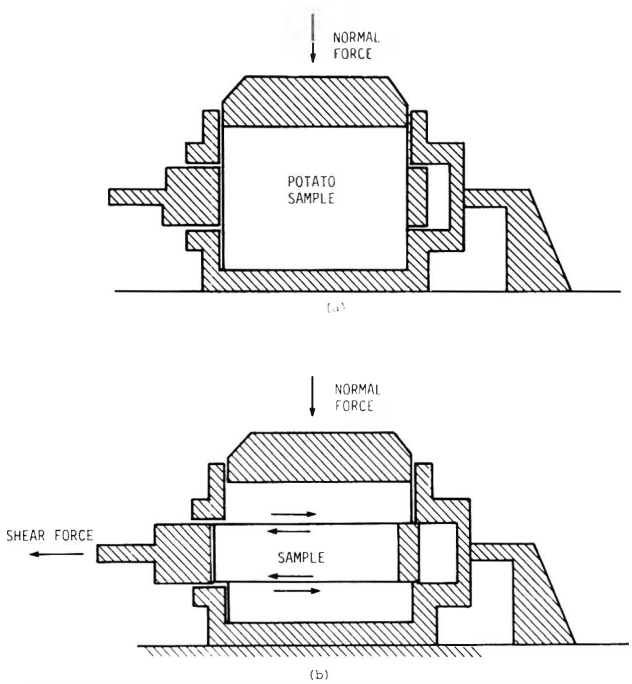

Fig. 2—Sample holder for double direct shear machine: (a) before and (b) after test.

τ, is:

$$\tau = \sigma \tan \psi \quad (1)$$

where σ is the normal stress and ψ is the obliquity angle or the angle between the resultant of the normal and shearing

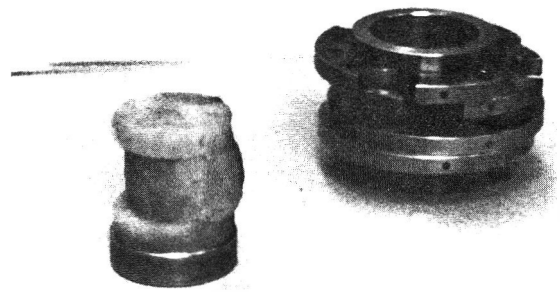

Fig. 3—Sample and holder following shear test.

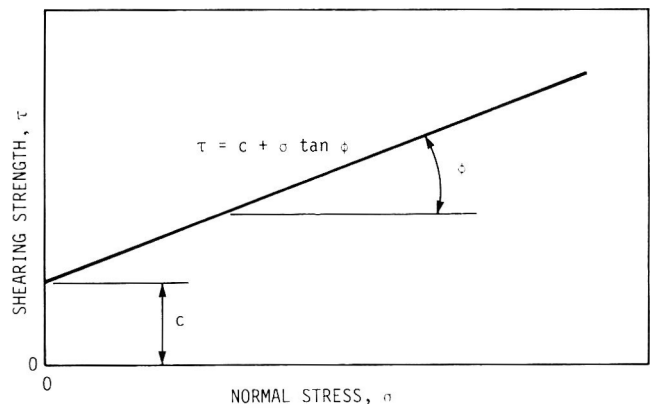

Fig. 4—Coulomb equation for shear strength of a material: τ = shearing stress; c = cohesion; σ = normal stress; ϕ = friction angle.

forces and the normal force. When the obliquity angle reaches its maximum or critical value, shear failure occurs. Thus, if the maximum obliquity angle is reached, the stresses on the failure plane are described by the equation:

$$\tau = \sigma \tan \phi \quad (2)$$

where ϕ represents the maximum obliquity and is referred to as the friction angle, ϕ. The tangent of the friction angle is called the coefficient of friction.

Coulomb showed empirically that a general equation to describe shear strength of particulates has two components, one in which the strength is independent of normal stress on the failure plane and the other, which is the friction component of Mohr. Thus, the strength of particulates can be characterized by the equation:

$$\tau = c + \sigma \tan \phi \quad (3)$$

where "c" is the cohesion and the other terms are as previously defined. A graph of this equation with the independent variable and the dependent variable as a straight line is shown in Fig. 4. The cohesion and the coefficient of friction can be thought of as shear strength parameters of a potato.

Peak and ultimate shear stress (g/cm^2) were plotted as the dependent variables, with normal stress as the independent variable. Slopes of the lines that best fit the data provided, the coefficients of friction; the intercepts provided the cohesions.

Overall comparison of mealy and waxy potatoes

Both potato types exhibited low coefficients of friction and high cohesions (Table 1). The coefficients of friction did not

Table 1---Means of shear strength parameters of potatoes, all samples and both machine speeds

	Waxy peak shear	Mealy peak shear	Waxy ultimate shear	Mealy ultimate shear
Coefficient of friction	0.20[a]	0.22[a]	0.22[a]	0.22[a]
Cohesion (g/cm²)	166[x,y]	237[z]	148[x]	174[y]

[a,x,y,z] Means with the same superscript are not significantly different based on a t-test ($P > 0.01$).

Table 2---Influence of shear rate on shear strength parameters of potatoes, 2.5 mm/min compared with 15.0 mm/min[a]

Ultimate shear means	2.5 mm/min	15.0 mm/min
Coefficient of friction	0.31[b]	0.07[c]
Cohesion (g/cm²)	146[y]	187[z]

[a] All samples were utilized for analyses.
[b,c,y,z] means with the same superscript are not significantly different based on a t-test ($P > 0.01$).

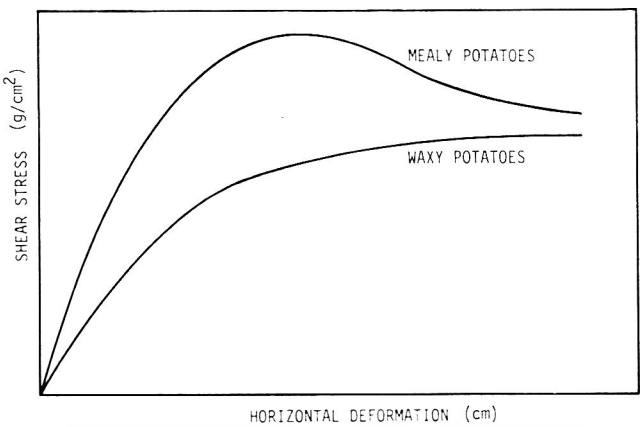

Fig. 5—Typical stress-strain curves generated by double direct shear of two potato types.

differ with type of potato or with peak or ultimate shear values. Cohesion of the mealy potatoes was significantly higher than cohesion of the waxy potatoes. Stress-deformation curves showed that mealy potatoes had a peak strength greater than ultimate. However, waxy potatoes exhibited nearly constant shear stress, with no clearly defined failure stress (Fig. 5). Examination of the waxy sample removed from the holder showed no clearly defined failure plane, whereas mealy samples exhibited a definite fracture surface. The stress-deformation curves and nature of the failing plane demonstrated more "plastic" behavior for the waxy potatoes, whereas the mealy potatoes were more "brittle". Ultimate values for the mealy potatoes were not much different from those of the waxy potato.

Cohesiveness has been defined as the strength of the internal bonds within the product (Szczesniak, 1963). The mealy potato was more cohesive when subjected to an initial force sufficient to cause failure; however once the structure was fractured, the strength behavior of the mealy potato differed little from the waxy potato. This may be correlated with observations that a mealy potato, when overcooked (Nonaka, 1980) or manipulated (Nonako and Timm, 1983), developed a gummy texture similar to a waxy potato. Starch exuded from damaged cells may have caused such change (Nonaka, 1980; Nonaka and Timm, 1983; Reeve, 1954; 1977).

Comparison of machine speed, all samples

Values for the two machine speeds at ultimate shear for cohesion and coefficient of friction were significantly different, with the slower machine speed giving a higher coefficient of friction and lower cohesion value (Table 2.). Peak values did not differ for either slope or intercept. Consolidation of the material become a factor at the slower rate of speed as the interstitial water was expelled.

Table 3---Means of shear strength parameters of bud and stem ends of potatoes, shear rate 15.0 mm/min.

	Peak shear		Ultimate Shear	
	Coefficient of friction	Cohesion (g/cm²)	Coefficient of friction	Cohesion (g/cm²)
Waxy, stem end	0.03[a]	188[w,x]	0.06[a]	172[w,x]
Waxy, bud end	0.11[a]	146[w]	0.09[a]	144[w]
Mealy, stem end	0.12[a]	267[z]	0.03[a]	236[y,z]
Mealy, bud end	0.10[a]	214[x,y,z]	0.13[a]	180[w,x]

[a,w,y,z] Means with the same superscript are not significantly different based on a t-test ($P > 0.01$).

Cohesion and coefficient of friction are parameters of particulate matter, not properties because, for a given material, they will vary depending upon test conditions. A particulate material that is sheared slowly will allow interstitial water to be squeezed from the pores, permitting the material to consolidate during shear. If the same material is sheared at a very fast rate, with insufficient time for consolidation, the shear strength will be described by:

$$\tau = c \qquad (4)$$

The coefficient of friction in Equation (3) is then equal to zero (Lambe, 1951).

Comparison of bud and stem ends of potatoes

Coefficients of friction were not significantly different between the bud and stem ends of potatoes sheared at a rate of 15.0 mm/min (Table 3). Cohesion of stem ends of mealy potatoes was significantly greater at both peak and ultimate shear than the stem ends of waxy potatoes. Bud ends of mealy potatoes at peak shear were more cohesive than bud ends of waxy potatoes; there was no difference at ultimate shear. The stem end of mealy potatoes was more cohesive than the bud end of mealy potatoes at ultimate shear. Stem and bud ends of waxy potatoes did not differ. Differences between potato ends in shear strength, cooking time to soften, sloughing, solids, sugars, bruise susceptibility and phase angles have been reported in the literature (Iritani et al., 1973; 1977; Nonaka, 1980; Peterson and Hall, 1974; Powers and Board, 1973; Reeve, 1954; Sayre et al., 1975).

CONCLUSIONS

THE MOHR-COULOMB MODEL used to characterize shear strength of particulate can be applied to describing the shear strength of cooked potatoes. Differences in cohesion of waxy and mealy potatoes and in bud and stem ends of mealy potatoes were observed.

These observations suggest that stress-deformation or stress-strain behavior of potatoes may be a more important quantitative assessment of potato texture than measures of strength or hardness. Additional testing may indicate the double direct shear as an appropriate measuring device for evaluating the texture of other food products.

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An Examination of Hedonic Response to *Tuber gibbosum* and Three Other Native Oregon Truffles

A. B. MARIN and M. R. McDANIEL

ABSTRACT

The aromas of four relatively unknown, native Oregon truffle species were hedonically rated and compared for preference by a large group of untrained subjects whose only communality was curiosity about fungi. More subjects liked the truffle aromas than disliked them, but generally, most subjects were indifferent or undecided over the novel truffle aromas. Male and female subjects responded differently to the truffle aromas: most males liked the aromas, while most females were indifferent or did not like them. For individuals who liked the truffle aromas, no significant difference in hedonic scoring was found between male and female populations. However, for those subjects that disliked the truffle aromas, females rated three of the four significantly less desireable than did males.

INTRODUCTION

TRUFFLES are the fruiting bodies of hypogeous fungi (fungi which fruit underground). Mature truffles produce aromas as a mechanism to attract animals to locate and consume them since these fungi are dependent on mycophagy for spore dispersal (Maser et al., 1978). Europeans have traditionally used pigs to assist them to "sniff out" certain truffle species which are highly prized and priced as gourmet foods because of their unique and enticing aromas. The compounds responsible for the unusual aromas of truffles have only recently been investigated and reported.

Claus et al. (1981) isolated and identified two odoriferous steroidal compounds from species of European truffles. These compounds, 5 α-androst-16-en-3-one (androstenone), and the corresponding alcohol, are also known to be porcine sex pheromones (Melrose et al., 1971; Claus and Hoffman, 1971). In light of this fact, it is not surprizing that pigs are able to detect truffles at a depth of nearly a meter underground (Claus et al., 1981).

Human response to androstenone has been studied because this compound was identified as an unpleasant odor occurring in pork (Griffiths and Patterson, 1970). At near threshold concentrations, androstenone was described as "sweet, fruity and perfume-like," while at higher concentrations, it was described as "sweaty, animal, urinous or ammonia-like." Also, women are reported to be more sensitive to the odor of androstenone and find it significantly more unpleasant than do men (Griffiths and Patterson, 1970; Koelega, 1980). The concentration of these steroids in European truffles is reported to be well above human thresholds (Claus et al., 1980). However, contribution of the steroids' odors to the overall aroma of these truffles has not been reported, nor has hedonic response to the aromas of European truffle species. Chemical analysis of the aroma of native truffle species used for this study has just begun (Marin et al., 1984; Marin, 1985), but they have not been analyzed for steroidal constituents.

This sensory study was undertaken to examine hedonic response of a large number of subjects to the aromas of four native truffle species. Knowledge and use of native truffles is being promoted through the efforts of the North American Truffling Society (NATS). As part of this effort, NATS presents a display of native truffles at the annual Fall Mushroom Show sponsored by the Oregon Mycological Society in Portland, Oregon. The truffle display attracts large numbers of individuals who are curious about these fungi and whose initial response to truffles, as noted in past years' displays, is to smell each specimen. The annual Mushroom Show and NATS truffle display, then, provided an appropriate experimental setting, and a large population of individuals who were willing and interested in being subjects for this study.

This sensory study was initiated in order to evaluate the extent of the variation in hedonic response between individuals and between truffle species. Considering the sex difference in hedonic response to the odor of androstenone, it seemed worthwhile to examine the results of this sensory study by sex to determine if there might be male/female differences in hedonic scoring of the truffle aromas evaluated. The purpose of this study, then, was to find out if people like or dislike truffles, to see if male and female segments of the population respond to truffle aromas in the same way, and to determine if there is a preference for any of the four truffle species.

MATERIALS & METHODS

Truffle sample preparation

Four truffle species, *Tuber gibbosum*, *Picoa carthusiana*, *Rhizopogon parksii*, and *Gautieria monticola*, were selected because of availability and distinct difference in aroma quality. Several mature specimens of each sample, raw fresh or frozen, were grated with a fine stainless steel grater and a subsample of approximately 3g placed in a 200 mL clear stemmed wine glass. Five grams of common table salt and 5 mL hot tap water, about 50°C, were added to each sample. Samples were thoroughly mixed, covered with a snug aluminum foil cap and allowed to cool to room temperature for sampling. Each glass of truffle slurry was labeled with a numbered specimen name. Three digit coding was not used for these samples since each specimen was identified by name.

Sensory testing

Individuals interested in the NATS truffle display were asked if they would fill out a ballot to rate truffle aroma. Subjects were instructed on sniffing procedure and how to fill out the ballot. In order to accomodate a large number of subjects, three identical testing stations were set up, each displaying the four different truffle slurries so that samples were available for random selection. Instructions on the

Table 1—Observed and expected frequencies of males and females who are likers, dislikers or indifferents to the aromas of native truffle[a]

Hedonic category	Males		Females		Total
	Observed	Expected	Observed	Expected	
DISLIKERS	23	25	34	31	57
INDIFFERENTS	42	63	101	79	143
LIKERS	56	32	17	41	73
Total	121		152		273

$\chi^2 = 44.34$ df = 2 $p < 0.001$

[a] LIKERS: subjects rating at least 3 of the 4 truffle aromas in the like range of the hedonic scale: ratings > 5; DISLIKERS subjects rating at least 3 of the 4 aromas in the dislike range: ratings < 5, and INDIFFERENTS subjects rating 2 of the truffle aromas as liked and the other 2 as disliked, or scoring the truffle as neither liking nor disliking the aroma: rating = 5.

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Table 2—Comparison of male and female hedonic score means for the aromas of four truffle species.

		Likers[a]					Dislikers[a]				
Truffle species	Sex	n	Mean	Std error	|T|	p>	n	Mean	Std error	|T|	p>
P. carthusiana	M	50	7.360	0.133	0.194	0.848	19	2.526	0.246	3.165	0.003
	F	14	7.429	0.327		NS	27	1.593	0.162	Significant	
T. gibbosum	M	56	6.982	0.129	0.712	0.486	23	2.696	0.203	2.182	0.034
	F	14	7.214	0.300		NS	34	2.088	0.191	Significant	
R. parksii	M	17	7.176	0.214	0.183	0.856	23	2.739	0.261	2.496	0.017
	F	17	7.117	0.241		NS	29	1.931	0.192	Significant	
G. monticola	M	53	7.736	0.132	0.291	0.773	7	2.714	0.286	1.119	0.279
	F	16	7.813	0.228		NS	18	2.278	0.266		NS

[a] LIKERS are subjects rating 3 or more truffles as liked; hedonic score > 5; DISLIKERS are subjects rating 3 or more truffles as disliked; hedonic score < 5.

aroma evaluation procedure were posted at each station.

Instructions for aroma evaluation posted with the samples were: (1) swirl glass; (2) remove cover; (3) sniff; (4) rate aroma desirability for each sample using a 9-point hedonic scale where 1 = dislike extremely and 9 = like extremely (Peryam and Pilgram, 1957); (5) indicate preferred sample; and (6) describe the truffle aromas. Experimental data obtained from each subject was a hedonic score for each truffle aroma, the subject's preferred sample and descriptors for the truffle aromas. The only background information requested on the ballot was the subject's sex. A total of 273 individuals, 152 females, and 121 males, completed the sensory testing ballot and 20 additional subjects indicated their preferred sample but did not rate each truffle aroma for desirability. Descriptors for the truffle aromas were given by 67 subjects. After sensory testing, ballots from the 293 subjects participating in the study were collected, the data interpreted and entered into a Primos 9750 computer for statistical analysis using SAS (SAS, 1985).

RESULTS & DISCUSSION

Hedonic Scores

To determine which subjects generally liked or disliked the aroma of truffles, three categories were defined from the hedonic scores:

LIKERS were defined as individuals who rated at least three of the four truffle aromas in the like range of the hedonic scale: ratings > 5;

DISLIKERS were defined as those who rated at least three of the four aromas in the dislike range: ratings <5; and

INDIFFERENTS were defined as individuals who liked two of the truffle aromas and disliked the other two, or scored the truffle aroma as neither liking nor disliking the aroma: rating = 5.

The distribution of subjects in these three categories was: LIKERS 26.7%, INDIFFERENTS 52.4%, and DISLIKERS 20.9%. That is, more than half the population are indifferent or undecided about liking truffle aromas while about 1/4 of the subjects liked the aromas and 1/5 did not.

These categories were further examined to see if the distribution of males and females in each was the same. A χ^2 value was calculated from the expected and observed frequencies of males and females in each category to determine if any apparent differences in the distributions were real or due to chance (O'Mahoney, 1986). Male and female observed and expected frequencies in each category are given in Table 1 as well as the calculated χ^2 value and associated significance level. Results indicate a highly significant sex effect; more males like truffles than do females. Two thirds of the females were indifferent about truffle aromas and only about 10% of them liked the aromas. However, only one third of the male subjects were indifferents and nearly half of all the males liked the truffle aromas.

To determine the extent of the observed sex effect, we examined male and female hedonic scoring for the likers and dislikers categories for each of the four truffle species. Male and female hedonic scores were compared for each truffle aroma by subjecting them to an unpaired T-test. Results are given in Table 2. There were no significant differences found between male and female likers' mean hedonic scores for any of the four truffles, but there were many more males than females in this category, as mentioned previously. However, for the dislikers, females rated three of the four truffle aromas as significantly more unpleasant than did male subjects. This hedonic response pattern is similar to that reported for androstenone (Griffiths and Patterson, 1970; Koelega, 1980) and might be expected if androstenone-like steroidal constituents were present in the aroma volatiles of the truffle samples disliked most intensely by these females.

Table 3—Truffle sample preference as indicated by percentage of population indicating aroma of one of four native truffles as favorite

Preferred truffle (in order of preference)	Population		
Truffle species	Males n = 133	Females n = 160	Total n = 293
Gautieria monticola	57.9%	50.0%	53.6%
Picoa carthusiana	20.3%	25.6%	23.2%
Tuber gibbosum	16.5%	16.3%	16.4%
Rhizopogon parksii	5.3%	8.1%	6.8%

Truffle aroma preference

Statistical analysis of the truffle aroma preference data is given in Table 3. *G. monticola*, the sample preferred by the most subjects, was also given a hedonic rating (mean of 7.75) which was significantly higher than scores given the other truffle aromas. This truffle aroma was also disliked by the fewest number of subjects—about 9% of the total subject population. Interestingly, this species is the only one for which no significant difference between male and female hedonic scoring was demonstrated.

Truffle aroma descriptors

The most frequently given descriptors for each of the truffle aromas are listed in Table 4. Less than 25% of the participants in the study chose to report descriptors, perhaps demonstrating the difficulty people have in describing odors (Engen, 1982). Nonetheless, the resulting list was compiled from those descriptors which were used by at least two individuals and so indicates some consensus. The aroma descriptors given illustrate some similarities and differences in the aromas of the four species.

CONCLUSIONS

THIS STUDY EVALUATES hedonic responses to the aromas of four native truffles species given by 293 individuals who demonstrated some interest and curiosity in truffles. As might be expected, there were more subjects in this sample population who liked the aroma of truffles than disliked them, but, in general, more than half of the subjects were indifferent or undecided about the novel truffle odors.

The hedonic response of male and female segments of the population to the truffle aromas was not the same. Almost half of the male subjects liked the truffle aromas, while only 10%

Table 4—Descriptors given for the aromas of each of four truffle species

	Truffle sample			
	G. monticola	*P. carthusiana*	*T. gibbosum*	*R. parksii*
Descriptors:	Sweet	Apple	Cheese	Earthy
	Mushrooms	Cheese	Nuts	Musty
	Cake	Nuts	Fungus	Spoiled wine
	Cheese	Coconuts	Potatoes	Stale nuts
	Buttery	Spoiled prunes	Bread	Saw dust
	Coconut	Wine	Fruity	Varnish
	Marmalade	Oysters	Wet dog	Bleach

of the female subjects liked them. Most females were indifferent or disliked the truffle aromas. No male/female differences were observed in hedonic scores for individuals who liked the truffle aromas. However, for subjects disliking truffle odors, females rated the aromas significantly lower than did males for 3 of the 4 truffle species. This negative response by females is of particular interest since this is the same response demonstrated for the odor of androstenone steroids - compounds which have recently been identified in the aroma profile of species of European truffles. It would, therefore, be interesting to chemically analyze the three native truffle species which generated this response for odoriferous steroidal constituents.

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Ms received 6/2/86; revised 2/24/87; accepted 3/24/87.

Taken in part from a thesis submitted to Oregon State University in partial fulfilment of the requirements for the M.S. degree.

Presented at the 45th Annual Meeting of the Institute of Food Technologists, Atlanta, GA, June 9 - 12, 1985.

The authors gratefully acknowledge the assistance of members of the North American Truffling Society in truffle and data collection, the financial support of the Oregon Mycological Society, and the assistance of Profs. M.R. McLellan and T.E. Acree, Cornell Univ., in the editing of this manuscript.

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Ms received 11/3/84; revised 12/26/85; accepted 3/11/87.

This is Journal Paper No. 11705 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa. Project No. 2584.

The authors are grateful to David L. O. Smith for designing and calibrating the apparatus; David F. Cox and Hugh Voigt for statistical analyses; Judith Kennedy and Bonnie Gorder for technical assistance; and the Civil Engineering and Food & Nutrition Departments, the Engineering Research Institute, and the Agriculture and Home Economics Station for funding.

Functionality of Defatted Corn Germ Proteins in a Model System: Fat Binding Capacity and Water Retention

C. S. LIN and J. F. ZAYAS

ABSTRACT

Functional properties of two corn germ protein (CGP) preparations, supercritical CO_2 ($SC-CO_2$) and hexane defatted, were studied in model systems using response surface methodology. The protein preparations had different fat and moisture content. The $SC-CO_2$ CGP was whiter (L), less red (a) and less yellow (b) in color measurement than hexane CGP. Temperature of incubation influenced the functionality of the $SC-CO_2$ and hexane-defatted CGP in model system. $SC-CO_2$ CGP had higher fat binding and water retention than hexane CGP. Fat binding decreased during the heat treatment for $SC-CO_2$ CGP. Water retention in both preparations increased as temperature increased to 70°C. The different functional properties of hexane CGP may be due to the extent of protein denaturation.

INTRODUCTION

THE IMPORTANCE of the functional properties of basic food components depends on the type of foods, i.e., fluid emulsion, powder, sausage emulsion or dough. Water retention and fat binding of proteins as basic components of foods determine the quality (juiciness, texture, binding of structure, appearance) and yield of the finished product.

The interaction of proteins with water is important to properties such as hydration, swelling, solubility, viscosity and gelation. Water retention and hydration are the first and the critical steps in imparting desired functional properties to proteins. However, in corn germ protein (CGP) preparations, carbohydrates also may play an active role.

Many properties of foods involve the interactions of proteins and lipids: formation of emulsions, fat entrapment in meat emulsions, flavor absorption and dough preparation. Fat absorption of proteins is affected by protein source, processing, composition of additive, particle size and temperature. Proteins of plant origin, for example, sunflower proteins, contain numerous nonpolar side chains that bind hydrocarbon chains, thereby, contributing to increased oil absorption. Although the mechanism of fat absorption has not been completely explained, fat absorption is attributed mainly to the physical entrapment of oil.

The functional property of water retention has been used as a criterion for selection of protein additives for food systems, especially meat. Measurements of water retention and fat binding may provide data for selecting a protein raw material.

Difficulties of determining the functionality of proteins in foods are related to the diversity of their properties and functions, as well as the interactions between different proteins and nonprotein components. The functional properties of corn protein preparations reflect the composition of the sample; the nature and reactivity of proteins; their native structure; and interactions with nonprotein components of CGP preparations, carbohydrates and lipids. They are affected also by environmental and processing conditions.

Kinsella (1979) summarized the functional properties of soybean proteins, i.e., solubility, water retention and binding, fat adsorption and emulsification. These functional properties demonstrate the desirable uses of soybean proteins in food applications. However, the uses of soybean proteins were limited because of the strong beany, grassy and bitter flavors associated with these proteins.

The CGP has considerable potential for use as a supplement in a variety of foods and as a new protein source. A good corn protein source can be obtained by drying corn germ meal at low temperatures and extracting the oil by solvent (Lucisano et al., 1984). At present, conventional oil extraction solvent (hexane) leaves certain lipids in the flour which reduce its quality (Phillips and Sternberg, 1979). Recently, Christianson et al. (1984) studied a supercritical CO_2 ($SC-CO_2$) method to extract oil from corn germ and obtained a food-grade quality, defatted, corn germ product.

Some functional properties of CGP have been reported (Lucisano et al., 1984). CGP is a carbohydrate-rich product. It stabilizes emulsions by absorbing or binding excess water, thus enabling more water to be added (Bhattacharya and Hanna, 1985; Luallen, 1985). However, in meat systems, the carbohydrates have been reported to neither participate in the emulsifying process nor improve the water holding capacity of meat emulsions (Mittal and Usborne, 1985).

Although studies have been reported on the refining processes and properties of corn gluten (Phillips and Sternberg, 1979) and corn germ flour (Blessin et al., 1979; Christianson et al., 1984) and their use in fortifying some foods (Lucisano et al., 1984) very little work has been done on the functional properties of the corn germ proteins.

Corn germ preparation is totally different from that of corn gluten meal. Proteins of the wet milled germ are modified during sulfurous acid steeping (Lucisano et al., 1984; Wu and Sexson, 1976). Commercially produced wet corn gluten meal is flash-dried at high temperature to produce dry corn gluten meal (60% protein and 10% moisture). In spite of its high protein and abundance of methionine, corn gluten meal has poor functional properties and nutritional quality (Bhattacharya and Hanna, 1985).

The objective of this study was to investigate the functional properties of two CGP preparations ($SC-CO_2$ and hexane defatted) in relation to their fat and water binding characteristics under subsequent processing for sausage batter.

MATERIALS & METHODS

Sample preparation

Both of the CGP preparations ($SC-CO_2$- and hexane-defatted) were obtained from the USDA Northern Regional Research Center (Peoria, IL). Homogeneous samples were obtained by milling process using a colloid mill (IKA-UNIVERSAL MUHLE A 20; Tekmar Company, Cincinnati, OH) for 45 sec with a water cooled jacket.

Color of the samples was measured on the ILLA L, a, b values by

Table 1—Composition and color measurements of $SC-CO_2$ and hexane-defatted corn germ proteins

Corn germ proteins	ILLA			Moisture	Fat	Protein
	L	a	b	%	%	%
$SC-CO_2$	89.82	1.75	5.73	4.25	1.62	19.44
Hexane	85.50	3.16	8.61	4.62	11.11	17.54

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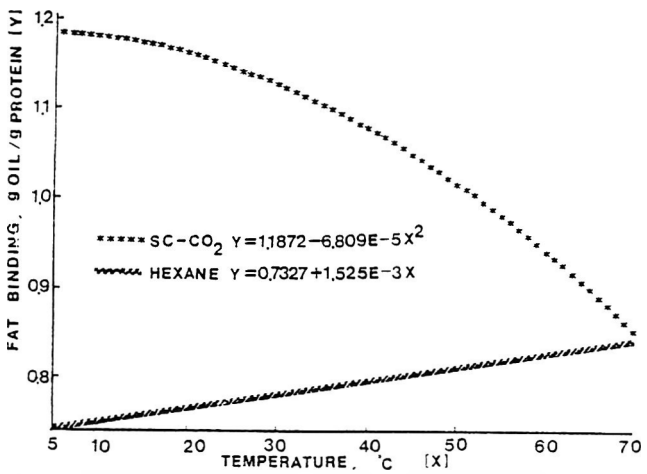

Fig. 1—Fat binding of SC–CO_2- and hexane-defatted corn germ proteins (10%) at different temperatures of incubation.

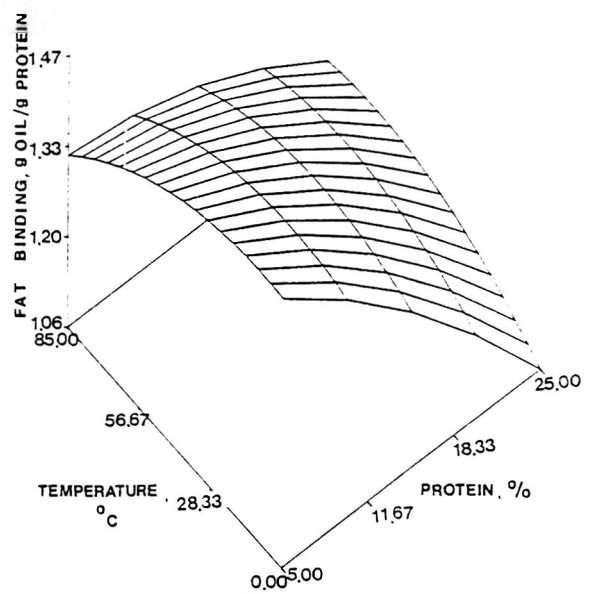

Fig. 2—Fat binding of SC–CO_2-defatted corn germ protein as a function of protein concentration and incubation temperature.

the Hunterlab spectrophotometer (Hunterlab D54, Hunter Associates Laboratory, Fairfax, VA).

Composition analyses

Moisture, fat and protein of CGP were analyzed following AOAC (1984) methods.

Fat binding

The fat binding index was determined by magnetically stirring 5–25% protein with 50 mL corn oil (Mazola, CPS International INC, Englewood Cliffs, NJ) in a beaker for 30 min and then incubating at temperature settings of 5°, 15°, 22°, 40°, 55° and 70°C. A range of temperatures (15–70°C) was used in response surface analysis. The mixture was transferred to two 50 mL centrifuge tubes and centrifuged at 3,000 rpm for 30 min. The excess oil was decanted. Fat binding index was calculated as the residue weight divided by the original weight (Phillips and Sternberg, 1979).

Water retention

The methods of Inklaar and Fortuin (1969) and Aoki et al. (1980) were used for water retention determination. Five grams corn germ was dispersed in 95 mL distilled water. The solution (pH 6.0 to 7.0) was mixed by magnetic stirrer at incubation temperatures ranging from

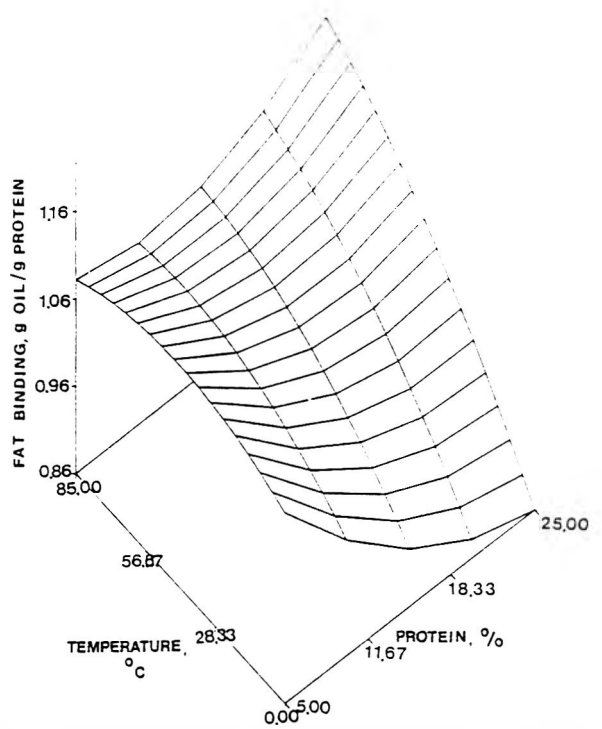

Fig. 3—Fat binding of hexane-defatted corn germ protein as a function of protein concentration and incubation temperature.

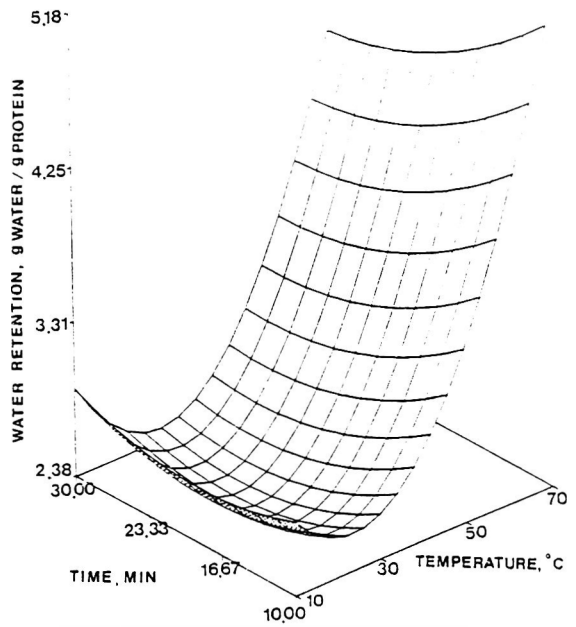

Fig. 4—Water retention of SC–CO_2-defatted corn germ protein as a function of incubation time and temperature.

10–70°C for 10 to 30 min. Two 50 mL centrifuge tubes filled with the above solution were placed in a tap water bath for 30 min for temperature equilibration. A 3,000 rpm centrifugation was used for 30 min. The supernatant was transferred into a 100 mL volumetric flask. The water binding value or hydration capacity was calculated as the difference between hydrated weight and original weight and expressed in grams of water retained by 1 gram of protein.

Statistical analyses

The ranges and interval of experimental parameters for response surface methodology (RSM) followed the designs of Box and Behnken (1960) and Box and Wilson (1951). Each experiment was replicated once for each treatment. Data analysis and graphic plotting were done

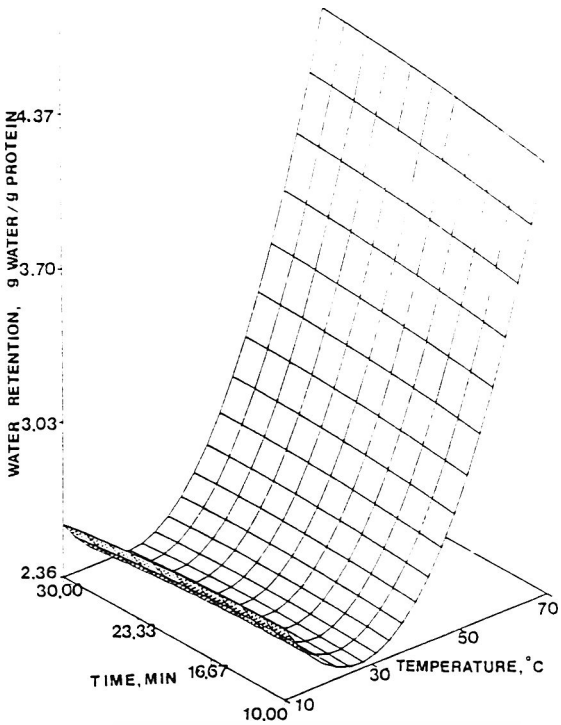

Fig. 5—Water retention of hexane-defatted corn germ protein as a function of incubation time and temperature.

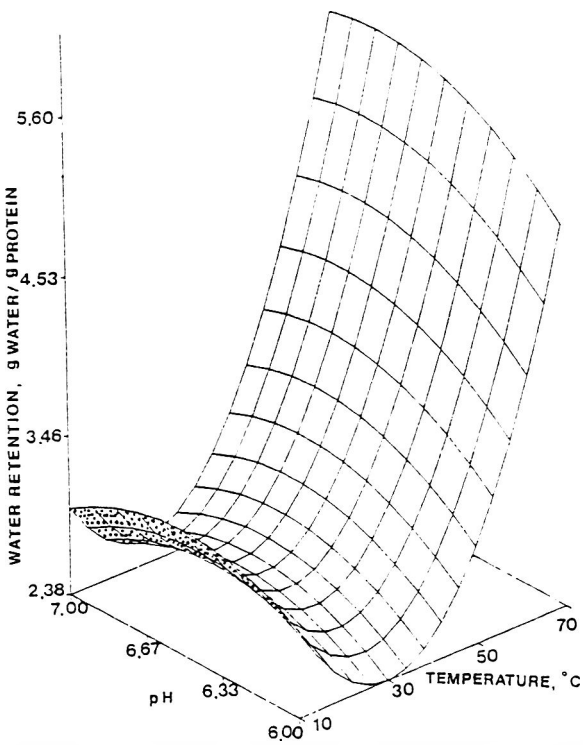

Fig. 6—Water retention of SC–CO_2-defatted corn germ protein as a function of pH and temperature of incubation.

with SAS programs (SAS, 1982). Quadratic models were used to create the 3-dimensional response surface. In response surfaces, independent variables are located along the traditional X and Y-axes, respectively, whereas the response variable is at Z-axis perpendicular to the X-Y axes. Shaded areas indicate the bottom side of saddles. Regression analysis was conducted by the method of Steel and Torrie (1980) for simple regression models.

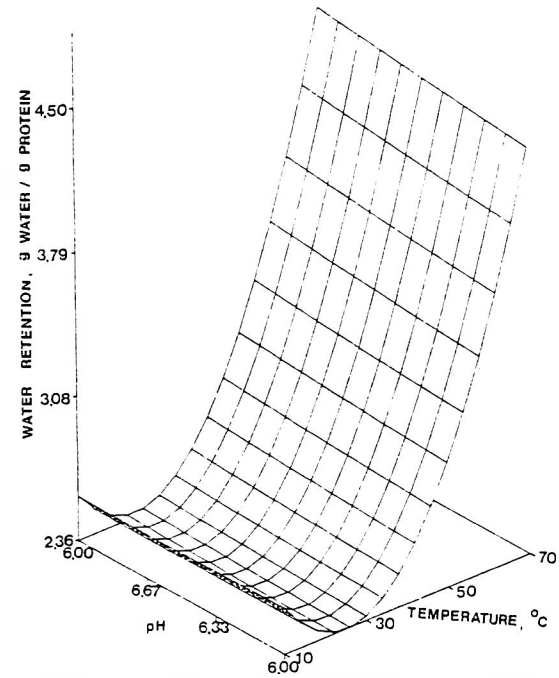

Fig. 7—Water retention of hexane-defatted corn germ protein as a function of pH and temperature of incubation.

RESULTS & DISCUSSION

THE COLOR MEASUREMENTS of SC–CO_2-defatted CGP and hexane-defatted CGP are shown in Table 1. The processing technology influenced the color of the preparations. The SC–CO_2 CGP was whiter (L), less red (a), and less yellow (b) in color than hexane CGP. The higher percentage of fat and fat oxidation may contribute to the yellow color of hexane CGP. Interaction between oxidized fat and protein may cause additional denaturation of the hexane defatted CGP. Specificity of the SC–CO_2 and hexane defatting processes influenced the composition of the CGP preparations (Table 1). Hexane-defatted CGP preparations had a significantly higher fat content than SC–CO_2 CGP preparations. The higher level of fat extraction by the SC–CO_2 method will significantly increase the shelf life of the preparations.

Fat binding index

Binding of fat with other food components, particularly proteins and carbohydrates, influenced the textural and other properties of food quality. The ability to bind oil was significantly higher for SC–CO_2 CGP than for hexane CGP at a lower range of temperatures (Fig. 1). The higher fat binding capacity of SC–CO_2 CGP may be due to the lower fat content in the preparation. Both proteins reached the same final level of fat binding ability after 30 min of incubation at 70°C. As temperature increased from 0° to 70°C, fat absorption decreased. Possibly, the high viscosity of the system at lower temperatures contributed to greater ease of fat entrapment.

The process of incubation for 30 min at 70°C enhanced the denaturation of SC–CO_2 CGP and loss of its fat-binding properties. Although pressure-induced denaturation of SC–CO_2 CGP has been reported (Christianson et al., 1984), hexane-defatted CGP had significantly greater nitrogen solubility index. Less fat binding in the hexane defatted CGP can be explained by higher original fat content in hexane-defatted CGP than SC–CO_2 CGP. (Fig. 1). Sausage batter is processed by comminution, with an end point temperature of 10–18°C. Because of that, the fat binding capacity of CGP at this range of temperatures had practical application. The higher fat binding capacity of the SC–CO_2 CGP may affect structural properties of the batter and binding of the fat in the finished meat products.

The fat binding capacity as a function of temperature for SC–CO_2 CGP can be expressed by the equation:

$$Y = 1.1872 - 6.809E\text{-}5X^2 \qquad (P<0.001 \text{ and } R^2 = 0.795);$$

For hexane CGP, the equation was:

$$Y = 0.7327 + 1.525E\text{-}3X \qquad (P<0.001 \text{ and } R^2 = 0.646);$$

where Y = fat binding and X = temperature of incubation.

Response surface analysis of SC–CO_2 CGP (Fig. 2) showed a maximum point at 0°C incubation temperature and 5% protein concentration under test conditions. Fat binding (g of oil/g of protein) decreased as protein concentration increased and decreased as temperature increased at 5% protein concentration. The high concentration of CGP meant less oil in the ratio, which decreased the availability of fat for CGP to bind. However, it increased as temperature increased at 25% protein concentration.

Lower overall responses were established for hexane CGP (Fig. 3) than SC–CO_2 CGP. Hexane CGP reached a maximum point at high temperature and high protein concentration. Temperature became a significant factor for fat binding at high protein concentration. At low temperature, fat binding decreased as protein concentration increased. These results agreed with Fig. 1, which shows that SC–CO_2 CGP was sensitive to heat and both protein preparations reached the same level of fat binding value at 25% protein concentration and at 85°C.

The quadratic models for fat binding were:

$$Z_1 = 1.5243 - 2.97E\text{-}4Y - 1.033X - 2.53E\text{-}5Y^2 + 1.455E\text{-}4XY - 3.262E\text{-}4X^2$$
$$(P<0.001,\ R^2 = 0.831)$$

$$Z_2 = 1.195 + 8.25E\text{-}4Y - 2.475E\text{-}2X + 1.8E\text{-}4XY - 2.0E\text{-}5Y^2 + 4.49E\text{-}4X^2$$
$$(P<0.01,\ R^2 = 0.910)$$

where Z_1 = fat binding of SC–CO_2 CGP and Z_2 = fat binding of hexane CGP, X = protein concentration, Y = incubation temperature.

Water retention

Water-binding capacity is important in many applications of protein flours, concentrates and isolates. Because CGP will be utilized in sausage production, the water retention of SC–CO_2 and hexane CGP were determined over a range of temperatures corresponding to various sausage processing conditions.

Water retention was higher for SC–CO_2 CGP than for hexane CGP (Fig. 4 and 5). SC–CO_2 and hexane CGPs had pH of 6.0 and 6.3, respectively. Both proteins had a similar response surface, which was low at low incubation temperature around 20–25°C. The maximum value of water retention was obtained after 10 min incubation at 70°C. Water binding increased significantly at the range of temperatures 35–70°C. This was probably due to increased protein solubility. Carbohydrates in the sample might play a significant role in water retention, i.e., starch swelling increased along with temperature. A longer time of incubation was needed by hexane CGP. The highest water retention was at 70°C after 30 min of incubation time. The water retention surfaces show the curvilinear nature of the response to both temperature and time of incubation. Incubation time affected the water retention of SC–CO_2 CGP, with a lower value around the center of tested range. Overall, no significant time effect on water retention was obtained for either CGPs.

The pH of solution affected the water retention of SC–CO_2 CGP; water retention slightly increased as pH increased from 6.0 to 6.8 (Fig. 6). The increase of water retention was evident at all temperatures studied (10–70°C). There was no pH effect on the water retention of hexane CGP (Fig. 7).

The quadratic models for water retention of SC–CO_2 CGP and hexane CGP were:

$$Z_3 = -28.8509 - 7.3E\text{-}2Y + 10.2317W - 9.61E\text{-}2X + 1.5E\text{-}3Y^2 + 4.0E\text{-}3YW - 0.8033W^2 - 7.0E\text{-}4XY + 7.7E\text{-}3XW + 1.2E\text{-}3X^2$$
$$(P<0.001,\ R^2 = 0.973)$$

$$Z_4 = 3.8432 + 6.5E\text{-}3Y - 0.22W - 6.95E\text{-}2X - 1.6E\text{-}4Y^2 + 1.667E\text{-}2W^2 + 1.0E\text{-}3X^2 - 1.0E\text{-}3WY + 1.8E\text{-}4XY + 2.3E\text{-}3XW$$
$$(P<0.001,\ R^2 = 0.999)$$

where Z_3 = water retention of SC–CO_2 CGP and Z_4 = water retention of hexane CGP, X = incubation temperature, Y = time of incubation, W = pH.

CONCLUSIONS

OVERALL PATTERN showed that in model systems, SC–CO_2 CGP had better functional properties than hexane CGP. Hexane CGP had more yellow color, more fat residue and more protein denaturation by heat during sample preparation. SC–CO_2 CGP had higher fat binding (more lipophilic) and water retention. At the end of thermal treatment, both CGPs had similar fat binding. The fat binding of SC–CO_2 CGP was most efficient at low protein percentage in the mixture. A high water retention was obtained, with no significant time effect on either CGP, in the period of 10 to 30 min. With high temperature and alkaline conditions, both CGPs will have higher protein solubility than with low temperature and pH. Because of the better functional properties in a model system, particularly higher fat binding capacity and water retention, SC–CO_2 CGP was recommended for practical utilization. Studies of other properties of CGP such as emulsion stability and emulsion capacity are necessary.

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Ms received 12/24/86; revised 3/7/87; accepted 3/9/87.

Contribution No. 87-174-J, Dept. of Foods & Nutrition, Kansas Agricultural Experiment Station, Manhattan, KS 66506.

Chemical (NaOH) Peeling as Viewed by Scanning Electron Microscopy: Pimiento Peppers as a Case Study

JOHN D. FLOROS, HAZEL Y. WETZSTEIN, and MANJEET S. CHINNAN

ABSTRACT

Scanning electron microscopy (SEM) was used to examine microstructural changes of pimiento peppers (*Capsicum annum* L. cv. 'Truhart') treated with different NaOH (lye) solutions (1, 4, and 9%), maintained at 80°C, for various times (1, 2, and 3 min). Photomicrographs indicated that NaOH removes the epicuticular and cuticular waxes, diffuses uniformly into the fruit where it breaks down epidermal and hypodermal cell walls, and solubilizes the middle lamella causing separation of the skin. In severe treatments the lye also dissolves the parenchyma cells of the mesocarp resulting in considerable loss during processing.

INTRODUCTION

PEELING is one of the major operations in the processing of most fruits and vegetables. It is also a high-cost operation in terms of labor, and when coupled with coring, it accounts for approximately 60% of the total labor in processing (Gould, 1983). Among the different peeling methods employed by the processing industry, chemical peeling using NaOH (widely known as lye peeling) is one of the most common and oldest methods.

The first commercial use of lye for peeling was in the production of hominy, followed by that of peaches (Cruess, 1958). Today the use of lye for peeling fruits and vegetables is widespread due to its economy, simplicity, and labor-saving advantages (Lucas, 1967), but it continues to face many problems due to major processing losses of edible part. In the case of commodities with high volume/weight ratio (e.g., green bell peppers, pimientos) peeling loss can be as high as 60% (Heaton, 1984). This acute problem of high product loss has been the subject of many investigations, but little literature exists on the fundamentals of the mechanism of lye peeling. A better understanding of the underlying mechanism could lead to improved processes in practical applications. Reeve (1976) discussed some changes in the structure of fruits and vegetables as affected by lye-peeling, and Walter and Schadel (1982) considered changes caused in sweet potato tissue due to heat mediation during lye-peeling. Cruess (1958) suggested that the lye solubilizes the pectic substances in the middle lamella, thereafter causing separation of the skin. it has also been reported that the sodium hydroxide solution selectively attacks and breaks down the "outer cuticular tissue" of fruits and vegetables leaving the inner flesh untouched (Anon., 1964).

Researchers have employed scanning electron microscopy (SEM) to examine changes during flour milling and wheat processing (Moss, 1985), differences between firm and soft cucumber pickles (Walter et al., 1985), and for observations of the cell structure in fresh and processed carrots and green beans (Grote and Fromme, 1984). Use of SEM provides a unique insight by locating and confirming changes at the microstructural level.

The purpose of this study was to evaluate critically the peeling mechanism by investigating ultrastructural changes occurring during a chemical (lye) peeling process. Pimiento peppers were chosen as a model, because they possess a relatively tough skin (exocarp) and a characteristic hydrophobic waxy surface (Worthington, 1981) making the peeling difficult.

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MATERIALS & METHODS

Lye treatments

Pimiento peppers (*Capsicum annuum* L.) of the 'Truhart' cultivar, supplied by Cherokee Products Co. Haddock, GA, were sorted for uniform size (5.5 – 7.5 cm diameter, 7.5 – 10.5 cm height) and maturity (red to deep-red color). Batches of six peppers were subjected to lye treatments of varying concentration and time (Table 1). Lye solutions were maintained at 80 ± 1°C throughout all treatments as it has been reported that temperature is the least important factor in a lye-peeling process (Floros and Chinnan, 1987). Immediately after lye treatment, fruits were washed with pressurized spray water for 2 min.

Preparation for SEM

Each lye-treated batch was divided into two groups of three peppers. Pericarp tissue blocks (approximately 2 × 3 × 4 mm) obtained from the middle wall region of the fruit were prepared for SEM by one of the following two methods:

Fixation and critical point drying (CPD). Tissue blocks were fixed at 0°C for at least 2 hr with 2% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.2). Slight vacuum was applied for the first hour of tissue fixation. The samples were washed twice in the same buffer, dehydrated in graded series of ethanol (20, 35, 50, 60, 75, 85, 95, 100%), and dried through CO_2 using a Polaron critical point dryer.

Freeze-drying. Pericarp tissue blocks were dehydrated overnight in a Virtis freeze-dryer (Model 10-MR-TR, Gardiner, NY).

All samples were mounted on aluminum stubs using double-sticking tape, sputter-coated with gold-palladium, and examined using a Phillips 505 SEM at 20 kV.

RESULTS & DISCUSSION

Microstructural characteristics of fresh peppers

A cross-section of the outer pericarp layers for the nontreated (control) fruits is presented in Fig. 1A and shows that the exocarp (ex), commonly known as skin, is distinct from the mesocarp (me) which constitutes the edible part of the fruit. The latter is composed of large thin walled parenchymatous polygonal cells of varying size. The exocarp (Fig. 1B) is made

Table 1—Experimental design for lye-peeling treatment of pimiento peppers

Treatment #	Concentration % NaOH	Time min
1	1	1
2	1	2
3	1	3
4	4	1
5	4	2
6	4	3
7	9	1
8	9	2
9	9	3
Control	0	0

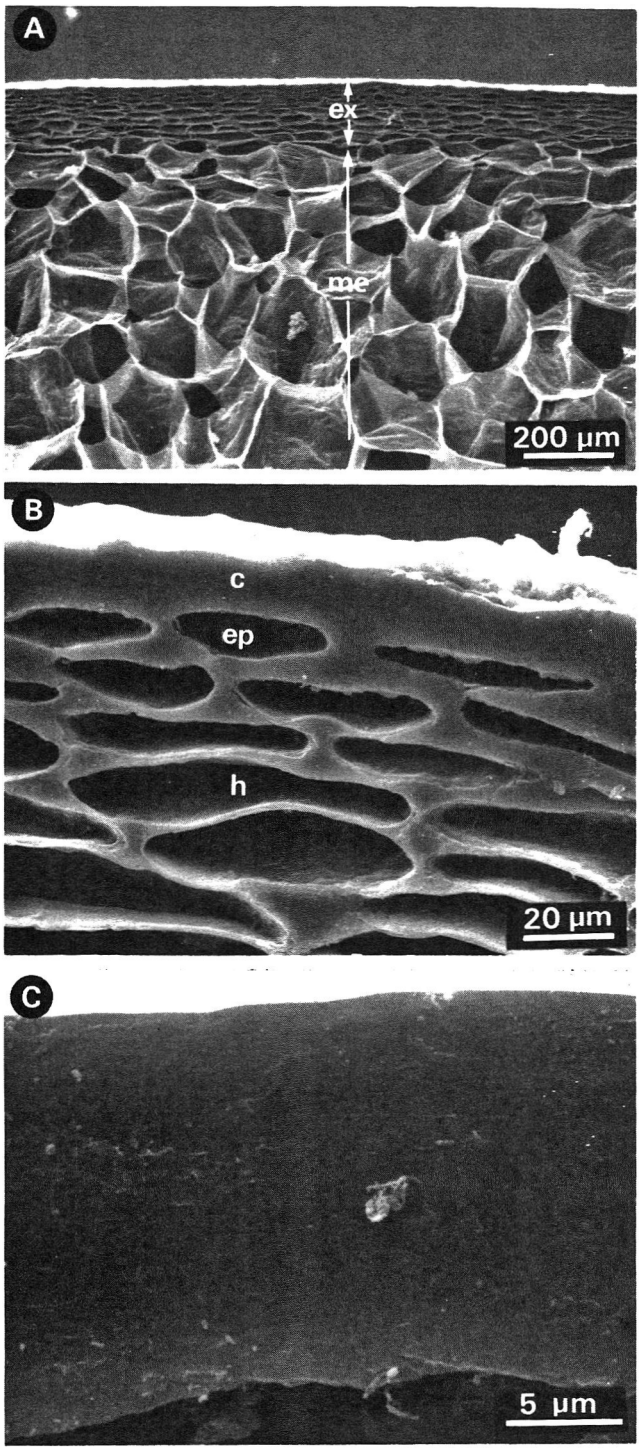

Fig. 1—Electron photomicrographs of fresh pimiento pepper tissue: (A) Cross-section of the outer portion of the fruit wall (pericarp) showing the exocarp (ex) and the mesocarp (me); (B) Higher magnification of the exocarp in cross-section showing the cuticle (c), the epidermis (ep) with one layer of small cells and the hypodermis (h) consisting of several cell layers; (C) Higher magnification of the cuticle showing a smooth surface line without crystalline wax formations.

up of smaller cells with a single epidermal layer (ep) and several (5 to 8) layers of collenchymatous hypodermal cells (h) with tangentially thickened walls. The outer surface of the fruit was covered with a thick continuous cuticle (c). The cuticular surface was generally smooth and amorphous, without crystalline wax formations (Fig. 1C).

The component primarily responsible for the structure, rigidity, and ultimately the texture of plant tissue, is the cell wall.

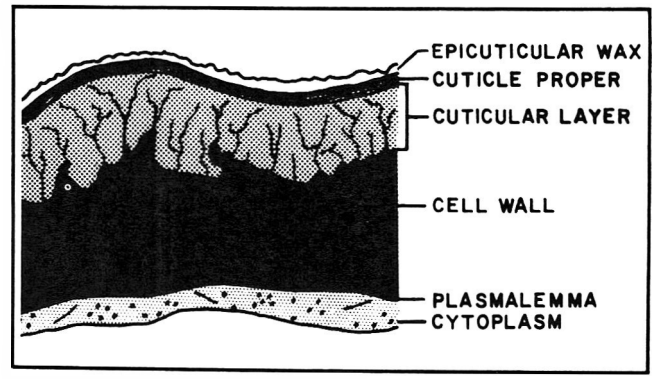

Fig. 2—Idealized model of the plant cuticle.

Cell walls are composed of polysaccharides (primarily cellulose and hemicelluloses) and glycoproteins (Alberts et al., 1983). The substance which functions as a "glue" to hold the cells together, the middle lamella, is located between cell walls and it consists of pectic substances mainly polygalacturonic acid. Another structural component of plants, the "cuticle" (Fig. 2), which exists as a thin continuous extracellular membrane has a fundamental protective role as a barrier between the plant and its environment, and it is physically and chemically heterogeneous (Holloway, 1982a; Price, 1982).

In a simplified way (Fig. 2), epicuticular wax makes up the exterior part of the cuticle and is composed of soluble waxes deposited on the surface. The cuticle proper lies beneath the epicuticular wax, and is a region formed of cutin and cuticular waxes. Below the cuticle proper is the cuticular layer(s) which is (are) composed of cutins, cuticular wax, and incrustations of cellulosic wall material (Norris and Bukovac, 1968). Baker et al. (1982) studied the composition of the tomato fruit cuticle and found that triterpenols, flavonoids, naringenin and hydrocarbons (mainly hentriacontane) were the principal components of the epicuticular waxes, while fatty acids (C_{16} and C_{18}) were the main components of the intracuticular waxes. The cutin was found to consist primarily of positional isomers of dihydroxyhexadecanoic acid. Norris (1974) found a cuticle thickness of 4.5 ± 0.5 µm for the tomato fruit.

Microstructural changes during processing

Surface action of lye. Anticlinal cell walls of epidermal cells were indistinct in surface views of control fruit (Fig. 3A) due to the presence of surface waxes and/or cuticle. Action of lye on the surface of the peppers resulted in increased visibility of cell outlines as the treatment severity increased (Fig. 3).

A considerable reduction in the thickness of the outer epidermal wall was not observed with increased severity of lye action. The thickness of the epidermal walls of treated fruits were similar to the control (Figs. 1B and 1C) suggesting that total removal of the cuticle and cell wall digestion did not take place. Instead, increased visibility of cell outlines, observed in Fig. 3 (B, C and D), can be attributed to dissolution of waxes and a probable change in the physical and chemical structure of the cuticle. Slight reduction in the outer epidermal wall thickness is explained by the existence and the relative thickness of the cutin and the cellulose network in the cuticle. The action of lye probably involves dissolution of the epicuticular and cuticular waxes leaving most of the cutin matrix and the cellulose network unaltered.

Cutins, which form the main structural component of plant cuticles, are high molecular weight polyesters readily depolymerized by common reagents used to cleave ester bonds. Holloway (1982b) reported that under reflux conditions plant cutins exhibit a low rate of depolymerization in aqueous KOH, while in alcoholic KOH rapid depolymerization is observed. However, even the action of such extreme KOH treatments

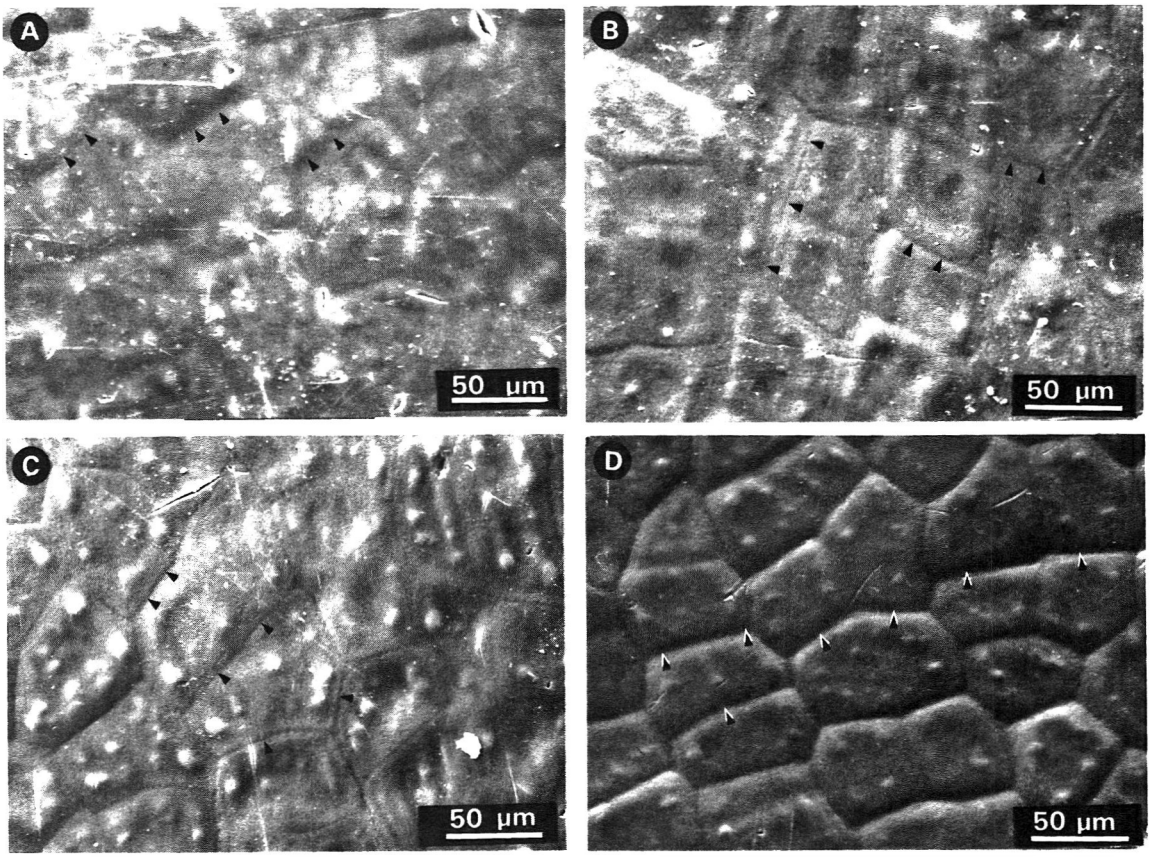

Fig. 3—Electron photomicrographs of the exocarp surface of control and lye-treated fruit showing the effect of treatment on the visibility of epidermal cells (arrows indicate anticlinal cell walls): (A) Nontreated peppers; (B) 4% NaOH for 1 min; (C) 9% NaOH for 2 min; (D) 9% NaOH for 3 min.

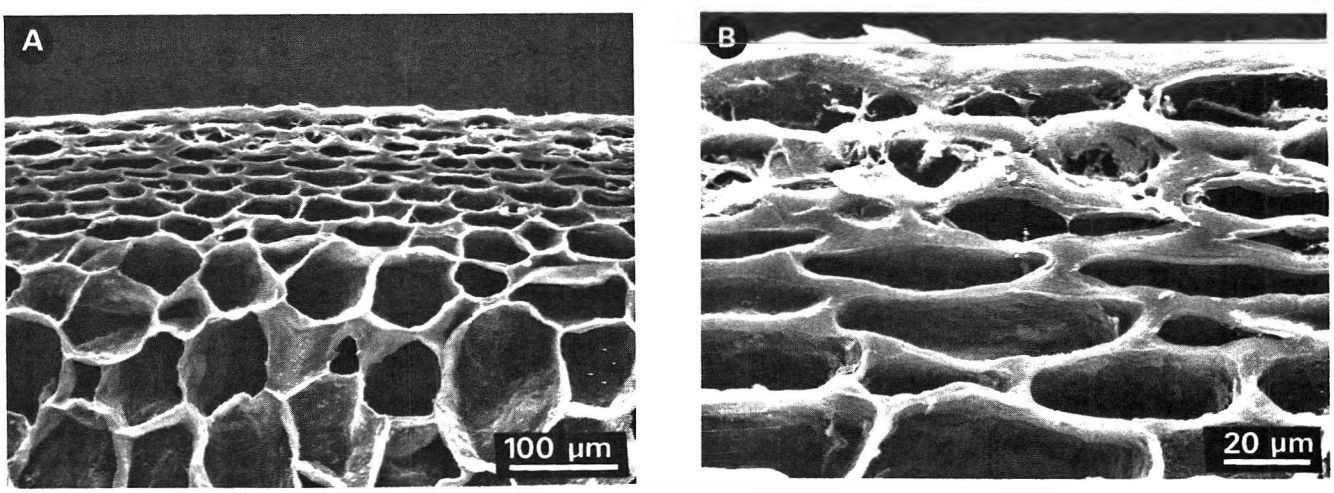

Fig. 4—Disruption of epidermal and hypodermal cells with 4% lye for 2 min shown under two magnifications.

(alcoholic) is not enough to depolymerize the resistant cellulosic wall components (Roelofsen, 1952; Hulsbruch, 1966; Holloway, 1982a). Worthington (1981) used 10% KOH to extract surface waxes from pimiento peppers. In this study NaOH solutions were used, which exhibit properties similar to KOH. Epicuticular waxes and portions of the cuticle proper (composed of cutin and waxes) may likely have been dissolved by the alkali treatment. Removal of cutins in the inner cuticular layer is also possible and would explain the increasingly evident anticlinal cell wall outlines (Figs. 3A through 3D) with longer or more severe alkali treatment. The resistance to depolymerization of the cellulosic wall components in the cuticular layer would account for the observed consistency in the thickness of the outer epidermal wall. Similarly, lye penetration through the cuticle is supported by reports on other chemicals (e.g. herbicides, pesticides, nutrients) which enter the cuticle after their application on the surface (McFarlane and Berry, 1974; Price, 1982; Holloway, 1982a; Glenn et al., 1985).

The technique for preparation of SEM specimens involved dehydration with ethyl alcohol. Worthington (1981) used 10% KOH in ethyl alcohol for the extraction of waxes from the surface of the pimiento peppers. Therefore, it was possible that wax removal took place due to the combination of lye treatment and ethanol dehydration. To confirm or reject this possibility, SEM specimens prepared with a freeze-drying technique were also examined. Freeze-drying resulted in severe tissue distortion, but photomicrographs corresponding to both SEM preparation techniques were similar as far as wax

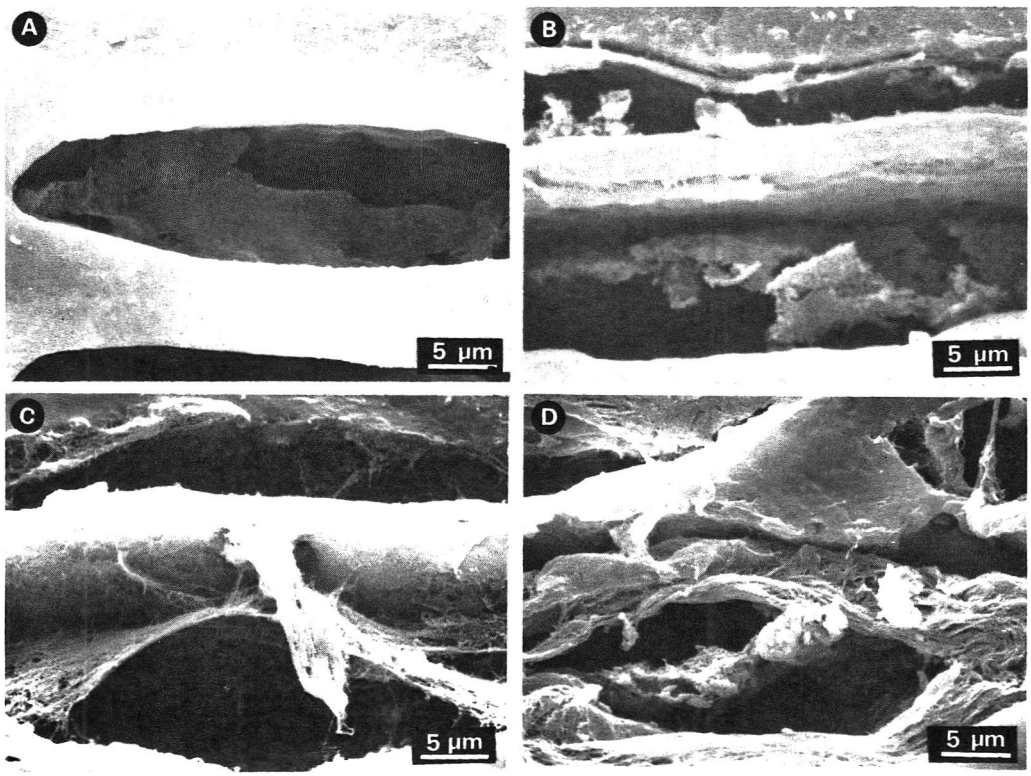

Fig. 5—Highly magnified hypodermal cells as affected by different treatments: (A) 1% NaOH for 1 min; (B) 1% NaOH for 2 min; (C) 4% NaOH for 2 min; (D) 9% NaOH for 1 min.

deposition and wax removal are concerned. Therefore, at least for the resolution required in this study, wax removal can be attributed solely to NaOH treatment.

Penetration of lye and mode of action inside the fruit. As the lye penetrated the cuticle and moved further into the interior of the fruit, disruption of epidermal and hypodermal cells took place (the outermost three cell layers in Fig. 4). The penetration effect of lye was uniform and a distinct boundary of disrupted cells was observed (Fig. 4). The boundary of disrupted cells moved deeper as the severity of lye treatment increased. These observations suggest that movement of lye was due to diffusion. Other chemicals and ions have also been reported to transfer from the surface to the interior of the fruit via diffusion (Price, 1975; Hoch, 1979; Price, 1982; Glenn et al., 1985).

The degree of degradation and the loss of integrity of cell walls caused by the lye action was proportional to the severity of lye treatment applied (Fig. 5). When fruits were treated with 1% NaOH for 1 min the cell walls of the epidermal and hypodermal cells of the exocarp were partly disrupted but their structure, shape and overall appearance were not greatly affected (Fig. 5A). When the time of treatment was increased to 2 min a total disruption of the cell walls was observed and signs of cell collapsing were evident (Fig. 5B). Application of higher concentrations of lye resulted in loss of integrity of cell walls (Fig. 5C) and solubilization of the middle lamella (Fig 5D). From biochemical investigations, McNeil et al. (1984) reported that the pectic and hemicellulosic polysaccharides can be solubilized by chelating agents and KOH. Lye action observed in Fig. 4 and 5 can be readily explained by the chemical reaction between NaOH and the polysaccharides of cell wall and middle lamella. The basic lye solution solubilizes the pectic and hemicellulosic polysaccharides leaving the cellulose microfibrils as an empty network with weak structure, which in turn results in cell collapsing.

The weakening and dissolution of cell wall constituents, as well as the solubilization of the middle lamella, eventually caused the separation of the exocarp (skin) from the mesocarp (Fig. 6A). The separation occurred mostly at the boundary of hypodermis and mesocarp, which could be explained on the basis of wall thickness of different cell layers and the amount of pectic substances in the middle lamella. Although the exocarp cells come in contact with the lye first, their thick walls and the presence of large amount of pectic substances in the middle lamella make them more resistant to disintegration. On the contrary, rapid disintegration of the parenchymatous cells of the mesocarp was observed, even though these cells come into contact with a reduced strength lye solution (due to larger distance from the surface and prior chemical reaction with epidermal and hypodermal cells). This is due to their thinner cell walls and the smaller amount of pectic substances in the middle lamella.

Treatment with 9% NaOH for 2 min resulted in separation of the skin at the boundary between hypodermis and mesocarp (Fig. 6A). All the hypodermal cell layers (6–8) were present even if the cell walls were extensively disrupted. Increasing the treatment time to 3 min also resulted in separation of the skin but with only two layers of cells attached (Fig. 6B). This indicates that separation will occur between hypodermis and mesocarp when 9% solution is applied for 2 min, but if lye action continues for another min (3 min total time) the lye would disrupt and break down more hypodermal cell layers, which are dissolved and washed away, as well as more parenchymatous cell layers from the mesocarp resulting in some loss of edible fruit (peeling loss). The same phenomenon takes place even at lower concentrations. Treatment of 4% NaOH for 3 min resulted in separation of the skin with only three cell layers attached (Fig. 6C), indicating that the time of treatment is a very important factor in the peeling process and that a short-time, high-concentration process would be more effective (lower peeling loss). This is also supported by optimization studies (Floros and Chinnan, 1987) where it was found that processing time is the most significant factor in a peeling process, followed by NaOH concentration and temperature.

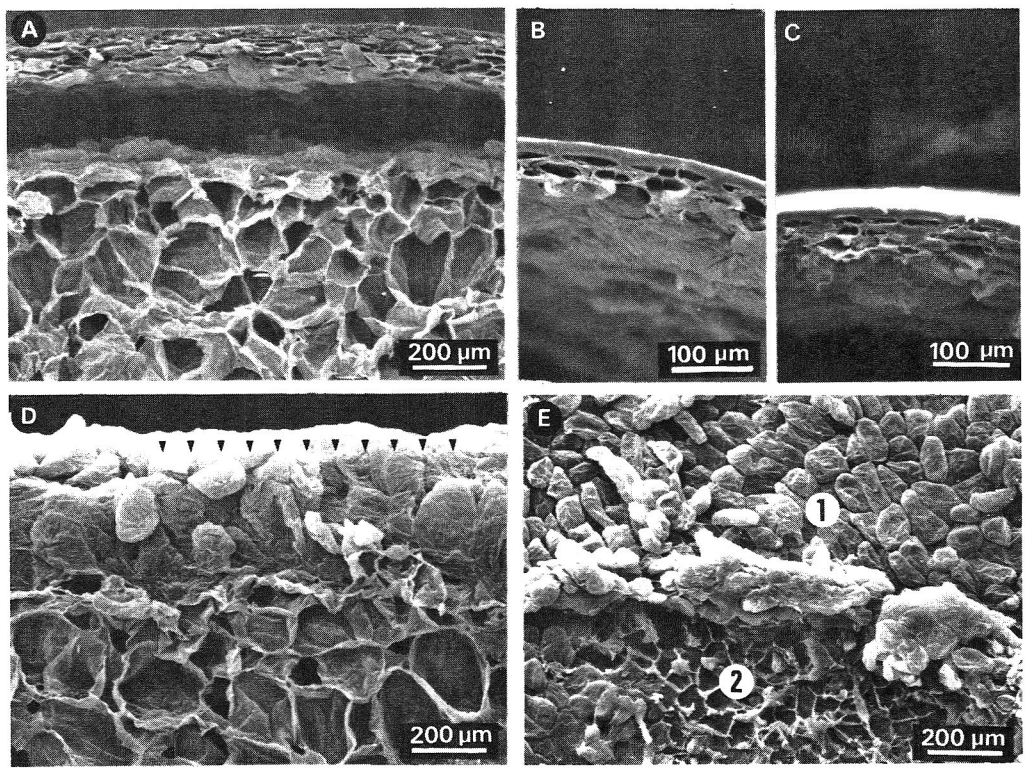

Fig. 6—Photomicrographs of the exocarp and/or mesocarp for selected lye treatments which resulted in separation of exocarp (skin): (A) Cross-section of the outer portion of fruit treated with 9% NaOH for 2 min showing separation of exocarp from the mesocarp; (B) Cross-section of the exocarp of a fruit treated with 9% NaOH for 3 min; (C) Cross-section of the exocarp of a fruit treated with 4% NaOH for 3 min; (D) Cross-section of the mesocarp of a fruit treated with 9% NaOH for 3 min (Arrows indicate region where separation was due to solubilization of middle lamella); (E) View of the inner surface of the separated exocarp of a fruit treated with 9% NaOH for 3 min. The two regions indicate separation due to solubilization of middle lamella (region, 1), or due to cell wall breakdown (region, 2).

The major reason for skin separation was the solubilization of middle lamella as evidenced by the entire cell outlines at the separation area (arrows in Fig. 6D and region 1 in Fig. 6E). However, skin separation also took place due to cell wall breakdown (region 2 in Fig. 6E) which is evident from viewing the inner surface of the separated skin.

SUMMARY & CONCLUSIONS

AN EVALUATION of the events during a lye peeling process has been described at the ultrastructural level. The mode of action of lye has been determined using pimiento pepper as a case study.

The hot lye-solution dissolves and removes the epicuticular and cuticular waxes of the outer pericarp surface, penetrates the skin and diffuses uniformly into the fruit. The diffusion of lye causes breakdown of the epidermal and hypodermal cells and solubilization of the pectic substances in the middle lamella, separating the skin from the edible part of the fruit. More severe action of lye causes destruction of cell walls especially of the mesocarp cells resulting in increased peeling loss.

The severity of the lye action, and the depth of penetration depends primarily upon the concentration of the solution and the time it is permitted to act on the fruit. If the lye-peeling process is carried out satisfactorily, diffusion and action of lye will be controlled and cell wall degradation effects will be restricted to epidermal and hypodermal regions with a minimum effect on parenchymatous mesocarp cells. If the lye solution is applied for too long a time, or it is highly concentrated, severe action will take place deeply inside the flesh, and some loss of the edible part of the fruit will occur.

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Relationship Between Pectic Compositions and the Softening of the Texture of Japanese Radish Roots During Cooking

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ABSTRACT

The pectins of Japanese radish roots were fractionated by successive extraction with three reagents: diluted HCl, sodium acetate buffer, and sodium hexametaphosphate solutions. The tissue, softened by thermal maceration, contained more HCl-soluble pectin (pA) than the sodium acetate buffer soluble pectin (pB). The degree of esterification (DE) of pA was higher than that of pB. With $CaCl_2$, pB formed some precipitates, but pA did not. The elution patterns of pA and pB by DEAE-cellulose column chromatography were different. A clear relationship between other dietary fiber (hemicellulose, cellulose and lignin) and softening of roots was not observed. The composition of pectins, especially DE, appeared to have an effect upon the texture of the Japanese radish softened during cooking.

INTRODUCTION

PECTIN is the main component of middle lamella. It contributes to adhesion between parenchyma cells of vegetables and mechanical strength of tissues. Maceration of vegetable tissues seems to be brought about mainly by the degradation of pectin (Doesburg, 1965). Softening of vegetables during cooking is considered to be affected considerably by the properties of pectic substances. The low methoxyl pectins are difficult to break down in hot neutral solutions by trans-elimination (Albersheim et al., 1960; Konishi et al., 1974). Therefore, the vegetables which have a comparatively larger amount of low methoxyl pectin may be difficult to soften during cooking. Pectic substances of vegetable tissues are heterogeneous. The composition of pectic substances seems to vary with the kind of vegetables (Kertetz, 1951; Fuchigami, 1987).

A new method of fractional extraction of pectic substances of vegetable tissues under mild conditions has been reported (Fuchigami and Okamoto, 1984). The extraction was done successively with these three agents: hydrochloric acid, sodium acetate buffer, and sodium hexametaphosphate solutions. By using this method, three pectins, which were different from each other in the degree of esterification (DE), specific viscosity and degree of precipitation with $CaCl_2$ were extracted.

The present study was concerned with the relationship between pectic compositions, especially DE of pectin or dietary fiber and the softening of Japanese radish roots during cooking.

MATERIALS & METHODS

Sample preparation

Roots of fresh Japanese radish (*Rapanua sativas* L.) were peeled, decorticated with a cork borer to produce cylinders of internal xylem tissue 1 cm in diameter and cut into segments 5.0 mm long. The middle parts of 39 roots (or 58 roots for pH) were used for this experiment. Twelve disks (5.0g) of roots were dropped into boiling distilled water (10 mL) to inactivate enzymes. After the roots were cooked in the test tubes for 30 min in a boiling waterbath, they were rapidly cooled in tap water for 10 min. Firmness of the 12 disks was measured by the Curdmeter (II0 Electric Co. M-301A, 2-23-1 Yoyogi, Shibuya-ku, Tokyo, Japan) and expressed as breaking strength (dyne/cm^2) at the point where the disks collapsed. pH of the solution was measured after cooking.

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Extraction of pectic substances

Ten gram samples cut into tiny pieces were homogenized in a Waring Blendor for 2 min with 90g 0.01N HCl (pH 2.0), and then the homogenated sample was adjusted to pH 2.0 with 1N HCl. The first extraction was done at 35°C for 24 hr in an incubator (Sakura Seiki Co., 3-9 Honmachi, Nihonbashi, Chuoh-ku, Tokyo, Japan). Homogenates were filtered through a glass filter 3G2. The residue was extracted 4 or 5 times until no pectin could be detected. The extractants were centrifuged and the supernatants were dialyzed against distilled water at 5°C. The first extracts were designated as pectin A (pA). Subsequently, the residue from the extraction with HCl solution was extracted 4 or 5 times with 0.1M sodium acetate buffer solution (pH 4.0) for 24 hr at 35°C. The second extracts were designated as pectin B (pB). The last extraction was done with 2% sodium hexametaphosphate solution (pH 4.0) at 90°C for 3.5 hr. The last extracts were designated as pectin C (pC).

Analysis of extracts

Each dialyzed fraction was treated in the following way. Total pectin was determined by the carbazole method (Galambos, 1969). DE of pectin was measured by the gas chromatographic procedure of Bartolome and Hoff (1972).

Specific viscosities were measured in an Ostwald viscosimeter at 20°C. To avoid the effect of Ca^{-2}, 1 mL extracts of pA and pB at concentration of 0.2% anhydrouronic acid were added to 0.5mL 0.14M sodium oxalate solution (pH 6), and 0.5 mL 1M acetate buffer solution (pH 6).

To check for precipitation of pectic substances by $CaCl_2$, 0.5 mL extracts of pA and pB at concentration of 0.2% anhydrouronic acid were added to 0.5 mL 5M $CaCl_2$.

The DEAE-cellulose column chromatography of pectic substances was carried out by the method of Hatanaka and Ozawa (1968). Pectic substances (about 5 mg in 0.02M sodium acetate buffer, pH 6.0) were added to a DEAE-cellulose column (2.0 × 5.0 cm) equilibrated with the same acetate buffer. The column was first washed with equilibrating buffer and then eluted successively with 0.1–1.0M acetate buffer of pH 6.0 (linear gradient) and 0.1N NaOH. Pectinic acid, acid-insoluble pectic acid, and pectic acid were prepared for comparison with pA, pB and pC from citrus pectin by the method of Hatanaka and Ozawa (1968). The fractions were monitored by the phenol-sulfuric acid method (Dubois et al., 1956). The amount of uronic acid was determined by the carbazole method. The amount of neutral sugar was calculated by the method of Hatanaka and Ozawa (1968).

Neutral sugars were analyzed by gas chromatography (Kusakabe et al., 1977). The pectic substances were hydrolyzed with sulfuric acid at pH 1 at 120°C for 60 min. Neutral sugars in the hydrolysate were reduced with sodium borohydride, and the amount of the alditol-acetates was determined by gas liquid chromatography; column, 3% ECNSS-M, 3 × 1500mm glass column; column temperature, 180°C; carrier gas, N_2, 60 mL/min; detector, FID; internal standard, methyl-β-D-glucoside.

The other dietary fibers (hemicellulose, cellulose and lignin) were determined by the method of Van Soest and Wine (1968).

Minerals (Ca, Mg, K and Na) were determined by atomic absorption spectrophotometry (Kohara et al., 1982).

RESULTS & DISCUSSION

THE BREAKING STRENGTH (BS) of raw or cooked Japanese radish roots was 231.6 ± 31.2 × 10^5 and 19.0 ± 9.4 × 10^5 dyne/cm^2, respectively. The degree of softening (DS) was indicated as 100 − BS after cooking ÷ raw BS × 100%. The mean (n = 39) of DS was 91.6 ± 41%. A good relationship ($r = 0.618$, $n = 58$, $p<0.01$) was observed between the pH of solution after cooking for 30 min and DS (Fig.1). Rise in pH increased DS. Vegetable tissues were usually kept firm when they were cooked at pH 4 and were rapidly softened by cooking either above pH 5 or below pH 3 (Doesburg, 1965; Fuchigami, 1983). Pectic substances released from vegetables during cooking were the lowest at pH 4 and increased both above the pH 5 level and below the pH 3 level. Pectic substances released from vegetables during cooking (above pH 6) gave positive results by the thiobarbituric acid test (Fuchigami, 1983). Trans-elimination of pectins increased with rising pH and methoxylation when they were heated above pH 5 (Konishi et al., 1974). These results suggested that enhanced softening above pH 5 was due to degradation of pectin by the trans-elimination mechanism. Since the amount of the intercellular components such as organic acids seems to affect the pH of solution during cooking, these components may also affect DS.

The amounts of minerals, ash, moisture and dietary fiber (hemicellulose, cellulose, and lignin) are shown in Table 1. There was no correlation between the amount of minerals, ash or moisture and DS. The amount of hemicellulose depended on the species of Japanese radish, but no correlation existed between the amount of dietary fiber and DS. The amount of ash, minerals or dietary fiber did not correlate with the BS of the raw tissues, but that of moisture correlated with BS ($r = -0.474$, $p<0.05$). Possibly, softening of vegetables during cooking was affected more by the composition of properties of pectic substances than that of the other dietary fiber.

A method, previously developed to extract fractionally pectic substances from vegetable tissues (Fuchigami and Okamoto, 1984), was used to investigate the relationship between the pectic composition and DS. The amount and properties of pectic substances of Japanese radish roots extracted with the three agents are shown in Table 2.

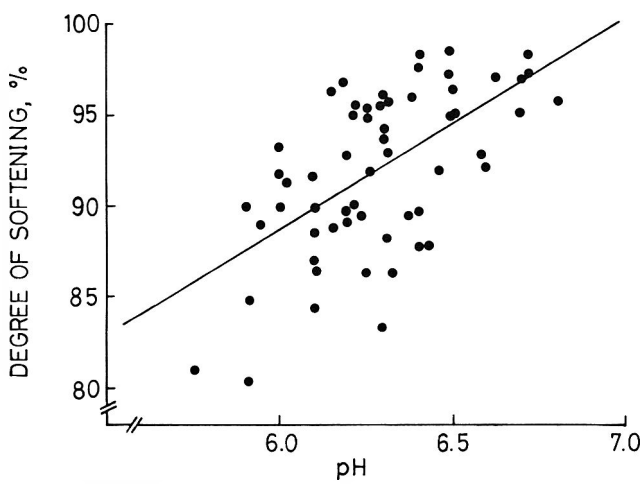

Fig. 1—Relationship between the degree of softening and pH of solution after cooking. Y = 19.25 + 11.6X, r = 0.618 (p<0.01), n = 58. The degree of softening (DS) was calculated, 100 − breaking strength after cooking (BS) ÷ raw BS × 100%.

While dipping tissues in a diluted HCl solution (pH 2.0) at 35°C, 26~70% pectic substances were extracted from the tissues due to removal of polyvalent cations (Fig.2). A solution of 0.01N HCl was used as calcium and magnesium sequestering agent. About 92.2% Ca and 95.5% Mg were extracted from the Japanese radish roots at 35°C after 24hr (Fuchigami and Okamoto, 1984). Pectic acid and pectinic acids with a low degree of esterification are usually precipitated by addition of acid: therefore, it is difficult to extract these pectic substances at pH 2.0. HCl-soluble pectic components (pA) were highly methyl-esterified (about 58%). Addition of $CaCl_2$ to pA did not cause precipitation. Therefore, PA was easily extracted. Possibly, methoxyl groups lay randomly in polygalacturonic acid chains or the polygalacturonic acid chain without the methoxyl group was not present as a block.

After repeating the extraction with diluted HCl until no detection of pA could be found, the residue was extracted with 0.1M acetate buffer solution (pH 4.0) at 35°C; 27.6–66.9% of pectins were found in this fraction. PB was the low-methoxyl pectin (DE, about 40.0%). With the addition of $CaCl_2$, pB formed precipitates, but pA did not. PB may have some groups of the polygalacturonic acid chain with no methoxyl group. Specific viscosity values of pB were higher than those of pA. This suggested that the molecular weight of pB was higher than that of pA. This might be one of the causes of the high solubility of pA.

About 92.3% of the pectic substances were extracted from Japanese radish roots by the use of 0.01N HCl and 0.1M acetate buffer solution at 35°C. The quantity of pectin C, which was extracted with 2% sodium hexametaphosphate solution (pH 4.0) at 90°C for 3.5 hr, was considerably small (about 7.7%). This strongly suggests that pectins linked with insoluble hemicelluloses through covalent bonds (Keegstra et al., 1973) are absent or in minute quantities in Japanese radish cell walls. Pectic substances in the cell walls seemed to be insoluble and stable because of polyvalent cations such as Ca^{2+}. There was a distinct positive correlation ($r = 0.689$, $n = 39$, $p<0.01$) between the percent pA and DS (Fig. 2). There was a positive correlation between DE of the pectin in Japanese radish roots and DS ($r = 0.506$, $n = 39$, $p<0.01$). The tissue softened by thermal maceration contained more pA than pB. DE of pA was higher than that of pB. Therefore, pA was more easily broken down in a hot neutral solution by the trans-elimination mechanism, and the tissue with the greater amount of pA decreased in intercellular adhesion strength.

There was a negative correlation ($r = -0.690$, $n = 39$, $p<0.01$) in the total amount of pB plus pC vs DS (Fig.3), and there was also a negative correlation between the percent pB or pC / total pectin vs DS ($r = 0.625$, $p<0.01$; $r = 0.406$, $p<0.01$), respectively. Japanese radish roots with less thermal maceration had more pB and pC than pA. They did have a comparatively larger amount of low methoxyl pectin which was difficult to break down in hot neutral solutions. A considerable amount of pB remained undissolved after cooking (Fuchigami, 1986a): therefore, these tissues stayed firm.

PA markedly decreased while pB increased by preheating Japanese radish roots at 60°C for 2hr. A similar tendency was observed with dried tissues (Fuchigami, 1986a) and alkaline

Table 1—Composite analysis of Japanese radish roots (fresh weight basis)

Mineral	mg%		%	Dietary fiber	mg%
Calcium	21.9 ± 4.2	Ash	0.5 ± 0.14	Hemicellulose	252.4 ± 149.8
Magnesium	8.2 ± 2.7	Moisture	94.9 ± 0.8	Cellulose	500.6 ± 106.3
Potassium	154.3 ± 60.9			Lignin	47.7 ± 21.8
Sodium	21.7 ± 7.8			Pectin	409.7 ± 121.9

Table 2—Amount and some properties of pectic substances in Japanese radish roots (n = 39)

Types of pectin	Amount of pectin (Anhydrouronic acid, mg%)	Percentage of pA, pB and pC	Specific viscosity	Degree of esterification (DE), %	Precipitated by $CaCl_2$
pA[a]	179.2 ± 46.7	45.0 ± 9.9	0.268 ± 0.122	58.8 ± 7.4	−
pB[b]	198.7 ± 56.0	47.3 ± 8.0	0.432 ± 0.153	40.0 ± 5.2	+ +
pC[c]	31.8 ± 19.0	7.7 ± 4.6			

[a] pA: Extraction with 0.01N HCl (pH 2.0) at 35°C.
[b] pB: Residues of pA extracted with 0.1M sodium acetate buffer solution (pH 4.0) at 35°C.
[c] pC: Residues of pB extracted with 2% sodium hexametaphosphate (pH 4.0) at 90°C.

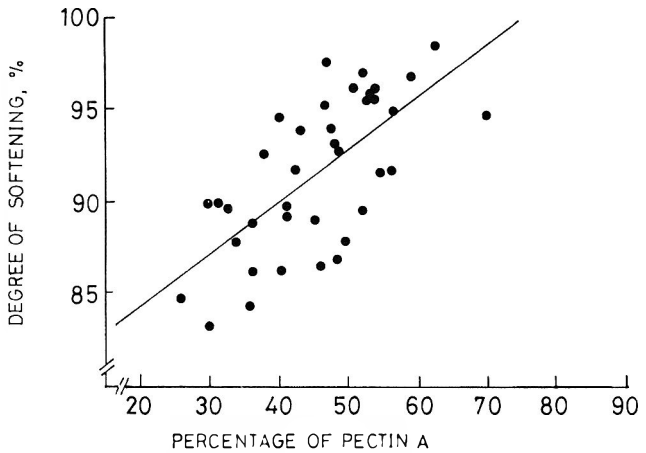

Fig. 2—Relationship between the degree of softening and the percent pectin A. Y = 78.65 + 0.29X, r = 0.689 (p<0.01), n = 39.

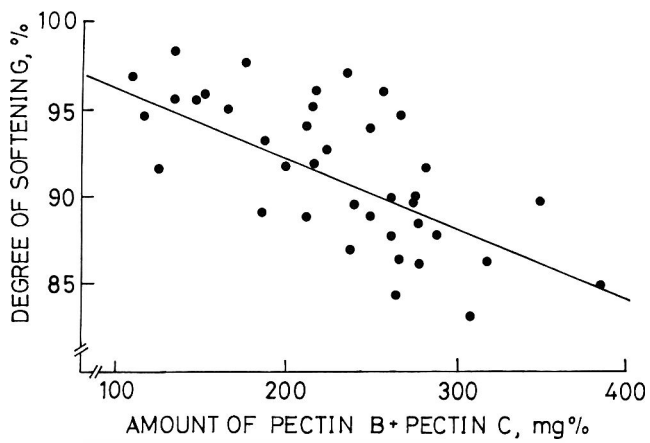

Fig. 3—Relationship between the degree of softening and the amount of pectin B + pectin C. Y = 101.5 − 0.043X, r = −0.69 (p<0.01), n = 39.

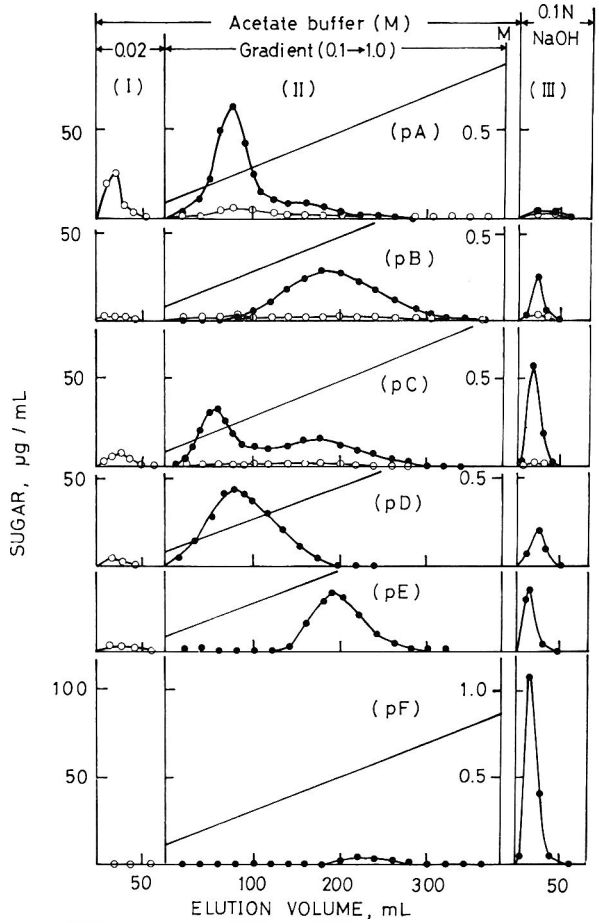

Fig. 4—DEAE-cellulose column chromatogram of various pectic substances. pA, pectin A; pB, pectin B; and pC, pectin C extracted from Japanese radish roots (see Table 2). pD = pectinic acid purified with ethyl alcohol from citrus pectin. pE = acid-insoluble pectic acid saponified at 0°C for 90 min after boiling with 2% HCl for 2 hr. pF = pectic acid saponified at 0°C for 90 min. ○, neutral sugar; ●, galacturonic acid.

saponified tissues (Fuchigami, 1986b). This phenomenon was caused by the demethoxylation of pectin which was done by pectin methylesterase or alkaline saponification. These pretreated tissues were more difficult to soften than raw tissues when they were cooked at 98°C. The breakdown of pectins within raw tissues by trans-elimination proceeded more rapidly than that in pretreated tissues. This indicated that the composition of pectic substances in vegetables was important for the softening of tissues during cooking.

To investigate the properties of these pectins in detail, pA, pB and pC were fractionated into neutral and acidic fractions by DEAE-cellulose chromatography. The elution patterns of pA, pB and pC by DEAE-cellulose chromatography were different (Fig. 4). Neutral sugar is usually eluted in a 0.02M acetate buffer (I). Fraction I (100% neutral sugar) of pA contained 11.8%, but those of pB and pC contained only small quantities. The composition of these neutral sugars is shown in Table 3. Arabinose was about 62 ~ 69% of the neutral sugars in fraction I.

The next fraction (II) was eluted with a linear gradient of increasing concentration of acetate buffer (pH 6). The pA peak was detected at about 80ml. This pattern was similar to that of pectinic acid purified from citrus pectin. The pB peak was detected at about 180 mL. This pattern was the same as that of acid-insoluble pectic acid. Pectin C had two peaks. About 81 ~ 86% pectic substances was eluted in fraction II. Since the low methoxylpectin was usually eluted later in fraction II, DE of pB was lower than DE of pA (Fig. 4). Neutral sugar/pectic substances in fraction II was pA, 17.2%; pB, 16.9%; and pC, 14%, respectively. The percent arabinose/neutral sugars was pA, 59.2%; pB, 53.8%; and pC, 35.1%; conversely, that of galactose was pA, 19.6%; pB, 22.0%; and pC, 40.5%, respectively.

Finally, pectic substances were eluted with 0.1N NaOH.

Table 3—Monosaccharide composition of polysaccharide fractions separated by DEAE-cellulose column chromatography

Types of pectin	Fraction[a] by DEAE	Percent of fraction	Neutral[b] sugar (%)	Composition of monosaccharides (%)					
				Rha[c]	Ara[d]	Xyl[e]	Man[f]	Gal[g]	Glc[h]
pA	I	11.8	100	2.3	68.3	2.7	8.1	5.8	12.7
	II	81.7	17.2	2.3	59.2	1.6	10.3	19.6	7.0
	III	6.4	32.1	14.7	14.7	13.0	18.4	31.5	7.6
pB	I	3.3	100						
	II	85.6	16.9	7.4	53.8	8.5	4.9	22.0	3.4
	III	11.0	13.7	18.0	26.7	8.7	19.8	15.6	11.3
pC	I	4.9	100	0.8	61.7	5.6	4.7	9.5	17.6
	II	80.8	14.0	7.8	35.1	3.6	11.1	40.5	1.9
	III	14.3	3.7	8.7	17.3	21.6	18.7	26.0	7.8

[a] Fraction obtained by DEAE-cellulose column chromatography (see Fig. 4).
[b] Neutral sugar (%) = neutral sugar ÷ pectic substances (galacturonic acid + neutral sugar) × 100.
[c] Rhamnose
[d] Arabinose
[e] Xylose
[f] Mannose
[g] Galactose
[h] Glucose

The amount of pectic substances eluted in fraction III was as follows: pC > pB > pA, respectively.

The amount of pA in fraction III was small. The percent neutral sugar/pectic substances was pA > pB > pC, respectively, with small amount of pC. The pectic acid, saponified at 0°C, and eluted in fraction III was hardly eluted in fraction II. The average polymerization grade (43.7) of acid-insoluble galacturonic acids was smaller than that of pectic acid. Therefore, pectic substances having no methoxyl group and a comparatively larger molecular weight were eluted in fraction III. Since pB and pC had both a comparatively large fraction III and a lot of pectin which was eluted later in fraction II, pB and pC were comparatively low-methoxyl pectins. These pectins were difficult to break down by the trans-elimination mechanism; therefore, the tissues containing more pB and pC than pA in themselves were difficult to soften by thermal maceration.

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Ms received 12/15/86; revised 3/6/87; accepted 3/6/87.

A part of this research was supported by scientific research fund from the Ministry of Education.

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Ms received 2/20/87; revised 4/18/87; accepted 4/28/87.

Presented at the 46th Annual Meeting of the Institute of Food Technologists, Dallas, TX, June 15–18. The authors express their appreciation to Ms. Hazel David for technical assistance.

Nitrogenous Constituents of Selected Grain Legumes

S. S. DESHPANDE and S. S. NIELSEN

ABSTRACT

Nitrogenous constituents of four legume species and their varieties were investigated. The total N of whole seeds ranged from 3.16–4.21%, of which 8.3–14.5% was nonprotein N. Water- and salt-soluble proteins together constituted 72–94% of the total seed protein. The mean protein contents of the water- and salt-soluble fractions were 67% and 87%, respectively, while the carbohydrates associated with them ranged from 17–30% and 4–14%, respectively. The albumin:globulin ratios were characteristic of the variety. The SDS-PAGE patterns for proteins of *Phaseolus* showed a more complex profile than those of the other legume species investigated.

INTRODUCTION

MOST STUDIES on legume proteins have focused on the globulin fractions, the major storage proteins comprising 50–75% of the total seed protein (Romero et al., 1975; Chakraborty et al., 1979). In contrast, only a few studies have been conducted on the albumins and other proteins of legumes (Sefa-Dedeh and Stanley, 1979; Bhatty, 1982; Marquez and Lajolo, 1981). Unlike the globulins, albumins are considered as primarily enzymic or nonstorage proteins. Recent studies, however, have indicated that the albumins of germinating pea cotyledons are degraded, and thus may also serve a similar function as the globular storage proteins of the legumes (Murray, 1979).

Additonal basic information is needed on the legume proteins as related to digestibility, particularly for the albumin and the globulin fractions. This study represents the first is a series of investigations designed to determine the characteristics of legume proteins that give rise to their poor digestibility. The main purpose of the present investigation was to evaluate the relative proportions of water- and salt-soluble proteins and TCA-soluble nonprotein nitrogen (NPN), and to compare certain properties of these proteins in different genotypes and species of food legumes.

MATERIALS & METHODS

THE SOURCE of dry beans of *Phaseolus vulgaris* type was reported earlier (Deshpande et al., 1982). Tendergreen French beans were from W. Atlee Burpee Co., Warminster, PA. Garbanzo (chickpea), Black-eye peas, Baby Lima and Large Lima beans were a gift from Dr. William Isom. The scientific names and the seed types of these legumes are summarized in Table 1.

Whole bean flours were obtained by grinding the hand-cleaned, sound beans in a UDY Cyclone mill (0.4 mm screen). Unless mentioned otherwise, all chemicals used were of reagent grade.

Classification of nitrogenous constituents

Typical nitrogen solubility studies were carried out using Osborne's (1907) classical fractionation scheme by a modification of the technique of Lund and Sandstrom (1943). Duplicate samples of 2g bean flours were sequentially extracted with distilled water, 2% NaCl, 60% aqueous ethanol and 0.2% NaOH. Two consecutive extractions (1:10 flour:solvent ratio, w/v) were carried out using each of these four solvents for 2 hr (determined to be optimal time in preliminary studies)

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Table 1—Legume seed types selected for the study

Sample no.	Common name	Scientific name	seed type
1	Navy	*Phaseolus vulgaris*	Sanilac
2	Great Northern		D76067
3	Small White		D 76063
4	Pink bean		Viva
5	Pinto		UI 111
6	Cranberry		Michigan Improved Cranberry
7	Light Red Kidney		Manitou
8	Dark Red Kidney		Charlevoix
9	Small Red		UI 36
10	Black bean		Black Beauty
11	French bean		Improved Tender-green
12	Large Lima	*Phaseolus lunatus*	Lee
13	Baby Lima		Mezcla
14	Black-eye Peas	*Vigna unguiculata*	California #5
15	Chickpea (Garbanzo)	*Cicer arietinum*	UC #5

per extraction at room temperature (25–27°C). The samples were centrifuged at 25,000 X g for 30 min after each extraction. The supernatants from similar extractions were pooled, made to volume and filtered through Whatman #4 filter paper prior to nitrogen analyses.

Water- and salt-soluble protein fractions

The above studies indicated that water and salt together solubilized over 80% of the total seed nitrogen depending upon the legume. Hence the water-soluble and salt-soluble proteins were selected for further studies. These protein fractions were obtained by sequential extraction of 25g bean flour with water and salt as described above. The residue remaining after the salt extraction was discarded. The supernatants were dialyzed for 48 hr against distilled water at 4°C and lyophilized.

The water-soluble proteins, on analysis by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), showed cross-contamination with the major storage protein (viz., phaseolin or the G1 globulin) of dry beans. To demonstrate that salt was essential for the solubility of the latter, legume flours of selected varieties were dialyzed against distilled water for 48 hr, centrifuged (25,000 X g, 30 min) to separate the precipitated globulins from the water-soluble proteins, and lyophilized. The true albumin:globulin ratios were obtained in a similar fashion.

Protein determination

The total N, TCA-soluble NPN, and the nitrogen contents of each fraction were measured by the micro-Kjeldahl method (AOAC, 1975). A conversion factor of 6.25 was used for calculating the protein content.

Carbohydrate analysis

Total carbohydrates (expressed as glucose equivalents) associated with the water- and salt-soluble proteins were assayed by the phenol-sulfuric acid method (Dubois et al., 1956).

SDS-PAGE

SDS-PAGE separation was performed according to Laemmli using gradient slab gels of 7.5–20% monomer acrylamide concentrations (Sathe et al., 1983). Glycoproteins were detected using the periodic acid-Schiff's (PAS) stain (Dubray and Bezard, 1982). Low MW marker protein kid containing phosphorylase b (94 Kdalton), bovine serum albumin (67 kdalton), ovalbumin (43 kdalton), carbonic anhydrase (30 kdalton), soybean trypsin inhibitor (20.1 kdalton) and alpha-lac-

Table 2—Total, nonprotein (TCA-soluble), and protein nitrogen of grain legumes[a]

Legume Variety	Total N g 100 g beans	Nonprotein N		Protein N	
		mg/100g bean	% of Total N	g/100g beans	% of Total N
Dry beans					
Navy	3.54	292	8.3	3.24	91.7
Great Northern	3.58	521	14.5	3.06	85.5
Small White	3.30	371	11.2	2.93	88.8
Pink Bean	3.47	441	12.7	3.03	87.3
Pinto	3.79	453	11.9	3.34	88.1
Cranberry	4.17	458	11.0	3.71	89.0
Light Red Kidney	4.13	448	10.9	3.68	89.1
Dark Red Kidney	4.10	485	11.8	3.61	88.2
Small Red	3.83	452	11.8	3.38	88.2
Black Bean	3.76	481	12.8	3.28	87.2
French Bean	3.68	395	10.7	3.29	89.3
Lima beans					
Large Lima	4.21	454	10.8	3.75	89.2
Baby Lima	3.61	340	9.4	3.27	90.6
Black-eye Peas	4.07	451	11.1	3.62	88.9
Chickpea	3.16	291	9.2	2.87	90.8
Mean	3.76	422		3.34	
LSD[b] (P = 0.05)	0.257	36.8		0.185	

[a] Data expressed on a dry weight-basis.
[b] Least significant difference. Differences of two means exceeding this value are significant.

talbumin (14.4 kdalton) proteins was from Pharmacia Fine Chemicals, Piscataway, NJ.

Statistical analyses

Analyses of variance and Pearson's correlation coefficients between different parameters were calculated according to the procedures of the Statistical Analyses System (SAS, 1979). Least significant differences were used for multiple mean comparison tests.

RESULTS & DISCUSSION

DATA on the total N, TCA-soluble NPN and the protein N of different legumes investigated are summarized in Table 2. The total N of whole seeds ranged from 3.2–4.2% on a dry weight basis. The NPN accounted for 8.3–14.5% of the total N. Among the varieties studied, the Great Northern beans contained significantly higher NPN. The NPN of the legumes correlated positively with the total N of the seed (r = 0.62, significant at 1% level). The large NPN content of legumes may influence the estimated protein by at least 2–4% on seed weight basis, consequently leading to erroneous estimations of protein intake in the diet. The protein N for all the varieties investigated ranged from 2.87–3.75%. The mean protein content corrected for the NPN was 20.9% with the chickpeas and the Small White beans having the lowest average value of about 18%. Data on total N in the present study are in agreement with previously published reports on several legumes (Chakraborty et al., 1979; Marquez and Lajolo, 1981; Bhatty, 1982).

The Osborne classification of legume protein nitrogen by sequential extraction of whole bean flours is presented in Table 3. The water- and salt-soluble proteins together accounted for 63–83% of the total N with a mean value of 73.2%. When corrected for the NPN, these values ranged from 72–94% of the protein nitrogen with an average recovery of 82.6% of the total proteins. Lima beans, black-eye peas and chickpeas contained significantly higher water-soluble N than the 11 genotypes of dry beans. They also had proportionately lower salt-soluble proteins. The range of values for the 60% ethanol-soluble proteins was very small, the average for all the species investigated being 1% of the protein N. However, dilute alkali solubilized 2–18% of the protein N remaining after the first three solvent extractions. With the exception of Cranberry, the pigmented varieties of dry beans had more protein solubilized in alkali than the white seed types. The mean protein N recovery with the four different solvents was about 94%.

Most studies on various legume species have been limited to water-soluble ("albumins") and salt-soluble ("globulins") proteins, since they constitute over 80% of the total protein. Recently, Bhatty (1982) reported a range of 62.7–71.9% for the salt-soluble N of 8 different legume species with a mean recovery of 93.3%. Of these, the albumins and the globulins constituted 8.1–14.1% and 30.5–42.0%, respectively, of the total meal N. Murray (1979), on the other hand, reported a wide range of 14–42% for the albumin fraction of several pea varieties. Bhatty (1982) suggested that the discrepancies in the published data are most likely due to cross-contamination of the water- and salt-soluble proteins. In fact, this probably was the main reason for the high water-soluble protein values observed in the present study. The native ionic strength of the bean flours investigated and the low flour:solvent ratios (1:10, w/v) may have solubilized some globulin proteins in water.

Despite similar protein recoveries, the pigmented varieties of dry beans yielded more alkali-soluble N. These varieties were also rich in tannins (Deshpande et al., 1982). The interactions of tannins with proteins are well documented in the literature (Deshpande et al., 1984). The tannins in the pigmented varieties may account for the fact that the quantity of the salt-soluble proteins for some of these varieties investigated was lower than that reported in the literature. Clearly, the true "globulin" contents cannot be measured accurately by the Lund and Sandstrom (1943) classification procedure due to the effects of endogenous tannins present in the pigmented dry beans as well as the native ionic strength of the bean flour dispersions in water.

The differences in the protein solubility properties were further illustrated when expressed as a ratio of water-soluble:salt soluble N (Table 4). This ratio differed widely between different varieties and legume species. Among the dry beans, Pinto, Cranberry and the Great Northern beans had relatively more salt-soluble N, whereas Small White and Small Red beans had nearly an equal distribution of water- and salt-soluble N. This ratio for the remaining seed types was greater than one. The other three legume species investigated had significantly higher ratios than those obtained for *P. vulgaris*. The two lima bean seed types had similar ratios.

Since the major storage protein, phaseolin, of the *P. vulgaris* species was also soluble in water, the true albumin:globulin ratios were somewhat different (Table 4). Yet, differences were observed for the 11 dry bean varieties. With the exception of Navy, Black and the two Kidney bean types, this ratio was less than one, while the four other species investigated had

Table 3—Classification of legume protein nitrogen based on solubility property[a]

Legume/Variety	Water-soluble		2% NaCl-soluble		60% EtOH-soluble		0.2% NaOH-soluble		Protein N recovery
	% of Total N	% of Protein N	% of Total N	% of Protein N	% of Total N	% of Protein N	% of Total N	% of Protein N	% of Protein N
Dry beans									
Navy	42.7	46.5	32.4	35.4	1.02	1.11	7.1	7.7	90.7
Great Northern	31.3	36.6	43.9	51.3	0.81	0.95	5.5	6.4	95.3
Small White	33.7	37.9	34.9	39.4	0.79	0.89	7.4	8.3	86.5
Pink Bean	36.9	42.2	32.4	37.1	0.95	1.09	11.6	13.2	93.6
Pinto	25.8	29.3	48.7	55.3	1.06	1.20	12.0	13.7	99.5
Cranberry	31.9	35.9	51.6	58.0	0.58	0.65	2.3	2.5	97.1
Light Red Kidney	40.4	45.3	32.2	36.2	0.87	0.98	11.0	12.3	94.8
Dark Red Kidney	40.4	45.8	29.5	33.5	1.00	1.13	8.1	9.2	89.6
Small Red	33.7	38.2	33.9	38.4	0.86	0.98	13.4	15.2	92.8
Black Bean	45.7	52.4	33.0	37.9	0.82	0.94	11.0	12.6	103.8
French Bean	32.0	35.8	36.7	41.1	0.90	1.00	9.6	10.8	88.7
Lima beans									
Large Lima	65.7	73.7	14.1	15.8	0.69	0.77	4.8	5.4	95.8
Baby Lima	63.1	69.7	13.8	15.2	0.75	0.83	7.0	7.7	93.4
Black-eye Peas	48.1	54.1	26.6	29.9	0.86	0.97	9.8	11.0	96.0
Chickpea	47.0	51.8	16.3	20.0	1.17	1.29	17.0	18.7	91.8
LSD[b] (P = 0.05)	2.9		2.1				1.8		

[a] Mean of duplicate determinations on a dry weight basis.
[b] Least significant difference. Differences of two means exceeding this value are significant.

Table 4—Water:salt soluble nitrogen and albumin:globulin ratios of grain legumes[a]

Legume Variety	Water soluble protein N g 100 g beans	Salt soluble protein N g/100 g beans	Water:salt soluble	Albumins gN/100g beans	Globulins gN/100 beans	Albumin:Globulin
Dry beans						
Navy	1.51	1.15	1.32	1.28	1.32	0.97
Great Northern	1.12	1.57	0.71	0.99	1.72	0.58
Small White	1.11	1.15	0.96	1.01	1.19	0.85
Pink bean	1.28	1.12	1.14	1.12	1.33	0.84
Pinto	0.98	1.85	0.53	0.89	2.01	0.44
Cranberry	1.33	2.15	0.62	1.18	2.25	0.52
Light Red Kidney	1.67	1.33	1.25	1.52	1.52	1.00
Dark Red Kidney	1.66	1.21	1.37	1.57	1.36	1.15
Small Red	1.29	1.30	0.99	1.14	1.38	0.82
Black Bean	1.72	1.24	1.38	1.50	1.39	1.08
French Bean	1.18	1.35	0.87	1.06	1.51	0.71
Lima beans						
Large Lima	2.77	0.59	4.65	2.28	0.96	2.38
Baby Lima	2.28	0.50	4.57	1.17	0.93	1.91
Black-eye Peas	1.96	1.08	1.81	1.75	1.16	1.51
Chickpea	1.49	0.52	2.88	1.41	0.48	2.94

[a] Data expressed on a dry weight basis.

more albumin N. Poulter (1981) reported an even larger variation in the albumin:globulin ratios (0.44–28.20) for eight cultivars of bambara groundnut (*Voandzeia subterranea*), a lesser known grain legume grown in the African subcontinent. The significance of such ratios in terms of their relative protein digestibility remains to be determined.

The protein and carbohydrate contents of the water- and salt-soluble fractions prepared from these legumes are summarized in Table 5. The mean protein contents of these two fractions were approximately 67% and 87%, respectively. The total carbohydrates associated with these two fractions ranged from 17–30% and 4–14%, respectively, with the mean values of 22.6% and 8.7% for the different legumes investigated. The high carbohydrate content of the water-soluble proteins accounts for the presence of certain high MW hydrophilic polysaccharides such as gums, pectins and starch. These compounds cannot be easily removed by dialysis. Marquez and Lajolo (1981) reported 8% bound carbohydrates with the albumin fraction of Carioca dry bean. On the other hand, globulin proteins of legumes are reportedly glycosylated (Pusztai, 1965; Pusztai and Watt, 1970; Chang and Satterlee, 1981). Although their role is still undefined, the presence of bound carbohydrates in legume storage proteins has been suggested as a possible barrier to proteolysis, therefore, contributing to the overall low digestibility of these proteins (Semino et al., 1985).

Table 5—Protein and carbohydrate contents of albumin and globulin fractions of grain legumes[a]

Legume/Variety	Albumins		Globulins	
	Protein %	Total carbohydrates[b] %	Protein %	Total carbohydrates %
Dry beans				
Navy	75.40	21.45	94.39	5.55
Great Northern	64.10	21.41	80.21	7.46
Small White	59.20	22.81	85.84	6.67
Pink bean	68.82	25.05	85.88	7.10
Pinto	68.78	17.53	95.33	6.93
Cranberry	63.58	27.03	87.28	6.62
Light Red Kidney	72.06	19.16	86.05	13.76
Dark Red Kidney	62.31	27.45	80.71	11.18
Small Red	61.94	29.71	89.61	7.84
Black Bean	63.62	26.59	91.89	11.80
French Bean	73.64	18.20	82.24	10.28
Lima beans				
Large Lima	70.28	19.79	82.72	12.59
Baby Lima	70.35	17.25	85.39	10.27
Black-eye Peas	70.61	17.24	88.81	8.05
Chickpea	62.31	27.55	84.82	4.15
LSD[c] (P = 0.05)	1.39	0.74	0.91	0.85

[a] Data expressed on a dry weight basis.
[b] Expressed as glucose equivalents.
[c] Least significant difference. Differences of two means exceeding this value are significant.

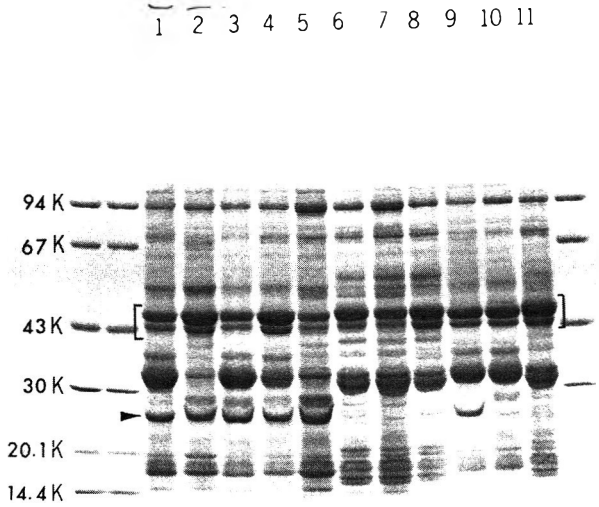

Fig. 1—SDS-PAGE of water-soluble proteins of dry beans. The brackets ([]) show the major storage globulin (phaseolin) as a cross-contaminant in the water-soluble fraction. The arrow indicates the protein subunit not present in all the varieties studied. Sample numbers on top of the gel are as given in Table 1 and 14.4–94 Kilodaltons are the molecular weights of the standard protein markers.

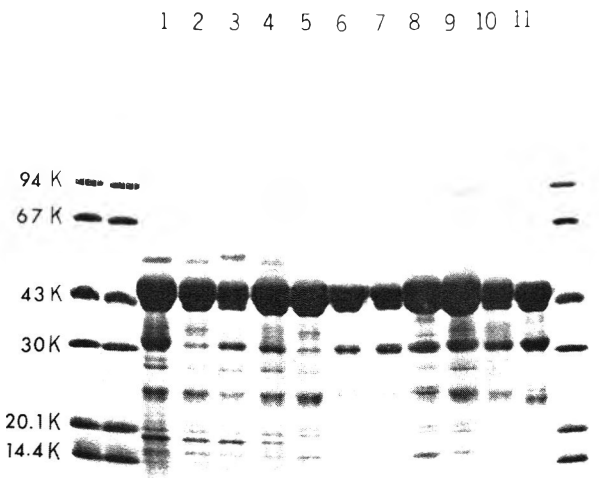

Fig. 2—SDS-PAGE of salt-soluble proteins of dry beans. Samples were overloaded to show the minor protein subunits. Sample numbers on top of the gel are as given in Table 1 and 14.4–94 kdaltons are the molecular weights of the standard protein markers.

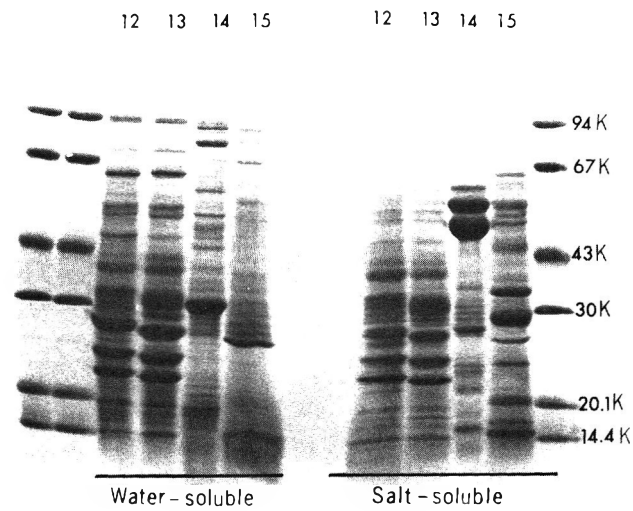

Fig. 3—Water- and salt-soluble proteins of Baby Lima (#12), Large Lima (#13), Black-eye peas (#14) and chickpea (#15). The numbers 14.4–94 kdaltons indicate the molecular weights of the standard protein markers.

The SDS-PAGE patterns of the water- and salt-soluble proteins of different legumes investigated are shown in Fig. 1 to 3. Among the *P. vulgaris* group, depending upon the variety, the water-soluble fraction was composed of at least 24–38 subunits with the apparent MW range of 11–114 kdaltons (Fig. 1). The major storage protein of these beans, phaseolin or G1, was clearly soluble in water, although the extent of its solubility was different for different varieties investigated. Although the overall pattern appeared to be similar for these beans belonging to the same species, the relative proportions of at least some of the subunits showed distinct differences. One protein subunit of 28 kdalton MW (indicated by an arrow in Fig. 1) and which stained positively for the PAS reagent was conspicuously absent in Cranberry, Black, French and the Kidney beans. The subunit profile in the MW range of 15–21 kdalton also showed marked differences, whereas, with the exception of Great Northern and Pinto beans, the remaining varieties showed the presence of 3–5 bands in the MW range of 29–32 kdaltons. These proteins also gave a positive test for the PAS reagent. Felsted et al. (1981) have shown proteins in this region to be isolectins and mitogenic in 62 cultivars of *P. vulgaris*. The Great Northern and Pinto beans showed patterns similar to those classified by these researchers to be isolectins of Group II category, while the rest exhibited electrophorectic patterns characteristic of Group I isolectins. Besides the presence of several small MW protein subunits, the salt-soluble fraction of *P. vulgaris* comprised predominantly of phaseolin, its major storage protein (Fig. 2).

Among the four remaining legume species investigated, the electrophoretic profiles of the two lima bean varieties, although markedly similar, were different from those of *P. vulgaris* types (Fig. 3). Both varieties had 3 major subunits in the range of 23–28 kdaltons and two subunits of 33 and 40 kdaltons. The black-eye peas had only one major subunit of MW 30–32 kdalton in their water-soluble fraction, whereas the chickpeas had predominately low MW protein subunits. The latter also had the simplest water-soluble protein profile among the legumes investigated (Fig. 3). The salt-soluble protein profile for the two lima beans was similar to their water-soluble protein profile, but was characterized by the absence of certain high MW protein subunits. The black-eye peas contained two major subunits in the MW range of 55–58 kdaltons, the former predominating. The salt-soluble proteins of chickpeas were characterized by the presence of several major subunits in a broad MW range of 15–60 kdaltons, and were more complex in nature compared to its unusually simpler water-soluble protein profile.

The water-soluble protein fractions of all the *P. vulgaris* varieties indicated cross-contamination with the major storage globulin. To eliminate the effects of native salts present in the beans, the whole flours were first extracted with NaCl and the extracts were dialyzed extensively against distilled water. The SDS-PAGE patterns of true water-soluble ("albumins") and the precipitated "globulins" thus obtained are shown in Fig. 4. The albumin fraction was conspicuous by the absence of the major storage globulin (indicated by the arrow). Anomalous behavior, however, was observed for several protein sub-

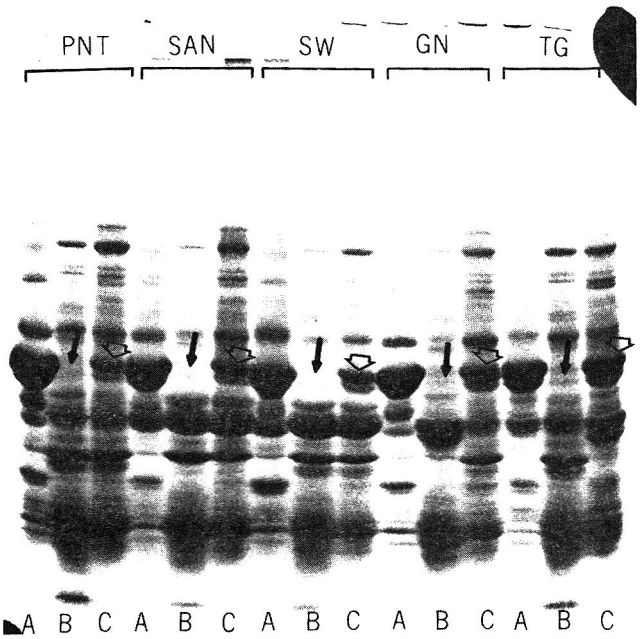

Fig. 4—True globulin (A) and albumin (B) protein profiles of Pinto (PNT), Sanilac (SAN), Small White (SW), Great Northern (GN) and French Tendergreen (TG) beans as compared to their water-soluble (C) protein fractions. The dark arrows indicate the absence of phaseolin in the true albumin fraction, and the open arrows show that phaseolin is soluble to some extent in the bean flour-water suspensions.

units as indicated by their presence in both the fractions, particularly in the lower MW region. It is difficult to judge from these preliminary studies whether (a) they represent different proteins, (b) the same proteins which may remain soluble in water in association with other proteins, or (c) proteins which may precipitate with globulins as aggregates. Based on these data, it was difficult to quantitate the true albumin and globulin content of these legumes. In recent studies with these legumes, albumins have been identified that fit the classical definition of globulins (Bollini and Chrispeels, 1978; Youle and Huang, 1978). Any quantitation of legume albumins and globulins should therefore only be considered estimates at best.

CONCLUSIONS

THE TOTAL SEED N of four legume species and their genotypes investigated varied from 3.2–4.2%. The nonprotein N comprised a significant portion of the meal N. The water- and salt-soluble proteins together accounted for 63–83% of total N. Lima beans, black-eye peas and chickpeas had significantly greater water-soluble N compared to *P. vulgaris* seed types. The native ionic strength of bean flour dispersions and the presence of endogenous tannins influenced the protein solubility. The albumin:globulin ratios were characteristic of the variety. *Phaseolus* seed types had more complex protein profiles compared to those of the other species investigated.

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Ms received 11/10/86; revised 4/27/87; accepted 5/8/87.

Presented at the 46th Annual Meeting of the Inst. of Food Technologists, Dallas, TX, June 15–18, 1986.

Journal Paper No. 10,900 of the Purdue Agricultural Experiment Station.

We sincerely thank Dr. William Isom, USDA Cooperative Extension, Univ. of California, Berkeley, CA, for kindly supplying certain legumes for this study. The project was supported in part by the U.S. Dept. of Agriculture Grant 8500772.

In Vitro Enzymatic Hydrolysis of Phaseolin, the Major Storage Protein of *Phaseolus Vulgaris* L.

S. S. DESHPANDE and S. S. NIELSEN

ABSTRACT

Susceptibility of the major storage protein, phaseolin, to different commercially available proteinases was studied *in vitro* for 17 varieties of *Phaseolus vulgaris* L. using electrophoretic techniques. Among serine proteinases, trypsin, subtilisin and pronase E readily hydrolyzed the native phaseolin, while chymotrypsin was less effective. Native phaseolin was markedly resistant to a carboxyl proteinase (pepsin) and somewhat resistant to a thio proteinase (papain). Major breakdown products of native phaseolin subunits were of a similar size irrespective of the enzyme used, and had an approximate MW of 22–25 kilodaltons. SDS-PAGE indicated that heated phaseolin from all the varieties investigated was effectively hydrolyzed by subtilisin, pronase E, pepsin and papain within 30 min, while chymotrypsin and trypsin exhibiting restricted specificity were slightly less effective.

INTRODUCTION

LEGUME PROTEINS are of poor nutritional value unless subjected to heat treatment. The improvement is generally attributed to the inactivation of the heat-labile proteinase inhibitors and phytohemagglutinins. Several lines of evidence now suggest that the proteinase inhibitors in *Phaseolus vulgaris* probably do not play a major role as a growth inhibitor (Jaffe and Vega Lette, 1968; Palmer et al., 1973). If these and the lectins which interfere with the amino acid availability are destroyed by heat treatment, one should be able to predict the nutritive value on the basis of amino acid composition. Evidence in the literature, however, indicates that this is not the case for legumes. The deficiency of sulfur-amino acids alone does not seem to be the only factor which limits the nutritive value of cooked legumes, since no definite correlation was observed between the sulfur-amino acid contents of cooked legumes and their nutritive value in terms of PER (Kuppuswamy et al., 1958). Liener (1976) suggested that such lack of correlation may be attributed to the inherently low digestibility of legume proteins and the availability of amino acids even after cooking.

To better understand the digestibility of legume proteins, considerable attention has been directed toward phaseolin, the major storage protein of the common bean, *P. vulgaris*. Phaseolin is the major globulin protein in the seed and comprises about 50% of the total protein in mature seeds (Ma and Bliss, 1978). The resistance of native phaseolin to proteolytic attack by mammalian digestive enzymes *in vitro* has been demonstrated (Romero and Ryan, 1978; Chang and Satterlee, 1981; Liener and Thompson, 1980), and it is considered to be an important factor contributing to the poor nutritive value of the unheated bean. This inaccessibility to enzymes has been attributed to the structural constraints of phaseolin (Romero and Ryan, 1978) and its compact structure (Chang and Satterlee, 1981), to the stability imparted to its three-dimensional structure by its carbohydrate moiety (Pazur and Aronson, 1972), and to steric hindrances of the proteinase by the saccharide chain (Marsh et al., 1977; Chang and Satterlee, 1981; Semino et al., 1985). Other factors suggested for the poor *in vitro* digestibility of legume proteins include starch complexed with protein, product inhibition during digestion, protein-protein and protein-mineral interactions, and the presence of phytic acid and polyphenols (Boonvisut and Whitaker, 1976; Galemback et al., 1977; Azari and Feeney, 1958; Kleczkowski and Van Kammen, 1961; Deshpande, 1985).

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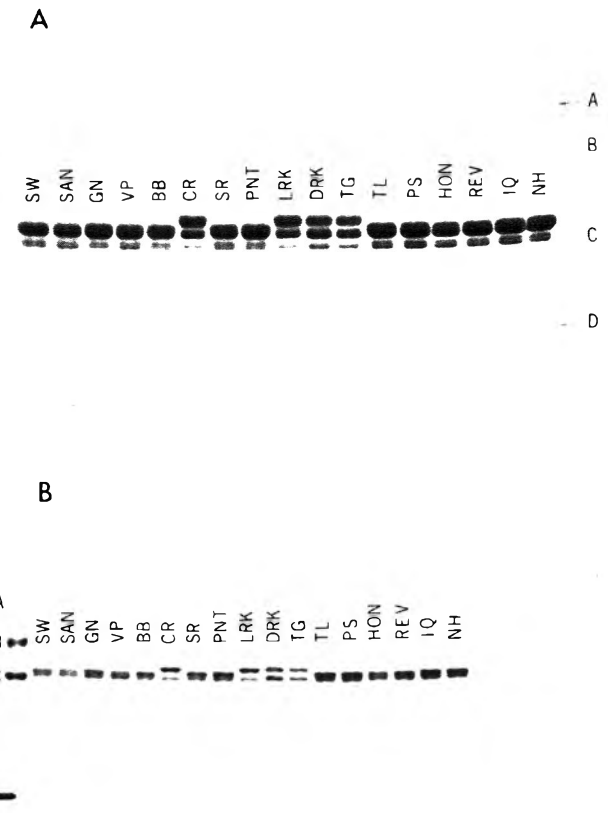

Fig. 1—SDS-PAGE patterns of phaseolin isolated from 17 varieties of P. vulgaris L. Variety abbreviations and the MW markers are as given under Materials & Methods. (A) Method of Laemmli (1970) as modified by Ma and Bliss (1978); (B) Method of Fling and Gregerson (1986)

Since legumes are seldom eaten raw, the study of protein digestibility in its native or unheated form is inconsequential from a nutritional point of view. Studies in the literature on heated phaseolin have most often focused on trypsin digestion, but data are conflicting. Two studies have found heated phaseolin to be highly susceptible to trypsin digestion (Liener and Thompson, 1980; Bradbear and Boulter, 1984). Other studies have shown improved digestibility upon heating, but limited changes in protein structure (Chang and Satterlee, 1981) and still a much lower percentage of peptide bonds hydrolyzed compared to bovine serum albumin (Romero and Ryan, 1978).

The main purpose of this investigation was to study the *in vitro* susceptibility to different proteinases of both native and heated phaseolin isolated from 17 varieties of dry beans using electrophoretic techniques.

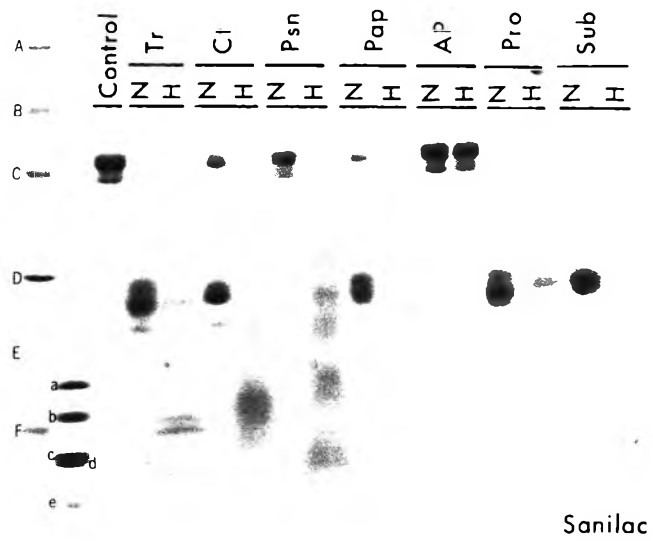

Fig. 2—SDS-PAGE patterns of phaseolin isolated from Sanilac beans subjected to 30 min digestion with different proteinases. N and H indicate the native and heated phaseolin. Tr, Ct, Psn, Pap, AP, Pro and Sub denote digestions carried out with trypsin, chymotrypsin, pepsin, papain, aminopeptidase, pronase E and subtilisin, respectively. The letters A–F and a–e are MW markers as described under Materials & Methods.

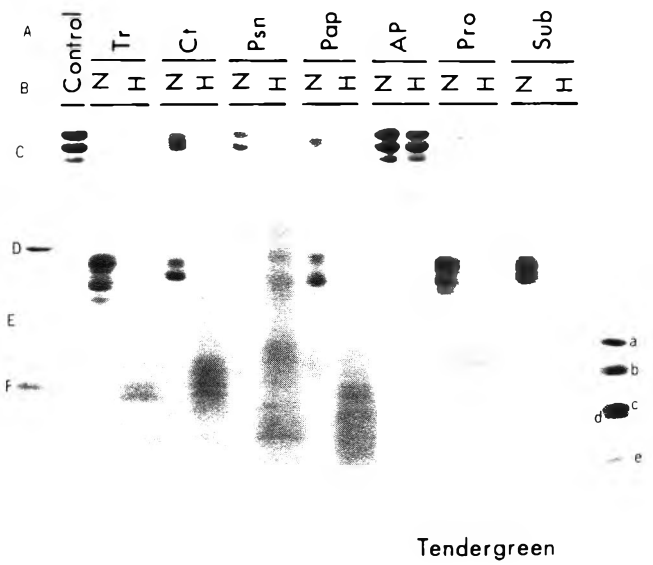

Fig. 3—SDS-PAGE patterns of phaseolin isolated from Tendergreen beans subjected to 30 min digestion with different proteinases. The abbreviations are as described for Fig. 2.

MATERIALS & METHODS

ALL BEANS studied belonged to the *Phaseolus vulgaris* L. group. The source of the beans of Small White (SW), Sanilac (SAN), Great Northern (GN), Viva Pink (VP), Black Beauty (BB), Cranberry (CR), Small Red (SR), Pinto (PNT), Light Red Kidney (LRK), Dark Red Kidney (DRK) and French Tendergreen (TG) was reported earlier (Deshpande and Nielsen, 1987a). The beans of Tamazu Lapa (TL), Porrillo Sinetico (PS), Honduras 46 (HON), Revolution 79 (REV), Icta Quetza (IQ) and Negro Huasteco 81 (NH) were a gift from Dr. R. Bressani, INCAP, Guatemala. Whole bean flours were obtained by grinding the hand-cleaned sound beans in a UDY Cyclone mill (0.4 mm screen).

The following enzyme preparations were obtained from Sigma Chemical Co., St. Louis, MO.: Trypsin (from bovine pancreas, Type III-S, 13,000 units/mg protein on BAEE, lot 65.5–8190); chymotrypsin (from bovine pancreas, Type II, 48 units/mg protein on BAEE, lot 15F–8160); bacterial protease (subtilisin Carlsberg, from *Bacillus subtilis*, Type VIII, 11.9 units/mg on casein, lot 104F–0099); pronase E (from *Streptomyces gresius*, Type XIV, 5.8 units/mg on BAEE, lot 113f–8135); aminopeptidase (from porcine intestinal mucosa, 100 units/g on L-leucine beta-naphthylamide, lot 80F–80101); and pepsin (from porcine stomach mucosa, 3,200 units/mg protein, lot 64F–8080). Unless mentioned otherwise, all chemicals used were of reagent grade.

Preparation of phaseolin

Phaseolin was isolated by the method of Hall et al. (1977), who referred to their preparation as the G1 fraction. The isolation procedure was repeated to precipitate the phaseolin three times before it was dialyzed against distilled water and lyophilized.

Digestion of phaseolin

Phaseolin was dissolved in the appropriate buffer at 2.5 mg/mL and aliquots (500 μ Lowry protein) were reacted with the proteinases. The buffer systems selected for the different proteinases used included 50 mM Tris-HCl, pH 8.1 containing 20 mM $CaCl^2$ for trypsin and chymotrypsin; 50 mM phosphate buffer, pH 7.5, for subtilisin and pronase E; 50 mM phosphate, pH 7.1, for aminopeptidase; 50 mM phosphate, pH 6.2, for papain; and 50 mM HCl for pepsin. Heated phaseolin samples were prepared by solubilizing it in the appropriate buffer and heating the sealed test tubes in a boiling water bath at 99°C for 30 min. All native (unheated) digestion assays were conducted at 37°C with a 10:1 phaseolin:enzyme ratio, while a 100:1 ratio was used for the heated phaseolin. The digestion reaction (30 min, except for the time-course studies) was stopped by adding the sample buffer for SDS-PAGE and immediately heating the tubes at 99°C for 3 min prior to electrophoresis. For each enzyme, all assays were carried out at least in duplicate, and the gels shown are for a typical run.

SDS-PAGE

SDS-PAGE was performed using either the method of Laemmli (1970) as modified by Ma and Bliss (1978) (15–25% linear gradient), or the method of Fling and Gregerson (1986). The MW marker proteins phosphorylase b (94 kdalton, A), bovine serum albumin (67 kdalton, B), ovalbumin (43 kdalton, C), carbonic anhydrase (30 kdalton, D), soybean trypsin inhibitor (20.1 kdalton, E), alpha-lactalbumin (14.2 kdalton, F), and the low MW kit containing myoglobin polypeptide backbone (16.95 kdalton, a), myoglobin fragment I + II (14.4 kdalton, b), fragment I (8.16 kdalton, c), fragment II (6.21 kdalton, d), and myoglobin fragment III (2.51 kdalton, e) were from Sigma Chemical Co., St. Louis, MO.

RESULTS & DISCUSSION

THE SDS-PAGE PATTERNS of phaseolin isolated from 17 varieties of *P. vulgaris* are shown in Fig. 1. The procedure of Ma and Bliss (1978) resolved two bands for most varieties and three bands for Tendergreen, the two Kidney bean types and Cranberry (Fig. 1A). The procedure described by Fling and Gregerson (1986) resolved three to four bands for each variety (Fig. 1B). The two subunits with lower MW (48 kdalton and 45 kdalton) in the four varieties mentioned above were present in all the remaining varieties. However, the highest MW subunit (51 kdalton) showed variable electrophoretic mobility depending upon the variety. Brown et al. (1981) classified domesticated *P. vulgaris* varieties into types 'S', 'T', and 'C' on the basis of their protein subunit composition. However, six new phaseolin types recently have been identified in studies on nondomesticated common bean germplasm (Romero-Andreas and Bliss, 1985). In the present study, Tendergreen, Cranberry and the two Kidney bean types appeared to be the 'T' type, while the others were of the 'S' type. None seemed to be of the newly identified M1–M6 types described by Romero-Andreas and Bliss (1985).

The SDS-PAGE patterns of both the unheated and heated

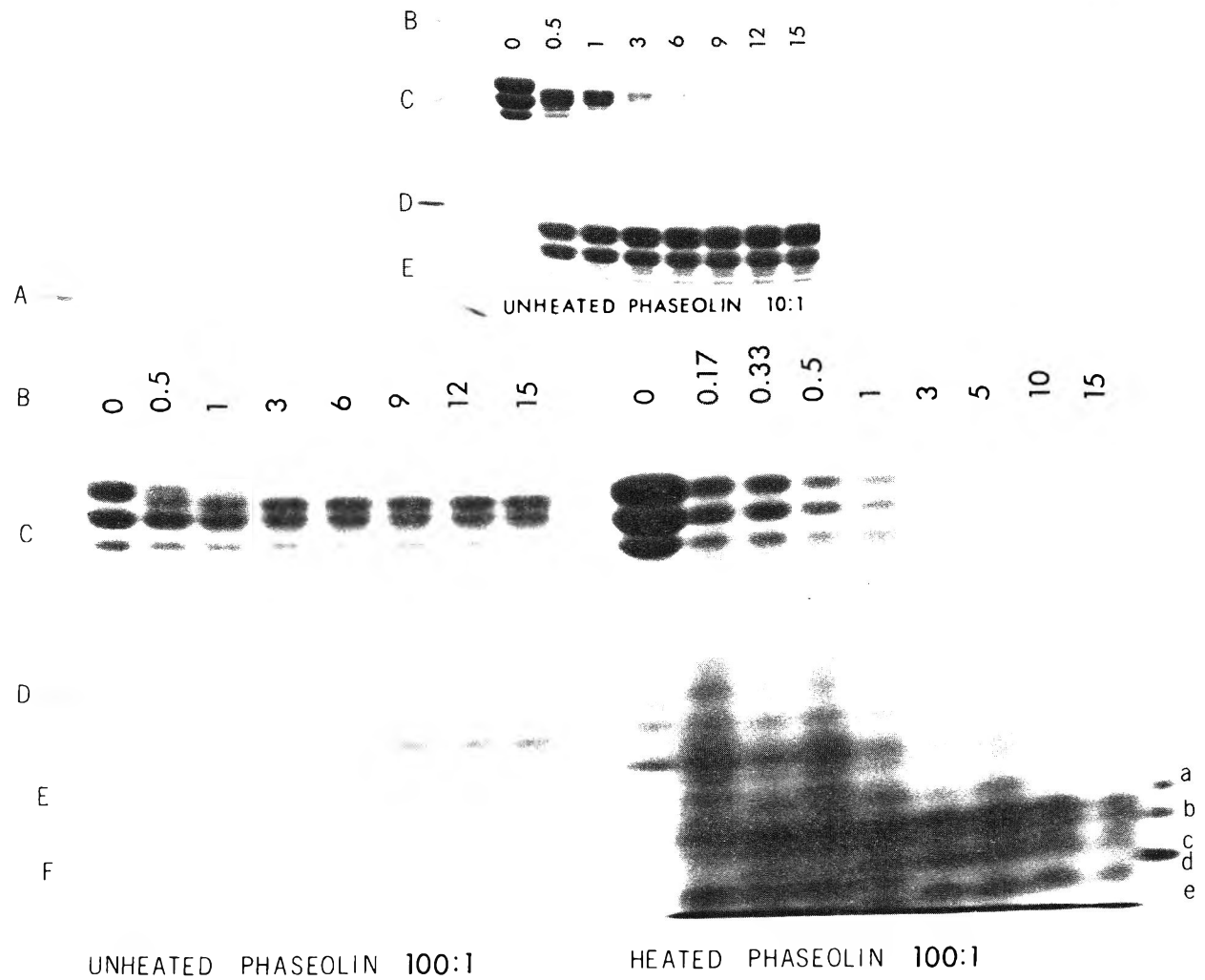

Fig. 4—Time-course of trypsin digestion of heated and unheated phaseolin from Tendergreen beans. The numbers on top of each lane denote the time of digestion in min. The inset shows the breakdown patterns of native phaseolin with a 10:1 protein:trypsin ratio. The letters A–F and a–e are MW markers as described under Materials & Methods.

phaseolin from two representative seed types, Sanilac and Tendergreen beans, subjected to digestion with different proteinases, are shown in Fig. 2 and 3. Similar profiles were obtained for the remaining varieties investigated (not shown here). The three subunits of native phaseolin completely disappeared within 30 min digestion with trypsin. These were degraded to polypeptides with MW between 20–30 kdaltons. Heated phaseolin, when reacted with trypsin, was completely degraded in 30 min to polypeptides in the range of 6–14 kdalton MW for all the varieties investigated. These polypeptides were not further broken down even after extensive digestion (data not shown). The original subunits of native phaseolin for all types of *P. vulgaris* investigated were only partially degraded by chymotrypsin (Fig. 2 and 3). The breakdown products, however, were of a similar size. As with trypsin, chymotrypsin readily hydrolyzed all types of heated phaseolin to smaller, nondistinct polypeptides.

The results obtained with papain digestion of native and heated phaseolin were similar to those obtained with chymotrypsin. The degradation of native phaseolin by pronase E and subtilisin was similar to that by trypsin (Fig. 2 and 3). Unlike chymotrypsin and papain, these two enzymes completely degraded the heated phaseolin. Incubation with aminopeptidase appeared to have no effect on the subunit size of either the native or the heated phaseolin. Native phaseolin was also markedly resistant to pepsin, but was readily hydrolyzed once heated (Fig. 2 and 3).

The SDS-PAGE patterns of the time-course of trypsin hydrolysis of both the native and heated phaseolin are shown in Fig. 4. The slight increase in the electrophoretic mobility of the largest subunit during the initial stages may suggest that the hydrolysis probably begins from the carboxyl end of the molecule. This is further substantiated by the inability of a general purpose aminopeptidase to act upon the native phaseolin, as well as the fact that the amino-terminal of the native phaseolin is not readily accessible for sequencing (unpublished data). Once the process is initiated, the central susceptible region of the molecule appeared to be exposed to trypsin, thus giving the characteristic breakdown products of approximately half the original size. This susceptible region of phaseolin, therefore, must contain a variety of amino acids such that the protein can be cleaved by enzymes with different specificities. The smallest subunit of the native phaseolin appeared to be the most susceptible for trypsin hydrolysis. Even in the heated phaseolin, the central portion of the molecule seemed to be the preferred sites during the initial stages of trypsin hydrolysis.

Data presented here on phaseolin susceptibility to trypsin hydrolysis are in agreement with two previous reports in the literature. Liener and Thompson (1980) showed that the major storage protein isolated from Sanilac navy bean was markedly resistant to *in vitro* digestion with pepsin, trypsin and chymotrypsin unless subjected to heat treatment. Under their ex-

perimental conditions, the native phaseolin was more susceptible to trypsin than to chymotrypsin, while pepsin treatment resulted in little breakdown. These authors, however, did not show the SDS-PAGE patterns for the enzyme hydrolyzed heated phaseolin. The phaseolin isolated from *P. vulgaris* L. c.v. Dwarf White was only partially digested by trypsin in its native form, but was fully digested after heat denaturation (Bradbear and Boulter, 1984). Both groups of researchers found that trypsin cleaves native phaseolin, generating polypeptides in the MW range of 20–30 kdaltons. Thus, while the native phaseolin appeared to be quite resistant to complete hydrolysis, heated phaseolin was degraded to small MW polypeptides by a variety of types of enzymes. Subsequent studies (Deshpande and Nielsen, 1987b), however, have revealed that phaseolin may not be hydrolyzed rapidly or completely enough in the presence of other bean constituents to allow absorption in the digestion tract. This could at least partially explain the inconsistency between the actual nutritive value of legume proteins and that expected from their amino acid compositions.

CONCLUSIONS

The major storage protein, phaseolin, isolated from 17 dry bean varieties was highly susceptible to a number of proteolytic enzymes *in vitro*. None of the theories proposed in the literature regarding the resistance of this protein to mammalian proteases seemed to have a sound base. Neither the compact structure and structural constraints nor the steric hindrances by its carbohydrate moiety to proteinases seemed to have any adverse effect on phaseolin digestion. Among the enzymes tested, heated phaseolin was effectively hydrolyzed by subtilisin, pronase E, pepsin and papain within 30 min digestion, while chymotrypsin and trypsin being restricted in their specificity were slightly less effective.

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Journal Paper No. 10,898 of the Purdue Agricultural Experiment Station.
The project was supported in part by the U.S. Dept. of Agriculture Grant 8500772.

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The authors are thankful to Cryovac Div., W.R. Grace Co., Duncan, SC, for providing equipment and material to conduct this research.

In Vitro Digestibility of Dry Bean (*Phaseolus Vulgaris* L.) Proteins: The Role of Heat-Stable Protease Inhibitors

S. S. DESHPANDE and S. S. NIELSEN

ABSTRACT

In vitro susceptibility to trypsin of whole bean extracts, and water- and salt-soluble proteins of 11 dry bean (*Phaseolus vulgaris* L.) varieties were investigated. Whole bean proteins were rapidly digested at a 10:1 protein:trypsin ratio. Salt-soluble proteins of beans were more readily and completely hydrolyzed by trypsin than their water-soluble proteins. Of the 8 fractions obtained by DEAE-cellulose column chromatography of Great Northern bean albumins, three were identified to contain heat-stable trypsin and chymotrypsin inhibitory activity. Proteins in these fractions seemed to protect phaseolin from rapid hydrolysis by proteases by virtue of both their inhibitory activity as well as protein:protein interactions.

INTRODUCTION

IN RECENT YEARS, resistance of the major storage protein (phaseolin) of dry beans to proteolysis has been increasingly implicated as the major cause for their poor nutritional value (Liener and Thompson, 1980; Chang and Satterlee, 1981; Sathe et al., 1983). This is a significant shift from the viewpoint of factors such as protease inhibitors and lectins, which traditionally have been cited as the major reasons for the observed detrimental nutritional effects of legumes in laboratory animals (Liener, 1978; Jaffe and Vega Lette, 1968). The theories proposed for this resistance of phaseolin to proteolysis include its structural constraints and compact structure, stability imparted to its three dimensional structure by its carbohydrate moiety, and thus the steric hindrances of the proteases by the saccharide chain (Romero and Ryan, 1978; Chang and Satterlee, 1981; Pazur and Aronson, 1972; Semino et al., 1985). This controversy regarding the resistance to proteolysis may, in fact, stem the fact that many of such studies were conducted with native phaseolin, a situation not pertinent to the *in vivo* conditions when legumes are invariable consumed by humans after some form of cooking. Results described in our previous study (Deshpande and Nielsen, 1987a) clearly indicated that none of these theories hold true for heated phaseolin which was digested *in vitro* by a variety of types of proteases.

The inconsistency in the literature about legume protein digestibility, however, still cannot be completely explained by heat treatment alone. For example, Sathe et al (1983) observed that phaseolin in heated protein extracts derived from germinated Great Northern bean cotyledons was digested to only a limited extent by trypsin. Factors such as the use of different protein:enzyme ratios, the relative activity of different enzyme preparations and the techniques employed for measuring *in vitro* digestibility may also add to this complexity Further, to represent a major determinant of nutritional quality, phaseolin needs to be resistant to a number of different proteases. Based on our earlier observations (Deshpande and Nielsen, 1987a) this is clearly not the case. Recent evidence also suggests that both phytic acid and tannins do not inhibit phaseolin digestibility *per se* by trypsin at levels commonly occurring in dry beans (Reddy et al., 1987; Deshpande and Nielsen, unpublished data). In view of all these factors, the scope of our investigations on legume protein digestibility was further extended. The major objective of the present investigation was to study phaseolin digestibility in relation to total extractable proteins as well as the water- and salt-soluble proteins of dry beans. Data presented here are based on studies exclusively carried out on heated protein systems.

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MATERIALS & METHODS

THE SOURCE of the 11 dry bean varieties (all belonging to *Phaseolus vulgaris* L.) and enzymes was reported earlier (Deshpande and Nielsen, 1987a,b). All other chemicals used were of reagent grade.

Whole bean flours were obtained by grinding sound beans in a UDY Cyclone mill (0.4 mm screen).

Protein fractions

In an earlier study (Sathe et al., 1983), little difference was observed in the electrophoretic patterns and subsequent tryptic hydrolysis of Great Northern bean proteins extracted under different conditions (0.5M NaCl: 0.05M Tris-HCl buffer, pH 8.1; and 0.05M Tris-HCl buffer, pH 8.1, containing 0.5M NaCl). Whole bean flour proteins were therefore extracted with 0.05M Tris-HCl buffer (pH 8.1) for 3–4 hr at 25°C with occasional mixing. The bean flour:solvent ratio was 1:10 (w/v). The extracts were centrifuged at 5,000 X *g* for 30 min, filtered, and then aliquots of supernatants were analyzed for biuret protein using appropriate blanks.

The water- and salt-soluble protein fractions, prepared by sequential extraction as described earlier (Deshpande and Nielsen, 1987b), were also solubilized in the same buffer and adjusted to a know biuret protein concentration.

Trypsin digestion

All extracts were heated in small sealed test tubes at 99°C in a boiling water bath for 30 min. In one study, the samples were heated for 30, 60, 90 and 120 min to investigate the effect of heating time on the breakdown pattern of bean proteins. Digestion was carried out individually in the same small test tube by adding trypsin to the heated extracts (2 mg protein) so as to give either a 10:1 or 100:1 (w/w) protein:trypsin ration in a total assay volume of 0.25 mL. The tubes were sealed with parafilm and incubated at 37°C for up to 2 hr. At the end of the required period, the reaction was stopped by adding 0.25 mL sample buffer (0.05M Tris-HCl, pH 6.8; 1% SDS, 30% glycerol, 2% beta-mercaptoethanol and 0.01% bromophenol blue), and immediately heating the tubes at 99°C for 3 min prior to SDS-PAGE.

Fractionation of Great Northern bean albumins

SDS-PAGE separation studies of the three systems described above indicated water-soluble proteins to be somewhat resistant to trypsin hydrolysis under the assay conditions used. Therefore, the true albumins of Great Northern beans were chosen for further studies. Albumins were prepared by extracting the whole bean flours with 0.5M NaCl (1:10, w/v, flour:solvent ratio). The supernatant obtained after centrifugation (5,000 X *g*, 30 min) was dialyzed extensively against deionized distilled water for 2 days at 4°C, centrifuged to remove the water-insoluble proteins and then lyophilized.

The true albumins thus obtained were fractionated using DEAE-cellulose (DE-52, Whatman Ltd., Great Britain) column chromatography. A DEAE-cellulose column (2.6 X 19.2 cm) was equilibriated with 20 mM Tris-HCl, pH 8.1, washed with the same buffer and developed in a linear gradient made with 500 mL each of this buffer

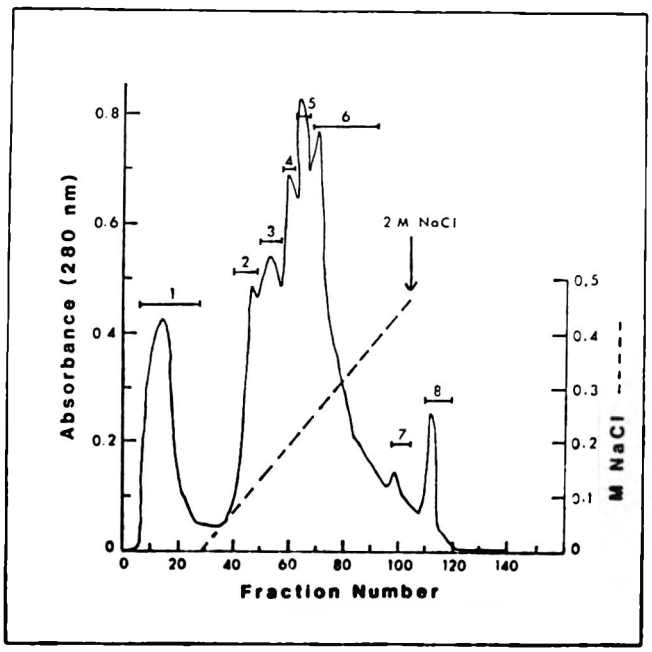

Fig. 1—DEAE-cellulose column chromatography of true albumins of the Great Northern beans. The horizontal bars with numbers indicate the different fractions pooled.

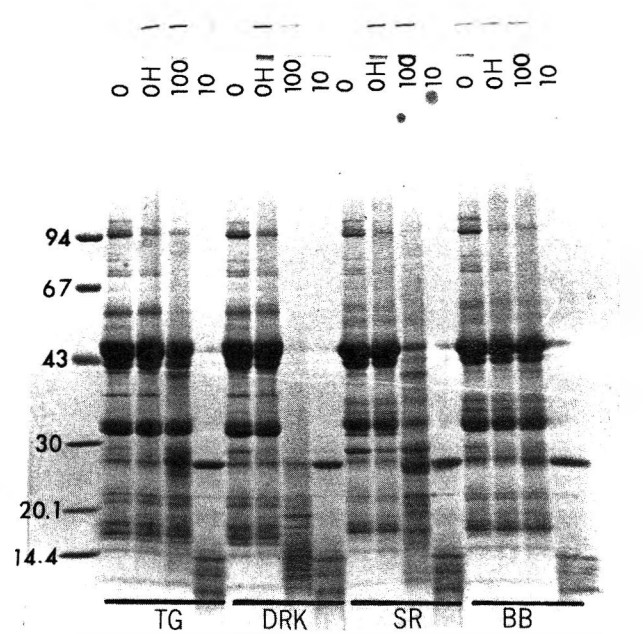

Fig. 2—SDS-PAGE of whole bean flour extracts (heated for 30 min at 99° C) subjected to trypsin digestion. O, OH, 100 and 10 at the top of each lane indicate the native control, the heated control, and the heated samples digested with 100:1 and 10:1 protein:trypsin ratios, respectively, and 14.4–94 kdaltons are the molecular weights of standard protein markers. Variety abbreviations are: French Tendergreen (TG), Dark Red Kidney (DRK), Small Red (SR) and Black bean (BB).

and the same buffer containing 0.5M NaCl. The flow rate was 30 mL/hr, and 10 mL fractions were collected. The protein concentration of each fraction was monitored by absorbance at 280 nm. Eight different fractions were pooled as shown in Fig. 1, dialyzed against water and lyophilized.

Enzyme inhibitory activity

Enzyme inhibitory activities of the eight fractions were measured by a modification of the method described by Kunitz (1947). The enzyme and the fraction (both unheated and previously heated in a boiling water bath for 30 min) were mixed in 1:1 (w/w) ratio and incubated for 15 min at 37°C. The reaction was initiated by the addition of Tris-HCl buffer (50 mM final concentration, pH 8.1 and containing no calcium) and casein reagent. At the end of 30 min, the reaction was stopped by the addition of TCA, samples were centrifuged, and the absorbances of the supernatants read at 280 nm using appropriate reagent and sample blanks. Based on these data, fraction 4, 5 and 6 (as described in Fig. 1) were selected for further studies. Heat stability of inhibitors in these fractions was also studied as a function of heating time. In one experiment, the samples after heating were allowed to stand at 4°C for 20 hr to study regeneration of the inhibitory activity. Trypsin inhibitory activity was also studied as a function of varying ratios of the inhibitor and trypsin concentration, both in the absence and presence of calcium (2 mM).

Phaseolin digestibility was further investigated in the presence of these crude inhibitor fractions. Phaseolin and the inhibitor fractions were mixed in a 3:1 ratio in Tris-HCl buffer, heated for 30 min at 99°C and then digested using four different protein:enzyme ratios as described earlier.

SDS-PAGE

SDS-PAGE, using gradient slab gels of 7.5–20% monomer acrylamide concentration, was performed according to Laemmli (1970).

RESULTS & DISCUSSION

THE SDS-PAGE separation patterns of heated whole bean extracts subjected to trypsin digestion are shown in Fig. 2. Although gels are shown only for representative varieties, the qualitative subunit patterns in the 60 kdalton MW region and lower were similar for both the unheated and heated control samples of all the varieties investigated. However, certain high MW subunits (particularly those above 70 kdalton) were conspicuously absent in the heated control samples. Some proteins in these samples also failed to enter the gel indicating some sort of protein aggregation took place during heating. The hydrolysis was strongly dependent upon the protein:enzyme ratios. Variety differences were also evident. Based on band width and intensity as judged visually, phaseolin (subunits MW 43–51 kdalton) in the heated whole bean extracts was 30–90% hydrolyzed with a 100:1 protein:trypsin ratio (Fig. 2). Subunits in the MW range of 31–35 kdalton were hydrolyzed comparatively more slowly. The major hydrolysis products appeared in the MW range of 25–30 kdalton. A lower protein:trypsin ratio (10:1) resulted in over 95% hydrolysis of bean proteins within 1 hr.

The salt-soluble fraction of bean proteins was digested more readily by trypsin compared to the corresponding water-soluble proteins (Fig. 3). With the exception of certain low MW (20 kdalton and lower) polypeptides, the salt-soluble proteins were completely hydrolyzed within the first hour of digestion even at 100:1 protein:trypsin ratio. Phaseolin was over 90% hydrolyzed. Based on our earlier study (Deshpande and Nielsen, 1987a), the undigested breakdown products appear to be derived from phaseolin. These were completely degraded by other nonspecific proteases to small MW fragments so as not to appear on the gel in that study. In the present study, even after 2 hr, several major water-soluble proteins were less readily hydrolyzed. Protein subunits in the isolectin MW region (32–34 kdalton) seemed to be the most resistant to trypsin digestion. Phaseolin, when present as a contaminant in the water-soluble fraction, was more slowly degraded in most varieties investigated compared to its rapid hydrolysis in the salt-soluble fraction. This was the first indication that there were proteins present in the water-soluble fraction that slowed phaseolin hydrolysis when lower trypsin concentrations were used in the assay. The major degradation products of the water-soluble proteins had MW of 30 kdalton and less (Fig. 3).

Our first assumption was that the 30 min heat treatment at 99°C might not have been sufficient for the complete denaturation of the water-soluble proteins. Hence, these proteins from

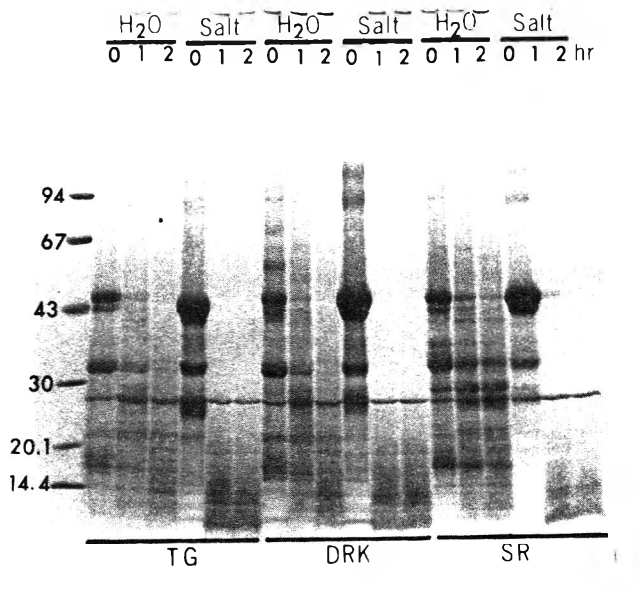

Fig. 3—SDS-PAGE of the trypsin digests of water (H^2O) and salt-soluble proteins of dry beans.), 1 and 2 hr indicate the time of trypsin (100:1) digestion. Variety abbreviations are as shown for Fig. 2. 14.4–94 kdaltons are the molecular weights of standard protein markers.

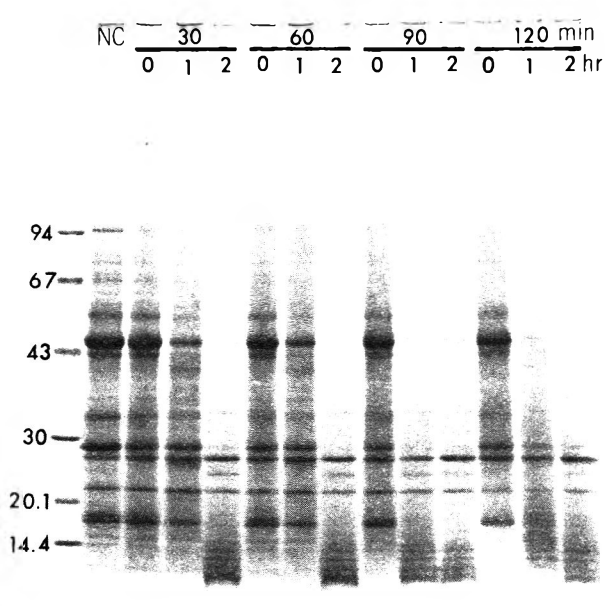

Fig. 4—Effect of heating on trypsin digestion of water-soluble proteins of the Great Northern beans. NC is the native unheated control. 30, 60, 90 and 120 min are the times the samples were heated at 99°C, prior to trypsin (100:1) digestion for 0, 1 and 2 hr, and 14.4–94 kdaltons are the molecular weights of standard protein markers. Variety abbreviations are as shown in Fig. 2.

the Great Northern beans were heat-treated for up to 2 hr prior to trypsin digestion. Heating for up to 60 min showed little differences in the qualitative electrophoretic patterns of the trypsin digests (Fig. 4).. However, when heated for 90 min and beyond, the phaseolin present in these fraction was over 95% hydrolyzed. Also, in each case, at least some proteins failed to enter the gel. This indicated two possibilities: (1) that certain unusually heat-stable trypsin inhibitors may be present in the water soluble proteins; or (2) that certain proteins may form aggregates with phaseolin and thus protect it from being hydrolyzed by trypsin.

To determine which proteins from the water-soluble fraction were responsible for this observation, the true albumins (from which over 95% of the phaseolin was removed) of the Great Northern beans were fractionated using DEAE-cellulose column chromatography. Of the eight fractions pooled as shown in Fig. 1, trypsin- and chymotrypsin-inhibitory activities were present in fractions 4–6. None of these eight fractions (both heated and unheated) inhibited papin, subtilisin, aminopeptidase and pronase E activity.

Table 1—Heat stability of trypsin and chymotrypsin inhibitors from Great Northern beans[a]

Sample	Trypsin	Chymotrypsin
Fraction 4		
Unheated	70.7	18.7
15′ heated[b]	62.7	6.2
30′ heated[b]	50.4	0
30′ heated[c]	60.1	ND[e]
Autoclaved[d]	56.0	2.0
Fraction 5		
Unheated	84.7	38.9
15′ heated	74.1	23.0
30′ heated	59.2	0
30′ heated	68.5	ND
Autoclaved	70.9	19.6
Fraction 6		
Unheated	35.3	0
15′ heated	31.4	0
30′ heated	21.7	0
30′ heated	23.7	ND
Autoclaved	40.7	0

[a] Values represent % inhibition as compared to the respective controls. A 1:1 (w/w) fraction:enzyme ratio was used.
[b] Heated in a boiling water bath at 99°C.
[c] Left standing at 4°C for 20 hr prior to measuring the inhibitor activity.
[d] At 121°C for 5 min.
[e] Not determined.

Data on the heat stability of trypsin and chymotrypsin inhibitory activities in fractions 4–6 are presented in Table 1. The major inhibitory activity was eluted in fraction 5. These values for trypsin and chymotrypsin inhibitory activities ranged from 35–85% and 0–39%, respectively. Fraction 6 contained no chymotrypsin inhibitory activity, while that present in the other two was completely lost on heating for 30 min at 99°C. Both fractions 4 and 5 retained some of the original chymotrypsin inhibitory activity on autoclaving at 121°C for 5 min. The trypsin inhibitory activity was more heat stable; 30–70% and 40–60% of the original activity was retained even after heating for 30 min at 99°C and on autoclaving, respectively. Inhibitors in these 3 fractions seemed to reassociate and regain at least some of their activity over a period of time (Table 1).

Trypsin inhibitory activity was also studied as a function of protein:trypsin ratios, both in the presence and absence of calcium (Table 2). At lower ratios, a greater inhibitory activity was observed in the absence of calcium, while for ratios above 2, maximum inhibition was observed in the presence of calcium. The reasons for these observation are unknown at present. It is likely that at certain calcium levels, protein:protein interactions may lead to changes in their inhibitory activities. This is further substantiated by the fact that both in the presence and absence of calcium, maximum inhibition occurred only at specific concentrations, and that exceeding these levels actually lowered trypsin inhibition.

Susceptibility of phaseolin to hydrolysis in the presence of these inhibitors was also evaluated as a function of trypsin and chymotrypsin concentrations (Fig. 5 and 6). Phaseolin was completely degraded in the absence of these inhibitors by the enzymes even at lower concentrations (Fig. 5A and 6A). It was less readily digested by trypsin in the presence of fractions 5 and 6 at 100:1 and 50:1 protein:trypsin ratios as compared to fraction 4. However, at higher levels of trypsin (25:1 and 10:1 ratios), phaseolin was more readily degraded. Although the three fractions had lower chymotrypsin inhibitory activities, phaseolin was not as readily digested by chymotrypsin as with trypsin in the presence of these proteins (Fig. 6A and

Table 2—Trypsin inhibitory activity of Great Northern bean albumin fractions[a]

Protein fraction:Trypsin ratio	Fraction 4		Fraction 5		Fraction 6	
	With Ca^{++}	Without Ca^{++}	With Ca^{++}	Without Ca^{++}	With Ca^{++}	Without Ca^{++}
0.5	10.8	37.3	14.6	32.1	0	22.9
1.0	39.0	44.3	60.5	67.4	0	28.9
2.0	38.2	71.1	82.4	80.7	9.1	26.7
4.0	75.0	71.8	78.4	78.4	3.7	8.1
5.0	74.4	66.7	78.8	68.7	0	9.9
10.0	69.9	48.5	70.8	63.6	0	52.5

[a] Values represent % inhibition of respective controls. All fractions were heated for 30 min in a boiling water bath prior to measurement of inhibitor activity.

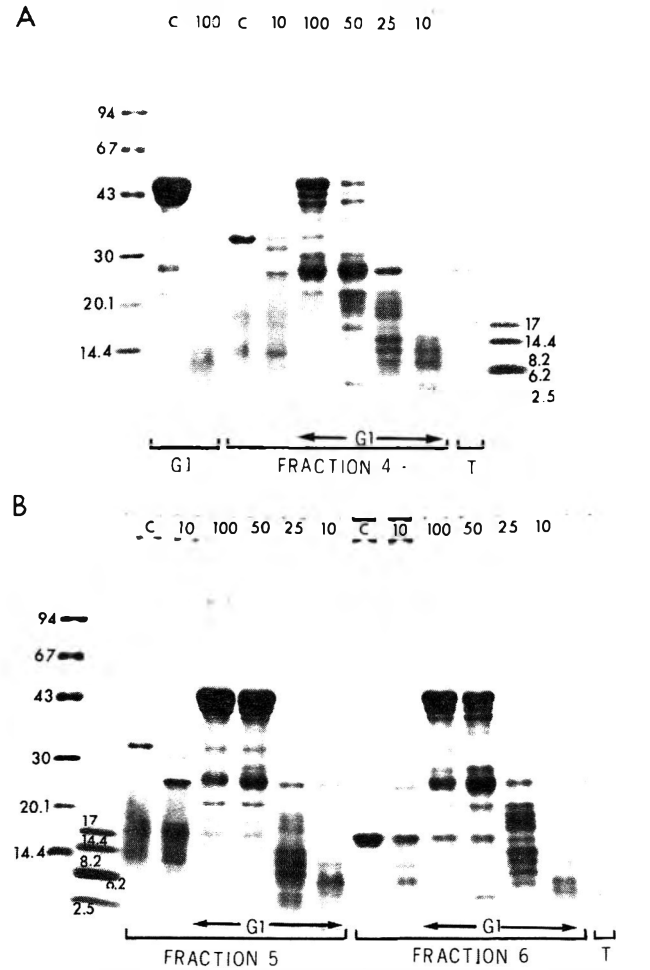

Fig. 5—Phaseolin (G1 protein) of the Great Northern beans digested with trypsin in the presence and absence of albumin fractions 4 (A), 5 and 6 (B) described in Fig. 1. C and T indicate the control samples of trypsin. 10, 25, 50 and 100 represent digestions carried out with 10:1, 25:1, 50:1 and 100:1 (w/w) protein:trypsin ratios. 2.5–17 and 14.4–94 kdaltons are the molecular weights of the standard protein markers.

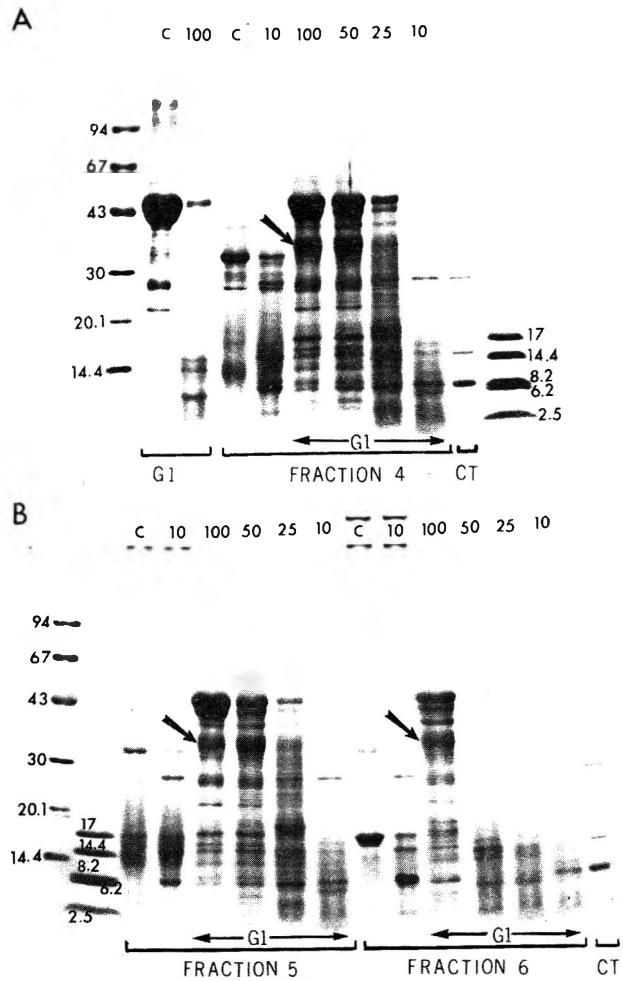

Fig. 6—Phaseolin (G1 protein) of the Great Northern beans digested with chymotrypsin in the presence and absence of fractions 4 (A), 5 and 6 (B) described in Fig. 1. C and CT indicate the control samples and chymotrypsin, and 10, 25, 50 and 100 represent digestions carried out with 10:1, 25:1, 50:1 and 100:1 (w/w) protein: chymotrypsin ratios. 2.5–17 and 14.4–94 kdaltons are molecular weights of the standard protein markers.

6B). Moreover, the breakdown pattern of phaseolin treated with chymotrypsin was completely different in the presence and absence of these fractions. The major initial breakdown products appeared in the MW range of 33–38 kdalton (indicated by an arrow in Fig. 6A and 6B), suggesting that in addition to enzyme inhibitory activity, proteins in these three fractions may form aggregates with phaseolin. Such aggregates appear to be digested by chymotrypsin in a characteristic manner. However, at a 10:1 ratio, even chymotrypsin completely degraded phaseolin.

Similar to our results, Marquez and Lajolo (1981) also reported a drastic reduction in the digestibility of bean albumins upon heating. They suggested, as possible causes, a steric impediment or a blockage of amino acid residues specific for enzyme action although they did not rule out the possibility of complexation of the protein by some phenol or quinone. They also observed appearance of high MW aggregates which did not penetrate the gel. Two other studies suggest the possible involvement of disulfide linkages in forming such aggregates. Romero and Ryan (1978) observed that the increases in trypsin hydrolysis caused by the heat treatment of phaseolin were substantially larger than those for similarly treated BSA, a protein known for its high cys + met content. Recently, Bradbear and Boulter (1984) reported that the denatured trypsin inhibitor of dry beans was poorly hydrolyzed *in vitro* even though other proteins under similar conditions were fully digested. It is quite likely that the resistance to trypsin hydrolysis of the water-soluble proteins of dry beans in the present study at lower

enzyme concentrations may be due to the stability conferred upon them by disulfide bonding. Excessive denaturation may also cause refolding of the proteins in such a way as to decrease their digestibility. Our data also suggest the possibility of some sort of protein-protein interactions involving the major storage protein of beans.

Data presented here give additional insights into studying legume protein digestibility. As mentioned earlier, controversies regarding the so-called inherent resistance of legume proteins to proteolysis may arise from the fact that such studies were largely carried out with native phaseolin. Heated phaseolin is highly susceptible to a variety of types of enzymes. It is also apparent from our studies that even in the heated systems, selection of parameters such as the protein system used (whether phaseolin alone or in combination with other bean proteins), use of varying protein:enzyme ratios, and other factors such as divalent cations by different researchers may lead to misleading observations. Although heat-stable inhibitors in the present study seemed to influence the overall protein hydrolysis to some extent, the total bean proteins were completely hydrolyzed to very low MW fragments in a reasonable time span at trypsin levels high enough to overcome the inhibition. While these studies were carried out in model systems, beans are seldom prepared and consumed in this way. Thus, these same proteins may behave differently when beans are cooked in a more complex environment. Also, to represent a major detriment to legume protein digestibility, these inhibitors must be resistant to a host of other non-specific proteases. This is clearly not the case. There is a probability of these inhibitors being fully digested by other enzymes prior to their encountering trypsin in the digestive tract. Further studies on the isolation and characterization of these proteins from cooked whole beans are underway. Although pancreatic hypertrophy is often reported in laboratory animals fed on legumes (probably as a direct result of the presence of such proteins), it is difficult to imagine that the digestive system in man has not adapted itself to such a situation since the introduction of legumes as a major food source parallels human civilization and the dawn of agriculture.

CONCLUSIONS

SUSCEPTIBILITY of phaseolin to hydrolysis in relation to different protein fractions of 11 dry bean varieties was investigated. The whole bean flour proteins were rapidly digested at a 10:1 (w/w) protein:trypsin ratio. Under similar conditions, the salt-soluble proteins of dry beans were more susceptible to hydrolysis than their corresponding water-soluble fractions. Fractionation of the true water-soluble proteins of the Great Northern beans using DEAE-cellulose column chromatography yielded 8 different fractions. Of these, three were identified to contain heat-stable trypsin and chymotrypsin inhibitor activities. Proteins in these three fractions seemed to protect phaseolin from rapid hydrolysis by enzymes, effects that were largely overcome at higher enzyme levels.

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Ms received 11/10/86; revised 4/27/87; accepted 5/8/87.

Presented in part at the 46th Annual Meeting of the Institute of Food Technologists, Dallas, TX, June 15–18, 1987.

Journal Paper No. 10897 of the Purdue Agricultural Experiment Station.

We thank Dr. S.K. Sathe, Dept. of Foods & Nutrition of this university for assistance in column chromatography.

This project was supported in part by the U.S. Dept of Agriculture Grant 8500772.

Nutrient Composition, Protein Quality, and Sensory Properties of Thirty-Six Cultivars of Dry Beans (*Phaseolus vulgaris* L.)

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ABSTRACT

Thirty-six cultivars representing eight types of dry beans (*Phaseolus vulgaris* L) were analyzed chemically for content of moisture, fat, ash, total nitrogen, iron, zinc, calcium, magnesium, potassium, phosphorus, thiamin, riboflavin, and amino acids. Protein quality was evaluated using *Tetrahymena pyriformis* W. Cooked beans were subjected to sensory evaluation and shear-force measurement. Nutrient concentrations were similar to values reported by earlier investigators. Protein quality ranged from 60% (Red Kidneys) to 90% (Pintos) that of casein. Pintos Nodak and Fiesta and Hyden Navy beans were most acceptable in flavor and texture; Bonus Small White was least acceptable. Cooperation between bean breeders and nutritionists will enable early screening of segregating hybrid populations for desirable nutritional and sensory qualities.

INTRODUCTION

DRY LEGUME SEEDS are important foods in the diets of peoples throughout the world, especially those living in technically underdeveloped tropical and subtropical areas (Molina et al., 1975). Despite relatively low digestibility (Koehler et al., 1986), dry beans are an important source of protein for countries having short supplies of animal protein. Beans are also good sources of thiamin, zinc, (Murphy et al., 1975), iron (Sgarbieri et al., 1979), and potassium (Meiners et al., 1976b).

New cultivars of *Phaseolus vulgaris* are continually being developed and released. The economic value of a new cultivar (cv.) depends on its resistance to disease, its yield, rate of maturity, and seed size, its nutritional quality, and the flavor and texture of the cooked food. The nutritional contribution, however, is only recently becoming a serious consideration in breeding programs (Koehler and Burke, 1981).

Bean breeding programs have approached the problem of improving the nutritional value from various directions. Bressani et al. (1973) urged increasing the protein content and improving the digestibility through breeding trials. Bliss and Hall (1977) and Ma and Bliss (1978) investigated increasing the amounts of methionine in high protein genotypes using protein fractionation. They warned of the low negative correlations between percentage protein vs methionine content and between percentage protein and seed yield, traits affected by genetic selection. Ghaderi et al. (1984) and Hosfield and Uebersax (1980) studied fifteen independent quality traits, most of which were genetically controlled.

The objectives of this study were to investigate the nutrient composition, protein quality, and sensory and textural properties of 36 dry bean cultivars, including 25 developed and released in the last 15 years.

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MATERIALS & METHODS

Source and preparation of dry beans

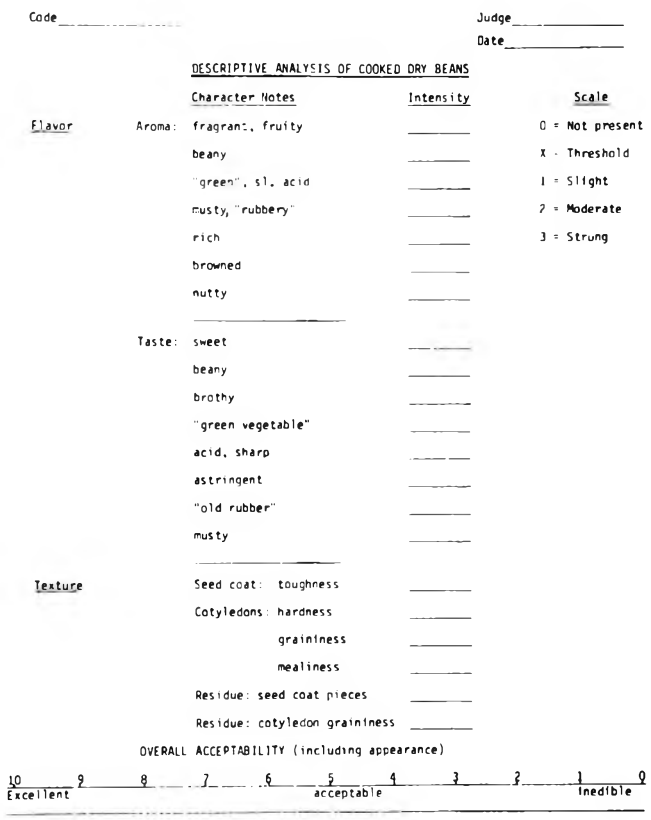
Commercially released cultivars representing Small White, Navy (Pea), Great Northern, Red Kidney, Pink, Red Mexican, Pinto, and Black beans were grown by personnel of the U.S. Dept. of Agriculture, ARS/Washington State University (WSU) Irrigated Agriculture Research and Extension Center, Prosser, WA. One often-used breeding line, Pinto JM-126, was included.

Beans were seeded the middle of May in rows 56 cm apart, in soil classified as fine sandy loam. Irrigation water was applied in alternate interrow ditches at 5- to 7-day intervals. The soil nitrogen level was minimal (20-40 kg/hectare), but phosphorus and potassium were applied as needed to obtain optimal levels according to soil tests. Bean

*Table 1—Proximate composition[a] of 36 dry-bean cultivars (*Phaseolus vulgaris*)*

Cultivar	H_2O (%)	Fat (%)	Ash (%)	N (%)
Small White				
Aurora	9.5	1.8	5.0	3.92
Bonus	9.6	1.7	4.8	4.03
Chief	10.2	1.8	4.6	4.11
Fleetwood	10.4	2.1	3.7	3.77
Navy				
Hyden (NW-230)[b]	9.9	1.7	4.0	4.00
NW-395[b]	9.6	2.0	4.9	3.61
Sanilac	9.7	1.8	4.3	3.74
Great Northern				
Harris	9.9	1.3	3.8	4.18
GN-1140	9.5	1.7	4.0	3.68
UI-59	10.8	1.5	4.4	4.08
Red Kidney				
Red Kloud	10.1	1.4	3.2	3.78
Royal Red	10.9	1.5	5.2	5.15
Pink				
Gloria	8.2	1.5	4.1	3.62
Roza	8.3	1.3	4.4	3.68
Sutter	10.2	1.5	4.5	3.88
Viva	9.2	1.4	4.5	3.67
Harold (gh-1053)[b]	10.0	1.4	4.2	3.69
Victor (6R-122)[b]	10.5	1.2	4.2	4.02
Red Mexican				
Rufus	10.4	1.5	4.4	3.77
NW-59[b]	8.9	1.4	5.6	3.82
NW-63[b]	10.4	1.3	4.2	3.77
UI-36	9.0	1.3	5.5	3.80
Pinto				
Columbia	10.4	2.8	4.7	3.72
Fiesta	10.3	1.4	3.6	3.68
Holberg (6R-364)[b]	9.6	1.8	3.9	3.56
Nodak (gh-370)[b]	10.3	1.4	3.6	3.80
Olathe (COLO-3439)[b]	10.9	1.6	3.7	3.60
Pindak (6R-354)[b]	8.4	1.2	4.1	3.73
Othello (gh-215)[b]	10.2	1.7	3.5	3.36
JM-126[c]	10.5	1.5	3.9	3.85
NW-410[b]	10.3	1.4	4.1	3.68
NW-590[b]	11.1	1.6	4.0	3.14
UI-111	9.2	1.5	4.3	3.76
UI-114	10.5	1.5	4.1	3.80
WYO-166	10.6	1.5	3.9	3.48
Black				
Turtle soup	9.9	1.6	4.6	4.07

[a] Dry basis; means of triplicate analyses.
[b] Recently released cvs.
[c] Recently released much-used breeding line.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DESCRIPTIVE ANALYSIS OF COOKED DRY BEANS

		Character Notes	Intensity
Flavor	Aroma:	fragrant, fruity	
		beany	
		"green", sl. acid	
		musty, "rubbery"	
		rich	
		browned	
		nutty	
	Taste:	sweet	
		beany	
		brothy	
		"green vegetable"	
		acid, sharp	
		astringent	
		"old rubber"	
		musty	
Texture		Seed coat: toughness	
		Cotyledons: hardness	
		graininess	
		mealiness	
		Residue: seed coat pieces	
		Residue: cotyledon graininess	

Scale: 0 = Not present; X = Threshold; 1 = Slight; 2 = Moderate; 3 = Strong

OVERALL ACCEPTABILITY (including appearance)

10 9 8 7 6 5 4 3 2 1 0
Excellent — acceptable — inedible

Fig. 1—Score sheet used for sensory evaluation.

plants were cut in early September, dried in the field, and harvested when they reached a moisture content of 10 to 12%. Ten pounds of seeds of each cultivar were collected randomly and stored at IAREC in paper bags at 10°C and 50 percent relative humidity no longer than 30 days.

On receipt at the WSU Agricultural Research Center, seeds were sealed in plastic bags and stored at room temperature. Thirty gram aliquots of each cultivar were ground in a Wiley Mill (Arthur H. Thomas Company, Philadelphia) and a mortar until all powder passed through a 60-mesh sieve. Bean powders were stored at room temperature in airtight, light-proof containers until analyzed (no longer than 2 wk). Concentrations of nutrients determined were calculated on the basis of 100g bean powder, dry basis.

Chemical analyses

Moisture, fat, mineral ash and total (Kjeldahl) nitrogen were determined according to procedures 14.003; 7.003, 7.055-7.056; 14.006; and 47.021-47.023 respectively of the Association of Official Analytical Chemists (AOAC, 1980).

Thiamine was assayed by AOAC fluorometric method 43.024-43.029, using Taka-Diastase (Pfaltz and Bauer, Inc., Stanford, CT) or Mylase 100 (U.S. Biochemicals Corp., Cleveland, OH) to release bound thiamin and Bio-Rex 70 resin (Bio-Rad Laboratory, Richmond, CA) for purification. Riboflavin was determined by AOAC fluorometric procedure 43.039 (AOAC, 1980).

Minerals were determined by atomic absorption spectroscopy using a Techtron Model AA-4 instrument, according to AOAC procedures 7.091–7.094 (zinc, iron, magnesium), 3.006-3.009 (potassium) and 2.109–2.113 (calcium), except for phosphorus, which was assayed by molybdovanadate method 7.120-7.123. Certified commercial standards (Fisher Scientific Co.) were run with every determination.

Amino acid contents of bean powder were determined by the WSU Bioanalytical Laboratory after 24-hour hydrolysis with 6N HCL according to the method of Spackman et al. (1958), using a Beckman Model 121MB Amino Acid Analyzer. Cystine and methionine were determined after treatment with performic acid reagent and measured as cysteic acid and methionine sulfone, respectively. Tryptophan is destroyed by acid hydrolysis and was not determined.

Microbiological estimation of protein quality

The protozoan *Tetrahymena pyriformis* W (American Type Culture Collection, Rockville, MD) was used as test organism to evaluate protein quality of the raw bean powder (Evancho et al., 1977). All estimations were done on triplicate bean samples, each leading to triplicate aliquots. Growth of organisms was measured by counting in a hemacytometer. Data were expressed as Relative Nutritive Value (RNV), calculated as follows:

$$RNV = \frac{\text{Number of organisms per mL of sample}}{\text{Number of organisms per mL of casein control}} \times 100$$

Sensory evaluation of cooked beans

Seeds (200g) of each cultivar were soaked for 15 hr at room temperature in 1L distilled water, then were cooked in the soak water in covered 1.8L casseroles at 160°C until "just tender," not soft, as judged by the chief investigator. Cooked bean samples were randomized and presented hot in foil-covered 50 mL beakers to 10 trained panelists. Each cultivar was tested 50 times (10 panelists X 5 replications); the panelists evaluated aroma, taste and texture characteristics using modified Descriptive Analysis plus a rating scale for overall acceptability. The score sheet used (Fig. 1) was developed by a panel of experienced tasters to include flavor descriptors detected in cooked beans.

Instrumental measurement of cooked-bean texture

Texture (cookability) was measured (Koehler and Burke, 1981) by means of an Allo-Kramer shear-press provided with a 500 lb (227 kg) proving ring under 50 lb (22.7 kg) of pressure at range 20. Shear readings were made on triplicate 50 g aliquots of the same cooked beans prepared for sensory evaluation.

Statistical analyses

Data were subjected to Analysis of Variance and Duncan's New Multiple Range Test, where applicable.

RESULTS & DISCUSSION

Proximate composition

Concentrations of moisture, fat, ash and total nitrogen are presented in Table 1, and are means of data from the harvest years for which analyses were made. Proximate composition concentrations varied from cultivar to cultivar, but were similar to values published by Meiners et al. (1976a), Sgarbieri et al. (1979), and Swanson et al. (1977) for pink, white and red beans, and in USDA Handbook 8 (Watt and Merrill, 1963) for white and red beans. Nitrogen in pinto beans was slightly greater than that found by Swanson et al. (1977) but less than that reported by Rockland et al. (1977). Varriano-Marston and DeOmana (1979) analyzed black beans that contained 3.5% nitrogen.

Minerals

Concentrations of iron, zinc, calcium, magnesium, potassium and phosphorus (dry-weight basis) are presented in Table 2. Values for iron and zinc were corrected when reassessment analysis showed contamination from the Wiley Mill of 9.99% (iron) and 10.70% (zinc). Iron varied from 4.5 mg (Othello pinto) to 8.8 mg/100g (Bonus small white); the mean for all cultivars was 6.89 mg ± 1.02 SD. Iron values in Table 2 were similar to or smaller (compared on wet basis) than concentrations given by Meiners et al. (1976b) and Fordham et al. (1975) for Navy, Great Northern, Red Kidney and Pinto beans. Iron values compared on dry basis to those reported by Augustin et al. (1981) were similar for Small White, Navy and Red Kidney beans, but were slightly greater for Great Northern, Pink, Pinto, Small Red and Black Turtle Soup beans. Iron in beans is in nonheme form, however, and is only 0.8 to 5.2% available (Sgarbieri et al., 1979; Lynch et al., 1984).

Zinc concentrations (Table 2) in the 36 cultivars varied very

*Table 2—Mineral[a] and vitamin[b] contents of 36 dry bean cultivars (*Phaseolus vulgaris*)*

Cultivar	Fe	Zn	Ca	Mg	K	P	Thiamin	Riboflavin
	mg/100g							
Small White								
Aurora	8.65	3.4	233	180	1526	502	1.010	0.175
Bonus	8.75	3.5	195	158	1303	403	0.727	0.218
Chief	7.53	3.1	175	176	1617	489	0.752	0.202
Fleetwood	6.52	3.0	184	146	1118	396	0.827	0.205
Navy								
Hyden (NW-230)	7.76	2.9	240	170	1335	398	0.842	0.212
NW-395	5.67	3.0	170	188	1366	500	0.751	0.204
Sanilac	6.33	2.7	238	172	1383	492	0.821	0.168
Great Northern								
Harris	7.60	3.1	212	158	1215	388	0.906	0.185
GN-1140	5.49	2.4	231	158	1207	406	0.670	0.157
UI-59	6.70	2.9	211	176	1336	476	0.753	0.186
Red Kidney								
Red Kloud	5.16	3.1	131	128	1038	385	0.944	0.169
Royal Red	7.76	3.8	163	143	1648	405	0.596	0.238
Pink								
Gloria	7.48	2.9	164	156	1326	412	0.807	0.224
Roza	7.24	2.8	142	155	1371	427	0.794	0.259
Sutter	6.87	2.8	143	189	1458	458	0.698	0.221
Viva	6.48	2.6	182	170	1380	426	0.812	0.199
Harold (gh-1053)	6.63	2.6	133	153	1346	438	0.643	0.130
Victor (6R-122)	8.18	2.8	126	145	1344	403	0.399	0.188
Red Mexican								
Rufus	7.52	2.7	142	161	1392	493	0.926	0.199
NW-59	8.21	3.2	162	139	1374	432	0.815	0.216
NW-63	6.31	2.9	122	160	1498	493	0.824	0.183
UI-36	8.03	3.4	156	144	1320	459	0.856	0.209
Pinto								
Columbia	6.53	2.7	166	176	1377	612	0.452	0.165
Fiesta	5.32	2.5	127	144	1285	375	0.809	0.186
Holberg (6R-364)	6.11	2.9	159	155	1225	424	0.772	0.132
Nodak (gh-370)	6.97	3.0	133	145	1173	362	0.691	0.171
Olathe (COLO-3439)	4.47	2.7	173	163	1376	506	0.614	0.154
Pindak (6R-354)	6.05	3.0	140	170	1386	520	0.920	0.156
Othello (gh-215)	4.47	2.5	130	128	1164	390	0.592	0.193
JM-126	8.22	2.9	150	151	1217	453	0.678	0.203
NW-410	6.62	2.6	154	167	1408	479	0.602	0.186
NW-590	8.27	2.6	154	163	1316	466	0.623	0.172
UI-111	6.31	2.8	154	175	1552	566	0.775	0.177
UI-114	6.55	2.8	162	161	1379	486	0.631	0.188
WYO-166	6.48	2.4	173	161	1360	548	0.447	0.192
Black								
Turtle Soup	6.91	2.9	225	171	1468	614	1.140	0.154

[a] Means of triplicate analyses; dry basis.
[b] Means of duplicate analyses; dry basis.

little, mean 2.88 ± 0.30, from a low of 2.4 mg (in GN-1140 and WYO-166 Pinto) to 3.8 mg/100g (in Royal Red Kidney). These values are similar to or slightly greater (compared on wet basis) than concentrations reported by Meiners et al. (1976b), and are similar to values compared on dry basis published by Rockland et al. (1979) and Augustin et al. (1981) except for Black Turtle Soup beans (4.4 mg). Although zinc is less available for absorption from vegetable proteins than from meats, dry beans are a significant source of dietary zinc (Murphy et al., 1975).

Calcium concentrations varied conspicuously according to bean types. Much greater amounts were found in Small White and Great Northern beans (means 197 mg and 218 mg respectively) than in beans with colored seedcoats (means Red Kidney, 147 mg; Pink, 148 mg; Red Mexican, 146 mg; Pinto, 152 mg/100 g beans). The exception was Black Turtle Soup (225 mg). These calcium values are slightly greater than concentrations reported by Meiners et al. (1976b), Fordham et al. (1975), and Augustin et al. (1981), but a similar relationship is seen among bean types.

Magnesium concentrations in Table 2 are generally smaller for all types of beans than values reported by other researchers. Red Kidney beans had very small quantities of magnesium (mean 136 mg). Phosphorus concentrations in the 36 cultivars were similar for Small White and Pink beans, smaller for Great Northern, Red Kidney and Pinto beans, and greater for Navy, Red Mexican and Black Turtle Soup beans, when compared to concentrations reported by Meiners et al. (1976b), Fordham et al. (1975), and Augustin et al. (1981). Potassium concentrations in the 36 cultivars were smaller than concentrations reported by other researchers except for Navy beans, varying from 1038 mg (Red Kloud Kidney beans) to 1552 mg/100g (Pinto UI-111). Dry beans are good sources of dietary phosphorus and potassium.

Vitamins

As might be expected from work by Augustin et al. (1981), thiamin concentrations in the 36 cultivars (Table 2) exhibited low variability (mean 0.748 mg ± 0.15 SD). Black Turtle Soup contained the greatest quantities of thiamin (1.14 mg), followed by Red Mexican beans (mean 0.855 mg ± 0.05). Pinto cultivars varied within their type, with the smallest mean concentration (0.66 mg/100g). Beans are a significant dietary source of thiamin.

Riboflavin values (Table 2) exhibited very little variation (mean 0.189 mg % ± 0.03). Great Northern, Pinto and Black Turtle Soup beans contained smaller amounts of riboflavin

Table 3—Essential amino acids (EAA)[a] in 36 dry-bean cultivars (mg amino acid/g protein, wet basis)

Cultivar	Histidine	Iso-leucine	Leucine	Lysine	Total S-cont.[b]	Total aromatic[c]	Threonine	Valine
Small White								
Aurora	31	47	82	72	20	95	52	53
Bonus	33	40	68	73	16	116	47	45
Chief	33	43	72	69	17	90	50	49
Fleetwood	25	41	74	63	22	79	43	48
Navy								
Hyden (NW-230)	28	45	80	68	22	89	41	52
NW-395	36	46	79	74	19	116	52	51
Sanilac	31	45	79	71	19	94	47	52
Great Northern								
Harris	35	54	90	79	25	97	46	65
GN-1140	30	48	84	73	23	92	42	62
UI-59	30	47	85	72	21	93	46	54
Red Kidney								
Red Kloud	27	44	78	66	22	87	44	54
Royal Red	30	46	77	66	18	84	56	46
Pink								
Gloria	33	45	77	73	17	96	48	48
Roza	34	48	77	78	20	100	51	52
Sutter	35	46	82	80	22	116	52	58
Viva	29	46	78	69	21	87	45	88
Harold (gh-1053)	31	48	84	74	20	93	41	54
Victor (6R-122)	27	40	68	63	14	75	33	47
Red Mexican								
Rufus	30	45	80	72	19	101	47	50
NW-59	35	43	72	74	19	116	51	54
NW-63	32	46	81	75	19	107	52	45
UI-36	38	44	79	83	20	146	52	56
Pinto								
Columbia	39	49	86	89	21	112	55	51
Fiesta	33	49	85	76	21	93	49	64
Holberg (6R-364)	34	50	90	81	23	97	45	70
Nodak (gh-370)	27	43	77	64	19	84	38	53
Olathe (COLO-3439)	36	49	86	77	21	105	47	53
Pindak (6R-354)	30	47	84	71	21	90	43	70
Othello (gh-215)	29	46	80	71	23	87	41	52
JM-126	31	49	85	72	19	94	40	56
NW-410	33	46	79	75	20	98	43	50
NW-590	34	49	86	79	22	99	47	56
UI-111	34	46	83	75	20	98	46	61
UI-114	33	45	79	71	21	96	43	55
WYO-166	37	47	83	78	18	104	48	52
Black Turtle	31	48	87	75	21	95	49	56
EAA pattern for high quality protein (FNB, 1975)	17	42	70	51	26	73	35	48

[a] Tryptophan was not determined.
[b] Methionine + cystine.
[c] Phenylalanine + tyrosine.

(means 0.176 mg %, 0.175 mg % and 0.175 mg % respectively) than the other five types of beans (means each 0.20 mg %). Beans are not a significant source of dietary riboflavin.

Amino acids

Essential amino acid (EAA) composition of the 36 cultivars is given in Table 3. Concentrations of seventeen amino acids were determined; data on those not reported here are available on request. All cultivars tested were deficient in methionine plus cystine, when compared to the EAA pattern for high-quality proteins of the Food and Nutrition Board, National Research Council (FNB, 1975). However, Great Northern beans Harris and GN-1140 and Pintos Holberg and Othello came close to meeting the FNB standard. Small deficiencies were seen in valine, leucine, iso-leucine and threonine. All cultivars were very good sources of lysine, and should be useful complements for lysine-deficient cereal proteins.

Protein quality and quantity

Protein quality of foods for people is most accurately evaluated by people, but experiments with human subjects are costly and time-consuming. Microbiological estimations of protein quality give a measure of protein availability, and are comparatively inexpensive, quick and efficient. Resulting data have been found to correlate well ($r = 0.9$) with rat Protein Efficiency Ratio (PER) values (Wang et al., 1979). The protozoan *Tetrahymena pyriformis* W has a requirement for EAA similar to that of humans and rats (Hsu et al., 1978). Growth of *Tetrahymena* organisms supported by the bean protein was compared to growth supported by casein, and is expressed as Relative Nutritive Value (RNV).

Table 4 presents protein quantities and RNV data for the 36 cultivars. Protein quantities ranged from 19.6 to 32.2% (dry basis).

Pinto beans had the greatest protein quality. Columbia, Holberg, Othello, NW-590, UI-111 and WYO-166 had RNV values of 90 or greater and the other pintos RNV $\geq$ 80, except for Fiesta (RNV = 72). The least protein quality was found in Red Kidney beans (mean RNV = 59). Black Turtle Soup had RNV = 68, Red Mexican mean RNV = 70, and Small White beans mean RNV = 70. Both protein quality and digestibility were found to be low in raw beans (Koehler et al., 1986). Harper (1983) emphasized that in low-protein diets especially (as in developing countries) amino acids must be adequately balanced for efficient use in synthesis of tissue proteins. Bean protein quality could be improved by adding methionine

Table 4—Protein quantity and quality of 36 dry-bean cultivars

	Protein[a] (%)	Protein quality[b] RNV[c]
Small White		
Aurora	22.2	68
Bonus	22.8	67
Chief	23.1	69
Fleetwood	21.1	76
Navy		
Hyden (NW-230)	22.5	75
NW-395	20.4	78
Sanilac	22.4	64
Great Northern		
Harris	23.6	82
GN-1140	20.8	88
UI-59	22.8	74
Red Kidney		
Red Kloud	21.3	60
Royal Red	28.7	58
Pink		
Gloria	20.7	72
Roza	21.0	75
Sutter	21.7	82
Viva	21.3	76
Harold (gh-1053)	20.7	79
Victor (6R-122)	22.5	78
Red Mexican		
Rufus	21.8	66
NW-59	21.7	69
NW-63	21.1	74
UI-36	21.6	72
Pinto		
Columbia	20.8	90
Fiesta	20.6	72
Holberg (6R-364)	20.1	92
Nodak (gh-370)	21.3	82
Olathe (COLO-3439)	20.1	88
Pindak (6R-354)	21.4	80
Othello (gh-215)	18.9	91
JM-126	21.6	88
NW-410	20.6	88
NW-590	17.5	90
UI-111	21.3	92
UI-114	21.2	81
WYO-166	19.4	93
Black		
Turtle soup	22.9	68

[a] Wet basis; means of triplicate analyses; N × 6.25.

[b] Means of triplicate estimations.

[c] RNV (Relative Nutritive Value) = $\frac{\text{No. organisms per mL of sample}}{\text{No. organisms per mL of casein}} \times 100$

and cystine, or by complementing the amino acids in beans by mixing with cereals or other proteins (with soybeans, Sgarbieri et al., 1981; semolina flour, Bahnassey et al., 1986; sesame, Nielsen et al., 1983).

Sensory evaluation and texture measurement

Conspicuous sensory characteristics of taste and texture of cooked beans evaluated by Descriptive Analysis are presented in Table 5. Aroma characteristics were judged as well, but in most samples these were similar to the taste judgments. Shear-force measurements of the texture of these same cooked beans, and their overall acceptability ratings, are also listed in Table 5.

The acceptability rating for each cultivar should reflect the cumulative effect of its aroma, taste, and texture appraisal as well as its cookability (shear-force) measurement. Nodak and Fiesta pintos and Hyden navy beans were judged most acceptable. These cultivars had conspicuous sweet, nutty, and rubbery tastes and were lacking in undesirable texture characteristics. The least acceptable cultivars, Bonus small white, Roza and Gloria pink, and Royal Red Kidney beans, on the other hand, were judged to taste predominantly green, astringent, and hay-like, flavor characteristics considered less desirable. The texture characteristics evaluated by the sensory panel were all undesirable ones. The cultivar most easily cooked, Chief, was occasionally judged "mealy." The firmest beans, Columbia and WYO-166 pintos and Royal Red kidney beans, were judged to have undesirable texture characteristics such as tough seed coat, grainy cotyledons, and seed coat and cotyledon residues. Most of the cultivars having good cookability as measured by low shear-force values had few or no undesirable sensory texture characteristics. An exception to this relationship was Fiesta pinto, a firm bean without undesirable sensory texture characteristics.

A few cultivars were consistently found to be difficult to hydrate and cook to an acceptable texture. Bonus small white beans were conspicuous in this "hard bean phenomenon". Cookability of beans has been reported (Salunkhe, 1982) to depend upon such factors as growing conditions, handling and storage, and chemical composition. Much research has been and is being conducted on these aspects and their interrelationships but no satisfactory solution to the hard bean problem has yet been found.

Usually, genetic improvement of dry beans has taken into consideration production and marketing criteria such as seed yield, size, rate of maturity, disease resistance and appearance of the raw and canned product. Seldom have genetic differences in nutritive value and sensory quality been recognized and utilized. Recently released cultivars Hyden, NW-395, Harold, Victor, NW-59, NW-63, Holberg, Nodak, Olathe, Othello, Pindak, JM-126 (released breeding line), NW-410 and NW-590 were subjected to these criteria, and possess nutritive and sensory qualities equal or superior to those of older cultivars. (Release reports are available on request.)

Improved nutrition, flavor and texture of dry bean cultivars should be marketable improvements. Quick and effective methods of testing early generations of segregating hybrid populations for improved nutritive and sensory qualities would encourage and facilitate work by breeders. The need for increased and on-going cooperation between nutritionists, food processors, and bean breeders is apparent.

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*Table 5—Sensory evaluations and shear-force measurements of 36 dry bean cultivars (*Phaseolus vulgaris*)*

Cultivar	Overall acceptability[A,B]	Conspicuous sensory characteristics		Shear force[B]
		Taste	Texture[B]	
Small White				
Aurora	6.41[bcde*]	Green, rubbery	2, 3, 5, 6	40.4[bcdefghi]
Bonus	4.82[f]	Green	1, 2, 3, 5, 6	49.1[hijk]
Chief	6.35[cde]	Sweet, musty, rubbery	4	25.5[a]
Fleetwood	6.96[abcd]	—[D]	1, 4	47.7[ghij]
Navy (Pea)				
Hyden (NW-230)	7.73[a]	Sweet, nutty	3	45.1[defghij]
NW-395	6.70[abcde]	Sweet, green, acid, astr.	—[D]	36.6[abcdefgh]
Sanilac	7.13[abcd]	Sweet, musty, astr., green, rubbery, acid	—[D]	33.4[abcdef]
Great Northern				
Harris	7.63[abc]	Sweet, brothy	6	46.2[fghij]
GN-1140	7.07[abcd]	Sweet, green, musty, brothy, astringent	1, 2, 3, 5, 6	44.7[defghij]
UI-59	7.23[abcd]	Sweet, musty, astr.	1, 2, 3, 5, 6	41.5[cdefghij]
Red Kidney				
Red Kloud	7.13[abcd]	Brothy, astr., hay-like	1, 5	62.0[klm]
Royal Red	6.08[def]	Green, astr., hay-like	1, 3, 5, 6	64.6[lm]
Pink				
Gloria	5.43[ef]	Green, astringent	5, 6	47.7[ghij]
Roza	4.79[f]	Green	1, 2, 3, 5, 6	53.4[ijkl]
Sutter	7.26[abcd]	Sweet, green, astringent	2, 3	35.1[abcdefg]
Viva	7.27[abcd]	Sweet, green	1, 2, 3, 5, 6	38.0[abcdefgh]
Harold (gh-1053)	7.46[abc]	Green, acid, rubbery	1, 2, 5	38.9[bcdfgh]
Victor (6R-122)	7.64[abc]	Green, acid, musty	—[D]	43.9[cdefghij]
Red Mexican				
Rufus	7.28[abcd]	Astringent, rubbery, musty	1, 2, 5	45.8[efghij]
NW-59	6.94[abcd]	Sweet, green, acid, rubbery	—[D]	32.1[abcd]
NW-63	7.08[abcd]	Green, acid, rubbery, musty	3, 6	32.6[abcd]
UI-36	6.79[abcd]	Green, acid	—[D]	41.2[cdefghij]
Pinto				
Columbia	7.01[abcd]	Green, astringent, rubbery	1, 3	67.7[m]
Fiesta	7.93[a]	Nutty	—[D]	61.1[klm]
Holberg (6R-364)	6.94[abcd]	Green, acid, rubbery	1, 3, 5, 6	37.6[abcdefgh]
Nodak (gh-370)	7.98[a]	Rubbery, musty	2, 3, 6	52.5[ijkl]
Olathe (COLO-3439)	6.90[abcd]	Green, musty, rubbery, astr.	1, 2, 3, 5, 6	52.7[ijkl]
Pindak (6R-354)	7.72[ab]	Sweet, musty, rubbery, astr.	[D]	32.9[abcde]
Othello (gh-215)	7.56[abc]		—[D]	40.4[bcdefghi]
JM-126	7.70[ab]	Sweet, acid, green, musty	1, 2, 3, 6	53.6[ikl]
NW-410	7.38[abcd]	Green, acid, musty	1, 2, 3, 6	38.7[bcdefgh]
NW-590	7.18[abcd]	Sweet, acid, rubbery	—[D]	37.9[abcdefgh]
UI-111	7.40[abc]	Sweet, green		41.1[cdefghij]
UI-114	7.49[abc]	Sweet, green, musty rubbery, astringent	1, 2, 3, 5, 6 —[D]	46.6[ghij]
WYO-166	6.94[abcd]	Green, astringent		65.1[lm]
Black				
Turtle soup	6.99[abcd]	Sweet		30.8[abc]

[A] Overall acceptability rated on a scale of 0 (inedible) to 10 (excellent).

[B] Values having the same letter superscripts are not significantly different according to Duncan's New Multiple Range Test ($P = 0.05$).

[C] Texture characteristics: (1) tough seed coat; (2) hard cotyledons; (3) grainy cotyledons; (4) mealy; (5) seed coat residue; (6) grainy cotyledon residue.

[D] No conspicuous characteristics.

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Ms received 11/24/86; revised 3/9/87; accepted 3/25/87.

Scientific Paper No. 7631, College of Agriculture & Home Economics Research Center, Washington State Univ., Pullman, WA 99164-6240. Project 0317.

Surface Active Properties of Proteins: Effects of Progressive Succinylation on Film Properties and Foam Stability of Glycinin

S.H. KIM and J.E. KINSELLA

ABSTRACT

Succinylation of glycinin at 25, 50, 70, and 98% of available amino groups progressively increased hydrophobicity, viscosity and exposure of aromatic amino acids. Maxima in surface pressure (at pH 4–8), film–yield stress, and film elasticity were observed at 25% succinylation where the maximum in foam stability was also observed. Excessive succinylation (>50%) reduced these parameters because the high net negative charges may have impaired protein:protein interactions in films.

INTRODUCTION

THE PHYSICAL PROPERTIES and functional attributes of soy proteins have been extensively studied (Kinsella and Shetty, 1979; Kinsella et al., 1985). The functional properties of the major component namely, glycinin or 11S protein (350–360,000 daltons) may be limited by its relatively stable oligomeric structure. The limited flexibility of glycinin may restrict is more widespread use in foods, particularly for applications requiring surface activity, e.g., foaming and emulsification, where molecular flexibility is important in facilitating orientation and spreading at an interface and forming cohesive interfacial films (MacRitchie, 1978). Appropriate modification of glycinin may be used to improve its surface active properties. Thus, reduction of component disulfide bonds increased molecular flexibility and foaming properties (Kim and Kinsella, 1986a, b). Modification of the net charge on glycinin may be a feasible approach for improving its functional behavior, particularly its surface active properties; for example, succinylation of soy protein isolate improved its solubility and emulsifying activity (Franzen and Kinsella, 1976). Because succinylation causes unfolding and dissociation of soy protein and since net charge on proteins greatly affect their ability to form cohesive films in foams or emulsions, this study was conducted to determine the effects of progressive succinylation on the film and foaming properties of soy glycinin.

MATERIALS & METHODS

ANALYTICAL GRADE chemical reagents were obtained from Eastman Kodak (Rochester, NY), Sigma Chemical Co. (St. Louis, MO), or Fisher Scientific (Rochester, NY). Doubly distilled deionized water was used for preparation of all solutions.

Preparation of soy glycinin

Glycinin was prepared from defatted, low heat treated soy flour (Central Soya, Ft. Wayne, IN) by the procedure of Than and Shibasaki (1978). The defatted flour (50g) was suspended in 1L 0.03M Tris-HCl buffer, pH 8.0, containing 2mM 2-mercaptoethanol. The solution was stirred for 1 hr at 24°C and then centrifuged at 15,000 x *g* for 20 min at 20°C. The supernatant was adjusted to pH 6.4 with 2N HCl and centrifuged at 15,000 x *g* for 20 min at 2°C. The glycinin precipitate was washed with Tris-HCl buffer pH 6.4 and then dispersed in Tris-HCl buffer, pH 8.0. Aliquots of 2 N NaOH were added while stirring until the protein was fully dissolved (pH 8.0). The protein solution was dialyzed against water adjusted to pH 8.0 at 4°C and lyophilized.

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Succinylation of glycinin

Succinylation of the glycinin was performed as detailed in the method of Franzen and Kinsella (1976). To the glycinin (5g in 70 mL) solution in 0.1M phosphate buffer (pH 8.2), known amounts (0.025, 0.05, 0.125, 0.25, and 0.5g) of succinic anhydride were added in small (10 mg) increments. The solution was stirred and the pH maintained above 8.1 by adding 3N NaOH at 25°C. After the pH stabilized at 8.0, following the addition of the succinic anhydride, the solution was dialyzed for 24 hr at 5°C against distilled water adjusted to pH 8 to remove excess reagent and then lyophilized.

The extent of succinylation was determined by titrating the free amino groups using trinitrobenzenesulphate (TNBS) (Kim and Kinsella, 1986b). Changes in structure of the glycinin were determined as detailed previously (Kim and Kinsella, 1986a). Thus, viscosity was determined using an Ostwald viscometer, hydrophobicity by cis-parinaric acid binding, and changes in conformation were monitored by changes in the ultraviolet absorbance and fluorescence spectra of the proteins (Kim and Kinsella, 1986a,b).

To determine the effects of succinylation on film and foaming properties, the experiments were designed and conducted as described in previous papers (Kim and Kinsella, 1985, 1986a,b; Waniska and Kinsella, (1985).

Surface pressure. The surface pressure development was monitored using a Wilhelmy plate attached to a Cahn electrobalance (Waniska and Kinsella, 1985).

Surface viscosity. The viscosity (n_s) and yield stress (f_s) of the interfacial film, which reflect the cohesiveness and strength of protein films (magnitude and force of protein:protein interactions), were determined using a special cylindrical rotator attached via torsion wire to a Brookfield viscometer (Kim and Kinsella, 1985).

Film elasticity. The change in surface pressure occurring in response to change in surface area of a film or film elasticity was monitored by tensiolaminometry (German et al., 1985).

Foam stability. Foams were made using the column aeration technique of Waniska and Kinsella, (1979) and foam stability was determined from the rate(s) of drainage, a reliable index of stability (Halling, 1981). Plotting log of the volume of liquid in the foam against time yields an apparent rate constant for drainage or half life of foam (Kim and Kinsella, 1985). All data represent the mean values of triplicate analyses.

RESULTS & DISCUSSION

THE SUCCINYLATION of the lysine residues in glycinin increased progressively with concentration of succinic anhydride, and 25, 50, 80, and 98% succinylation were obtained at ratios of 0.1, 0.15, 0.30, and 1.0 succinic anhydride reagent to glycinin, respectively. These were comparable to the results of Rao and Rao (1979) for glycinin and to the succinylation of other proteins where 100% succinylation was achieved at relatively low ratios of reagent to protein (Franzen and Kinsella, 1976; Kinsella and Shetty, 1979; Means and Feeney, 1971). These results indicate that most of the ϵ – amino group of lysine and N-terminal amino acids were accessible to succinic anhydride at pH 8.0, or that initial succinylation of these

altered the protein conformation and enhanced the availability of additional amino groups.

The net charge on glycinin increased with succinylation, i.e. cationic amino groups were changed to anionic carboxyl groups following succinylation, and upon 100% succinylation the negative charges were increased by approximately 160 units per mole of glycinin at pH 8.0 (Kim and Kinsella, 1986). From the electrometric titration data of Catsimpoolas et al. (1971), glycinin carries a net negative charge of about 260 electron units per mole at pH 7.8. Therefore, the increase represents a significant increase in net negative charge of glycinin at pH 7.0. This was accompanied by changes in the solubility and conformation of glycinin. The solubility of glycinin was significantly improved by succinylation as observed previously (Franzen and Kinsella, 1976). Glycinin, succinylated above 25% was completely soluble at all pH values in the pH range 4 to 8.

Marked changes in viscosity, hydrophobicity and absorbances occurred following succinylation (Table 1) reflecting extensive conformational changes and dissociation of the oligomeric glycinin. The increase in viscosity reflects changes in the hydrodynamic volume probably caused by some unfolding of the glycinin. The alteration of the net charge by 160 units upon succinylation destabilized the oligomeric structure of glycinin causing dissociation into half molecules i.e. hexameric subunits and repulsive intrapeptide charges may have caused unfolding of acidic and basic subunits, thereby increasing the viscosity, as observed also by Rao and Rao, (1979).

The changes in the UV and fluorescent spectra together with shifts in λ maxima were consistent with unfolding of glycinin and exposure of hydrophobic aromatic residues to the polar aqueous phase. These data are consistent with hydrophobicity changes and the shifts as observed with the progressive succinylation of arachin (Shetty and Rao, 1978) and soy protein (Damodaran and Kinsella, 1981). The decrease in fluorescence intensity of glycinin above 50% succinylation indicated reassociation of the unfolded polypeptides to bury some of the tryptophan residues (Kim and Kinsella, 1986a,b).

Surface film properties

Surface properties are related to structural characteristics of proteins, hence the surface properties and foam stability of the native and succinylated soy 11S globulins were determined. Surface pressure after 5 min adsorption at pH 6 significantly increased with 25% succinylation and progressively decreased with further succinylation, but even at the highest level of succinylation the surface pressure was higher than that of native soy 11S globulin (Fig. 1). The low surface pressure of the native glycinin was consistent with the observation of Tornberg (1978a, b).

The rate of surface pressure development was affected by rates of diffusion and adsorption of proteins at the air water interface (MacRitchie 1978). The fifteen-fold increase in surface pressure at 25% succinylation reflects the increased solubility, and possibly enhanced diffusion, of the modified glycinin molecules which dissociated upon limited modification (Rao and Rao, 1979). The adsorption at the interface may be related to the increased hydrophobicity (Table 1) which, thermodynamically, should favor orientation of the protein at the gas or air interface.

The decreased pressure with increasing succinylation most likely reflected electrostatic repulsion between the highly charged adsorbed layer and the molecules newly arriving at interface. This retarded further adsorption and reduced protein:protein interactions in the interfacial film.

The rheological properties of an interfacial film reflects its mechanical strength which can be estimated by viscosity and surface yield stress. These are important properties for the formation of stable foams. The apparent surface yield stress of succinylated glycinin varied with extent of succinylation and pH (Fig. 2). At pH 4 and 5, where unmodified glycinin was insoluble, the succinylated protein showed relatively high values, especially at pH 5. Significantly, at pH 6 and 8, surface yield stress decreased with extent of succinylation. This coincided with the high charge density which probably minimized protein:protein interaction resulting in expanded films lacking cohesiveness and mechanical strength. In contrast, the low net charge at pH 5 facilitated greater hydrophobically-driven protein:protein interactions resulting in condensed, stronger more cohesive films, formation of which were facilitated by succinylation-induced dissociation of the native structure (Fig. 2). This pH effect is consistent with the behavior of other proteins which show maximum film strength close to the isoelectric pH (pI) (Halling, 1981; Kinsella, 1981, Graham and Phillips 1979; Kim and Kinsella 1985). Succinylation of soy proteins shifted the pI by approximately 0.4 pH units i.e. from 4.6 to 4.2 (Franzen and Kinsella, 1976).

The values for surface yield stress of succinylated glycinin films are comparable to those obtained for bovine serum albumin and reduced glycinin films (Kim and Kinsella, 1986a,b), but lower than these obtained for native ribulose phosphate carboxylase (Barbeau and Kinsella 1986) a large globular molecule.

Film elasticity influences the capacity of an interfacial film to expand and accomodate localized stresses and is a parameter related to foam stability. The dynamic film elasticity of glycinin increased with succinylation at 20 and 50% (Fig. 3), but then decreased upon further succinylation and paralleled the surface yield characteristics. These reflect the cohesiveness of the film and, therefore, extensive succinylation, by introducing excessive negative charges, apparently impaired the formation of cohesive films.

Because the above properties are related to foam stability, this parameter was also examined. Foam stability, expressed as half lifetime of liquid in the foam (drainage stability), increased with the lowest level of succinylation tested (25%) but further succinylation decreased stability (Fig. 4). However, even at the highest level of succinylation, foam stability was higher than that of native soy 11S globulin. These data reveal a relationship between the film properties and foam stability and show that limited succinylation, i.e. up to 25%, generally improves the foaming properties of glycinin. These observations corroborate these obtained with disulfide reduced glycinin (Kim and Kinsella, 1986a,b).

In conclusion, limited succinylation, by increasing solubility and causing some dissociation and perhaps unfolding of glycinin, improved film formation and film properties, resulting in more stable foams. Extensive modification, by causing com-

Table 1—Changes in molecular and conformational properties of glycinin following progressive succinylation[a]

Parameter	Succinylated (%)				
	0 (Native)	25	50	80	>98
Relative hydrophobicity	225	330	350	315	290
Relative viscosity × 10^{-2}	25	80	120	160	>800
UV absorbance (275 nm)	0.88	0.78	0.75	0.71	0.68
Relative fluorescence (336 nm)	46.0	48.0	55.0	51.0	46.0

[a] Determined as described by Kim and Kinsella (1986 a,b).

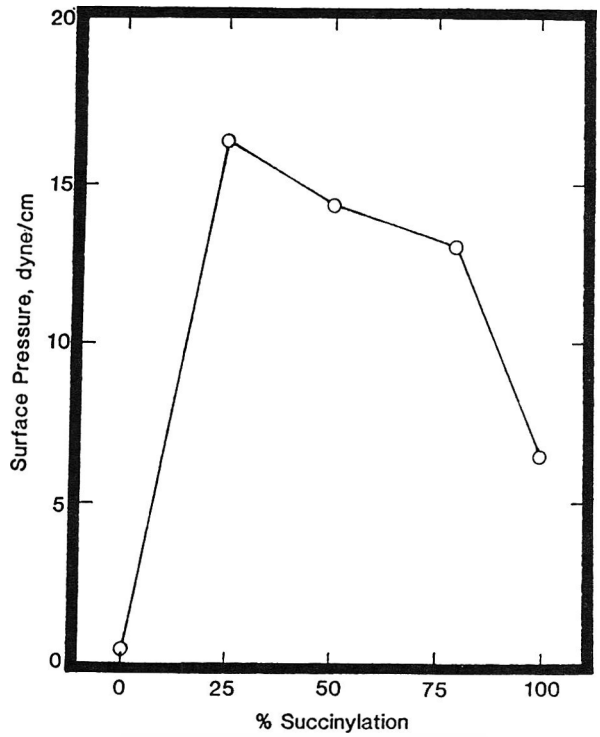

Fig. 1—Effects of increasing succinylation on surface pressure after 5 min of adsorption of native and succinylated glycinin. Protein concentration was 5 x 10⁻³% (w/v) in 20 mM phosphate buffer.

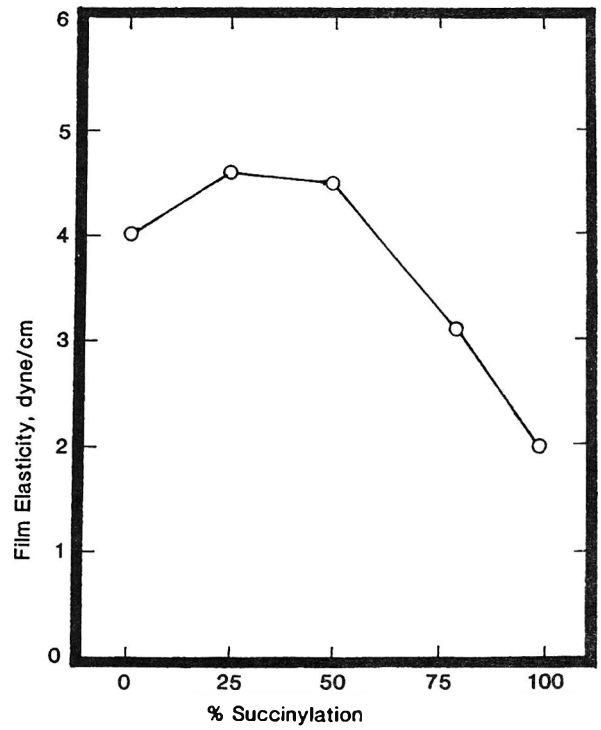

Fig. 3—Effects of increasing succinylation on film elasticity of glycinin. Protein concentration was 0.1% (w/v) in 20 mM phosphate buffer (pH 6).

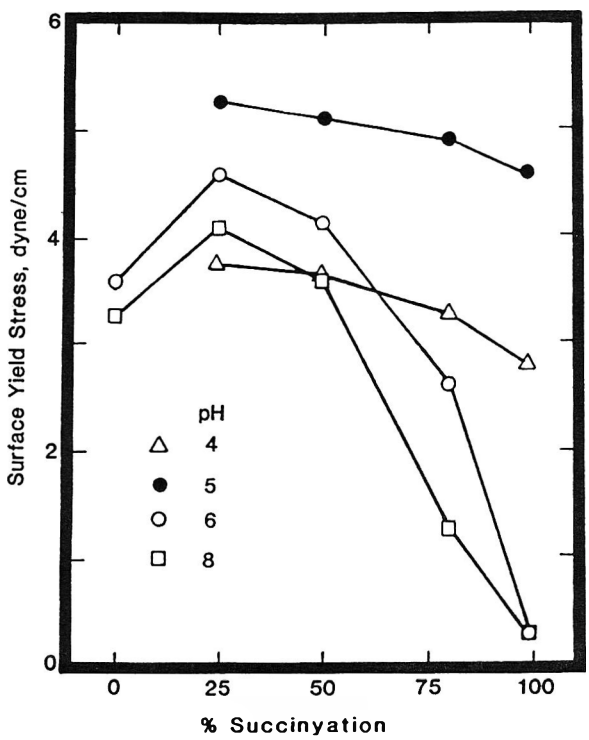

Fig. 2—Effects of increasing succinylation and pH on surface yield stress of native and progressively succinylated glycinin; the concentration was 0.1% (w/v) in 20 mM buffer (citrate, pH 4 and 5; phosphate, pH 6 and 8).

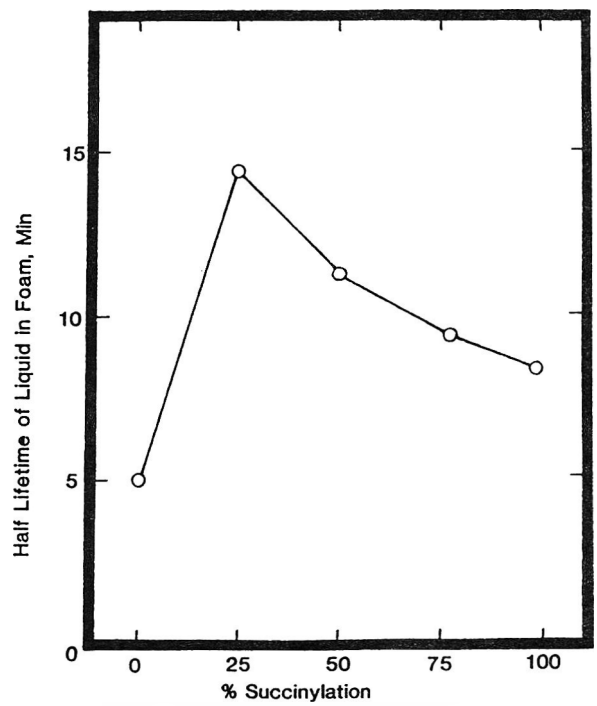

Fig. 4—Effects of increasing succinylation on foam stability of glycinin. Protein concentration was 0.1% (w/v) in 20 mM phosphate buffer (pH 6).

plete dissociation and greatly increasing net negative charge, reduced protein:protein interactions in the interface and inpaired formation of strong films. These observations are in agreement with the criteria established for good foaming proteins (Graham and Phillips, 1979; Halling, 1981; Joly 1972a,b; MacRitchie 1978).

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Inactivation of Soybean Lipoxygenase by Microwave Heating: Effect of Moisture Content and Exposure Time

S.H. WANG and M.C.F. TOLEDO

ABSTRACT

A microwave oven (2450 MHz) was used as heating source to inactivate soybean lipoxygenase. Variables studied were initial moisture level of the soybean and time of heating. After exposure to microwave, the temperature, the moisture loss, the residual lipoxygenase activity and the nitrogen solubility index (NSI) of the soybeans were determined. No linear relationship was observed between lipoxygenase activity and NSI. Complete inactivation of lipoxygenase in soybeans with moisture content between 26.9 and 56.8% was reached after 210 sec of microwave heating, corresponding to NSI between 49.4 and 39.2%, respectively. The beans with their innate moisture (8.7%) had approximately 98% inactivation of lipoxygenase and 56.1% of NSI after 240 sec of exposure.

INTRODUCTION

THE ACTION of lipoxygenase during the rupture of soybeans results in the formation of compounds with objectionable beany flavors, which are responsible for the low acceptability of some soybean products, in special "soymilk" (Wilkens et al., 1967; Mustakas et al., 1969; Khaleque et al., 1970; Kon et al., 1970; Nelson et al., 1971; Ashraf and Snyder, 1981; Rice et al., 1981). Investigators have identified a range of oxidation products of linoleic acid generated by lipoxygenase action, including aldehydes, ketones and alcohols (Goossens, 1974; Huang et al., 1982; Wilkens and Lin, 1970). Most of these products have undesirable odors, especially ethyl vinyl ketone, which, according to Mattick and Hand (1969) has a raw, green bean odor. Therefore, the inactivation of lipoxygenase in the intact soybean to prevent the development of undesirable flavor has received attention in recent years.

The procedures normally proposed to improve soymilk flavor include hot water grinding (Wilkens et al., 1967), bleaching (Nelson et al., 1976) and grinding at low pH followed by cooking (Kon et al., 1970). The products obtained by these processes have a better flavor, but high losses of protein solubility may occur, decreasing the extractibility of the protein in water.

The possibility of using dielectric heating to improve nutritional quality of soybeans has been suggested (Borchers et al., 1972; Wing and Alexander, 1975; Pour-El et al., 1981). Pour-El et al. (1981) showed that dielectric heating at 42 and 2450 MHz is as efficient as commonly practiced moist toasting in improving the biological properties of soybean at natural moisture level. As stated by Minett and Witt (1976) radio frequency 3–300 MHz) and microwave (above 300 MHz) are both produced by electromagnetic radiation and, although not identical, are basically similar in their effects. Under certain circumstances both produce heat uniformily throughout the mass of a suitable electrically nonconducting material, resulting in rapid temperature rises.

The present study was performed to investigate the influence of the moisture content of soybean and the time of microwave heating on the lipoxygenase inactivation and loss of protein solubility of whole soybeans. We have hypothesized that the shorter time needed to generate heat with microwaves, as compared to conventional processes, would decrease the loss of protein solubility, making the soybean appropriate for further milk preparation.

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MATERIAL & METHODS

FIELD GRADE SOYBEANS (1983) of the Sant Rosa variety were provided by the "Instituto Agronômico", Campinas, SP., Brazil. Their composition on a dry basis was: protein-38.9%, fat-22.7%, ash-4.9%, crude fiber-5.8% and carbohydrate-27.7%.

The moisture, protein, fat and ash contents were determined according to the AACC approved methods (1969) 44-31, 46-12, 30-25 and 08-16, respectively. Crude fiber analysis was conducted according to the method of Van de Kamer and Van Ginkel (1952) and carbohydrate level was estimated by difference.

Soybeans with various moisture contents were obtained by soaking the beans in water (soybean: H_2O 1:4 w/v) at 28°C, for 15, 30, 45, and 60 min.

Microwave treatment

Approximately 150g whole soybean were placed in Pyrex petri dishes (15 cm diameter by 2 cm high) and submitted to microwave heating at 2450 MHz for 30, 60, 90, 120, 150, 180, 210, or 240 sec in a domestic Sanyo microwave oven, Model 9003-B. For more uniform heating of the soybeans, each exposure was interrupted at its midpoint for 4–5 sec and the petri dish was rotated around its vertical axis, as suggested by Pour-El et al. (1981).

Soybean temperature and moisture loss

The temperature of the soybeans after microwave heating was determined with three thermocouples type T (copper-constant No 36) inserted in three different points of the sample in the petri dish immediately after exposure, and the temperature read directly from a Kaye Digestrip III Potentiometer. The mean temperature for each exposure time was calculated by averaging the six highest temperatures observed in each sample (duplicate). The moisture determination was performed using approximately 5g soybeans withdrawn from the petri dish and placed in pre-weighed small can (triplicate).

Residual lipoxygenase activity

Immediately after microwave exposure the soybeans were frozen in liquid nitrogen and kept in a freezer at −20°C until analyses were done, in order to lower the temperature of the soybeans quickly and avoid any further effects of the heating. The same nitrogen imersion was done with nonmicrowaved soybeans containing 8.7% moisture, which served as a control.

Both exposed and nonexposed samples were lyophilized, ground, defeated in a Soxhlet apparatus and ground again to about 100 mesh size soybean flour. Approximately 5g flour were extracted with 200 mL water (30°C) in a magnetic shaker for 2 hr and the aqueous extract was used as a source of enzyme. Lipoxygenase activity was determined using linoleic acid as a substrate in borate buffer, pH 9.0 (Surrey, 1964). The residual lipoxygenase activity was calculated based on the enzymatic activity of the control sample as 100%.

Nitrogen solubility index (NSI)

The determination of NSI was performed using the same aqueous extract prepared for lipoxygenase activity determination, according to AACC approved method 46-23 (1969).

Statistical analyses

Two-way ANOVA were done to determine the effect of the exposure time and initial moisture content on the loss of moisture.

RESULTS & DISCUSSION

THE SOYBEANS, after soaking, had moisture contents of 26.9, 38.8, 47.0, and 56.8% (d.w.). These beans and the controls with their natural moisture content (8.7%, d.w.) were used in all experiments.

Temperature elevation and moisture loss

The mean observed temperatures to which soybeans samples were raised by being exposed to microwave during various periods of time are shown in Fig. 1. Only the soybeans at 8.7% moisture continually increased in temperature with increase of time up to 240 sec. At 120 sec, the temperature of the soybeans was about 80°C, which is much lower than 190°C obtained by Pour-El et al. (1981) after heating soybean with 7.6% moisture for the same period of time. Considering that the same microwave frequency was used in both studies, the discrepancy could be due to different operating characteristics of the microwave ovens. The samples with higher moisture contents showed an increase in temperature to about 100°C. This value remained constant for approximately 30 sec and then the temperature started rising again, at a slower rate. This agrees with Sale (1976) that during microwave heating of wet food the temperature rises to 100°C, then the continued generation of heat boils off free water. According to the same author, when the free water is gone there is nothing to hold the temperature at 100°C and the temperature can rise rapidly. The rate of heating was, in general, greater for the samples with higher initial moisture content, although no appreciable differences were observed among the soybeans with moisture contents equal to or greater than 38.8%. This suggests that there may be a critical moisture above which the effects of the increase of moisture upon the dielectric properties of the product may be neglected. However, these relationships are not yet clear and little information is available on such effects. The lower temperatures reached by the wet samples at the end of the microwave treatment (240 sec), as compared to the sample with 8.7% moisture, may be due to the higher water content of those soybeans, which required part of the absorbed energy to evaporate the free water in the soybeans instead of raising the temperature.

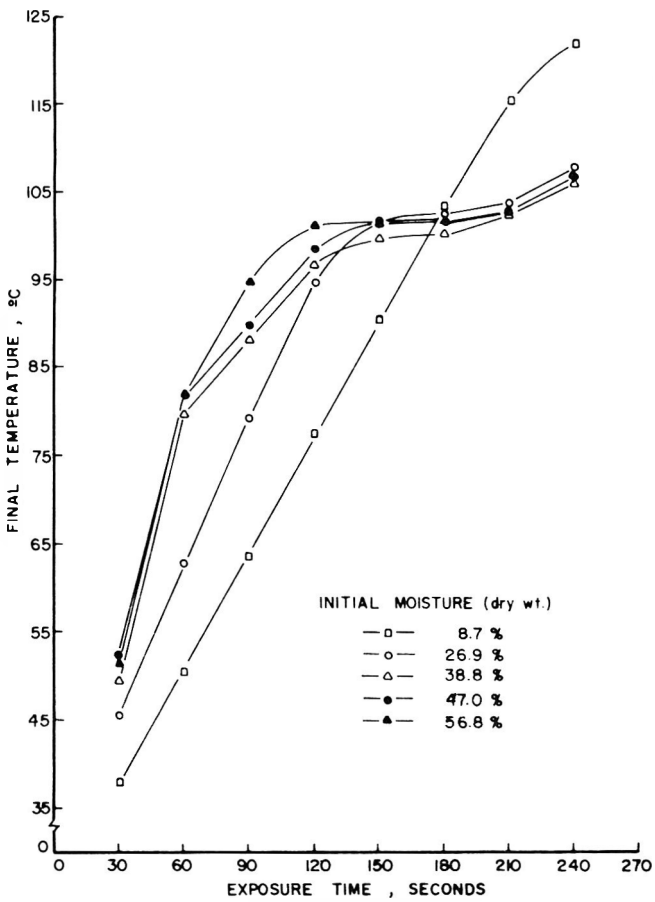

Fig. 1—Temperature of soybeans as a function of exposure time to microwave.

The moisture loss increased with the time of microwave exposure, especially for the samples with higher initial moisture contents (Table 1). According to Maheshwair et al. (1980) the moisture loss of soybeans during microwave treatment can be taken as an index of the rate of microwave heating. Pour-El et al. (1981) preferred to use absorbed energy as an indicator of the heating effect. The same authors considered that energy absorption in the soybeans during dielectric heating is attributable to two principal processes: the elevation of the soybean temperature and the vaporization of water in the soybean. In our study, both moisture loss and temperature elevation were used as indicators of microwave heating. The moisture loss was more representative of the heating, as it increased continuously during the heating process (Table 1). Only the soybeans with natural moisture content presented a linear relationship between moisture loss and temperature elevation during microwave exposure (Fig. 2). The temperature of the wet products remained around 100°C during the evaporation of the free water, as previously discussed (Fig. 2).

This compromise between moisture loss and soybean temperature during microwave heating is important in that both will affect the enzyme inactivation and/or the denaturation of proteins.

Lipoxygenase inactivation

Lipoxygenase activity in all samples was greatly reduced by the microwave treatments (Fig. 3). However, in the first 30 sec of exposure an increase in enzyme activity was observed, especially in the sample with 38.8% moisture. The same enhancement of soybean lipoxygenase activity was found by Pour-

Table 1—Moisture loss from soybeans after exposure to microwave for various periods of time

Initial moisture (%)	Moisture loss (% dw) after different exposure times (sec)[a]							
	30	60	90	120	150	180	210	240
8.7	0 dG	0 cG	0.3 dF	0.5 dF	0.7 dD	1.0 eC	1.2 eB	1.5 eA
26.9	0 dG	1.6 bF	4.0 cE	5.2 cE	8.1 cC	8.4 dC	14.0 dB	15.0 dA
38.8	1.9 bG	5.6 aF	9.3 bE	12.9 bD	13.4 bD	17.3 cC	18.6 cB	20.5 cA
47.0	3.8 aH	5.6 aG	9.5 bF	13.1 bE	14.1 bD	18.3 bC	21.6 bB	23.2 bA
56.8	1.1 cH	5.1 aG	11.1 aF	14.3 aE	16.5 aD	20.9 aC	29.5 aB	32.9 aA

[a] Means followed by different lower case letters in columns and capital letters in rows, differ statistically by the Tukey test at 5% probability level.

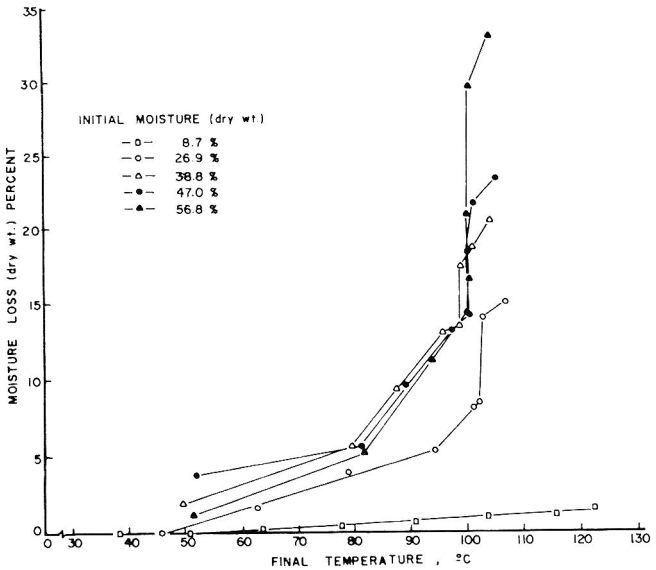

Fig. 2—Moisture loss as a function of the final temperature of soybeans with various moisture levels exposed to microwave.

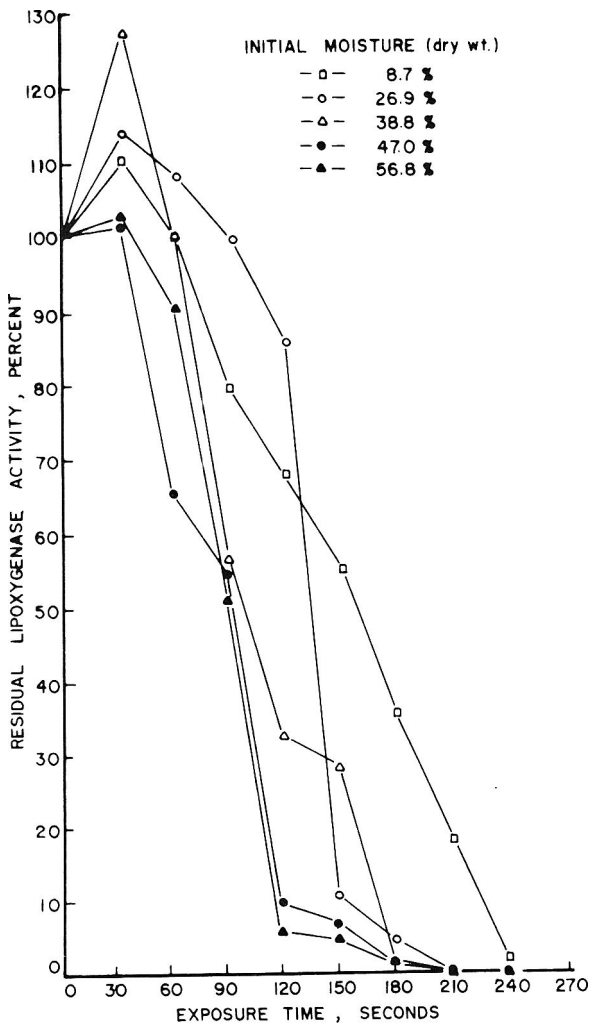

Fig. 3—Residual lipoxygenase activity of soybeans as a function of exposure time to microwave.

El et al. (1981) in the beginning of dielectric heating. This mechanism has not been explained, but we agree with the possibility suggested by Pour-El et al. (1981) that this might

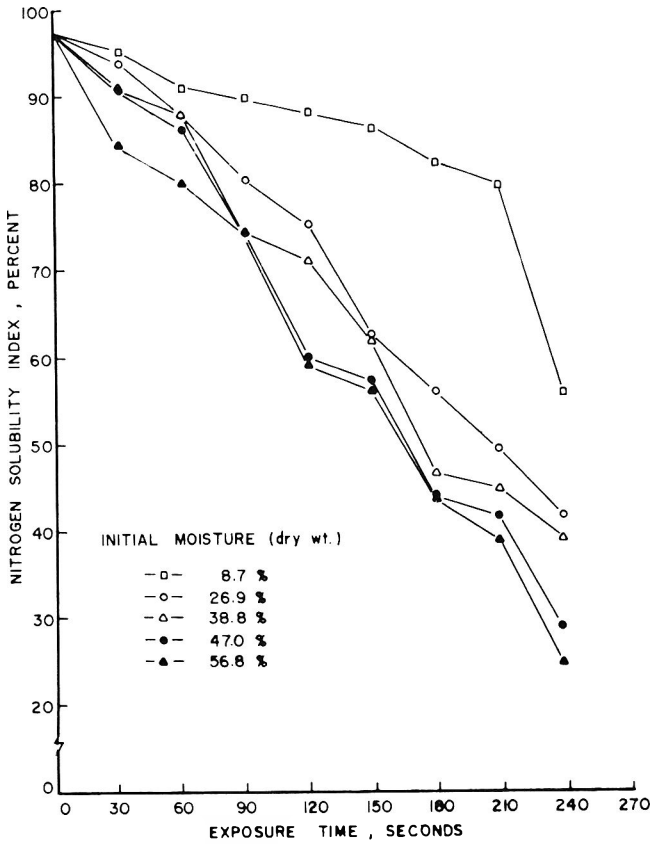

Fig. 4—Nitrogen solubility index of soybeans as a function of exposure time to microwave.

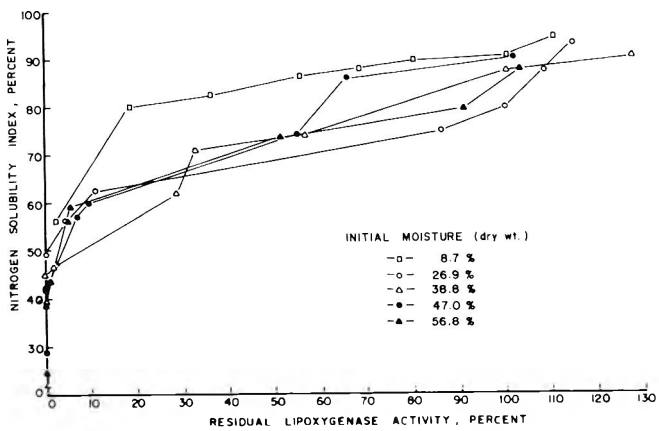

Fig. 5—Relationships between nitrogen solubility index and residual lipoxygenase activity of soybeans exposed to microwave.

result from increased enzyme extractibility during the assay rather than an increase in activity. Lipoxygenase inactivation was favored in the samples that contained higher moisture contents. Small differences, not statistically significant, were observed in the rate of enzyme inactivation among the soybeans with 38.8, 47.0, and 56.8% moisture. These results agree with the similar, greater rate of heating shown by these samples in the first 120 sec of heating (Fig. 1). Although the soybeans at 8.7% moisture reached a temperature of 122.3°C at the end of the microwave treatment (Fig. 1), a residual enzyme activity of 2.3% could be detected at this point (Fig. 3). For the wet samples, complete inactivation of lipoxygenase occurred when the soybean temperature was around 100°C. This confirms that not only is the temperature of the soybean important for enzyme inactivation, but the moisture content of the bean also plays an important role, since it will result in higher energy absorption.

Nitrogen solubility index (NSI)

The decrease in NSI during microwave heating is shown in Figs. 4 and 5 as a function of the exposure time and the residual lipoxygenase activity, respectively. As for the lipoxygenase inactivation, the increase in the soybean moisture and time of heating favored the decrease in NSI. This behavior can also be seen as a consequence of the generation of heat by the wet samples resulting in a higher degree in protein denaturation. No linear relationship was observed between NSI and lipoxygenase activity, although both decreased during the microwave heating. The complete inactivation of lipoxyenase corresponded to NSIs of 49.4, 44.9, 41.8, and 39.2% for soybeans at 26.9, 38.8, 47.0, and 56.8% initial moisture, respectively. The soybeans with natural moisture content had 56.1% NSI for a residual enzyme activity of 2.3%. These NSI values are higher than the ones that have been found in conventional treatment of soybean. Mustakas et al. (1969), applying wet heat, direct steam and dry heat + wet heat to soybeans, found 96.0, 99.2, and 96.0% of lipoxygenase inactivation corresponding to 31, 28 and 14% of NSI, respectively.

These results confirm that during microwave heating the time needed for enzymatic inactivation is shorter, and the retention of protein solubility is higher in comparison with conventional methods. Several investigators consider protein extractibility of the soybeans as a fundamental property for producing an acceptable soymilk (Bourne et al., 1976; Nelson et al., 1976; Smith and Beckel, 1946; Wilkens and Hackler, 1969).

Thus, we can postulate that microwave treatment of soybeans at their natural moisture content (8.7%) could provide suitable material for soymilk processing. Although the use of wet soybeans would require a shorter microwave exposure to inactivate lipoxygenase, the greater decrease in NSI would provide a less acceptable milk.

Studies on the flavor improvement and on the suitability of the microwave-treated soybeans to be used in soymilk production have been conducted in our laboratory. We believe that this method of treatment deserves further investigation to explore its industrial applicability.

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Ms received 8/2/86; revised 2/20/87; accepted 2/27/87.

The authors gratefully acknowledge the financial support from FAPESP.

Barley Neutral Lipid Changes During the Fuel Ethanol Production Process and Product Acceptability from the Dried Distillers Grains

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ABSTRACT

Changes in lipids were traced from the parent barley through the fuel production process to the finished dried distillers grains (DDG). Free fatty acids (FFA) increased from 6% to 22% while the triacylglycerols (TAG) decreased from 73% to 56% during processing. Minor quantities of ethyl esters of fatty acids, not found in the parent barley, were formed during fermentation. The fatty acid profile of the mono- and diacylglycerol and TAG bands remained constant over processing, indicating that these fractions were attacked by nonspecific hydrolysis. During processing, the FFA composition changed with an increase in saturated fatty acids and a decrease in unsaturated fatty acids. Consumer taste panelists preferred commercial and ground barley supplemented granolas over granolas containing either full fat or defatted barley DDG. Trained taste panel results indicated that defatting the DDG did not result in improved product acceptability.

INTRODUCTION

DRIED DISTILLER'S GRAINS (DDG) are by-products of either fuel alcohol production or the brewing/distilling industries. Attempts have been made to incorporate DDG into human foods, particularly baked goods. The maximum amount of brewers DDG which could be incorporated and still yield an acceptable product was 15%-20% flour replacement in cookies (Tsen et al., 1982). Barley DDG from fuel ethanol production are acceptable at much lower levels in baked products (Eidet et al., 1984) due in part to flavor. At least a portion of the flavor problem is associated with the lipid component. Dawson et al. (1984) reported equal consumer panel preferences for oatmeal cookies, containing 15% defatted-barley DDG and standard oatmeal cookies. Thin layer chromatography and gas chromatography indicated that the DDG lipid contained high levels of free fatty acids and reduced amounts of unsaturated fatty acids when compared to literature values of lipid composition reported for barley. The purpose of this experiment was three fold: (a) to confirm changes in the neutral lipid fraction observed by Dawson et al. (1984), (b) to determine the point(s) in the ethanol production process where changes in neutral lipids occurred, and (c) to evaluate the acceptability of DDG in both granola (unleavened) and granola bar (chemically leavened) products.

MATERIALS & METHODS

PIROLINE BARLEY (a two row malting and feed barley) was the feedstock used for alcohol production at a fuel ethanol plant in Montana. Sampling of the barley took place at seven separate stages during processing (Table 1). Two separate fermentations were sampled. Samples were immersed in reagant grade hexane (Baker Chemical, Phillipsburg, NJ) at the fuel ethanol plant and shipped to the laboratory.

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Lipid analysis

Hexane-covered samples were stirred for 15 min by hand and then the hexane extracts were filtered through Whatman GF/A glass fiber filter paper. The lipid in hexane was concentrated under vacuum in a 40°C water bath. The resultant concentrated lipid extracts in hexane were place in individual 4-dram vials, purged with nitrogen and frozen until analyzed.

Analytical thin-layer chromatography (TLC) of the lipid samples and of neutral lipid standards (18-4 Nu-Check Prep, Elysian, MN) were run concurrently on silica TLC plates (0.25 mm thick and activated at 110°C for 4 hr) and developed in hexane/ether/acetic acid (85/15/1, v/v/v) (Walsh et al., 1967). The spots were visualized by spraying lightly with $H_2SO_4 - 5\%$ $K_2Cr_2O_7$ solution and charring at 120°C.

Quantitative preparative silica TLC plates (0.7 mm thick and activated at 110°C for 6 hr) were run in triplicate (25 ± 5mg/plate) for each sample. These TLC plates were developed in the same solvent system as the analytical plates. The bands were visualized by spraying with 0.5% rhodamine 6G in 50% aqueous ethanol and viewing under ultraviolet (UV) light. While under the UV light, five fractions were marked with a scriber. Using a razor blade, each band was thoroughly scraped from the glass plate into a funnel plugged with glass wool covered ca. 0.5cm deep with 100-200 mesh silica gel. The lipid was eluted with diethyl ether into a tared round bottom flask. After removing the ether by vacuum rotary evaporation, the lipid was weighed to nearest mg, transferred in hexane to a vial equipped with a teflon lined cap, blanketed with nitrogen, sealed with teflon tape and stored in the freezer for 1 month.

Analytical TLC plates were run on an aliquot of the fractions to determine the purity of each fraction. The TAG band usually was preparatively rechromatographed on a 0.25mm thick silica plate (Baker Chemical, Phillipsburg, NJ) and treated the same as the thick preparative TLC plates.

Gas chromatography

An aliquot of each lipid fraction was pipetted into a tared vial, dried under nitrogen and reweighed. Each dried lipid fraction was diluted with toluene. FFA were esterified to methyl esters by co-injection with 1 μL (*m*-trifluoromethylphenyl)trimethyl ammonium hydroxide in water (Meth Prep I, Applied Science, State College, PA). Mono- and diacylglycerols and TAG were converted to fatty acid methyl esters by transesterfication with (*m*-trifluoromethylphenyl) trimethyl-

Table 1—Temperature, pH, and time elapsed at the selected sampling stages in fuel alcohol fermentation

Sample number	Temp of Sample (°C)	pH of sample	Time elapsed (hr)
1. Whole grain	NA[a]	NA	0
2. Ground grain	NA	NA	1
Grain and H_2O	60,50[b]	5.4,5.3[b]	8
H_2O, grain and amylase enzyme	84,83	4.9,5.7	12
3. Mashed, cooled grain slurry	33,38	5.2,4.2	24
4. End of 47hr fermentation	36,37	3.5,3.7	71
5. End of distillation	60,62	3.1,3.7	83
6. After centrifugation	43,47	NA	83.2
7. Dried distillers grain[c]	29,32	NA	83.5

[a] Not available.
[b] Temperature and pH of two separate batches at the time of sampling.
[c] Drying temperature 370°C.

Table 2—Product formulations

Ingredient	Product		
	Granola	Granola bar	Oatmeal cookies[a]
Rolled oats(g)	174	–	432
DDG[b], defatted DDG or ground barley(g)	42	–	37.5
All purpose flour(g)	–	250	212.5
Granola[c](g)	–	694	–
Brown sugar(g)	66	424	424
Granulated sugar(g)	–	62	196
Honey(g)	64	–	–
Oil (mL)	75	–	–
Vegetable shortening(g)	–	282	282
Egg(mL)	–	96	96
Water (mL)	–	120	120
Sesame seeds(g)	28	–	–
Vanilla (mL)	2.5	10	10
Baking soda(g)	–	4	4
Soy lecithin(g)	5	–	–
Salt(g)	–	12	12

[a] Dawson et al. (1984).
[b] DDG—dried distillers grain.
[c] One of 4 types—barley, DDG, defatted DDG, or commercial.

ammonium hydroxide in methanol (Meth Prep II Applied Science, State College, PA) added to an aliquot of each fraction. Three injections were made for each FFA and TAG sample. Mono- and diacylglycerols were injected one time.

The methyl/ethyl esters were prepared for GC injection by two different procedures. The first method, to determine if ethyl esters were present, was to dilute the sample (1 mg lipid/100 μL) in toluene and to inject to see if any peaks matched the standard fatty acid methyl esters. The second procedure was to transesterify to methyl esters as used for MAG-DAG and TAG and determine whether modification to fatty acid methyl esters had occurred. The presence of ethyl esters was confirmed by gas chromatography-mass spectrometry on a 30m DB-225 (J&W Scientific, Inc, Folsom, CA) in a Varian 3700 GC (Varian Assoc., Sunnyvale, CA) coupled with a VG-MM16 mass spectrometer (VG Inc. Manchester, England). The temperature was programmed from 100 to 200°C at 5°C/min.

Fatty acid methyl esters were analyzed on a 2m × 5mm ID glass column of 10% DEGS on 80-100 mesh Chromosorb W-AW (Ultra Scientific, Hope, RI). Helium flow was set at 40ml/min, injector temperature at 180°C, and detector temperature at 240°C. All samples were run isothermally at 185°C. The column was mounted in a Varian model 3700 (Varian Instruments, Sunnyvale, CA) gas liquid chromatograph (GLC), equipped with a Hewlett-Packard Model 3380A electronic integrator (Hewlett-Packard, Avondale, PA). Peak identification was determined by using retention time comparison with a known standard ester mixture (Hormel Institute, Austin, MN) containing palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), and linolenic (18:3) methyl esters. The quantity of individual components was expressed as percent total quantity of the five fatty acids recorded on the chromatogram.

Total lipid, protein, neutral detergent fiber, and acid detergent fiber content of the DDG were determined using methods reported by Dawson et al. (1984).

Product ingredient preparation

The DDG was ground using a seed grinder (Lab Construction Co., Kansas City, MO) set at zero. Half the material was reserved for baking while the remainder was defatted with reagent grade hexane (Baker Chemical, Phillipsburg, NJ) using the method of Dawson et al. (1984). Piroline barley, from the same lot used to produce the DDG, was ground in the same manner as for DDG. All materials were placed in freezer bags and held at −20° for 3 wk.

Product preparation

All product formulations are shown in Table 2. The ground, defatted DDG, full fat DDG or Piroline barley made up 7.5% by weight of the granola product while honey and brown sugar, in equal quantities, made up 25% of the product weight. The commercial granola was Nature Valley Granola (General Mills, Inc., Minneapolis, MN).

Granola bars (Table 2) were prepared by adapting the oatmeal cookie formula used by Dawson et al. (1984). The granola bars contained the same weight of sugar (33%), baking soda (0.2%) and slightly more DDG or ground barley (2.4% as opposed to 2%) incorporated by Dawson et al. (1984). All products were placed in freezer bags and stored at −20°C until evaluation. Storage time for granola used in the consumer panel was 3 wk, for trained panels 8 weeks and for granola bars 3 wk.

Sensory testing

The granola was removed from storage and allowed to thaw at room temperature (24°C) in the bags overnight. The ground barley, full fat DDG and defatted DDG granolas were evaluated against a similar commercial product with the same sugar content (Pennington and Church, 1985). For consumer evaluation, the granolas were placed in 30 mL plastic cups on coded trays served buffet style. Untrained panelists (consumers) were asked to rate each sample in the series using a 5 point facial hedonic scale. Room temperature distilled water was provided for drinking.

A total of 163 people participated. The age of the panelists ranged from 3 to 61 yr (mean age was 21.8 yr ± 28.85) with 41% males and 59% females participating. Improperly completed hedonic scales were eliminated from the samples before analysis. Data were analyzed using the Chi Square test and the effect of age and sex on preference was determined. Further statistical analysis included the likelihood ratio test (G^2) using log-linear models (Snedecor and Cochran, 1980).

A trained taste panel consisting of 9 women and 3 men (mean age 25.8 ± 4.52 yr and all nonsmokers) evaluated the 4 granolas and the granola bars using the triangle test and a 9 point hedonic scale. Testing for each product required 2 days. Half the panel received the first set of triangle tests while the remaining panelists received the second set of triangles on day one. On day 2, the testing sets were reversed. Panelists were seated in individual cubicles with 25 watt red lights providing the only illumination. Distilled water was provided for drinking.

Triangle tests results were analyzed using the tables provided by ASTM (1968). Hedonic scores were analyzed using a three way analysis of variance and the least significant difference test (Snedecor and Cochran, 1980).

RESULTS & DISCUSSION

CHEMICAL ANALYSIS of the DDG was as follows: lipid 2.7%, protein 22.6%, neutral detergent fiber 51.5% and acid detergent fiber 20.1%. These values are similar to those obtained by Dawson et al. (1984).

Lipid analysis

The five bands obtained on preparative TLC compared in retention to (1) mono- and diacylglycerols (MAG/DAG-R^f 0–0.06) in a broad band, (2) free fatty acids (FFA-R^f0.06–0.1), (3) triacylglycerols (TAG-R^f0.13–0.30), (4) fatty acid methyl

Table 3—Lipid composition of Piroline barley as it passes through a fuel alcohol fermentation plant (Percent by weight)

No.	Sample	MAG/DAG	FFA	TAG	Fatty acid ethyl esters	Sterol wax esters
1.	Whole grain	7 ± 1[a]	6 ± 2	73 ± 10	–	14 ± 7
2.	Ground grain	8 ± 1	5 ± 1	83 ± 1	–	5 ± 2
3.	Mashed, cooled grain slurry	11 ± 1	43 ± 9	39 ± 2	–	7 ± 3
4.	End of fermentation	14 ± 5	45 ± 5	32 ± 4	8 ± 1	7 ± 2
5.	After distillation	12 ± 1	62 ± 12	19 ± 11	4 ± 0	3 ± 2
6.	After centrifugation	13 ± 4	45 ± 13	33 ± 16	6 ± 1	5 ± 0
7.	Dried distillers grain	12 ± 2	22 ± 2	56 ± 7	8 ± 3	3 ± 2

[a] Means ± standard deviations of two separate fermentations (n = 3 for each fermentation).

Table 4—Triacylglyceride fatty acid composition of Piroline barley as it passes through a fuel alcohol fermentation plant

No.	Sample	Fatty acid % by weight				
		16:0	18:0	18:1	18:2	18:3
1	Whole grain	18 ± 0[a]	2 ± 1	19 ± 0	55 ± 1	5 ± 0
2	Ground grain	19 ± 0	2 ± 0	19 ± 0	55 ± 0	5 ± 0
3	Mashed, cooled grain slurry	19 ± 0	2 ± 0	19 ± 0	56 ± 0	4 ± 0
4	End of fermentation	18 ± 0	2 ± 0	19 ± 0	56 ± 0	4 ± 0
5	After distillation	18 ± 1	2 ± 0	20 ± 1	56 ± 1	4 ± 1
6	After centrifugation	19 ± 0	2 ± 0	20 ± 1	55 ± 0	5 ± 0
7	Dried distillers grain	19 ± 0	2 ± 0	20 ± 0	54 ± 1	6 ± 1

[a] Mean ± standard deviation of two separate fermentations (n = 3 for each fermentation).

Table 5—Free fatty acid composition of Piroline barley as it passes through a fuel alcohol fermentation plant

No.	Sample	Fatty acid % by weight				
		16:0	18:0	18:1	18:2	18:3
1	Whole grain	26 ± 1[a]	4 ± 0	9 ± 1	52 ± 2	9 ± 2
2	Ground grain	23 ± 1	3 ± 1	9 ± 0	59 ± 2	7 ± 1
3	Mashed cooled grain slurry	33 ± 3	2 ± 1	7 ± 1	55 ± 2	4 ± 1
4	End of fermentation	33 ± 1	4 ± 0	9 ± 0	51 ± 0	3 ± 1
5	After distillation	29 ± 4	2 ± 1	8 ± 1	58 ± 1	4 ± 1
6	After centrifugation	32 ± 1	3 ± 1	7 ± 1	54 ± 2	4 ± 1
7	Dried distillers grain	31 ± 1	4 ± 1	9 ± 1	52 ± 1	4 ± 1

[a] Mean ± standard deviation of two separate fermentations (n = 3 for each fermentation).

Table 6—Goodness of fit (G^2) scores of granola ratings for the consumer taste panel

Granola[a]	G^2	Statistical significance
DDG vs COM	5.02	NS[b]
DDG vs DF	6.70	NS
BAR vs COM	3.96	NS
DDG vs BAR	8.11	NS
DF vs BAR	11.62	P≦ 0.02
DF vs COM	12.39	P≦ 0.01
DF + DDG vs BAR + COM	12.89	P≦ 0.01

[a] Granola types as follows: DDG-dried distillers grains, DF – defatted dried distillers grains, BAR - barley, COM - commercial.
[b] NS - nonsignificant.

or ethyl esters (R^f0.30–0.43) and (5) sterol/wax esters (R^f0.43–0.60). Analytical TLC plates indicated that minor quantities of sterols and aliphatic alcohols might be present in the first band, and minor quantities of hydrocarbons might be in the fifth band. These bands were rechromatographed. The fourth band did not show in the unfermented grain samples but was present in measurable quantities after fermentation.

Fatty acid methyl esters and fatty acid ethyl esters had similar mobility in the TLC solvent used. GC analysis of the compounds in band #4 gave six peaks that had retention times slightly longer than the retention times of the common fatty acid methyl esters in a standard. Transesterification of band #4 compounds with Meth Prep II to methyl esters and GC of the product gave peaks with retention times matching with six of the methyl esters of common fatty acids. Comparison of the retention times of methyl esters of common fatty acids and the band #4 compounds indicated that the band #4 compounds were the ethyl esters of the following fatty acids: linoleic (18:2) 54 ± 3%, palmitic (16.0) 30 ± 3%, oleic acid (18:0) 11 ± 5%, linolenic (18.3) 3 ± 1% and stearic (18:0) 2 ± 1%. The identification of ethyl esters was confirmed by gas chromatography/mass spectrometry. These esters may hae been the result of ethylation of free fatty acids or the transesterification of fatty acid glycerol esters from the ethanol in the system. The amount and composition of the fatty acid ethyl esters was relatively constant following their appearance during fermentation.

As the barley lipids passed through the fuel alcohol production process, considerable hydrolysis of esters occurred beginning with the initial grinding procedure when the sterol was esters decreased by 72% (between sample 1 and 2, Table 3). Barley contains a steryl ester hydrolase (Hughs and Goad, 1983) which would have been active following the grinding procedure. The 10% increase in TAG following grinding was not due to synthesis but was a proportional increase resulting from the loss of the sterol wax esters.

Hydrolysis of glycerol esters occurred in the wetting and alpha-amylase digestion stage (between sample 2 and 3, Table 3) and during distillation (between samples 4 and 5, Table 3). The TAG value dropped to about half the original percentage during mashing and to about one quarter the original during distillation with a corresponding increase in free fatty acid level. Fatty acid analysis of TAG (Table 4) indicated that the fatty acid composition of the TAG fraction did not change appreciably during processing. The decrease in TAG levels was apparently the result of random hydrolysis. The ester linkages of grain lipids are subject to hydrolysis resulting from enzymes, thermal stress, or chemical action (Galliard, 1983).

Although barley contains lipases, it is doubtful that enzymatic hydrolysis played a prominent role as the optimum pH and temperature for this reaction 7.5 and 45–48°C, respectively, (Rinke, 1964) were exceeded early in processing (Table 1). Further, Rinke (1964) reported that barley lipase activity was inhibited at moistures exceeding 40%. Thus any hydrol-

Table 7—Mean hedonic scores and distribution of granola ratings for consumer taste panel

Granola	Rating distribution (%)[a]			Mean	Standard deviation
	Above average[b]	Average	Below average[c]		
Commercial	69	21	10	3.8	± 1.1
w/Ground barley	68	16	16	3.9	± 1.4
w/Dried distillers grains	58	28	14	3.9	± 1.2
w/Defatted dried distillers grains	57	25	18	3.7	± 1.2

[a] Statistically significant at p≤ 0.02.
[b] Above average—hedonic score above 3.
[c] Below average—hedonic score below 3.

Table 8—Triangle test results for evaluation of granola and granola bars

Pair	Granola	Granola bars
w/Ground Barley vs w/Defatted DDG[a]	N.S.[b]	P≦0.05
w/Ground barley vs w/DDG	N.S.	N.S.
w/Ground barley vs Commercial	P≦0.001	P≦0.05
Commercial vs w/Defatted DDG	P≦0.001	P≦0.05
Commercial vs w/DDG	P≦0.001	P≦0.01
w/DDG vs w/Defatted DDG	N.S.	N.S.

[a] Dried distiller's grains.
[b] Nonsignificant.

Table 9—Analysis of variance for trained taste panel hedonic scores for granola

Source	DF	SS	MS	F Value
Replicates	1	8.8	8.8	6.8*
Sampler	3	71.6	23.9	18.5**
Judges	11	126.6	115.0	8.9**
Judge & Sample	33	70.8	2.1	1.7*
Error	47	60.7	1.3	

* Statistical significance at P≦0.05
** Statistical significance at P≦0.001

Table 10—Analysis of variance for trained taste panel hedonic scores for granola bars

Source	DF	SS	MS	F Value
Replicates	1	9.4	9.4	7.2*
Samples	3	13.1	24.4	18.6**
Judges	11	130.2	11.8	9.0**
Judge & Sample	33	69.7	2.1	1.6
Error	57	61.6	1.3	

* Statistical significance at P≦0.05 level
** Statistical significance at P≦0.001.

Table 11—Analysis of trained taste panel mean hedonic scores for granola and granola bars using the least significant difference test

	Hedonic scores	
	Granola	abGranola bars
Samples	Mean ± S.D.	Mean ± S.D.
Commercial	6.7[a] ± 1.2	6.7[c] ± 1.2
w/Ground barley	5.1[b] ± 1.6	5.1[d] ± 1.6
w/DDG	4.7[b] ± 1.4	4.7[d] ± 1.4
w/Defatted DDG	4.5[b] ± 1.8	4.5[d] ± 1.8

[a,b] Granola samples with different superscripts are significantly different at P≦ 0.01 level.
[c,d] Granola bar samples with different superscripts are significantly different at P≦0.01 level.

ysis due to barley lipase would be confined to the grinding step and the short time period when the moisture in the mashing vat was below 40%.

Hydrolysis of TAG by yeast cells was unlikely (Taylor et al., 1979). They found that the TAG fraction of spent grains was not utilized by yeasts during fermentation.

As indicated in Table 1, acidic conditions were present early in processing and combined with heat available would have favored a nonspecific acid hydrolysis of lipid during the prolonged mashing and fermentation period and the shorter, higher temperature distillation period.

The moderate increase in the MAG/DAG band combined with the raise in the level of FFA indicated that hydrolysis of TAG tended to go to nearly completion once it started (Table 3). As with the TAG fraction, the MAG/DAG fatty acid profile was very stable throughout processing and very similar to the composition of the TAG band. Mean fatty acid composition of MAG/DAG over all processing stages was as follows: Palmitic acid (16:0) 22 ± 3%, stearic acid (18:0) 2 ± 1%, oleic acid (18:1) 14 ± 1%, linoleic acid (18:2) 58 ± 1% and linolenic acid (18:3) 4 ± 1%.

The level of FFA rose during mashing from 5% to 43% and achieved a maximum level of 62% during distillation and then fell through centrifugation and drying to 22% in the DDG (Table 2). The FFA profile, unlike the other fractions was not stable (Table 4). Palmitic acid (16.0) levels rose during processing leading to a more saturated fatty acid DDG than the original barley. Changes in FFA composition may have been due to selective destruction of the unsaturated FFA. This would explain why the fatty acid content of the FFA fraction (Table 5) differs from the fatty acid composition of the TAG fraction (Table 4).

Linoleic acid (18:2) rose, probably as a result of TAG hydrolysis following grinding and distillation and then dropped during mashing, fermentation and drying. Linolenic acid (18:3) fell chiefly as a result of grinding and mashing. The drop in linolenic acid was not matched by a drop in linoleic acid during grinding but both fell as a result of mashing. Barley lipoxygenase will hydrolyze linolenic acid but the preferred substrate is linoleic acid and given its instability to temperatures above 50°C (Lulai and Baker, 1976), it would have been inactivated between stages 2 and 3 (Table 1). Thus enzymatic hydrolysis of linolenic acid during mashing by lipoxygenase is unlikely.

Chemical oxidation of linoleic and linolenic acids would have been favored by the use of mild steel tanks during mashing and fermentation. Heavy metals such as iron are effective catalysts of both the initiation and secondary reactions of lipid oxidation (Lillard, 1978). Another possibility would be that the unsaturated fatty acids were utilized by yeast during fermentation (Taylor et al., 1979).

Centrifugation (between samples 5–6, Table 3) of the solids from the slurry resulted in a decrease in FFA. This is likely due to partial solubility of the FFA as salts but not the other neutral lipids in the water layer. The water layer was not analysed for lipids since we were interested in the lipids of the DDG product.

Drying also resulted in a drop in FFA (Table 3) but as the qualitative analysis of FFA and TAG indicated (Tables 4 and 5), there were no selective changes in fatty acid composition for either fraction between stages 6 and 7. The drying drum reached temperatures in the area of 400°C which is sufficient to volatilize free fatty acids (Nawar, 1985). The increase in TAG during stages 6 and 7 was not due to synthesis but was a proportional increase resulting from the loss of FFA.

A Chi square value of 23.10 for consumer taste panel scores indicated that granola type was significant at $p \leq 0.02$ while age and sex proved to be non-significant factors. Taste panel score distributions did not differ between defatted DDG and DDG granolas or between commercial and ground barley granolas but were statistically significantly different ($p \leq 0.01$) when commercial and ground barley granolas combined were compared to defatted DDG and DDG granolas combined (Table 6). As indicated by goodness of fit statistical analysis (G^2), the difference in hedonic rating distribution pattern was significant at $p \leq 0.02$ (Table 7). More panelists rated commercial and ground barley granolas above 3 (neutral) than rated DDG or defatted DDG above 3. As indicated in Table 7, there was little apparent difference in the means and standard deviations for these samples. The low and high scores tended to cancel each other and obscured the differences between samples.

The trained taste panel was able to differentiate between the commercial granola (sampled as granola or granola bars) and any other granola (Table 8). The level of statistical significances was higher ($p \leq 0.001$) for granola than for granola bars where it ranged from $p \leq 0.05$ to $p \leq 0.01$. This decrease in statistical significance may be a reflection of the lower concentration of DDG and ground barley in the granola bars (granola 7.5%, granola bars 2.4% by weight) or the higher concentration of sugar (granola bars 33%, granola 25% by weight).

The panel was unable to differentiate between granolas containing ground barley, defatted DDG or DDG. They were able to differentiate between ground barley and defatted DDG granola bars ($p \leq 0.05$). Taste panel comments indicated that some panelists thought that the defatted DDG granola bars were slightly crunchier than the more chewy ground barley granola bars.

Defatting could have removed some phospholipid resulting in a slightly different product texture. Panelists were again unable to differentiate between DDG and ground barley granola bars and between defatted DDG and DDG granola bars. Taste panel comments indicated that the defatted DDG and particularly the DDG samples were thought to be slightly bitter.

There was a difference ($p \leq 0.001$) in hedonic scores for both granola and granola bars (Tables 9 and 10). Hedonic means for granola and granola bars were identical (Table 11). The least significant difference test results indicated that commercial granola and granola bars were preferred over all other granolas ($p \leq 0.01$) and granola bars ($p \leq 0.01$).

Defatting the DDG did not result in improved taste panel scores. Dawson et al. (1984) found that DDG with 3% neutral lipid and 78% of neutral lipid as FFA could be made more acceptable by defatting. They postulated that high FFA levels resulted in off-flavor through the interaction of FFA with baking soda. The DDG tested in the baking soda leavened granola bars contained 2.7% neutral lipid and 22% of the neutral lipid as FFA. The DDG used in the granola bars has an FFA concentration of 0.6g/100g DDG as opposed to an FFA concentration of 2.3g/100g DDG used by Dawson et al. (1984). DDG used in this study apparently did not have a high enough concentration of FFA to affect product acceptability and defatting, therefore, did not improve product acceptability.

CONCLUSIONS

THE LIPIDS of Piroline barley underwent dramatic changes during fermentation of the carbohydrate to ethanol and subsequent separation of the ethanol and DDG. Free fatty acids were released from TAG and possibly other fractions and some of the FFA fraction appeared to be destroyed in the later stages of drying of DDG. A minor amount of fatty acid ethyl esters was formed during the fermentation process and appeared in the DDG. Changes in lipid composition did not affect the acceptability of the DDG in the products tested.

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Ms received 11/10/86; revised 2/17/87; accepted 4/10/87.

Submitted in partial fulfillment of requirements for a Master of Science Degree. The authors express their appreciation to Larry Blackwood for statistical assistance and to Jeanne Canavan and Lynn Gallager for help with product preparation.

Funded in part by the Montana Wheat Research and Marketing Committee. Montana Agricultural Experiment Station Journal Series Number J-1779.

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Ms received 12/29/86; revised 4/24/87; accepted 5/2/87.

Support of NSF grant #CBT-8506243 and a special award from General Foods Foundation to J.E. Kinsella are gratefully acknowledged.

Canola Phytase: Isolation and Characterization

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ABSTRACT

Two phytase isoenzymes were isolated from 8-day germinated canola cv Regent. Gel filtration chromatography of an ammonium sulfate fractionated extract on Sephadex G-100 produced one peak with phytase activity. The phytase fraction was separated into two isoenzymes by DEAE-cellulose chromatography. The optimum pH was 4.5–5.0 and 5.0 for the phytase isoenzymes 1 and 2, respectively. Both isoenzymes exhibited maximum activity at 50°C. Km values at pH 5.0 were 0.36 and 0.25 mM for phytase 1 and 2 isoenzymes, respectively, while molecular weight determination showed both fraction were identical with a molecular weight of 70,100 ± 4,000 daltons.

INTRODUCTION

PHYTIC ACID is the major storage form of phosphorus in mature grains and legumes. When present in the diet, it is known to complex divalent metal ions such as calcium, zinc, magnesium and iron rendering them nutritionally unavailable (Atwal et al., 1980; Davies and Nightingale, 1975; Forbes and Parker, 1977). The presence of high levels of phytic acid in canola seeds, a major oilseed crop in Canada, remains a problem for the use of the meal in human nutrition (Latta and Eskin, 1980). One way of reducing phytic acid is by phytase (myoinositol hexaphosphate phosphohydrolase, EC 3.1.3.8) which hydrolyses phytate to inositol and inorganic phosphorus. Phytase has been reported in a number of seeds including soybeans, dwarf beans, lettuce seeds, triticale, wheat and fababeans (Peers, 1953: Gibbins and Norris, 1963: Mandal et al., 1972; Mayer et al., 1961; Singh and Sedeh, 1979; Eskin and Wiebe, 1983). A recent study of phytase in six canola cultivars licensed in Western Canada indicated that the canola cultivar summer rapeseed (*Brassica napus*) Regent developed the most active phytase during germination (Lu et al., 1987). This study reports a more detailed examination and characterization of phytase in the canola cultivar Regent.

MATERIALS & METHODS

Sample preparation

Seeds of canola cultivar summer rapeseed (Brassica napus) Regent were provided by Dr. B. Stefannson, Dept. of Plant Science, Univ. of Manitoba. The dry seeds were sterilized in a 2% sodium hypochlorite solution for 15 min and then rinsed with distilled water for 30 min. The seeds were germinated at room temperature (20°C) and 65% relative humidity for 8 days, freeze-dried, and ground in a Wiley Mill using a 30-mesh screen.

Enzyme assay

Phytase activity was measured following the procedure of Lolas and Markarkis (1977). The enzyme was incubated at 50°C for 30 min with sodium phytate in sodium acetate buffer at pH 5.0. The reaction was terminated with 1.0 mL cold trichloroacetic acid (1.54M). The supernatant was determined for inorganic phosphorus (Pi) using the method of Chen et al. (1956). Phytase activity was expressed as release of μg Pi/mg protein/30 min and values were corrected against a control with boiled enzyme.

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Protein determination

Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. During the separation of phytase by column chromatography protein was monitored by following the absorbance at 280 nm.

Enzyme extraction

Phytase was extracted from canola seeds according to the procedure of Goel and Sharma (1979). Twenty grams germinated seeds were defatted by stirring with 200 mL of cold hexane in an ice bath with a magnetic stirrer for 30 min. The mixture was then filtered with suction and the meal was treated twice more with cold hexane and freeze-dried for 2 hr. The dry meal was then extracted with 0.1M sodium acetate buffer (pH 5.0) for 18 hr. After centrifugation for 1 hr at 10,000 × *g* at 2°C the supernatant was collected.

Partial purification

The first step involved treating the supernatant with polyvinyl pyrrolidone (PVP) for 2 min to remove interfering polyphenols and filtering through glass wool. The PVP-treated extract was then subjected to ammonium sulfate fractionation; the fraction between 40–70% saturation was dissolved in a minimum volume of 0.05M sodium acetate buffer (pH 5.0) and dialysed against the same buffer for 18 hr. The dialysed enzyme extract was then centrifuged for 30 min at 20,000 × *g* to remove any inactive protein precipitates.

Column chromatography

The dialysed enzyme extract was applied to the bottom of a Sephadex G-1000 column (1.5 × 86 cm) equilibrated with 0.05M sodium acetate buffer (20 mM NaCl, pH 5.0). The enzyme extract was eluted with 0.05M sodium acetate buffer (20 mM NaCl, pH 5.0) using an upward flow rate of 10 mL/hr and 3 mL fractions were collected. Aliquots of each fraction were assayed for both phytase activity and protein. Those fractions containing enzyme activity were pooled and concentrated by ultrafiltration on PM 10 membrane (Amicon). The concentrated enzyme fraction was then precipitated by ammonium sulfate at 100% saturation and centrifuged for 1 hr at 20.000 × *g*. The precipitate was then dissolved in 25 mM Tris-HCl buffer (pH 8.5) and dialysed for 18 hr against the same buffer in the cold room (4°C). The dialysed enzyme fraction was further concentrated by ultrafiltration.

The enzyme fraction obtained from gelfiltration was then applied to a DEAE-cellulose column (1.5 × 43 cm) equilibrated with 25 mM Tris-HCl buffer (pH 8.5). The column was washed with 200 mL of the same buffer to remove unabsorbed protein. The absorbed proteins were sequentially eluted with a linear gradient consisting of 0–0.15M NaCl in 25 mM Tris-HCl buffer (pH 8.5), and 0.15–0.5M NaCl in the same buffer. Five milliliter fractions were collected at a flow rate of 30 mL/hr. and aliquots from each fraction were analyzed for both phytase activity and protein.

Conductivity measurements were made at room temperature on every fifth fraction using a YSI model conductivity Bridge (Yellow Springs Instrument Co.) equipped with a type CDC 314 Conductivity probe (Radiometer).

Kinetic studies

Phytase fractions eluted from the DEAE-cellulose column were used for kinetic studies. The effect of pH, temperature, incubation time and substrate concentration were studied. Km and V_{max} were obtained from the Lineweaver-Burk plots using 8 substrate concentrations.

Molecular weight determination

Molecular weight of phytase was estimated by gel filtration on a Sephadex G-100 column (1.5 × 86 cm) at pH 5.0 (0.05M sodium acetate buffer, 20 mM NaCl, 4°C). Cytochrome C (MW 12,400 daltons), carbonic anhydrase (MW 29,000 daltons) and bovine serum albumin (MW 66,000 daltons) were used as calibration proteins. Blue dextran 2000 (MW 2 × 10^6 daltons) was used to measure the void volume of the column.

RESULTS & DISCUSSION

THE PURIFICATION of canola phytase is summarized in Table 1. Treatment with PVP to remove any interfering phenols resulted to negligible improvement in purification. Gel filtration of this fraction on Sephadex G-100 revealed a single peak with phytase activity (Fig. 1). Further purification by DEAE-cellulose column chromatography separated phytase into distinct fractions referred to as phytase 1 and phytase 2, respectively (Fig. 2). These peaks appeared at conductivity between 3500 and 13,000 μmho corresponding to a salt gradient of 50–220 mM NaCl. The peak at 60 mM NaCl (phytase 1) accounted for 92% of the total enzyme activity obtained by separation on the DEAE-cellulose column. The second peak at 130 mM NaCl (phytase 2) accounted for only 8% of the total activity. The overall purification was substantially lower compared to gel filtration reduced to a factor of 9.8 and 4.9-fold for phytase 1 and phytase 2, respectively. It is evident that considerable loss of enzyme activity occurred during ion exchange chromatography.

Table 1—Purification of phytase from canola seeds

Fraction	Total[a] phytase activity (μmole)	Total protein (mg)	Specific activity (μ mole/mg)	Purification (fold)	Recovery (%)
Hexane	1562.7	832.5	1.9	1.0	100
PVP	1515.0	719.8	2.1	1.1	96.9
Ammonium Sulfate (40–70%)	1269.8	103.8	12.2	6.4	81.3
Gel-filtration	1159.8	35.8	32.4	17.1	74.2
DEAE-Cellulose					
Phytase 1	481.3	25.7	18.7	9.8	30.8
Phytase 2	42.3	4.5	9.4	4.9	2.7

[a] Phytase activity is expressed as μmoles Pi released/30 min.

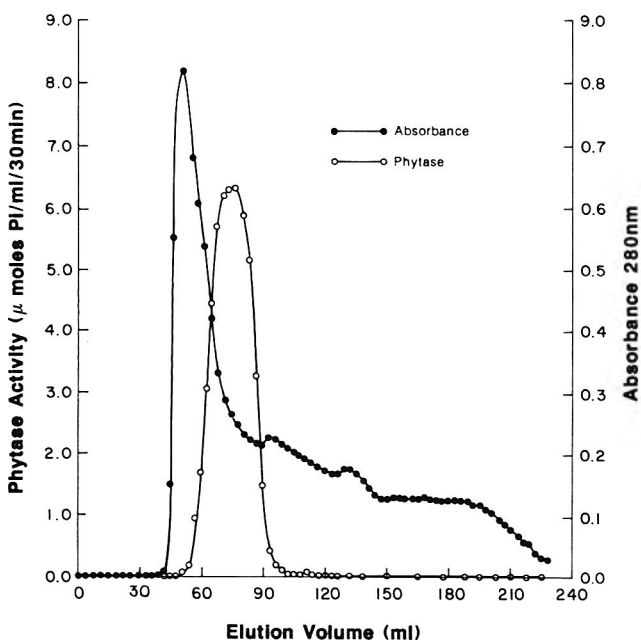

Fig. 1—Gel filtration chromatography on Sephadex G-100 of $(NH_4)_2SO_4$ fraction.

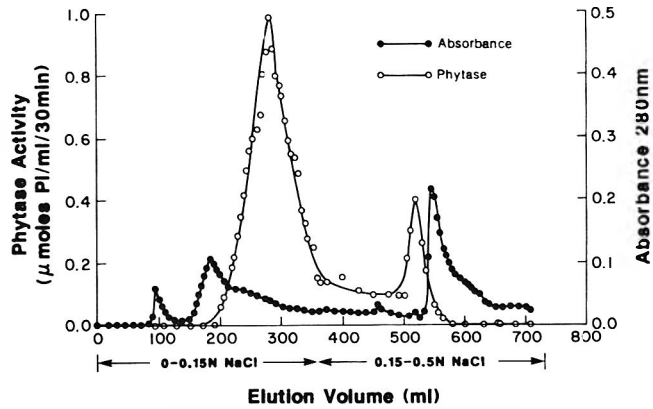

Fig. 2—DEAE-cellulose chromatography of the phytase fraction obtained from the Sephadex column.

Phytase fractions eluted from the DEAE-cellulose column exhibited the following properties. The pH optima were found to be 4.5–5.0 and 5.0 for phytases 1 and 2, respectively. These values were within the reported range (4.6–5.6) (Change, 1967; Chang and Schwimmer, 1977; Gibbins and Norris, 1963; Lolas and Markarkis, 1977; Nayini and Markakis, 1984; Wang et al., 1980). The optimum temperature for both phytase fractions was 50°C. This is similar to that reported for Navy bean phytase (Lolas and Markakis, 1977), corn seed phytase (Chang, 1967) and phytase from *Aspergillus oryzae* (Wang et al., 1980). Above 50°C the canola phytase 1 activity was reduced by 56 and 96% at 60° and 70°C, respectively. Phytase 2 appeared to be somewhat more heat stable exhibiting a decrease in activity of 33 and 65% at 60° and 70°C compared to phytase 1.

The K_m values for phytase 1 and 2 were 0.36 and 0.25 mM, respectively. This compared to 0.22 mM for phytase from small white beans (Chang and Schwimmer, 1977) and 0.21 mM for yeast phytase (Nayini and Markakis, 1984), 0.33 mM for wheat phytase (Peers, 1953) and 0.4 mM for mung bean phytase (Maiti et al., 1974). Phytase 1 and 2 showed V_{max} (μ moles/Pi/mg protein/30 min.) of 8.76 and 2.64, respectively. Phytase 1 activity increased with increasing substrate concentration reaching a maximum in the presence of 0.8 mM. Inhibition of phytase 1 activity was observed above a substrate concentration of 1.0 mM. In the case of phytase 2, however, no inhibition was evident in the presence of up to 3.0 mM substrate concentration. Complete or partial inhibition of phytase by substrate has been reported by other researchers (Chang and Schwimmer, 1977; Gibbins and Norris, 1963; Lolas and Markakis, 1977).

Molecular weight determination of phytase 1 and 2 showed both fractions were identical with molecular weight of 70,100 ± 4,000 daltons. This compared to 66,500 ± 4,000 daltons for phytase isolated from the germinated cotyledons of pumpkin seeds (Goel and Sharma, 1979) and 47,000 ± 2,000 daltons for phytase from wheat bran (Lim and Tate, 1973).

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Optimization of Extrusion Rice Bran Stabilization Process

C. J. KIM, S. M. BYUN, H. S. CHEIGH, and T. W. KWON

ABSTRACT

Effects of processing variables, production rate, number of die openings and initial moisture content on the discharge temperature and power input during rice bran extrusion with an autogenous single-screw extruder were analyzed. At extrusion temperatures above 128°C, all lipase activity in the bran was lost regardless of moisture content of the bran fed. Net specific energy input (NSE) varied with changes in die opening and moisture contents and total power efficiency was increased with increasing production rate without change in NSE value. Percolation rate through the extruded bran bed was increased to nine times over that of raw bran; the extraction time to reach 1% residual oil in extruded bran was reduced to 10 min from 100 min for the raw bran.

INTRODUCTION

RICE BRAN, a by-product of rice milling containing 15-22% oil, can serve as a potential edible oil source in rice producing countries. Unfortunately, its utilization is hampered seriously because, upon milling, neutral oil is rapidly hydrolyzed into free fatty acids (FFA) by the action of lipases in the bran. Inactivation of lipase or stabilization of bran immediately after milling is required. Once the bran is stabilized, it can be transported and stored for 30-60 days at ambient conditions without an appreciable increase in FFA content.

Various extrusion cookers have been developed and tested for stabilization (Enochian et al., 1981; Williams and Bear, 1965; Williamson, 1976; Viraktamath and Desikchar, 1971; Randall et al., 1985). Since rural rice mills run at small capacity and extraction of oil from rice bran after milling to obtain foodgrade rice oil in developing countries is impossible, extrusion machines of large capacity have a disadvantage on an economic and practical basis. Previously, two of the authors developed a small scale (400 kg/hr) low-cost extrusion cooker (LEC) of a single-screw design for stabilization of fresh milled rice bran in rural rice mills (Cheigh and Kim, 1984). Its chief advantages are very low capital investment, simplicity of design and operation, low maintenance, and inexpensive operation.

Fluctuation of moisture contents in raw bran causes different operational discharge temperature of the extrusion cooker during stabilization. This significantly affects the inactivation of lipases in the bran (Randall et al., 1985; Sayre et al., 1982). The machine used in this study can be used with all kinds of rice bran without any adjustment of moisture before stabilization. The present study was conducted to examine in detail the operational conditions necessary to stabilize the fresh rice bran having various moisture contents with our lowcost extrusion cooker.

MATERIALS & METHODS

Materials

Fresh rice bran, of the "Yushin" variety, a Japonica type brown rice, was collected at the cyclone separator from the bran millstream at a local rice mill, packed in a hemp bag and kept in a cold room (−20°C) until processed. The proximate composition of the rice bran was found to be 9.5% moisture, 20.1% crude oil (ether extract), 11.3% crude protein (N x 6.25), 41.3% soluble carbohydrate (NSE), 8.4% crude fiber, 9.4% ash and 4.5% FFA by determination with AOAC methods.

Extrusion stabilizer

The rice bran extrusion stabilizer, Model K 100303, Dai-Poong Co. Ltd. (Seoul, Korea) is equipped with a 30 HP electric motor and consists of a 100 mm diam. barrel equipped with 4 straight grooves, a single flighted screw with uniform pitch of 70 mm and flight height of 6.5 mm, and a flat multihole-die plate with 12 round die openings (3 mm diam x 25 mm length). The extrusion screw rotates at a fixed speed of 800 rpm. A feed screw having continuous variable speed capability of 0-150 rpm was arranged horizontally at the bottom of the feed hopper and perpendicular to and above the main screw. With this arrangement, the bran in the feed hopper can be fed into the extrusion screw in a controlled manner (Cheigh and Kim, 1984).

Extrusion stabilization

The theoretical conveying capacity of the extrusion screw used was 98.6 cc per revolution according to the solid-conveying theory of Chung (1971). The extrusion stabilizer has a maximum capacity of 1,600 kg/hr raw rice bran at open discharge. As expected, a reduction of the production rate was observed when the number of die openings was reduced. This reduction is due to the increasing pressure required to force the bran through the greater physical restriction, which increases the negative pressure flow in the screw channel. Therefore, the feed rate was controlled at values less than the maximum theoretical rate by a variable speed feed screw.

This study was a 3 x 3 x 3 full factorial design. The ranges of process parameters were (a) moisture level of feed bran — 9.5, 11.0, 15.0% wet basis; (b) number of die openings — 3, 6, 12; (c) production rate — 100, 200, 400 kg/hr. The desired moisture level was achieved by spraying water as a fine mist onto the rice bran in a Hobart mixer. Moistened bran was held overnight at 4°C to assume to equilibrium. The extruder was started with the 12-die opening configuration and a 100 kg/hr production rate and then changed to the desired operation condition by adjusting the number of die openings

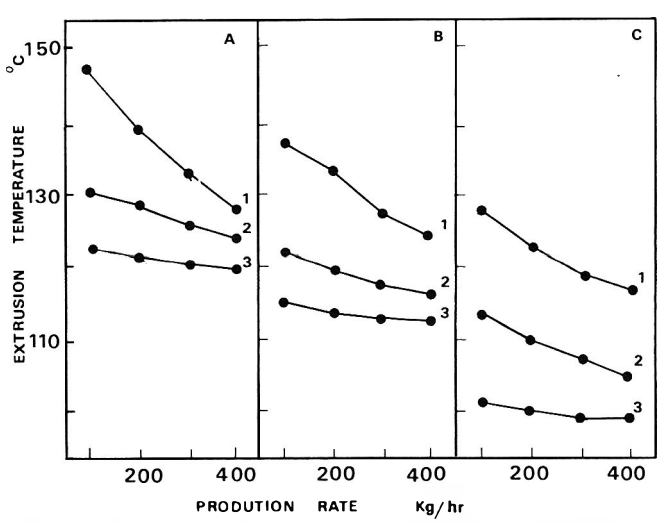

Fig. 1—Changes of discharge temperature as a function of production rate. Brans extruded at moisture level of 9.5 (A), 11.0 (B) and 15.0% (C) and at 3 (1), 6 (2), and 12 die opening (3).

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Table 1—Specific energy input and energy dissipated as heat during extrusion of rice bran

Mechanical conditions		Moisture content of bran fed (%)								
		9.5			11.0			15.0		
No. die openings	Product on rate (kg/hr)	SE	NSE	EDH	SE	NSE	EDH	SE	NSE	EDH
3	100	429.5	222.1	208.8	425.1	205.0	195.5	399.2	193.4	180.1
3	200	326.3	222.1	193.9	314.4	205.0	190.3	303.3	193.4	175.0
3	400	273.7	222.1	175.6	267.1	205.0	175.0	248.4	193.4	161.4
6	100	408.2	201.8	179.0	394.2	188.6	166.9	378.4	178.4	156.3
6	200	309.1	201.8	173.3	296.5	188.6	166.5	289.3	178.4	149.5
6	400	253.1	201.8	169.0	235.0	188.6	159.7	224.4	178.4	144.4
12	100	394.2	190.7	165.7	382.7	179.7	158.0	373.0	166.9	135.9
12	200	293.6	190.7	164.0	282.1	179.7	156.3	271.3	166.9	134.2
12	400	242.0	190.7	160.7	231.0	179.7	154.6	218.4	166.9	132.5

[a] Specific energy input (kJ/kg. was calculated by dividing the total energy input by the production rate.
[b] Net specific energy input was obtained from the slope of the curve of total energy input as function of production rate (Fig. 2).
[c] The amount of energy dissipated as heat (kJ·kg) was calculated by multiplying the temperature increase by the specific heat capacity from the data of Narain (1978).

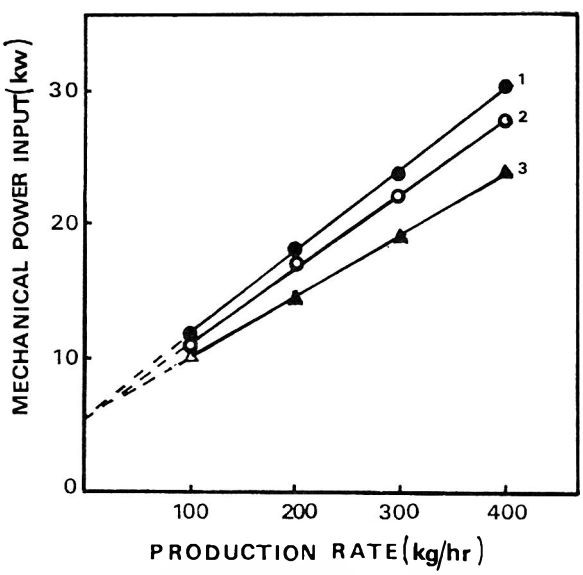

Fig. 2—Relation between production rate and total power input. Extruded at 9.5% moisture and 3-die opening (1), 11.0% moisture and 6-die opening (2), and 15.0% moisture and 12-die opening (3).

and the feed screw speed. Extruded hot bran was delivered onto the inclined sieve plate-air cooler where ambient air was blown over the bran as it tumbled through the sieve plate.

The extruder was allowed to reach steady state before samples and data were collected. Although the raw bran has a relatively high oil content (about 20%), no surging occurred within the range of these operating conditions. Attainment of steady state was declared when a variation of <1% of the extrusion temperature and mechanical energy input occurred after a 20 min period. Extrusion temperature was measured with J-thermocouple in direct contact with the extrudate before the exiting die. The open space between the final element of screw and die plate had a length of 5 mm. Mechanical energy additions were measured with a cumulative Wattmeter directly connected to the electric drive motor. Specific mechanical energy input was calculated by dividing the total mechanical energy input by the product throughout for the period of measurement.

Characteristics of extruded rice bran

Immediately after extrusion, bran samples were packed in polyethylene bags and stored in liquid nitrogen until analyses were completed. Sample moistures were determined by drying at 50°C for 16 hr in a vacuum oven. Bulk densities (g/cc) were determined by weighing a tared 1 liter container filled with bran as described by Narain et al. (1978). Lipase activity was assayed by a colorimetric method described by Kim et al. (1984) where the free fatty acids (FFA) released from a triolein emulsion with an extract of defatted bran per unit time at defined conditions was determined as the color developed from a complex between the FFA and cupric ion. Residual lipase activity in the stabilized bran was calculated, on a dry weight basis, as a percentage of the activity in raw bran. The extraction characteristics of extruded bran were also examined by percolation of 60°C n-hexane through a 90 cm column and the percolation rate of miscella (a mixture of n-hexane and extracted oil) and the extraction rate of oil were also measured.

RESULTS & DISCUSSION

Extrusion temperature

The changes in discharge extrusion temperature resulting from different moisture levels, number of die openings and

Table 2—Changes in bulk density, moisture content and residual lipase activity of the rice brans extruded at various conditions

Conditions			Bulk density[a] (g/cc)	Moisture after extrusion[a] (%)	Residual lipase activity[a,b] (%)
Moisture level (%)	No. die openings	Production rate (kg/hr)			
9.5	3	100	0.434 ± 0.041	6.02 ± 1.42	0.0
9.5	3	200	0.467 ± 0.031	6.31 ± 0.83	0.0
9.5	6	400	0.561 ± 0.091	7.03 ± 0.21	1.8 ± 0.34
9.5	12	200	0.603 ± 0.071	7.74 ± 1.01	2.4 ± 2.10
9.5	12	400	0.590 ± 0.041	8.32 ± 1.01	10.8 ± 3.11
11.0	3	200	0.484 ± 0.043	6.94 ± 0.81	0.0
11.0	3	400	0.503 ± 0.087	7.10 ± 1.01	0.6 ± 0.81
11.0	6	100	0.531 ± 0.021	7.06 ± 1.10	00.8 ± 0.34
11.0	6	400	0.576 ± 0.053	7.30 ± 0.38	16.8 ± 1.21
11.0	12	100	0.585 ± 0.067	8.01 ± 1.31	6.7 ± 0.81
15.0	3	100	0.503 ± 0.021	8.10 ± 0.01	0.0
15.0	3	400	0.582 ± 0.031	8.32 ± 0.41	8.1 ± 1.01
15.0	6	200	0.521 ± 0.021	8.54 ± 0.61	5.6 ± 2.30
15.0	12	100	0.490 ± 0.031	12.80 ± 1.10	9.6 ± 1.83
15.0	12	400	0.515 ± 0.019	12.91 ± 0.31	10.4 ± 1.73

[a] The data are averages of th-ee determinations listed as mean ± confidence interval with confidence coefficient 99.0%.
[b] Residual lipase activity was calculated on dry basis as a percentage of activity in raw bran.

production rates are presented in Fig. 1. The extrusion temperature decreased as the number of die openings increased because the extrusion stabilizer is a thermodynamically autogenous extruder cooker. Using 9.5% moisture feed, the extrusion discharge temperature dropped as the production rate increased from 100 to 400 kg/hr by 23°C with 3 die openings and 3°C with 12 die openings. This trend was similar for bran at higher moisture levels but the temperature drop decreased to 12° and 9°C using 3 die openings for 11% and 15% moisture, respectively. The increase of moisture in feed bran also decreased the discharge extrusion temperature as shown in Fig. 1. This was due to the decrease in viscosity of extrudate at higher moisture content which decreased the mechanical energy dissipated as friction heat. Therefore, more physical restriction or fewer die openings is required to attain the necessary extrusion temperature with higher moisture content rice bran.

In the autogenous extruder cooker operating under a fixed screw speed, the amount of frictional heat generation is dictated by the sheer stress and residence time (McKelvey, 1954). With fewer die openings, the amount of extrudate in the screw channel was increased so that the effective contact area of the extrudate with screw channel surface was increased. This is a main factor in the increase of the frictional heat. The length of filled screw channel was also increased as the production rate increased. However at a higher production rate, the reduction of residence time offset the effect of increased length as found in this study. Based on the above, the extruder used to stabilize bran would best be controlled by first adjusting the feed rate to achieve the designed discharge temperature followed by adjustments in the number of die openings.

Power requirement

Total power requirement was directly related to production. However, specific energy input (SE), the amount of total mechanical energy to extrude 1 kg bran, was reduced with increasing production rate as presented in Table 1. These data are replotted on Fig. 2 which shows clearly that the extruders no-load power was 5.73 kW regardless of extrusion conditions. Therefore, the SE does not indicate exactly the true power dissipated into the extrudate at each set of conditions. To get a better indication of the specific power dissipated into bran, the slope of each line in Fig. 2 was obtained by linear regression and shown in Table 1 as net specific energy input (NSE). The NSE value did not change according to the production rate and was controlled by a given combination of number of die openings and the feed moisture. According to the data of Narain et al. (1978), the specific heat of rice bran was 1.657, 1.699 and 1.810 kJ/kg for 9.5, 11.0 and 15.0% moisture content, respectively. Using these values, energy dissipated as heat (EDH) was obtained with the discharge temperatures measured (Table 1). The extrusion stabilizer used in this study showed very high net power efficiency (above 0.80), which was obtained from the ratio of EDH/NSE. The high net power efficiency was attributed to the high screw speed (800 rpm) and the minimization of slippage at the barrel wall by attaching 4 splines to the barrel. The product slippage is the main factor for reduced power efficiency of single screw extruder (Straka, 1985). The net power efficiency became less with increasing production rate and the extent of this reduction was larger at fewer die openings. This trend may be due to the increased pressure requirements of these conditions consuming part of the power. Total power efficiency (EDH/SE), however, increased from 0.433 to 0.653 without any changes in NSE as production rate increased from 100 to 400 kg/hr. Therefore, better input power efficiency was achieved when the production rate was maximized to the extent possible.

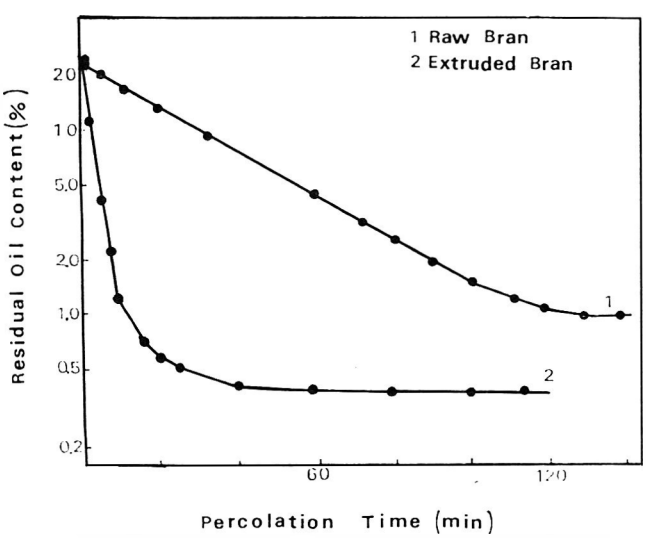

Fig. 3—Extraction curves for raw rice bran (1) and the rice bran extruded at discharge temperature of 128°C and initial moisture content of 11.0% (2).

Characteristics of extruded bran

All extruded brans were in a pellet form with a significant increase in bulk density and reduction in moisture (Table 2). Expansion in stabilized bran did not occur when discharged. The increase in bulk density noticed would contribute to changes in bed porosity through the agglomeration and compression of fine particles during extrusion. The moisture of raw bran was in the super-heated state just behind the die, which promotes vaporization immediately after extrusion into the atmosphere. At 9.5% moisture, the moisture content of extruded bran was decreased 6.01 to 8.32%, depending on the extrusion temperature. Higher moisture losses were related to higher extrusion temperatures.

At extrusion temperatures above 128°C, the lipase in the raw bran was completely inactivated. The lipase activity remaining in the extruded bran was inversely related to extrusion temperature regardless of the moisture level. Within the experimental range, the increase of moisture was not effective in the inactivation of lipase since the increase in moisture of bran fed decreased the extrusion temperature through reduction of the friction heat generation. The influence of production rate was also observed as expected, since at a higher production rate, the temperature profile along the barrel was lower than that at a low production rate. This agrees with the results of Sahgun and Harper (1981). The lower temperature profile obtained with a higher production rate resulted in lower lipase inactivation.

The percolation rate of miscella (ml/cm^2/min) through a 90 cm column of extruded bran was increased to 7.6 from 0.78 for the bed of raw bran. It was almost a 10-fold increase for the extruded bran compare to the raw bran. In practice, extraction time of extruded rice bran was also significantly reduced as shown in Fig. 3. Extraction time to reach 1% residual oil for the extruded bran was decreased to 10 min from 100 min for the raw rice bran.

CONCLUSION

WITHIN THE RANGE of moisture content between 9.5 and 15%, rice bran was able to be stabilized through extrusion at discharge temperature above 128°C with an autogenous single-screw extruder. Although total power efficiency was increased with increasing production rate without change in the net specific energy input, production rate was limited upto 200 kg/hr to obtain enough temperature to completely inactivate lipase activity in the raw rice bran. The optimum extrusion conditions were considered to be production rate of 200 kg/hr and number of die openings of 3. Extrusion under such conditions should

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Modelling of Wheat Drying in Fluidized Beds

S. A. GINER and A. CALVELO

ABSTRACT

Experiments on thin-layer drying of wheat were interpreted in terms of a kinetic model based on internal control for water transfer and absence of temperature gradients inside the kernel. The drying operation in a batch fluidized bed was modelled by assuming a perfect mixing of solids. The kinetic parameters obtained from the thin-layer runs introduced into this model allowed to interpret satisfactorily drying experiments performed in a batch fluidized bed. Predictions of the model showed that drying times could be decreased about four fold by raising the air temperature from 40°C to 70°C. It was also calculated that inlet air temperatures much higher than that of wheat damage could be used without deterioration of grains during drying. Predicted thermal efficiencies were low suggesting the recirculation of air as a means of improving them.

INTRODUCTION

EARLY HARVEST of wheat with moisture of about 25% requires artificial drying of the grain to obtain aqueous activities which inhibit both bacterial growth and spoilage reactions (Multon, 1984). It is considered that the safe water content in wheat should be below 14% wet basis (0.163 w/w dry basis) (Pomeranz, 1978).

In continuous drying equipments, grains form a bed which interacts with the drying air in concurrent, countercurrent or crossflow (Bakker-Arkema et al., 1978). However, control of grain temperature in this type of dryer is difficult, since there are usually important temperature and moisture gradients along different zones of the equipment. This leads to lack of uniformity in grain drying and to thermal damage of the grains, which can affect their viability as seeds and the industrial quality of the flour (Schreiber et al., 1981; Lupano and Añón, 1986).

Drying in fluidized beds offers an interesting alternative, since the thorough mixing which the solids undergo during fluidization ensures a good homogeneity of the thermal treatment of the product and a more stringent control of the grain temperature (Bakker-Arkema et al., 1978). Besides these advantages, there is also an excellent rate of heat and mass transfer between the grain and the surrounding air which results in shorter residence times.

Homogeneity in the thermal treatment of grain also allows the use of higher air temperatures, increasing production without detriment to quality. However, adoption of fluidized bed dryers requires the support of modelling studies to compare energy efficiencies, power consumption and maximum air temperatures among the various alternatives and respect to conventional dryers.

Modelling of wheat drying in fluidized beds requires information about fluidization characteristics of wheat, as well as expressions which describe the process of heat and mass transfer which take place between grain kernels and fluidizing air.

In the present work, the fluidization characteristics of wheat grains were determined, and the parameters of thin-layer drying were obtained experimentally. Drying curves in a batch fluidized bed were also obtained for different bed heights and temperatures of fluidizing air. Results for fluidized beds were interpreted by means of a model of heat and mass transfer based on simplified equations for the individual drying of grains.

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MATERIALS & METHODS

Wheat

Wheat (*Triticum aestivum*) var. "Marcos Juarez-INTA" with moistures between 0.13 and 0.16 (w/w dry basis) was used. Samples were wetted to a moisture of about 0.25 (w/w d.b.) by adding the necessary amount of water and leaving them at 20°C for 48-72 hr with occasional mixing of the grains.

Grain kernel shapes were assimilated to ellipsoids to obtain their average dimensions, and the 3 axes were measured in 10 kernel samples. Values obtained were a = 0.32 cm; b = 0.16 cm and c = 0.14 cm.

To verify that the chosen geometry represented the material under study, the mean volume of the ellipsoid, calculated on the basis of the axis measurements, was compared with picnometric determinations of the average grain volume; a satisfactory agreement was obtained. Surface area was calculated according to this geometry, and the value was increased by 7% to account for the indentation of the wheat kernel; the value obtained was 0.575 cm^2.

Picnometry

Average volume of the grains was determined by picnometry using a mixture of xylene isomers on samples containing 200 particles; $V_p = 3.16 \times 10^{-2}$ cm^3 was obtained. Grain moisture during determinations was 0.147 (d.b.). With the average volume, density of the grains was also determined, the value obtained being $\rho = 1.38$ g/cm^3.

Determination of water content

In order to use a technique simpler than the AOAC (1980) method without loss of accuracy, moisture determinations were carried out on 3-4 g samples formed by whole kernels dried in a conventional oven at 130°C for 2 hr. This technique was checked against the AOAC (1980) method which employs ground grain; the following correlation was obtained:

$$W_{AOAC} = 0.02793 + 0.9537\ W \quad (r^2 = 0.984) \qquad (1)$$

where W is the water content on a dry basis obtained by the whole grain method. Consequently, moisture determinations during drying experiments were carried out with this method, correcting the values by means of Eq. (1).

Equipment and experimental techniques

A fluidized bed of rectangular section (24.5 cm × 32.2 cm) with glass lateral walls to observe the behavior of the bed during fluid dynamic experiments was used.

In the drying experiments the walls were thermally insulated with plates of expanded polystyrene. Air was forced by means of a 5.5 HP centrifugal blower with a maximal flow rate of 9500 m^3/hr and a discharge pressure of 120 mm water. Air was then forced through 7 electrical resistances of 2000 W each, which could be connected in various ways along a 1 m long duct. The last resistance was operated by a proportional controller which was in turn governed by a temperature sensor located under the grid supporting the fluidized bed.

Air at a controlled temperature (± 0.5°C) first entered a pressure chamber, then passed through two perforated plates to obtain a uniform velocity profile and finally flowed upwards through a grid which formed the base of the fluidized bed. This grid was constituted by a perforated metallic plate with 22.5% free area and 1.5 mm holes in a triangular arrangement. The pressure drop caused by the three grids was large enough to obtain a uniform air flow through the bed. Pres-

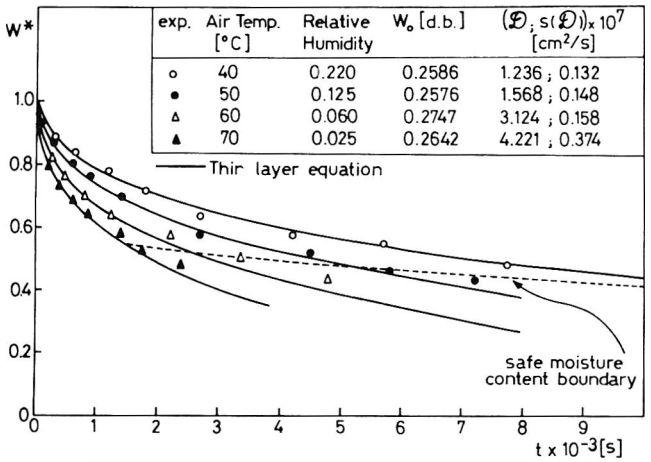

Fig. 1—Wheat drying in thin layer as a function of air temperature.

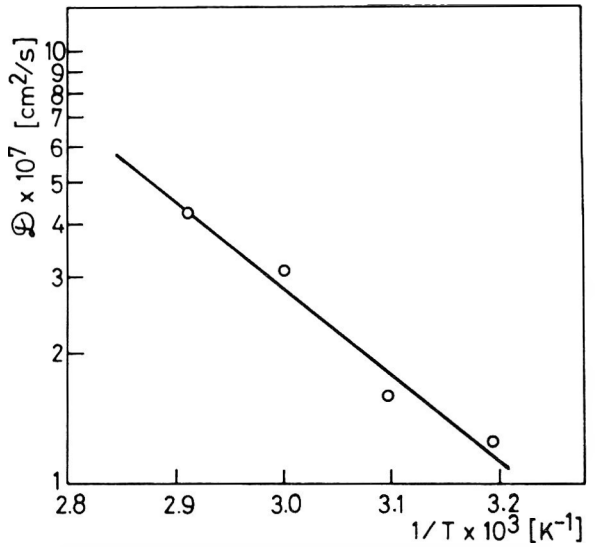

Fig. 2—Effect of temperature on the diffusion coefficient of water in kernel.

sure drop through the bed was obtained from readings of static pressures taken at inlets located on the lateral walls under the entrance grids connected to a liquid inclined manometer.

The air flow section was gradually decreased above the rectangular container to allow the measurement of the superficial air velocity with a previously standardized Alnor velometer.

Dry-bulb temperatures of air at the inlet and outlet of the fluidized bed were measured by means of copper-constantan thermocouples connected to a potentiometric recorder. Simultaneously, wet-bulb temperatures at the air inlet and outlet were measured using wet muslin-covered thermocouples. These thermocuples were connected differentially with the dry-bulb ones. The differences were amplified 100-fold and continuously recorded. In the fluid dynamic experiments, the pressure drop was determined as a function of the superficial velocity (V_o) for fixed bed heights between 3 and 10 cm. Bed expansion was also measured and was expressed in terms of the void fraction of the expanded bed as a function of the superficial velocity of air as described previously (Vázquez and Calvelo, 1980).

Temperature and moisture of inlet and outlet air and water content of grain samples obtained from the bed at different operation times were measured in the drying experiments. Samples were analyzed in duplicate; the differences from the mean were in general below 1%. Thin-layer drying experiments (bed thickness ≈ 0.5 cm) were carried out at different air temperatures. Drying experiments in fluidized beds were also performed at different inlet air temperatures (T_{g_i}) and different bed heights (H_o).

RESULTS & DISCUSSION

Thin-layer drying of wheat

To obtain the drying parameters of the wheat studied, thin-layer runs were performed, since this procedure ensured that all particles were in contact with air of constant temperature and humidity. To this purpose the same fluidized bed was used in all experiments, and air at different temperatures and velocity V_o = 150 cm/sec was forced upward through a layer of about 0.5 cm thickness (no significant differences were found in experiments in which V_o = 500 cm/sec).

Experimental results are shown in Fig. 1 in terms of the dimension-less water content $\overline{W}^* = (\overline{W} - W_e)/(W_o - W_e)$ where $\overline{W}$ is the mean water content of the grains at time t, W_o is the initial water content and W_e is the equilibrium content at the temperature and humidity of the circulating air. The latter value was calculated on the basis of the corresponding desorption isotherm (Bakker-Arkema et al., 1978). The dotted lower curve corresponds to the practical limits of wheat drying (safe moisture content $\overline{W}$ = 0.150).

Interpretation of drying curves was based on the solution of the microscopic mass balance (second Fick's law) assuming an internal control of water movement, which for short times predicts (Becker, 1959):

$$\overline{W}^* = \frac{\overline{W} - W_e}{W_o - W_e} = 1 - \frac{2}{\sqrt{\pi}} a_v \sqrt{D\,t} + B\, a_v^2\, D t \quad (2)$$

where B = 0.33, a_v is the area per unit volume of the wheat kernel, assessed according to the values of surface area and grain volume as a_v = 18.2 cm^{-1} and D is the diffusion coefficient dependent on temperature according to:

$$D = D_\infty \exp(-E/Rg\,T) \quad (3)$$

where E is the energy of activation of water diffusion in the wheat kernel.

Equation (2), is valid for $W^* > 0.25$, with less than 1% error; this criterion is fully met by the data included in Fig. 1. By using a non-linear regression program (Marquardt, 1963) and Eq. (2), values of D and its standard error for each temperature were obtained (Fig. 1). Those diffusion coefficients are plotted according to Eq. (3) in Fig. 2. The energy of activation and the pre-exponential factor obtained by least squares were E = 9330 cal/gmol and D_∞ = 0.375 cm^2/sec with standard errors of 655 cal/gmol and 0.0288 cm^2/sec, respectively, being r^2 = 0.977. Full lines on the experimental points in Fig. 1 correspond to the predictions of Eqs. (2) and (3) with the values of the described parameters. As can be seen, there was satisfactory agreement within the range of experimental conditions.

Fluidization of wheat

Beds formed by particles such as wheat grains of medium size and low sphericity (Ψ_e = 0.84) do not exhibit a clearcut passage from fixed to fluidized beds (Davidson and Harrison, 1971), but they show a transitional behavior. Thus, when the superficial velocity of the air was increased, the bed behaved as fixed up to values of V_o = 65 cm/sec.

Void fraction of the bed under these conditions was ϵ_o = 0.41. The transitional behavior, which extended up to V_o = 120 cm/sec was characterized by a partial fluidization with fluidized sectors in which bubbles were formed and zones where the bed was still fixed. On increasing the superficial velocity, V_o, the fluidized fraction increased. For values higher than V_o = 120 cm/sec, total fluidization was attained with aleatory formation of bubbles in the different bed sectors.

Based on the results described, velocities not lower than 120 cm/sec were chosen in the drying runs to obtain a total bed fluidization.

Wheat drying in batch fluidized beds

Theory. Assuming that a perfect mixing of the grains takes place in the fluidized bed and that air leaves the bed in thermal equilibrium with the solids, the following energy balance can be proposed (Becker and Isaacson, 1970)

$$m_s C_p \frac{dT}{dt} = m_s \lambda_d \frac{d\overline{W}}{dt} + \rho_g V_o S C_{p_g} (T_{g_1} - T) \quad (4)$$

where $m_s = \rho_s (1 - \epsilon_o) S H_o$ is the mass of dry solid; T, the grain temperature assumed equal to that of outlet air; T_{g_1}, the temperature of air entering the bed; C_{p_g} its specific heat; ρ_g its density; and λ_d, the mean heat of desorption of water in wheat grains; W is the mean water content of the grain (uniform throughout the bed), S the section of the bed and C_p the specific heat of the partially dehydrated grain expressed on a dry basis according to:

$$C_p = C_{p_s} + C_{p_W} \overline{W} \quad (5)$$

where C_{p_s} is the specific heat of the dry matter and C_{p_w} that of water.

Similarly, a balance of water in the air flowing through the bed leads to:

$$\rho_g V_o S (H_2 - H_1) = - m_s \frac{d\overline{W}}{dt} \quad (6)$$

Equations (2) to (6) were used by Becker and Isaacson (1970) to model continuous moving-bed commercial dryers. Thus, by replacing Eq. (5) in Eq. (4) and writing it in dimensionless form, the following equations are obtained.

$$K_1 (1 - K_3 \Psi) \frac{d\phi}{d\eta} + K_2 \phi = \frac{d\Psi}{d\eta} \quad (7)$$

$$\eta = a_v^2 D_o t; \Psi = \frac{W_o - \overline{W}}{W_o - W_s}; \phi = \frac{T_{g_1} - T}{T_{g_1} - T_o} \quad (8)$$

$$K_1 = \frac{C_{p_o} (T_{g_1} - T_o)}{\lambda_d (W_o - W_s)} \quad (9)$$

$$K_2 = \frac{\rho_g V_o S C_{p_g} (T_{g_1} - T_o)}{m_s \lambda_d (W_o - W_s) a_v^2 D_o} \quad (10)$$

$$K_3 = \frac{C_{p_W} (W_o - W_s)}{C_{p_o}} \quad (11)$$

where W_o and T_o are the initial water content and the temperature of the grain, respectively; $C_{p_o} = C_{p_s} + C_{p_W} W_o$ is the initial specific heat of the grain on a dry basis and D_o the diffusion coefficient evaluated at temperature T_o. Moreover, W_s has been taken as the mean arithmetic value of W_e between entrance and exit conditions of air $W_s = (W_{e_1} + W_{e_2})/2$.

Equation (7) shows the presence of two dependent variables, ϕ and Ψ. Eq. (2) is also required to solve the system. Nevertheless, it should be considered that equation (2) has been obtained by integration, assuming that D and W_s are constants. Consequently to apply it to a fluidized bed, mean values of these parameters should be used, since they are dependent on the temperature, T, and on the humidity and temperature, respectively, which in turn vary with the drying time.

To account for the dependence D (T), Eq. (2) is modified as follows.

$$\Psi = \frac{2 a_v}{\sqrt{\pi}} \sqrt{\int_o^t D \, dt} - B a_v^2 \int_o^t D \, dt \quad (12)$$

In turn, Eq. (3) can be written:

$$D = D_o \exp \left[K_5 (1 - \phi)/(K_4 - \phi) \right] \quad (13)$$

where $K_5 = E/R_g T_o$, and $K_4 = T_{g_1}/(T_{g_1} - T_o)$ (14)

If now Eq. (13) is replaced in (12), the following expression is obtained by derivation

$$\frac{d\Psi}{d\eta} = \frac{1}{U(\Psi)} \exp \left[K_5 (1 - \phi)/(K_4 - \phi) \right] \quad (15)$$

$$U (\Psi) = (1 - Z)/B Z \text{ and } Z = (1 - B \pi \Psi)^{1/2} \quad (16)$$

Equations (7) and (15) can be combined to give, respectively:

$$\frac{d\phi}{d\Psi} = \frac{1 - K_2 \phi U(\Psi) \exp \left[- K_5 (1 - \phi)/(K_4 - \phi) \right]}{K_1 (1 - K_3 \Psi)} \quad (17)$$

$$\frac{d\eta}{d\Psi} = U (\Psi) \exp \left[- K_5 (1 - \phi)/(K_4 - \phi) \right] \quad (18)$$

where, according to Becker and Isaacson (1970) the dimensionless water content, Ψ, has been taken as independent variable.

The corresponding initial conditions are

For $\Psi = 0$ $\eta = 0$ (19)

$\Psi = 0$ $\phi = 1$ (20)

For Equations (17) to (20) the times needed (as well as the corresponding temperatures) to attain a given water content in the grain can be calculated. On the other hand, the values of Ψ and ϕ obtained at each time can be used to evaluate the humidity of the outgoing air by means of the dimensionless form of Eq. (6).

$$H_2 = H_1 + \frac{K_6}{U(\Psi)} \exp \left[K_5 (1 - \phi)/(K_4 - \phi) \right] \quad (21)$$

where $K_6 = m_s (W_o - W_s) D_o a_v^2/\rho_g V_o S$ (22)

Values of humidity and temperature of the outlet air obtained in each step of the program were used to calculate a new W_s value, thus taking into consideration its variation along the drying time. The solution was obtained by means a method of Runge-Kutta-Gill (Lapidus, 1962) of fourth order programmed in a Series I IBM computer.

Comparison with experimental results

The mathematical model developed was compared with the results obtained with the described experimental equipment. Figure 3 shows the $\overline{W}/W_o$ ratio as a function of time for the range of inlet air temperatures generally used in wheat drying. Velocity of air, V_o, and bed height, H_o, were kept constant in the experiments. In this Figure, the predictions of the model for the same conditions are also included. Table 1 lists the parameters used in the prediction as well as the source corresponding to each of them. As can be seen, there was satisfactory agreement, thus showing that the model was adequate within the range of experimental temperatures. Figure 4 depicts the influence of bed height at a consultant inlet air temperature and superficial velocity. There was also in this instance a satisfactory agreement between the experimental values and the model. The lower curve corresponded to thin-layer ($H_o \approx 0$) results. As the bed height increased, the time needed to attain

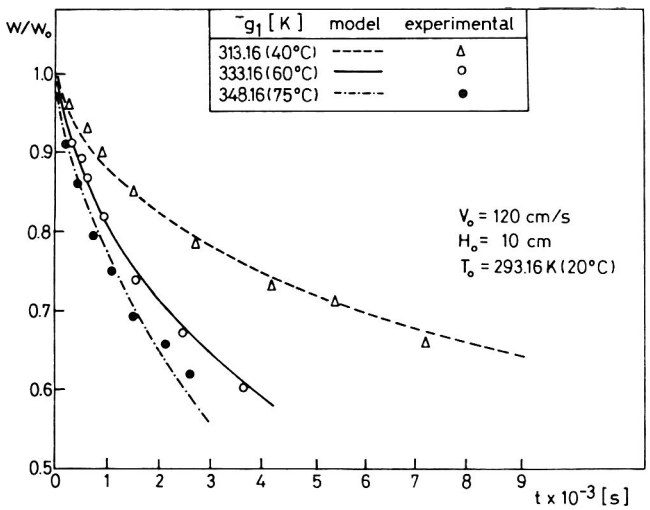

Fig. 3—Effect of inlet air temperature on drying curves for a batch fluidized bed dryer.

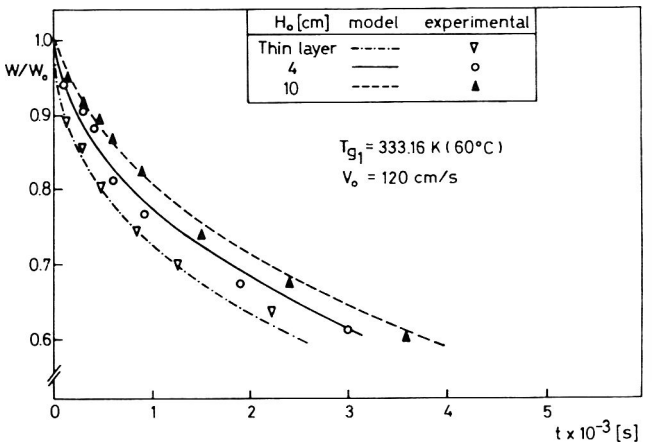

Fig. 4—Effect of bed height on drying curves for a batch fluidized bed dryer.

a given grain moisture also increased. It should be noted, however, that the influence of bed height on drying times was relatively small within the range studied.

Predictions of the model

The model described can be used to analyze the behavior of fluidized beds under conditions not exactly similar to those studied experimentally. Theoretical information is thus obtained on the effect of operation variables and yields, which are often difficult to assess experimentally.

Thus, Figure 5 shows the effects of bed height on drying time, t_s, defined as that necessary to lower grain moisture from an initial value, $W_o = 0.25$, to a final one, $W_f = 0.15$. Curves are provided for different inlet air temperatures (Fig. 5) within the range usually employed in industrial dryers. The increase in height is not paralleled by an equivalent increase in drying time, which favors, from the point of view of production rates, the use of greater heights. This alternative should be made compatible with the higher requirements of power for blowing.

It is also of interest to analyze the grain temperature as this determines the extent of damage underwent by wheat during drying. It is generally accepted that grain temperatures higher than 65°C lead to considerable damage, not only of germinative capacity, but also of the bread-making quality of flour (Schreiber et al., 1981; Lupano and Añón, 1986). However, due to the thorough mixing of solids that characterizes fluidized beds, temperature throughout the bed is uniform and different from that of the inlet air. Thus, higher inlet temperatures of air can be used, which lead to shorter drying times without danger of wheat damage during the operation.

Figure 6 shows the grain kernel temperatures at the end of drying, assumed to be equal to that of the outlet air, as a function of the inlet air temperature for different bed heights. For thin-layer conditions ($H_o \approx 0$) both temperatures were equal, since under those conditions air did not appreciably change its temperature on going through the bed. However, as the bed height increased, the possibility of using higher air temperatures without exceeding the 65°C limit occurred. Thus, for $H_o = 30$ cm, air temperature up to 75°C could be used, without damage to the grain during the operation. Moreover, if it is considered that Fig. 6 corresponds to the temperatures reached by the grain at the end of the operation (i.e., the highest), it would also be possible to shorten the drying times even more with no damage to the grain by using higher inlet air temperatures as the beginning of the operation and lowering them to the limits imposed by Fig. 6 at the end. Thus, model simulation of drying in a 30 cm high fluidized bed with an inlet air temperature of 95°C for the first 20 min, and lowering it to 75°C for the rest of the operation, predicts a reduction of the drying time to 75% of the original value, with grain temperatures not exceeding 63°C. Figure 7 shows the evolution of

Table 1—Parameters used

$C_p = C_{p_s} + C_{p_W} \overline{W}$	$C_{p_s} = 0.30$ cal g K $C_{p_W} = 1$ cal g K	(Becker and Isaacson, 1970)
$\rho_s = \frac{\rho \cdot \rho_W}{\rho_W (1 + \overline{W}) - \rho \cdot \overline{W}}$	$\rho = 1.38$ g cm^3 $\rho_W = 1$ g cm^3	(Becker, 1959)
$W_e = \frac{1}{100}\left[\frac{-\ln(1 - RH)}{\alpha[(T - 273.16) + \beta]}\right]^{\gamma}$	$\alpha = 1.23 \times 10^{-5}$ $\beta = 64.346$ $\gamma = 0.3909$	(Bakker-Arkema et al., 1978)
$\rho_g = P M_g R_g T_{g_1}$	$M_g = 29$ $R_g = 8.314 \cdot 10^7$ erg gmol K	
$C_{p_g} = 0.24$ cal g K $\lambda d = 610.66$ cal g	1	1. (Becker and Isaacson, 1970)
	$\epsilon_o = 0.41$ $a_v = 18.2$ cm^{-1} $D = 0.375$ cm^2 sec $E = 9330$ cal gmol	2 2. This work

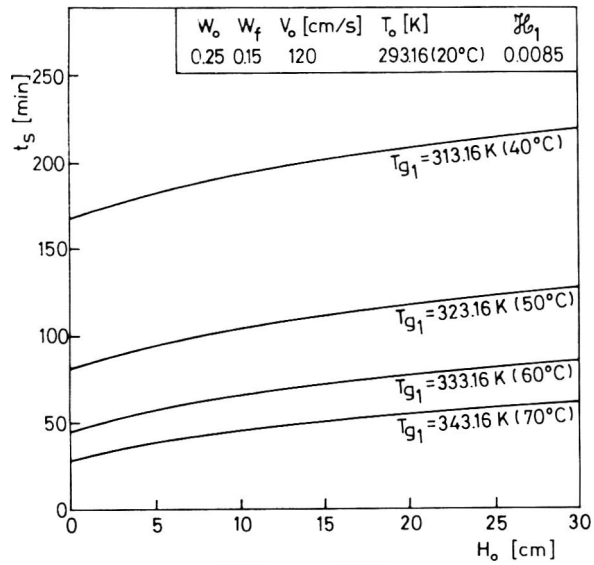

Fig. 5—Effect of bed height and inlet air temperature on drying time.

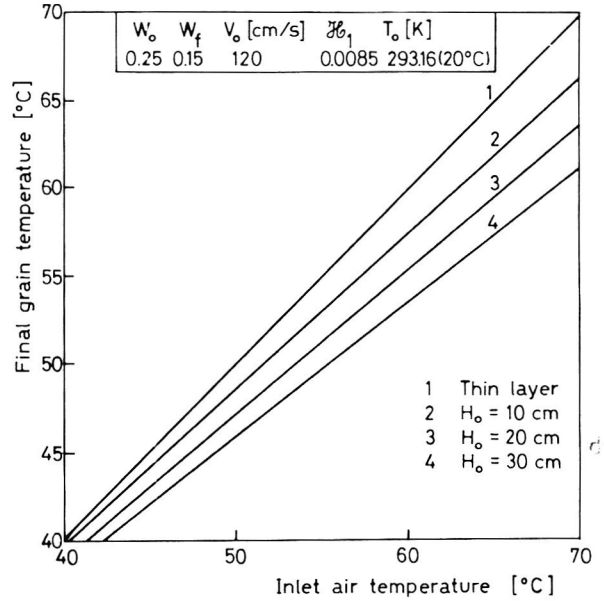

Fig. 6—Effect of bed height and inlet air temperature on the final grain temperature.

grain temperature and moisture content for both alternatives.

The possibility of adopting fluidized bed dryers is strongly dependent on an efficient use of energy. In this regard, it is possible to define the thermal efficiency of air/grain interaction as:

$$E_f = \frac{\text{Energy transmitted to the solid}}{\text{Energy incorporated in the drying air}} \qquad (23)$$

Efficiency E_f can be expressed in terms of the operation parameters as:

$$E_f = \frac{\rho_s (1 - \epsilon_o) H_o \left[\lambda d (W_o - W_f) + C_{p_m} (T_f - T_o)\right]}{60 \rho_g V_o C_{p_g} (T_{g_1} - T_a) t_s} \qquad (24)$$

By means of the predictions of the described mathematical model, it is possible to evaluate the thermal efficiency of the fluidized bed dryer by using Eq. (24).

Figure 8 depicts thermal efficiency E_f as a function of bed height and temperature of inlet air for normal wheat drying conditions. Bed height increased thermal efficiency, though not linearly, due to the indirect effect on t_s, as seen in Eq. (24). In a parallel fashion, even if an increase of T_{g_1} increases the enthalpy loss in the exit air, this effect is largely compensated by the shorter drying time, t_s, leading to a higher thermal efficiency, as can be observed in Fig. 8.

It should also be noticed that E_f values in Fig. 8 are remarkably low, showing that a large portion of the thermal energy supplied is lost in the outlet air, which represents a serious disadvantage for this type of system. Bearing in mind that T_{g_1} increases are limited by the need of preventing grain damage, and those of H_o by the blower power, it would be advantageous that enthalpy could be further exploited through recycling or division of the bed into sectors, with successive air passes through the sectors.

CONCLUSIONS

EXPERIMENTAL CURVES for thin-layer drying of wheat were used to obtain the parameters of a known kinetic model based on the existence of internal control for water transfer and absence of temperature gradients inside the kernel. Results were satisfactory; a feature to be noticed was the use of a surface water content obtained form desorption isotherms.

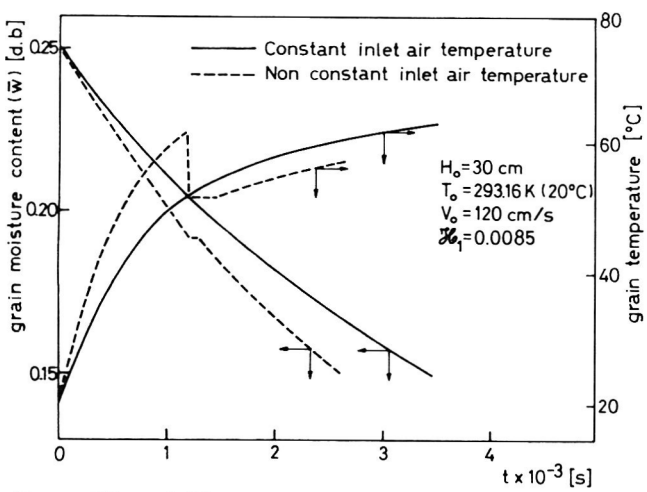

Fig. 7—Effect of different programs of the inlet air temperature on the drying time and grain temperature.

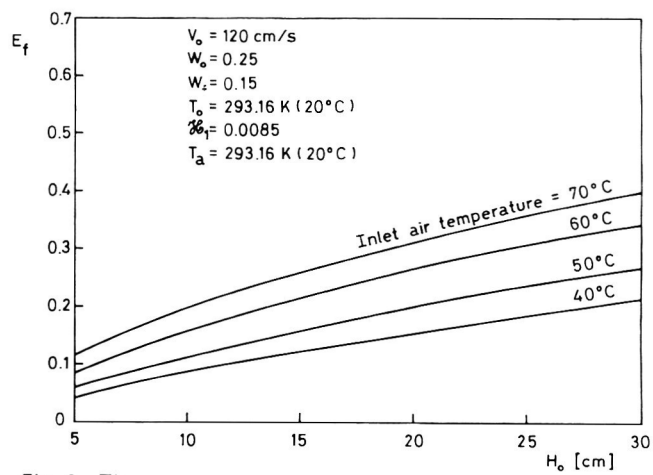

Fig. 8—Thermal efficiencies as a function of inlet air temperature and bed height.

The parameters of fitting were the diffusion coefficient and its temperature dependence, thus obtaining $D_\infty = 0.375$ cm^2/sec and E = 9330 cal/gmol.

The fluid dynamic experiments on fluidization showed that the air superficial velocity that ensured a good fluidization, and therefore, a good mixing of the grains was $V_o \geq 120$ cm/sec. Below this value there existed a partial degree of fluidization typical of products of low sphericity, which was not advisable for the drying operation.

Moisture contents and temperatures of exhaust air, as well as the evolution of grain moisture during the drying operation, were calculated by means of the respective mass and energy balances, together with the experimentally tested expression of thin-layer drying.

The model exhibited a satisfactory fit to experimental results obtained for wheat drying in a batch fluidized bed. It should be pointed out that no fitting parameters were used; instead, the kinetic information obtained from thin-layer and the properties of fluidized beds allowed satisfactory prediction of the experimental results for different bed heights and different temperatures of fluidizing air.

Model predictions showed that increases in drying time did not parallel those of bed heights, thus suggesting the use of deeper beds as a means to increase production. Temperature in turn had an important effect; drying times could be decreased about fourfold when the temperature was raised from 40°C to 70°C.

Fluidized bed dryers have good possibilities for controlling grain damage as the solids maintain a uniform temperature below the inlet air temperature. In this respect, with 30 cm bed heights, temperatures of inlet air near 10°C beyond that of grain damage could be used, with no danger of deterioration of solids during the drying operation. This would lead to shorter drying times and, therefore, to higher production rates.

Model simulations also showed that it was possible to reduce the operation times even more by using inlet air temperatures about 30°C higher than that of damaging temperature during the first drying stage, and reducing later air temperature to the specification value.

Analysis of thermal efficiency showed that fluidized bed dryers were of low efficiency. In this respect, the number of passes of air through the grain bed should be increased to take advantage of its enthalpy content.

NOMENCLATURE

a_v	Grain area per unit volume, cm^{-1}
B	Constant in Eq. (2); B = 0.33
C_p	Specific heat of solids at $\overline{W}$, cal/g K
C_{pg}	Specific heat of air, cal/g K
C_{pm}	Specific heat of solids at W_m, cal/g K
C_{po}	Specific heat of solids at W_o, cal/g K
C_{ps}	Specific heat of dry solids, cal/g K
C_{pw}	Specific heat of water, cal/g K
D	Diffusion coefficient of water in kernel at T, cm^2/sec
D_o	Diffusion coefficient of water in kernel at T_o, cm^2/sec
D_∞	Parameter in Eq. (3), cm^2/sec
E	: Activation energy for moisture diffusivity, cal/gmol
E_f	: Thermal efficiency
H_1	: Absolute humidity of inlet air
H_2	: Absolute humidity of exit air
H_o	: Bed height, under fixed bed conditions, cm
K	Kelvin temperature
K_1, K_2, K_3, K_4, K_5, K_6	Dimensionless group defined in Eq. (9), (10), (11), (14) and (22)
m_s	Mass of dry solid in the bed, g
M_g	Molecular weight of air
P	Atmospheric pressure, dyne/cm^2
R_g	Gas constant, erg/gmol K
RH	Relative humidity of air, decimal
S	Bed section, cm^2
t	Time, sec
t_s	Drying time (time to reduce moisture from W_o to W_f), min
T	Solid temperature, K
T_a	Room temperature, K
T_f	Temperature of solids at the end of drying, K
T_{g_1}	Inlet air temperature, K
T_o	Initial solid temperature, K
$U(\Psi)$	: Variable defined in Eq. (16)
V_o	Superficial air velocity, cm/sec
V_p	: Average particle volume, cm^3
$\overline{W}$	Average solid moisture, w/w (d.b.)
W_e	Equilibrium solid moisture, w/w (d.b.)
W_f	Final solid moisture, w/w (d.b.)
W_m	= $(W_o + W_f)/2$; medium solid moisture, w/w (d.b.)
W_o	Initial solid moisture, w/w (d.b.)
W_s	: Average equilibrium solid moisture, w/w (d.b.)
$\overline{W}^*$	Dimensionless solid moisture defined in Eq. (2), w/w/ (d.b.)
Z	Variable defined in Eq. (16)

Greek symbols

ϵ_o	: Bed void fraction, under fixed bed conditions
η	: Dimensionless time defined in Eq. (8)
λ_d	: Heat of desorption of water in wheat grains, cal/g
ρ	: Solid density, g/cm^3
ρ_g	: Air density, g/cm^3
ρ_s	: Dry solid density, g/cm^3
ρ_w	: Water density, g/cm^3
ϕ	: Dimensionless solid temperature defined in Eq. (8)
Ψ	: Dimensionless solid moisture defined in Eq. (8)
Ψ_e	: Grain sphericity

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Ms received 9/23/86; revised 3/20/87; accepted 4/14/87.

This study was supported by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), the Secretaría de Ciencia y Técnica (SECYT) and the Cosmisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC) of Argentina.

Flow Properties of Solutions of Oat β-Glucans

K. AUTIO, O. MYLLYMÄKI, and Y. MÄLKKI

ABSTRACT

The flow properties of aqueous solutions of oat β-glucans were studied using a Bohlin rheometer (concentric cylinder geometry) over the shear rate range 0.734–1500 1/sec at temperatures between 15 and 75 °C. The β-glucan powder concentrations employed were 0.20–1.56% (w/w). The power law model was satisfactory for describing the flow behavior of β-glucans over the shear rate range 18.6–232 1/sec. The magnitudes of activation energy were in the range 2.41–4.61 (kcal/mol). Sucrose decreased the flow behavior index and increased the consistency index of β-glucans and decreased the activation energy at lower β-glucan concentrations, these effects being most pronounced at a sucrose concentration of 25%. The D-value for solutions of β-glucans (0.65%) at 100 °C was 145 hr.

INTRODUCTION

OAT ENDOSPERM CELL WALLS are mainly composed of two chemically distinct groups of polysaccharides, namely pentosans and β-glucans. Oat grains have been reported to contain 2.7–5.4% (w/w) of β-glucans (McCleary and Glennie-Holmes, 1985). Differences in the β-glucan content were observed in different varieties and growing conditions.

Cereal β-glucans are composed of linear polysaccharide chains, the structural components being glucose units connected with β-(1-3) and (1-4)-linkages. It has been reported that oat β-D-glucan consists of about 70% of (1-4)-linked and about 30% of (1-3)-linked β-D-glucosyl residues (Clarke and Stone, 1963).

From the point of view of application in the food industry, one of the most important physical characteristics of cereal β-glucans is their viscosity. Only a few reports (Wood et al., 1977; Wood, 1984) on the molecular weights of oat β-glucans are available, but the data are difficult to compare because of variations in the starting materials and methodology used. Many viscosity data on cereal β-glucans are derived from single-point measurements. Since such measurements give no information on the flow behavior, evaluations may be contradictory.

The aim of this study is to provide detailed information about: (1) flow properties of β-glucans at various concentrations and temperatures; (2) effects of sugar and salt concentrations and loss of consistency of the solutions due to thermal degradation.

MATERIALS & METHODS

β-GLUCAN was extracted from oat bran of mixed Finnish commercial varieties. The β-glucan content of the bran (12.7%) was determined by the method described by McCleary and Glennie-Holmes (1985). Extraction was at pH 9.2 and 70 °C. Fifty grams bran were stirred in 1700 mL water for 1 hr. The solids were centrifuged in a Sorvall RC-5B centrifuge at 16300 × *g* for 20 min and the proteins precipitated from the clear supernatant by lowering the pH to 4.5 with 2M HCl. Precipitated proteins were centrifuged as above and the pH of the clear viscous supernatant was raised to 7.0 with 2M NaOH. β-Glucans were precipitated by rapidly mixing an equal volume of 2-propanol with the viscous supernatant. The fibrous precipitate was collected on a 0.105 mm sieve and washed with 2-propanol. The β-glucan was stored under a 2-propanol atmosphere in a glass jar. Before dissolving the β-glucan in water the 2-propanol was evaporated at 35 °C.

The molecular weight of the sample was determined at 30 °C by GP-HPLC μBondagel E-1000, E-500 and E-125 columns' connected in series. The fraction ranges of the columns are 2×10^6–5×10^4, 5×10^5–5×10^3, and 5×10^4–2×10^3 daltons, respectively. Dextran standards were used for the calibration of columns and water was used as the mobile phase. β-Glucan stock solution was freeze-dried and the β-glucan content in the dry powder was determined by the barley β-glucan assay procedure (Biocon Pty. Ltd., 31 Wadhurst Drive Boronia, Victoria 3155, Australia) (McCleary and Glennie-Holmes, 1985).

Measurements

β-Glucan solutions were prepared by solubilizing β-glucan precipitates in hot water, sucrose solutions (0–50%) or NaCl-solutions (0–10%) with a homogenizer (Bamix, Switzerland) and the dispersed air was removed in vacuum.

Viscosity measurements were performed with a Bohlin Rheometer system (Bohlin Rheology AB, Lund, Sweden) using a concentric cylinder system, (C-25).

Calculations

Flow properties were expressed by the power law

$$\tau = K\dot{\nu}^n$$

where τ is stress (N /m^2), $\dot{\nu}$ is shear rate (1/sec), K is a consistency index (Ns^n/m^2), and n is a dimensionless constant which indicates deviation from Newtonian flow. The magnitudes of K and n were determined from linear regression analysis of log τ vs log $\dot{\nu}$. The variation in apparent viscosity due to temperature (15–75 °C) was examined at different gum concentration using the Arrhenius model:

$$\eta_a = Ae^{(E_a/RT)}$$

where A is the frequency factor, e is the natural logarithm base, E_a is activation energy, and R is the gas constant. The slopes and intercepts of the regressions of the logarithm of apparent viscosity at 92.3 1/sec and 232 1/sec vs the inverse of absolute temperature were used to calculate the frequency factors and activation energies at each of the concentrations examined.

The heat-stability of β-glucan solutions were determined by sealing 10 mL 0.65% β-glucan solutions in tubes and heating at 100°C for 140 hr. The power-law constants during heat treatment were determined. The reduction in K due to heat treatment was characterized by the D-value (Rao et al., 1981).

RESULTS & DISCUSSION

AT ALL POWDER CONCENTRATIONS studied (0.2–1.6%, w/w on a dry weight basis; β-glucan content was 80%, w/w; M.W. 2×10^6 daltons, the other major component was protein), β-glucans were pseudoplastic, but did not exhibit time-dependant behavior. The power law held at shear rates between 18.6 and 232 1/sec. The power law parameters of the solutions are given in Table 1. The correlation coefficients for the applicability of the power law model ranged between 0.988 and 0.999. Increases in gum concentration increased the consistency coefficient and decreased the flow behavior index, in-

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Table 1—Power law constants for β-glucans in water and in 25% sucrose solution at 25°C

β-glucan powder conc (%, w/w)	n_w[a]	n_s[b]	K_w[a]	K_s[b]
			($N \cdot s^n/m^2$)	
0.20	0.84	0.83	0.04	0.06
0.45	0.62	0.53	0.57	1.49
0.67	0.50	0.40	3.40	7.28
0.89	0.30	0.29	12.2	21.3
1.00	0.28	0.26	24.2	31.2
1.10	0.24	0.23	36.1	40.3
1.22	0.20	0.21	46.9	50.7

[a] n_w and K_w are for water solutions.
[b] n_s and K_s are for 25% sugar solutions.

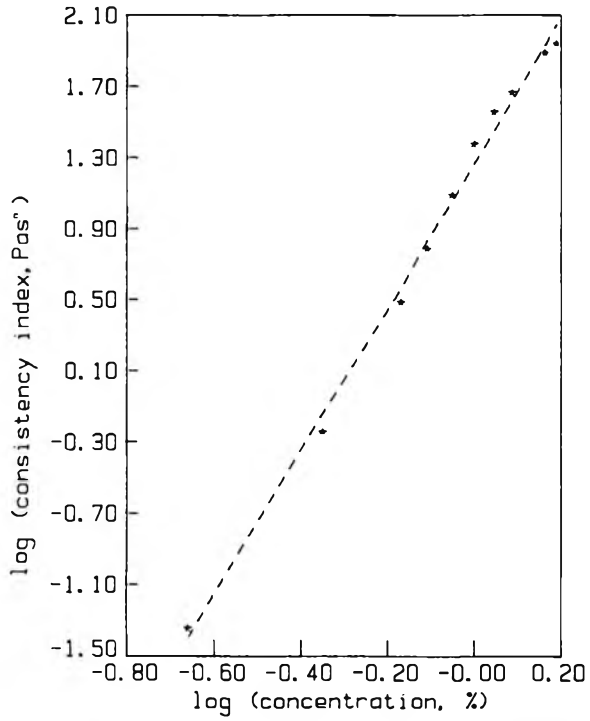

Fig. 1—Variation of consistency index of β-glucan solutions with concentration.

dicating that the solutions became more pseudoplastic as the concentration of β-glucan increased. A power type relationship was found between the consistency index and the concentration of β-glucan solutions (Fig. 1). Rao and Kenny (1975) observed a similar relationship for furcellaran, xanthan gum and guar gum.

Szczesniak and Farkas (1962) reported that gum solutions with high values of n tend to feel slimy in the mouth, whereas gum solutions having a low n value have a cleaner mouthfeel. At 25°C the magnitudes of n and K for 1.0% β-glucans were 0.27 and 24.2, respectively. Rao and Kenny (1975) reported n and K values of 0.170 and 20.34 for guar gum, and 0.222 and 3.68, respectively, for xanthan gum. At a concentration of 1.0% both guar gum and xanthan gum were more pseudoplastic than β-glucans, but the magnitude of consistency index K was higher for β-glucans, indicating higher apparent viscosity at a given shear rate. Sucrose increased the viscosity of β-glucans at low concentrations and at low shear rates, when the molecules are less oriented (Fig. 2) . Elfak et al. (1977) reported a similar effect for guar gum and locust bean gum solutions and suggested that added sugars at higher polymer concentrations restrict the hydration and extension of polymer molecules, thus causing a decrease in viscosity. In dilute gum solutions the increased polymer-polymer interactions compensate for the reduction in hydration. At 0.5% β-glucan concentrations, sucrose increased the viscosity by up to 50% (Fig. 3). At 75% sugar solutions the solubility of β-glucans was restricted, explaining the lower viscosity.

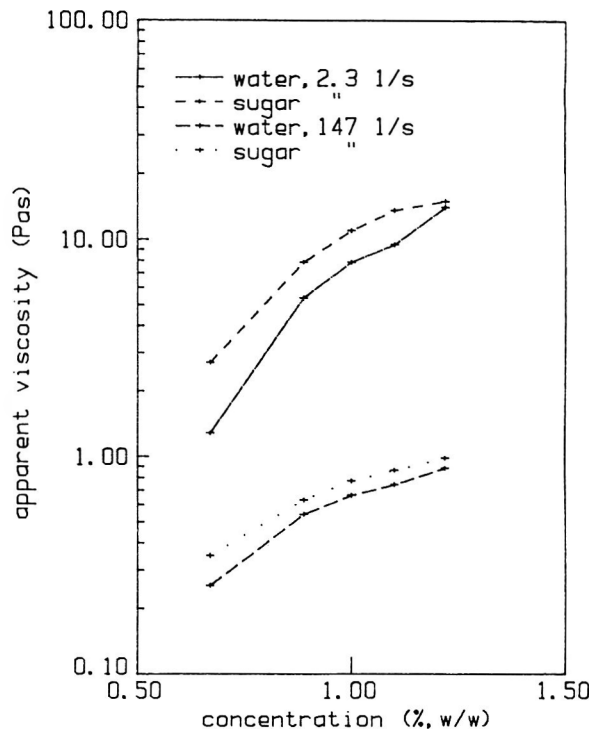

Fig. 2—Effect of β-glucan concentrations without and with sugar (25%) on apparent viscosity at two shear rates (1/sec).

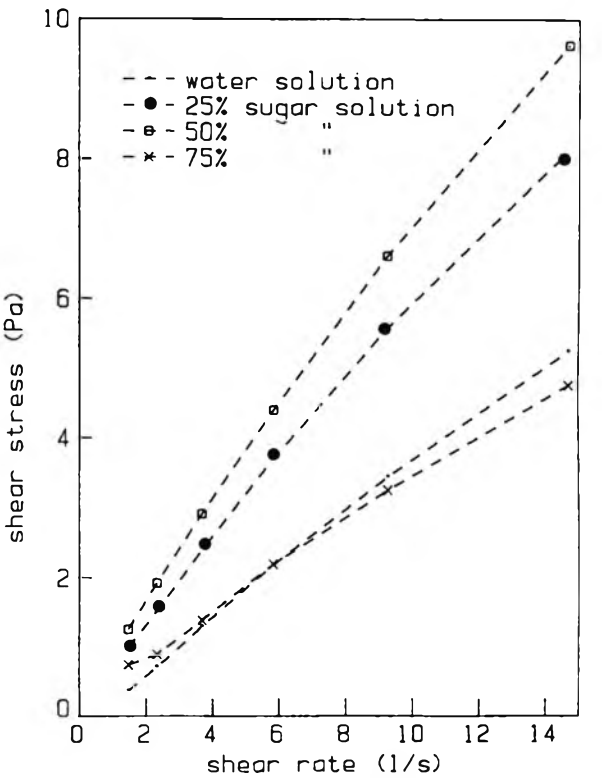

Fig. 3—Shear rate-shear stress diagram at different sugar solutions (β-glucan concentration 0.50%).

For β-glucan solutions the consistency index decreased from a value of 3.3 to 0.4 $N \cdot s^n/m^2$ while the flow behavior index increased from 0.53 to 0.79 during heating (140 hr, 100°C).

Table 2—Activation energies, frequency factors and coefficients of determination for β-glucans

	Shear rate					
	92.3 1/sec			232 1/s		
Conc[a] (%, w/w)	E (kcal/mol)	A (mPas)	r^2	E (kcal/mol)	A (mPas)	r^2
In water solution						
0.6	4.61	0.067	0.996	3.71	0.20	0.991
0.8	3.96	0.369	0.990	3.17	0.86	0.990
1.0	3.10	3.04	0.992	2.41	5.45	0.993
In 25% sucrose solution						
0.6	4.27	0.157	0.995	3.53	0.36	0.995
0.8	3.76	0.633	0.996	3.03	1.30	0.998
1.0	3.50	1.790	0.996	2.80	3.25	0.997

[a] Concentration of β-glucan in powder.

The D-value was found to be 145 hr and first order reaction constant 0.0159 hr^{-1}. Comparing the magnitudes of the D-values with those reported for guar gum and CMC-solutions, about 50 and 150 min, respectively (Rao et al., 1981), we note that β-glucan solutions are much more stable against heat-treatment.

The NaCl-concentrations studied (0–10%) had no effect on the power law constants of β-glucans, as would be expected for neutral polymers.

The Arrhenius model described well the effect of temperature on apparent viscosity at all concentrations. The activation energies, frequency factors and coefficients of determination are shown in Table 2 for both β-glucan- and β-glucan-sugar (25%) solutions at two shear rates. The magnitude of activation energy, E_a (kcal/mol) were dependent on the concentration and shear rate. E_a was high for β-glucans, indicating that temperature has a major effect on apparent viscosity. 25% sugar decreased the magnitudes of E_a at lower polymer concentrations (0.6 and 0.8%).

CONCLUSIONS

AT A POWDER CONCENTRATION of 1.0% β-glucans have a low flow behavior index and a high consistency index suggesting a good mouthfeel. The addition of sugars improves the pseudoplasticity of β-glucans, particularly at low polymer concentrations. The D-value for β-glucans was 145 hr indicating a good heat stability. The viscosity of β-glucan solutions temporarily decreased with increased temperature, suggesting that β-glucans might have applications in products in which high fluidity at elevated temperatures is desired followed by thickening on cooling.

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Ms received 9/18/86; revised 3/10/87; accepted 3/23/87.

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produce bran which would be in the form of pellet. The solvent extraction time to reach 1% residual oil in the extruded bran significantly reduced because of the 10-fold increase of percolation rate of miscella through the bed of extruded bran pellet.

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Ms received 10/6/86; revised 4/6/87; accepted 4/14/87.

Authors would like to thank Dr. J. M. Harper, Vice President for Research, Colorado State Univ., for valuable comments and English correction in preparing this manuscript.

Effect of Form of Enrichment and Iron on Thiamin, Riboflavin and Niacinamide, and Cooking Parameters of Enriched Spaghetti

P. T. BERGLUND, J. W. DICK, and M. L. DREHER

ABSTRACT

The effect of encapsulation, iron and low (LT) or high (HT) temperature drying on thiamin, riboflavin and niacinamide concentrations and on cooked spaghetti parameters of enriched spaghetti were studied. Thiamin, riboflavin and niacinamide were greater in the encapsulated enriched spaghetti. Cooked spaghetti had lower cooked weight but greater firmness when enriched with encapsulated vitamins. Thiamin, niacinamide and cooked weight were greater in cooked spaghetti when iron was added, but spaghetti was less firm. High temperature drying resulted in lower riboflavin and niacinamide in dried spaghetti but did not affect vitamin concentrations in cooked spaghetti. High temperature drying caused less cooking loss and greater firmness in cooked spaghetti.

INTRODUCTION

PASTA PRODUCTS in the United States are commonly enriched according to requirements detailed in the Federal Register (1980). However, Dexter et al. (1982) found that riboflavin in spaghetti was lost during processing and that all three B vitamins (thiamin, riboflavin and niacin) were rapidly lost during cooking. Woodcock et al. (1982) found that under experimental conditions used in their research, more than 50% of the riboflavin in macaroni was lost within one day due to light intensity and increased temperature. Furuya and Warthesen (1984) further studied riboflavin and reported that pasta packaged in paperboard cartons with or without transparent windows had approximately 100% riboflavin retention after one week compared to about 70% retention in pasta packaged in transparent bags. Ranhotra et al. (1985) analyzed pasta products for six B vitamins, including thiamin, riboflavin and niacin and concluded that one-half to three-fourths of the vitamins were retained following cooking. Iron is always added along with thiamin, riboflavin and niacin in enriched pasta (Federal Register, 1980). Therefore, it would be interesting to note whether the omission of iron has any effect on vitamin retention in cooked pasta as compared with the usual practice of iron addition.

Encapsulation, a particle-coating process, protects nutrients from heat, moisture and other materials and ensures maximum effectiveness in formulated foods (Anonymous, 1981). If encapsulation does protect nutrients added in enrichment from retrogradation and leaching during processing and cooking, it may be possible that encapsulated nutrients in dried or cooked spaghetti would be retained to a greater degree than unprotected or nonencapsulated forms of the nutrients. These authors found no studies reported on the use of encapsulated nutrients in enriched pasta.

Consideration must also be given to the effect of the added nutrients on sensory qualities of the food to which they are added (Quick and Murphy, 1982). Dexter et al. (1982) reported that the addition of an enrichment mixture at 10 mg/100g had little effect on spaghetti color properties, but the enrichment mixtures at 30 mg/100g altered color for spaghetti dried at low temperature and two high temperature cycles. Matsuo and Dexter (1980) concluded that increased surface dullness and browness were likely due to the iron contained in the enrichment mixture. Spaghetti cooking quality did not appear to be affected by enrichment.

High temperature drying of pasta products has been discussed by numerous workers (Anonymous, 1979; Dexter et al., 1981, 1982, 1983, 1984; Pavan, 1979, 1980; Resmini and Pagani, 1983; Wyland and D'Appolonia, 1982). Major emphasis, however, has been on bacteriological and quality aspects of spaghetti dried by high temperature. Dexter et al. (1982) reported one of the few studies concerning effect of high temperature drying on stability of vitamins in enriched pasta.

The purpose of this study was to relate vitamin encapsulation, iron addition or deletion, spaghetti drying temperatures and cooking to concentrations and retention of thiamin, riboflavin and niacinamide in cooked spaghetti enriched with encapsulated and unprotected vitamins and to cooked spaghetti quality.

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MATERIALS & METHODS

Enriched semolina

Commercial semolina, No. 1 Durakota, was obtained from the North Dakota State Mill and Elevator (Grand Forks, ND). All vitamins added in enrichment were obtained from Hoffman-LaRoche, Inc., Nutley, NJ. Ferrous sulfate, 100% potency, was obtained from Pennwalt Flour Service, Plymouth, MN. All nutrients met the upper limit of Federal Register requirements for enriched spaghetti on a 10.0% moisture basis (spaghetti, as purchased). The unprotected nutrients, standard vitamins, U.S.P., all 100% potency, were added as follows per batch (1570.9g semolina): thiamin mononitrate, 16.61 mg; riboflavin, 7.31 mg; niacinamide, 112.9 mg; and iron, 54.8 mg. The encapsulated vitamins, Rocoat formulations at 33% potency, were added in triple amount per batch to achieve 100% potency. The treatments were as follows: (1) encapsulated vitamins with iron; (2) encapsulated vitamins without iron; (3) unprotected vitamins with iron; and (4) unprotected vitamins without iron. All nutrients were blended thoroughly into approximately 200g preweighed semolina which was then blended with remaining semolina in a V blender.

Table 1—HPLC conditions for thiamin, riboflavin and niacinamide analyses

	Thiamin	Riboflavin	Niacinamide
Mobile phase	Isocratic Phase A	Gradient[a]	Isocratic Phase A
Detector	Fluorescence 364 nm excitation 425 nm emission	Fluorescence 450 nm excitation 495 nm emission	Ultra Violet 254 nm
Attenuation	64	64	.05
Flow rate (mL/min)	2.5	2.5	1.6
Chart speed (cm/min)	0.5	0.5	0.5
Injection (μL)	25	25	25
Retention time (min)	4.00	12.50	4.15

[a] Gradient conditions were as follows: initial, 100% Phase A; 11 min, 100% Phase B, curve 9; 15 min, 100% Phase A, curve 6

Table 2—Effect of enrichment form, iron, drying temperature and cooking on thiamin retention in enriched spaghetti (95% confidence interval n = 10)

	Encapsulated				Unprotected			
		Percent retention[a]				Percent retention[a]		
Treatment	mg/100g Mean[b]	Mean[b]	Std. error of mean	Confidence interval	mg/100g Mean[b]	Mean[b]	Std. error of mean	Confidence interval
Enriched semolina	1.1642				0.9974			
LT dry	0.8834	75.88	2.46	70.32-81.44	0.8884	89.07	2.02	84.47-93.65
HT dry	0.9158	78.66	1.94	74.27-83.05	0.8206	82.27	0.80	80.46-84.07
LT cooked								
With iron	0.4992	42.88	1.71	39.02-46.74	0.3849[c]	38.59[c]	2.87	31.98-45.20
Without iron	0.3766	30.23	1.59	26.64-33.82	0.4032	42.34	1.53	38.89-45.79
HT cooked								
With iron	0.4610	39.60	1.08	37.15-42.05	0.4074	40.85	2.65	34.86-46.84
Without iron	0.4440	35.64	1.16	33.01-38.27	0.3952[c]	41.50[c]	2.26	34.26-46.71

[a] Percent retention = $\frac{\text{Thiamin in sample (after processing)}}{\text{Thiamin in enriched semolina}} \times 100$

[b] Expressed on a dry basis

[c] n = 9

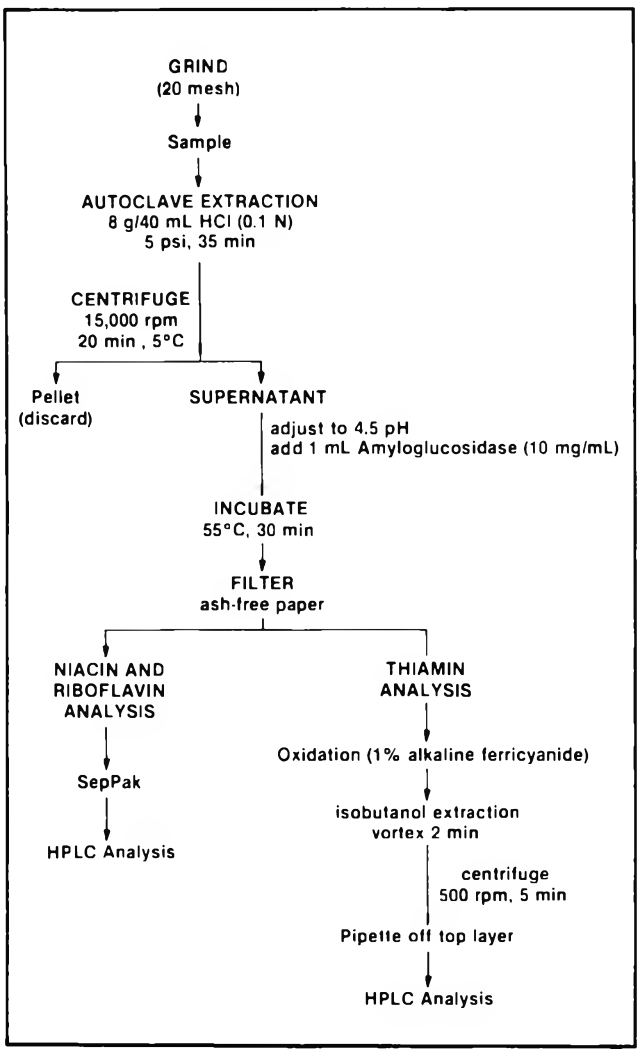

Fig. 1—Flow chart for vitamin analyses.

Table 3—Duncan's Multiple Range Test for thiamin, riboflavin and niacinamide levels in enriched spaghetti

	Mean[a] (mg/100g)		
Treatment	Thiamin[b]	Riboflavin[c]	Niacinamide[c]
Effect of enrichment form			
Encapsulated	0.7847[x]	0.2155[x]	3.3584[x]
Unprotected	0.7062[y]	0.1607[y]	3.1259[y]
Effect of iron in cooked spaghetti			
With iron	0.4395[x]	0.0984[x]	1.4925[x]
Without iron	0.4050[y]	0.0960[x]	1.3457[y]
Effect of drying temperature and cooking			
Enriched semolina	1.0808[x]	0.3068[w]	5.8339[w]
LT dry	0.8859[y]	0.2323[x]	4.1090[x]
HT dry	0.8682[y]	0.1995[y]	3.3021[y]
LT cooked	0.4451[z]	0.0967[z]	1.5039[z]
HT cooked	0.4342[z]	0.0953[z]	1.4791[z]

[a] Within each vitamin and each effect, values with the same letter are not significantly different ($P \geq 0.05$)

[b] n = 50 for effect of enrichment form;
n = 39 for iron; n = 20 for drying temperature and cooking.

[c] n = 30 for effect of enrichment form; n = 24 for iron;
n = 12 for drying temperature and cooking.

Spaghetti processing

Spaghetti was processed on a semicommercial (11.35 kg) pasta extruder (DeMaco) under the following conditions: extrusion speed, 20 rpm; jacket water temperature, 47°C; absorption, 31.5%; and vacuum, 457 mm of Hg.

Spaghetti was case-hardened prior to drying at either low or high temperatures in an experimental pasta dryer (Gilles et al., 1966). In the low temperature (LT) procedure, spaghetti was dried at 40°C, with the relative humidity decreasing linearly from 95 to 60% during the entire 18 hr drying cycle. The high temperature (HT) procedure utilized an initial temperature of 50°C with 95% relative humidity for 2 hr. Temperature was then increased to 70°C over 2 hr and held constant for 6 hr with relative humidity at 84% followed by dry-down at 60% relative humidity until equilibrium was obtained. The glass on the dryer doors was covered with brown paper in an attempt to minimize exposure to light. After drying cycles were completed, dried samples were wrapped in brown paper and stored up to two months under ambient room conditions in the dark until further analysis.

Spaghetti cooking

A modification of the procedure described by Dick et al. (1974) was used for cooking. Ten grams spaghetti were broken into lengths of approximately 5 cm and cooked in 300 mL boiling distilled water. Spaghetti was cooked to optimum cooking time, defined as the time required for the white core in the strands to disappear (12 min). Time for disappearance of the core had been established in a preliminary cooking by removing strands from the cooking water at intervals and crushing them between two glass plates. After cooking, the samples were allowed to drain in a Buchner funnel for 2.5 min.

Vitamin analyses

All cooked samples were freeze-dried in a shelf freezer dryer (Model USM-15, The Vitris Co., Gardiner, NY) for approximately 27 hr. Samples were prepared for vitamin analyses according to the flow chart presented in Fig. 1. All procedures were carried out in subdued light and storage was in foil-covered containers. The extraction procedure used was a modification of the AOAC (1980) Method 43.033 and methods used by Toma and Tabekhia (1979) and Kamman et al. (1980). A modification of the method described by Kwok et al. (1981) was used for analyses of thiamin, riboflavin and niacinamide by high performance liquid chromatography (HPLC) (Waters Associates, Milford, MA). The operating conditions for each vitamin are shown in Table 1. Mobile phase A consisted of 0.0025 M 1-hexanesulfonic acid

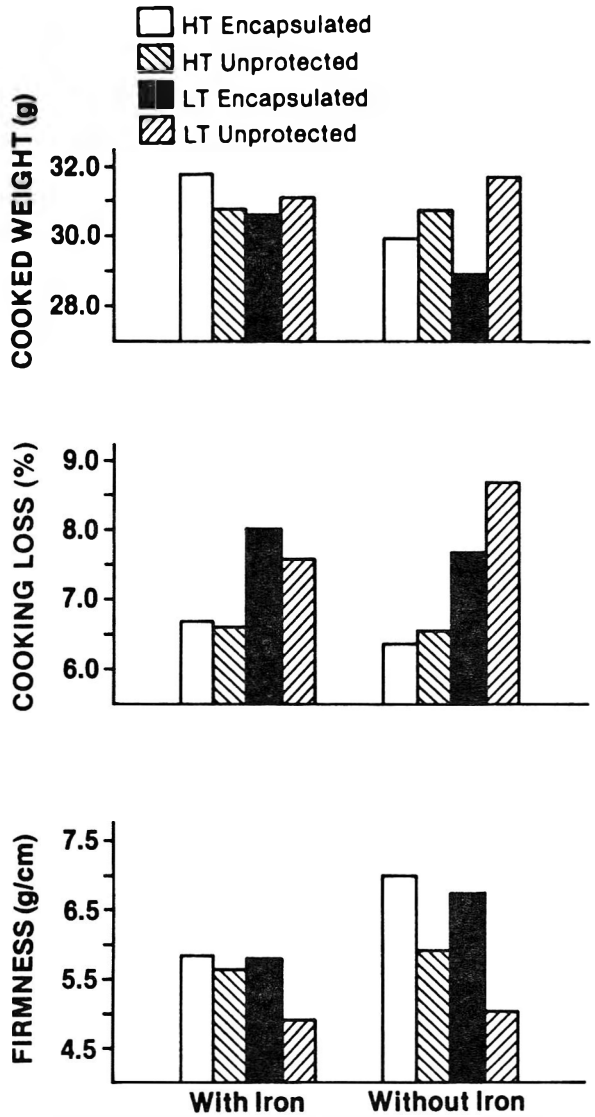

Fig. 2—Cooked spaghetti parameters: Effect of iron, drying temperature and enrichment form.

(B-6 PIC reagent, low UV, Waters Associates, Milford, MA) in 0.5% acetic acid. Mobile phase B consisted of 80% methanol. Prior to use, the mobile phases were filtered through a 0.45 um Fluoropore filter and degassed by vacuum. Standard curves for thiamin and niacinamide were developed by triplicate injections of working standard solutions of concentrations of 0.5, 1.0 and 2.0 μg/mL and 2.5, 5.0 and 10.0 μg/mL, respectively. Standard solutions for thiamin were oxidized to thiochrome prior to analysis by HPLC. The K (means of observations: μg/mL/area for thiamin and μg/mL/height for niacinamide) of each standard curve was determined and used to calculate the concentration of unknown samples by the following equations:

Thiamin

$$\text{Area of sample } (\mu g/mL) \times K \times \frac{43 \text{ mL}}{8 \text{ g}} \times \frac{1 \text{ mg}}{1000 \ \mu g} \times 100 = \text{mg}/100\text{g}$$

Niacinamide

$$\text{Ht of sample } (\mu g/mL) \times K \times \frac{43 \text{ mL}}{8g} \times \frac{1 \text{ mg}}{1000 \ \mu g} \times 100 = \text{mg}/100\text{g}$$

A working standard solution of 0.4 μg/mL was used for riboflavin. The Waters Associates data module calculated the response factor for riboflavin. The following equation was used for calculation of sample concentration of riboflavin:

$$\text{HPLC value } (\mu g/mL) \times \frac{\mu L \text{ Std injected}}{\mu L \text{ Sample injected}} \times \frac{43 \text{ mL}}{8g} \times \frac{1 \text{ mg}}{1000 \ \mu g} \times 100 = \text{mg}/100\text{g}$$

Spaghetti quality tests

Visual differences in spaghetti color were noted and compared to spaghetti samples of known color values as determined by a Hunterlab D-25 difference meter. Cooked weight was determined by weighing the drained spaghetti and was reported in grams. To determine cooking loss, the combined cooking and wash water was collected in a preweighed beaker, placed in an air oven at 100°C and evaporated to dryness. The residue was weighed and reported as percent dry spaghetti. Spaghetti firmness was measured with an Instron Universal tester by shearing two cooked spaghetti strands at a 90° angle with a specially designed plexiglass tooth as described by Walsh (1971). The force required to shear the spaghetti was measured and the results were reported as g cm. A higher value indicates a firmer product.

Analysis of data

Encapsulated and unprotected vitamins were the two forms of thiamin, riboflavin and niacinamide used to enrich the spaghetti. In addition to analyzing enriched semolina, the processing and cooking treatments studied were spaghetti dried at either low or high temperature both in the dry and cooked forms.

The General Linear Model (GLM) procedure of the Statistical Analysis System (SAS, 1982) computer package was used to perform statistical analyses. Differences in concentrations and retention of thiamin, riboflavin and niacinamide in the spaghetti due to addition or deletion of iron, drying temperature, cooking and form of enrichment were tested for significance using analysis of variance techniques. Additionally, differences in cooked weight, cooking loss and firmness of cooked spaghetti samples due to iron addition or deletion, drying temperature and form of enrichment were tested for significance using analysis of variance techniques. Duncan's Multiple Range Test was used as the a posteriori multiple comparisons test when the analysis of variance

Table 4—Effect of enrichment form, iron, drying temperature and cooking on riboflavin retention in enriched spaghetti (95% confidence interval n = 6)

	Encapsulated				Unprotected			
		Percent retention[a]				Percent retention[a]		
Treatment	mg/100g Mean[b]	Mean[b]	Std. error of mean	Confidence interval	mg/100g Mean[b]	Mean[b]	Std. error of mean	Confidence interval
Enriched semolina	0.3445				0.2690			
LT dry	0.2312	67.10	1.88	62.26-71.94	0.2334	86.74	1.21	83.64-89.84
HT dry	0.2240	65.02	1.24	61.83-68.22	0.1750	65.06	1.11	61-96-68.16
LT cooked								
With iron	0.1292	37.50	1.06	34.76-40.22	0.0615	22.86	0.89	20.57-25.15
Without iron	0.1263	37.99	0.97	35.51-40.47	0.0593	27.03	0.56	25.59-28.47
HT cooked								
With iron	0.1285	37.30	0.95	36.71-39.74	0.648	24.10	0.26	23.43-25.44
Without iron	C.1450	43.71	1.43	40.04-47.38	0.625	28.47	0.67	26.74-30.20

[a] Percent retention = $\frac{\text{Riboflavin in sample (after processing)}}{\text{Riboflavin in enriched semolina}} \times 100$

[b] Expressed on a dry basis

Table 5—Effect of enrichment form, iron, drying temperature and cooking on niacinamide retention in enriched spaghetti (95% confidence interval n = 6)

	Encapsulated				Unprotected			
		Percent retention[a]				Percent retention[a]		
Treatment	mg/100g Mean[b]	Mean[b]	Std. error of mean	Confidence interval	mg/100g Mean[b]	Mean[b]	Std. error of mean	Confidence interval
Enriched semolina	6.3757				5.2922			
LT dry	4.1817	65.59	1.23	62.43-68.75	4.0363	76.27	2.03	71.04-81.50
HT dry	3.5982	56.44	2.73	49.42-63.46	3.0060	56.80	1.14	53.87-59.73
LT cooked								
With iron	1.3663[c]	21.43[c]	0.52	20.15-22.71	1.6645	31.45	2.24	25.68-37.22
Without iron	1.2943	18.36	1.00	15.78-20.94	1.7488	31.45	1.53	27.52-35.38
HT cooked								
With iron	1.6020	25.13	0.74	23.20-27.03	1.3316[d]	25.16[d]	0.93	22.58-27.74
Without iron	1.1898	16.88	1.19	13.81-19.95	1.1497	20.67	1.85	15.90-25.43

[a] Percent retention = $\frac{\text{Niacinamide in sample (after processing)}}{\text{Niacinamide in enriched semolina}} \times 100$

[b] Expressed on a dry basis

[c] n = 7

[d] n = 5

Table 6—Effect of enrichment form, iron and drying temperature on cooked spaghetti parameters

	Mean[a]		
Treatment	Cooked wt (g)	Cooking loss (%)	Firmness (g cm)
Effect of enrichment form			
Encapsulated	30.19[x]	7.19[x]	6.34[x]
Unprotected	31.08[y]	7.37[x]	5.38[y]
Effect of iron			
With iron	31.07[x]	7.23[x]	5.53[x]
Without iron	30.20[y]	7.33[x]	6.19[y]
Effect of drying temperature			
HT cooked	30.67[x]	6.55[x]	6.09[x]
LT cooked	30.60[x]	8.01[y]	5.62[y]

[a] Within each cooking parameter and each effect, values with the same letter are not significantly different (P ≥ 0.05) according to Duncan's Multiple Range Test.

indicated significant difference in means. A level of significance of $P \leq 0.05$ is used throughout the analysis unless otherwise noted.

In order to compare across subsets of data to make multiple comparisons on vitamin retention, confidence intervals were calculated. Each individual comparison is a 95% confidence interval, however, the overall confidence attached to the confidence interval (experimentwise error rate) is probably lower than 0.95 and thus the overall error is higher than 0.05. Calculations for percent retention in Tables 2, 4, and 5 were based on vitamin levels of mg/100g in enriched semolina analyzed prior to further processing.

RESULTS & DISCUSSION

Thiamin

Enrichment form, iron, drying temperature and cooking had significant effects on thiamin retention ($P < 0.001$). Results from Duncan's Multiple Range Test are shown in Table 3. When values from enriched semolina and both drying temperature and cooked samples were combined, thiamin was greater ($P < 0.001$) in spaghetti enriched with encapsulated thiamin. Thiamin concentrations were greater ($P < 0.01$) in cooked spaghetti enriched with iron. Spaghetti dried at either temperature had similar but lower thiamin concentrations than the enriched semolina. Cooked spaghetti contained the least thiamin. These data are in agreement with Dexter et al. (1982) who also found that thiamin decreased ($p < 0.01$) during cooking. There were no differences in thiamin concentrations in cooked spaghetti dried either at LT or HT.

Differences in retention of thiamin were noted at the 95% confidence level for individual comparisons (Table 2). There was a greater percentage of unprotected thiamin retained in LT dried spaghetti than in HT dried samples. There were no differences in unprotected and encapsulated thiamin concentrations of HT dried spaghetti or in cooked spaghetti dried at HT.

The retention of encapsulated thiamin in LT dried, cooked spaghetti, without iron was less than that of unprotected thiamin with the same treatment; it was also less than thiamin retention in the encapsulated with iron, LT dried, cooked spaghetti. Spaghetti cooked for 12 min retained only 38–49% thiamin in either form compared to 75–89% retained in the dried samples. Dexter et al. (1982) found that the mean value of thiamin retained after 12 min of cooking was about 40% of that originally present in the uncooked spaghetti.

Riboflavin

Enrichment form, drying temperature and cooking affected riboflavin retention ($P < 0.001$); iron did not. The results from Duncan's Multiple Range Test for riboflavin are shown in Table 3. Encapsulated samples had greater ($P < 0.001$) riboflavin concentrations than did unprotected samples. Riboflavin concentrations were similar, however, in cooked spaghetti enriched with and without iron. The spaghetti dried at LT had more riboflavin than that dried at HT, although when samples dried at LT were separated by enrichment forms (Table 4), the encapsulated LT dried samples had similar concentrations of riboflavin as samples dried at HT. The reason for the higher concentration of riboflavin in the unprotected LT dried spaghetti compared to other dried samples cannot be explained by these authors. Dexter et al. (1982) also found that riboflavin was lost during all drying cycles, particularly during both HT cycles utilized in their study. Cooked samples had the least riboflavin. There was no difference in riboflavin concentrations in cooked spaghetti dried at LT or HT.

Differences in retention of encapsulated and unprotected riboflavin were noted at the 95% confidence level (Table 4) for all treatments except HT dried spaghetti. There were no differences in unprotected or encapsulated riboflavin concentrations for HT dried spaghetti. Retention of encapsulated riboflavin in cooked samples dried at both temperatures was greater than that of unprotected riboflavin, indicating that encapsulation might render riboflavin less accessible to solubilization in the cooking water or might protect riboflavin somewhat from ultraviolet light. It is possible that there was some riboflavin loss due to photodegradation during cooking. Dexter et al. (1982) found that the total riboflavin recovery, including spaghetti and cooking water was less than 60% of the original amount in uncooked spaghetti. The riboflavin retention in unprotected, LT dried, cooked spaghetti, without iron, was slightly greater than that of unprotected riboflavin in the cooked spaghetti with iron dried at the same temperature. The retention of unprotected riboflavin was only slightly greater in samples without the addition of iron. Spaghetti cooked for 12 min retained only about 37% of the encapsulated and 23–28% of the unprotected riboflavin compared with 65–89% for the dried samples. This

concurs with Dexter et al. (1982) who found that only about 30% of the riboflavin in the enriched semolina was retained in spaghetti cooked for 12 min.

Niacinamide

Niacinamide retention was affected by enrichment form, iron, drying temperature and cooking. The results from Duncan's Multiple Range Test for niacinamide are shown in Table 3. The niacinamide was greater ($P < 0.001$) in samples enriched with encapsulated niacinamide and in cooked spaghetti ($P < 0.05$) when iron was added. Dried and cooked samples had less niacinamide than enriched semolina. Spaghetti dried at LT had more niacinamide than spaghetti dried at HT. There was no difference in niacinamide concentrations between cooked samples dried at LT or HT.

Differences in retention (Table 5) of encapsulated and unprotected niacinamide were noted at the 95% confidence level for LT dried and cooked spaghetti samples. The retention of unprotected niacinamide was greater than that of encapsulated niacinamide in LT dried dry and cooked spaghetti. No differences in retention of encapsulated or unprotected niacinamide were observed for HT dried spaghetti. Retention of encapsulated niacinamide was greater in cooked samples when iron was added. The retention of unprotected niacinamide in LT dried, cooked spaghetti without iron was greater than for unprotected niacinamide in HT dried, cooked spaghetti without iron. Conversely, the encapsulated niacinamide retention in HT dried, cooked spaghetti with iron was slightly greater than in LT dried, cooked spaghetti with iron. No differences were noted between encapsulated and unprotected niacinamide retention in HT dried cooked spaghetti with iron. Likewise, the retention of encapsulated and unprotected niacinamide in HT dried, cooked spaghetti without iron, was not different. Niacinamide retained in samples cooked for 12 min ranged from 21–31% compared with 56–76% for dry samples. Dexter et al. (1982) reported that spaghetti cooked for 12 min retained 48% niacin originally present in the uncooked spaghetti.

Spaghetti and cooking quality

Visual comparisons of dried spaghetti to samples of known color value indicated that spaghetti with iron added had a lower average color score (8.1) than spaghetti without iron (8.6). There were no differences in average color scores for spaghetti dried at LT (8.4) or HT (8.4) or for spaghetti enriched with encapsulated (8.4) or unprotected (8.4) vitamins.

Duncan's Multiple Range Test (Table 6) and specific values of cooked spaghetti parameters (Fig. 2) show the effect of form of enrichment, iron and drying temperature on cooked spaghetti parameters. The cooked weight of spaghetti enriched with unprotected vitamins was greater than spaghetti enriched with encapsulated vitamins. Spaghetti enriched with iron had a greater cooked weight than spaghetti without iron. Drying temperature did not affect cooked weight but had a highly significant effect on cooking loss. Enrichment form and iron did not affect cooking loss. Spaghetti dried at HT had less cooking loss than spaghetti dried at LT. Effect of drying temperature on cooked weight and cooking loss confirm earlier reports of Dexter et al. (1981). All three variables affected cooked spaghetti firmness. Firmness scores for encapsulated enriched spaghetti were greater than for spaghetti enriched with unprotected vitamins. Spaghetti which was not enriched with iron had higher firmness scores than spaghetti enriched with iron. High temperature dried cooked spaghetti was firmer than LT dried cooked spaghetti. Wyland and D'Appolonia (1982), Dexter et al. (1981, 1982, 1983) and Manser (1980) reported similar findings regarding spaghetti firmness and drying temperature.

CONCLUSIONS

ALTHOUGH THE THIAMIN, riboflavin and niacinamide concentrations were greater in spaghetti enriched with encapsulated vitamins in this study, the use of encapsulated vitamins for enrichment of pasta is probably not warranted. The encapsulated vitamin concentrations were greater, but it is questionable if they were greater from a nutritional standpoint. The use of encapsulated nutrients would probably not be cost effective because of the greater cost of encapsulated vitamins and the necessity of using triple amounts of encapsulated vitamins due to their potency of 33%. Additionally, the small differences in cooked weight and firmness of encapsulated enriched spaghetti probably does not warrant use of encapsulated vitamins. Concentrations of thiamin and niacinamide in enriched spaghetti were greater when iron was added; riboflavin concentrations were similar regardless of iron addition. Thus, according to this study, the practice of including iron in the enrichment of pasta products increased or had no effect on vitamin concentrations; iron addition did not have a detrimental effect on concentrations of vitamins studied. Cooked spaghetti with iron had increased cooked weight and decreased firmness. Cooked spaghetti samples contained less thiamin, riboflavin and niacinamide than dried, "as purchased" spaghetti. Less than 50% of the three vitamins studied were retained in the cooked spaghetti samples.

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Ms received 8/25/86; revised 3/23/87; accepted 3/23/87.

North Dakota Agricultural Experiment Station, Journal Series No. 1560.

Kinetics of Corn Meal Gelatinization at High Temperature and Low Moisture

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ABSTRACT

The kinetics of corn meal starch gelatinization and melting was investigated. At 25% moisture, in the temperature range 110–140°C, Arrhenius equation parameters were determined by two calculable procedures which agreed well. Activation energy for gelatinization was calculated to be approximately 86.2 kJ g-mole. Further experiments in the moisture range 13.4–34.4% and temperature range 10–165°C, yielded results for endotherm enthalpies and melt temperature which agreed with literature reports.

INTRODUCTION

GELATINIZATION of starch during extrusion or extrusion-like processing is sometimes adopted as a quantifiable reaction to indicate "overall cook." The kinetics of starch gelatinization at high temperature and low moisture is of interest in developing a process model for extrusion or other processes in which starch is gelatinized and melted.

The disordering reactions of starch at sufficient moisture (molar ratio of water to anhydroglucose greater than 14) and low moisture (molar ratio of water to anhydroglucose less than 5) may be differentiated as gelatinization and melting, respectively (Donovan, 1985, 1979). Biliaderis et al. (1986) have elaborated the phenomena occurring during heating in a differential scanning calorimeter to also involve annealing and crystallization.

For potato starch, the gelatinization endotherm is manifested only when the molar ratio of water is greater than 4 (31% w/w water), and reaches a maximum and constant value at a molar ratio of 14 (58% water) (Collison and Chilton, 1974; Donovan, 1979). Reid and Charoenrein (1985) identified the minimum and maximum requirements for water in the gelatinization of corn starch to be 35% and 60% and of modified waxy maize to be 42% and 64%, respectively. Eliasson (1980) stated that the minimum water necessary for gelatinization of wheat starch is 33% w/w (approximately 4 molar ratio). Wootton and Bamunuarachchi (1979b) reported on three types of maize and wheat starches and listed the minimum moistures for gelatinization as 31% (w/w) for wheat, 31% for maize, 32% for waxy maize, and 34% for amylomaize. From the preceding literature, it is clear that the production of food by extrusion which uses meals with less than 30% moisture is conducted at a moisture level below the minimum needed for complete gelatinization.

The enthalpies of reaction of the gelatinization and melting endotherms have been shown to depend on the moisture content of the starch. Eliasson (1980) showed for wheat starch that the ΔH for the gelatinization endotherm increases up to a maximum value at sufficient water content and then is approximately constant, whereas the ΔH for the melting endotherm increases at low water content and then decreases again as sufficient water is provided for gelatinization to predominate. Donovan (1979) found the same phenomenon for the melting endotherm of potato starch, with the ΔH maximized at a molar ratio of about 4. For wheat starch, Eliasson (1980) reported an increase in gelatinization enthalpy from zero at 33% water up to about 13.8 J/g at 70% water. The melting endotherm reached a maximum of about 8.4 J/g at 45% water (approximately 7 molar ratio) and was zero at about 30% water and 90% water. Wootton and Bamunuarachchi (1979b) reported enthalpies for gelatinization of wheat starch ranging from 0.84 J/g at 33% water up to 19.7 J/g at 67% water. Differences between Eliasson's (1980) results and the results of Wootton and Bamunuarachchi (1979b) could be due to calorimeter scan rates or sample preparation. Stevens and Elton (1971) and Wootton and Bamunuarachchi (1979a) have reported enthalpies of gelatinization of various wheat starches ranging from 10.1 J/g to 19.7 J/g, of various maizes from 15.5 J/g to 31.8 J/g, of potatoes from 21.4 J/g to 27.6 J/g and rice from 14.2 J/g to 16.3 J/g. Biliaderis et al. (1980) reported enthalpies of gelatinization of various legumes from lows of 5.86 J/g (lentil), 11.3 J/g (pea) and 11.7 J/g (bean) at about 33% moisture to highs of 14.2 J/g (lentil), 14.7 J/g (pea) and 21.8 J/g (bean) at approximately 81% moisture.

The gelatinization endotherm for potato starch occurs at approximately 66°C, and the melting endotherm occurs at higher temperature (Donovan, 1979). The temperature of the melting endotherm for potato starch was found to depend upon moisture content such that the melting temperature increased as moisture decreased. Biliaderis et al. (1980) reported that for legume starches a lower temperature endotherm existed which depended on legume variety but which did not vary with moisture and that a second higher temperature endotherm existed. The second peak became predominant at low water contents. The lower temperature endotherm would correspond to gelatinization, and the higher temperature peak to melting.

The Flory-Huggins equation has been used to analyze the melting phenomenon of starch as a first-order phase transition between crystalline and amorphous states. The equation relates melting point of a polymer, heat of fusion of polymer and molar volumes of repeat unit and diluent (Lelievre, 1973). Gelatinization and melting processes do not satisfy the equilibrium thermodynamics assumption of Flory-Huggins theory (Slade and Levine, 1984), but nevertheless the equation is useful in describing the dependence of melting temperature on moisture (Biliaderis et al., 1986). Extrapolation of melting temperature to zero volume fraction of diluent gives the melting point of the most perfect crystallites. Literature values for the melting point of perfect crystallites for various starches are: wheat 210°C (Lelievre, 1973), wheat 204°C and 227°C (Burt and Russell, 1983), potato 168°C (Donovan, 1979), lentil 166°C, smooth pea 194°C, and adzuki bean 203°C (Biliaderis et al., 1980). The Flory-Huggins equation is used in this study to estimate the melting point of corn meal starch crystallites.

The kinetics of gelatinization has been reported on systems in which water is in excess, such as would be the case in rice cooking. For such cases, the temperatures investigated are less than 100°C which are much below the temperatures experienced in extrusion processing. Pseudo-first order kinetics has been assumed for starch gelatinization (Lund, 1984) although gelatinization is probably more correctly modeled as a set of reactions (Reid and Charoenrein, 1985). The Arrhenius model

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has been applied by Kubota et al. (1979), Bakshi and Singh (1980), Wirakartakusumah (1981), and Pravisani et al. (1985) to calculate an activation energy for gelatinization. Some studies use data taken at temperatures at which the extent of gelatinization is dependent on temperature, which is a dependence not strictly in accord with the Arrhenius model. Complete gelatinization is achieved by about 75°C (Lund, 1984). The study reported herein also uses an Arrhenius model to calculate kinetic parameters based on pseudo-first order reaction, but since the lowest temperature investigated is 80°C at which gelatinization is complete at sufficient time it is believed that the constraint concerning full extent of gelatininzation is satisfied.

Kubota et al. (1979) reported an activation energy (E_a) for gelatinization of rice starch of 58.6 kJ/g-mole which they believed was in agreement with their previous result of 79.5 kJ/g-mole. Bakshi and Singh (1980) reported E_a values of 77.6 kJ/g-mole below 85°C and of 43.8 kJ/g-mole above 85°C for rough rice and values of 103.3 kJ/g-mole below 85°C and 40.1 kJ/g-mole above 85°C for brown rice. Wirakartakusumah (1981) found E_a equal to 104.7 kJ/g-mole for rice by an isothermal method and E_a values in the range of 184.2 kJ/g-mole to 305.6 kJ/g-mole by a nonisothermal method. Pravisani et al. (1985) found E_a equal to 820.6 kJ/g-mole below 67.5°C and to 243.7 kJ/g-mole above 67.5°C for potato slices.

The present study was undertaken to provide starch gelatinization kinetics information which could subsequently be combined with shear effects, a subject of further study, to provide an overall model of extrusion cooking of starch-based foods with time and temperature, shear, and interaction effects. The experimental methods to be described do not include a shear component. Shear is an important contributor to starch degradation (Diosady et al., 1985) in certain extrusion operations. Mercier and Feillet (1975) showed that in the presence of high pressure and shear one can achieve increased starch susceptibility to α-amylase down to a moisture of 16.8% for corn-grits which is below the water content reported by other researchers as the minimum water needed for gelatinization in the absence of shear. While many extrusion operations involve significant shear, certain other extrusion operations rely on high pressure and high temperature cooking, rather than high shear, to gelatinize starch (Enterline, 1982). Preconditioning, which is generally a time-temperature reaction between dry feed and added moisture without much shearing action prior to extrusion, has been reported as being important in starch cooking (Harper, 1986). The results of this paper are applicable to preconditioning of ground starch meals for extrusion. Both Thompson et al. (1976) and Phillips et al. (1983) have conducted studies directed toward providing information on extrusion processing by using non-shear experimental means described by Thompson et al. (1976) as "extrusion-like". This research uses an experimental approach similar to Thompson et al. (1976) and Phillips et al. (1983), with certain modifications to increase heat transfer rate and utilize differential scanning calorimetry (DSC).

THEORY

THE LAG TIMES due to heating corn meal up to a constant reaction temperature, and to cooling it therefrom, require that a correction be made in data analysis to account for the fact that temperature increases and decreases were not step changes. Pseudo first-order kinetics was assumed, since the lowest temperature investigated (80°C) was above gelatinization temperature and experiments showed that gelatinization was complete (as measured by DSC) with sufficient time. A condition which is needed for first-order kinetics to be applied is that the extent of reaction is not a function of temperature (Reid and Charoenrein, 1985; Lund, 1984). Lund (1984) has reviewed other work in which pseudo first-order kinetics was assumed.

Lund and Wirakartakusumah (1984) proposed a data correction which adjusts for gelatinization extent being a function of temperature and for the first-order assumption to apply after the lag period. Rather than using the integrated form of the first-order reaction equation as:

$$\ln = \frac{[UG_t]}{[UG_i]} = kt \tag{1}$$

they modified the equation to incorporate the ultimate amount of ungelatinized starch at a given temperature condition $[UG_f]$ and the ungelatinized starch content at lag time $[UG_l]$ as:

$$\ln \frac{[UG_t - UG_f]}{UG_l - UG_f]} = kt \tag{2}$$

This form of analysis was applied to our data with the lag time taken from heating curves and the ungelatinized starch at lag time calculated from plots of data showing decrease of UG_t with time.

A more rigorous method of determining Arrhenius equation constants from experiments in which heating and cooling lags may be significant was presented by Deindoerfer and Humphrey (1959). The usual Arrhenius equation was assumed:

$$k = k_o \cdot e^{-(E_a/RT)} \tag{3}$$

Equations for hyperbolic, linear, and exponential heating and cooling curves were derived by Deindoerfer and Humphrey (1959). The physical situation of the present experiments corresponded to batch heating and cooling with isothermal heat source and sink, since the oil bath and ice bath used for heating and cooling were so much more massive than the filled thermal death time can (American Can Co. 208 × 006 can) in which reaction took place. Temperature changes in the baths were very small (less than 1°C). Heating and cooling curves for each temperature run were fitted to an equation of the form:

$$T = T_H \left(1 + \frac{T_o - T_H}{T_H} \cdot e^{-(UA/Mc)t} \right) \tag{4}$$

The only adjustable parameter in this equation is (UA/Mc) since the bath and initial temperatures define other parameters. The equation for the cooling curve is analogous to Eq. (4).

Substituting the temperature profile of Eq. (4) into the Arrhenius equation of Eq. (3), and integrating, yields an equation for the amount of reaction (i.e. decrease in ungelatinized starch) during heating:

$$\begin{aligned} \ln\left(\frac{UG_i}{UG_t}\right) = {} & \frac{k_o}{\left(\frac{U\cdot A}{M\cdot c}\right)} \cdot \left[E_1\left(\frac{E_a/(R\cdot T_H)}{1 + \frac{T_o - T_H}{T_H}} \right) \right. \\ & \left. - E_1\left(\frac{E_a/(R\cdot T_H)}{1 + \left(\frac{T_o - T_H}{T_H}\right) \cdot e^{-(U\cdot A/M\cdot c)\cdot t}} \right) \right] \\ & - \frac{k_o \cdot e^{-E_a/(R\cdot T_H)}}{(U\cdot A/M\cdot c)} \cdot \left[E_1\left(\frac{E_a/(R\cdot T_H)}{1 + \frac{T_o - T_H}{T_H}} - \frac{E_a}{R\cdot T_H} \right) \right. \\ & \left. - E_1\left(\frac{E_a/(R\cdot T_H)}{1 + \left(\frac{T_o - T_H}{T_H}\right) \cdot e^{-(U\cdot A/M\cdot c)\cdot t}} - \frac{E_a}{R\cdot T_H} \right) \right] \end{aligned} \tag{5}$$

The function E_1 is defined as:

$$E_1(z) = \int_z^\infty \frac{e^{-x}}{x} dx \tag{6}$$

Table 1—Results of heating corn meal in oil bath (All samples at 25% moisture)

Temperature (°C)	Enthalpy data: Ho d time (min)	Corrected time[a] (min)	Residual enthalpy[c] (J/g)	Heating/Cooling curve[b]: $\frac{T_a - T_{H/C}}{T_{H\,C}}$	(UA/Mc) min^{-1}	r^2
80	3.13	3.25	4.71 (0.24)	−0.155/0.294	0.091/0.072	0.90/0.9
	5.00	5.12	4.64 (0.28)			
	7.58	7.70	4.50 (0.06)			
	10.50	10.62	4.26 (0.40)			
	15.00	15.12	3.99 (0.06)			
	20.16	20.28	3.66 (0.09)			
110	2.63	2.80	2.28 (0.15)	−0.229/0.404	0.056/0.095	0.93/0.91
	4.08	4.25	1.79 (0.06)			
	5.16	5.33	1.52 (0.08)			
130	0.58	0.79	2.51 (0.09)	−0.264/0.469	0.034/0.123	0.98/0.88
	1.42	1.63	0.92 (0.18)			
140	0.62	0.93	1.25 (0.05)	−0.276/0.507	0.049/0.124	0.96/0.94
	0.70	1.01	1.13 (0.06)			

[a] Corrected time is based on $k = 1.28 \times 10^{11} \cdot e^{-(86.19\ kJ/g\text{-}mole)/RT}\ min^{-1}$.

[b] Heating cooling curve results refer to Eq. (4). Numbers on the left of the slant line are for heating curve, and on the right are for cooling curve. r^2 corresponds to fitting of heating cooling data with Eq. (4) and values given.

[c] Amount of residual enthalpy in ungelatinized corn meal is 5.08 (0.26) J/g. Amount of residual enthalpy in corn meal at final heating is 0.50 (0.08) J/g. Values in parentheses are standard deviations of means.

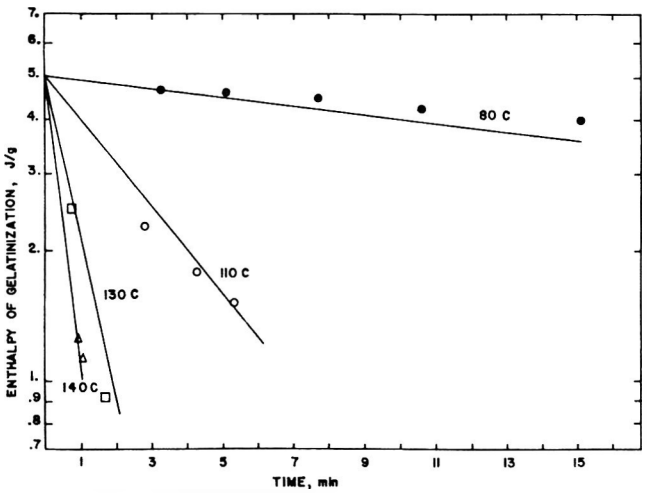

Fig. 1—Data showing decline in ungelatinized starch with heating of corn meal in TDT cans in a temperature controlled oil bath. Lines are calculated from Eq. (9). Moisture = 25%.

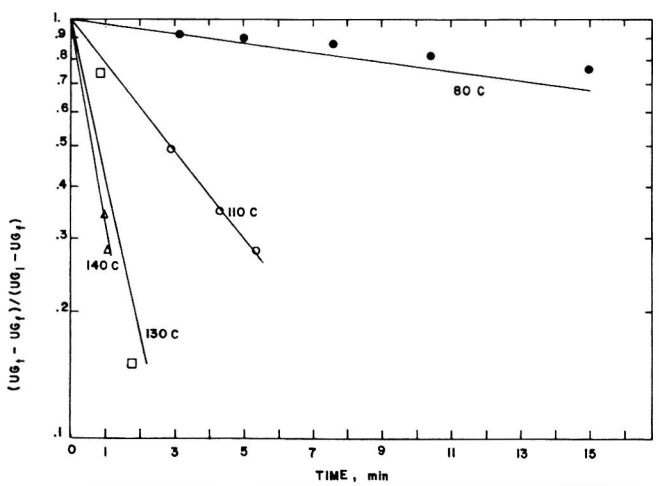

Fig. 2—Data transformed using lag correction of Lund and Wirakartakusumah (1984). Lines are calculated from Eq. (8). Moisture = 25%.

Values for the function E_1 are given by Deindoerfer and Humphrey (1959).

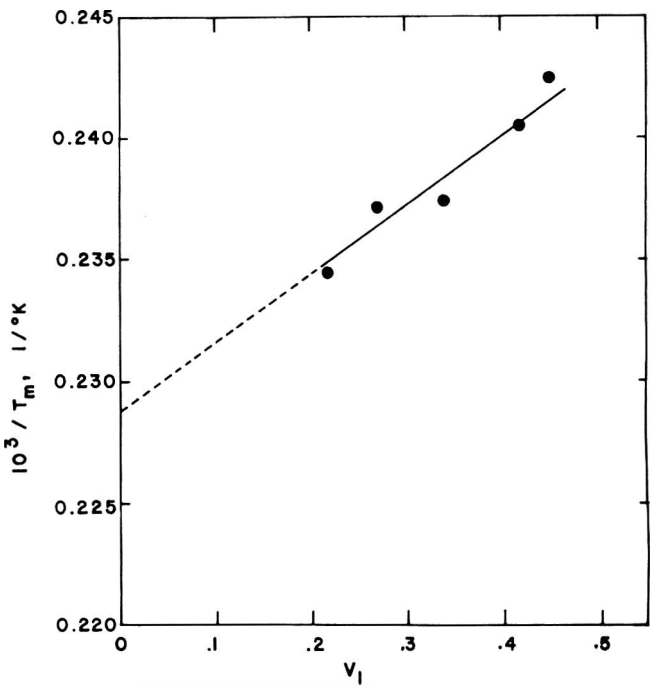

Fig. 3—Reciprocal plot according to Flory-Huggins (Lelievre, 1973) theory, yielding melting temperature of perfect corn meal starch crystallites.

An equation similar to Eq. (5) also applied to the reaction occurring during cooling. To apply Eq. (5), the E_a determined using Eq. (2) was first used and an amount of reaction attributable to heating and cooling was calculated. Then using Eq. (1), the time which would give an equivalent amount of reaction if the reaction had occurred at the constant reaction temperature (i.e., 80, 110, 130, or 140°C) was determined. This time was then added to the actual hold time to give a corrected hold time at each temperature. The calculated amount of reaction occurring during the holding period at constant temperature was determined using the right-hand side of Eq. (1). The calculation of Arrhenius constants was iterated through refinement of the heating and cooling lag effects on the amount of reaction until the constants converged to values which differed by less than 2%.

The DSC-4 (Perkin-Elmer, 1982) system included a kinetics software package. This software is used with controlled tem-

Table 2—Results for heating moisture-adjusted samples in the DSC-4 for kinetic analysis

	Moisture (%)	Temperature range (°C)	Enthalpy J/g	Activation energy kJ/g	Order
Gelatinization peak	13.4	118.2-126.2	0.56 (0.06)[a]	328.9 (50.1)[a]	0.5 (0.1)[a]
	17.0	103.3-129.7	1.11 (0.48)[a,b]	106.6 (65.1)[b,c]	0.6 (0.2)[a,b]
	25.0	84.9-118.9	1.97 (0.63)[b]	99.8 (21.5)[b]	0.7 (0.2)[a,b]
	31.5	87.2-128.7	7.46 (1.95)[c]	144.3 (16.3)[c]	0.8 (0.1)[b]
	34.4	74.5-125.4	9.24 (1.36)[c]	114.0 (8.4)[b,c]	0.8 (0.03)[b]
Melting Peak	13.4	143.9-153.4	0.47 (0.03)[d]	317.5 (86.5)[A]	0.5 (0.1)[A]
	17.0	137.3-148.6	0.52 (0.11)[d]	449.6 (147.2)[A,B]	0.8 (0.2)[B]
	25.0	133.1-148.1	1.44 (0.43)[e]	368.2 (56.3)[A]	0.8 (0.1)[B]
	31.5	131.7-142.7	0.75 (0.14)[d]	368.2 (187.8)[A]	0.8 (0.2)[B]
	34.4	131.4-139.4	0.35 (0.16)[d]	571.9 (73.6)[B]	0.7 (0.2)[A,B]

[a c] Means for each peak with a common superscript letter in a column do not differ significantly. Upper case letters correspond to 0.05 level. Lower case letters correspond to 0.01 level. Means were compared by the Tukey-Kramer method (Bancroft, 1968). Numbers in parentheses are standard deviations of means.

perature ramping, as in the experiments done in this study. The software uses the Arrhenius expression (Equation 3) in conjunction with a general rate equation:

$$\frac{d\alpha}{dt} = k\cdot(1-\alpha)^n \qquad (7)$$

and fits the data by multilinear regression to yield values of k_o, E_a, and n. Strictly, this procedure assumes that multiple reactions are not occurring, which may limit its applicability to gelatinization. The DSC software also gives enthalpy of the endotherm through peak analysis.

MATERIALS AND METHODS

CORN MEAL was purchased from ICN Nutritional Biochemicals (Cleveland, OH, Catalog #901411, Lot #15890). The typical analysis of this degerminated yellow corn meal provided by ICN Nutritional Biochemicals was: carbohydrate, 77.10%; protein, 8.0%; fat, 2.25%; fiber, 0.80%; ash, 0.90%; and water, 10.95%. In-house assays using standard methods (AOAC, 1980; AACC, 1983; Holst and Gehrke, 1975) resulted in the following analysis: carbohydrate, 79.67%; protein, 7.71%; fat, 1.18%; fiber, 0.62%; ash, 0.45%; and water, 10.37%. The corn meal contained 74.73% (s.d. 3.68%) starch as determined by a glucoamylase method (AACC, 1983; Method 76-11). All results in this paper are expressed on a total corn meal weight basis, except where noted. Before use, the corn meal was further milled through a Microjet mill with 0.5 mm screen (Micro Materials Corp., Model 10-ZM1).

Various moisture levels of corn meal were used. Potassium sorbate (0.2%) was added to the corn meal to inhibit mold growth during humidification. Corn meal was sprayed with water and equilibrated either over salt solutions at 25°C (Resnik et al., 1984) or in a temperature-humidity chamber (Hotpack Model 434304, Philadelphia, PA). Moisture level was determined gravimetrically after drying in a vacuum oven at 70°C.

Gelatinization and melting of starch were assayed using differential scanning calorimetry (Perkin-Elmer DSC-4). Samples were sealed in volatile sample pans. For experiments conducted in the hot oil bath, for which the DSC was only used to determine the amount of ungelatinized starch remaining based upon residual enthalpy per gram, 2.5 mg corn meal were added to 10 mg deionized water and sealed in the pan. Scan rate was 10°C/min. In experiments for which the DSC unit was used to study gelatinization and melting of moisture equilibrated samples up to 165°C, 5 mg of material were sealed in the volatile sample pans and the scan rate adjusted 2.5°C/min. This scan rate was selected for possible use with Perkin-Elmer stainless steel high pressure cells if such cells had been needed to hold pressure at 165°C. It was found that the volatile sample pans performed adequately for the low moisture samples at high temperature.

Time-temperature experiments were conducted by sealing moisture equilibrated corn meal in thermal death time cans and immersing the cans in a temperature-controlled hot oil bath. The general procedure was very similar to that of Phillips et al. (1983) who studied reaction kinetics of cowpea flour for extrusion applications and of Thompson et al. (1976) who studied lysine retention in extrusion-like processing.

The corn meal (5–6g) was placed in the TDT can into which a thin wire (30 gauge) copper-constantan thermocouple had been expoxied. A specially designed aluminum insert was placed on the meal and 2.1 MPa (300 psi) pressure exerted to pack the meal to a 2 mm height. This pressure was in the range experienced in an extruder. The compression of the meal was done to increase the effective thermal conductivity, and the aluminum insert was then sealed in the can so that the heat transfer distance was 2 mm across the corn meal sample. The cans were vacuum seamed (to 84 KPa or 25 in. Hg) in a Rooney seamer (Bellingham, WA). Analysis showed that no significant moisture loss occurred during the seaming. It was critical to remove the air that would otherwise expand during heating of the can and buckle the can to reduce the heat transfer rate. The thermocouple in the can allowed data logging of the heating and cooling curves, so that a correction for heating and cooling lag could be accomplished. The sealed TDT can with aluminum insert contained 0.09 g sample per cm^2 of heat transfer surface area, which is similar to the situation described by Thompson et al. (1976) whose retortable pouches contained 0.07g sample per cm^2 of surface area.

Heating of cans was done in a 0.025 m^3 oil bath (Precision Scientific No. 73410, Chicago, IL). Additional temperature control was achieved by use of a high temperature circulator (Lauda/Brinkman NS-HT, Sybron Corp., Westbury, NY) with external coils inside the oil bath. The oil was General Electric silicone oil SF-1154. A high speed stirrer (Lightnin Series 30, Mixing Equipment Co., Avon., NY) was added to increase agitation and heat transfer coefficient. The temperature difference to achieve rapid heat transfer into the cans was maintained high (not less than 50°C) by using a separate oil bath at very high temperature to heat the cans to a desired temperature before transferring to the temperature controlled bath. This smaller bath contained General Electric silicone oil SF-1265, and was heated to 190°C. This method of heating is similar to that of Phillips et al. (1983). Following the hold time at a given temperature, the can was rapidly transferred to an ice bath for quick cooling. The heated samples were reground before DSC analysis to provide more homogeneous sampling.

In the oil bath experiments, four to six cans were used at each time-temperature condition with analysis at least in triplicate. For the kinetics experiments conducted in the DSC, up to 12 replications were done at each condition.

RESULTS & DISCUSSION

TABLE 1 lists the times and temperatures used in oil bath heating of 25% moisture corn meal in TDT cans, as well as the corresponding mean of the residual endothermic enthalpy of each sample. The hold time refers to the actual time the sample was held at the constant reaction temperature, as determined from the temperature profile of each experiment. The corrected time adds to the hold time an equivalent time at the reaction temperature attributable to heating and cooling lags. This corrected time is that for the final iteration based on $k = 1.28 \times 10^{11} \cdot e^{-(86.19/RT)}$ (See Eq. 9). Table 1 also lists the Eq. (4) parameters used to fit the heating and cooling curves and the r^2 for the fitting of these curves. The heating/cooling curve values were determined by combining all heating/cooling data for a given reaction temperature.

The data points shown in Fig. 1 are those from the oil bath experiments at temperatures of 80°, 110°, 130°, and 140°C using 25% moisture corn meal as listed in Table 1. Standard deviations for each point are listed in Table 1. The time values are the corrected holding time after accounting for heating and cooling lags.

Transforming the data from Fig. 1 according to the lag cor-

rection of Lund and Wirakartakusumah (1984) yields points as shown in Fig. 2. Applying Eq. (2) yields an Arrhenius expression with constants as follows:

$$k = 1.47 \times 10^{11} \cdot e^{-(86.41 \text{ kJ/g-mole})/RT} \text{ min}^{-1} \quad (8)$$

with $r^2 = 0.951$ and standard error (S.E.) = 0.192. The lines in Fig. 2 are calculated using Eq. (8).

Applying the more rigorous analysis of Eq. (5) in successive iterations until the value of E_a differed by less than 2% from the preceeding trial yielded the following Arrhenius expression:

$$k = 1.28 \times 10^{11} \cdot e^{-(86.19 \text{ kJ g-mole})/RT} \text{ min}^{-1} \quad (9)$$

with $r^2 = 0.946$ and S.E. = 0.134. The lines drawn in Fig. 1 are calculated using Eq. (9).

The agreement between the values of the constants in Eq. (8) and (9) was remarkable, especially since the theories underlying the methods of data treatment were quite different. The agreement indicated that the kinetic constants given were correct, and that the two methods verified each other. The values shown in Eq. (8) are assumed to be more accurate since they result from a more rigorous analysis. The values of E_a were the same order of magnitude as those reported by other researchers for gelatinization activation energy.

The results of experiments in which the differential scanning calorimeter itself was used to heat various moisture-adjusted samples through the gelatinization and melting temperature range are listed in Table 2. The maximum melting temperature at each moisture level was used to calculate the melting point of perfect corn meal starch crystallites according to the Flory-Huggins theory (Lelievre, 1973). Extrapolating the melting temperature to zero volume of diluent water as shown in Fig. 3 yields a value of 165.4°C ($r^2 = 0.88$). This value for the melting point of perfect corn starch crystallites compares well with those for other starches which have been reported to be between 168°C and 227°C (Biliaderis et al., 1980; Burt and Russell, 1983; Donovan, 1979; Lelievre, 1973).

The general variation of reaction enthalpy with moisture that is shown in Table 2 is as described by Eliasson (1980). For the gelatinization peak, the enthalpy, which is listed as J/g of corn meal, increases with moisture. Dividing the gelatinization enthalpy at 34% moisture by the percentage starch in the corn meal (80%) gives a value for gelatinization enthalpy on a starch basis of 11.6 J/g which agrees well with literature values at low moisture. Higher moisture presumably would have shown the enthalpy value reaching a maximum and constant level.

The melting peak enthalpy passed through a maximum and then declined, as water increased. From 31.5% to 34.4%, the melting peak was not seen, while the gelatinization peak enthalpy increased significantly.

The kinetics analysis software of Perkin-Elmer was applied to the data, although the kinetic analysis truly applied only to cases in which a single reaction occurred. It is recognized that neither gelatinization nor melting are single reactions. The software uses a three parameter fitting procedure which simultaneously fits k_o, E_a, and n using Eq. (7). The results of this software analysis proved to be extremely dependent upon the user's specification of the baseline and calculation limits of the thermal curve. However, the trend of the results seemed consistent with literature values. Activation energies were of the order of 100 kJ/g-mole which roughly corresponded to published values, and in general the activation energy for the gelatinization peak decreased as moisture increased. The reaction order was calculated to be approximately 0.8, which might suggest a diffusional limitation of a first-order reaction. This would be reasonable for a system in which water was limited and in which gelatinization depended upon migration of available water molecules to starch granules and water into the granule. The reaction order decreased slightly as moisture content diffusion decreased, which would support the presumption of diffusion playing a greater role as water became more limiting. In an extruder where bulk mixing and shearing action are present, convective mass transfer should aid in reducing the mass transfer limitation of diffusion alone. Also, shearing physically degrades starch granules which would allow faster transfer of water to the interior starch molecules.

NOMENCLATURE

- A = surface area for heat transfer, m^2
- c = specific heat of medium, $cal \cdot g^{-1} \cdot {}^oK^{-1}$
- E_a = activation energy in Arrhenius equation, $J \cdot (g\text{-mole})^{-1}$
- ΔH = enthalpy of reaction $J \cdot g^{-1}$
- k = specific reaction rate, min^{-1}
- k_o = constant in Arrhenius equation, min^{-1}
- M = mass, g
- n = reaction order
- r = correlation coefficient
- R = gas constant, $8.314 \text{ J} \cdot (\text{g-mole}^{-1}) \cdot {}^oK^{-1}$
- t = time, min
- T = temperature, °K
- T_H = temperature of heat source, °K
- T_M = maximum melting temperature, °K
- T_o = initial temperature, °K
- U = overall heat transfer coefficient, $cal \cdot sec^{-1} \cdot m^{-2} \cdot {}^oK^{-1}$
- UG_f = amount of ungelatinized starch at final heating
- UG_i = amount of ungelatinized starch initially
- UG_l = amount of ungelatinized starch at lag time
- UG_t = amount of ungelatinized starch at time t
- z = argument of the exponential function E_1
- V_1 = volume fraction of water
- α = degree of chemical conversion

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Antioxidant Effect of Riboflavin Tetrabutylate in Emulsions

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ABSTRACT

The antioxidant effects of riboflavin tetrabutylate in emulsions in the dark and under irradiation were studied, as was the effect of racemic (*rac*) α-tocopherol on the antioxidant action. Riboflavin tetrabutylate had an antioxidant effect in the dark, but an oxidative effect when irradiated, perhaps because the activated oxygen and hydrogen peroxide produced induced lipid peroxidation. The effect of *rac* α-tocopherol on the antioxidant action of riboflavin tetrabutylate was synergistic in the dark. The results showed that riboflavin tetrabutylate was satisfactory as an antioxidant when the emulsion was protected from light and that the effect was increased by the addition of *rac* α-tocopherol.

INTRODUCTION

Riboflavin tetrabutylate was developed as a fat-soluble derivative of riboflavin, and is a legal food additive and a legal source of vitamin B_2 in Japan, although not in the United States. It is used to raise the vitamin B_2 level of fats and oily foods. Ingested riboflavin tetrabutylate is hydrolyzed in the tissue *in vivo* and quickly absorbed (Aoyama et al., 1985; Miyazawa et al., 1983b). When absorbed, riboflavin was claimed to limit lipid peroxidation in the tissue; the effect was increased by *rac* α-tocopherol (Miyazawa et al., 1983a, b). The *in vitro* antioxidant effect of riboflavin tetrabutylate is still being studied. Aoyama et al. (1985) evaluated the peroxidation of lard or palm oil mixed with riboflavin tetrabutylate and heated in the dark. They found that the compound had little antioxidant effect; *rac* α-tocopherol had no effect on the peroxidation. Totani et al. (1975) have studied the effect of riboflavin tetrabutylate on the autoxidation of fish oil, and reported that it acts as an antioxidant. Ohama and Yagi (1969) found that the addition of riboflavin tetrabutylate to linoleic acid which was allowed to autoxidize reduced the production of hydroperoxide. These results showed that the antioxidant effect of riboflavin tetrabutylate changes depending on the experimental conditions.

Since riboflavin tetrabutylate can be used in Japan as an additive to fatty and oily foods, where it forms an emulsion, *in vitro* antioxidant effects of the additive should be studied in a form as close as possible to that of the food. This study was done to examine the *in vitro* antioxidant effects of riboflavin tetrabutylate in a form that resembles the emulsion used in food systems. The potential synergistic effects of the antioxidant tocopherol and the stability of the emulsion system were also studied.

MATERIALS & METHODS

Preparation of emulsions

Linoleic acid (99% pure; Sigma Chemical Co., St. Louis, MO) was used as the fat in the emulsions. Given amounts of riboflavin tetrabutylate (Aldrich Chemical Co., Inc., Milwaukee, WI) and of synthetic *rac* α-tocopherol (more than 99% pure; Nakarai Chemicals, Ltd., Kyoto, Japan) were directly dissolved in the linoleic acid. For emulsification of this solution, a 1% solution of chondroitin sulfate sodium salt (Tokyo Kasei Kogyo Co., Ltd., Japan) in 40 mM phosphate buffer (pH 7.4) was used. The linoleic acid and 1% chondroitin sulfate solution were mixed in the ratio of 65:35 (v/v) and treated with an ultrasonic generator (Model UR 200P, Tomy Seiko Ltd., Japan) at the output of 90 W for 3 min to prepare an oil/water emulsion for the tests.

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Preparation of test sample

Fifty milliliters of the emulsion thus prepared were immediately placed in an Erlenmeyer flask with a ground-joint stopper, put in a constant-temperature bath set at 30°C, and stirred with a magnetic stirrer. Some of the flasks were kept in the dark, and some were irradiated a given intensity of 3000 lux from all directions in a box equipped with fluorescent light. Filters were used to allow only light of a given wavelength (450 nm). Samples were left in the dark for up to 8 days or irradiated for up to 100 min. At times shown in the figures and tables, 5.0 mL samples, were removed from the flask and assayed.

Assay of hydroperoxide

Hydroperoxide was calculated in terms of 2',7'-dichlorofluorescein (DCF). The 5.0 mL samples were centrifuged (10,000 × *g*, 30 min) to demulsify them. The linoleic acid of the supernatant was then measured for hydroperoxide by the method of Cathcart et al. (1984). First 1.0 mL of a 1.0 mM solution of DCF in ethanol and 2.0 mL of 0.01N NaOH were mixed and stirred for 30 min before being neutralized with 10 mL of 25 mM phosphate buffer (pH 7.2). Then 2.0 mL neutralized DCF solution were added to a solution of hematin in 25 mM phosphate buffer (pH 7.2; 0.01 mg DCF/mL) and 2.9 mL of this hematin-DCF solution and 10 μL of the linoleic acid sample were mixed and left at 50°C for 50 min before fluorometry treatment (excitation, 400 nm; emission, 470 nm) to measure DCF. This method measures hydroperoxide with more sensitivity than the iron rhodanide method usually used.

Assay of superoxide anion and hydrogen peroxide

Superoxide anion (O_2^-) was measured by the nitro blue tetrazolium (NBT) reduction method (Korycka-Dahl and Richardson, 1978). The precipitate of diformazan produced upon reduction of NBT by O_2^- was obtained by centrifugation at 3000 rpm for 10 min and dissolved in 4.0 mL of N',N'-dimethylformamide. Absorbance was measured at 560 nm to estimate the amount of O_2^- produced in the test sample from a calibration curve prepared with the use of a standard diformazan solution. Hydrogen peroxide (H_2O_2) was measured by the method of Munday (1985).

Assay of emulsion stability

The stability of the emulsion was assayed by the method of Aoki and Nagano (1975). First, the sample (5.0 mL) was put in a test tube with a ground-joint stopper and placed in a constant temperature bath (30°C) for 30 min. Then 2.5 mL of the sample were taken from the bottom of the test tube and freeze-dried. Moisture content was calculated from the weight difference, which was used to determine the emulsion stability percentage (ESP) of the sample by the method of Acton and Saffle (1970).

$$\text{ESP (\%)} = \frac{100 - M_{test}}{100 - M_{original}} \times 100$$

where ESP is the emulsion stability percentage, M_{test} is the actual moisture (%), and $M_{original}$ is the original moisture (%).

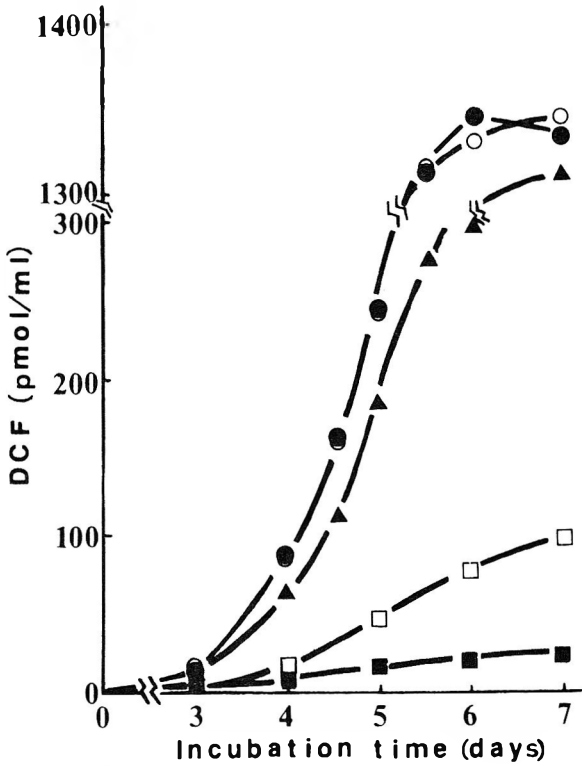

Fig. 1—Effects of riboflavin tetrabutylate on the oxidation of linoleic acid in an emulsion in the dark. Each point represents a mean of three trials. DCF, 2',7'-dichlorofluorescein. Symbols: ●, no riboflavin tetrabutylate; ○, 1.0 ppm riboflavin tetrabutylate; ▲, 10 ppm riboflavin tetrabutylate; □, 50 ppm riboflavin tetrabutylate; ■, 100 ppm riboflavin tetrabutylate.

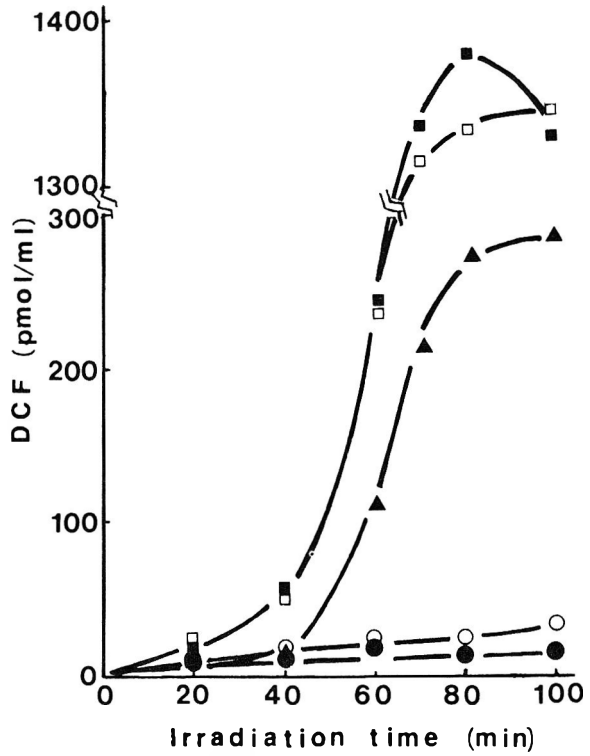

Fig. 2—Effects of riboflavin tetrabutylate on the oxidation of linoleic acid in an irradiated emulsion. Each point represents a mean of three trials. Abbreviation and symbols same as in the legend of Fig. 1.

Assay of hydroxyl radicals

The activated-oxygen quenchers listed in Table 2 were used to check for the formation of hydroxyl radicals in irradiated emulsions and to see if such radicals were involved in the peroxidation of the linoleic acid. The β-carotene was in the form of dispersable beadlets. These compounds, were added to already prepared emulsion samples in the concentrations shown in Table 2, and the emulsion was irradiated for 60 min with stirring as before.

RESULTS & DISCUSSION

IN EMULSIONS left in the dark, riboflavin tetrabutylate inhibited the oxidation of linoleic acid in a concentration-dependent way (Fig. 1). The effect was slight with 10 ppm and much stronger with 50 ppm; oxidation almost ceased with 100 ppm. In irradiated emulsions, riboflavin tetrabutylate accelerated oxidation in a concentration-dependent way (Fig. 2). The results showed that riboflavin tetrabutylate had an antioxidant effect in emulsions when protected from light.

Totani et al. (1975) have reported that autoxidizing lipids degrade riboflavin tetrabutylate by oxidation through activated oxygen. Ohama and Yagi (1969) have reported that the isoalloxazine nucleus of riboflavin tetrabutylate is degraded by activated oxygen and that riboflavin tetrabutylate decomposes peroxides. These reports do not mention protection of the samples from light. Riboflavin is photolyzed by activated oxygen (Toyosaki et al., 1983; 1984a, b). Activated oxygen produced by irradiation may decompose riboflavin tetrabutylate. Absorbance of the isoalloxazine nucleus while the sample was irradiated declined temporarily but returned to the original level after the sample was returned to the dark (data not shown).

The flavin pigment system acts as a photosensitizer, and thus is involved in lipid peroxidation through the activated oxygen produced by it (Kellogg and Fridovich, 1975; Tyler, 1975; Dederson and Aust, 1973). This suggested that the oxidation

Table 1—Generation of superoxide anion and hydrogen peroxide in emulsions

Concentration of riboflavin tetrabutylate (ppm)	O_2^- (μM/min)[a]		H_2O_2 (nM/min)[a]	
	Dark[b]	Light[c]	Dark[b]	Light[c]
0	0	0	0	0
1.0	0	1.6	0	2.7
10.0	0	9.0	0	5.8
50.0	0	18.5	0	8.0
100.0	0	30.8	0	19.6

[a] O_2^-, Superoxide anion; H_2O_2, hydrogen peroxide. Mean of measurement of duplicate samples.
[b] Kept in the dark for 8 days.
[c] Irradiated for 60 min.

of linoleic acid in the irradiated emulsions was accelerated because of the production of activated oxygen. Emulsions in the dark did not produce O_2^- or H_2O_2, but both were produced in irradiated emulsions, and their production increased with the concentration of riboflavin tetrabutylate (Table 1). This suggested that when the emulsion was irradiated, its comparatively rapid peroxidation arose from O_2^- and H_2O_2 produced during the irradiation. The activation mechanism was probably as follows. Riboflavin tetrabutylate is raised from the ground state to the excited state when irradiated with light. The excited compound is reduced by an electron donor; as our results showed, reduction can proceed even in the absence of such a donor. The reduced riboflavin tetrabutylate will return to its original triplet state in the presence of oxygen, which is activated to produce O_2^-. H_2O_2 is probably produced by the reaction between molecules of hydrogen oxide, by the reaction of HO_2 with O_2^-, or by the reaction of riboflavin tetrabutylate with O_2 (Toyosaki et al., 1984a; Pryor, 1976a). Whatever the pathway, O_2^- and H_2O_2 were produced in the irradiated emulsions. There is a report that O_2^- is not directly involved in lipid peroxidation but that hydroxyl radicals probably are (Pryor, 1976b). Another report (Pryor, 1976c) points out that the activity of H_2O_2

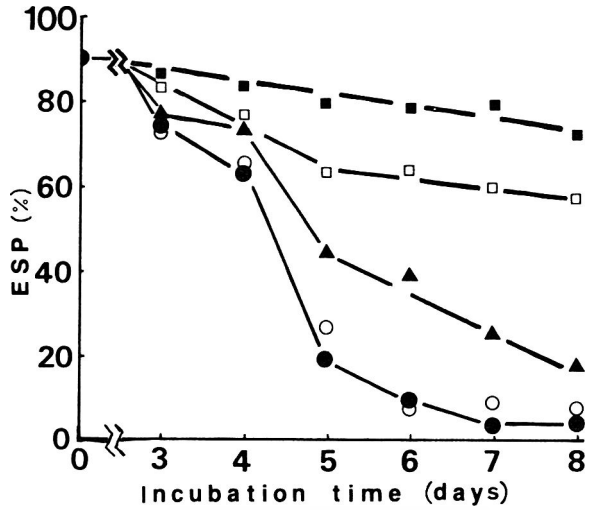

Fig. 3—Changes in the emulsion stability percentage (ESP) with the oxidation of linoleic acid in an emulsion in the dark. Each point represents a mean of three trials. Symbols same as in the legend of Fig. 1.

Table 2—Effects of quenchers on the generation of hydroxyl radicals in emulsions

Additions	DCF ratio (%)[a]
None	100
SOD[b] (5.0 μg/mL)	87
Catalase (20 μg/mL)	91
SOD[b] (5.0 μg/mL) + catalase (20 μg/mL)	74
Tryptophan (10 mM)	59
Mannitol (10 mM)	52
Histidine (10 mM)	89
β-Carotene (10 mM)	126

[a] DCF = 2',7'-Dichlorofluorescein. The DCF ratio is shown as the percentage of the fluorescence intensity (excitation, 370 nm; emission 475 nm) of the control, which was an emulsion containing 100 ppm riboflavin tetrabutylate. Mean of measurements of duplicate samples, irradiated for 60 min.

[b] SOD = superoxide dismutase.

is not high enough to induce lipid peroxidation in an unsaturated fatty acid, but that lipid peroxidation occurs because of the optical radical cleavage of the 0-0 bond of H_2O_2 with the formation of hydroxyl radicals that then abstract hydrogen atoms from the fatty acid. We concluded from these findings that the peroxidation of linoleic acid in the irradiated emulsion in this experiment was probably induced by hydroxyl radicals. The peroxidation of linoleic acid was inhibited by the addition of superoxide dismutase (SOD) or catalase; the inhibition was increased by their simultaneous addition (Table 2). The addition of tryptophan or mannitol also inhibited peroxidation. These findings suggest that hydroxyl radicals were involved in the peroxidation of linoleic acid. The addition of histidine or β-carotene had no such effect, which suggested that singlet oxygen was not involved in the peroxidation. Hydrogen peroxide and activated oxygen was produced in the emulsion containing riboflavin tetrabutylate by irradiation, increasing lipid peroxidation. Emulsions in the dark were more stable with high concentrations of riboflavin tetrabutylate (Fig. 3). When the emulsion was exposed to light, the results were opposite; the emulsion was less stable at high concentrations (Fig. 4). These results were in agreement with conclusions made from the findings shown in Fig. 1 and 2. In the dark, the stability of the emulsion improved as the concentration of added riboflavin tetrabutylate increased, controlling lipid peroxidation. However, in the irradiated emulsion, the stability decreased as peroxidation increased, perhaps because peroxidation made the oil phase of the emulsion unstable.

Tocopherols are antioxidants available in nature. Miyazawa et al. (1983b) have shown that *rac* α-tocopherol and riboflavin tetrabutylate act synergistically as antioxidants *in vivo*. It is

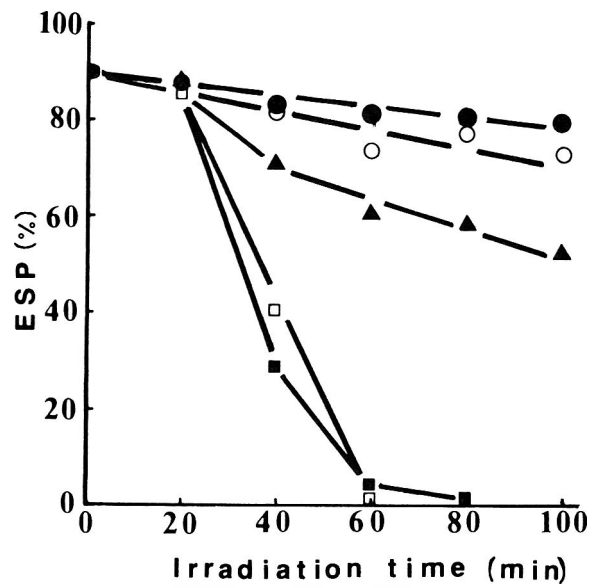

Fig. 4—Changes in the emulsion stability percentage (ESP) with the oxidation of linoleic acid in an irradiated emulsion. Each point represents a mean of three trials. Symbols same as in the legend of Fig. 1.

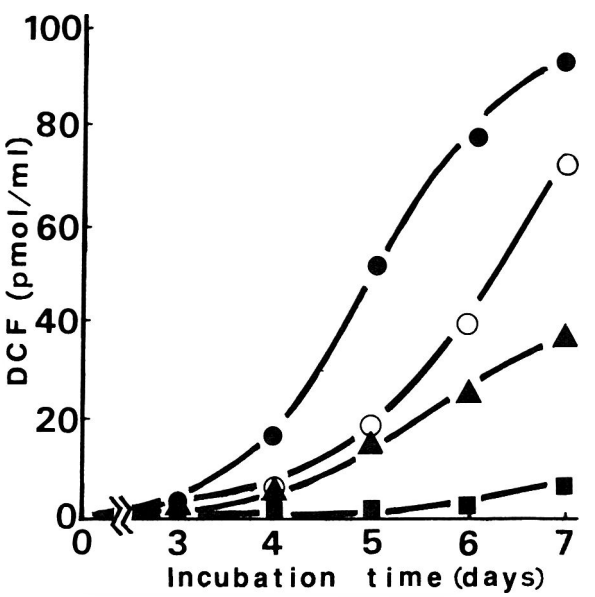

Fig. 5—Effects of rac α-tocopherol on oxidation of linoleic acid in an emulsion in the dark. Each point represents a mean of three trials. DCF, 2',7'-dichlorofluorescein. Symbols: ●, riboflavin tetrabutylate, 50 ppm; ○, rac α-tocopherol, 25 ppm; ▲, riboflavin tetrabutylate at 50 ppm + rac α-tocopherol at 10 ppm; ■, riboflavin tetrabutylate at 50 ppm + rac α-tocopherol at 25 ppm.

not certain if their antioxidant effects are synergistic *in vitro*. In the dark, the antioxidant effect of riboflavin tetrabutylate in emulsions at 50 ppm or more increased with the addition of 25 ppm *rac* α-tocopherol to a much higher level than that obtained with either compound used alone. This suggested that riboflavin and *rac* α-tocopherol acted synergistically under these conditions (Fig. 5). In conclusion, riboflavin tetrabutylate in emulsions was a strong antioxidant in the dark, but it accelerated oxidation in the light. These results, combined with the known antioxidant effect of riboflavin tetrabutylate *in vivo* (Miyazawa et al., 1983a, b) and *in vitro* (Totani et al., 1975), indicated that peroxidation could be prevented satisfactorily if the emulsion was shielded from light.

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This project was supported by a grant from the Natural Science and Engineering Research Council (NSERC) of Canada.

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Presented at the 47th Annual Meeting of the Institute of Food Technologists, Las Vegas, NV, June 16-19, 1987.

The authors recognize the assistance of Wendell Kerr of Calreco's Statistics group in analyzing data to provide kinetic constants, of Christine Barboza in preparing the manuscript, and of Joseph Cullen and Milo Nielsen, directors of Engineering & Packaging and of Research Services, respectively, in authorizing this research.

Water-Oil Absorption Index (WOAI): A Simple Method for Predicting the Emulsifying Capacity of Food Proteins

R.J. De KANTEREWICZ, B.E. ELIZALDE, A.M.R. PILOSOF and G.B. BARTHOLOMAI

ABSTRACT

An index for describing the relative hydrophilic-lipophilic character of proteins was proposed based on the measurement of the "spontaneous water and oil uptake." The water-oil absorption index (WOAI) showed a defined correlation with emulsifying capacity (EC) for most proteins making it possible to predict EC in a simple way. Maximum emulsion capacity was achieved when the WOAI was nearly two, that is when the protein absorbed twofold more water than oil. Proteins with WAOI greater than two had increasingly hydrophilic characteristics and concomitantly increasingly low EC. Proteins with WOAI lower than two showed increasingly lipophilic characteristics and concomitantly increasing low emulsifying capacity. The finding of this study supports the concept that emulsifying capacity of proteins depends on the suitable balance between the hydrophilic and lipophilic characteristics, rather than merely high values for each one.

INTRODUCTION

THE EMULSIFYING PROPERTY is an important functional property of a protein. Two main approaches have been used: this property may be expressed as emulsifying capacity or emulsion stability. The former measures the maximum oil addition until phase separation occurs, whereas the latter measures the tendency for the emulsion to remain unchanged.

Generally, emulsifying property and aqueous solubility of proteins are related (Kinsella, 1976). However, many reports cite evidence that emulsifying properties and aqueous solubility are not well correlated (Aoki et al., 1980; Flint and Johnson, 1981; Mc Watters and Cherry, 1975; Mc Watters and Holmes, 1979a,b; Wang and Kinsella, 1976).

Hydrophobic interactions perform important roles in protein functionality (Aoki et al., 1981; Kato and Nakai, 1980; Kato et al., 1981; Keshavarz and Nakai, 1979; Kinsella, 1979; Li-Chan et al., 1984, 1985; Nakai et al., 1980). Both hydrophobicity and solubility have been used as indices of emulsifying properties of proteins. Emulsifying properties were positively correlated to solubility and hydrophobicity but Li-Chan et al. (1985) showed that some square and interaction terms have significant contributions in the multiple regression models indicating that optimum emulsifying properties require an optimum balance of dispersibility, hydrophobicity and structural parameters and not just high values for an individual parameter.

Optimal emulsifying properties may be related to a balance of hydrophilic and lipophilic properties of the protein. The hydrophil-lipophil balance (HLB system) introduced by Griffin (1949) has been extensively used to characterize properties of surfactants or emulsifiers. The HLB of a protein is an empirical quantity estimated by comparing the stability of emulsions prepared using various oils whose "required" HLB numbers were known (Van Eerd, 1971; Aoki et al. 1981). The HLB numbers seem to be meaningless as absolute values. The procedure is difficult and time-consuming.

Hydrophilic properties have been expressed in terms of solubility or dispersibility of the protein and the lipophilic property as hydrophobicity. It is generally accepted that at the oil-water interface the protein molecule unfolds orienting the hydrophilic segments towards the water phase and holding it, with the lipophilic segments towards the oil phase. Thus, the physicochemical property that best describes this affinity of the protein for water at the interface may be the water absorption capacity in terms of the "spontaneous uptake of water" (Hermansson, 1972), rather than solubility. Similarly, the ability of the protein to take part in lipophilic interactions, could be determined by the measurement of the "spontaneous oil uptake." The objective of this study was to develop an index to describe the relative hydrophilic-lipophilic character of proteins for use in predicting their-emulsifying properties. This index was based on the measurements of the "spontaneous water and oil uptake" of a protein. Its usefulness in predicting the emulsifying capacity of food proteins was investigated.

MATERIALS & METHODS

Materials

Commercial soybean protein isolates were used: Proteinmax 90 NB was from Sambra S.A. (Sao Paulo, Brazil) and Purina Protein 710, 500 E and 760 were from Ralston Purina Co. (St. Louis, MO). The pH of 1% dispersions was 6.9–7.1. Bovine albumin (AB) was from Sigma Chemical Co. (St. Louis, MO). Whey Protein concentrate (75%) (WPC) from New Zealand Milk Products, Inc., sodium caseinate (SC), from Lab. Argentinos Farmesa S.A., Argentina; gelatin (G) (food grade) was from Stauffer Rioplatense S.A., Argentina.

Bean protein isolate (BPI) was prepared according to Pilosof et al. (1982); meat salt soluble proteins (MSSP), according to Acton and Saffle (1970) and freeze-dried; egg white powder (EW), by freeze drying fresh egg white; pumpkin protein isolate (PUPI), according to Vigo et al. (1973). Commercial corn oil from Refinerías de Maíz SAICF, Argentina was used.

Absorption capacities

The spontaneous uptake of water by protein powders was determined according to Pilosof et al. (1985) using the device proposed by Torgersen and Toledo (1977). The spontaneous uptake of oil by protein powders was determined in the same way as water uptake. A glass microfiber Whatman GF/C filter was used in the Millipore unit instead of common filter papers; the apparatus was filled with oil.

Emulsifying capacity (EC)

For EC determinations all proteins were dispersed in distilled water except for MSSP which was dispersed in a 3% NaCl solution. EC was determined by a modification of the method proposed by Quaglia et al. (1981). Protein solutions and oil were combined at different ratios maintaining the total volume equal to 50 mL. The oil phase was increased at 1 mL increments until a further increase caused phase inversion manifested by an abrupt change in color and viscosity. Emulsification was carried out by mixing protein solutions and oil (4–

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5°C) with a Griffin and George stirrer Model L 42 at a speed of 6000 rpm for 3 min while maintaining the temperature constant at 4–5°C. EC was defined as mL oil/g protein before the mixture where phase inversion occurred.

RESULTS & DISCUSSION

Spontaneous water and oil uptake

Characteristic spontaneous water uptake curves are shown in Fig. 1 . Since all the samples were chemically stable in the presence of water, equilibrium occurred and the water uptake curves levelled off at the water absorption capacity (WAC in mL H_2O/g protein).

Since Hermansson (1972) adopted the Baumann apparatus for measuring the spontaneous uptake of water by food proteins, the method has been increasingly used (Torgersen and Toledo, 1977; Kuntz et al., 1978; Romo, 1980; Urbanski et al., 1983; Wallingford and Labuza, 1983). Pilosof et al. (1985) used this method to determine the water uptake of animal and vegetable proteins and starchy materials. They developed equations for expressing fluid uptake as a function of time.

When the device used for the determination of the water uptake was used for oil, curves in Fig. 2 were obtained. In all cases spontaneous equilibrium was reached after 3 to 12 min. Rate of oil absorption was rapid initially and decreased as equilibrium approached. The volume of oil/g protein at equilibrium represented the oil absorption capacity (OAC). The water and oil absorption capacities of the proteins tested are shown in Table 1. The water-oil absorption index (WOAI) defined as the ratio of WAC to OAC is also shown in Table 1.

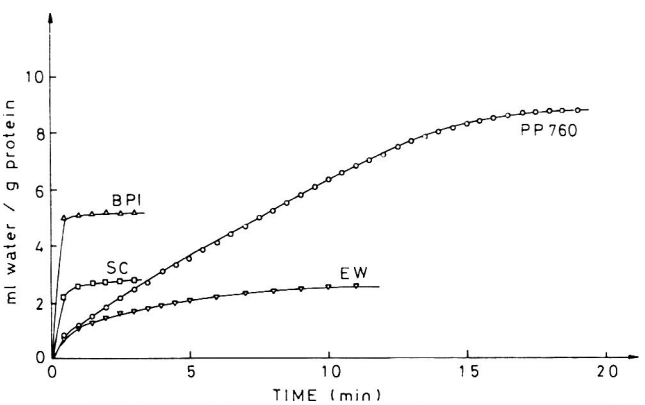

Fig. 1—Spontaneous water uptake by different proteins. BPI: bean protein isolate; EW: egg white powder; PP760: Purina protein 760; SC: sodium caseinate.

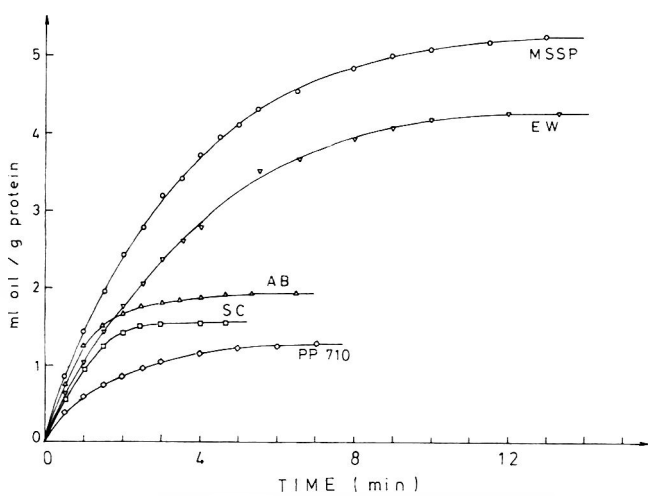

Fig. 2—Spontaneous oil uptake by different proteins. AB: bovine albumin; EW: egg white powder; MSSP: meat salt-soluble protein; PP710: Purina protein 710; SC: sodium caseinate.

Table 1—Water and oil absorption capacities of proteins and the WOAI

Protein	WAC (mL water/g)	OAC (mL oil/g)	WOAI[a] (mL water/mL oil)
Whey protein concentrate	0.20	0.97	0.20
Meat salt soluble proteins	1.48	5.25	0.28
Bovine albumin	0.65	1.90	0.34
Egg white	2.58	4.22	0.61
Pumpkin protein isolate	2.89	4.66	0.62
Bean protein isolate	5.16	4.07	1.27
Caseinate	2.80	1.55	1.80
Purina protein 710	2.48	1.26	1.97
Purina protein 500 E	7.90	1.80	4.39
Purina Protein 760	8.75	1.45	6.03
Proteinmax 90 NB	19.90	1.90	10.47
Gelatin	11.60	0.93	12.47

[a] WOAI was calculated as the ratio of WAC to OAC.

Each protein had different WAC and OAC. Proteinmax 90 NB had the highest WAC but low OAC. Meat salt soluble proteins on other hand, had the greatest OAC. The relative hydrophilic-lipophilic character of the proteins was reflected by the WOAI which ranged from 0.20 for whey protein concentrate to 12.47 for gelatin.

Correlation between the emulsifying capacity and the WOAI

The classical approach to evaluating the emulsifying capacity of a protein is based on the technique of Swift et al. (1961) which involves dropwise addition of oil to a vigorously agitated protein-water dispersion until emulsion inverstion occurs as indicated by a sudden drop in viscosity. In this procedure the total emulsion volume and blending time varies with the emulsification capacity of the protein. In the present study the volume of the emulsions and the emulsifying times were maintained constant.

In Fig. 3, the emulsifying capacity of the proteins (1% dispersions) is shown as a function of the WOAI. Emulsification capacity increased with increasing WOAI to reach a maximum when WOAI was nearly two that is when the protein absorbed two times more water than oil per unit weight. When proteins absorbed water greater than twice the amount of oil (WOAI>2) the hydrophilic-lipophilic balance shifted towards the hydrophilic side and emulsifying capacity decreased with increasing WOAI.

Regression equations for the descending and ascending straight lines in Fig. 3 (excluding gelatin, WPC and MSSP) were, respectively:

$$EC = 422.5 - 26.7\ (WOAI);\ r = -0.956$$

$$EC = 192.2 + 96.1\ (WOAI);\ r = 0.981$$

Both correlation equations are significant at $P = 0.01$. The magnitude of the slopes in the regression equations indicates that emulsification capacity of lipophilic proteins are affected more by WOAI than the hydrophilic proteins.

WPC and MSSP deviated from the trend exhibited by the other proteins. The high concentration of lactose in the WPC and NaCl in the MSSP may result in decreased water uptake (López de Ogara et al., 1987) and reduced WOAI. In addition solutes can modify the surface charge of proteins and the interfacial tension between water and oil.

Looking specifically at the results of soy proteins in Table 1, it can be seen that PP500E and Proteinmax 90 NB, two denatured soy proteins, showed similar oil absorption capacities; however, the latter absorbed more than twofold water than the former. This transfers the hydrophilic-lipophilic balance of Proteinmax 90 NB towards the hydrophilic side (a higher WOAI) leading to a very low emulsifying capacity as can be seen in Fig. 3. The key for best emulsifying capacity appeared to be

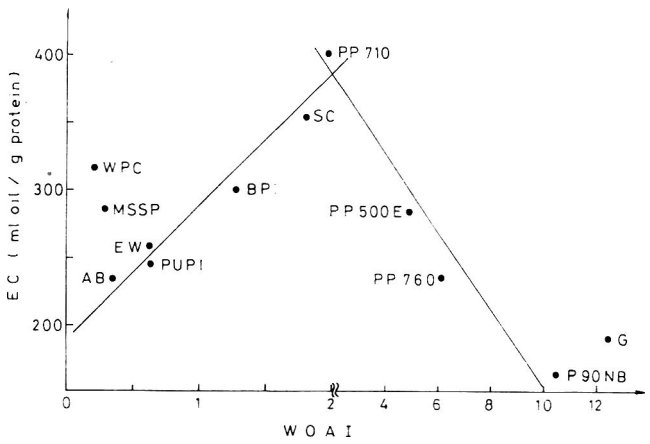

Fig. 3—Correlation between the emulsifying capacity of proteins and their water-oil absorption index (WOAI). AB: bovine albumin; BPI: bean protein isolate; EW: egg white powder; G: gelatin; MSSP: meat salt-soluble protein; PUPI: pumpkin protein isolate; P90 NB: Proteinmax 90 NB; PP710: Purina protein 710; PP760: Purina protein 760; PP500 E: Purina protein 500 E; SC: sodium caseinate; WPC: whey protein concentrate.

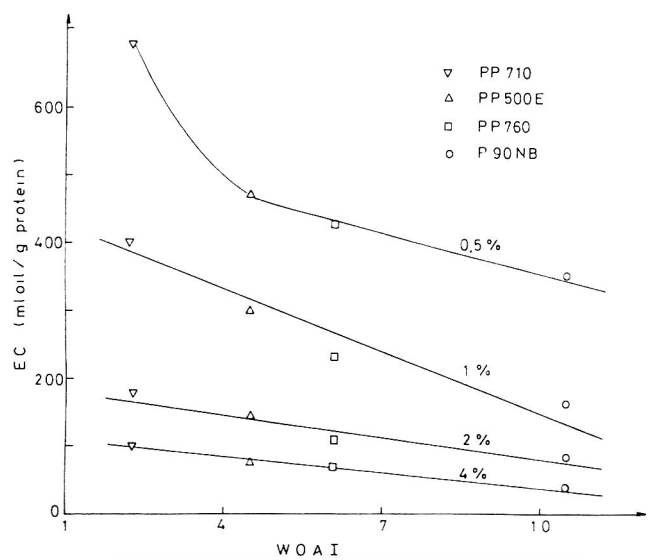

Fig. 4—Effect of protein concentration on the correlation between emulsifying capacity and WOAI of soy proteins. PP710: Purina protein 710; PP500E: Purina protein 500 E; PP760: Purina protein 760; P90NB: Proteinmax 90 NB.

the denaturation of soy proteins in conditions in which an excessive increase of the WAC was prevented.

PP 710 showed the best hydrophilic-lipophilic balance corresponding to the peak of the curve in Fig. 3. This protein has reduced molecular weight because of hydrolisis, greatly reducing WOAC (Table 1).

EC of soy proteins depends on concentration (Kinsella, 1979). In order to investigate the effect of protein concentration on the correlation between EC and WOAI, the EC of soy proteins as a function of concentration was determined. As shown in Fig. 4 increasing soy protein concentration from 0.5 to 4%, a lower dependence of the EC on the WOAI was observed.

CONCLUSIONS

THE RESULTS of this study supported the concept that the emulsifying capacity of proteins depended on the suitable balance between the hydrophilic and lipophilic characteristics rather than merely high values for each one.

Emulsifying capacity could be predicted by the simple calculation of WOAI by measuring the spontaneous water and oil uptake, which were the best indicators of the ability of a protein to participate in hydrophilic and lipophilic interactions.

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Ms received 8/25/86; revised 3/4/87; accepted 3/26/87.

The authors acknowledge the financial support from the Subsecretaría de Ciencia y tecnologia; the Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina, and the Comisión de Investigaciones Científicas de la Provincia de Buenos Aires.

Oxidation of Ascorbic Acid in Copper-Catalyzed Sucrose Solutions

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ABSTRACT

The effect of sucrose on the oxidation of ascorbic acid (A) in Cu-catalyzed (1, 2.5, and 5 ppm) and noncatalyzed solutions was studied at 30°C in samples shaken at various speeds. In unbuffered solutions, all sucrose levels tested (10–40%) were protective of A without shaking; however, all sucrose levels tested were destructive of A at the highest speed of shaking. This observation was much more pronounced in Cu-catalyzed reactions. Sucrose enhanced the catalytic action of Cu on the oxidation of A. In 0.1M acetate-buffered sucrose solutions this destructive effect of sucrose was slightly diminished due to the Cu-complexing effect of the buffer salt.

INTRODUCTION

ASCORBIC ACID and sucrose often coexist in natural food components and in synthetic food ingredients. The oxidation of ascorbic acid in the presence of sugars has been the subject of several investigations (Birch and Pepper, 1983; Lin and Agalloco, 1979; Kyzlink and Cïda, 1970; Miller and Joslyn, 1949a,b; Chamrai, 1941). In general, it has been established by previous investigators that sucrose exerts a protective action on nonenzymic and enzymic oxidation of ascorbic acid. Chamrai (1941) first suggested that the protective effect of sucrose in an acid medium was due to its effect in reducing the rate of oxygen diffusion from the air, and in an alkaline medium, to its copper binding power. Obviously, viscosity, an important physical property of sucrose solutions, can decrease the solubility of oxygen in a medium and the rate of oxygen diffusion from the air (Joslyn and Supplee, 1949) thus protecting ascorbic acid from rapid oxidation. Miller and Joslyn (1949a) studied the rate of oxidation of ascorbic acid under conditions of limited oxygen concentration and under conditions where oxygen was bubbled through the solution in the presence of several sugars. They reported that the extent of protection by the sugars increased with an increase in sugar concentration. In the limited oxygen samples, oxygen solubility and the diffusion rate of oxygen were limiting factors which were not expected to be involved in the oxygenated samples. The mechanism of protection by sugars in the oxygenated samples was proposed to be the formation of cupric-sugar complexes, but the sugars tested, in concentrations up to 40 per cent, suppressed the catalytic power of only an amount of copper equivalent to approximately one ppm. Kyzlink and Cïrda (1970) presented additional evidence that increasing the concentration of sucrose in a copper-catalyzed solution leads to the retardation of the oxidation process when sufficient oxygen was supplied. However, their calculated second order reaction constant indicated that the added sucrose showed a marked, but not dominating, catalyzing effect on the oxidation of ascorbic acid. The above researchers and others claimed that sufficient or even surplus oxygen was applied to their samples. This claim was apparently based on two assumptions, (1) the oxygen dissolution was faster than its disappearance through any possible reaction, and (2) the oxygen bubbling rate was fast enough to replenish the oxygen consumed through the reaction system.

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Although the ability of polyhydroxy compounds and food carbohydrates to form complexes with copper is well recognized (Cross et al. 1985; Briggs et al. 1981; Bourne et al. 1971), there are few data available on the chemistry of the cupric-sucrose complex. Recently, Hsieh and Harris (1987) found that the copper activity or the effective concentration of copper in sucrose solutions was higher than that in the absence of sucrose. On the basis of this observation, it would be expected that the catalytic power of copper on the oxidation of ascorbic acid could be enhanced by the presence of sucrose. Another complicating factor in many of the previous works on copper-catalyzed oxidation reactions of ascorbic acid was the use of buffers which interact to different extents with cupric ions to form complexes (Joslyn and Miller, 1949). Because of the intricate factors involved in these systems, most of the early workers reported results from which no fruitful conclusion on the mechanism of protection by sucrose on the oxidation of ascorbic acid could be made. The purposes of the present study were: (1) to investigate the influence of oxygen supply, copper concentrations, and buffer on the rate of oxidation of ascorbic acid in sucrose solutions, and (2) to define more clearly the physical and chemical roles of sucrose on ascorbic acid stability.

MATERIALS & METHODS

Materials

Sucrose, L-ascorbic acid, 2,6-dichlorophenol-indophenol, and sodium bicarbonate were obtained from Sigma Chemical Co. (St. Louis, MO). Glacial acetic acid, cupric nitrate standard solution, metaphosphoric acid, and anhydrous sodium acetate were purchased from American Scientific Products (Ocala, FL). All chemicals were analytical grade and used without further purification. Deionized distilled water was used to prepare all of the solutions in this study.

Methods

This experiment was based on a factorial design with three factors: concentration of sucrose solution (0, 10, 20, 30 and 40% w/v); concentration of copper (0, 1, 2.5 and 5 ppm); and shaking speed (no shaking, 80 oscillations/ min and 200 oscillations/min). Sucrose solutions were prepared by dissolving the required amount of sucrose in water or in 0.1M sodium acetate-acetic acid buffer at pH 3.2. A 2 mL aliquot of each of the above solutions was placed into a 50 ml flask thus providing ample air exposure and oxygen accessibility. One tenth mL of 0, 20, 50 and 100 ppm cupric nitrate standard solution was added to each sample to make the desired copper levels. Samples were placed in a 30°C water bath shaker until temperature equilibrium was reached and solutions were saturated with air oxygen. Then 0.1 mL 2% L-ascorbic acid standard solution was added immediately to each sample and the timing of the reaction was begun. After the addition of ascorbic acid the pH of the sample solutions was 3.2. The oxygen supply rate for samples was manipulated by shaking at zero speed (no shaking); low speed (80 oscillations/min); and high speed (200 oscillations/min). After 30 min, 5 mL metaphosphoric/acetic acid (3%/8%) were added to each sample to prevent further oxidation of ascorbic acid. The ascorbic acid concentration was determined by titration with 2,6-dichlorophenol-indophenol reagent (AOAC, 1970) and expressed as a percentage of the original ascorbic acid concentration. All samples were analyzed in triplicate. The entire experiment was repeated three times. Statistical analysis of variance was used to determine the significance of factors. Duncan's New Multiple Range

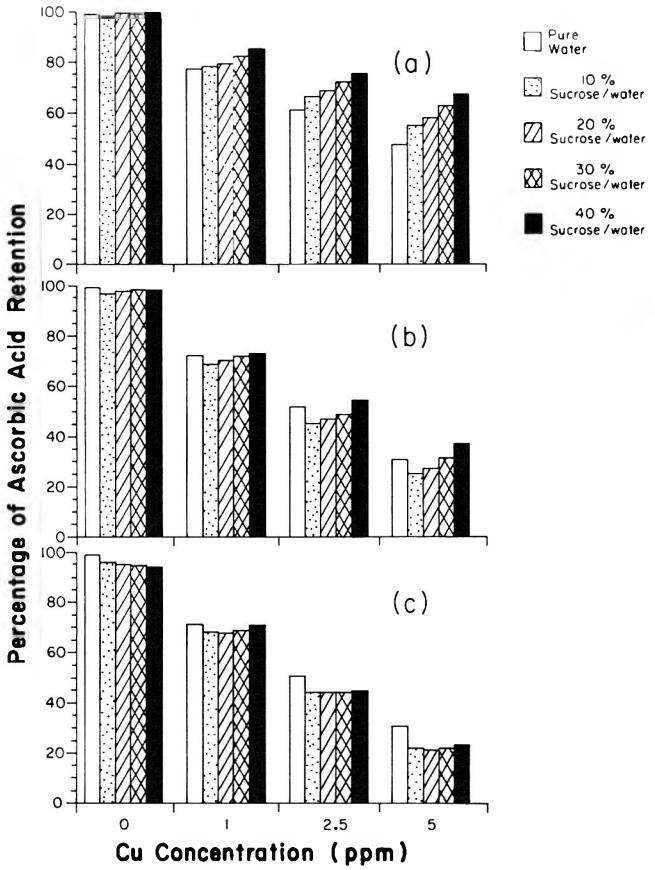

Fig. 1—Percentage of ascorbic acid retention in unbuffered solutions of sucrose (0–40%) and Cu (0–5 ppm) at pH 3.2. Samples were (a) unshaken, (b) shaken at 80 oscillations/min, and (c) shaken at 200 oscillations/min in a water bath at 30°C for 30 min.

Test was performed to determine significant differences among treatment means. Significant differences were accepted at the 5% level of probability.

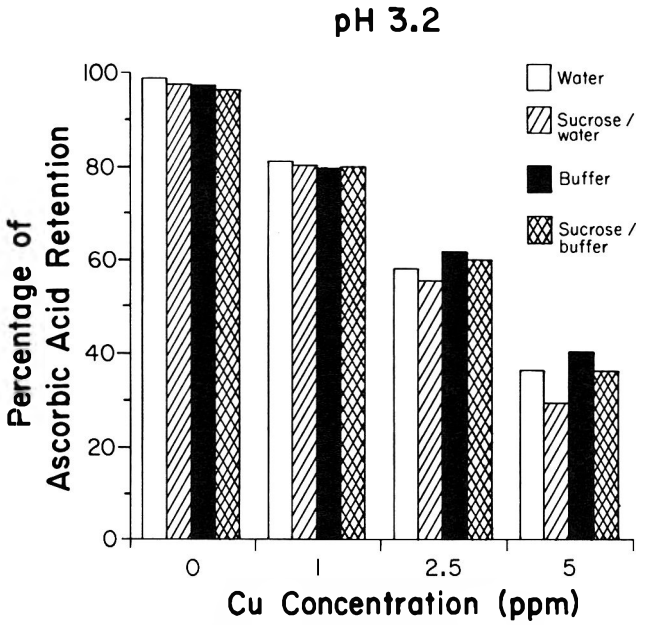

Fig. 2—Percentage of ascorbic acid retention in 01.M acetate buffered and unbuffered solutions at pH 3.2 in the presence of various concentrations of Cu and in the presence and absence of 10% sucrose. Samples were shaken at 200 oscillations/min in a water bath at 30°C for 30 min.

Table 1—Comparison of the ratios of the percentage of ascorbic acid retention in 10% sucrose solutions to that in solutions without sucrose in acetate buffered and unbuffered reactions at pH 3.2[a]

Cu conc (ppm)	Solution	
	Unbuffered	Acetate buffered
0.0	0.985	0.991
1.0	0.992	1.005
2.5	0.957	0.972*
5.0	0.806	0.900**

[a] Samples were shaken at 200 oscillations/min in water bath at 30°C for 30 minutes.
* This value was significant at $p < 0.05$ as compared with the control unbuffered solution.
** This value was highly significant at $p < 0.01$ as compared with the control unbuffered solution.

RESULTS & DISCUSSION

The percentages of ascorbic acid retention in unbuffered solutions under three conditions of oxygen supply, in the absence and presence of four levels of sucrose, and in the absence and presence of three levels of copper are presented in Fig. 1.

Under a nonshaking condition (Fig. 1a), there was no significant difference in ascorbic acid retention among water and sucrose solutions in the absence of copper. However, all sucrose solutions showed significantly higher ascorbic acid retention than their respective water counterparts at all levels of copper-catalyzed reactions. This protective effect of sucrose increased with increasing concentrations of sucrose and became marked at higher concentrations of copper. Ascorbic acid retention in water and sucrose solutions significantly decreased as the copper concentration increased. In the presence of the copper catalyst, the oxidation rate was much faster than that in noncatalyzed reactions, therefore the oxygen demand of the reaction system was also much greater. Under nonshaking conditions, oxygen supply was totally dependent on the rate of oxygen diffusion from the surface of the sample. It has been reported that the size of the sample influenced the rate of reaction due to diffusion of oxygen from the surface into the solution (Miller and Joslyn, 1949b). In the present study, a 2 ml aliquot was thinly spread in a 50 ml flask in order to maximize the air exposure surface area for oxygen access and to minimize the effect of viscosity of sucrose solutions. The amount of oxygen continuously diffused from air in the above condition may have been enough for slow noncatalytic oxidation but not enough for rapid catalytic oxidations. Data in Fig. 1a suggest that under limited oxygen supply viscous sucrose solutions restrict the rate of oxygen diffusion into liquid and in turn protect ascorbic acid from rapid oxidation. As copper concentration and thus reaction rate increased, the oxygen diffusion became more apparent as a limiting factor. Therefore, the extent of this protection increased as the viscosity of the solution, i.e. the concentration of sucrose, increased and as the rate of reaction, i.e. the concentration of copper, increased.

When the oxygen supply of samples was increased by shaking at 80 oscillations per min (Fig. 1b), the situation changed. In the absence of copper, ascorbic acid retention in sucrose solutions was slightly, but not significantly, lower than that in absence of sucrose except in the 10% sucrose solution which showed a significant lower retention. In the presence of copper, there was a significantly lower ascorbic acid retention in the less viscous 10% and 20% sucrose solutions than that in their blank counterparts. The more viscous, higher concentrations of sucrose solutions exhibited better retention and gradually tended to be protective of ascorbic acid as the level of copper increased. The data suggest that the destructive effect of sucrose at lower levels was overcome as viscosity increased at higher levels of sucrose. This implies that at 80 oscillations per min shaking the oxygen supply was still a limiting factor for ascorbic acid oxidation.

Further increasing of the oxygen supply by shaking at 200 oscillations per minute (Fig. 1c), caused all sucrose solutions to exert a significant destructive action on ascorbic acid. Thus, the protective effect of sucrose formerly attributed to viscosity was overcome by 200 oscillations per min of shaking. In the absence of copper, ascorbic acid retention was slightly lower in sucrose solutions than in solutions containing no sugar and the retention decreased as the concentration of sucrose increased. This again suggests that sucrose had a destructive effect on ascorbic acid if oxygen was not a limiting factor. The slightly destructive effect of sucrose is probably due to trace amounts of metal impurities which act as a catalyst in ascorbic acid oxidation. Analytical grade sucrose contains less than 5 ppm total metal impurities. In copper-catalyzed reactions, sucrose showed a marked significant destructive effect on ascorbic acid and the degree of this destructive effect was increased with increasing concentrations of copper. It appears that sucrose enhanced the catalytic action of copper on the oxidation of ascorbic acid. According to Hsieh and Harris (1987), the copper activity or the effective concentration of copper was increased in sucrose solutions due partly to the bulking effect of sucrose in solution and partly to the lowered water activity. This increased copper activity is perhaps the most important factor contributing to the destructive effect of sucrose on ascorbic acid stability in copper-catalyzed solutions. It is likely that this destructive effect would increase as the concentration of sucrose increases if oxygen is not a limiting factor. In copper-catalyzed reactions, the differences in ascorbic acid retention among various concentration of sucrose solutions almost disappeared, indicating that oxygen is still a limiting factor in this experiment. Regardless of how fast the oxygen is supplied to the system by shaking, the rate of oxygen diffusion into solution would not be fast enough to replenish the oxygen consumed in the reaction system when the concentration of copper or the concentration of sucrose solution is very high.

Data were also analyzed to determine the effect of shaking speed on the ascorbic acid retention in various experimental solutions. There was no significant difference in ascorbic acid retention caused by shaking for water solutions containing no sucrose and no copper. The other solutions showed significant change affected by shaking either in the presence of sucrose or copper or both, and the degree of significance increased as the levels of copper and sucrose increased. This result provides further evidence that under certain conditions, the amount of oxygen supplied to the solution may be sufficient for relatively slow oxidations of ascorbic acid. Results of the present study indicate that previous observations reported in the literature may be complicated by the restricted availability of oxygen and the combinations of solution properties involved. The present study shows that if the viscosity effect can be eliminated and if the oxygen is not a limiting factor sucrose actually enhances the oxidation of ascorbic acid; furthermore, this effect increases as the concentrations of copper and sucrose increase.

For the purpose of comparing the stability of ascorbic acid in buffered and unbuffered solutions at the same pH level, a similar experiment with the 200 oscillations per min shaking was carried out in 0.1M sodium acetate-acetic acid buffer at pH 3.2 in the absence and presence of 10% sucrose. The value of pH 3.2 was chosen so that the pH of the buffered and unbuffered ascorbic acid solutions was identical. Results are illustrated in Fig. 2. Although the retention of ascorbic acid was reduced only slightly, it was significantly lower in buffered than in unbuffered solutions in the absence of copper or at a very low level of copper (1 ppm). This indicates that the acetate buffer salt may have a slight catalytic effect. However, at higher levels of copper ($\geq$2.5 ppm), ascorbic acid in buffered solutions tended to be protected due to the copper complexing power of acetate buffer salt (Joslyn and Miller, 1949). Data in Fig. 2 suggested that although the acetate buffer itself has some degree of catalytic effect on the oxidation of ascorbic acid, the protective characteristic of the buffer would be dominant at higher levels of copper due to the copper complexing ability of acetate buffer salt. In the presence of 10% sucrose solutions, a similar trend was observed. However, in both sucrose buffered and unbuffered solutions, the ascorbic acid retention was consistently lower than that in their blank counterparts, except in the buffered solution containing 1 ppm of copper. The extent of this destructive effect can be illustrated by calculating the ratio of the percentage of ascorbic acid retention in 10 per cent sucrose solutions to that of solutions containing no sucrose (Table 1). Results suggest that in copper-catalyzed reactions, this destructive effect of sucrose was less pronounced in buffered than in unbuffered sucrose solutions because part of the copper was complexed by the acetate buffer salt (Joslyn and Miller, 1949).

Thus it can be concluded as follows: viscous sucrose solutions physically retarded the rate of oxygen diffusion into liquid and, thus, protected ascorbic acid from rapid oxidation. When the viscosity effect of sucrose was diminished by vigorous shaking, sucrose lowered the retention of ascorbic acid, especially in solutions containing copper. The enhanced catalytic power of copper in sucrose solutions on the oxidation of ascorbic acid was probably due to the bulk of sucrose that relatively increased the copper activity, and to the decreased water activity in solution. In the 0.1 molar acetate buffered solutions, sucrose also lowered the retention of ascorbic acid, however, this effect was partially suppressed in copper-catalyzed reactions due to copper complexing by the buffer salt. Chemically, sucrose itself may have no effect on the stability of ascorbic acid; however, even the trace levels of metal impurities found in analytical grade sucrose may contribute a slight catalytic effect on the oxidation of ascorbic acid.

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Ms received 9/15/86; revised 2/24/87; accepted 3/5/87.

Depolymerization of Starch by High Pressure Extrusion

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ABSTRACT

High pressure extrusion (13,000–40,000 psi), using a French Pressure Cell and hydraulic press, was evaluated for the degradation of potato starch. Rapid decreases in both relative and intrinsic viscosities of starch colloidal solutions (0.5–2.5%, w/v) were evident, particularly following the first extrusion. However, maximum reducing powers formed (1.15 dextrose equivalents) were negligible even after 5 consecutive extrusions of a 0.5% starch solution. The significant decreases in viscosities of starch solutions were due to depolymerization of the starch molecules into smaller fractions with little reducing power. However, the use of high pressure extrusion combined with heat (97°C) and 3N HCl significantly enhanced degradation of a 2.5% starch solution to yield 52.1 dextrose equivalents.

INTRODUCTION

ETHYL ALCOHOL (ethanol) fuels have been considered one of the most suitable substitutes for petroleum. This solvent is now produced in the United States by fermentation of high starch-containing crops (Sachs, 1980). When raw materials containing polysaccharides are used for alcohol fermentation by yeast, they are first converted to fermentable sugars using enzymes, inorganic acids, irradiation (Saeman et al., 1952; Han et al., 1981; Azhar et al., 1982), ultrasonic techniques (Szent-Györgyi, 1933; Azhar and Hamdy, 1979), and ball milling (Millett et al., 1976). Combinations of acid hydrolysis and physical extrusion have been used (Brenner et al., 1981; Noon and Hochstetler, 1981). However, acid hydrolysis requires corrosion-proof equipment, and yields undesirable by-products (Kerr, 1950; BeMiller, 1965; Humphrey, 1979; Azhar and Hamdy, 1981a; Azhar et al., 1981). On the other hand, enzymes used for hydrolysis are slow and expensive (Kooi and Armbruster, 1967; Azhar and Hamdy, 1981b). Physical treatment has also been used for pretreatment of cellulosic materials prior to enzyme action (Han et al., 1981; Carr and Doane, 1984; Bertran and Dale, 1985). As saccharification is the key step for the conversion of polysaccharides to fermentation substrates, processes that improve hydrolysis with minimal side reactions need to be explored. The objective of this study was to examine the effect of very high pressure using the French Pressure Cell, alone or in combination with hydrochloric acid and heat, on starch depolymerization to fermentable sugars.

MATERIALS & METHODS

Starch fractionation and depolymerization

Powder reagent grade soluble potato starch (Fisher) was used, and the desired concentration in deionized water was made by heating starch at 100°C for 5 min. The most effective solvent system for fractional precipitation of undegraded and degraded (depolymerized) colloidal starch solution was determined using 25, 50, 75, and 90%, (v/v) ethanol, methanol, isopropanol, acetone, and a mixture of ethanol and acetic acid (60:40, v/v ratio). Aqueous colloidal potato starch solution (0.4g) was prepared and the desired volume of solvent added to yield a final volume of 40 mL. The starch fractions precipitated (overnight at 4°C) were then centrifuged, dried to a constant weight under vacuum, and weighed.

The French Pressure Cell, FPC, (American Instrument Co., Silver Spring, MD) used for the depolymerization of starch consisted of a piston and thick-walled cylinder, a base plate for the cylinder equipped with an adjustable orifice, and a hydraulic press capable of continuously depressing the piston while maintaining constant desired high pressure within the cylinder. Upon extrusion of the starch solution through the orifice, the pressure rapidly decreased to atmospheric and mechanically disrupted the particles in the sample. Starch solution (35–40 mL) was introduced into the cylinder, the piston installed, and the valve controlling the orifice closed. The cell was then pressurized to the desired level using the hydraulic press, and the valve slowly opened. Unless specified, all experiments were conducted at room temperature (24°C) and viscosities as well as reducing powers (reported as dextrose equivalent values) were determined to monitor the degree of depolymerization.

Viscosity measurement

Relative viscosity (η_{rel}) and intrinsic viscosity [η] of undegraded and/or depolymerized colloidal starch solutions were measured in a water bath (25 ± 0.05°C) using a Cannon-Fenske viscosimeter (Induchem Lab Glass Co., Roselle, NJ). The η_{rel} was determined using the following equation:

$$\eta_{rel} = (\eta/\eta_o) = (t \times d)/(t' \times d') = t/t', \text{ if } d = d'$$

where η is the viscosity of the solution, and η_o that of the solvent (water), t is the efflux solution time (sec) in the viscosimeter and t' that of the solvent (water), and d is the solution density and d' that of the solvent (water). The [η] values were determined for all aqueous undegraded and depolymerized starch fractions using concentrations ranging from 0.2 and 0.6 g/dL. From η_{rel}, the specific viscosities (η_{sp}) were obtained:

$$\eta_{sp} = \eta_{rel} - 1$$

The quantity (η_{sp}/C), where C is grams dry weight of the depolymerized starch per dL, was calculated and plotted vs. C, from which the [η] was obtained by extrapolation (Foster, 1965):

$$[\eta] = \lim_{C=o} \eta_{sp}/C$$

The reducing sugars liberated from starch solution were determined spectrophotometrically based on the reduction of 3,5-dinitrosalicylic acid (Bernfeld, 1955), and reported as dextrose equivalent values (DEq), expressed as follows:

$$DEq = \frac{\text{Reducing powers of sample measured as dextrose}}{\text{Sample dry wt}} \times 100$$

Separation and identification of the sugars present in the depolymerized starch utilized a descending unidimensional chromatographic technique on Whatman filter paper No. 1, 1-butanol-pyridine-water (60:40:30, v/v, respectively) as solvent, and p-anisidine hydrochloride reagent solution (Pridham, 1956).

Effect of molecular weight and concentration

Undegraded and depolymerized starch solutions (2.5%) were fractionated (at 4°C) into three fractions designated as A', B', and C' for the undegraded and A, B, and C for the depolymerized starch using a 60:40 (v/v) mixture of ethanol-acetic acid at levels of 20, 40, and 60% (v/v), respectively. The starch fractions were dried under vacuum, weighed, and the [η] of each fraction determined. Fractions A', B', and C' represented high, medium, and low molecular weight starches as determined by their [η].

Various starch concentrations (0.5, 0.75, and 1.0%, w/v), were

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extruded at 20,000 psi five consecutive times. Viscosities and reducing powers (DEq) of the resulting solutions were determined following each extrusion.

Effect of acidification

Starch colloidal solution (2%, w/v) was mixed well with an equal volume of 2N HCl, and the mixture (now 1N) immediately extruded at 20,000 or 40,000 psi five consecutive times at 2–4°C. Following each extrusion, an aliquot of the resulting solution was neutralized with 2N NaOH and analyzed for reducing powers (DEq). A control (not acidified) starch solution was treated in the same manner, and the DEq present determined.

Starch solution (0.5% w/v) was hydrolyzed for 5 min in 1N HCl at 97°C, neutralized, extruded at 20,000 psi, and examined for DEq formed.

Effect of acidification and heating

Both HCl (desired normality) and starch solutions were preheated (97°C) separately, mixed well, and immediately extruded at 13,000 psi using a preheated (97°C) cylinder of the FPC. The effluent was rapidly cooled to 4°C, neutralized, and examined for DEq. A control starch solution was treated similarly but without extrusion and assayed for its DEq value after neutralization.

RESULTS & DISCUSSION

Effect of high pressure extrusion

A rapid decrease in the η_{rel} of a starch colloidal solution (2.5%, w/v) was observed after each high pressure extrusion (13,000 psi) particularly following the first one (Fig. 1A). Reducing powers (as DEq) also increased slowly with repeated extrusion but the values were very small, even after five extrusions (0.07 after the 2nd and only 0.18 after the 5th extrusion).

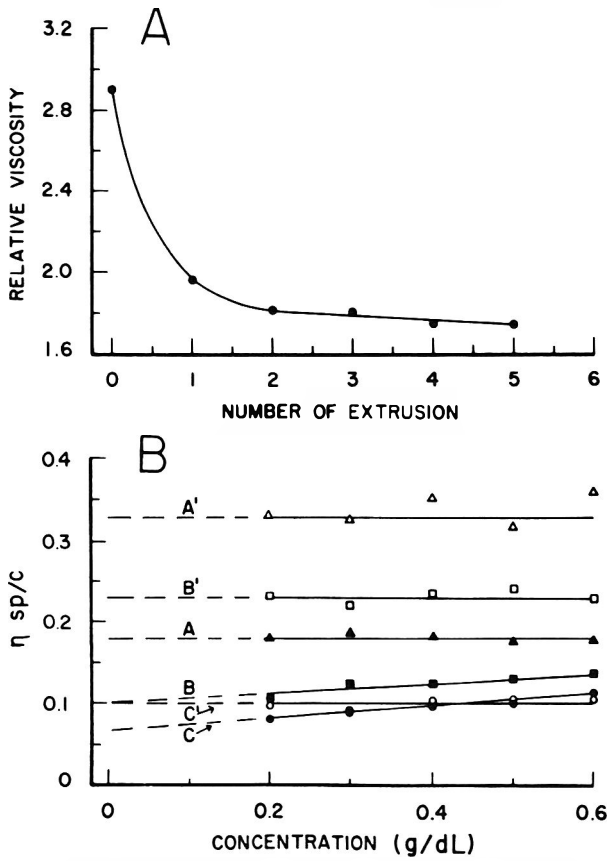

Fig. 1—Effect of high pressure extrusion (13,000 psi) on the degradation of starch as measured by its relative viscosity (A) and on starch fractions as determined by the intrinsic viscosities (B). The [η] was determined by extrapolation to zero starch concentration. The starch solution (2.5%, w/v) was extruded five consecutive times and then fractionated into A, B, and C, where A', B', and C' represent the corresponding starch fractions, respectively, before extrusion.

Ethanol-acetic acid mixture (60:40, v/v) at the 50% level precipitated 92.9% of the starch and, therefore, was considered the most effective solvent for fractionation of the undegraded starch, followed by isopropanol (Table 1). At the 75% level, both solvents were effective and 100% of the starch was recovered, whereas at 90%, all solvent systems exhibited similar precipitation capacities. Hamdy (1953) reported that isopropanol (99%) was an effective solvent for precipitation of dextran polymer and that a mixture of ethanol-acetic acid (60:40, v/v) was the poorest. This may be due to the difference in conformation structure between the starch and the dextran polymers.

The data for fractionation of the undegraded starch revealed the presence of diverse molecular weight molecules, as evidenced from the intrinsic viscosity data (Fig. 1B). As expected, the highest molecular weight (high [η]) starch was precipitated at the lowest solvent concentration. The [η]s of the 3 fractions (A', B', and C') of the undegraded starch, precipitated at 20, 40, and 60% levels of the ethanol-acetic acid mixture, were 0.33, 0.23, and 0.10, respectively (Fig. 1B). Fraction A' represented 91% of the total starch precipitated, whereas fractions B' and C' comprised 3 and 6% of the total, respectively.

A starch solution (2.5%) was also fractionated after depolymerization at 13,000 psi (5 consecutive times). Three fractions (A, B, and C) were obtained at 20, 40, and 60% levels of the ethanol-acetic acid mixture (60:40, v/v), respectively. The [η] values for fractions A and B (Fig. 1B), were markedly reduced from the original values of 0.33 and 0.23 for the undegraded starch to 0.18 and 0.10, respectively, indicating decrease in molecular weights. The C fraction was not affected, having respective intrinsic viscosities of 0.10 and 0.08 before and after depolymerization. The significant changes in the relative viscosities of the starch solution following extrusion (Fig. 1A) were due mainly to depolymerization of larger starch molecules (fractions A and B). The weight percent of fraction A decreased from 91% to 60% (A'), whereas the percentages of fractions B and C increased from 3% (B') and 6% (C') to 22% and 19%, respectively. This indicated that the large molecules (fraction A') were depolymerized to smaller molecules thereby increasing the proportions of fractions B and C.

High pressure extrusion (20,000 psi) markedly decreased the η_{rel} of various starch solutions (0.5, 0.75, and 1.0% w/v) particularly after the first extrusion (Fig. 2A). However, after the second extrusion, the rate of change was much slower, with very little change thereafter. The results for the effect of high pressure extrusion (20,000 psi) on the [η] of a 1% (w/v) starch solution are shown in Fig. 2B. A rapid decrease in the [η] value was noted from 1.13 before extrusion to 0.32 after the first one, whereas on the third and fifth extrusions, the [η] values were 0.21 and 0.17, respectively. The intrinsic viscosity measurement used in this investigation is one of the simplest, hence the most utilized, of physical measurements capable of providing useful information on the molecular weight(s) such as undegraded and depolymerized starch fractions and on the

Table 1—Effect of various precipitating solvent systems on percent recovery of potato starch fractions[a]

% Solvent used	Percent recovery of starch fractions				
	Ethanol	Methanol	Ethanol[b] acetic acid	Isopropanol	Acetone
25	29.9	29.6	9.1	16.0	28.0
50	72.9	69.2	92.9	82.2	69.6
75	90.3	70.2	100.6	104.6	95.0
90	101.1	99.6	102.0	103.0	102.5

[a] Results are average of two experiments.
[b] Ethanol: acetic acid mixture (60:40, v/v ratio).

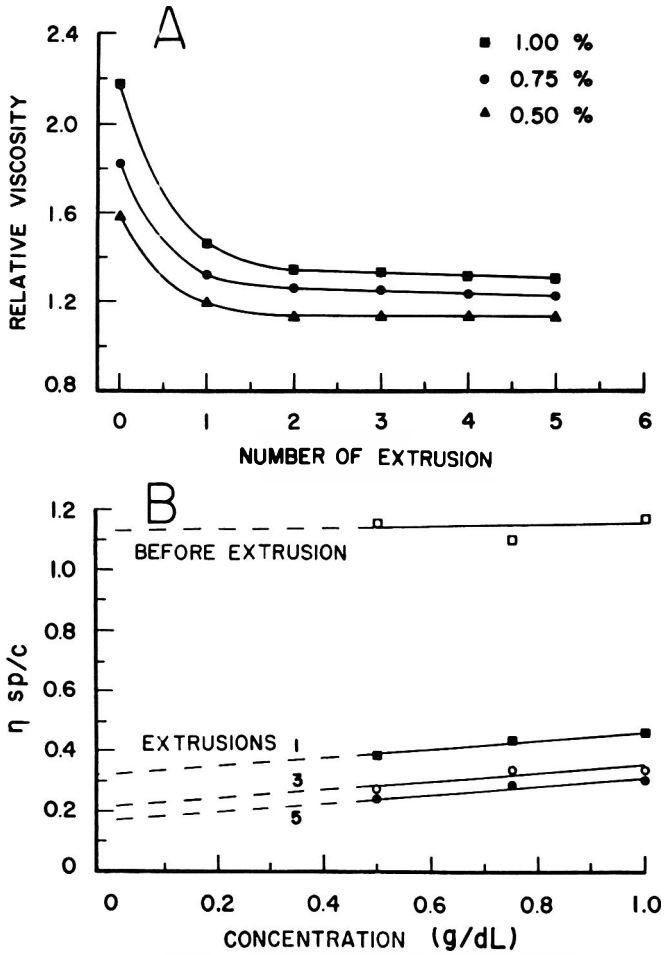

Fig. 2—Effect of high pressure extrusion (20,000 psi) on the depolymerization of different concentrations of starch solutions as followed by the relative viscosities (A) and the intrinsic viscosities (B). The [η] was obtained by extrapolation to zero starch concentration.

shape of polymers in solution (Hamdy, 1953; Foster, 1965). Hamdy (1953) reported that the intrinsic viscosity of a colloidal solution can be used as a guide to the molecular size for large rod-like colloical molecules, and that smaller molecules exhibit lower [η]s than do larger ones. This relationship is expressed in the following equation:

$$[\eta] = K \cdot M^a$$

where K and the exponent a are empirical parameters and M is molecular weight. It is important to note that K and a must be established by molecular weight measurements. Killion and Foster (1960) reported that the average molecular weight of starch as determined by light scattering measurement was 1.9 $\times 10^6$. Cowie (1960) regarded the conformation of linear amylose in aqueous solution as a stiff coil, whereas Rao and Foster (1963) established it as a relatively stiff, wormlike coil consisting essentially of an imperfect or deformed helical backbone. The rapid decreases in both η_{rel} and [η] observed in this investigation were attributed to rapid depolymerization by high pressure extrusion of the potato starch granules into fragments of smaller molecular weights.

Table 2—Effect of high pressure extrusion (20,000 psi) on degradation of various potato starch levels, as measured by dextrose equivalent (DEq)

Starch (%, w/v)	DEq formed following extrusion (number)				
	1	2	3	4	5
0.50	0.00	0.72	0.83	1.08	1.15
0.75	0.00	0.36	0.55	0.72	0.72
1.00	0.04	0.54	0.58	0.63	0.72

The results for the effect of high pressure extrusion on the DEq values of various concentrations (0.50, 0.75, 1.00%) of starch are recorded in Table 2. Although the reducing powers (DEq) of all levels of starch increased with repeated extrusion at 20,000 psi, the resultant DEq was 1.15 or less. However, neither glucose nor maltose was chromatographically detected in the depolymerized starch solution. All the starch solutions extruded using high pressure were tested for the degree of starch depolymerization using the iodine reaction. The blue color of the starch-iodine complex after the 5th extrusion was not significantly altered from its initial value. An increase in DEq value that is dependent on the terminal aldehyde group of the starch molecule will occur upon breaking the α (1-4)- or α (1-6)-linkage of starch molecules, resulting in decreased molecular weights. However, the low reducing powers (<1.15 DEq), positive iodine test for depolymerized starch solution, and the absence of glucose or maltose in the solution containing depolymerized starch indicated that a very small concentration was hydrolyzed to fermentable sugars. The decrease in both η_{rel} and [η] of the starch colloidal solutions extruded at high pressure is probably due to simple breakdown of large starch molecules into smaller starch fragments with no reducing power. This physical degradation of the starch was similar to that reported by Azhar and Hamdy (1979) for ultrasonic degradation where no reducing sugars were detected following the sonication. Leach (1965) stated that the swollen starch granules during gelatinization become increasingly susceptible to disintegration by mechanical shear. Azhar and Hamdy (1979) also observed depolymerization of potato starch by sonication and concluded that depolymerization as evidenced by the decreases in viscosity may lead to configurational modification of the starch granules rather than the true alteration of the individual polymers.

Effect of high pressure extrusion and acid

Since hydrochloric acid (HCl) is effective in the hydrolysis of starch to fermentable sugars (BeMiller, 1965; Azhar and Hamdy, 1981a; Kim and Hamdy, 1985), the effect of the combined treatment of 1N HCl and high pressure on the rate of starch depolymerization was studied. Extrusion of acidified starch solution at 20,000 psi and 2–4°C did not enhance the depolymerization rate (Table 3) above that of acid alone. However, at 40,000 psi and 2–4°C, HCl increased the rate of depolymerization and considerable reducing powers were formed following repeated extrusion.

The effect of high pressure on partially acid-hydrolyzed starch was also examined. Starch (0.5%) was hydrolyzed in 1N HCl for 5 min at 97°C, and the acid-hydrolyzed starch (after neutralization) extruded at high pressure (Table 4). High pressure extrusion was not effective in degrading partially hydrolyzed

Table 3—Comparative effects of combined high pressure extrusion and acidification (1N HCl) of potato starch on the relative viscosity (η_{rel}) and on the dextrose equivalent (DEq) values of the depolymerized starch

Number of extrusions	Starch solution	Value for η_{rel}[b]	DEq (at psi)[a]	
			20,000	40,000
0	Aqueous	2.17	0.00	0.00
	HCl	2.10	0.00	0.00
1	Aqueous	1.46	0.04	0.22
	HCl	1.39	0.26	1.09
2	Aqueous	1.34	0.54	0.40
	HCl	1.36	0.97	3.50
3	Aqueous	1.33	0.58	0.49
	HCl	1.34	1.12	5.05
4	Aqueous	1.31	0.63	0.58
	HCl	1.33	1.47	7.08
5	Aqueous	1.30	0.72	0.58
	HCl	1.32	2.02	8.62

[a] Starch solutions (2%, w/v) were extruded at 2-4°C and desired pressure and the results are the average of two experiments.
[b] The relative viscosity was determined at 25 ± 0.05°C.

Table 4—Effect of high pressure extrusion (20,000 psi) on the depolymerization of partially acid-hydrolyzed potato starch as measured by the dextrose equivalent (DEq)[a]

Number of extrusions	DEq value
0	39.6
1	40.0
2	41.0
3	41.7
4	41.7
5	42.0

[a] Results are the average of two experiments.

Table 5—Effect of high pressure extrusion (13,000 psi) combined with hydrochloric acid and heat on the degradation of potato starch as determined by dextrose equivalent value of the degraded starch[a]

Normality of HCl	Dextrose equivalent	
	0 psi	13,000 psi
1	4.8	16.9
2	14.7	37.1
3	21.6	52.1

[a] The starch (2.5%, w/v) in HCl was extruded at 13,000 psi for 2 min. The initial temperature of starch in acid solution was 97°C but decreased to 70°C after the extrusion. The average of 3 experiments is reported.

starch beyond that of acid alone. The increase (from 39.6 to 42.0 DEq) due to the extrusion was not of important value.

Effect of high pressure in combination with HCl and heat

Results of the depolymerization of starch solution at high pressure combined with various normalities of HCl and heat are summarized in Table 5. High pressure in combination with 1N HCl and 97°C increased the DEq values of the starch solution from 4.8, at 0 psi, to 16.9 at 13,000 psi. This increase was more pronounced using 2N and 3N HCl. Previous experiments at 2–4°C (Table 3) showed that 40,000 psi increased the reducing powers of the starch solution only slightly, from a DEq value of zero to 1.09 DEq in 1N HCl after the first exposure. These results also indicated that, at an elevated temperature (97 – 70°C), high pressure extrusion combined with HCl treatment degraded the starch solution more efficiently. This efficient degradation procedure was also used by Noon and Hochstetler (1981) on cellulose. These authors reported that sawdust (40% moisture), when subjected to 600 psi at 226.7°C in 1.8% (w/v) sulfuric acid, yielded 17.12% glucose and 26.08% total sugar during only 5 sec of hydrolysis. However, such a high temperature enhanced the production of undesirable end products such as hydroxymethyl furfural which is known to inhibit yeast during alcohol fermentation (Kim and Hamdy, 1985; and Azhar et al., 1982).

CONCLUSIONS

HIGH PRESSURE alone (up to 40,000 psi) did not depolymerize starch solution to the desired level of fermentable sugar. However, high pressure (13,000 psi) in combination with heat (97°C), and acid (1N HCl) improved the depolymerization of starch (2.5%, w/v) to fermentable sugars, reaching a value of 16.9 DEq after one extrusion. Further degradation was accomplished using 2N and 3N HCl leading to DEq values of 37.1 and 52.1, respectively. The potential of high pressure in combination with acid and heat for degradation of polymers such as starch, with reasonable cost and no side reactions, merits further attention.

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Ms received 1/30/87; revised 4/17/87; accepted 5/5/87.

Sensory Quality of Selected Sweeteners: Unbaked and Baked Flour Doughs

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ABSTRACT

Sucrose, fructose, aspartame, acesulfame K, sodium saccharin, and calcium cyclamate were studied in unbaked and baked shortbread-type cookies. Sweeteners were evaluated for initial, maximum, and residual sweetness intensity and nonsweet aftertaste for effects of lemon and vanilla flavor on sweetness character. Type of sweetener influenced sweetness quality in unbaked and baked cookies. Intensities and sweetness profiles for the six sweeteners differed between unbaked and baked cookies, particularly for aspartame. Sweetness of sucrose and saccharin were perceived similarly in baked cookies, but they differed in quality and intensity of nonsweet aftertaste. Flavor did not affect initial, maximum, or residual sweetness but nonsweet aftertaste was less intense in lemon and plain unbaked cookies than in vanilla-flavored unbaked cookies.

INTRODUCTION

MANY RESEARCHERS have studied relative sweetness and effects of basic tastes on sweetness intensity in simple solutions and dispersions (Fabian and Blum, 1943; Gregson and McCowen, 1963; Hyvonen et al, 1977; Cardello et al, 1979). Sweetness character in food systems that contain a high percentage of fat or that undergo heat processing has been studied to a lesser extent.

The most relevant studies have been concerned with carbohydrate sweeteners or a combination of sweeteners exposed to various baking methods. Hardy et al. (1979) compared sucrose to fructose in sugar cookies and white (vanilla) cake. Hess and Setser (1983) evaluated chocolate, lemon, orange, or spice flavored cakes sweetened with aspartame or aspartame and fructose and baked conventionally or in the microwave. Overall sweetness was the parameter evaluated by the panelists in the aforementioned studies.

Little information has been published on perceived sweetness of aspartame, saccharin, or other intense sweeteners in food systems, particularly baked products. All ingredients must be considered when characterizing sweetness quality. The objective of this study was to investigate effects of thermal processing on perception of sweetness attributes of some nutritive and non-nutritive sweeteners with and without flavorings in a simple but complete food system, a shortbread-type cookie.

MATERIALS & METHODS

Sample preparation

A shortbread-type cookie was prepared from hydrogenated shortening (Crisco, Procter and Gamble, Cincinnati, OH), distilled water, flour (Gold Medal, General Mills, Minneapolis, MN), sweetener, and flavoring according to the formula in Table 1. Lemon or vanilla liquid flavorings (#4267 Lemon Natural Oil and #3454 Vanilla, Warner Jenkinson Flavors, St. Louis, MO) were used. Control samples were designated "plain" and contained no flavoring. Granulated sucrose (C and H), chemically pure crystalline β-D-fructose (Sigma Chemical Co., St. Louis, MO), unbulked aspartame (G.D. Searle, Chicago, IL), acesulfame K (Hoescht, AG., Frankfort, W. Germany), sodium saccharin (Sherwin Williams, Cincinnati, OH), and calcium cyclamate (Abbott Laboratories, Chicago, IL) were used. The sucrose cookie contained 25% sweetener. Difference tests and scaling techniques were employed during preliminary work to determine approximate equisweet concentrations of all non-sucrose sweeteners (Redlinger and Setser, 1987).

Each sweetener was dissolved prior to incorporation into the shortening to ensure even blending and to prevent panelists from discerning differences based on crystal or granule size. After addition of the sweetener solution and flavoring, the mixture was whipped on high speed with a portable rotary mixer (Sunbeam, Model 3-7x). Flour was blended in by hand until the mass was homogeneous.

Unbaked cookie doughs were placed in coded plastic bags and refrigerated overnight at 7 ± 1°C. On the day of evaluation, small samples (approximately 5–8g) were placed in coded plastic 1 oz. cups, capped with foil, allowed to come to room temperature (25 ± 1°C),

Table 1—Formulations[a] for cookie based on percentage total system (w/w) and percentage flour weight

		% Total system				%
	Amount	Sweetener	Flour	Shortening	Water	Flour wt
Flour	115.0					100.0
Shortening	75.0					65.22
Water	20.0 mL					17.39
Flavoring	0.3 mL					0.26
Sweetener						
Sucr	70.00	25.00	41.07	26.79	7.14	60.87
Frc	54.50	20.60	43.48	28.36	7.56	47.39
Asp	0.47	0.22	54.64	35.63	9.50	0.41
AsfK	0.47	0.22	54.64	35.63	9.50	0.41
Sac	0.47	0.22	54.64	35.63	9.50	0.41
Cycl	1.06	0.50	54.49	35.53	9.48	0.92

[a] Formulation is for plain cookie, although amounts of shortening, flour, water, and flavoring used in each system were the same, percentages varied slightly according to sweetener used.

Table 2—F-values and probabilities[a] for sweetness quality determined by sensory analysis of unbaked and baked cookies

		Sensory parameters			
Source of variation	DF	Initial sweetness	Maximum sweetness	Residual sweetness	Nonsweet aftertaste
Unbaked Cookies					
Replication (R)	2	3.08 (NS)	4.06 (NS)	0.84 (0.01)	0.33 (NS)
Flavor (F)	2	3.12 (NS)	0.85 (NS)	1.04 (NS)	7.13 (NS)
Sweetener (S)	5	47.25 (0.0001)	44.48 (0.0001)	49.42 (0.0001)	70.18 (0.0001)
F × S	10	0.43 (NS)	0.74 (NS)	0.58 (NS)	4.06 (0.0014)
Baked Cookies					
Replication (R)	3	1.22 (NS)	0.65 (NS)	1.58 (NS)	1.24 (NS)
Flavor (F)	6	0.56 (NS)	3.10 (NS)	1.08 (NS)	0.61 (NS)
Sweetener (S)	5	102.37 (0.0001)	122.09 (0.0001)	81.87 (0.0001)	48.27 (0.0001)
F × S	10	1.07 (NS)	0.62 (NS)	1.28 (NS)	0.50 (NS)

[a] Probabilities are given in parentheses; NS - not significant.

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Table 3—Mean[a] sensory values[b] pooled for all sweeteners for lemon, vanilla, and plain flavored unbaked and baked cookies

	Sensory parameters							
	Initial sweetness		Maximum sweetness		Residual sweetness		Nonsweet aftertaste	
Flavor	Unbaked	Baked	Unbaked	Baked	Unbaked	Baked	Unbaked	Baked
Lemon	22.95a	21.82a	33.97a	32.06a	21.43a	18.92a	16.43b	17.11a
Vanilla	22.38a	22.67a	33.17a	33.71a	22.16a	19.68a	29.14a	17.85a
Plain	20.44a	21.87a	32.91a	31.80a	20.94b	19.38a	16.87b	19.10a
LSD (0.05)	2.92	2.52	2.88	2.44	2.37	1.45	2.99	5.07

[a] Three replications with six sweeteners for a total of 18 observations mean; means in same column with same letter are not significantly different ($p < 0.05$); no comparison intended among columns or between systems.

[b] Sixty point computer scale with 60.0, highest and 0.0, lowest, equivalent to 6 inch QDA scale with 1 inch = 10 points.

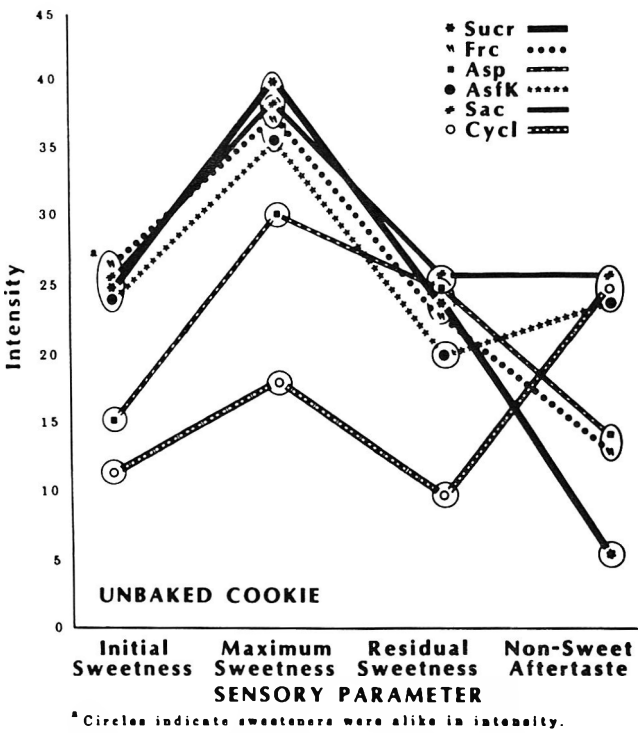

Fig. 1—Sensory profiles for sweeteners in unbaked cookies when flavors were pooled. Evaluations for initial and maximum sweetness were made approximately 5 and 20–35 sec, respectively, after ingestion. Residual sweetness and nonsweet aftertaste were evaluated about 30 sec after swallowing. Sucr = sucrose, frc = fructose, asp = aspartame, asfK = acesulfame K, sac = sodium saccharin, and cycl = calcium cyclamate.

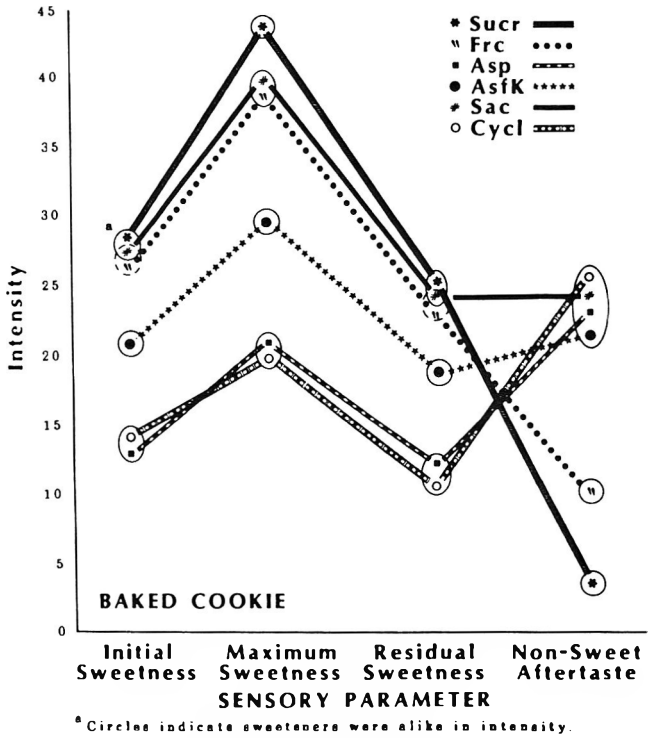

Fig. 2—Sensory profiles for sweeteners in baked cookies when flavors were pooled. Evaluations for initial and maximum sweetness were made approximately 5 and 20–35 sec, respectively, after ingestion. Residual sweetness and nonsweet aftertaste were evaluated about 30 sec after swallowing. Sucr = sucrose, frc = fructose, asp = aspartame, asfK = acesulfame K, sac = sodium saccharin, and cycl = calcium cyclamate.

and presented to panelists. Small stainless steel knives were used to sample the cookie dough according to protocol established by panelists during training.

Dough to be baked was mixed on day 1. The dough was weighed, divided in half, placed in plastic bags, and stored in the refrigerator (7 + 1°C). The cookies were baked on day 2 and evaluated on day 3. On the day of baking, the dough was brought to room temperature (25 ± 1°C) and rolled out with a wooden rolling pin between two sheets of wax paper over a plastic mold. The cookies were trimmed of excess dough and placed on a 30 × 35 cm (12 × 14 in) CushionAire insulated aluminum baking sheet. Each unbaked cookie measured approximately 14–15 cm in diameter and 1 cm in thickness. A plastic template was used to score each cookie into eight individual, equal-sized portions. Cookies were baked in a rotary hearth electric oven at 163°C (325°F) for varying lengths of time to approximate the color of the sucrose reference. Invariably, the fructose cookie was darker than any other cookie, but sufficient baking time was necessary to prevent raw starch flavor. Fructose and sucrose cookies were baked for 25 and 35 min, respectively, (to approximate endpoint temperatures of 140° and 145°C), aspartame, acesulfame K, saccharin, and cyclamate cookies were baked for 50 min to approximately 155°C. After cookies were removed from the oven, they were cut immediately and allowed to cool on the baking sheet. Cookies were then placed on styrofoam plates and stored in odor-free, plastic bags at room temperature (25 ± 1°C) overnight.

Each panelist received one eighth of the total baked cookie (one wedge) on a coded, plastic-wrapped, watch glass. Panel members evaluated cookies by sampling the innermost point of the wedge because this portion of the cookie underwent minimal browning compared to the outer edge. Tasting one small part of the total cookie helped ensure uniformity in judging samples.

Sensory and data analysis

Food systems were evaluated for four parameters of sweetness intensity with time on a 60-point computerized scale. Initial sweetness was defined as intensity of sweetness perceived when sample was first experienced, or approximately 5 sec after ingestion. Maximum sweetness, the most intense sweetness perceived, was assessed during manipulation and occurred approximately 20–35 sec after ingestion, depending upon the sweetener. Residual sweetness was the sweetness that lingered after swallowing and was evaluated about 30 sec after the sample was swallowed. Intensity of nonsweet aftertaste was noted about 30 sec after swallowing, and intensity and identity of non-sweet tastes and/or feeling factors prior to and after swallowing were recorded. Further details of evaluation and data analysis are described in Redlinger and Setser (1987). The experimental design for each food system was the same as in the previous study, a 3 by 6 flavor by sweetener treatment combination in a split plot design. Analysis of

variance and least significant differences were used to ascertain significant effects.

RESULTS & DISCUSSION

NONSWEET AFTERTASTE in unbaked cookies was the only attribute for which interaction of sweeteners and flavors was significant ($p < 0.001$) (Table 2). Highly significant differences existed among sweeteners for all components of sweetness quality; initial, maximum, and residual sweetness and non-sweet aftertaste, in both unbaked and baked cookies (Table 2).

When mean scores for all sweeteners were pooled (Table 3) no significant differences were found among flavors for initial, maximum, or residual sweetness. Type of flavor affected only non-sweet aftertaste in unbaked cookies (Table 3). Nonsweet aftertaste was less predominant ($P < 0.05$) in lemon and plain cookies than in vanilla-flavored cookies. According to panelists' comments, bitter and medicinal notes were obvious in the vanilla-flavored unbaked cookies with acesulfame K, saccharin, and cyclamate.

As with aqueous and lipid model systems (Redlinger and Setser, 1987), sensory scores were plotted (Fig. 1 and 2) so that overall sweetener profiles for each sweetener could be observed. Crammer and Ikan (1986) observed that taste profiles of any sweetener need to be similar to sucrose: a fairly quick sweetness followed by a sharp cut-off. Therefore, plots of sweetness intensities of the four parameters can be compared to note similarities and differences on the sweeteners' profiles to sucrose. Generally, order of sweetness intensity in unbaked cookies was: fructose $\geq$ saccharin $\geq$ sucrose $\geq$ acesulfame K $>$ aspartame $>$ cyclamate. In baked cookies, fructose and sucrose were interchanged, otherwise, the order was similar: sucrose $\geq$ saccharin $\geq$ fructose $>$ acesulfame K $>$ aspartame $\geq$ cyclamate.

From the profiles one can observe that no sweetener behaved exactly like sucrose, especially after swallowing. However, sweetness profiles for sucrose, fructose, saccharin, and acesulfame K in the unbaked systems were shaped similarly, and aspartame and cyclamate had profiles that paralleled each other. The nonsweet aftertastes for saccharin, cyclamate, and acesulfame K were similarly high. In this system, non-sweet aftertastes of aspartame and fructose were relatively low and of the same intensity ($p < 0.05$), and sucrose had a significantly lower non-sweet aftertaste than all other sweeteners.

In baked cookies, profiles were strikingly similar in shape for all sweeteners for initial, maximum, and residual sweetness. The differences in intensity reflect, in large part, difficulties in obtaining isosweet systems because of inherent differences in the sweetness qualities of each of the materials and the manner in which each interacts with other ingredients present. Consistently, cyclamate was less sweet ($p < 0.05$) than any other sweetener, except aspartame, in the baked cookie. As with solutions and creams (Redlinger and Setser, 1987), the inherent bitterness of cyclamate offset sweetness and high levels could not be used. Baking for varying lengths of time was necessary to avoid influence of raw starch flavor in some systems and of browned compounds in others. Thus, one should concentrate on changes in the character of the profiles with baking. For example, profiles of sucrose, fructose, saccharin, and cyclamate were similar in shape and sweetness intensities for the unbaked and baked cookie. Differences between aspartame and acesulfame K, two sweeteners described similarly (Arpe, 1978; O'Brien and Gelardi, 1981; Rymon-Lipinski and Huddart, 1983), were especially obvious.

Acesulfame K's profile was shaped similarly in both systems but intensity decreased with heat, whereas, both shape and intensity of aspartame profiles changed dramatically. According to the manufacturer, acesulfame K is stable over the temperature and pH ranges normally encountered in food preparation and processing. Although a loss in sweetness was noted from unbaked systems sweetened with acesulfame K, the difference appeared small compared to aspartame.

In the unbaked cookie, nonsweet aftertaste of aspartame was low: no greater than fructose. However, in the baked cookie, aspartame aftertaste was much higher than its respective residual sweetness and as intense as the non-sweet aftertaste of the other three intense sweeteners. The aftertaste was described primarily as bitter, although medicinal and metallic were noted by some panelists. Possibly some interaction occurred with the proteins, lipids, or other ingredients in the system at elevated temperatures. The decrease in sweetness intensity of aspartame from unbaked system was expected, because aspartame generally is considered too unstable for use in foods requiring prolonged heat exposure (McCormick, 1975; Beck, 1978). At extremes of temperature or pH, aspartame decomposes to diketopiperazine, with a concurrent loss of sweetness. Initial, maximum, and residual sweetnesses of aspartame-sweetened cookies were low but some sweetness existed. Scientists at Searle (Beck, 1978) found that baked products made with aspartame retained some sweetness, particularly when microwave-baked.

The heat stability of saccharin and acesulfame K and the similarities of their profiles to sucrose in the baked system give them a decided advantage over aspartame. However, each of the intense sweeteners had more ($p<0.05$) aftertaste than the carbohydrate sweeteners and none of them participated in browning reactions.

SUMMARY & CONCLUSIONS

TYPE OF SWEETENER influenced sweetness quality, as exhibited by profiles in unbaked and baked cookies. No sweetener (fructose, aspartame, acesulfame K, sodium saccharin, calcium cyclamate) was perceived exactly like sucrose. Intensities for sucrose, fructose, acesulfame K, saccharin, or cyclamate sweetness varied slightly before and after baking, but aspartame was noticeably different. Sweetnesses of sucrose and saccharin were perceived similarly in baked cookies, but the two differed in quality and intensity of nonsweet aftertaste. Flavor did not affect initial, maximum, or residual sweetness in unbaked cookies but non-sweet aftertaste was less intense in lemon and plain unbaked cookies than in vanilla-flavored unbaked cookies.

Results from this study illustrate that sweeteners have unique profiles which are influenced by the type of food system. Information in the literature on sweetness in solutions or dispersions should be reevaluated to compare systems with added ingredients or thermal processing.

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Hollow Fiber Ultrafiltration of Maple Sap: A Performance Study

GUY POULIOT and JACQUES GOULET

ABSTRACT

In an attempt to develop new products from maple sap, membrane technology was used to fractionate and concentrate macromolecular components (10,000 daltons or larger). Permeate fluxes increased with transmembrane pressure, reaching a maximum at 190–200 kPa, at 8–10°C. The flux profiles were similar to those of protein solutions and fruit juices. This method of clarification was successfully applied to obtain a clear "cold sterilized" sap. Application of ultrafiltration to maple farms or industries are discussed.

INTRODUCTION

THE MAIN USE of maple sap (*Acer saccharum* Marsh.) is as the starting material for the production of genuine pure maple syrup and various sugar-based by-products. Very little research has been done to develop new products from it. Efforts by producers and researchers have always begun with the syrup and resulted in new foodstuffs that are not highly competitive in the market of confectionery products.

Studies of the composition of maple sap are scarce. The proximate composition is well established, i.e., water (92–99%), carbohydrates and minerals. The dry matter consists chiefly of sucrose (95%) and of other sugars (Marvin, 1957). Haq and Adams (1961) detected minute quantities of polysaccharides; traces of nitrogen compounds are also present (Pollard and Sproston, 1954). Except for some reports on the presence of enzymatic activities by Bois and Nadeau (1938) and Meusse (1949), no other data are available on the detailed chemical composition of this raw material, which is surprising, given its economic importance in Quebec, New England and Ontario.

The concentration of maple sap by reverse osmosis has already been investigated by Lonsdale (1972) and has been successfully integrated into maple farming (Timbers et al., 1974; Allard, 1981). This membrane filtration process is used to obtain a three fold (3X) concentration of fresh sap, from 2.5–3.0°Brix to 8.0°Brix. This permits a reduction of the evaporation time required to reach a syrup-like consistency and leads to major energy savings (Allard, 1981).

The fractionation of maple sap by ultrafiltration was recently suggested by Goulet and Pouliot (1984) as a mean of recovering undenatured macromolecules. This technology is already established in the dairy industry (Glover et al., 1978), the beverage industry (Heatherbell et al., 1977) and other food industries (Bambridge et al., 1977). Ultrafiltration of maple sap would also improve its microbiological and biochemical qualities for syrup and other high quality sugar-based products.

This work was designed to study the performance of a hollow fiber ultrafiltration (UF) system for maple sap fractionation over a two year period. The effects of sap pre-concentration by reverse osmosis (RO), of transmembrane pressure, cleaning cycles, temperature and the volume concentration factor were investigated in particular, in light of their effect on flux and fouling of the system.

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MATERIALS & METHODS

THE PILOT-SCALE UNIT was installed in an experimental maple sugar production facility to process large volumes of raw and RO concentrated maple sap as would be the case on an industrial scale. Experiments were performed on an ultrafiltration unit (Model HF 2SSS, Romicon Inc., Woburn, MA) with two 2.5 m^2 hollow fiber cartridges (HF–26.5–43–PM10, Romicon Inc., Woburn, MA). The unit was operated as a recirculating 113 L topped-off batch system (Fig. 1).

The sap (2.5–3.0°Brix) was collected under vacuum from tapholes without formaldehyde pellets and kept in stainless steel bulk tanks until concentration by RO to 8.0°Brix prior to continuous atmospheric evaporation. The fresh or concentrated sap (3–8°C) was continuously added to the tank at a rate matching the permeate flux, while the concentrate stream was returned to the tank through a valve used to control the transmembrane pressure. The permeate stream was used to feed the reverse osmosis unit or the continuous evaporators during production tests (concentration) and was recirculated to the feedtank during the pressure-flux tests (without the process in). Ultrafiltration was performed before and after reverse osmosis of the raw sap.

Standardized cleaning procedures were repeated before every production test and also between the preset concentration factors during every pressure-flux test. The procedures were started by flushing with tap water at 50°C for 20 min, to rinse off most of the viscous material, then washed with enough commercial phosphoric acid (Detac-2, Ecochimie Ltd., Longueuil, Qc) to reach pH 2.5 for 15 min, followed by tap water to remove the acid and 0.5% sodium hydroxide-detergent solution (Caustek L, Ecochimie Ltd., Longueuil, Qc) to recondition the membrane cartridges. The cartridges were finally sterilized by flushing with sodium hypochlorite (0.02%) before rinsing with cool tap water (15°C). During the second season the cleaning procedures were improved by doing both the caustic wash and the sterilization at the same time to benefit from the synergistic effects of chlorine and hydroxide as proposed by the manufacturer of the unit. All solutions were prepared with food grade chemicals and used at 50°C (unless otherwise specified) with an average transmembrane pressure of 150 kPa. The procedure was completed by thoroughly rinsing with tap water (4°C) to restore operating conditions.

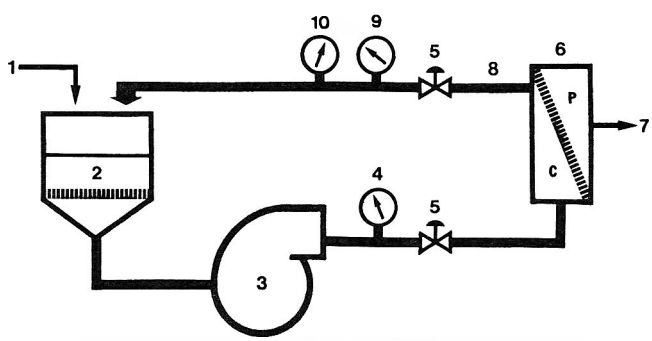

Fig. 1—Schematic diagram of the hollow fiber ultrafiltration apparatus: process in (1), feedtank (2), feed pump (3), inlet pressure gauge (4), valves (5), UF cartridge (6), permeate line (7), retentate line (8), outlet pressure gauge (9), and temperature gauge (10).

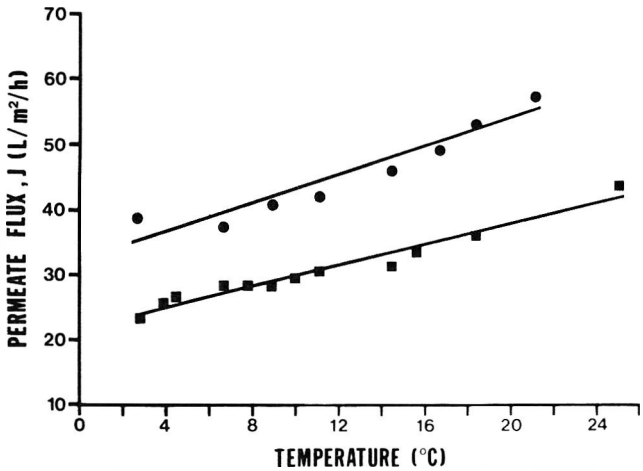

Fig. 2—Relationship between temperature and permeate flux of maple sap at 96.5 kPa (■) and 172.5 kPa (●).

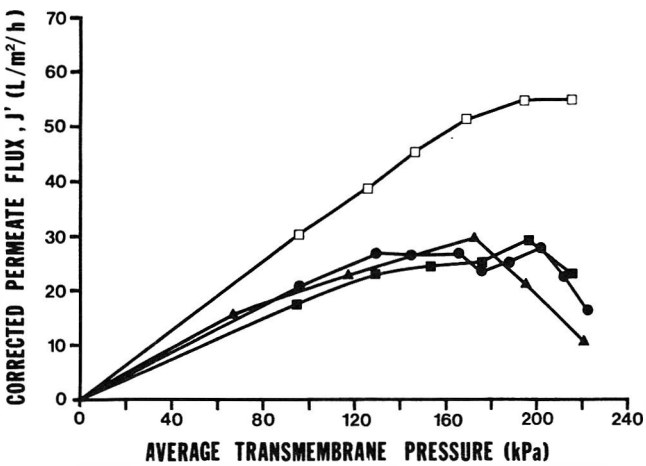

Fig. 3—Effect of transmembrane pressure on ultrafiltration permeate fluxes (corrected at 10°C) of maple sap at several concentration factors: 1 (□), 48 (■), 79 (●) and 108 (▲).

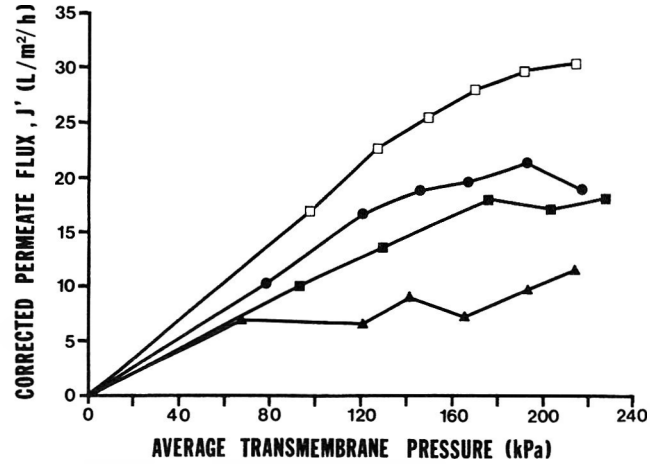

Fig. 4—Effect of transmembrane pressure on ultrafiltration permeate fluxes (corrected at 10°C) of RO concentrated maple sap (8.0°Brix) for several concentration factors: 1 (□), 15 (■), 23 (●), and 35 (▲). Their corresponding values from original raw sap are 3, 48, 72, and 102, respectively.

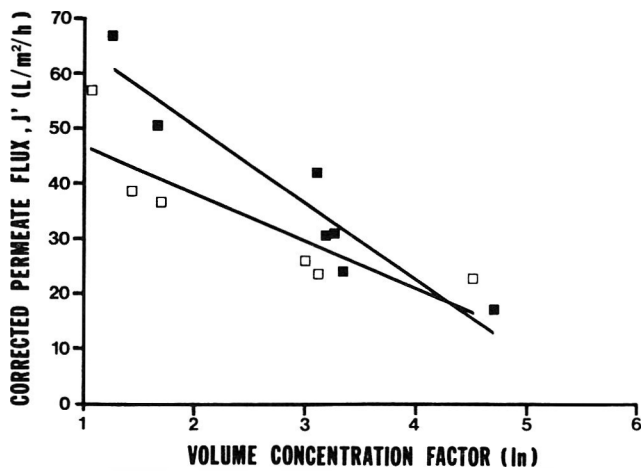

Fig. 5—Effect of volume concentration factor on corrected permeate flux (20°C) at 162 kPa for maple sap at the beginning, 03–14–85 (□) and at the middle of the production season, 03–23–85 (■).

RESULTS & DISCUSSION

Temperature effect

During the production tests the temperature of the concentrate stream was stable at 8–10°C due to the constant addition of cold sap. However, the temperature rose during the pressure-flux and concentration-flux tests due to the recycling of the permeate stream. As reported by many authors for various feeds (Breslau et al., 1975; Kirk et al., 1983; Chiang and Cheryan, 1986), permeate fluxes increased linearly with temperature (relationship shown in Fig. 2), the permeate fluxes were corrected accordingly to a standard value of 10°C or 20°C. It was then assumed that each correction factor also had a linear relation to the average transmembrane pressure and, therefore, the corrected permeate fluxes at 10°C or 20°C were obtained. This way, the real effects of transmembrane pressure and concentration are shown, since all fluxes are compared on the same basis.

Transmembrane pressure effects

Transmembrane pressure is defined as the average of the inlet and outlet pressures. Figures 3 and 4 show that permeate flux increases initially with applied transmembrane pressure but the flux rate gradually decreases with further increase of transmembrane pressure. This gradual decrease of flux rate with pressure is usually attributed to the concentration polarization which causes a build-up of macromolecules formed on the membrane surface (Chiang and Cheryan, 1986).

Kirk et al. (1983) reported that flux stabilizes to a maximum and then decreases with further increase in pressure for pear juice, as observed for maple sap (Fig. 3) which is in contrast to the observations made with protein dispersions (Cheryan, 1977). This behavior is attributed to pectic substances which are structurally different from protein. Composition of maple sap (Pouliot and Goulet, 1986) indicates the presence of polysaccharides which is confirmed by several authors (Porter et al., 1954; Haq and Adams, 1961).

The pattern observed for RO concentrated maple sap, however, is quite similar to that of protein solutions (Fig. 4). This can be explained by the higher protein-polysaccharide ratio in the retentate which is composed of 17.5% protein (Goulet et al., 1985).

Since the optimum transmembrane pressure for maximum flux changed with the concentration of the feed stream, it is suggested that the transmembrane pressure may be adjusted towards the end of the process (Fig. 3 and 4).

Concentration effects

Figures 5 and 6 show that permeate fluxes decrease linearly with the logarithm of the concentration and follows the rela-

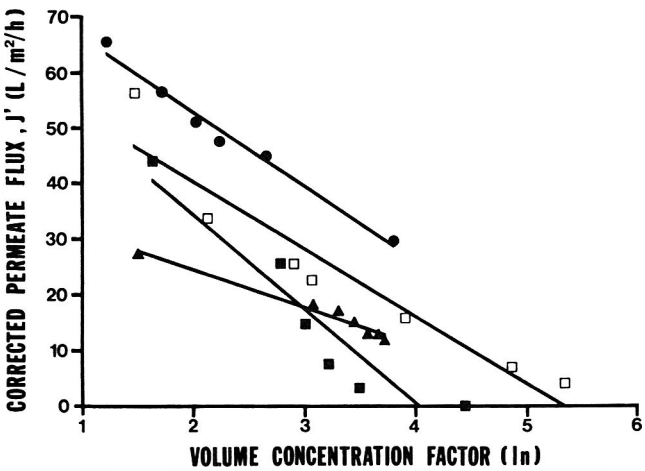

Fig. 6—Effect of volume concentration factor on corrected permeate flux (20°C) at 180 kPa for RO concentrated maple sap (8.0°Brix) at the middle, 03–28–85 (□), 03–29–85 (■), 04–01–85 (▲) and at the end of the production season, 04–12–86 (●).

tionship suggested by Breslau et al. (1975) for cottage cheese whey,

$$J = K_1 - K_2 \ln VCF$$

where J is the ultrafiltration flux, VCF is the volume concentration factor, and K_1 and K_2 are experimental constants. Heatherbell et al. (1977) and Kirk et al. (1983) also reported this logarithmic flow-concentration relationship for apple and pear juice, respectively. Although the curves may not appear to be well-fitted, the correlation coefficient (R) varied from 0.94 to 0.99 indicating that the logarithmic model was appropriate, except for one curve (03–14–85) for which R was 0.85. An improvement both in flux and correlation coefficients (0.99) mainly due to the improved cleaning procedures was noted in 1986 (Fig. 6).

Cleaning cycles

The cleaning procedures of the UF hollow fibers are crucial for maximum fluxes and product microbiological quality (Breslau et al., 1984). In only one season the average fluxes were almost doubled (1.7 X) as a result of improved use of chemicals. The conditions were modified according to the recommendations of the manufacturer of the unit. The first flushing was very important and must be done with warm water (50°C) but not hot enough to cause cooking of the particles onto the membrane. To remove most of the clogged material, the stream must not be recirculated. Since maple sap has a high mineral content, an acid wash is needed to demineralize the unit (Pouliot and Goulet, 1986).

The real improvement occurred through the synergistic effect of chlorine and alkali, which may be explained by the increased activity of sodium hypochlorite in strong sodium hydroxide solution. Independent studies by the manufacturer have revealed the presence of wash-resistant clogging material that could be removed only by stronger oxidizing agents. This modification remained within the membrane chemical resistance specifications.

Processing with reverse osmosis

The RO concentration of the raw maple sap was done at a permeation rate of 15 L/m²/hr, whereas UF rates were of the order of 35 L/m²/hr, at the same normal operating conditions of 190–200 kPa and 8–10°C. Preconcentration of the sap by RO prior to UF would increase the overall ultrafiltration performance by a factor of 2.03, slightly less than the RO preconcentration factor. On the other hand, ultrafiltration of the raw sap prior to RO concentration did not significantly improve the overall fluxes. In terms of performance it may be preferable to concentrate maple sap by reverse osmosis before performing ultrafiltration. However, microbiological and biochemical considerations would require that ultrafiltration be conducted preceding the reverse osmosis operation, because ultrafiltration performs cold sterilization of the sap allowing longer RO processing without the necessity of cleaning.

CONCLUSIONS

HOLLOW FIBER ULTRAFILTRATION was well suited to fractionating and concentrating macromolecules (polysaccharides and proteins) in maple sap. The use of 10,000 dalton exclusion range membranes permitted the clarification of even very turbid sap and produced a crystal clear permeate. Operation at transmembrane pressures of 190–200 kPa (inlet valve open) appeared to be optimal and gave maximum permeate fluxes. The latter were higher with regular sap than with reverse osmosis concentrated sap. Economic factors were not investigated; therefore, new optimum process parameters might emerge.

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The authors express their gratitude to Mr. Gaston Allard and all the personnel of the "Centre Acéricole Expérimental" in St-Norbert (Arthabaska), Québec, for their valuable assistance during the experiments.

This research was supported by a "Conseil de recherche et Services Agricoles du Québec" research grant and scholarship.

Effects of Immobilization and Permeabilization Procedures on Growth of *Chenopodium rubrum* Cells and Amaranthin Concentration

DIETRICH KNORR and JOCHEN BERLIN

ABSTRACT

Cultures of *Chenopodium rubrum* were subjected to permeabilization and immobilization procedures to examine their potential for pigment production. Amaranthin content of culture media was highest for cultures treated with dimethylsulfoxide (DMSO) or dissolved chitosan. Labelling of the cells with L-(U-^{14}C) tyrosine revealed that amaranthin was released from the cells but degraded rapidly. Product degradation in chitosan immobilized cells was delayed by 12–24 hr. Sufficient cell growth was observed in cultures treated with 0.77 mg chitosan per gram fresh biomass, with 0.42 ml.g^{-1} DMSO, or when immobilized in a complex Ca-alginate-chitosan gel system. All other treatments resulted in inhibition of growth.

INTRODUCTION

RECENT increasing attention has been directed towards the production of food ingredients via plant cell cultures (Baladrian et al. 1985; Knorr et al., 1985; Moshy, 1985; Sahai and Knuth, 1985;). Most of these efforts concentrate on flavor production (Dziezak, 1986a,b,; Moshy, 1985; Nickel, 1980) and relatively little information has been published on the use of plant cell cultures for pigment production (Fujika and Hara, 1985; Weller and Lasure, 1981). This is somewhat surprising considering that food colors comprise a major portion of the food ingredient market. However, the first industrial scale process for recovery of secondary metabolites from cell culture has been installed for the production of shikonin, a pigment and anti-inflammatory agent (Curtin, 1983). Betalains, especially the red-violet betacyanins, have been examined for food application (Weller and Lasure, 1981; Pasch et al., 1975; Von Elbe et al., 1974) and an interesting approach for pigment concentration and purification has been provided by Pourrat et al. (1983).

Accumulation of betalains in beet cultures has been reported by Constabel and Nassif-Makki (1971) and by Weller and Lasure (1981). Berlin et al. (1986) found cell suspension cultures of *Chenopodium rubrum* to accumulate the betacyanins amaranthin, celosianin, and betanin reaching concentrations of 0.3 to 0.4% betacyanins on a dry weight basis after two to three weeks of cultivation. Amaranthin accounts for more than half of the betacyanin concentration in *C. rubrum* cultures (Berlin et al., 1986) and amaranthin and isoamaranthin are the only betalains present in *Amaranthus caudatus* (French et al., 1974) and *A. tricolor* (Piatelli et al., 1969), making amaranthin a prime candidate for pigment production. This is also of interest because amaranths are currently being reestablished as valuable food crops and vegetable plants (Teutonico and Knorr, 1985).

Although chemical synthesis of betacyanins has been achieved (Hermann and Dreiding, 1977), their isolation from natural sources would be more desirable and economical (Berlin et al., 1986). Recent estimates suggest that with advanced immobilization techniques, production costs for secondary metabolites from cultured plant cells can be decreased to approximately $25 to $50 per kg (Sahai and Knuth, 1985; Moshy, 1985).

However, there have been indications that immobilization and permeabilization procedures necessary to achieve effective production of secondary metabolites and their mass transfer into the culture medium (Knorr et al., 1985), do affect the composition of secondary metabolites from cultured plant cells and may result in bioconversion of the desired compounds (Berlin, 1985; Schiel et al., 1984). With regards to betacyanin decolorization, Wasserman et al. (1983) suggested a peroxidative mechanism being responsible for betanin decolorization of red beets (*Beta vulgaris* L.) and Elliot et al. (1983) described an enzyme involving oxidation as being involved in betacyanin decolorizing in *A. tricolor* seedlings.

In this study, we attempted to examine the effects of various immobilization and permeabilization procedures on the concentration of amaranthin in *C. rubrum* cells and culture media, as well as the potential effects of such procedures on amaranthin decolorization and cell biomass accumulation.

MATERIAL & METHODS

SUSPENSION CULTURES of *Chenopodium rubrum* were maintained on MX-medium (MS + 2M 2,4-d) as described by Berlin et al. (1986). Two grams of cell biomass were transferred to 35 mL MX-medium and grown under a 16 hr photoperiod (fluorescent light Osram L33W/22-2 Fluora) on a shaker operating at 110 rpm for four days until the beginning of the immobilization and permeabilization experiments (average cell biomass 4.68g per 35 mL of medium). Treated cultures were maintained under above environmental conditions until sampling.

Chitosan (Nacell Na-500) was purchased from Nanyo Kasei Co., Ltd. (Tokyo), alginic acid was from Sigma and L-(U-^{14}C) tyrosine from Amersham Buchler GmbH & Co., KG, (Braunschweig, FRG). All other chemicals were from Merck or Sigma.

Chitosan used for permeabilization experiments was dissolved at 1.0% in 1.5% acetic acid and autoclaved at 121°C for 20 min. Concentrations used were 0.35 mL per flask or 0.70 mL per flask. Dimethylsulfoxide (DMSO) was used in concentrations of 2.0 mL, or 4.0 mL DMSO per flask, and untreated chitosan flakes were added at concentrations of 0.4g and 0.8g per flask of 35 mL medium.

Calcium alginate gel beads of approximately 2.5 mm diameter were prepared from 3% aqueous solutions of alginic acid and 100 mM $CaCl_2$ x 2 H_2O as counterion solution and adjusted to a cell density of 33g per 100g biocatalysts using all the cell biomass per flask. The counterion solution and the water used for washing of the biocatalysts contained, after completion of the permeabilization procedure, 9.3 μg amaranthin per flask. The biocatalysts were formed using a peristaltic pump for droplet formation producing 65 to 70 beads per min. Chitosan beads of approximately 1.0 mm diameter kindly provided by K.D. Vorlop were produced after Vorlop and Klein (1981) and autoclaved in the 2% aqueous tripolyphosphate (pentasodium salt) solution. These beads were added to the cell biomass at a concentration of 2.0g per flask with the addition of one mL 3.0% ascorbic acid, or without the antioxidant. Complex polysaccharide gels were produced using solutions of 3% alginic acid and 3% sodium hexametaphosphate which were combined with the cell biomass. This mixture was then dropped, via the peristaltic pump, into a mixture of 1% chitosan in

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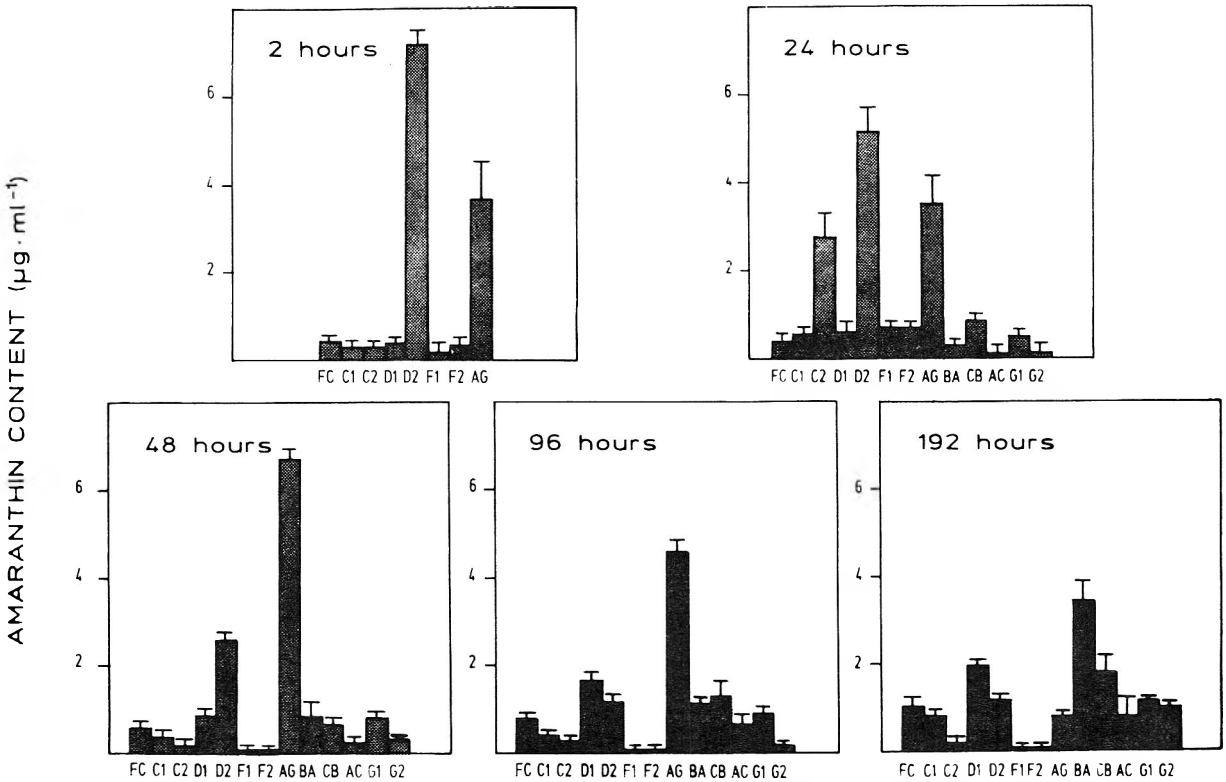

Fig. 1—Amaranthin concentrations of culture media of C. rubrum *cultures after various immobilization and permeabilization treatments. Same codes as in Table 1.*

Table 1—Summary of immobilization and permeabilization treatments of Chenopodium rubrum *cells*

Code	Treatment per flask (35 mL medium)
FC	untreated cells
C1	addition of 0.35 mL 1% dissolved chitosan
C2	addition of 0.70 mL 1% dissolved chitosan
D1	addition of 2.0 mL dimethylsulfoxide (DMSO)
D2	addition of 4.0 mL dimethylsulfoxide (DMSO)
F1	addition of 0.4g chitosan flakes
F2	addition of 0.8g chitosan flakes
AG	immobilization in Ca-alginate, 33% cell loading
BA	addition of 2.0g chitosan beads plus 1 ml 3% ascorbic acid
CB	addition of 2.0g chitosan beads
AC	immobilization in complex alginate-chitosan gel system, 33% cell loading
G1	immobilization in chitosan gels, addition of 1 mL 10% acetic acid and 0.5 mL 1N NaOH, 25% cell loading
G2	immobilization in chitosan gels, 25% cell loading

1.5% ascorbic acid and 100 mM of $CaCl_2$ x $2H_2O$ resulting in a cell loading of 33%. Counterion solution and washing water after completion of the immobilization experiments were colorless. Chitosan gels were produced from 1% chitosan dissolved in 1.5% ascorbic acid, using 2% aqueous tripolyphosphate as counterion solution which had been adjusted to pH 11. Chitosan had been autoclaved in distilled water prior to dissolving to avoid changes in viscosity. To examine the effects of harsh immobilization procedures, 1 mL 10% acetic acid and 0.5 mL 1N NaOH were added in one case, which resulted in partial decolorization of the cells during immobilization. Chitosan gel beads without such treatment accumulated 1.03 mg amaranthin per flask in the combined counterion and washwater solution. Cell loading of the chitosan gel beads was 25%. Duplicate or triplicate samples were prepared for each treatment at each sampling point. A summary of the various treatments is provided in Table 1.

Betacyanin concentration of the culture medium and of a 50% methanol extract of cell biomass was determined spectrophotometrically at 537 nm, using a molar extinction coefficient for amaranthin of 5.66 x 10^4 (Berlin et al., 1986, Piatelli et al., 1969).

Cell biomass accumulation was determined by transferring 1.0g cell biomass (or an equivalent of 1.0g cell biomass in the case of F1, F2, AG, BA, CB, AC, G1, G2) into 35 mL fresh MX medium and measuring fresh weight of total cell biomass after 14 days. Dry cell biomass was determined by oven drying for 12 hr at 70°C.

RESULTS & DISCUSSION

Amaranthin concentration

Amaranthin concentrations of culture media from 2 to 192 hr of treatment are given in Fig. 1. After 2 hr of treatment, only the culture medium of D2 and AG showed higher amaranthin concentrations than that of the freely suspended cells. After 24 hr of treatment, amaranthin concentration in C2 media also increased markedly and small increases were observed in the remaining cultures. After 48 hr, F1 and F2 reached zero values while maximum amaranthin concentration of 6.80 ± 0.26 $\mu g \cdot mL^{-1}$ was found for AG medium which decreased during the next 144 hr to 0.79 ± 0.13 $\mu g \cdot mL^{-1}$ medium. It should be noted that the amaranthin concentrations measured in the various media never exceeded 17% of the total amaranthin content present within the cell biomass of one flask. Amaranthin concentrations in 50% methanol extracts of cell biomass are given in Fig. 2. Overall amaranthin concentration decreased over time with already low values for C2, F1, F2, AG, BA, CB, G1 and G2 after 24 hr. Initially higher levels for C1, D1, and AC were still elevated after 96 and 192 hr of treatment. C1 maintained the highest levels at 2.92 ± 0.37 $mg \cdot g^{-1}$ dry matter at 96 hr and 1.94 ± 0.20 $mg \cdot g^{-1}$ at 192 hr.

Amaranthin degradation

Amaranthin concentration in cell extracts and culture media of the various *C. rubrum* treatments are provided in Table 2 as percentages of untreated freely suspended cells. Substantial concentrations were present in extracts of C1, C2, D1, D2, F1, and AC. However, these concentrations decreased in the

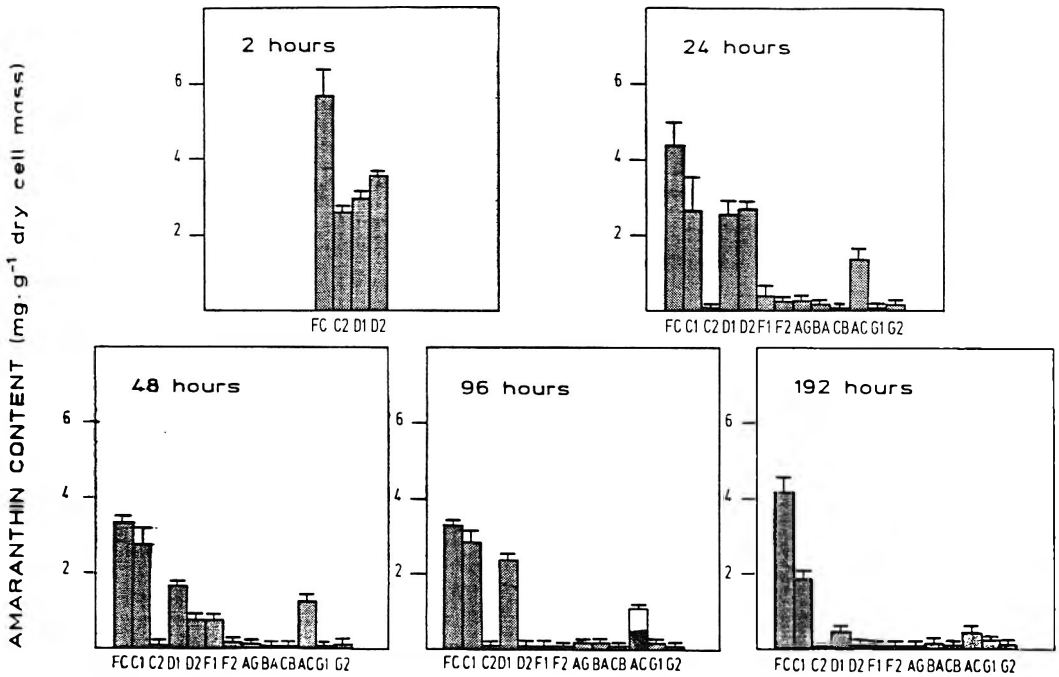

Fig. 2—Amaranthin concentration of C. rubrum *cell extracts after various immobilization and permeabilization treatments. Same codes as in Table 1.*

case of C2 after 2 hr of treatment and for D2 and F1 after 48 hr. In culture media notable amaranthin concentrations were only found for CB, C2 and D2, indicating a sufficient permeabilizing effect of these treatments. The maximum release value of 3175% (as compared to FC values) for D2 at 2 hr equaled 18.2% of the total amaranthin concentration available. After 24–48 hr of treatment, pigment concentration decreased drastically.

These data suggest that time dependent conversions of amaranthin did occur and were especially drastic for C2, D2, F1, F2, G1, and G2. Only C1 and AC were subject to less amaranthin degradation, up to 96 hr. The addition of ascorbic acid as antioxidant in BA slightly reduced decolorization as compared to CB. This observation is in agreement with findings on the oxidative decolorization of betacyanins in *Beta vulgaris* and *A. tricolor* (Elliot et al., 1983; Wasserman et al., 1983).

Permeabilization of free and immobilized cells resulted in partial release of betacyanins from the cells into the medium, but there was also a significant degradation of amaranthin in *Chenopodium rubrum* cells without excretion of the products (Berlin et al., 1986; Strack et al., 1986). Therefore, the absence of amaranthin in the medium of some of the permeabilized cells could result from rapid degradation. To clarify this point, *C. rubrum* cells were labelled with L-(U-^{14}C) tyrosine (Berlin et al., 1986) which was in part incorporated into the betacyanins during the three days prior to treatment with chitosan (C_2) or DMSO (D_2). Of the radioactivity incorporated, 35–40% remained extractable from the cells with 50% MeOH. The cells treated with dissolved chitosan (C2) were decolorized within 4–6 hr. Up to 80% of the radioactivity extractable with 50% MeOH was found in the culture medium. But the highest amaranthin content in the medium reached only 19% of the initial value. This indicates that a large portion of amaranthin was indeed released from the cells but was degraded either before or after release. After 48 hr of treatment, only traces of betacyanins were left in the medium and the cells, but the radioactivity in the medium remained unchanged. In the case of DMSO (D2), a similar initial release of radioactivity into the medium was noted. However, part of this radioactivity was reabsorbed and was most likely bound to the cell material. After 24 hr of treatment, 30.9% of the radioactivity, but 58.1% of amaranthin, was found in the cells. The medium contained

Table 2—Percentages of amaranthin concentrations in cell extracts and culture media of Chenopodium rubrum *cultures as compared to untreated freely suspended cells*[a]

Treatments	Duration of experiments (hr)				
	2	24	48	96	192
C1		60.4⁻	82.8⁻	88.2⁻	48.2⁻
		124.2*	62.6*	55.1*	72.8*
C2	45.4⁻	0.8⁻	1.0⁻	0.6⁻	0.6
	137.5*	660.1*	414.0*	38.90*	16.2*
D1	61.7⁻	58.8⁻	51.5⁺	72.2⁻	11.7⁻
	1475.0*	150.3*	150.0*	226.0*	179.4*
D2	61.7⁻	60.6⁻	22.9⁻	1.1⁻	0.6⁻
	3175.0*	1182.0*	464.6	161.1*	105.6*
F1		11.8⁻	23.2⁻	1.8⁻	0.8⁺
		150.3*	0.5*	0.4*	0.1*
F2		3.2⁻	5.5⁻	0.5⁻	0.8⁺
		152.3	0.5*	0.4*	0.2*
AG		6.0⁻	4.6⁻	16.3⁻	1.4⁻
		794.1*	1201.5*	604.9*	0.2*
BA		6.1⁻	1.8⁻	6.9⁻	7.9⁻
		213.9*	196.4*	145.9*	283.0*
CB		0.7⁺	3.1⁺	4.9⁻	4.9⁺
		766.7*	138.8*	171.1*	21.0*
AC		44.5⁺	45.5⁺	35.2⁺	14.6⁻
		99.3*	50.6*	80.5*	62.4*
G1		0.7⁻	1.9⁻	6.4⁻	10.9⁻
		465.1*	175.0*	126.5*	96.6*
G2		6.5⁻	7.8⁻	3.8⁻	3.6⁻
		165.3*	55.9*	29.3*	85.7*

[a] Amaranthin values of untreated cells per flask at 0 hr: extract 1398.8 μg, culture medium 8.2 μg

* Culture medium

⁻ Cell extract

15% of amaranthin and 25.2% of the radioactivity. The loss of radioactivity extractable with 50% MeOH as well as the loss of amaranthin showed that in the presence of this concentration of DMSO, further reactions such as binding of degradation products to the cell material occurred.

Growth of treated cells

The observed degradation of amaranthin will make it difficult to develop effective production processes for amaranthin

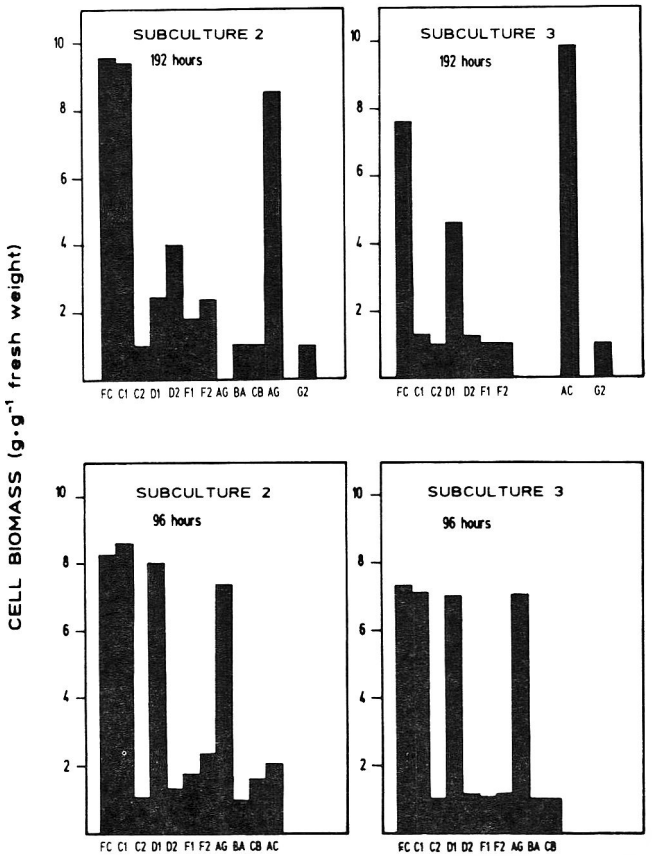

Fig. 3—Cell biomass of C. rubrum *cultures subjected to various immobilization and permeabilization procedures for 96 and 192 hours and subsequent subculturing for 3 cell cycles. Same codes as in Table 1.*

from cultured cells until methods to inhibit the degradation are found or unless pigments are extracted prior to degradation (Wiley and Lee 1978). However, the sensitivity to degradation may not hold true for many other metabolites released from cultured plant cells. Thus, the question of maintenance of cell viability after such permeabilization treatments is of general importance.

Cell biomass data of *C. rubrum* cultures treated for 96 and 192 hr and subsequently subcultured during three cell cycles are given in Fig. 3. The samples C1, D1, AG and AC were the only cultures providing cell biomass accumulation comparable to FC controls. F2 and D2 at 192 hours showed some cell growth until the second subculture. All other treatments resulted in no accumulation of cell biomass over the two week cell cycles, suggesting a severe effect of those treatments on cell viability.

These permeabilization treatments can be regarded as extraction and might actually prove useful as such in the recovery of plant metabolites from batch cultures. It needs to be seen whether it will be possible to find a solvent concentration and optimum duration of treatment which allow efficient release without damaging the cells too severely. Indeed, the permeabilizing agent will not only act on the tonoplast and plasma membrane but also on all other compartments of the cell. Despite the recent claims of Lundberg et al. (1986) that DMSO permeabilized/immobilized cells of *Catharanthus* may release their products without showing severe growth inhibition, there seems to be no experimental data available indicating that chemical induced release of metabolites at significant levels is tolerable for cells. Consequently, optimization of such treatments, development of new permeabilization/immobilization procedures, or the selection of cultures resistant to the adverse effects of permeabilization need to be sought.

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Ms received 9/18/86; revised 2/24/87; accepted 3/7/87.

The authors recognize the assistance of C. Mollenschott and B. Martin and are thankful to R.A. Teutonico and M. Beaumont for comments. Parts of this work were sponsored by the U.S. National Sea Grant College Program under Grant No. NA83AA-D-00017, Project No. R/N-9 and parts were supported by the Gesellschaft für Biotechnologische Forschung, Braunschweig, FRG.

Recovery of Glutamic Acid from Fermentation Broth by Membrane Processing

WEN SHIUH KUO and BEEN HUANG CHIANG

ABSTRACT

Recovery of glutamic acid from the fermentation broth by membrane processing was investigated. The broth was ultrafiltered (UF), and the retentate was diafiltered (DF) to wash out the remaining product. The dilute DF permeate was concentrated by reverse osmosis (RO). Recovery of glutamic acid by UF (R_{uf}) was a function of the volume concentration ratio (VCR); $R_{uf} = 1.052 - 1.048/VCR$. High VCR, however, decreased UF flux (J) ($J = 14.2 - 5.4 \ln VCR$). The rate of recovery by DF was a function of the turnover ratio. A linear relationship between flux and pressure was found during RO. Separation of glutamic acid from bacterial cells by membrane processing could improve the efficiencies of subsequent evaporation and crystallization processes.

INTRODUCTION

MONOSODIUM GLUTAMATE (MSG), the sodium salt of glutamic acid, is widely used in foods as a flavor enhancer. The world's production of MSG currently reaches 350 thousand tons per year (Kawakita and Saeki, 1986), and is produced mostly by microbial fermentation. The recovery of glutamic acid from fermentation broth is achieved conventionally by concentration, crystallization and centrifugal separation processes (Kawakita and Saeki, 1986). The presence of bacterial cells in the broth, however, would reduce the efficiency of these unit operations.

Membrane processing is a potential tool for separating bacterial cells from the fermentation product, thus improving the efficiency of recovery processes. The applications of membrane processing to fermented products have been reported (O'Sullivan et al., 1984; Tsuda et al., 1983; Beaton, 1980). This article reports on the possibility of using membrane processing for MSG production. Our purpose was to evaluate the feasibility of using ultrafiltration (UF), diafiltration (DF) and reverse osmosis (RO) for recovering glutamic acid from fermentation broth, and to investigate the effects of membrane processing on the performance of downstream recovery operations.

MATERIALS & METHODS

Fermentation broth preparation

The glutamic acid fermentation broth was supplied by Ve Wong Co. (Taipei, Taiwan), produced by growing *Brevibacterium* sp. in a medium composed of sugar cane molasses, ammonia, phosphoric acid and other minor constituents at 30°C for approximately 30 hr. The proximate composition and some physical and chemical properties of the broth are listed in Table 1. The fermentation broth was pasteurized at 82°C for 1–2 sec before processing.

Process equipment

The De Dnaske Sukkerfabrikker (DDS) module-20 UF/RO unit (Copenhagen, Denmark) was used for membrane processing. The membrane module is a plate-and-frame system and can be installed with up to 40 segments of membranes, providing a total of 0.72 m^2 membrane area. In this study, however, only 32 segments of UF membranes, which gave 0.576 m^2 of membrane area, were used for UF and DF, and 8 segments, providing 0.144 m^2 of membrane area, were used for RO. The unit was equipped with a constant speed piston pump to provide feed for the module. A by-pass device installed between the pump outlet and the membrane module inlet can be used to adjust the flow rate.

The DDS GR10PP UF membrane with a molecular weight cutoff of 500,000 daltons was used for UF and DF. The recommended maximum operating pressure and temperature for the membrane were 10 bar and 80°C, respectively. For RO operation, the DDS HR-98 membrane was used. The permeability of a 0.2% NaCl solution with HR-98 membrane is less than 1.5%. The pressure and temperature limits for the membrane were 80 bar and 80°C, respectively.

Membrane processes

All membrane processes were conducted at 30 ± 1°C, the operating temperature for the fermentation. Preliminary studies indicated that the trend of increasing the UF permeation rate with increasing transmembrane pressure [$(P_{in} + P_{out})/2$] reached a plateau around 7.5 ± 0.5 bar; accordingly, the operating pressure for both UF and DF was fixed at 7.5 ± 0.5 bar. The pump of the UF/RO unit provided a constant feed flow rate of 7.2 L/min during processing. However, when the concentration of the broth reached approximately 30% (wt/wt) or higher, the significant pressure drop across the membrane module would require adjustment of the by-pass valve to maintain a constant transmembrane pressure. This, inevitably, reduced the flow rate.

Under the predetermined processing conditions, the fermentation broth was concentrated by UF to various volume concentration ratios

Table 1—Some physical and chemical properties of the glutamic acid fermentation broth

Total solids, (wt/wt)	21.8%
Glutamic acid, g/100 mL	9.9
Total sugar, g/100 mL	3.0
Ash, %(w/w)	2.5
Cell content (dry basis), g/100 mL	2.0
Cell content (wet basis), g/100 mL	6.8
Viscosity (30°C), cps	2.8
Specific gravity	1.1
pH	6.9

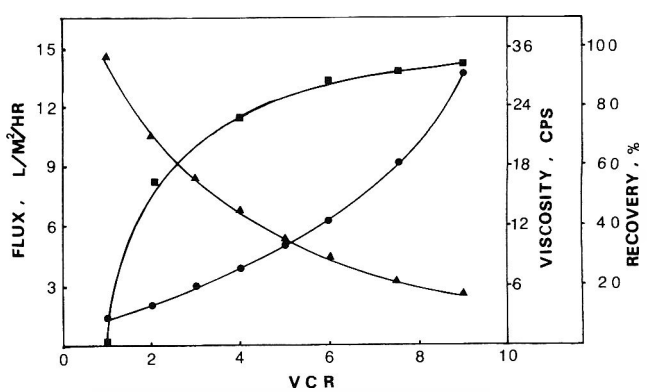

Fig. 1—Effect of the volume concentration ratio on the UF flux (▲), the retentate viscosity (●) and the percentage recovery (■).

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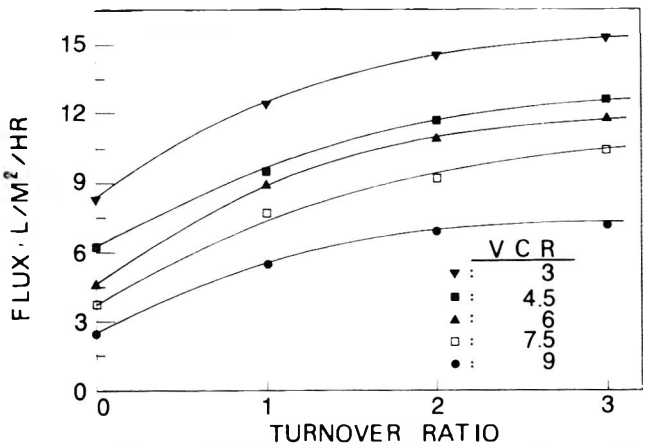

Fig. 2—Relationship between the flux and turnover ratio during DF of the UF retentates at various concentration ratios.

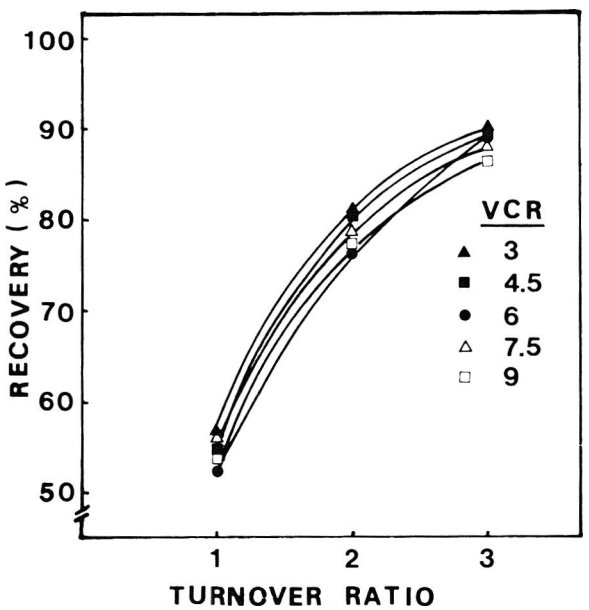

Fig. 3—Effect of the turnover ratio on the recovery of glutamic acid during DF.

Table 2—BOD, COD, and residual glutamic acid content of waste liquor after crystallization

	BOD[a] (ppm)	COD[b] (ppm)	Glu. conc. (g/dL)
Original broth	51500[c]	149900[c]	5.5[c]
UF permeate	43000[d]	124500[d]	2.3[d]

[a] BOD = biological oxygen demand
[b] COD = chemical oxygen demand
[c d] Means within columns followed by the different letters are significantly different at the 5% level.

(VCR) (VCR = V_t/V_o; V_t = initial volume of feed, V_o = Final volume of UF retentate), and the permeate flux was measured with a rotameter. The recovery of glutamic acid during UF (R_{uf}) was estimated by the formula:

$$R_{uf} = V_p \times C_p / V_i \times C_i \quad (1)$$

where V_p = volume of UF permeate; C_p = glutamic acid concentration (w/v) in UF permeate; C_i = glutamic acid concentration (w/v) in the feed.

Experiments for DF were conducted on the UF retentates of VCR 3, 4.5, 6, 7.5, and 9. The constant volume batch diafiltration mode was used, in which the diafiltering water was added to the feed tank at a rate equal to UF permeate flux to maintain a constant volume. Details of the operation and theory of DF can be found elsewhere

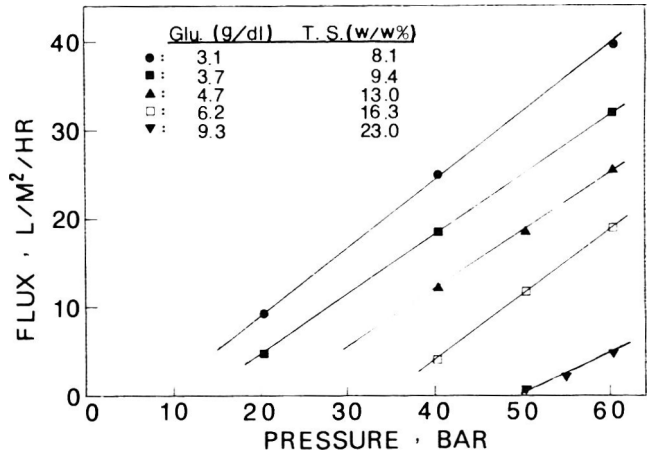

Fig. 4—Effect of the operating pressure and the feed concentration on the permeate flux of RO.

(Beaton and Klinkowski, 1983). During DF, the permeation rates at turnover ratios 0, 1, 2 and 3 were measured with a rotameter. The turnover ratio (N) was defined as;

$$N = V_p/V_o \quad (2)$$

where V_p = permeate volume at time t, which also equals the added water volume at time t; V_o = constant retentate volume during DF. The recovery of glutamic acid during DF (R_{df}) was estimated using the formula;

$$R_{df} = 1 - C_f/C_o \quad (3)$$

where C_o = glutamic acid concentration of retentate at the start of DF operation; C_f = the glutamic acid concentration of retentate at time t during DF.

The feed for RO was the DF permeate. The operating pressure was arbitrarily chosen at 60 bar, which is 75% of the maximum allowable operating pressure for the membrane. During concentration, the retentate was recycled to the feed tank while discharging the permeate, and the flux was measured at various VCR.

Evaporation process

A rotary vacuum evaporator (Model N1, Tokyo Rikakikai Co., Ltd. Japan) equipped with aspirator (model A-2S), water bath (Model SB-35), and cooler (Model CA-101), was used. The processes were conducted at 40°, 50°, and 60°C, 25 mm Hg absolute pressure, 90 rpm rotating speed, and 4 ± 1°C cooler temperature. Five hundred milliliters of solution were concentrated. Changes of product volume at various time intervals during processing were measured to determine the rate of evaporation.

Crystallization process

To simulate industrial conditions, the fermentation broth and UF permeate were concentrated by evaporation to approximately 2.5-fold until the glutamic acid content reached 25 g/100mL. Hydrochloric acid (5.5N) was then slowly added to the solutions at 30°C under constant agitation until the pH reached 3.2, the isoelectric point of glutamic acid. After crystallization was completed, the solutions were allowed to stand for 30 min at 30°C to precipitate the crystals. Samples were taken from the supernatents to analyze the Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD), and residual glutamic acid content.

Analytical procedures

The total solids were determined according to AOAC (1980) method. The viscosity of the solutions at 30°C was measured by a rotational viscometer (Rotovisco RV 100, Haake, Inc., W. Germany) with a NV sensor system. For the glutamic acid quantitation, the solution was diluted with deionized-distilled water to 100-fold, centrifuged at 800 × *g* for 15 min, and the supernatant was analyzed for glutamic acid content with an amino acid analyzer (Model 4150, LKB Bioch-

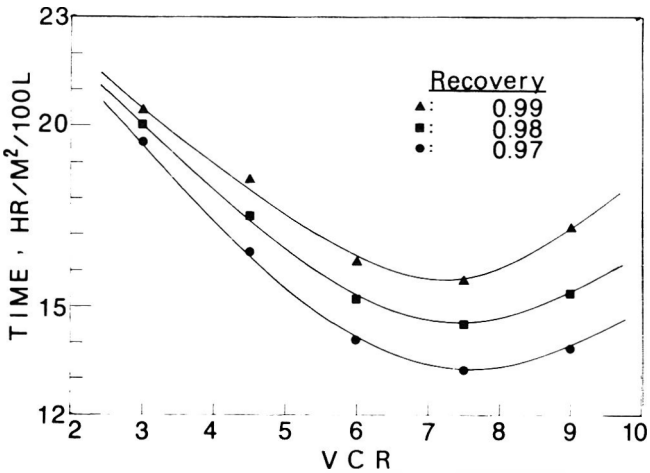

Fig. 5—Overall membrane processing time as a function of the washing point VCR for various rates of recovery.

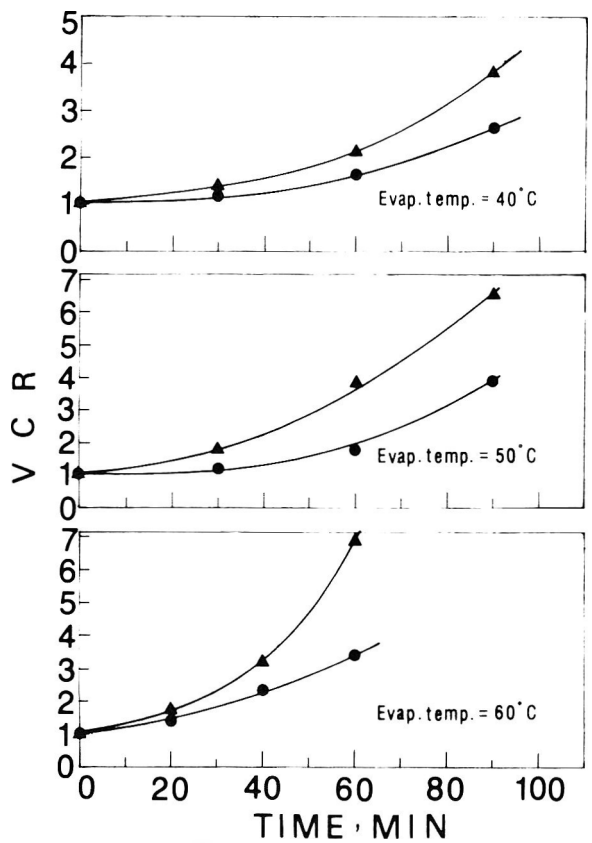

Fig. 6—Rates of evaporation of the fermentation broth (●) and the UF permeate (▲) at evaporation temperatures of 40°, 50°, and 60°C.

rom. Co., Ltd. UK) equipped with an Ultropac 11 resin (Na form) column (LKB Biochrom Co.); eluted with sodium citrate buffer (pH 3.2, 4.25 and 6.45) at a flow rate of 50 mL/hr. The flow rate of ninhydrin was 25 mL/hr. The column temperature ranged from 50–80°C, and the response was determined by a spectrophotometer at 570 nm. The Biological Oxygen Demand (BOD) was analyzed by measuring the difference of dissolved oxygen (DO) between the initial sample and the sample solution being incubated for 5 days at 20°C (Tahara, 1977). The DO was determined using the azide modification of Winkler's procedure (Rand, 1975). The Chemical Oxygen Demand (COD) was determined by measuring the consumption of $KMnO_4$ during oxidation (Ito, 1972).

RESULTS & DISCUSSION

Performance of UF

The relationships among permeate flux, retentate viscosity, and volume concentration ratio (VCR) are shown in Fig. 1. The flux decreased as VCR increased, and their relationship could be described by the formula (Breslau and Kilcullen, 1977):

$$J = 14.2 - 5.4 \ln VCR \quad (4)$$

where J is flux in L/hr/m^2. This equation fits the experimental data well. The coefficient of determination (r^2) was 0.99.

The UF membrane was expected to retain the bacterial cells because the rod-like glutamic acid-producing bacteria have an average diameter of 0.5 μm and are 1 to 2 μm in length, much larger than the pores (ca. 0.02 μm) of the membrane. The increase in cell concentration and other retained solids during UF was responsible for the increase of retentate viscosity (Fig. 1). The increase in viscosity and solids content, in turn, caused flux decline.

The retentivity of the UF membrane with glutamic acid was practically zero, thus the cell-free glutamic acid could be recovered in the UF permeate. As expected, the higher the concentration ratio the higher recovery for glutamic acid was accomplished by UF (Fig. 1), and the recovery (R_{uf}) could be predicted using the following equation with satisfactory accuracy (r^2 = 0.99):

$$R_{uf} = 1.052 - 1.048/VCR \quad (5)$$

Although the R_{uf} increased with increasing VCR, the low permeate flux at high VCR may entail processing difficulties. Therefore, DF process mode should be used at a certain VCR of UF process to "wash out" the remaining product in the UF retentate, thus improving the process performance.

Performance of DF

The effect of the turnover ratio and the washing point VCR on the flux during DF are illustrated in Fig. 2. As expected, low washing point VCR resulted in higher flux. Increasing the usage of diafiltration fluid (i.e. high turnover ratio) would increase the removal of membrane-permeable solutes, reducing both the solids concentration and viscosity of the UF retentate, thus increased flux.

Recovery of glutamic acid by DF was a function of the turnover ratio as shown in Fig. 3. Eq. (6) derived from the material balance also predicts the same phenomenon (Beaton and Klinkowski, 1983).

$$R_{df} = 1 - \mathrm{Exp}(-V_p/V_o) \quad (6)$$

It was also noted, however, that the R_{df} values predicted by Eq. (6) were often 5% to 15% higher than the experimental values. In addition, our data seemed to show a tendency that under the same process conditions of DF the higher the cell concentration (i.e. high washing point VCR) the lower the recovery. It was suspected that the glutamic acid might adsorbed on the bacterial cells in the broth, which interfered with the diafiltration process. Further work is needed in this area.

Performance of RO

The concentration of glutamic acid in the DF permeate was relatively low. For a turnover ratio of 3 the glutamic acid concentration in the DF permeate was approximately 3 g/100 ml with a total solids content of 8% (wt/wt). The dilute glutamic acid solution could be concentrated by reverse osmosis to facilitate later processes. The DDS HR-98 membrane was chosen for the RO process because it retains nearly 100% glutamic acid based on our preliminary tests. The relationship between permeate flux and operating pressure at various feed

concentrations is shown in Fig. 4. The flux increased linearly with increasing pressure, but decreased with increasing concentration. Based on the simple equation, $J = A(\Delta P - \Delta\pi)$, where A = rate coefficient (L/hr/m^2/bar), ΔP = operating pressure (bar), $\Delta\pi$ = osmotic pressure (bar), one can estimate the values of $\Delta\pi$ at various solid contents by extrapolating the linear plots of J vs. ΔP to zero flux. The predicted $\Delta\pi$ increased linearly with increasing total solids and can be expressed by;

$$\Delta\pi = 1.85\ C - 13.67 \quad (7)$$

where C was the total solids in % (wt/wt).

Process optimization

Recovery of glutamic acid from fermentation broth by membrane processes may involve UF, DF and RO. The objective of optimization was to minimize the process time requirement to achieve the desired ratio of recovery. During UF, increasing VCR would increase the recovery of glutamic acid, which, however, also prolongs the UF processing time. Decreasing VCR during UF, on the other hand, would require a high turnover ratio during DF to achieve the desired recovery, which not only increases the DF processing time but also increases the load of the subsequent RO concentration process. Therefore, determination of an optimal VCR during UF (i.e. the washing point for DF) is the key for optimizing the overall process. However, the overall process time depends on the membrane area used for the processing and the volume of the feed. To illustrate the determination of the optimal washing point, the experimental data were used to estimate the time required for processing 100 liter fermentation broth with 1 m^2 each of UF, DF and RO membrane. The volume of the product, i.e. UF permeate plus RO retentate, was also assumed to be 100 liter. The results of the calculation are given in Fig. 5. The time required to reach a desired rate of recovery decreased initially with increasing washing point VCR until it reached a minimum, thereafter the trend was reversed. The relationships between the processing time (T) and washing point for 97%, 98% and 99% recoveries of glutamic acid could be described by the equations:

$$T = 0.286\ VCR^2 - 4.431\ VCR + 30.503\ (r^2 = 0.99) \quad (8)$$

$$T = 0.273\ VCR^2 - 4.098\ VCR + 30.064\ (r^2 = 0.99) \quad (9)$$

$$T = 0.245\ VCR^2 - 3.556\ VCR + 29.163\ (r^2 = 0.94) \quad (10)$$

respectively. Their minimum process times were 13.3 hr at VCR = 7.8; 14.7 hr at VCR = 7.5 and 16.3 hr at VCR = 7.2 respectively.

Effect of membrane processes

In normal industrial practice, the glutamic acid fermentation broth is often concentrated by evaporation before adding acid to crystallize the product. Comparison of the performance of the evaporation process between the fermentation broth and the UF permeate is shown in Fig. 6. Since the microorganisms and macromolecules have been removed by UF, the rate of heat transfer during evaporation for the UF permeate was higher than that for the fermentation broth as a result of better heat convection. Based on this experiment it was estimated that by concentrating the solutions to 2.5-fold at 50°C, which is the normal evaporation condition used in MSG industry, the process time required for the UF permeate was only 64% of that for the fermentation broth.

Microscopic examinations revealed that typical pyramidal shape α-form glutamic acid crystals could be obtained from the concentrated UF permeate and RO retentate, demonstrating that the membrane processes did not affect the normal crystallization process.

After concentration and crystallization, the resulting waste liquor often contains a significant quantity of glutamic acid and has high values of BOD and COD. Removal of the microorganisms from the broth prior to glutamic acid recovery processes could decrease BOD and COD of the waste liquor, and increase the efficiency of crystallization as shown in Table 2. The improvement of crystallization efficiency for UF permeate could be attributed to less impurities in the solution after UF process.

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Ms received 10/14/86; revised 2/17/87; accepted 3/4/87.

Defining a Taste by a Single Standard: Aspects of Salty and Umami Tastes

R. ISHII and M. O'MAHONY

ABSTRACT

Descriptive analysis of foods involves the communication of sensory concepts which are defined using standard stimuli. The concept created by a single NaCl standard stimulus was measured as a set of probabilities. It was found not to be the same for each judge. Disagreement between judges can thus be caused by variations in conceptualization, as well as sensation. This has important implications for the definition of terms in descriptive analysis. The concept was found not to generalize to strong monosodium glutamate (MSG) stimuli which raises questions about reports that MSG tastes salty at high concentrations. Effects on the concept of context, time and variation of standards were also noted.

INTRODUCTION

THE DESCRIPTIVE ANALYSIS of flavor is a widely used technique in the sensory analysis of foods; there are several procedures available (Amerine et al., 1965; Cairncross and Sjöström, 1950; Caul, 1957; Stone et al., 1974). All use a system whereby particular flavor 'notes' are defined for panelists by using standard stimuli. In this way, an ad hoc language is created for the description of the flavor characteristics of the food product. To understand fully the mechanisms involved in the creation of a 'flavor language', it is necessary to consider the formation of sensory concepts.

According to current theory (Miller and Johnson-Laird, 1976), a sensory concept like 'redness' is formed by a two-part process: abstraction and generalization. For the first part, the concept of redness is abstracted from red and nonred stimuli. For the second, this concept is generalized or broadened beyond those sensations used in the abstraction process. Then, stimuli colored shades of red that have not been seen before, can be categorized as falling within the concept; they have 'redness'. The concept is given a label, 'red', for purposes of communication between those who share the concept. The same reasoning can be applied to the formation of taste or flavor concepts like 'salty', 'sweet' or 'fruity'. In terms of concept formation, the presentation of standards prior to flavor descriptive analysis is an attempt to align the concepts of the panelists and give them commonly agreed labels. However, the efficacy of such procedures has not been investigated. For instance, it is not known whether the presentation of a single standard is sufficient to align the concepts of panelists enough for the precision required in sensory analysis.

The present study investigated whether the presentation of a single standard NaCl solution was sufficient to form the same 'salty' concept in a group of panelists, such that they would include or exclude further taste stimuli from the concept in the same way.

As a further refinement, a taste concept was treated as a 'probabilistic cloud' with fuzzy boundaries rather than a clearly defined space with sharp boundaries. Thus, some sensations were visualised as having a 100% probability of falling within the concept, while others had lower probabilities or even a 100% probability of falling outside. In this study, the probabilities of a set of stimuli falling within a concept, defined by a single NaCl solution, were examined for each panelist. Variability in these probability values between panelists, indicated lack of alignment of their concepts. In further experiments, investigations were made regarding changes in the concept with time, effects of variation in the use of standards, context effects and whether the concept included strong monosodium glutamate (MSG) stimuli

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MATERIALS & METHODS

Stimuli

Experiments I—III. Aqueous single solute solutions of 50 mM, 100 mM, 300 mM, 800 mM, 1500 mM NaCl, 192 mM KCl, and 208 mM sodium citrate were prepared, as well as binary solute mixtures of strength: 53 mM fructose/270 mM NaCl, 265 mM fructose/150 mM NaCl, 96 mM KCl/150 mM NaCl, 1.5 mM citric acid/195 mM NaCl, and 60 mM MSG/240 mM NaCl. A solution of 100 mM NaCl was colored yellow and given a lemon odor (3 drops yellow coloring, 2 drops lemon flavoring added to 1L solution) and a second 100 mM NaCl solution was colored orange and given a vanilla odor (2 drops red and 2 drops yellow coloring, 2 drops vanilla flavoring added to 1L solution). The fourteen stimuli were dispensed in 10 mL volumes (Oxford adjustable dispensor, Lancer Co., St. Louis, MO) and presented at constant room temperature (22–24°C) in 1 oz (29.6 mL) paper cups (Lily Portion cups, No. 100; Lily Tulip Inc., Toledo, OH). Solutions were prepared in Millipore purified water (Milli-RO 4/5 Filtration and Reverse Osmosis System in series with a Milli-Q system: ion exchange and activated charcoal; Millipore Corp., Bedford, MA). NaCl, KCl, citric acid, sodium citrate were reagent grade (Mallinckrodt Inc., Paris, KY), MSG was standard grade (U.S. Biochemicals Corp., Cleveland, OH) and fructose was Baker Grade (Baker Chemical Co., Phillipsburg, NJ). The colorings were from the Food Color Kit (Safeway Stores, Oakland, CA). The flavorings were: 'vanilla extract' and 'lemon artificial flavor' (Safeway Stores, Oakland, CA).

Experiment IV. Solutions of 80 mM, 300 mM, 1000 mM NaCl and 80 mM, 300 mM, 1000 mM MSG, 192 mM KCl, 530 mM fructose, were prepared and presented at constant room temperature (22°C), in the same way as for Experiment I.

Color discs

Color discs were used for a color sorting task. They were circular mounted plastic chips from the Farnsworth-Munsell 100-Hue Test for Color Discrimination (Munsell Color, MacBeth, A Division of Kollmorgen Corp., Baltimore, MD). The discs chosen had the following numbers: 5 (orange-flesh), 18, 19, 20, 21 (yellowish-green), 43 (blue-green), 50, 51, 52 (blue), 63, 64, 65 (purple), 76 (reddish-purple).

Judges

Twelve students from the University of California, Davis (6M, 6F, 19–25 yr) were used in Experiments I—III; ten of these (4M, 6F, 19–22 yr) returned for Experiment IV. All judges were naive to taste experiments.

Procedure

Control experiments. Before Experiment I, all 12 judges were given difference tests to ensure that they could discriminate clearly between the stimuli used in Experiments I to III. They were required to distinguish between all adjacent concentrations of NaCl and be-

tween 300mM NaCl and all the remaining stimuli. Before Experiment IV, further testing ensured that judges could discriminate between adjacent MSG concentrations and between the weakest and strongest MSG and NaCl stimuli. In all cases, judges were required to distinguish to a criterion of nine triangle tests without error (binomial $p < 0.001$). Because the judges were naive to tasting, some required prior practice and orientation sessions.

For the control sessions, judges first cleaned the mouth with at least 3 (3–10) tapwater rinses. They then 'warmed up' (O'Mahony, 1972) by tasting the two stimuli to be discriminated, alternately, three times; three water rinses were taken before tasting each simulus. Judges were allowed to repeat the 'warm-ups' until they felt confident to proceed to the triangle tests; no judges required more than three 'warm-ups'. After 'warming-up', judges were asked to describe the differences between the stimuli and their descriptions were used to explain the differences that would occur in the following triangle tests. Judges then performed 9 triangle tests, rinsing the mouth with water 3 times before each stimulus. In the triangles, the stronger or the non-NaCl stimulus was always the odd stimulus; sometimes this procedure is called a 3-alternative forced choice (3-AFC) procedure (Frijters, 1980, 1981; Frijters et al., 1980). At the end of the triangles, the subject rested approx. 5 min prior to performing the 'warm-up' for the next set of triangle tests. Judges who did not reach the criterion of 9 correct triangles were allowed to repeat the test later; no judge exceeded three attempts. The only exception was the NaCl-KCl difference test where one subject (M, 21 yr) required four sets of triangles and another (F, 19 yr) required five 'warm-ups' and seven sets of triangles. These judges were not eliminated because their initial performance appeared to be caused by naivety; after practice, they performed successfully.

Before Experiment I, judges had 3–8 orientation and/or control sessions (total time ranged 3–8.5 hr); before Experiment IV they had 1–2 control sessions (total time ranged 1.5–2.5 hr).

Experiment I. Judges first performed a color sorting task. The judge was presented with an array of 13 Farnsworth-Munsell color discs on a white tray and required to sort them into conceptual categories, having the same or similar color, according to how each judge felt they should be grouped together. The judge was told that the color discs were all different colors and that they could be sorted into as many categories as desired, including single disc categories. The subject was told that there was no correct answer and that the sorting pattern was a matter of opinion.

After sorting the color discs, the idea of color and taste concepts was introduced to the judge. It was explained how each judge had sorted the chips into their color conceptual categories and it was indicated how colors fell inside or outside the concepts obtained. The judge was then given 300 mM NaCl and told that it was the standard that defined a taste concept, that is was prototypical, at the centre of the conceptual space. The judge was then given all the other stimuli and required to state whether they fell within or outside the taste concept. The concept was not given a name so as to avoid language connotations. Judges responded verbally: 'within the concept,' 'within the concept-not sure,' 'outside the concept,' 'outside the concept-not sure.' From these data, R-index values (O'Mahony, 1979a, 1983, 1986) were calculated for each simulus for each judge, giving the probability of the stimulus falling within the taste concept defined by the 300 mM NaCl. Strictly, the R-index gives the probability of distinguishing a stimulus from 300 mM, in terms of inclusion within the concept, when the two stimuli are given in paired comparison. An R-index of 100% indicates that the stimulus was conceptually completely distinguishable from the NaCl standard; it was outside the concept. An R-index at the chance level of 50% indicated that the stimulus could not be distinguished from 300 mM NaCl in terms of the concept; it was always included within the concept. Intermediate values indicated that the stimulus was sometimes included within the concept and sometimes outside it. In signal detection terms (Green and Swets, 1966), the R-index gives the probability of distinguishing an extra-conceptual signal from the background noise of the concept.

All the 14 stimuli (including 300 mM NaCl) were presented eight times each in random order. Included among the stimuli was an extra set of 300 mM NaCl stimuli, which acted as the referent 'noise' stimuli for the R-index computation (O'Mahony, 1979a). Thus, there were 15 stimuli presented 8 times each, giving a total of 120 stimulus presentations.

When the standard was tasted initially, judges were allowed to taste it as often as they wished (2–6 times). It was available for reference throughout the experiment; 11 subjects used it, tasting it 1–10 times. The stimuli were presented in random order with the modification that 50 mM or 100 mM NaCl never followed 800 mM or 1500 mM NaCl so as to avoid extreme intensity reduction by adaptation (O'Mahony, 1979b). Before responding to each stimulus, subjects were required to report whether it had a taste; water blanks were introduced randomly into the stimulus order as a control, to ensure that these were reported as having 'no taste' and the other stimuli reported as having a taste. Judges rinsed the mouth at least three times before tasting each stimulus; it was also ensured that six rinses were taken after tasting 1500 mM and 800 mM NaCl and before 50 mM NaCl or the water blanks. This was achieved by casually suggesting that it was worth taking some more rinses to keep the mouth clean and by taking this number of rinses after other stimuli at random so as not to give judges clues about the next stimulus to be presented. The experiment was performed in one session (session lengths ranged 55–115 min).

Experiment II. The procedure was the same as for Experiment I, except that the color sorting was not performed and that several standards were presented to define the concept: 300 mM and 50 mM NaCl, and the 100 mM NaCl colored orange with vanilla odor were given as stimuli falling within the concept: the 800 mM NaCl, 53 mM fructose/270 mM NaCl and 96 mM KCl/150 mM NaCl mixtures were given as stimuli falling outside the concept. Experiment II was performed at least one week (1–3 wk) after Experiment I in a single session (session lengths ranged 45–55 min).

Experiment III. The procedure was the same as for Experiment I, except the color sorting was omitted. The experiment was performed at least 1 wk (1–2 wk) after Experiment II, in a single session (session lengths ranged 48–76 min).

Experiment IV. The procedure was the same as for Experiment I except that the stimuli presented after the 300 mM NaCl standard were: 80 mM, 300 mM, 1000 mM NaCl; 80 mM, 300 mM, 1000 mM MSG; 192 mM KCl, 530 mM fructose. Stimuli were presented five times each in random order and six rinses were required after tasting the 1000 mM NaCl and MSG stimuli. The experiment was performed at least 1 wk (1–5.5 wk) after Experiment III in a single session (session lengths ranged 10–4 min).

RESULTS & DISCUSSION

Experiment I

R-indices were calculated for each stimulus for each judge. An R-index gives the probability of discriminating a given stimulus, conceptually, from the 300 mM NaCl standard, should they both be presented in paired comparison. An R-index of 100% indicates that the stimulus was completely distinguishable; it fell outside the concept. An R-index of 50% represents chance discrimination; it was conceptually identical with the standard. Table 1 gives these values; for clarity, values of 100% are represented by dashes.

It can be seen from Table 1 that no judge had the same distribution of R-indices; no judge included or excluded the stimuli from his concept in the same way; no judge had formed the same concept from the 300 mM NaCl standard; this was a significant majority trend (binomial $p < 0.006$). It can also be seen that for judges 1–9, who excluded some stimuli completely from the concept ($R = 100\%$), no judges agreed on which set of stimuli should be excluded. Again, this was a significant majority trend (binomial $p = 0.004$). Only judges #2 and #10 had all their R-indices differ by no more than 10 points.

The concept formed was probabilistic, with 54.8% of the judgements indicating that a stimulus was neither completely in ($R = 50\%$) nor completely out ($R = 100\%$) of the concept. Such judgements indicated that a stimulus was sometimes judged as within the concept and sometimes outside it. Such shifts in judgement were caused by changes in conception; the control experiments indicated that they were not caused by difficulties in discrimination. Further evidence of changes in conceptual judgement is seen by the fact that the 300mM NaCl stimulus was only judged as conceptually identical to the standard ($R = 50\%$) by one judge (#9), a significant minority trend (binomial $p = 0.006$). In fact, five judges (#3, 6, 7, 9, 12) rated other stimuli as conceptually closer to the standard than the 300 mM NaCl (R-index smaller than the R-index for 300 mM NaCl). Such shifts in conceptualization are also suggested by physiological evidence (Skarda and Freeman, 1987).

The concepts formed around the 300 mM NaCl did not ex-

Table 1—R-indices[a] indicating the probabilities (below 100%)[b] of distinguishing a stimulus, conceptually, from 300 mM NaCl

Stimuli	Judges											
	1	2	3	4	5	6	7	8	9	10	11	12
1500 mM NaCl	—	—	—	75	—	—	50	90.6	43.8	93.8	87.5	31.2
800 mM NaCl	—	—	—	93.8	87.5	97.7	50	92.2	50.8	93.8	87.5	38.3
300 mM NaCl	75.8	56.3	75	62.5	56.3	77.3	62.5	62.5	50	54.7	51.6	52.3
100 mM NaCl	98.4	—	—	—	87.5	—	62.5	92.2	96.1	93.0	87.5	74.2
50 mM NaCl	—	—	—	—	—	—	68.5	82.8	99.2	93.8	87.5	85.9
100 mM NaCl, orange color, vanilla odor	—	—	—	—	93.8	—	—	—	—	93.8	85.2	87.5
100 mM NaCl, yellow color, lemon odor	—	—	87.5	—	—	95.3	—	89.1	97.7	93.0	87.5	87.5
208 mM sodium citrate	—	—	—	—	—	—	—	—	—	93.8	87.5	87.5
192 mM KCl	—	—	—	—	—	—	—	—	—	93.8	87.5	87.5
96 mM KCl/150 mM NaCl	—	81.3	68.8	93.8	93.8	71.1	56.3	—	88.4	82.0	71.1	52.3
60 mM MSG/240 mM NaCl	—	87.5	—	—	—	—	—	79.7	96.9	93.8	87.5	80.5
1.5 mM citric/195 mM NaCl	—	—	—	—	—	95.3	87.5	95.3	—	93.8	87.5	87.5
265 mM fructose/ 150 mM NaCl	—	—	—	—	—	91.4	—	—	—	93.8	87.5	87.5
53 mM fructose/ 270 mM NaCl	—	—	93.8	93.8	93.8	89.1	56.3	96.9	75.8	90.0	87.5	80.5

[a] The higher the R-index above 50%, the lower the probability of the stimulus falling within the concept.
[b] R-indices of 100%, representing complete exclusion from the concept are represented by —.

tend their boundaries broadly across the other stimuli; this can be seen by the generally high R-index values that indicate that stimuli tended to be excluded from the concept (68% are above 90; a majority trend, binomial $p < 0.0006$). This is again reflected by the generally high mean R-index values for this experiment, given in Table 2. This indicated a relatively small degree of concept generalization from the 300 mM NaCl stimulus.

The data indicated that the presentation of a single standard was not sufficient to align the taste concepts of the judges; a more thorough procedure was required. Thus, variations in description given to a stimulus could be not only due to variation in the stimulus but also due to variation in the use of the descriptive term. This has important implications for descriptive analysis, not only in the definition of descriptive terms but also in the interpretation of the results. As with scaling, variation in the data can be due not only to differences in sensation but also due to differences in use of the psychophysical tool. Just as a between-judges experimental design (often caused by judge substitution) should be avoided for scaling, it should also be avoided for descriptive analysis.

Experiment II

This experiment investigated whether giving a set of stimuli as standards raised or lowered the R-index for a set of target stimuli, compared to when only the 300mM NaCl standard was given. The mean R-indices for these target stimuli in Experiments I and II are given in Table 2. Specific hypotheses were developed regarding whether R-index values would be raised or lowered.

The 50mM NaCl and 100mM NaCl, colored orange with vanilla odor, were given as standards for the concept in this experiment; in Experiment I they were not. It was hypothesized that they would thus be included more within the concept; their R-index values would be expected to decrease. From Table 3, it can be seen that the mean R-indices were decreased significantly; reduced R-indices were recorded for all subjects (binomial $p < 0.006$).

In the same manner, it would be hypothesized that standards given as examples of stimuli that were not in the concept would tend to be excluded more; their R-indices would increase. For two stimuli (800 mM NaCl, 53 mM fructose/270 mM NaCl) the means increased insignificantly. It may be hypothesized that the lack of significance may have been due to a partial 'ceiling effect' in the already high values. For the third stimulus (96 mM KCl/150 mM NaCl) the mean decreased.

The prior hypotheses were generated about how the judges would respond to standards per se. Further hypotheses were generated regarding how judges generalized to other stimuli from these standards.

It was hypothesized that because 50 mM NaCl and 300 mM NaCl were given as standards, the intermediate 100 mM NaCl would be interpolated as being within the concept. It was further hypothesized that because the 100 mM NaCl, with the orange color and vanilla odor, was given as a standard, the concept would generalize to the same solution colored yellow with a lemon odor. In both cases, a decrease in R-indices would be hypothesized. Table 2 indicates that the mean values decreased significantly; reductions in R-index occurred for at least $11/12$ judges (binomial $p < 0.006$).

It may be hypothesized that judges would also generalize from standards given as examples of stimuli falling outside the concept, to more extreme stimuli. A more extreme stimulus is one that is more different from those standards that fell within the concept. Thus, 800 mM NaCl would be expected to exclude the stronger 1500 mM NaCl; 53 mM fructose/270 mM NaCl would be expected to exclude the sweeter 265 mM fructose/150 mM NaCl; 96 mM KCl/50 mM NaCl would be expected to exclude the more potassium tasting 192 mM KCl. For all these stimuli, R-indices would be hypothesized to increase significantly. Table 2 indicates this did not occur; for KCl the reverse occurred. It may be hypothesized that this could have been caused by a partial ceiling effect.

The noise level stimulus for the R-indices calculate here was 300 mM NaCl. This was to make the R-indices comparable to the values in Experiment I. Yet, the true noise level, the true

Table 2—Mean[a] R-index values for selected stimuli from Experiment I and Experiment II

Stimuli	Experiment I	Experiment II	Significance of difference (t test)
50 mM NaCl	93 ± 10	53 ± 17	$p < 0.001$
100 mM NaCl, orange color, vanilla odor	97 ± 5	44 ± 15	$p < 0.001$
800 mM NaCl	83 ± 22	91 ± 9	$p > 0.2$
53 mM fructose/270 mM NaCl	88 ± 12	89 ± 12	$p > 0.2$
96 mM KC1/150 mM NaCl	78 ± 15	69 ± 14	$p = 0.076$
100 mM NaCl	91 ± 12	50 ± 19	$p < 0.001$
100 mM NaCl, yellow color, lemon odor	95 ± 6	71 ± 23	$p < 0.01$
1500 mM NaCl	81 ± 25	92 ± 8	$p > 0.1$
265 mM fructose/150 mM NaCl	97 ± 5	92 ± 8	$p > 0.1$
192 mM KCl	97 ± 5	92 ± 8	$p = 0.046$
300 mM NaCl	61 ± 10	57 ± 6	$p > 0.2$

[a] Each mean ± standard deviation represents scores from 12 judges.

Table 3—Consistency of the concept formed by the 300 mM NaCl standard in Experiments I and III; consistency is indicated by mean R-indices, their ranges and the number of judges for whom the R-index did not change

Stimuli	Mean R-Indices[a]		No. of judges[b] whose R-index was unchanged
	Experiment I	Experiment III	
1500 mM NaCl	81 (31-100)	97 (88-100)	5
800 mM NaCl	78 (38-100)	92 (50-100)	4
300 mM NaCl	61 (50-76)	62 (50-100)	0
100 mM NaCl	91 (63-100)	78 (50-100)	2
50 mM NaCl	93 (69-100)	86 (50-100)	3
100 mM NaCl, orange color, vanilla odor	97 (85-100)	90 (50-100)	5
100 mM NaCl, yellow color, lemon odor	95 (88-100)	84 (56-100)	4
208 mM sodium citrate	97 (88-100)	97 (88-100)	10
192 mM KCl	97 (88-100)	96 (88-100)	9
96 mM KCl/150 mM NaCl	78 (69-94)	84 (63-100)	0
60 mM MSG/240 mM NaCl	96 (81-100)	95 (50-100)	7
1.5 mM citric/195 mM NaCl	96 (88-100)	93 (56-100)	5
265 mM fructose/150 mM NaCl	97 (88-100)	95 (88-100)	3
53 mM fructose/270 mM NaCl	84 (31-100)	97 (88-100)	8

[a] Each mean with range in parenthesis, represents scores from 12 judges.
[b] Number of judges from twelve.

center of the concept is not easily predicted from the set of standards given. This would have caused some R-index values to fall below 50%, as can be seen from Table 2. Further, this may have been a partial cause of the target R-indices not being reduced as hypothesized. However, Table 2 does show no significant change in the mean R-index for the 300 mM NaCl stimulus, indicating that the center of the concept did not change radically in Experiment II.

The data indicate that the use of more than one standard could bring about improvement. Certainly, a stimulus intermediate between two standards would tend to be included more in the concept, while admittal of a stimulus with color and odor into the concept caused the other stimulus with color and odor to be admitted. It is not clear whether this effect was caused by a concept being formed which included all colored and odorous stimuli or one which included the taste of 100 mM NaCl while odor and color were irrelevant. Thus, it would seem that the generalization step in the concept formation process can be influenced; the concept can be broadened by the use of extra standards. The opposite process did not appear to be successful with the present stimuli. Standards defining stimuli as not to be included within the concept appeared not to be effective in limiting generalization of the concept. The lack of success in the present study may be due to the context of these particular stimuli rather than any inherent lack of efficacy of such standards. Certainly, further investigation into the limitation of conceptual boundaries would seem worthwhile.

Experiment III

Because the same standard was presented as in Experiment I, it can be hypothesized that the same taste concept would be specified; the R-indices would remain the same. This was clearly not the case from the mean values and their ranges indicated in Table 3. Only the mean and range for sodium citrate remained the same, because a significant majority of judges kept the same score (10/12, binomial $p = 0.039$). While a majority of judges kept the same R-index for some stimuli, this majority was always nonsignificant (binomial $p > 0.146$). No judges kept all their R-indices within 10 points of each other. Although the ranges of the R-index values increased in Experiment III (mean ranges 30, $S = 22$ vs 37, $S = 17$), the increase was not significant (t test $p > 0.2$). This indicated that the judgements for any given stimulus did not on average tend to become more discrepant.

The change in the taste concept indicated a dynamic aspect of the concept in that it changed over time. The formation of concepts in Experiment II may have been a contributing factor, although it may be hypothesized that the period of time between Experiments I and III was long enough to allow conceptual shift. Physiological evidence (Skarda and Freeman, 1987) would also suggest a dynamic aspect to conceptualization.

Experiment IV

The R-indices for the stimuli are shown in Table 4. In can be seen that the two stronger MSG concentrations were excluded from the concept ($R = 100\%$) by a significant majority (9/10) of the judges (binomial $p = 0.02$). 80mM MSG was excluded by 8/10 judges (binomial $p = 0.109$). As expected, fructose and KCl tended to be excluded. The R-indices for KCl changed for 4 judges (two by more than 10 points) from the values in Experiment I. In the context of these stimuli, a significantly higher proportion of subjects had an R-index of 50% for the 300mM NaCl stimulus than in Experiment I (6/10 vs 1/12, binomial comparison of proportions $p = 0.015$). Context would seem to have a significant effect on the concept formed. Interestingly, one judge (#7) judged all NaCl solutions to be totally within the concept and all others to be outside.

The taste quality of gustatory stimuli alters with concentration; this gustatory analog of the Bezold-Brücke phenomenon is well documented (Bartoshuk, 1968; Bartoshuk et al., 1964, 1974; Cardello and Murphy, 1977; Dzendolet and Meiselman, 1967; Murphy et al., 1981; O'Mahony and Bramwell, 1976; O'Mahony and Stevens, 1975; O'Mahony et al., 1976a, 1976b, Peryam, 1960, 1963). The taste of MSG also changes with concentration; at higher concentration it has been reported as being more 'salty' (Bartoshuk et al., 1974; Peryam, 1963). However, in these studies, the term 'salty' was not defined by the use of standards and so it was difficulty to interpret exactly what was meant by MSG becoming 'salty'. If 300 mM NaCl defined the salty concept, then higher concentrations of MSG were not included within the concept; according to this definition, they were not 'salty.' It may be asked why MSG was described as becoming 'salty.' In the studies reporting this, judges were only allowed to use the words: 'sweet,' 'sour,' 'salty,' and 'bitter' (these taste concepts were not defined by standards). It may be hypothesized that 'salty' was chosen not because MSG tasted like NaCl but that it was chosen by default, the other descriptive terms being more inappropriate (O'Mahony and Ishii, 1987). In fact, Japanese researchers call the taste of MSG 'umami' (Kawamura and Kare, 1986; Yamaguchi, 1979; Yamaguchi and Kimizuka, 1979; Yamaguchi and Takahashi, 1984; Yamaguchi et al., 1971) while the name in the marketplace is 'Ajinomoto taste' (O'Mahony and Ishii, 1986). Yet, the 'umami' taste is generally defined as that taste imparted by the broths made from kombu, shiitake or katsuobushi (Kuninaka, 1981; O'Mahony and Ishii, 1985). In this way, MSG has an umami taste at lower concentrations but the taste quality changes at higher concentrations. How this taste

Table 4—R-indices[a] indicating the probabilities (below 100%)[b] of distinguishing a stimulus, conceptually, from 300 mM NaCl

Stimuli	Judges[c]									
	1	3	4	6	7	8	9	10	11	12
1000 mM NaCl	90	80	—	—	50	80	80	—	—	50
300 mM NaCl	50	60	52	76	50	50	50	96	50	50
80 mM NaCl	82	80	—	—	50	82	60	—	—	80
192 mM KCl	—	80	—	—	—	—	—	—	—	—
530 mM fructose	—	80	—	—	—	—	—	—	—	—
1000 mM MSG	—	80	—	—	—	—	—	—	—	—
300 mM MSG	—	80	—	—	—	—	—	—	—	—
80 mM MSG	98	80	—	—	—	—	—	—	—	—

[a] The higher the R-index above 50%, the lower the probability of the stimulus falling within the concept.
[b] R-index values of 100%, representing complete exclusion from the concept, are represented by —.
[c] The judges were used in Experiment I and are identified by the same numbers as in Table 1.

change is described depends on the definition of descriptive terms.

To describe a stimulus as having some 'salty' character only makes sense if the 'salty' concept is defined in some way. If this is not done, the statement means little more than saying that the taste has changed. This is not a matter of mere semantics, it is a matter of the conceptual organization of the sensory instrument and of understanding exactly how much valid information the instrument really is providing.

CONCLUSION

IT SHOULD NOT BE assumed that presentation of one standard to define a taste concept will result in judges having the same concept; the procedure did not produce a high degree of conceptual alignment. The area of concept definition for descriptive analysis remains unresearched, despite its importance for the sensory evaluation of foods; it requires further investigation. The use of more than one standard to define a flavor concept would seem a promising approach although the definition of the boundaries of the concept remains problematical. The theoretical approach of concept formation provides a novel approach to understanding the mechanisms involved in descriptive analysis. It clarifies the data concerning the changes in taste as MSG increases in strength. A further novel aspect of this study was that it provided the first probabilistic measure of a taste concept.

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Ms received 6/19/86; revised 10/31/86; accepted 2/27/87.

This research was supported by the Umami Manufacturers Association of Japan. The authors thank the following for their help: Shunsuke Hiraguri, Hide Ishii, Paul Maldonado, Gwen Newhall, Sharlene Vite, Benjamin Weitzel, and Karen Wilkins.

Temporal Changes in Serum Cholesterol Levels of Rats Fed Casein and Skim Milk Powdered Diets

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ABSTRACT

The temporal cholesterolemic patterns to skim milk powder (SMP) and reprocessed SMP (RSMP) diets were compared to diets containing casein or laboratory rat chow over a 24-day period. SMP was hypocholesteremic relative to casein in rats fed a 1% dietary cholesterol. Reprocessing of SMP resulted in an apparent loss of the relative hypocholesterolemic reponse of native SMP. Amino acid analysis of SMP and RSMP, showed only marginally lower lysine levels than casein; however, the lysine/arginine ratio was higher in SMP than either in casein or RSMP. Available lysine content in SMP was higher than in RSMP, suggesting occurrence of nonenyzmatic browning reactions. Although a similar cholesterolemic response was observed in casein and RSMP, the available lysine content of these two protein sources were markedly different, suggesting that reduced available lysine alone was not totally responsible for the lower cholesterolemic response of SMP, relative to casein.

INTRODUCTION

THE IMPACT of diet and nutrition on the etiology of hypercholesterolemia and atherosclerosis has been the subject of considerable investigation and review (Kritchevsky, 1970; Hopkins and Williams, 1981). It is well known that both dietary protein (Carroll and Hamilton, 1975; Hamilton and Carroll, 1976) as well as various individual amino acids (Solomon and Geison, 1978; Kritchevsky, 1979; Katan et al., 1982) can have an effect on the level of circulating cholesterol. Studies conducted with rats fed various protein diets have shown that a casein diet increased serum cholesterol levels more so than a soybean protein diet (Yadav and Liener, 1977; Hevia and Visek, 1979b; Nagata et al., 1980). Other workers have reported no differences in the serum cholesterol level of rats fed these two diets (Sautier et al., 1979). Similarly, in man, dietary plant proteins were found to be hypocholesterolemic in one study (Sirtori et al., 1979), but not in another (Holmes et al., 1980). These apparent contradictions in experimental results may be explained on the basis that the absorption of dietary cholesterol into the circulation is known to be affected by many components of the diet (Kritchevsky, 1979).

Foods have a complex composition of numerous individual nutrients. Milk, which contains about 2.8% casein, 3.8% fat and 140 mg cholesterol/L is a prime example of a multinutrient food. Milk has been suggested as a possible health hazard on the basis of a correlation between consumption and coronary heart disease (Segall, 1977). Other studies have suggested that milk products may in fact actually reduce serum cholesterol levels in both animal and human subjects (Mannilow and McLaughlin, 1975; Howard, 1977; Kritchevsky et al., 1979). The mechanism on how milk products may regulate serum cholesterol levels has not been elucidated, however.

Since skim milk powders (SMP) include both high concentrations of proteins rich in lysine and reducing sugars, nonenzymatic browning can occur upon high temperature processing or under improper storage conditions (Labuza and Saltmarch, 1981). Such reactions can bring about the destruction of specific amino acids (All-Talib and Fox, 1983) as well as the structure of the proteins (Powrie and Nakai, 1981). It is of interest that Annand (1967) proposed that heated milk was a primary factor in atherogenesis.

In the present study with rats, the cholesterolemic effect of skim milk powder and casein diets with and without dietary cholesterol supplements was examined. For each rat on a specific diet, total serum cholesterol (TSC) levels were monitored during a 24-day period for the purpose of examining individual temporal changes. In a separate experiment, the cholesterolemic response associated with SMP was compared to SMP which had undergone reprocessing to yield RSMP. The quantitation of both amino acid content as well as available lysine was performed in these particular protein sources for the purpose of addressing the relative cholesterolemic responses to diets with known amino acid composition.

MATERIALS & METHODS

Animals

Male Wistar rats were obtained from the University of British Columbia's animal colony. All rats were fed a commercial diet (5001 Purina Lab Chow diet, Ralston Purina Co., St. Louis, MO) which was ground and fed in powdered form. On the fifth day after feeding, attempts were made to randomize the animals according to body weight (208–232g, Expt. 1 and 222–246g, Expt. 2) into groups of 6 animals each. Animals were housed individually in plastic cages with wire mesh flooring under conditions of controlled temperature (20–23°C) and lighting (alternating 12-h periods of light and dark).

Diets

The composition of the various test diets is shown in Table 1. Two experiments were conducted to examine the effects of these diets on the cholesterolemic response of rats. In experiment 1, rats were fed either a control diet (Rat Chow) or one of three experimental diets which included either casein, skim milk powder (SMP) or reprocessed skim milk powder (RSMP), all of which contained 1% added choles-

Table 1—Composition of the experimental diets

	Experiment 1			Experiment 2	
	Casein	SMP[a]	RSMP[a]	Casein	SMP
Ingredient	g/100g			g/100g	
Casein[b]	20.0	—	—	20.0	—
SMP[c]	—	50.0	—	—	50.0
RSMP	—	—	50.0	0	0
DL-methionine	0.3	0.3	0.3	0.3	0.3
Cornstarch	15.0	10.4	10.4	15.0	10.4
Sucrose	48.85	23.85	23.85	49.85	24.85
Fiber[d]	5.0	5.0	5.0	5.0	5.0
Corn oil	5.0	4.6	4.6	5.0	4.6
Mineral mixture[e]	3.5	3.5	3.5	3.5	3.5
Vitamin mixture[f]	1.0	1.0	1.0	1.0	1.0
Choline bitartrate	0.2	0.2	0.2	0.2	0.2
L-lysine HCl	0.15	0.15	0.15	0.15	0.15
Cholesterol	1.0	1.0	1.0	—	—

[a] SMP, skim milk powder; RSMP, reprocessed skim milk powder.
[b] Approx. 90% protein (Sigma Chemical Co., St. Louis, MO).
[c] Pacific Milk Division, Fraser Valley Milk Producers Association, Vancouver, B.C.
[d] Alphacel, non-nutritive, bulk, ICN Nutritional Biochemicals, Cleveland, OH., containing finely ground cellulose.
[e] AIN Mineral mixture 76, ICN Nutritional Biochemicals.
[f] AIN vitamin mixture 76, ICN Nutritional Biochemicals.

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terol. Skim milk powder was obtained from Pacific Milk Division, Fraser Valley Milk Producers' Association, Vancouver, B.C. RSMP was prepared by spray drying reconstituted SMP 3 times using a spray drier (A/S Niro Atomizer, Copenhagen, Denmark) under the following conditions: inlet temperature = 225°C, outlet temperature = 100°C, pressure = 110 psi. In experiment 2, the experimental diets with either casein or SMP did not contain added cholesterol. Both experiments were terminated after 24 days of feeding. All diets were fed in powdered form. Diets and water were provided *ad libitum*. The rats were weighed at the beginning of the study and at weekly intervals thereafter.

Analytical procedures

Blood samples were taken at intervals (3 to 7 days) between 0900 and 1000 hr under light anesthesia. The samples were obtained from the artery of the midventral surface of the tail without anticoagulant. Serum was then prepared by low-speed centrifugation at about 23°C and stored at −20°C until the time for assay. Cholesterol in the serum was measured enzymatically (Siedel et al., 1983) using a kit supplied by Boehringer-Mannheim, GmbH (Mannheim, West Germany).

The amino acid composition of casein, SMP and RSMP was determined by a Beckman System 6300 High Performance Amino Acid Analyser with a prepacked Beckmann System 6300 High Performance column. Available lysine was determined by using the 2,4,6-trinitrobenzenesulfonic acid method as outlined by Kakade and Liener (1969). All analyses were performed in triplicate.

Statistical analysis

All data were expressed as the mean ± SEM. Analysis of variance was used to determine individual treatment differences. The Newman-Keuls multiple range test ($p<0.05$) (Steel and Torrie, 1960) was used when the means were significantly different.

RESULTS

THE AMINO ACID composition of casein, SMP and RSMP protein sources are presented in Table 2. The spray drying (3 times) of reconstituted SMP caused about 14% decrease in total lysine and a reduction in the lysine/arginine ratio from 2.23 for SMP to 1.97 for RSMP. The observed decrease in total lysine in RSMP was not accompanied by a marked reduction in protein content (Table 3); however, this process reduced the amount of available lysine from 3.83 g/100g SMP to 2.88 g/100g RSMP.

Table 2—Amino acid composition of casein, SMP, RSMP[a]

Amino acid[b]	Casein	SMP	RSMP
	g/100g amino acid		
Aspartic acid	6.9	7.5	7.5
Threonine	4.0	4.2	4.1
Serine	4.9	4.9	4.8
Glutamic acid	18.0	18.9	18.6
Proline	10.4	9.8	9.7
Glycine	1.9	1.9	1.9
Alanine	3.0	3.2	3.2
Cystine	0.4	0.8	0.8
Valine	6.7	6.6	6.5
Isoleucine	5.5	5.5	5.5
Leucine	9.4	10.0	9.9
Tyrosine	5.5	4.6	4.7
Phenylalanine	4.2	4.0	4.2
Lysine	7.9	7.8	6.7
Arginine	3.8	3.5	3.4
L/A[c] ratio	2.08	2.23	1.97

[a] SMP, skim milk powder; RSMP, reprocessed skim milk powder.
[b] Chemical analysis using a Beckman system 6300 High Performance Amino Acid Analyser, on a prepacked Beckman system 6300 High Performance Column.
[c] L/A = Lysine:Arginine

Table 3—Available lysine and protein of casein and skim milk powder[a]

Preparation	Available lysine (g/100g sample)	Protein (%)
Casein	6.82	95.6
SMP	3.83	35.9
RSMP	2.88	36.3

[a] Values represent the average of triplicate analysis.

Animals receiving specific diets containing cholesterol supplements exhibited a numerical difference in body weight gain in casein fed (130 ± 4g) animals compared to SMP (115 ± 5g), RSMP (114 ± 4g) and rat Chow (121 ± 6g). These differences, however, were not significant. In diets without the addition of cholesterol, rats fed casein exhibited a greater ($p<0.05$) weight gain (119 ± 3g) compared to those fed either SMP (99 ± 3g) or a commercial rat chow diet (108 ± 2g).

Figure 1 shows the temporal pattern of total serum cholesterol (TSC) response in rats fed casein, SMP and rat Chow diets without added cholesterol. Sharp increase in TSC concentrations reaching higher ($p<0.05$) levels was observed in both the casein and SMP diets, relative to the rat Chow diet throughout the course of the experiment. In addition, rats fed the SMP diet exhibited a higher ($p<0.05$) TSC concentration than that observed in casein fed rats after 2 wk; however this increase appeared to be transitory and there was no significant difference in TSC levels between these two dietary treatments at the conclusion of the experiment.

Casein and SMP diets containing 1% added cholesterol brought about an increase ($p<0.05$) in TSC concentrations over the experimental period (Fig. 2.). Unlike the cholesterolemic responses observed in diets without added cholesterol, the TSC concentrations in cholesterol supplemented diets continued to rise gradually throughout the 24-day experiment. A difference ($p<0.05$) in TSC among the various dietary treatments was also observed at specific durations of the feeding trial. For example, TSC concentrations were consistently higher ($p<0.05$) in cholesterol fed rats receiving casein than SMP or lab Chow fed rats, respectively, at the 1 wk feeding interval and at the conclusion of the experiment. It was also noteworthy that in all cases, rats on the RSMP-cholesterol diet exhibited higher ($p<0.05$) TSC levels than rats fed the SMP-cholesterol diet. It would appear, therefore, that the reprocessing of SMP to yield RSMP resulted in a loss in the hypocholesterolemic property of SMP.

DISCUSSION

THE RAT is known of its ability to maintain cholesterol homeostasis through control mechanisms regulating cholesterol synthesis and absorption as well as excretion of bile acids (Wilson, 1964; Mathe and Chevallier, 1979). Our experiments

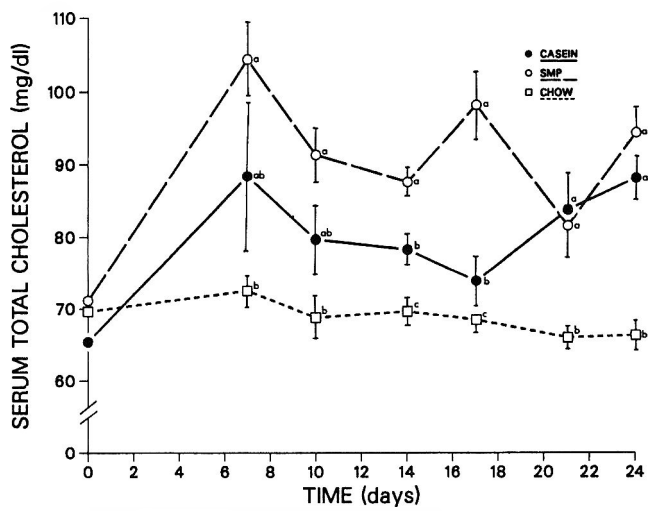

Fig. 1—Time course of serum cholesterol concentrations in rats fed noncholesterol formulated diets containing casein, SMP with Purina Lab Chow as a control. Results are expressed as means ± SE for six animals per group. Values represent the mean ± SEM of six animals. Symbols with different letters denote statistical significance ($p<0.05$).

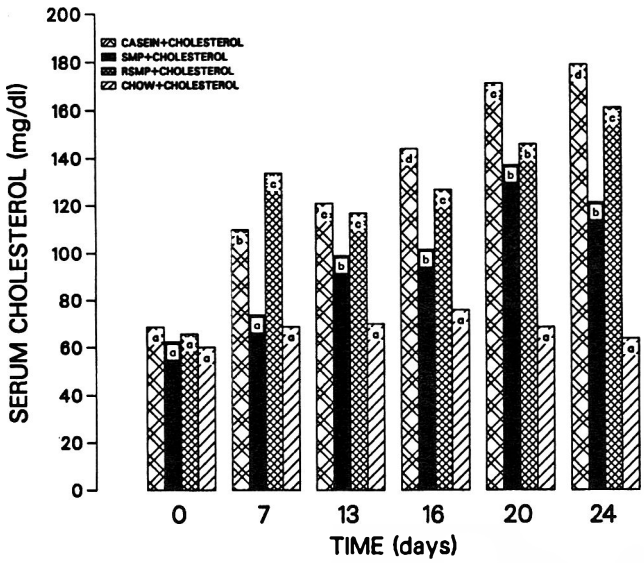

Fig. 2—Time course of serum cholesterol concentrations in rats fed cholesterol supplemented formulated diets containing casein, SMP and RSMP with Purina Lab Chow as a control. Results are expressed as means ± SE for six animals per group. Values represent the mean ± SEM of six animals. Bars with different letters denote statistical significance ($p<0.05$).

clearly indicated the hypercholesterolemic nature of casein, both in the presence and absence of added dietary cholesterol. However, the mechanism for this hypercholesterolemic property is largely unknown. In the present study, rats fed casein and SMP diets without added cholesterol showed initial increases in TSC levels which remained relatively constant throughout the experimental period. In experiments where rats were fed casein and SMP diets containing 1% added cholesterol, a gradual increase in temporal TSC was observed throughout the course of the 24-day period in these diets. These results support previous studies which have reported significant increases in TSC concentrations in rats fed diets containing 0.5% cholesterol (Mathe and Chevallier, 1979). The hypercholesterolemia observed in rats fed casein was especially exacerbated in cholesterol supplemented diets, indicating that the effect of dietary protein became more pronounced when cholesterol was included in the diet. It is noteworthy that Chow-fed rats failed to exhibit an increase in TSC in both cholesterol-free and supplemented diets, respectively. The protein source in these particular diets is largely derived from plant sources, namely soybean, oats, and alfalfa and there is considerable evidence to support the view that vegetable proteins are hypocholesterolemic in a variety of animal species (Kritchevsky et al., 1959; Hamilton and Carroll, 1976; Forsythe et al., 1980) as well as human subjects (Hodges et al., 1976; Sirtori et al., 1979). It should also be noted that SMP containing diets included approximately 25% lactose. In a previous study, Leir and Tolensky (1975) demonstrated enhanced fecal losses of fat and nitrogen in post weaning rats fed diets containing 10–30% lactose, which may partially explain the lower body weight gains observed in rats fed the SMP diet in this study. However, the fact that TSC concentrations were not different between animals fed casein and the SMP diets, respectively, suggests that the concentrations of lactose consumed by rats in this study were not a factor in the cholesterolemic responses attributed to SMP diets.

In formulated diets not containing cholesterol supplements, SMP was shown to be initially hypercholesterolemic with respect to casein. However, very little difference was observed between these two protein sources at the end of the 24-day experiment. In contrast to this observation, Kritchevsky et al. (1979) and Mannilow and McLaughlin (1975) observed a reduction in TSC in rats fed skim milk. Since the diets used by Kritchevsky et al. (1979) consisted of a commercial rat Chow, supplemented with liquid milk, the results obtained herein with strictly formulated diets, preclude a direct comparison with the former study. A relative hypocholesterolemic effect of SMP to that observed with casein was, however, observed in rats fed diets containing cholesterol. It has been stated that the calcium (Howard, 1977), lactose (Marks and Howard, 1977), orotic acid (Bernstein et al., 1975) and inhibitors of cholesterol biosynthesis (Kritchevsky et al., 1979) are implicated in the reduced cholesterolemic effect of milk. More recently, Sautier et al. (1983) reported that whey protein when compared to casein has the ability to decrease TSC in rats, to concentrations comparable to soybean and sunflower proteins. It follows, therefore, that since SMP contains both casein and whey proteins, diets containing SMP would have a modulating effect on cholesterolemia, when compared to diets containing casein as the sole source of protein.

The possibility that additional factors associated with the SMP protein sources contributed to lower TSC in comparison to casein was examined in this study by reprocessing the SMP to yield RSMP. Dairy products are particularly sensitive to nonenzymatic browning since they represent both the high concentrations of lactose and proteins with high lysine level. However, the high temperature obtained during processing or prolonged storage, coupled with their water content represents favorable conditions for the Maillard reaction (Labuza and Saltmarch, 1981). In the present study, rats fed a cholesterol-supplemented diet containing RSMP exhibited a cholesterolemic response which was greater than the SMP fed rats. Amino acid analysis of the three protein sources in the presence study revealed a notable decrease in the lysine content of RSMP when compared to SMP. This observation, coupled with the reduced availability of lysine strongly suggest that nonenzymatic browning occurred during the reprocessing of SMP. Al-Talib and Fox (1983) have demonstrated that improper storage of skim milk resulted in a 40% reduction of available lysine. By reprocessing SMP under the conditions used in this study, a 25% reduction in available lysine was observed. Without the addition of dietary cholesterol.

The lysine/arginine ratio has been considered to be an important factor in cholesterol homeostasis on the basis of antagonism between these two basic amino acids (Kritchevsky, 1979). In the present study, the lysine/arginine ratio in SMP (2.23) was higher that in either casein (2.08) or RSMP (1.93). Previous studies performed by Jarowski and Pytelewski (1975) have also reported a lowering of serum cholesterol in rats fed diets specifically supplemented with lysine. Additionally, other researchers have demonstrated that the cholesterolemic response associated with a diet can be modified by the structure of the protein (Huff and Carroll, 1980; West et al., 1984). It is interesting to note that the Maillard browning reaction in addition to causing a reduction in the content and availability of certain amino acids has also been shown to alter protein structure (Kato et al., 1981) and reduce net utilization and the biological value of the protein (Öste and Sjödin, 1984). The extent that these alterations may have on whole body cholesterol homeostasis needs further investigation.

In summary the results of this study show that SMP had a relatively hypocholesterolemic effect when compared to casein in rats that were fed cholesterol supplemented diets only. The spray drying (3 times) of reconstituted SMP to produce RSMP caused a higher cholesterolemic response in rats compared to SMP. Such an observation can be partially explained on the basis of different amino acid composition and lysine availability. The possible role of specific protein structure in elucidating the cholesterolemic responses observed herein cannot be ruled out.

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Portions of this research were supported with funds to W.D. Powrie from the Natural Sciences and Engineering Research Council of Canada.

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Presented at the 46th Annual Meeting of the Institute of Food Technologists, Dallas, TX, June 15–18, 1986. Contribution No. 86–506–J from Kansas Agricultural Experiment Station, Manhattan, KS 66506. Taken in part from the dissertation of P. Redlinger in partial fulfillment of the requirements for the Ph.D. degree. The authors express sincere thanks to G.D. Searle and Co.; Hoescht, AG; Abbott Laboratories; The Pillsbury Company; and Warner Jenkinson for providing sweeteners and flavorings for use in this study.

Mineral Binding Characteristics of Lignin, Guar Gum, Cellulose, Pectin and Neutral Detergent Fiber under Simulated Duodenal pH Conditions

STEPHEN R. PLATT and FERGUS M. CLYDESDALE

ABSTRACT

Effective stability constants (K_{eff}) and amount of Fe bound by lignin, cellulose, guar gum, low and high methoxy pectin and NDF were determined under simulated duodenal pH conditions. Guar gum bound 8.4 μmol Fe per g fiber with the greatest affinity ($K_{eff} = 6.27 \times 10^6$) of any of the fibers studied; NDF, high methoxy pectin, and one of the binding sites of lignin bound 5.0, 5.0, and 19.5 μmol Fe, respectively, per g fiber, with a $K_{eff} > 10^5$, and cellulose bound Fe so weakly that an effective stability constant could not be calculated. Lignin had two distinct binding sites for Fe, Zn, and Cu with the high affinity sites binding in the following order: Fe > Cu > Zn, but with twice as much Cu bound as either Fe or Zn.

INTRODUCTION

THERE ARE A NUMBER of reports of positive effects associated with an increase in dietary fiber intake. Positive effects cited include a reduction in the incidence of constipation, ischemic heart disease, diverticular disease of the colon, appendicitis, varicose veins, colon-rectal cancer, diabetes, obesity, and gallstones (Kelsay, 1978; Spiller, 1978; Eastwood, 1984). However, there also may be a number of adverse effects associated with an increase in fiber consumption, including a reduction in mineral availability (Reinhold et al., 1976; Ali et al., 1981; Kelsay, 1981). Reviews of the literature (INACG, 1982; Reinhold, 1982) indicate that dietary fiber decreases iron absorption, especially when consumed in diets rich in cereals and legumes. However, dietary fiber may have less of an effect on iron absorption when consumed as part of a nutritionally balanced diet (Caprez and Fairweather-Tait, 1982; Van Dokkum et al., 1982; Sandberg et al., 1982; Ifon, 1981). Nevertheless, there appears to be some controversy over the actual constituents of dietary fiber which may be involved.

A majority of the in vitro studies have evaluated the fiber constituents which affect iron absorption based on the percent iron bound under pH conditions simulating those of the small intestine (Camire and Clydesdale, 1981; Thompson and Weber, 1979; Reinhold et al., 1981). However, such results do not provide information on the iron-binding strength of fiber constituents in the presence of any side reactions which may occur in the gastrointestinal tract due to the consumption of a mixed diet containing various competing ligands and minerals.

A number of minerals have been found to compete for the binding sites of fiber components thus creating the potential for subsequent effects on availability (Platt and Clydesdale, 1985; 1986; Lee and Garcia-Lopez, 1985; Berner and Hood, 1983). Further, ligands such as ascorbic acid, citrate, EDTA, and phytate, have been shown to decrease the amount of iron bound by fibers under pH conditions simulating those of the small intestine (Reinhold et al., 1981; Leigh and Miller, 1983).

The purpose of this study was to determine the relative binding strengths and number of binding sites for lignin, cellulose, guar gum, neutral detergent fiber (prepared form Soft White Wheat Bran), and low and high methoxy, pectin with Fe under simulated duodenal pH conditions. In addition, the relative binding strengths and number of binding sites for lignin with Fe(II), Cu(II), and Zn(II) were determined and compared, under similar conditions.

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MATERIALS & METHODS

Reagents

All reagents were of analytical grade and solutions were prepared with double distilled deionized water. All glassware was acid washed with concentrated HCl and then thoroughly rinsed with double distilled deionized water (DDW).

Tyrode (intestinal) buffer contained the following in 2L DDW: NaCl (16.0g); KCl (0.4g); $MgSO_4 \cdot 7H_2O$ (0.52g); $NaH_2PO_4 \cdot 2H_2O$ (0.13g); glucose (2.0g); $NaHCO_3$ (2.0g); $CaCl_2$ (0.4g) and NaN_3 (0.4g).

Neutral Detergent Fiber (NDF) solution was prepared as described by Goering and Van Soest (1982) and contained the following in 1L DDW: Disodium EDTA (18.61g), ($Na_2B_4O_7 \cdot 10H_2O$ (6.81g), Na_2HPO_4 (4.56g). The pH of the final solution was adjusted to pH 6.9–7.1 with H_3PO_4 if necessary.

α-Amylase solution for the NDF determinations was prepared by mixing 5g α-amylase powder (Sigma A6880) with 100mL buffer solution at pH 7.00 (61 mL 0.1M Na HPO_2 + 39 mL 0.1M NaH_2PO_4) for 15 min. The mixture was then centrifuged at 2,335 x *g* for 10 min and filtered through a coarse glass frit filter.

Hydroxylamine HCl a 10% solution of hydroxylamine HCl was prepared by dissolving 10g hydroxylamine HCl (Fisher Scientific Co., MA) in 100 mL 95% ethanol.

Bathophenanthroline disulfonic acid (BPDA) BPDA solution for ferrous iron determination was prepared by dissolving 100 mg BPDA in 500 mL tyrode buffer. The pH was adjusted to 5.00 with 12 and 0.1n HCl.

BPDA solution for ionic iron determination was prepared by dissolving 100mg BPDA in 50 mL of 10% hydroxylamine HCl solution and diluted to 500 mL with tyrode buffer. The pH was adjusted to pH 5.0 with 10 and 0.1N NaOH.

Fiber sources

Cellulose sigma cell (type 100); guar gum and sodium phytate (corn crystalline) were purchased from Sigma Chemical Co., St. Louis, MO. Lignin (Kraft pine lignin polymer, Indulin AT), was provided courtesy of Westvaco, Charleston Heights, SC. Commercial citrus pectin, molecular weight 150,000 – 300,000 daltons, was purchased from Nutritional Biochemical Corp, Cleveland, OH. This starting material had a high degree of esterification. It was purified by washing three times with 70% ethanol once with 95% ethanol, and twice with acetone and then air dried at room temperature.

Pectin with a low degree of esterification was obtained through the courtesy of Mr. William Strohsnitter and Dr. M. Atallah of the Dept. of Food Science & Nutrition, Univ. of Massachusetts, Amherst. It was prepared by the enzymatic deesterification of high methoxy pectin. High methoxy pectin (100g) was prepared as a 1% (wt/vol) solution; the pH was adjusted to 7.0±0.1 with NaOH and maintained throughout the deesterification reaction by adding NaOH. The volume of NaOH required to neutralize the free carboxyl ions produced in 100g pectin deesterified to 40% esterification, was calculated prior to preparation. A concentrated enzyme solution (pectin pectyl hydrolase, E.C. 3.1.1.11 Sigma Chemical Co., St. Louis, MO), containing enough

units of enzyme to complete the reaction within 2 h, was then added to the stirring mixture and the enzymatic reaction was allowed to proceed until the total calculated volume of NaOH was added to the mixture. At this point 2.5 volumes of 95% ethanol was added to the mixture to stop the reaction and precipitate the pectin. The pectin was filtered, washed three times with 70% ethanol, once with 95% ethanol, and twice with acetone, then air dried at room temperature. The methyl ester content of the two types of pectin was determined using the procedure described by Hinton (1940).

Neutral Detergent Fiber (NDF) was prepared from Soft White Wheat Bran (obtained from the AACC and held under refrigeration until use) using a modification of the method of Goering and Van Soest (1970). Wheat bran samples (10.00g) were refluxed for 60 min with 500 mL neutral detergent fiber solution in the presence of 2 mL decahydronaphthalene and 2.5g Na_2SO_3. The contents were then filtered through a course glass frit filter and the residue washed with boiling DDW (< 2L). The residue was digested with 100 mL α-amylase solution in 400 mL DDW at 55°C for 90 min, filtered as before, and washed 3 times with hot DDW and twice with acetone.

Procedure

Fiber systems were prepared either by heating pectin and guar gum until completely dissolved or by suspending lignin, cellulose, and NDF in Tyrode buffer (0.5g dry wt of each fiber source in approximately 75 mL Tyrode buffer). The pH of each system was adjusted to 5.0 with 12N and 0.1N HCl. Ferrous sulfate solutions (5 mL) were then added to the systems to produce a range of iron levels from 0–10mg. The volume of each system was made up to 100 mL with Tyrode buffer (pH 5.00±0.05). Duplicate samples (40 mL) were sealed in centrifuge tubes and equilibrated for 30 min in a shaking water bath at 37°C. An aliquot from each system was analyzed for total, soluble, ferrous, and ionic iron.

The above procedure was repeated for lignin using Zn ($ZnSO_4 \cdot 7H_2O$) and Cu ($CuSO_4 \cdot 5H_2O$), at molar levels equivalent to those used with iron. These systems were analyzed for total and soluble minerals.

Analysis of minerals

Atomic absorption standards, reagents, and apparatus used for the analysis of minerals have been described by Platt and Clydesdale (1985).

To determine endogenous minerals, fiber samples (1.00g) were digested with 20 mL concentrated HNO_3 for 15 min, in 100 mL digestion flasks. The samples were cooled, made up to volume with DDW in volumetric flasks and analyzed for minerals using appropriate AAS lamps and standards.

To determine total minerals a 10 ml aliquot of each sample was pipetted into 100 mL digestion flasks containing 20 mL HNO_3, heated for 15 min. cooled and made up to volume with DDW in volumetric flasks. The samples were then analyzed as above.

To determine soluble minerals a 40 mL aliquot of each appropriate sample was centrifuged at a relative centrifugal force of 2,335g for 10 min. Immediately after centrifugation, the supernatant was decanted from the insoluble fraction and filtered through ashless Whatman #41 filter paper. A 10 mL aliquot of the filtered supernatant was digested with 20 mL HNO_3 and analyzed for soluble mineral by AAS, as discussed previously.

Ionic and ferrous iron

Ionic and ferrous iron were determined using a modification of the method of Miller et al. (1981).

The bathophenanthroline disulfonic acid (BPDA) solutions were prepared in the same medium (Tyrode buffer) as the fiber suspensions to insure minimal destruction of the iron-fiber complexes on transfer.

One to 5 mL of the fiber systems were added to 25 mL volumetric flasks containing 10 mL of appropriate BPDA solutions. The flasks were brought to volume with Tyrode buffer (pH 5), shaken, filtered through Whatman #41 filter paper and the filtrate analyzed spectrophotometrically at 535nm, after 2 min, for iron.

The above procedure was repeated in the absence of BFDA to determine whether the fiber itself absorbed at 535 nm.

Scatchard analysis

From the measurements of total, endogenous, soluble mineral, and ionic and ferrous iron the following relationships were established to obtain data for Scatchard analysis.

Iron-fiber data

Added iron = Total iron — endogenous iron
Free iron (F) = Ferrious iron(Fe(II) — Endogenous Fe(II)
Bound iron (B) = Added iron — Fe(II) — Fe(III)

Fe(II), Zn(II), Cu(II)—lignin data

Added mineral = Total mineral — Endogenous mineral
Free mineral (F) = Soluble mineral
Bound mineral (B) = Added mineral — soluble mineral

Where: The total, soluble and endogenous mineral were determined by AAS; The ferrous iron was determined by BPDA; The ferric iron (Fe(III)) was determined by difference: total ionic iron — Fe(II).

The endogenous iron content of the fibers on a dry wt. basis was as follows: NDF (84 μg/g); cellulose (18 μg/g); guar gum (37 μg/g); High methoxy pectin (138 μg/g); Low methoxy pectin (70 μg/g); lignin (104 μg/g).

The endogenous mineral content of lignin on a dry wt basis was as follows: Fe (104 μg/g); Cu (<5 μg/g); Zn (15 μg/g).

The intrinsic binding constants and number of binding sites per g fiber were determined by means of Scatchard plots where B/F was plotted on the ordinate and B on the abscissa. The slope was a measure of the effective stability constant ($-F_{eff}$) and the point where the binding line crossed the abscissa was a measure of the amount of mineral bound by the respective binding sites.

Statistics

The curves of best fit for the data points in Fig. 2–10 were calculated using appropriate statistical programs (HP-67/97 Math Pac I, 1980).

RESULTS & DISCUSSION

Preliminary Experiments

In preliminary studies it was shown that the adjustment of pH of the various fiber systems, in the presence of Fe(II) from pH 2 (simulating gastric pH conditions) to pH 5 (simulating intestinal pH conditions)—with either sodium hydroxide or sodium bicarbonate—resulted in the formation of insoluble iron hydroxides, which could interfere with the estimates of iron bound to fiber. Therefore it was decided to forego the sequential treatment and add iron solutions to the fiber systems in Tyrode buffer at pH 5 and readjust any minimal pH changes with 0.1N NaOH to pH 5. Data were not obtained for fibers in the presence of Fe(III), since ferric sulfate rapidly formed a precipitate at pH 5 (Fig. 1).

The effectiveness of BPDA as a nondisruptive technique to measure free iron was assessed in the presence of an insoluble fiber (lignin). There was no significant difference between data from BPDA and AAS obtained for free iron in the presence of lignin (Fig. 2).

Effects of buffer, mineral concentration, and valence on binding

When Fe(II) was added to lignin, guar gum, NDF, high methoxy pectin, low methoxy pectin and cellulose in amounts

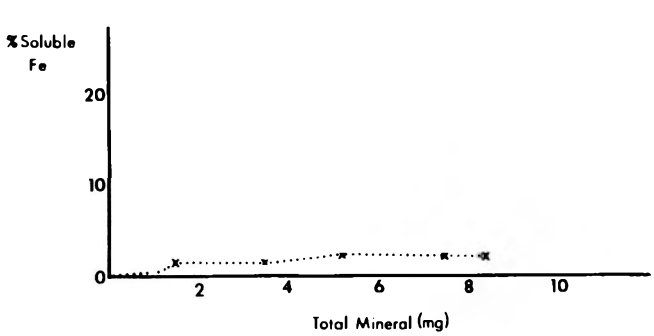

Fig. 1—Solubility of ferric sulfate in Tyrode buffer (pH 5). As determined by Atomic Absorption Sepctrophotometry (AAS).

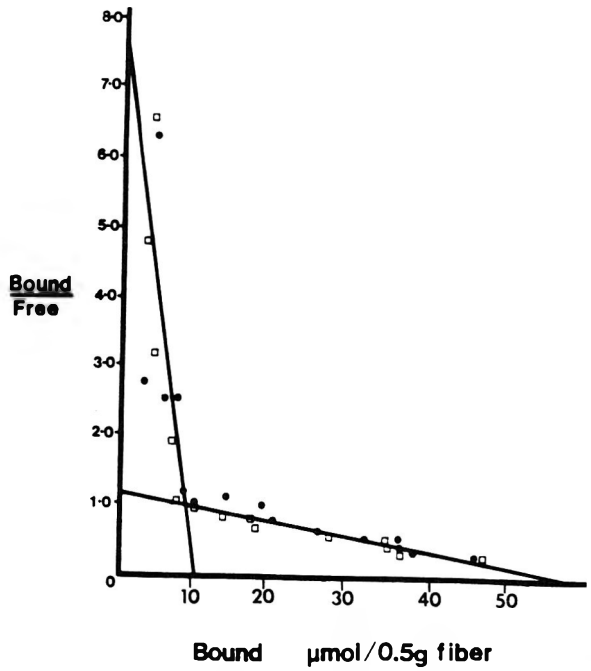

Fig. 2—Scatchard plot of the binding of Fe(II) by lignin under simulated duodenal (pH 5) conditions determined by Bathophenanthroline disulfonic acid (BPDA) (○———○) and by AAS (□———□).

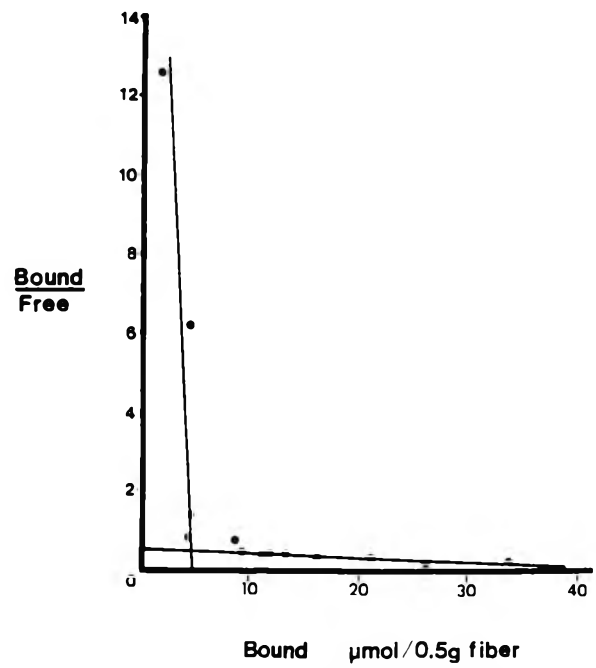

Fig. 3—Scatchard plot of the binding of Fe(II) by guar gum under simulated duodenal pH conditions (pH 5). As determined by BPDA.

ranging from 0-10 mg the resultant binding curves showed that the amount of iron bound to each fiber approached a plateau. These data were converted to Scatchard plots (Fig. 2–7).

The binding curves in Fig. 8 resulted from the addition of each of the minerals, Fe(II), Zn(II) and Cu(II) to lignin. Controls were performed to investigate the effects of Tyrode buffer alone (pH 5) on mineral solubility. Only copper formed an insoluble precipitate in the presence of buffer. Therefore, the lignin-Cu data were corrected for insoluble hydroxide formation, as shown in Fig. 8, prior to being incorporated in a Scatchard analysis. Plots for the Cu and Zn Scatchard data are shown in Fig. 9 and 10, respectively.

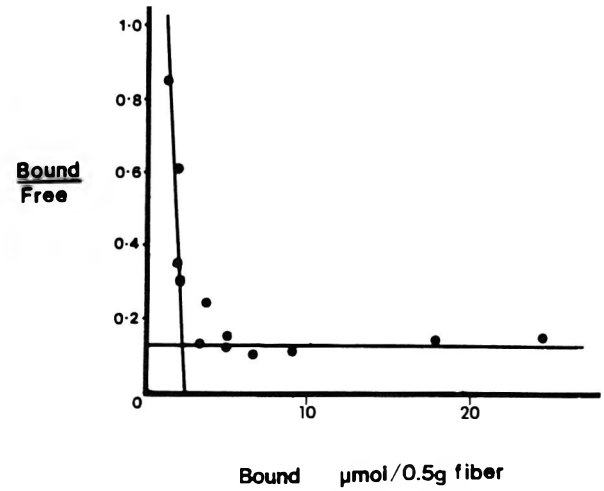

Fig. 4—Scatchard plot of the binding of Fe(II) by a high methoxy pectin, under simulated duodenal pH conditions (pH 5). As determined by BPDA.

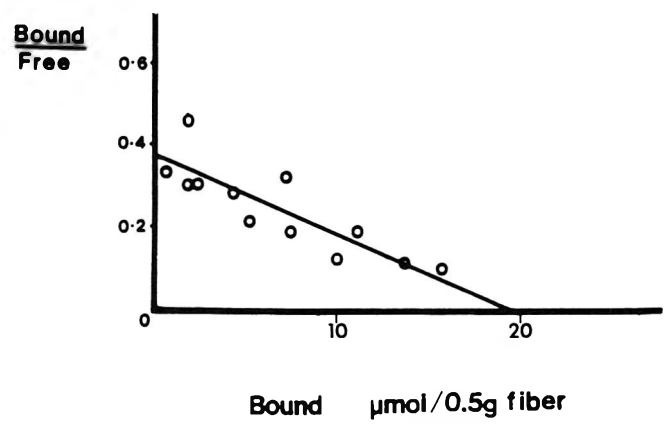

Fig. 5—Scatchard plot of the binding of Fe(II) by a low methoxy pectin under simulated duodenal pH conditions (pH 5). As determined by BPDA.

Binding characteristics of fiber components

The mineral-binding characteristics of the fiber components studied are summarized in Table 1. The binding is either specific or nonspecific. Nonspecific binding usually refers to the composite binding of ligand to all components possessing much lower affinity (at least 100× lower, preferably more) than the specific binder of interest (Chambers and McGuire, 1975). The effective stability constants (K_{eff} for each of the specific binding lines were determined according to Scatchard (1949). The amount of mineral bound by each group of binding sites was determined by calculating the point at which each binding line intercepted the abscissa.

Lignin had two specific binding sites for iron (Fig. 2) which supports previous findings by Fernandez and Phillips (1982a) and Platt and Clydesdale (1984). The two groups of binding sites bound 19.5 µmol and 118.9 µmol of Fe(II) per g fiber, respectively.

From the shape of the binding curve with guar gum and Fe(II) in Fig. 3, it is apparent that guar gum demonstrated both specific and nonspecific binding. The specific binding sites of guar gum bound ferrous iron more tightly than any of the other

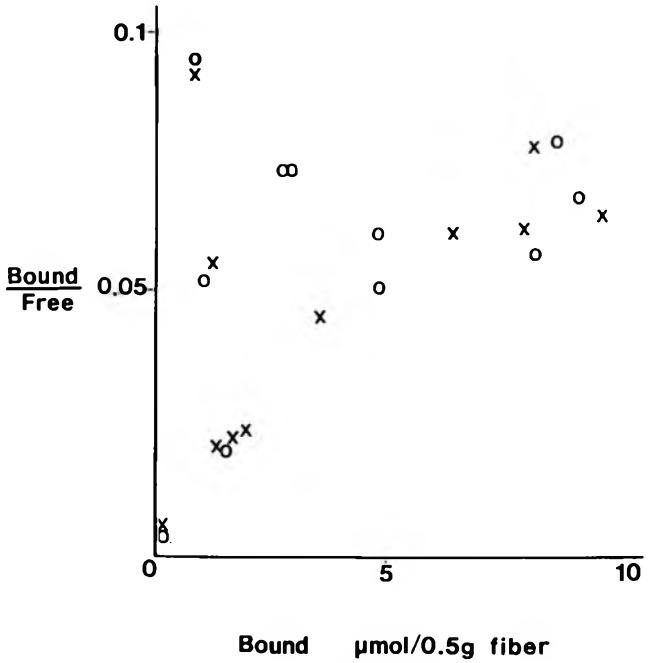

Fig. 6—Scatchard plot of the binding of Fe(II) by cellulose (○——○) and a control (x—x), under simulated duodenal pH conditions (pH 5). As determined by BPDA.

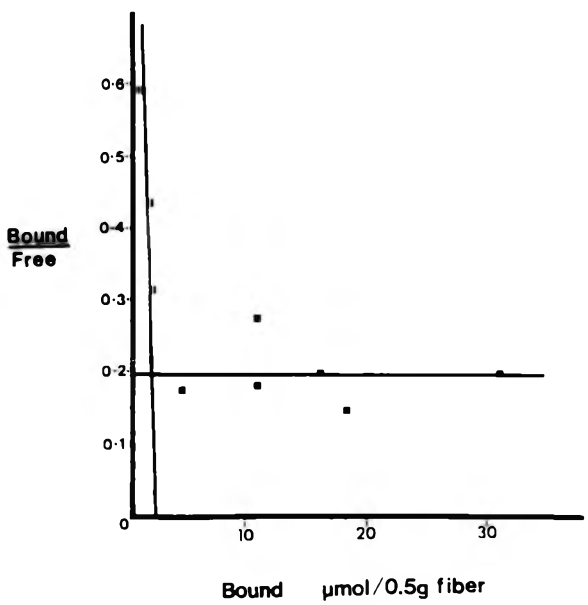

Fig. 7—Scatchard plot of the binding of Fe(II) by Neutral Detergent Fiber, under simulated duodenal ph conditions (pH 5). As determined by EPDA.

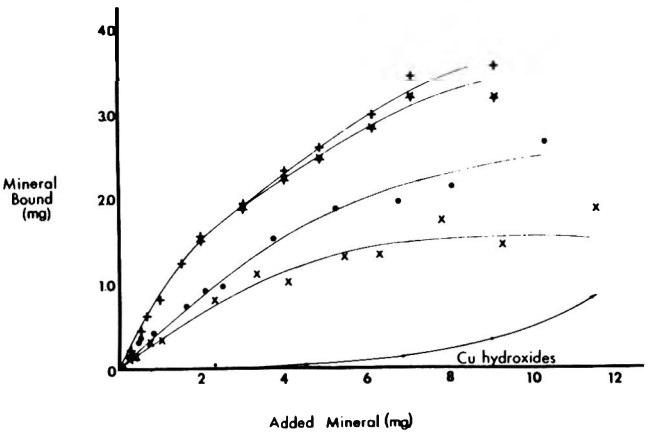

Fig. 8—Binding of each of the minerals, Fe(II) (○——○), Cu(II) (+——+), and Zn(II) (X——X), by lignin, under simulated duodenal pH (pH 5) conditions. The Cu(II) data was corrected for Cu hydroxide formation with buffer alone (★——★). AS determined by AAS.

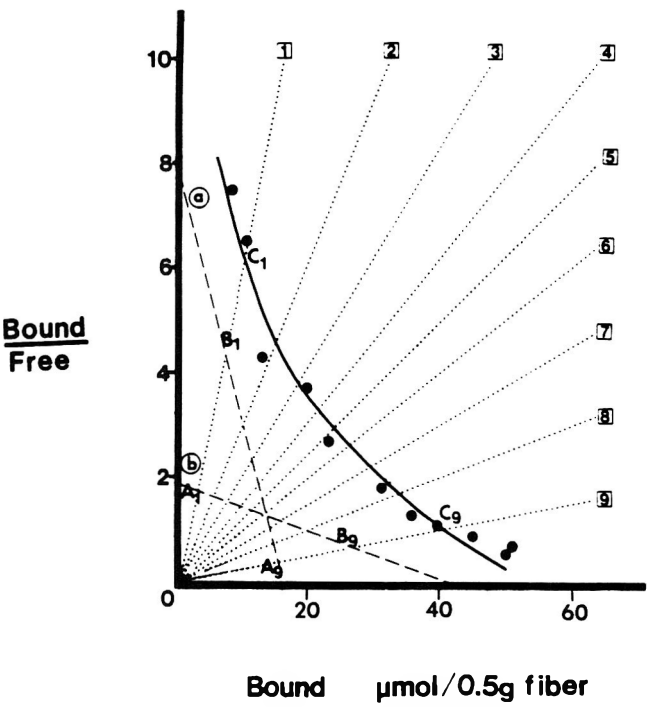

Fig. 9—Scatchard plot of the binding of Cu(II) by lignin, under simulated duodenal pH (pH 5) conditions. The solid line is the curve of best fit for the Scatchard data. The dotted lines 1-9 are the radial lines used to calculate the "binding lines" a and b using the method of Pennock (1973). A_1, B_1, C_1 and A_9, B_9, C_9 are the points on the radial lines which enable binding lines to be drawn to satisfy the equation $OA_1 = B_1C_1$.

fibers (as indicated by the values of the effective stability constants (K_{eff}) in Table 1). These in vitro results are consistent with other findings. Wobling et al. (1980) demonstrated that guar gum decreased iron absorption in tied-off jejunal segments of both normal and iron-deficient rats and decreased iron absorption, in vivo, in normal rats. Zemel and Zemel (1985) demonstrated that when guar gum (0.5%) was added to soy systems it significantly reduced iron solubility by 60.7% after undergoing a digestion with pepsin-HCl followed by the addition of bicarbonate, and digestion with pancreatin in the presence of bile extracts.

The fact that the high methoxy pectin had two types of binding curves (Fig 4) with both being different from the curve found with low methoxy pectin (Fig 5), may have resulted from differences in the physical structure of the two pectins (Rees, 1970, 1972; Atallah and Melnik, 1982). Pectins with a high degree of esterification form elastic gels with only moderate cross-linking between the pectin chains which allows water and solutes to distribute interstitially and on the surface of the gels. Pectins with a low degree of esterification form rigid gels and may exclude the mobility of solutes. Thus, differences in the iron-binding characteristics of the two pectins may be due to differences in their physical structure rather than differences in the amounts of available carboxyl groups.

As indicated in Fig. 6, there was no specific binding line

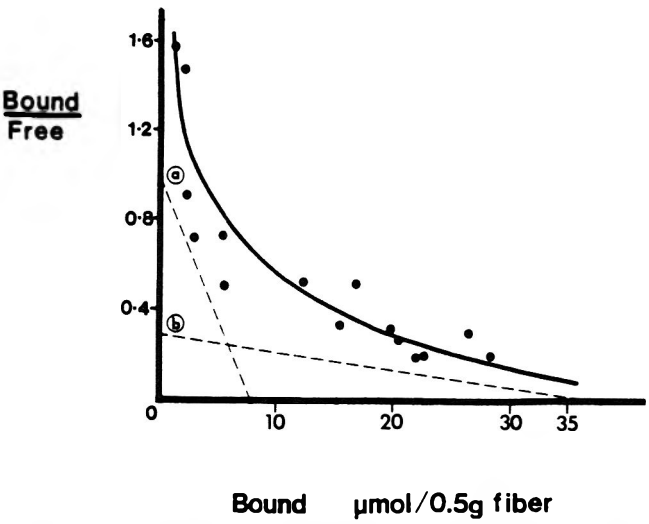

Fig. 10—Scatchard plot of the binding of Zn(II) by lignin, under simulated duodenal pH (pH 5) conditions. The "binding lines" a and b were calculated from the experimental curve using the method described by Pennock (1973).

for cellulose with Fe(II). Therefore, it was impossible to calculate an effective stability constant. The fact that cellulose did not strongly associate with Fe under the simulated duodenal pH conditions in this study support previous finding in which cellulose was shown to neither decrease iron absorption in vivo (Fernandez and Phillips, 1982b; Ranhotra et al., 1979) nor to form strong complexes with iron in vitro (Fernandez and Phillips, 1982a; Platt and Clydesdale, 1984).

Any iron other than free ionic iron in the Tyrode buffer (Fig 6), may have resulted from the formation of iron hydroxides.

NDF, prepared from Soft White Wheat Bran, had both specific and nonspecific binding groups (Fig. 7). The high affinity binding sites of NDF bound Fe(II) about 0.5x as strongly as the high affinity sites of lignin and bound 0.28mg Fe(II) per g NDF under these conditions. Reinhold et al., (1981), using Scatchard analysis, demonstrated that NDF prepared from wheat firmly bound 0.38mg iron (II) per g NDF at pH 6.45. The fact that Reinhold et al., (1981) found more iron bound at pH 6.45 than this study did at pH 5.00 is consistent with other reports that the amount of iron bound to NDF increased above pH 5.00 (Lee and Garcia-Lopez, 1985).

Lee and Garcia-Lopez (1985), based on Scatchard analysis, have suggested that NDF (prepared from cooked pinto beans) had only one type of binding site for Fe(II), one for Cu(II) and two for Zn(II). However, these conclusions were made from a limited number of points (Klotz, 1982).

Since lignin was shown to bind appreciable amounts of iron in this study and in previous in vitro studies (Camire and Clydesdale, 1981; Fernandez and Phillips, 1982a), it was chosen as a model fiber component to compare the binding of each of the transition metals Fe(II), Zn(II) and Cu(II) under duodenal pH conditions. The initial binding data are shown in Fig. 8. Scatchard diagrams (Fig. 2, 9 and 10) constructed on the basis of these data suggest that lignin has more than one type of binding site for these minerals. Rosenthal (1967) has presented a method for the determination of binding parameters from such data. If the solid line plot in Fig. 9 represents two non-interactive binding sites for Cu with lignin, the binding parameters for these sites can be calculated from two binding lines (a and b) which satisfy the relationship:

$$OA_j = B_jC_j \qquad (1)$$

where j = 1 to 9 for all the radial lines in Fig. 9.

The binding lines (a and b) in Fig. 8 and 9 were determined by a trial and error procedure, using a "simple calculator" described by Pennock (1973). Using this method, the slopes and intercepts were adjusted until the relation in Eq. (1) was satisfied for all the lines through the experimental curve. Approximate binding lines were first defined by a simple overlay and then more accurate determinations of the binding lines were made using dividers to satisfy the conditions in Eq. (1).

The data generated for lignin-binding with Cu, Zn, and Fe in Table 1 show that there are two binding sites for each of the transition elements. A high affinity group and a low affinity group. The high affinity binding sites of lignin bound Fe approximately 1.6x more strongly than copper, and 6.3x more strongly than zinc. However, the high affinity groups bound more copper than either the sites for Fe or Zn. The low affinity groups of lignin bound Cu approximately 2.2x more strongly than Fe and 5.8x more strongly than Zn.

SUMMARY & CONCLUSIONS

THE RESULTS of this study and the work of Fernandez and Phillips (1982a) do not support the conclusions of Reinhold et al. (1981) that the iron bound by acid detergent fiber (mainly cellulose and lignin) is largely accounted for by cellulose. The present study indicates that the binding by cellulose was nonspecific, and therefore weak, whereas there was substantial binding by the two specific binding sites of lignin.

The mechanism by which these fibers bind iron was not explored in this research. However, it may be that the fibers that form strong complexes with transition metals do so by forming multidentate complexes. For example, the hydroxyl and methoxy groups, characteristic of lignin, are similar to catechol which react with iron to form hexadentate complexes of high stability (Fernandez and Phillips, 1982a; Fieser and Fieser, 1965).

Lignin, but not cellulose and low methoxy pectin, is a potent binder of ferrous iron under pH conditions approximating those

Table 1—A summary of the binding characteristics of the fibers obtained from the mineral fiber data in Fig. 2–7, 9, and 10.

Fiber-mineral system	Characteristics of binding sites	K_{eff}	Bound mineral (μmol per g fiber)
Control + Fe(II)	Nonspecific	—	—
Cellulose + Fe(II)	Nonspecific	—	—
Guar Gum + Fe(II)	(a) Specific	6.27×10^6	8.4
	(b) Nonspecific	—	—
NDF + Fe(II)	(a) Specific	3.43×10^5	5.0
	(b) Nonspecific	—	—
High methoxy pectin + Fe(II)	(a) Specific	5.90×10^5	5.0
	(b) Nonspecified	—	—
Low methoxy pectin + Fe(II)	Specific	1.84×10^4	39.9
Lignin + Fe(II)	(a) Specific	$7.68 + 10^5$	19.5
	(b) Specific	2.07×10^4	118.9
Lignin + Cu(II)	(a) Specific	4.67×10^5	32.3
	(b) Specific	4.58×10^4	81.6
Lignin + Zn(II)	(a) Specific	1.21×10^5	15.4
	(b) Specific	7.84×10^3	72.2

in the proximal intestine. These results support the observations of Fernandez and Phillips (1982a). However, they postulated a common binding site in cellulose, psyllium mucilage and lignin which this study was unable to demonstrate in cellulose and lignin and in the other fibers investigated. This is not surprising in view of the lack of similarity in structure amongst the fibers studied.

The values for the effective stability constants for the binding sites of the fibers investigated agree with the majority of in vivo and in vitro findings in the literature which have shown that lignin (Fernandez and Phillips, 1982b; Gillooly et al., 1984) and guar gum (Wobling et al., 1980; Zemel and Zemel, 1985) inhibit iron absorption. Pectin decreased iron absorption in some studies (Fernandez and Phillips, 1982b; Monnier et al., 1980) but not in others (Fernandez and Phillips, 1982b; Gillooly et al., 1984; Mansoor Baig et al., 1983; Lei et al., 1980; Cook et al. 1983) and cellulose had little or no effect on iron absorption (Fernandez and Phillips, 1982b; Gillooly et al., 1984; Monnier et al., 1980; Buth and Mehta, 1983).

Lignin had two binding sites with Fe, Zn, and Cu with the high affinity sites binding these transition metals in the following order: Fe > Cu > Zn, but with twice as much Cu bound as either Fe or Zn.

This study illustrates the need to consider the effects of mineral-mineral competition on the thermodynamics of binding in mixed diets because such reactions might ultimately have an effect on bioavailability.

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Received 9/4/86; revised 4/8/87; accepted 4/17/87.

Paper No. 2842. Massachusetts Agricultural Experiment Station, Univ. of Massachusetts at Amherst. This research was supported in part from Experiment Station Project No. NE116.

The authors thank Dr. M. Atallah and Mr. W. Strohsnitter for their help and advice in the preparation of pectin samples.

Effect of the Source of Fiber in Bread-Based Diets on Blood and Liver Lipids in Rats

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ABSTRACT

Fiber in 12 types of breads was tested for effect on blood and liver lipid levels in normolipidemic young rats. Breads provided the same, 4.1%, level of total, but variable levels of soluble, fiber in the diets. Over one-fifth of the total dietary fiber in corn tortillas, oatmeal bread, white bread and two multigrain breads containing vegetable powder was soluble fiber. Diet based on white bread was the only 100% bread diet; sucrose was added to the other 11 diets to equalize the content of available carbohydrates. Results showed that soluble fiber was negatively correlated with serum cholesterol (r, −0.38) and serum triglycerides (r, −0.36) but positively correlated with liver cholesterol (r, 0.53); the reverse was true for three other experimental variables, namely sucrose and casein added to the diets and weight gains of the animals. This latter observation apparently negated, to some extent, the hypolipidemic effect of certain bread types tested.

INTRODUCTION

THE ABILITY of various natural and purified fiber sources to lower elevated blood lipid levels, a recognized risk factor in atherosclerosis, has been studied extensively. While water-soluble fiber fractions appear to exert a hypocholesterolemic effect in man and animals (Jenkins et al., 1979; Kirby et al., 1981; Chen et al., 1981; Bock and Ranhotra, 1984; Kritchevsky, 1985), insoluble fiber fractions appear not to show this effect (Ranhotra, 1973; Truswell and Kay, 1975; Connell et al., 1975; Kritchevsky, 1985; Cadden, 1986).

With the exception of oats, water-insoluble fiber fractions are the predominant fractions in cereal grains including wheat. Wheat is the major component of several high-fiber and other types of white and variety breads. These breads often also contain flours and fractions from other cereal and noncereal grains such as rye, barley, oats and soy. Some products also contain purified cellulose, cellulose derivatives and dehydrated vegetables. Vital wheat gluten is often used to enable the inclusion of these various ingredients in bread.

Because the ratio of insoluble versus soluble fiber fractions in nonwheat ingredients used in breads often differ, these bread products may have variable effects on blood (and liver) cholesterol and triglyceride levels even when the intake of total fiber from various bread products is maintained constant. Using rats as the test model, this possibility was examined with 12 different types of breads widely consumed in the United States.

MATERIALS & METHODS

Test breads

The 12 bread types tested are listed in Table 1. All were purchased locally but represent the national brands. These products were air dried, finely ground and then used to formulate test diets (Table 2).

Test diets

Diets were formulated to be equal in content of total dietary fiber, protein, fat and moisture (Table 2); they also contained about the same level of available carbohydrates. Diets were complete in all micronutrients required by the rat (NRC, 1978).

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Animals

Male weanling rats (6 rats/diet) of the Sprague-Dawley Strain (Harlan Sprague-Dawley, Indianapolis, IN) were housed individually in mesh bottom stainless steel cages in a controlled environment. Each rat was fed the same amount of total diet which was adequate and gradually increased during the test period. Rats were weighed at weekly intervals. At the end of the 3-wk test period, all animals were fasted overnight, anesthetized lightly and blood was drawn by heart puncture. The clotted blood was centrifuged to obtain serum which was then frozen. Livers were also removed, blotted dry, weighed and homogenized in a Fisher Dyna-mix homogenizer. The volumes of the homogenized livers were then recorded and suitable aliquots were saved for cholesterol analysis.

Analytical

Finely ground breads (and casein) were analyzed for moisture, protein (Kjeldahl), fat (acid hydrolysis), and ash using the standard AACC (1977) methods. Total dietary fiber (TDF) was determined by the recently approved method of Prosky et al. (1985); the incorporation of additional steps (filtration and precipitation) in this method allowed the determination of soluble and insoluble fiber components (Table 1). Serum total cholesterol (CH) was determined by the method of Kim and Goldberg (1969). Liver CH was determined by the method of Abell et al. (1952). Serum triglycerides (TG) were determined by a colorimetric procedure using a kit obtained from Sigma Chemical Co. The data were subjected to Duncan's (1955) multiple range test and correlation analysis (Snedecor and Cochran, 1980).

RESULTS AND DISCUSSION

TWELVE DIFFERENT TYPES of breads were tested. Three types (whole wheat bread, corn tortillas and German rye bread) represent three different grains used almost in totality. Four other types were mixed grain/multigrain products with two of these also containing powdered vegetables and, thus, were likely high, along with oatmeal bread, in soluble fiber. Three other bread types contained purified (cellulose) or natural (wheat

Table 1—Composition of test breads (as-purchased basis)

	Composition[a] (%)				
Bread	Moisture	Protein (N × 5.7)	Fat	Ash	Total fiber[b]
A White	36.7	7.8	2.8	1.8	3.0 (34.1)
B Cellulose containing	41.5	8.2	3.4	2.1	9.9 (11.4)
C Oatmeal	36.3	9.1	5.1	2.3	3.7 (32.6)
D Tortillas, corn	43.8	5.0	3.7	1.4	4.3 (21.7)
E Rye, German	36.8	10.2	3.8	2.2	8.3 (17.9)
F Cracked wheat	35.2	10.0	3.6	2.1	6.7 (14.1)
G Bran containing	39.1	9.2	3.6	2.1	5.4 (15.1)
H Whole wheat, 100%	39.7	10.5	5.3	2.1	8.1 (13.6)
I Mixed grain	40.3	9.9	3.3	1.9	5.6 (10.7)
J Multigrain	39.5	10.6	3.5	2.4	9.6 (13.1)
K Multigrain + DPV-I[c]	38.3	7.4	3.1	4.6	3.4 (40.6)
L Multigrain + DPV-II[c]	38.8	9.0	3.4	4.5	4.9 (24.4)

[a] Available carbohydrates values (not listed) equal the sum of components listed subtracted from 100.

[b] Total dietary fiber. Values within parenthesis are soluble fiber contents expressed as percent of total fiber.

[c] Dried powdered vegetables (DPV). DPV-I is a light and DPV-II is a dark product. Vegetables used included: carrot, pumpkin, lettuce, artichoke, celery, cabbage, parsley, cauliflower and kelp.

bran or cracked wheat) fiber sources. Only white bread was a refined product.

Fiber in breads

On an as-purchased basis, TDF in test breads ranged from 3.0 to 9.9% and soluble fiber, as percent of TDF, from 10.7 to 40.6% (Table 1). In addition to oatmeal and two multigrain breads (breads K and L), white bread and corn tortillas also contained a sizable portion of TDF as soluble fiber. For white bread, this may reflect the compositional makeup of fiber fractions in the wheat endosperm and/or the contribution of resistant starch formed during baking. As much as 30% of the TDF in white bread is now considered as resistant starch (Berry, 1986).

Fiber in diets

White bread was lowest in the content of TDF. At the maximum possible use level, it permitted only 4.1% TDF in the diet with a protein content of 11.8%. Other breads were used at variable levels to keep the TDF content of all diets constant at 4.1%. Casein and sucrose were used in the diets to equalize the contents of protein and available carbohdyrates, respectively. Since these two diet components induce hyperlipidemic effect, the extent of this effect was assessed in relationships to parameters measured (Tables 3 and 4).

Growth response of rats

All diets were not only isoproteinous but, as calculations show, also isocaloric. For this reason and because the animals were fed the same amount of total diet, the intake of usable energy between groups was identical. The growth response of animals, however, differed ($P<0.01$) between groups (Table 3). This occurred primarily because the amount of casein (Table 2), and thus the quality of protein, in diets varied.

Table 2—Composition of Test Diets[a]

	Components (g 100g)					
Bread[b]	Bread	Casein	Corn oil	Water	Sucrose	Others[c]
A	93.82	–	1.84	–	0.14	4.2
B	26.28	9.45	4.22	4.86	50.99	4.2
C	74.95	1.02	–	2.08	17.75	4.2
D	58.07	7.64	2.13	2.11	25.85	4.2
E	33.55	7.27	3.76	4.69	46.53	4.2
F	42.31	5.96	3.43	4.09	40.01	4.2
G	49.70	4.87	2.88	3.52	34.83	4.2
H	32.70	6.98	2.97	4.73	48.42	4.2
I	46.64	4.58	3.20	3.82	37.56	4.2
J	27.76	8.00	4.12	5.03	50.89	4.2
K	81.35	2.25	1.87	0.61	9.72	4.2
L	55.18	4.22	2.81	2.61	30.98	4.2

[a] Diets made with finely ground breads. All diets contained 11.8% protein (N × 6.25), 5.6% fat, 7.0% moisture and 4.1% total dietary fiber (TDF).
[b] See Table 1 for description.
[c] Include vitamin mix (AIN mix 76 from U.S. Biochemicals, Cleveland, OH), 1; and mineral mix (in sucrose base), 3.2. Mineral mix contained (mg): Ca, 500; P, 400; Fe, 3.5; Cu, 0.5; I, 0.015; Mg, 40; Mn, 5; Se, 0.01; and Zn, 1.2.

Serum cholesterol

Several dietary factors, including cholesterol, fat, protein and fiber affect blood CH levels. In these studies, diets contained no CH, and the same level of fat and protein. They also had the same level of available carbohydrates, except that the ratio of starch (in bread) and sucrose (added) differed, and the same level of fiber, except that the source (bread) of fiber differed.

At the end of a 3-wk test period, serum CH levels in rats averaged between 119 and 178 mg/dL (Table 3). Cholesterol levels were the lowest on diets made with oatmeal bread (diet C), tortillas (diet D) and white bread (diet A). These breads were all high in soluble fiber which likely exerted a hypocholesterolemic effect (Kirby et al., 1981; Chen et al., 1981; Kritchevsky, 1985) through mechanisms not well understood but likely involving increased fecal excretion of bile acids (Kirby et al., 1981; Kritchevsky, 1985). Hypocholesterolemic effects of two multigrain breads which were also high in soluble fiber was either not seen (diet L) or was only modest (diet K). Apparently other interacting factors, as correlations in Table 4 show, negated the hypocholesterolemic effect of soluble fiber in these two diets. Serum CH levels observed on other test breads suggest that diets high in insoluble fiber would not induce, in confirmation of other studies (Ranhotra et al., 1973; Connell et al., 1975; Kritchevsky, 1985; Cadden, 1986), a hypocholesterolemic effect.

Besides soluble fiber, sucrose and casein in the diet and weight gain of the animals were the other three experimental variables which likely also influenced the serum CH levels. While soluble fiber influenced CH levels negatively (r, −0.38), the reverse was true for sucrose, casein and weight gains (Table 4). Sucrose and weight gains were not an influencing factor in response obtained on white bread. As such, the hypocholesterolemic effect of white bread was likely due to soluble fiber and the absence of casein in the diet.

Serum triglycerides

In addition to CH, elevated serum TG levels have also been implicated as metabolic contributors to atherosclerosis (Kritchevsky, 1985). Although serum TG levels on all diets were in the normal range (Bock and Ranhotra, 1984), they averaged the lowest, below 60 mg/dL, on diets made with white bread and a multigrain bread containing vegetable powder (Table 3). These two diets (Diet A and K) also contained the highest

Table 3—Tissue lipids in rats fed bread-based diets[a,b]

			Serum		
Diet[c]	Body wt gain (g)	Liver wt (g)	Cholesterol (mg/dl)	Triglycerides (mg/dl)	Liver cholesterol (mg/g)
A	38 ± 2	2.3 ± 0.3	123 ± 7^{e,f}	57 ± 19^{c}	5.5 ± 0.9^{b,c}
B	55 ± 2	2.7 ± 0.2	143 ± 11^{c,d}	77 ± 10^{a,b}	5.5 ± 0.6^{b,c}
C	37 ± 1	1.7 ± 0.2	119 ± 18^{d,e,f}	69 ± 17^{a,b,c}	6.0 ± 0.8^{a}
D	54 ± 2	2.4 ± 0.2	122 ± 10^{f}	86 ± 8^{a}	5.1 ± 0.5^{c,d}
E	56 ± 2	2.5 ± 0.2	142 ± 14^{c,d}	87 ± 22^{a}	4.4 ± 0.5^{d}
F	54 ± 3	2.1 ± 0.1	153 ± 9^{b,c,d}	72 ± 15^{a,b,c}	4.6 ± 0.6^{c,d}
G	52 ± 2	2.3 ± 0.1	146 ± 16^{c,d}	72 ± 16^{a,b,c}	5.1 ± 0.6^{c,d}
H	56 ± 2	2.9 ± 0.2	151 ± 15^{b}	79 ± 9 ^{a,b}	4.6 ± 0.9^{c,d}
I	51 + 2	2.6 ± 0.1	165 ± 18^{a,b}	73 ± 6^{a,b,c}	4.2 ± 0.3^{d}
J	57 ± 1	2.3 ± 0.2	154 ± 13^{b}	64 ± 7^{b,c}	4.6 ± 0.6^{c,d}
K	40 ± 1	2.3 ± 0.2	140 ± 11^{c,d,e}	58 ± 11^{c}	6.1 ± 1.2^{a,b}
L	47 ± 2	2.7 ± 0.2	178 ± 14^{a}	65 ± 12^{b,c}	4.9 ± 0.4^{c,d}

[a] Values are averages (6 rats diet) ± SD. Averages with same superscripts in each column are not significantly different ($P>0.05$) by Duncan's Multiple Range test.
[b] Each group of rats was fed a total of 159g diet rat which contained 6.5g of TDF.
[c] See Tables 1 and 2 for description of breads and diets.

Table 4—Correlation coefficients among experimental variables and parameters measured[a]

Variables	Parameters measured		
	Serum cholesterol	Serum triglycerides	Liver cholesterol
Soluble fiber in diet	−0.38	−0.36	0.53
Sucrose in diet	0.41	0.35	−0.45
Casein in diet	0.29	0.69	−0.53
Wt gain of animals	0.30	0.35	−0.49

[a] Correlation (r) values are based on observations on individual rats.

level of soluble fiber (Tables 1 and 2), which is negatively correlated with serum TG (Table 4), and they were low in sucrose. Sucrose, in contrast to starch, is reported to be more lipidemic (Little et al., 1979) as the correlation coefficient listed in Table 4 seems to suggest. Because the weight gain (negatively correlated with serum TG) of animals fed diets A and K was low, the hypotriglyceridemic effect of soluble fiber in diet A and K was perhaps little negated by sucrose or weight gain; the absence (diet A) or low level (diet K) of dietary casein may have also contributed to the hypotriglyceridemic effect observed on diets A and K.

Liver cholesterol

In several species of animals, soluble fiber sources have been shown to lower serum as well as liver CH levels (Kritchevsky, 1985). While breads high in soluble fiber appear to exert a hypocholesterolemic effect in these studies, an accompanying liver CH-lowering effect was not seen (Table 3). Liver CH levels, averaging between 4.2 and 6.1 mg/g, were, in fact, negatively correlated (r, −0.61) with serum CH levels; such may not be the case in studies where animals are made sufficiently hypercholesterolemic initially. In a study reviewed by Kritchevsky et al. (1984), liver CH levels in rats fed oatbran were higher as compared to rats fed wheat bran or cellulose. The negative correlation between serum and liver CH may be the consequence of excess CH in blood being transported to the liver for excretion from the body. This transport usually involves high-density-lipoprotein CH (not measured) which is reported to be elevated when soluble fiber is fed to the animals (Chen et al., 1981). Alternatively, the synthesis of hepatic CH may have been affected by fiber (Qureshi et al., 1980) evolving into a correlation as observed. Liver TG was not measured but may have followed a pattern similar to CH.

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Ms received 2/10/87; revised 3/27/87; accepted 3/27/87.

Presented at the 47th Annual Meeting of the Institute of Food Technologists, Las Vegas, NV, June 16-19, 1987.

Studies were supported by AIB's Capital Fund for basic research and, in part, by a fellowship awarded to Paul Bright by the International Wheat Gluten Association.

Bioavailability of Copper Bound to Dietary Fiber in Mice and Rats

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ABSTRACT

The bioavailability of copper (Cu) was compared in mice or rats fed diets containing wheat bran-bound Cu and adequate Cu (unbound) or deficient Cu with cellulose or wheat bran. Cardiac and hepatic Cu content were comparable in mice fed bran-bound or adequate Cu and greater than mice fed deficient Cu. Cardiac Cu content was comparable in rats fed bran-bound Cu and adequate Cu and greater than rats fed deficient Cu. Hepatic Cu content, however, was less in rats fed bran-bound Cu than adequate Cu and greater in both than deficient Cu. Both rats and mice utilized dietary Cu bound to wheat bran, suggesting that mineral-fiber interactions may not decrease bioavailability when dietary mineral is adequate. Tissue Cu content in Cu-deficiency was lower in animals fed wheat bran compared to cellulose, suggesting that the type of fiber may exacerbate effects of mineral deficiency.

INTRODUCTION

INTEREST IN DIETARY FIBER has rekindled after epidemiological findings suggested that many of Western man's diseases could be prevented by increasing dietary fiber (Burkitt et al., 1974; Kelsay, 1978; Toma and Curtis, 1986). According to the Institute of Food Technologists' Expert Panel (IFT, 1979), dietary fiber relieves constipation, "probably" prevents diverticular disease, and "possibly" reduces serum cholesterol and other Western-risk diseases. This information coupled with the recommendation by the U.S. Dept. of Health & Human Services, USDA, and many health professions (Anonymous, 1985c) to increase complex carbohydrates and dietary fiber has increased the popularity of dietary fiber among the health conscience public. After reviewing the physiological actions of dietary fibers, Kelsay (1978) and Eastwood and Kay (1979), however, contend that the relative etiological importance of fiber in Western diseases is still unclear.

The known physiological responses elicited by components in dietary fiber (Sosulski and Cadden, 1982; Schneeman, 1986) are based on four physical properties — bacterial degradation, water-holding capacity, adsorption of organic molecules, and cation exchange capacity. This latter property may decrease the bioavailability of certain nutrients like minerals because dietary fiber sources such as wheat brans can interact with minerals in human diets (Dintzis et al., 1985) forming relatively insoluble complexes (Eastwood, 1984). The clinical significance of mineral-fiber interactions remains controversial (Dintzis et al., 1985; Toma and Curtis, 1986). Decreased availability is most likely if mineral intake is marginal and the fiber intake is high (Ali et al., 1981; Kelsay and Prather, 1983). Different fibers, however, vary in both their ability to bind minerals (Thompson and Weber, 1979, 1982; Rendleman and Grobe, 1982; Rendleman, 1982; Clydesdale and Camire, 1983) and their metabolic consequence (Lei, 1977; Reinhold et al., 1975; Ismail-Beigi et al., 1977; McHale et al., 1979; Lei et al., 1980; Cadden et al., 1983; Festa, 1985; Turland et al., 1985).

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One mineral that may be adversely affected by interactions with fiber is copper, an essential trace mineral required for humans at an estimated dietary intake of 2–3 mg/day (Food & Nutrition Board, 1980). U.S. diets are reported to provide less than 2 mg of copper daily, indicating that mild dietary inadequacy may occur frequently (Klevay et al., 1980; Sandstead, 1982; Anonymous, 1985a). Copper bioavailability, that dietary copper absorbed and utilized, may be decreased not only by marginal copper intake but also by other dietary factors that can interact with copper (Anonymous, 1986). Excess zinc, molybdenum, cadmium, protein, fructose, and fiber can decrease the bioavailability of copper (Sandstead, 1982; Anonymous, 1985b, 1986; Jiang, 1986).

Few studies, however, have investigated the effects of dietary fiber on copper bioavailability. The purpose of this study was to determine the bioavailability of copper bound to wheat bran compared to unbound copper ($CuSO_4$). This bioavailability was determined in both mice and rats to compare the species specificity of the effects of dietary fiber. Two fiber sources, cellulose and wheat bran, were used in this study because cellulose has little affinity for for copper (Tsai and Lei, 1979; Thompson and Weber, 1982; Jiang, 1986) while wheat bran has a high ion exchange capacity and readily binds copper (Thompson and Weber, 1982; Moorman et al., 1983). Bioavailability was assessed by copper status as indicated by body weights, tissue weights, food consumption and tissue contents of copper. This assessment evaluates the two components of bioavailability, absorption and utilization, together not separately.

MATERIALS & METHODS

Preparation of wheat bran and bran-bound Cu

Red Spring wheat bran was defatted overnight in a Soxhlet apparatus using chloroform-methanol (2:1), air-dried and ground using a hammer mill (screen mesh size number 040). The ground bran was passed serially through U.S. standard screens number 20 to 100. Bran retained on number 100 screen was used for the diets. Endogenous minerals were removed from defatted bran by washing with hydrochloric acid (1.0%) followed by washing with distilled-deionized water. Endogenous minerals were minimal in cellulose so acid extraction was omitted (data not shown). To bind Cu to the bran, a slurry of acid-washed wheat bran in excess of 100g and Cu (Fisher Certified Atomic Absorption Reference Solution) in excess of 5000 μg for mouse diets and 20,000 μg for rat diets were stirred overnight in an acid-washed plastic bottle using a temperature controlled shaker bath. The slurry was poured into an acid-washed porcelain filter funnel using a nylon filter cloth, washed repeatedly with distilled-deionized water and air-dried. Wheat bran bound 52.5 (for mouse diets) and 259 (for rat diets) μg Cu/g bran as analyzed by atomic absorption spectroscopy.

Dietary composition

Mice and rats were fed purified diets varying in the fiber source (wheat bran or cellulose) and the content and form of Cu. Diets contained cellulose with and without $CuSO_4$ acid-washed wheat bran with and without $CuSO_4$ or wheat bran with bound Cu. The composition of the diets shown in Table 1 (mice) and Table 2 (rats) met the nutritional requirements for each species, respectively, except for the diets without $CuSO_4$. As analyzed by atomic absorption spectroscopy,

Table 1—Composition of mouse diets[a]

Ingredients[b]	Cellulose diet		Wheat bran diet		Bran bound diet
	+Cu	−Cu	+Cu	−Cu	Cu
	(% weight)				
Skim milk (34.9% protein)	40.1	40.1	40.1	40.1	40.1
Cerelose	48.4	48.4	48.4	48.4	48.4
BHT	tr	tr	tr	tr	tr
Corn oil	3.0	3.0	3.0	3.0	3.0
AIN vitamin mix[c]	1.0	1.0	1.0	1.0	1.0
Mineral mix[d]	4.0	4.0	4.0	4.0	4.0
Cr_2O_3[e]	0.2	0.2	0.2	0.2	0.2
Choline chloride	0.2	0.2	0.2	0.2	0.2
Cellulose	3.0	3.0	0	0	0
Wheat bran[f]	0	0	3.0	3.0	0
Bran-bound Cu[g]	0	0	0	0	3.00
Copper sulfate	0.0008	0	0.0008	0	0

[a] Kcal g = 3.82.
[b] All ingredients from United States Biochemical Co., Cleveland, OH.
[c] AIN. (1977).
[d] Mineral mix (components as % of the mix): $CaHPO_4(2H_2O)$—70.85; NaCl—9.53; K_2CO_3—12.37; $MgSO_4(7H_2O)$—4.11; $FeSO_4(H_2O)$—0.64; $MnSO_4(H_2O)$—0.31; $ZnSO_4(7H_2O)$—0.13; $CoCl_2(6H_2O)$—0.10; NaF—0.02; KI—0.03; Na_2O_4Se—0.0006; $CrCl_3$—0.038; Cerelose (as filler), 1.92.
[e] Marker
[f] Acid-washed Red Spring Wheat Bran.
[g] 52.5 μg Cu g acid-washed wheat bran, provides 2 μg g diet.

Table 2—Composition of rats diets[a]

Ingredients[2]	Cellulose diet		Wheat bran diet		Bran bound diet
	+Cu	−Cu	+Cu	−Cu	Cu
	(% weight)				
Skim milk (34.9% protein)	34.4	34.4	34.4	34.4	34.4
Cerelose	54.1	54.1	54.1	54.1	54.1
Corn oil	3.0	3.0	3.0	3.0	3.0
BHT	tr	tr	tr	tr	tr
AIN vitamin mix[c]	1.0	1.0	1.0	1.0	1.0
Mineral mix[d]	4.0	4.0	4.0	4.0	4.0
Cr_2O_3[e]	0.2	0.2	0.2	0.2	0.2
Choline Chloride	0.2	0.2	0.2	0.2	0.2
Cellulose	3.0	3.0	0	0	0
Wheat bran[f]	0	0	3.0	3.0	0
Bran-bound Cu[g]	0	0	0	0	3.0
Copper Sulfate	0.0018	0	0.0018	0	0

[a] Kcal g = 3.82.
[b] Source of ingredients United States Biochemical Co., Cleveland, OH.
[c] AIN. (1977).
[d] Mineral mix (components as percent weight): $CaHPO_4(2H_2O)$—70.85; NaCl—9.53; K_2CO_3—12.37; $MgSO_4(7H_2O)$—4.11; $FeSO_4(H_2O)$—0.64; $MnSO_4(H_2O)$—0.31; $ZnSO_4(7H_2O)$—0.13; $CoCl_2(6H_2O)$—0.10; NaF—0.02; KI—0.03; Na_2O_4Se—0.0006; $CrCl_3$—0.038; Cerelose-(filler)1.92.
[e] Marker.
[f] Acid-Washed Red Spring Wheat Bran.
[g] 259 μg Cu g acid-washed wheat bran, provides 8 μg g diet.

the Cu-adequate cellulose and wheat bran diets provided 2 ppm copper for mice and 6 ppm for rat diets; the bran bound Cu diets (3% wheat bran) provided 2 ppm Cu for mice and 8 ppm Cu for rats; and the Cu-deficient diets provided 0.8 ppm Cu for mice and rats. The dietary requirement of mice has been shown to be 2 ppm for Cu (Weber and Lei, 1982), while the dietary requirement of rats is 6 ppm for Cu (National Research Council, 1978). The murine diet contained 6% more skim milk and 6% less cerelose than the rat diet did.

Animals

Animals were housed individually in suspended, stainless-steel cages and maintained at 22°C with 12 hr each of light and dark. Food and distilled-deionized (D.D) water were provided *ad libitum*. Fifty male Charles River D-1 weanling mice from the University of Arizona stock were randomly assigned to one of the five diets. There were no significant differences among initial body weights of the five groups of mice (data not shown). Food consumption was measured twice weekly for 4 wk; mice were weighed weekly for 5 wk. Sixty male weanling rats (Hilltop Sprague-Dawley) were fed rat chow for 1 wk. At the end of the first week rats were weighed, and the ten extremes were eliminated from the experiment. The remaining 50 rats were randomly assigned to one of the five diets. The average initial body weights were different among the five groups (Table 3). Food was weighed twice weekly for 3 wk; rats were weighed weekly for 4 wk.

Sample collection and analyses

After 5 wk, mice were decapitated. Hearts and livers were removed, opened, blotted, weighed, oven dried for 3 days and weighed again. After 4 wk, rats were fasted for 5 hr and anesthetized with ether. Hearts and livers were removed, blotted, weighed, freeze-dried and weighed again.

Tissues were wet-ashed according to the method of Tsai and Lei (1979). The samples were then diluted to suitable volumes for trace mineral determinations by flame atomic absorption spectrophotometry (Hitachi 180-70). The standard solutions were prepared daily from certified atomic absorption standard (Fisher Scientific, NJ). Standard curves were determined after every 10 samples with average correlation coefficients of 0.99995. Precision was determined within runs (< 5%) and between runs (< 10%). The background was 0.05 absorption units for copper. Blanks were run for all tissues to determine possible contamination. Tissue Cu was expressed as μg per g dried tissue or total μg per organ to correct for differences in tissue fluid content. Plasma Cu content could not be determined for mice because of inadequate plasma sample or rats because of inadvertent clotting of plasma samples.

Data, expressed as mean ± SD, were analyzed by analysis of variance (ANOVA) and least significant difference (LSD) (Steel and Torrie, 1960). Differences were significant when $p < 0.05$.

RESULTS & DISCUSSION

Body and tissue weights

Body weights, weight gain and food consumption of mice were comparable among Cu-adequate (cellulose, wheat bran or bran-bound Cu) and Cu-deficient (cellulose and wheat bran) diets (Table 3). These results agree with previous findings in mice fed Cu-deficient diets (Weber and Lei, 1982; Kincaid and Carlton, 1982). Rats fed Cu-deficient diet with cellulose or wheat bran, however, had significantly lower (10%) body weights compared with rats fed Cu-adequate diets with wheat bran or bran bound-Cu but had comparable body weights with rats fed Cu-adequate diet with cellulose (Table 3). Similar results are reported by Lei (1977) and Allen et al. (1982). Total weight gain of rats fed Cu-adequate diets with wheat bran or bran bound-Cu was similar and significantly greater (13%) than that of rats fed Cu-deficient (cellulose or wheat bran) or Cu-adequate (cellulose) diets. Rats fed the Cu-adequate (cellulose) diet consumed significantly less (6%) food than rats fed the Cu-deficient (cellulose) diet or the bran bound diet.

Cardiac weights in mice were comparable on a dry weight or % body weight basis among the Cu-adequate (cellulose, wheat bran or bran-bound Cu) and Cu-deficient (cellulose and wheat bran) diets (Table 4). Cardiac weights on a wet basis were significantly greater in mice fed the Cu-deficient cellulose (14%) and wheat bran (23%) diets and the bran bound-Cu (14%) diet compared to those mice fed Cu-adequate (cellulose or wheat bran) diets. Cardiac hypertrophy, a well-established symptom of Cu deficiency, may be due to an increased mitochondrial portion of the myocardial fibers (Alfaro and Heaton, 1973) and should be evident by increased dry and wet weight of the tissue. By this criterion, cardiac hypertrophy was not present in mice consuming copper deficient diets in this study, but cardiac fluid accumulation was present in the mice fed Cu-deficient and bran-bound Cu diets. The bioavailability of bran-bound Cu in mice can not be determined from these results because the characteristic cardiac hypertrophy did not occur in mice.

Unlike mice, rats fed both cellulose and wheat bran Cu-deficient diets had significantly enlarged hearts as percent of body weight (18–30%) and dry weight (13%) compared to Cu-adequate rats (Table 4). Such enlargement was characteristic

Table 3—Body weight, weight gain, and food consumption of mice and rats fed bran-bound copper, copper adequate or copper deficient diets[a]

	Cellulose diet		Wheat bran diet		Bran diet
	+Cu	−Cu	+Cu	−Cu	Bound Cu
Mice					
Final body weight (g)	31.1 ± 2.7	31.9 ± 2.5	31.9 ± 1.5	31.6 ± 3.0	31.0 ± 2.6
Weight gain (g)	22.1 ± 2.4	22.8 ± 2.6	23.0 ± 1.3	22.4 ± 2.8	21.9 ± 2.7
Food consumption (g/day)	4.2 ± 0.3	4.3 ± 0.3	4.5 ± 0.2	4.2 ± 0.3	4.3 ± 0.2
RATS					
Body weight (g)					
Initial	74.1 ± 4.2[e]	66.6 ± 4.3[c]	61.7 ± 2.9[d]	61.4 ± 2.7[d]	70.4 ± 2.6[b]
Final	192.6 ± 11.2[bc]	188.6 ± 13.7[c]	203.5 ± 16.5[be]	186.0 ± 14.7[ce]	210.0 ± 12.6[e]
Weight gain (g)	118.5 ± 9.4[b]	122.3 ± 14.1[b]	138.7 ± 14.8[e]	126.5 ± 13.4[b]	139.6 ± 12.7[e]
Food consumption (g/day)	12.2 ± 0.3[b]	13.0 ± 0.9[e]	12.8 ± 0.6[be]	12.9 ± 1.5[be]	13.5 ± 0.8[e]

[a] Weanling animals were fed purified diets for 5 (mice) and 4 (rats) weeks. Values represent mean ± SD for 10 animals per diet.
[b-e] Values for a given parameter not sharing a superscript differed significantly ($p<0.05$) by ANOVA and LSD (Steel and Torrie, 1960).

Table 4—Tissue weights and total copper content of mice and rats fed bran-bound copper, copper adequate or copper deficient diets[a]

	Cellulose diet		Wheat bran diet		Bran diet
	+Cu	−Cu	+Cu	−Cu	Bound Cu
MICE					
Heart					
Wet wt (g)	0.13 ± 0.02[b]	0.16 ± 0.02[e]	0.14 ± 0.02[b]	0.16 ± 0.02[e]	0.16 ± 0.02[e]
% Body wt	0.43 ± 0.06	0.50 ± 0.04	0.45 ± 0.06	0.48 ± 0.04	0.52 ± 0.04
Dry wt (g)	0.037 ± 0.004	0.041 ± 0.004	0.039 ± 0.003	0.042 ± 0.006	0.038 ± 0.005
Total Cu (μg)	0.75 ± 0.02[e]	0.62 ± 0.05[b]	0.84 ± 0.03[e]	0.41 ± 0.03[c]	0.87 ± 0.07[e]
Liver					
Wet wt (g)	1.56 ± 0.26[b]	1.27 ± 0.16[c]	1.60 ± 0.32[b]	1.59 ± 0.21[b]	1.84 ± 0.18[e]
% Body wt	4.99 ± 0.45[b]	3.98 ± 0.37[c]	5.02 ± 0.99[b]	5.50 ± 0.76[be]	5.96 ± 0.38[e]
Dry wt (g)	0.52 ± 0.09[e]	0.38 ± 0.05[b]	0.55 ± 0.04[e]	0.54 ± 0.09[e]	0.56 ± 0.06[e]
Total Cu (μg)	3.65 ± 0.20[e]	2.04 ± 0.14[b]	3.79 ± 0.17[e]	1.72 ± 0.11[b]	3.54 ± 0.43[e]
RATS					
Heart					
Wet wt (g)	0.63 ± 0.04[c]	0.79 ± 0.07[e]	0.70 ± 0.07[b]	0.70 ± 0.6[b]	0.70 ± 0.05[b]
% Body wt	0.33 ± 0.03[c]	0.43 ± 0.03[e]	0.34 ± 0.02[c]	0.38 ± 0.02[b]	0.33 ± 0.02[c]
Dry weight (g)	0.14 ± 0.01[c]	0.16 ± 0.01[e]	0.15 ± 0.02[bc]	0.17 ± 0.01[e]	0.16 ± 0.01[be]
Total Cu (μg)	2.31 ± 0.11[b]	1.00 ± 0.13[c]	2.46 ± 0.25[be]	0.91 ± 0.11[c]	2.53 ± 0.36[e]
Liver					
Wet wt (g)	7.17 ± 0.49[b]	7.87 ± 1.24[be]	8.48 ± 0.92[e]	7.43 ± 0.56[b]	8.16 ± 0.57[e]
% Body wt	3.70 ± 0.20[c]	4.20 ± 0.50[e]	4.20 ± 0.20[e]	4.00 ± 0.20[be]	3.90 ± 0.10[c]
Dry wt (g)	2.16 ± 0.17[c]	2.33 ± 0.38[bc]	2.56 ± 0.29[e]	2.30 ± 0.18[bc]	2.51 ± 0.17[be]
Total Cu (μg)	10.43 ± 2.44[b]	5.09 ± 1.11[c]	12.40 ± 1.33[e]	1.88 ± 0.37[d]	4.80 ± 0.64[c]

[a] Weanling animals were fed purified diets for five (mice) and four (rats) wk. Values represent mean ± SD for 10 animals per diet.
[b-e] Values for a given parameter not sharing a superscript differed significantly ($p<0.05$) by ANOVA and LSD (Steel and Torrie, 1960).

of the cardiac hypertrophy of Cu deficiency. In rats fed bran-bound Cu, the heart-to-body weight ratio was comparable to that of Cu-adequate (wheat bran) diet, thus indicating the availability of this bound Cu. Dry cardiac weights in rats fed bound Cu, however, were intermediate between that of Cu-adequate and Cu-deficient (wheat bran) rats. On a dry-weight basis, the bound copper was available but less available than the free Cu.

Hepatic wet weights were significantly reduced (20%) in mice fed the Cu-deficient (cellulose) diet compared to the Cu-adequate (cellulose) diet (Table 4) but did not differ in mice fed either the Cu-adequate or Cu-deficient wheat bran diets. This agrees with previous work showing comparable hepatic weights in Cu-adequate and deficient mice (Weber and Lei, 1982). Mice fed the branbound Cu had significantly greater hepatic wet weights than did mice fed any other diet but had hepatic dry weights comparable to mice fed the Cu adequate diets.

Hepatic wet weights were significantly reduced (12%) in rats fed a Cu-deficient (wheat bran) diet compared to Cu-adequate (wheat bran or bran-bound Cu) diets (Table 4). Hepatic weights were comparable in rats fed Cu-adequate (wheat bran) and bran-bound Cu diets. However, hepatic weights as percent body weights (Table 4) did not differ among the Cu-adequate (wheat bran and bran-bound Cu) and Cu-deficient (wheat bran) diets. Hepatic dry weights were comparable in rats fed Cu-adequate wheat bran or bran-bound Cu but were significantly ($p<0.05$) lower (10%) in Cu-deficient (wheat bran) compared to Cu-adequate (wheat bran) diets. These results support the bioavailability of Cu bound to fiber.

Consistently, hepatic weights were significantly lower (10%) in Cu-adequate (cellulose) rats compared to Cu adequate (wheat bran) rats. Furthermore, liver-to-body weight ratios were significantly greater (10%) in rats fed Cu-deficient (cellulose) diet compared to the Cu-adequate (cellulose) diet. An increased liver-to-body weight ratio has also been reported for rats consuming Cu-deficient cellulose diets (Allen et al, 1982; Alfaro and Heaton, 1973).

Tissue copper content

Hearts from mice consuming Cu-deficient (cellulose or wheat bran) diets contained significantly less Cu per gram dried tissue (26%) and 54.6%, respectively) than hearts from mice consuming a Cu-adequate (cellulose, wheat bran or bran-bound Cu) diet (Fig. 1A). Hearts from mice fed bound copper had the same Cu content as that of mice fed Cu-adequate (cellulose or wheat bran) diets and significantly greater Cu content per gram than that of mice on Cu deficient (cellulose or wheat bran) diets. Similar results were seen in total Cu content (μg per heart, Table 4) with significantly less (44 and 53%) in hearts from mice fed Cu-deficient diets (cellulose and wheat-bran) and comparable content in hearts from mice fed Cu-adequate diets (cellulose, wheat bran and brand-bound). Copper concentration and total content in murine hearts were strongly influenced by dietary copper and clearly showed that copper bound to wheat bran was utilized to maintain the cardiac copper.

Also in rats (Fig 1B), cardiac copper content per gram dried tissue was significantly ($p<0.05$) decreased (62–65%) in Cu-deficient (cellulose and wheat bran) compared to Cu-adequate

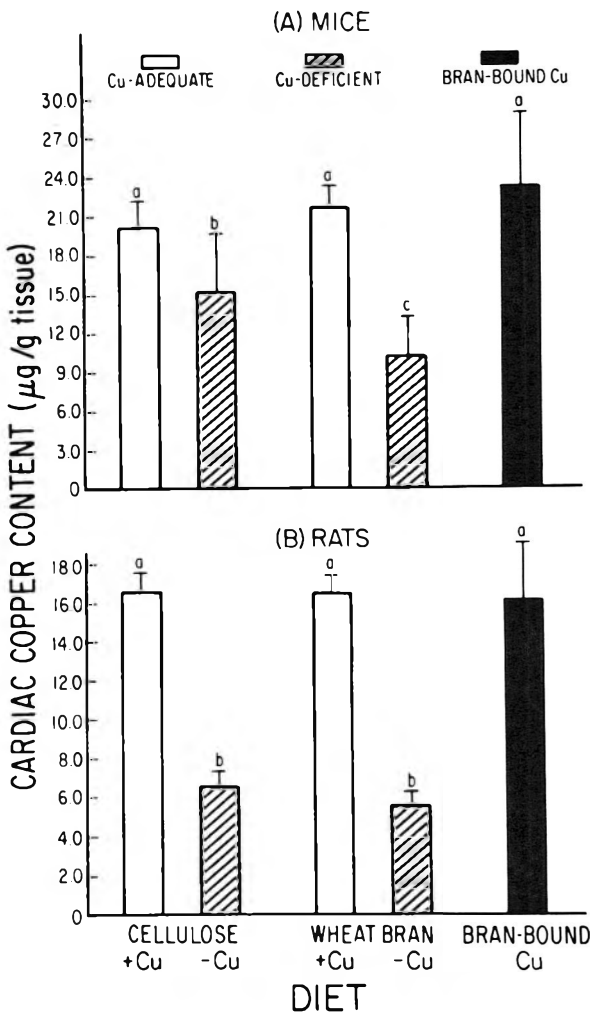

Fig. 1—Cardiac copper content in mice and rats fed a bran-bound copper diet. Male weanling mice (A) or rats (B) were fed purified Cu-adequate (cellulose or wheat bran), Cu-deficient (cellulose or wheat bran) or bran-bound Cu diets for 5 (mice) or 4 (rats). Values (μg Cu per gram dried tissue) represent mean ± SD of 10 animals per diet.
[a-c]Values not sharing a superscript differed significantly ($p<0.05$) by ANOVA and LSD (Steel and Torrie, 1960).

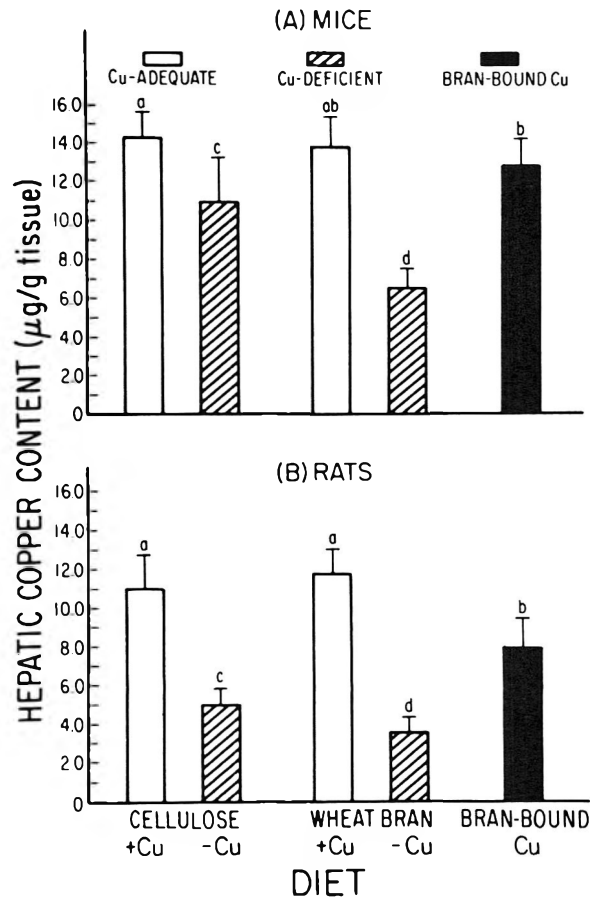

Fig. 2—Hepatic copper content in mice and rats fed a bran-bound copper diet. Male weanling mice (A) or rats (B) were fed purified Cu-adequate (cellulose or wheat bran), Cu-deficient (cellulose or wheat bran) or bran-bound Cu diets for 5 (mice) or 4 (rats) wk. Values (μg Cu/gram dried tissue) represent mean ± SD of 10 animals per diet.
[a-d]Values not sharing a superscript differed significantly ($p<0.05$) by ANOVA and LSD (Steel and Torrie).

(cellulose, wheat bran and bran-bound Cu) diets. Rats fed bran-bound Cu had comparable cardiac Cu content as Cu-adequate rats. Again, similar results were seen in total Cu content (μg per heart, Table 4) with significantly less (51 and 82%) in hearts from rats fed Cu-deficient diets (cellulose and wheat bran) and comparable content in hearts from those fed Cu-adequate diets (cellulose, wheat-bran and bran-bound). This similar cardiac Cu content demonstrated that bran-bound Cu was as available for absorption and utilization as Cu in a free form.

Mice fed Cu-deficient (cellulose or wheat bran) diets had significantly lower hepatic Cu per gram (24 and 53%, respectively) compared to Cu-adequate (cellulose, wheat-bran or bran-bound Cu) diet (Fig. 2A). Total Cu content (μg per liver, Table 4) was also significantly lower (44 and 53%) in livers from mice fed Cu-deficient diets (cellulose and wheat-bran). Decreased hepatic Cu content is a sign of Cu deficiency in mice (Weber and Lei, 1982). Mice fed bran-bound Cu had the same hepatic Cu content per gram as those fed Cu-adequate wheat bran diet and slightly less (10%) than those fed Cu-adequate cellulose diet. Total Cu content (μg per liver), however, was comparable in all mice fed Cu-adequate diets (cellulose, wheat-bran and bran-bound, Table 4). Similarly, rats fed Cu-deficient (cellulose and wheat bran) diets had significantly decreased hepatic content per gram (55 and 70%, respectively) and total Cu content per liver (51 and 82%) compared to Cu-adequate (cellulose, and wheat bran) to bran-bound Cu diets (Fig. 2B and Table 4). In contrast to mice, rats fed bran-bound Cu had significantly reduced hepatic Cu content per gram (32%) and total Cu content per liver (54%) compared to rats fed Cu-adequate (cellulose or wheat bran) diets (Fig. 2B and Table 4). Thus, rats fed bran-bound Cu had a hepatic Cu-content intermediate between Cu deficient and adequate rats. Liver is the storage organ for copper, (Eastwood, 1984). If copper absorption was marginal, then copper mobilization might have been increased in rats fed bran-bound Cu to maintain plasma levels. An increased Cu mobilization would lower hepatic content of copper possibly with no apparent ill effects. Maintaining cardiac copper may be critical for survival because sudden death results from cardiac rupture caused by decreased cardiac Cu content (Anonymous, 1985b). Preferential maintainance of cardiac Cu content may occur when Cu is marginal.

Bioavailability of bran-bound copper

Both mice and rats utilized dietary Cu when bound to wheat bran, although not always to the extent that unbound dietary copper was utilized. Cardiac Cu contents were comparable in mice and rats fed bran-bound Cu or Cu-adequate diets, but hepatic Cu contents were significantly decreased with bran-bound Cu compared to Cu-adequate (cellulose) diets in mice

and Cu adequate (cellulose and wheat bran) diets in rats. Hepatic Cu contents, however, were significantly greater in bran bound Cu diets than Cu-deficient (cellulose and bran) diets in mice and rats. In this study bran-bound Cu was provided in sufficient quantity to meet the requirements for Cu. Whether bran-bound Cu provided at marginal intakes could be utilized as well as free dietary Cu is unknown but could be evaluated by assessing copper status. Fiber-mineral interactions may not affect mineral bioavailability when dietary intake is adequate.

These results also demonstrated that copper utilization differed between species and was dependent upon the dietary fiber source. Future studies of mineral-fiber interactions may need to select a species in which Cu requirement and metabolism are similar to humans. Of particular interest are the effects of fiber source. In the Cu-deficient diets, wheat bran had a greater effect than cellulose on cardiac Cu content (mice) and hepatic Cu content (mice and rats). In contrast, tissue Cu contents were similar regardless of fiber source when copper was adequate. These results suggested that the inclusion of a dietary fiber source with a high mineral binding capacity such as wheat bran might adversely affect copper status only when dietary intake was marginal.

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Ms received 10/14/86; revised 3/23/87; accepted 3/23/87.

This study was supported in part by Univ. of Arizona Experiment Station No. 582. Presented in part at the 1985 meeting of the Federation of American Societies of Experimental Biology in Anaheim [Rockway, S.W., Brannon, P.M. and Weber, C.W. (1985). Copper exogenously bound to wheat bran availability in mice or rats. Fed Proc 44: 1501 (abst)] This study was submitted in partial fulfillment of the requirements for the Ph.D. degree to the Graduate Group in Nutritional Sciences, Univ. of Arizona by S.W. Rockway.

A Research Note

Comparison of Methods Used for Measuring pH in Muscle Tissue

MORSE B. SOLOMON

ABSTRACT

Two methods widely accepted for measuring muscle/meat pH are: (1) homogenizing muscle tissue in 5 mM iodoacetate and (2) spear-tip penetrating probe electrode. These two methods were compared with a third method to evaluate the precision of these methods for measuring muscle pH. The third method involved homogenizing tissue in deionized water and measuring pH within 3–5 sec of homogenization. It was verified that pH values measured within 3–5 sec of homogenization using deionized water were statistically equivalent ($P>0.05$) to those measured using the iodoacetate procedure or a spear-tip probe electrode.

INTRODUCTION

THE pH OF MUSCLE is considered to be one of the more important factors affecting meat quality and is associated with changes in: lean color, carcass grading characteristics, tenderness, microbial spoilage, water-holding capacity and binding properties of comminuted and restructured meats. Currently accepted methods for measuring muscle/meat pH are: (1) homogenizing muscle tissue in 5 mM iodoacetate (Io) solution (Bendall, 1973) and (2) direct measurements using a spear-tip (Sp) penetrating combination electrode (Carter et al., 1967).

Bager and Peterson (1983) compared the accuracy of the direct spear-tip probe method with the iodoacetate homogenization method (for skeletal muscle) and found both methods yielded the same precision for pH determinations. On the contrary, Dutson (1983) reported that the iodoacetate procedure yielded higher pH measurements than measurements obtained using a spear-tip probe electrode. Furthermore, he stated that this was true in the case of both stimulated and nonstimulated beef carcasses. The objective of the present study was to compare the precision of the widely accepted methods for measuring muscle pH with a third method commonly used in our laboratory. This method involves homogenizing tissue in deionized water (Wa) and measuring pH within 3–5 sec of homogenization.

MATERIALS & METHODS

EIGHTEEN RAM LAMBS (6 months of age) were used for this study. Nine lambs were electrically (ES) stimulated (45V, 60 Hz direct current) for 90 sec within 3 min of exsanguination. Carcasses were chilled at 2°C after being dressed. The pH of the longissimus muscle (LM), proximal to the aponeurosis of the LM with the gluteus medius was measured at 1, 3, 5 and 24 hr postmortem. Three methods for measuring muscle pH were compared. A Cole-Parmer spear-tip penetrating electrode (Sp) attached to the Orion model 211 digital pH meter was one method used for measuring pH. To obtain representative pH readings, a minimum of three readings were taken within the muscle with the Sp electrode.

Longissimus muscle samples (approximately 6g) were also removed from each carcass at 1, 3, 5 and 24 hr postmortem. Within 30 sec after removal, two 2-g samples were homogenized using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). One 2-g sample was homogenized in 5 mM iodoacetate (Io = method described by Bendall, 1973); the other 2-g sample was homogenized in deionized water (Wa). The pH of both homogenates was measured using a combination pH electrode attached to an Orion model 211 digital pH meter. The Wa homogenate was measured first, within 3-5 sec of homogenization and the Io homogenate after completing the measurements for the corresponding Wa homogenate. Intact muscle pH was measured prior to the removal of the samples for homogenization and in close proximity to these samples.

Data were analyzed by the analysis of variance technique (SAS, 1982) to determine the significance of variation among the different methods evaluated for measuring muscle pH. Total correlations were computed within the entire sample of data.

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RESULTS & DISCUSSION

A COMPARISON of longissimus muscle pH values as measured by the different methods for measuring pH in stimulated and nonstimulated lamb carcasses is presented in Table 1. The results of this study indicated that it was possible to obtain accurate measurements of muscle pH using the Io, Sp or Wa techniques. The three methods yielded the same relative level of precision (C.V. = 1.92) for measuring muscle pH. Muscle pH values measured at 1, 3, 5 and 24 hr postmortem using the Wa method were equivalent ($P>0.05$) to those measured using the Io or Sp method. Furthermore, no difference ($P>0.05$) was found between the level of accuracy for the Io or Sp method. One should note that when using the Wa method it is very important to measure the pH of the homogenate as soon as possible after homogenizing the tissue (e.g., within 5 sec).

Bager and Petersen (1983) compared the accuracy of the direct spear-tip probe method to the iodoacetate method and also found both methods to be reliable in yielding the same precision for muscle pH determination. However, Dutson (1983) found significant differences between pH values obtained by the Io and Sp methods. They observed higher pH values when using the Io compared to the Sp method. Furthermore, they found greater differences between the Io and Sp method at early postmortem times in electrically stimulated muscles. The opposite was true (less difference between the Io and Sp method at early postmortem times) for nonstimulated muscles.

In the present study, prior to performing the comparisons, a preliminary comparison of two different spear-tip direct probe electrodes was conducted. One probe electrode consisted of a glass electrode encased in a stainless steel sheath while the other probe electrode was a sealed glass gel-electrode. Results obtained with the stainless steel encased electrode were extremely inconsistent compared with the glass gel-electode and therefore, the glass gel-electrode was relied on for reporting results in the present study. Perhaps, the type of probe electrode used can account for some of the differences found between the present study and the study by Dutson (1983).

According to Bendall (1973), homogenizing muscle tissue in dilute iodoacetate lowers the ionic strength and thus has the disadvantage that the pKs of the muscle buffers are altered. Bendall (1973) recommended using iodoacetate in 150 mM potassium chloride, which results in the same ionic strength

Table 1—Longissimus muscle pH values (means, SD) as measured by iodacetate (Io), probe (Sp) electrode and deionized water (Wa) techniques from stimulated and nonstimulated lamb carcasses

Time, hr Post-stimulation	Nonstimulated[a] pH method[b] Io	Sp	Wa	Stimulated[a] pH method[b] Io	Sp	Wa
1	6.75 ± 0.08	6.80 ± 0.05	6.76 ± 0.07	6.46 ± 0.20	6.49 ± 0.25	6.42 ± 0.22
3	6.57 ± 0.13	6.60 ± 0.10	6.61 ± 0.12	6.14 ± 0.18	6.19 ± 0.15	6.13 ± 0.15
5	6.38 ± 0.12	6.36 ± 0.08	6.33 ± 0.14	5.85 ± 0.08	5.85 ± 0.08	5.82 ± 0.07
24	5.70 ± 0.08	5.73 ± 0.08	5.69 ± 0.11	5.61 ± 0.06	5.64 ± 0.09	5.63 ± 0.07

[a] N=9

[b] No significant difference (P>0.05)

as mammalian muscle. However, the use of iodoacetate whether in water or potassium chloride solution to arrest glycolysis causes unavoidable pH shifts, resulting in an alkalinization of about 0.2 pH units at pH of 7.0, about 0.1 pH unit at a pH of 6.0 and falls to zero at the ultimate pH (Bendall, 1973). Even when taking into account the potential pH shifts described by Bendall (1973), the three methods yielded the same level of precision for measuring muscle pH.

Bendall (1973) also reported that using a probe electrode inserted into an intact muscle permits accurate pH measurements yet introduces another set of necessary correlations. In particular, problems with the temperature gradient in the probe itself and of superimposed potentials which may arise from the membrane potential of the muscle may exist. These problems have been shown to be species related, e.g., beef vs. pork. In the present study the pH meter was adjusted to the corresponding muscle temperature at the time of sampling. Simple correlation coefficients for the different methods are presented in Table 2. The various methods were highly related to one another for measuring muscle pH. This was true from 1 through 24 hr postmortem.

Table 2—Simple correlation coefficients between the different pH methods

Time, hr Post-stimulation	pH method comparison Io—Sp[a]	Io—Wa[b]	Sp—Wa[c]
1	0.97**	0.98**	0.98**
3	0.98**	0.98**	0.96**
5	0.97**	0.97**	0.98**
24	0.85**	0.94**	0.78**

[a] Io compared with Sp.

[b] Io compared with Wa.

[c] Sp compared with Wa.

** P<0.01

CONCLUSIONS

VALUES for muscle pH using the Wa method were equivalent (P>0.05) to those measured using the Io or Sp techniques. This suggested that when measuring muscle pH, the method of choice would depend to a great extent on the time and technical help available as well as the type of study being performed.

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Ms received 6/1/87; accepted 6/1/87.

A Research Note

Stability of Polyunsaturated Fatty Acids after Microwave Cooking of Fish

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ABSTRACT

The stability of fish oils high in polyunsaturated fatty acids (PUFA) in fresh fish of low, medium and high fat content was investigated using microwave oven cooking. Cooked and uncooked portions of butterfish, mullet, mackerel and sardines were extracted, the lipid recovered and fatty acid composition determined. The effect of cooking was minimal with no detectable difference in total lipid between cooked and uncooked samples. Most important, PUFA were virtually unaffected by the overall cooking and the cooked fish retained their original PUFA composition and content.

INTRODUCTION

RECENTLY, interest has been growing in fish and fish products as sources of polyunsaturated fatty acids (PUFA), mainly of the n-3 family. This interest stems, in large part, from those studies which have suggested that n-3 PUFA may have an important role in prevention and management of cardiovascular disease (Glomset, 1985; Goodnight et al., 1982; Kromkout et al., 1985; Norum and Drevon, 1986), and from those studies which have suggested that PUFA of the n-3 family may decrease the risk of cancer development at certain sites (Caroll and Braden, 1984; Herold and Kinsella, 1986). These reports have prompted reevaluation of dietary sources, such as fish, for PUFA; the stability of PUFA during fish processing must also be investigated. Most information about the PUFA content of fish is only available for raw, concentrated, canned, or dried-fish and fish products. The effect of cooking on the relative proportions and concentrations of PUFA in fish has not been studied.

The two most common methods of cooking fish, deep-fat frying and broiling, are not particularly amenable to scientific investigation. In deep-fat frying, multiple external variables include varieties of fat used in frying and possible exchange and interaction between cooking fat and fish oils. Broiling is difficult to control experimentally because of a thermal layering effect; that is, thickness of fillets and distance from the heat source cause differences in heat throughout the thickness of the fillet, and because broiling is also usually done with additional oils added to the fish. Microwave cooking, used in this study, has the advantages that no additional oils need be added, the heat source can be standardized, and microwave ovens are readily available.

The aim of the current study was to determine whether heat destroys a relatively greater proportion of these PUFA than their saturated or monounsaturated counterparts.

MATERIALS AND METHODS

FOUR SPECIES of fresh fish were used in this study. Butterfish is of low (<5%), mullet and mackerel of medium, and sardines of high (>10%) fat content (Exler and Weichrauch, 1976; Stansby, 1969, 1973). Samples consisting mainly of flesh (white muscle), were taken from a minimum of four fresh fish. They were mixed and portions from each type fish were placed in glass dishes and cooked in a microwave oven (Minutemaster, Litton Microwave Cooking Products, Minneapolis, MN) for 6 min at a medium high power level. Moisture was determined by heating an accurately weighed (4–5g) representative amount in a pre-weighed dish in an oven at 110°C until constant weight was obtained. Aliquots, (6–10g, accurately weighed) of uncooked and cooked fish treated separately, were cut into small pieces and homogenized in 10 mL chloroform/methanol (1:1 v/v) according to the method of Bligh and Dyer (1959). To prevent oxidation, t-butyl hydroquinone was added to all samples during processing. After extraction, the resulting lipid fraction was weighed and aliquots removed for analysis. Lipid samples were converted to their fatty acid methyl esters as previously described (Hearn et al., 1987). Analysis of methyl esters was performed by gas-liquid chromatography (Hearn et al., 1987).

Further evidence for the identity of PUFA was obtained by chromatography on silver nitrate impregnated silica gel plates (Hearn et al., 1987).

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RESULTS

THE TOTAL FAT CONTENT of the fish, both before and after cooking is listed in Table 1 by weight percent. Butterfish was low in fat content and mackerel, mullet, and sardines had medium fat content compared to levels for other fish given in the literature (Exler and Weichrauch, 1976; Stansby, 1973). There was no decrease in the percentage of extractable lipid calculated on a dry weight basis showing no evidence for fat cooking out and being lost.

The individual fatty acid composition of fish, before and after cooking, is summarized in Table 2. Only those fatty acids that were consistently detected at the level of 0.5% or more of the total given in Table 2. As expected (Exler and Weichrauch, 1976; Stansby, 1969; Stansby, 1973), C18:3, C20:5, C22:5, and C22:6 were present in high concentrations in the species examined.

The SP2310 liquid phase gave good separation of the fatty acid methyl esters with the important exception that C20:5w3 co-chromatographed with C22:1. However, when the samples were chromatographed on the more polar phase, the two fatty acid methyl esters were clearly separated.

Table 1—Moisture and fat composition of raw and cooked fish[a]

	Raw		Cooked	
Fish	Moisture	Fat	Moisture	Fat
Butterfish	76.8 ± 2.8	2.8 ± 0.8 (9.0)[b]	51.8 ± 2.6	4.2 ± 0.9 (9.2)
Mackerel	73.7 ± 1.3	6.2 ± 0.6 (23.8)	49.8 ± 2.8	11.2 ± 1.2 (22.4)
Mullet	75.5 ± 2.3	5.6 ± 0.3 (25.5)	50.5 ± 3.3	9.8 ± 1.0 (24.2)
Sardines	71.2 ± 2.6	9.7 ± 0.8 (32.3)	49.2 ± 2.7	16.5 ± 1.3 (33.9)

[a] Moisture and fat contents are averages ± one standard deviation, in weight percent (wt/wt), for six samples.

[b] Values in parenthesis are the average of two determinations of percent fat (wt/wt) on a dry weight basis.

Table 2—Fatty acid composition[a] in fish before and after microwave cooking

Fatty acid[b]	Fish							
	Butterfish		Mackerel		Mullet		Sardines	
	Before	After	Before	After	Before	After	Before	After
14:0	3.3 ± 0.6	3.9 ± 0.3	2.8 ± 0.3	3.1 ± 0.2	4.1 ± 0.2	3.9 ± 0.3	3.4 ± 0.1	3.6 ± 0.2
15:0	1.8 ± 0.1	2.3 ± 0.5	1.2 ± 0.5	1.4 ± 0.1	3.4 ± 0.5	3.4 ± 1.0	1.3 ± 0.0	1.3 ± 0.1
16:0	17.8 ± 0.5	17.5 ± 0.2	17.5 ± 0.8	18.0 ± 0.4	11.8 ± 0.8	11.5 ± 0.6	14.5 ± 0.4	14.0 ± 0.2
16:1	6.6 ± 1.2	8.1 ± 0.8	6.0 ± 0.2	5.9 ± 0.4	9.4 ± 1.2	9.0 ± 1.0	7.0 ± 0.1	7.8 ± 0.1
18:0	4.3 ± 0.9	6.3 ± 0.8	5.8 ± 0.4	6.4 ± 0.2	10.3 ± 0.6	10.4 ± 0.8	4.9 ± 0.4	5.1 ± 0.2
18:1	23.1 ± 3.5	22.6 ± 1.6	7.8 ± 0.8	7.7 ± 0.4	12.1 ± 0.3	11.0 ± 1.4	15.4 ± 0.4	15.9 ± 0.1
18:2n-6	2.3 ± 0.5	1.5 ± 1.0	1.9 ± 0.3	1.6 ± 0.2	5.7 ± 0.4	5.4 ± 1.6	1.4 ± 0.4	1.5 ± 0.3
18:3n-3	3.1 ± 2.3	2.3 ± 1.3	---	---	---	---	---	0.6 ± 1.0
20:1	4.4 ± 0.2	5.1 ± 0.3	4.1 ± 0.1	4.7 ± 0.2	1.6 ± 0.2	1.9 ± 0.4	4.2 ± 0.8	4.7 ± 0.3
18:4n-3	---	---	2.4 ± 0.3	2.2 ± 0.4	1.2 ± 0.2	1.2 ± 0.4	3.8 ± 0.4	3.3 ± 0.2
20:2	2.9 ± 0.6	2.8 ± 1.1	---	---	1.1 ± 0.3	1.2 ± 0.2	---	---
20:3n-3	0.9 ± 0.3	1.1 ± 0.2	0.7 ± 0.1	0.6 ± 0.1	1.5 ± 0.3	---	---	---
20:4n-6	5.8 ± 0.5	5.4 ± 0.8	6.9 ± 1.2	7.4 ± 0.3	3.5 ± 0.4	3.3 ± 0.4	1.9 ± 0.1	1.9 ± 0.2
22:1	0.6 ± 0.2	.3 ± 0.1	0.7 ± 0.1	1.0 ± 0.1	---	---	0.8 ± 0.2	0.9 ± 0.2
20:5n-3	5.1 ± 1.4	5.0 ± 1.1	11.2 ± 0.9	11.9 ± 0.3	11.2 ± 0.6	11.4 ± 0.6	11.3 ± 1.0	12.0 ± 0.4
24:1	---	---	2.4 ± 0.6	2.5 ± 0.5	0.8 ± 0.2	0.8 ± 0.4	---	---
22:4n-6	3.1 ± 0.8	3.5 ± 0.8	---	---	---	---	0.8 ± 0.2	---
22:5n-6	2.7 ± 0.3	2.9 ± 0.8	0.7 ± 0.4	0.7 ± 0.2	---	0.7 ± 0.1	1.6 ± 0.1	1.3 ± 0.4
22:5n-3	2.7 ± 0.3	2.6 ± 0.7	4.1 ± 0.2	3.9 ± 0.1	5.7 ± 0.7	5.8 ± 0.4	2.5 ± 0.2	2.4 ± 0.2
22:6n-3	10.4 ± 0.3	10.6 ± 0.8	22.8 ± 0.4	22.1 ± 1.4	7.7 ± 1.0	7.7 ± 0.4	25.8 ± 0.8	26.0 ± 0.5

[a] Values are given as weight percentages of total fatty acid methyl esters. They represent averages from six determinations ± one standard deviation.

[b] In this notation for fatty acids, the first number indicates chain length, the number after the colon indicates the number of methylene interrupted double bonds, and the number following the n indicates the number of carbon atoms beyond the terminal double bond.

Differences between percent fatty acids, before and after cooking, were analyzed by a Student paired t-test for selected PUFA. The results of the statistical analyses (not shown) showed no significant differences.

DISCUSSION

UNDER the conditions described, the sought-after PUFA are not destroyed in the process of microwave cooking. This applies to the fish fatty acids as they exist in the fish products.

These results agree with similar work which studied the effects of cooking by baking, pan frying and deep fat frying on the lipid content and composition of freshwater species. Mai et al. (1978) concluded that lipid changes in cooked freshwater fish are least in fillets with high levels of lipid. Likewise Gall et al. (1983) reported that grouper (lean fish) cooked in the microwave oven had significantly lower level of the polyunsaturated 22:6, and significantly higher amounts of 17:0, 22:0 and 20:2. However, no significant differences were detected in the fatty acid composition of red snapper, pompano and spanish mackerel (medium to high fat content).

Research has already shown that isolated fish oils, obtained from sardines and mackerel, for example, are extremely unstable to heating (Olcott, 1962); this instability is mainly attributed to the larger amounts of the glycerides of the highly unsaturated fatty acids that are present. In addition, recent studies made it clear that autooxidized or thermally polymerized fish oils show acute toxicity as compared to other oils and fats (Billek, 1983).

Further experiments are required to gain a thorough knowledge of how fish components interact during heating to protect fish PUFA during cooking. It will suffice to say, on the basis of our work, that an event apparently does occur during cooking that protects PUFA from oxidation and degradation.

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Ms received 12/8/86; revised 5/4/87; accepted 5/19/87.

A Research Note

In Vitro Metabolism of Aflatoxin B_1 with Microsomal Enzymes in the Presence of Selected Nutrients

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ABSTRACT

High pressure liquid chromatography was used to evaluate the effects of several naturally occurring food components (selenium, vitamins A, E, B_6 and C) on the *in vitro* metabolism of aflatoxin B_1 (AFB_1). AFB_1 was incubated with a liver microsomal enzyme metabolizing system with varying concentrations of each nutrient. The following nutrients and levels either inhibited or reduced the metabolism of AFB_1: sodium selenite (25 μg/mL), d- α-tocopherol (25, 250 and 2500 μg/mL), pyridoxine hydrochloride (2.5 μg/mL), L-ascorbic acid (25 and 2500 μg/mL) and mixture containing 500 μg/mL of each chemical. Retinol acetate at levels of 2.5 and 25 μg/mL increased the level of AFB_1 metabolized.

INTRODUCTION

MOST CHEMICAL CARCINOGENS undergo metabolism by liver microsomal enzymes to form activated metabolites that are capable of forming covalent bonds with DNA (Bhattacharya et al., 1984). Metabolism of aflatoxin B_1 (AFB_1), a potent hepatocarcinogen, by the mixed-function oxidase system a complex organization of cytochrome-coupled 0_2- and NADPH- dependent enzymes of liver cells, has been established (Busby and Wogan, 1981). AFB_1 is activated by these microsomal enzymes to yield AFB_1 2–3 epoxide which is the ultimate electrophilic carcinogenic metabolite capable of DNA-adduct formation (Svoboda and Reddy, 1982; Hsieh et al., 1983; Bhattacharya et al., 1984). AFB_1 consumption has a high correlation with the incidence of hepatic carcinomas and to a lesser degree to colon and gastrointestinal carcinogenicity (Rieman and Bryan, 1979).

The nutritional status of an animal may alter its response to toxic substances and metabolism. Bhattacharya et al. (1984) reported an inhibition of *in vitro* DNA-AFB_1 adduct formation in the presence of selenium, riboflavin, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), retinol and retinol derivatives. Other studies note that vitamin E (Draper and Bird, 1984), vitamin B_6 (Shapiro and Gillhorn, 1951), and vitamin C (Shamberger, 1984) may be possible antimutagenic agents. Rosin and Stich (1983) observed a suppression of AFB_1 metabolites when AFB_1 was incubated with chlorogenic acid and a liver S-9 metabolizing system.

Compounds in food that are capable of altering the metabolism of carcinogens, such as AFB_1, may play a vital role in the prevention and inhibition of carcinogen modification. This study was devoted to the evaluation of the effect of several naturally occurring nutrients in the human diet upon metabolism of AFB_1.

MATERIALS & METHODS

Test chemicals

Retinol acetate, d-α-tocopherol acid succinate, ascorbic acid, sodium selenite (Sigma Chemical Co., St. Louis, MO) and pyridoxine hydrochloride (Purepac Pharmaceuticals, Elizabeth, NJ) were examined for their ability to alter the metabolism of AFB_1 by microsomal enzymes. Levels of 2.5, 25, 250, and 2500 μg/mL of each compound, and a mixture containing 500 μg/mL of each compound were used. Vitamins were dispersed in dimethylsulfoxide (DMSO) while sodium selenite was dissolved in distilled water. AFB_1, dissolved in HPLC grade chloroform, was used at a concentration of 13 μg/assay.

AFB_1 metabolism

Microsomal enzyme fractions (liver S-9) from 1254 Aroclor induced rats were obtained from Litton Bionetics (Charleston, SC). Diluted solutions of the liver S-9 plus co-factors were prepared by the method of Maron and Ames (1983). AFB_1 was metabolized by incubating 0.5 mL of S-9 solution with 13 μg AFB_1 at 37°C for 60 min. The effect of nutrients on AFB_1 metabolism was determined by mixing 13 μL of each concentration of test chemical with separate mixtures of S-9 and AFB_1. To determine the extent of binding by AFB_1 to test chemicals, each chemical was mixed and incubated with 13 μg AFB_1. No S-9 solution was added. Each test chemical was also mixed and incubated with separate portions of S-9 solution with no AFB_1 present to determine if compounds which might interfere with chromatographic analysis were formed. After incubation, mixtures were heated at 105°C for 30 sec to halt any further enzymatic action. Three replications of each assay were performed.

Aflatoxin extraction

Aflatoxin was extracted with three equal volume chloroform washes and evaporated to dryness. After reconstitution with 1.0 mL chloroform, the samples were filtered using 0.45 μm Acro LC13 disposable filters (Gelman Science, Ann Arbor, MI). Recovery of 63% AFB_1 was determined by incubating 13 μg AFB_1 with 0.5 mL S-9 solution denatured by heating for 30 sec at 105°C.

Chromatographic analysis

AFB_1 was separated using a Waters 501 HPLC pump with a μ-bondapack C-18 column (30 cm × 3.9 mm) (Waters Associates, Milford, MA) and quantitated with a 3392 A Hewlett Packard integrator (Hewlett Packard Co., Avondale, PA). Samples applied to the column in triplicate, were eluted isocratically at a flow rate of 1.0 mL/min. Column effluent was monitored by a Lambda-Max Model 481 LC spectrophotometer (Waters Associates, Milford, MA) at 365 nm. The solvent system used was water-saturated chloroform:cyclohexane:acetonitrile:2-propanol (1000:300:40:26) (Pons, 1976). The retention time of AFB_1 was compared to a known standard of AFB_1.

Statistical analyses

Data were analyzed statistically with the IBM 370 SAS package (SAS Institute, 1985). Duncan's multiple range test was applied to the means of treatments when F values were significant at the 5% level.

RESULTS & DISCUSSION

INCUBATION of 13 μg AFB_1 with a liver microsomal enzyme system in the absence of test chemicals resulted in the metabolism of 30% of the AFB_1. High-pressure liquid chromatography (HPLC) separation of AFB_1 gave a peak eluting

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Table 1—Amount of AFB_1 after exposure to various concentrations of selenium, vitamin E, vitamin B_6, vitamin C and vitamin A[a]

Chemical conc (μg/mL)	Selenium		Vitamin E		Vitamin B_6		Vitamin C		Vitamin A	
	μg AFB_1	% AFB_1 Metabolized[b]	μg AFB_1	% AFB_1 Metabolized	μg AFB_1	% AFB_1 Metabolized	μg AFB_1	% AFB_1 Metabolized	μg AFB_1	% AFB_1 Metabolized
0.0	5.7[d]	30	5.7[e]	30	5.7[d]	30	5.7[e]	30	5.7[c]	30
2.5	6.0[d]	27	6.6[de]	20	8.2[c]	—	6.5[de]	21	3.4[d]	59
25.0	8.2[c]	—[f]	8.2[c]	—	6.7[d]	18	8.2[c]	—	2.8[d]	66
250.0	4.3[d]	48	7.2[d]	12	5.7[d]	30	5.0[e]	39	4.3[cd]	48
2500.0	4.8[d]	41	8.2[c]	—	5.7[d]	30	7.7[cd]	6	5.5[c]	33

[a] 8.2 μg value represents approximately 67% recovery from the assay which initially contained 13 μg AFB_1.
[b] % metabolism = [(8.2 − x/8.2] × 100; where x denotes μg AFB1 recovered after assaying.
[c,d,e] Means bearing a common superscript in each column do not differ significantly ($p > 0.05$) by the Duncan's Multiple Range test.
[f] No AFB_1 was metabolized.

at approximately 2.75 min; however, other than the solvent peak no additional peaks were detected at 365 nm.

No evidence of binding of the test compounds and AFB_1 was detected since there was no decrease in the amount of toxin detected in assays containing toxin and each test chemical but lacking the S-9 fraction. Combinations containing each test compound and the S-9 fraction with no AFB_1 present revealed that no substances were produced which interfered with the chromatographic analysis of subsequent samples containing toxin.

All trace nutrients significantly altered the level of AFB_1 metabolized compared to when no test nutrients were present. Sodium selenite (selenium) at a level of 25 μg/mL inhibited metabolism of AFB_1 totally; however, all other levels had no significant effect (Table 1). A lower concentration of selenium may reduce the amount of AFB_1 metabolized by the microsomal enzymes, while AFB_1 in the presence of higher levels of selenium and the S-9 fraction is metabolized. Therefore, the alteration in metabolism of the toxin may be influenced by particular concentrations of the nutrient.

Levels of 25, 250 and 2500 μg/ml of d- α-tocopherol acid succinate (Vitamin E) also inhibited metabolism of AFB_1 (Table 1). Inhibition of enzymatic activity is indicated since there was no change in the amount of AFB_1 in the control samples containing vitamin E but not the S-9 fraction.

AFB_1 metabolism was inhibited in the presence of 2.5 μg/mL pyridoxine hydrochloride (vitamin B_6), the lowest level tested in the incubation mixture (Table 1). Addition of higher levels of vitamin B_6 did not significantly alter the AFB_1 metabolized. Possibly lesser amounts of vitamin B_6 have the capability of altering the enzymatic modification of AFB_1.

Addition of 2500 μg/mL L-ascorbic acid (vitamin C) in the reaction mixture resulted in only 6% AFB_1 metabolism. Vitamin C at 25 μg/mL resulted in no AFB_1 metabolism. The remaining levels of vitamin C were not effective against AFB_1 (Table 1).

Retinol acetate (vitamin A) at levels of 2.5 and 25 μg/mL resulted in an increase in AFB_1 metabolism (Table 1). Although the vitamin A levels seemed to enhance AFB_1 metabolism, this may not be an adverse situation. The metabolites yielded may be water soluble metabolites capable of being readily excreted in the bile and/or urine of animals rather than the electrophilic 2,3 epoxide believed to be the active carcinogenic metabolite of AFB_1. Since water soluble extracts were not analyzed in this study, additional research into this possibility is needed.

The reaction of AFB_1 to a mixture containing 500 μg/mL of each test chemical resulted in total inhibition of AFB_1 metabolism. The effects may be due solely to only one or a few of the components in the reaction mixture or to a combined effect of all the test compounds. A previous study indicated that various compounds in combination may enhance the effect of other known protective factors. For example, Draper and Bird (1984) observed an enhanced protective effect of vitamin E on selenium against DMBA-induced tumorigenesis.

From this study, it appears that certain nutrients found normally in the diet may alter the metabolism of AFB_1 by the liver S-9 enzyme fraction. This modification is dependent on the concentration of the test chemical exposed to a mixture of toxin and S-9 fraction. In some instances, levels 10-fold greater than those which reduce the metabolism of AFB_1 into the more mutagenic epoxide form, actually caused an increase in metabolism. To determine if this type of action is potentially beneficial as an antimutagenic effect, additional information concerning what type of metabolites are formed is necessary.

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Ms received 9/12/86; revised 5/26/87; accepted 6/1/87.

A Research Note

Sensory Evaluation and Acceptability of Cookies Enriched with Sweet Lupine Flour (*Lupinus albus* cv Multolupa)

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ABSTRACT

Cookies enriched with 0, 5, 10, 15, 20, and 25% full-fat sweet lupine flour (FFSL) were evaluated by a sensory panel using the rank of preference and paired comparison tests. Cookies with 0, 5, and 10% FFSL were preferred while those containing 20 and 25% FFSL were rejected ($p \leq 0.01$). Studies conducted with school children showed similar acceptability for 0 and 10% FFSL-containing cookies which was different ($p = 0.05$) from those containing 20% FFSL. Fortification of the basic formula with 10% FFSL was recommended on the basis of acceptability.

INTRODUCTION

SWEET LUPINE (*Lupinus albus* cv Multolupa) is a nontoxic legume with good protein and energy value (Ballester et al., 1984). It may be incorporated as flour in food products of daily consumption such as bread (Wittig de Penna et al., 1987), noodles (Gardiman and Wittig de Penna, 1984) and milk substitutes (Ivanović et al., 1983). As reported before (Ballester et al., 1986) the best nutritional quality was obtained with the 20% full-fat sweet lupine flour (FFSL) formula ($p \leq 0.01$) among the other levels assayed 0, 5, 10, 15 and 25%.

The present study was designed to evaluate the sensory quality and acceptability of the cookies containing 0, 5, 10, 15, 20, and 25% FFSL.

MATERIALS & METHODS

Materials

Cookies containing 0, 5, 10, 15, 20, and 25% FFSL as replacement of wheat flour were prepared according to the following basic formula: all purpose wheat flour (65.84%); vegetable shortening (9.01%); malt syrup (1.29%); calcium phosphate ($CaHPO_4$) (0.17%); vanillin (0.09%); sugar (15.45%); salt (NaCl) (0.26%); sodium bicarbonate ($NaHCO_3$) (0.43%) and water (7.37%). The sweet lupine flour containing 0.02% total alkaloids was a full-fat commercial sample obtained from the Campex von Baer, located in Gorbea (Chile). Wheat flour was purchased at the local market. According to Manley (1983) this type of cookie may be classified as rotary molded short dough.

Preparation of cookies

Wheat flour, FFSL, $NaHCO_3$, $CaHPO_4$ and vanillin were mixed in a NCR planetary type mixer. Melted hydrogenated fat and sugar solution (2:1) and a part of the water were added into the mixing bowl. This was then made up into a smooth paste with the gradual addition of the remaining water. The finished dough was shaped in a cookie molder and baked on sheets for 10 min at 270°C in an electric oven. Baked cookies were cooled to room temperature (20°C) and then packed in 250 ± 5g polyethylene bags.

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Sensory evaluation

Cookies packaged in polyethylene bags and stored in cardboard boxes at 20°C and 55–60% relative humidity were evaluated within a maximum of 1 wk after baking. A ranking test in two steps and then a paired comparison test were used to measure the preferences for each formula. The tests were performed according to the methods proposed by Wittig de Penna (1981) and were carried out with 3 panels of 20, 31 and 20 trained judges, respectively. The samples were served in series of 5, 3 and 2 samples, respectively, evaluating one set at a time, in small dishes coded by 3 digits in random order. A set corresponds to a random combination of samples presented at one time. Lightly sweetened coffee was used as neutralizer between samples (Jellinek, 1985; Matzik and Kettern, 1983; Brümmer, 1982; Hauptmann, 1982).

Acceptability

This evaluation was carried out with an untrained panel of 74 school children between 9 and 11 years of age. The samples were presented in a three compartment polyethylene bag, each one coded with three digits in random order. In each compartment the following products were placed: cookies prepared with the standard formula (0% FFSL) and those containing 10 and 20% FFSL. The children were requested to rate each sample for appearance and taste characteristics on a seven-point hedonic rating scale, where 1 represented "dislike very much" and 7 "like very much." Between sample evaluation, children rinsed their mouths with tap water (20°C), performing 10 replicates during 2 wk. The three samples were evaluated between 10 and 11 A.M., each of the 10 days.

RESULTS & DISCUSSION

Sensory evaluation

As shown in Fig. 1, the cookie containing 5% FFSL was widely accepted, due to its good appearance and crust color (more yellow than 0% cookie), while that containing 25% was rejected ($p \leq 0.01$). At the 5% level of significance the 20% FFSL cookie was also rejected. However, cookies containing 0, 10 and 15% lupine flour were enclosed in the zone of doubt To resolve this doubt it was necessary to use ranking and paired

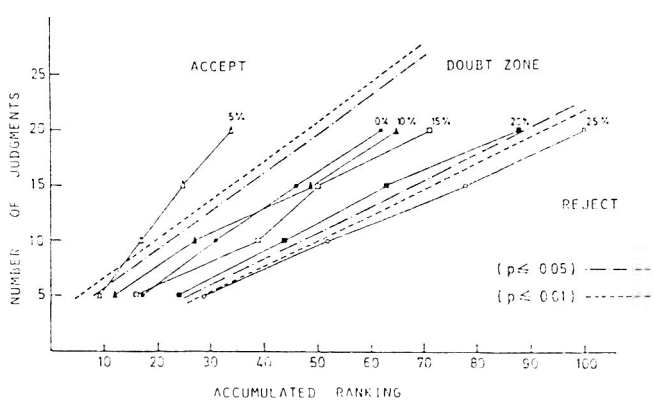

Fig. 1—Preference ranking test (n = 20) for 0, 5, 10, 15, 20, and 25% FFSL cookies.

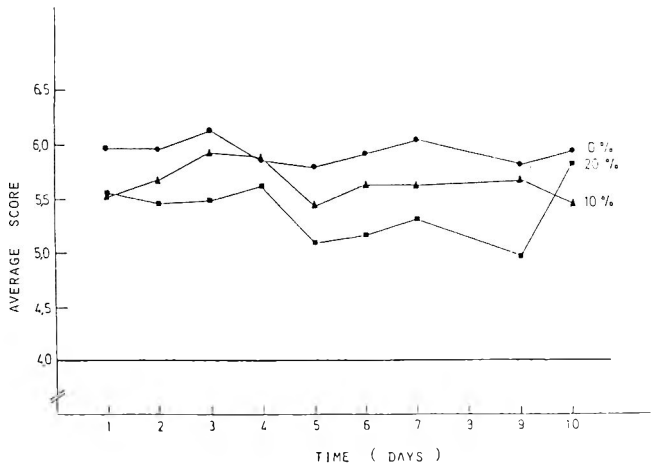

Fig. 2—Acceptability by school children (n = 74) for 0, 10, and 20% FFSL cookies.

comparison tests to discriminate preferences. The 10% FFSL cookie was preferred ($p \leq 0.001$).

School children acceptability

As shown in Fig. 2 the acceptability of the cookies was well above the minimal level of acceptance of 4.0. The values fell in a range between 4.95 and 6.15. The judgments for the 10% FFSL cookie were closer to the standard except for days 1 and 10. The analysis of variance did not show differences ($p \geq 0.05$) among the 0, 10 and 20% FFSL cookies.

From these results it can be concluded that the order of preferences for the cookies enriched with different FFSL levels was 5, 0, 10, 15, 20, and 25%. The acceptability of cookies containing 10% FFSL was similar to that of the standard. These findings agree with other reports about supplemented bread. Repetsky and Klein (1981) found that bread with 2.5 and 5% yellow-field pea flour was judged between fair and good by a trained panel. Hallab et al. (1974) studied Arabic bread supplemented with up to 10% soybean flour, finding a high level of acceptability when compared to unsupplemented bread. Briley et al. (1979) found similar results working with 10 and 15% sunflower flour supplementation. Nevertheless, Elgedaily et al. (1982) reported that bread baked with different soy protein isolates was considered, by the panelists, inferior to the perceived ideal bread, although acceptable. We feel that the cookie enriched with 10% FFSL may be recommended for introduction into school children feeding programs. The results of this investigation showed the feasibility of enrichment of cookies, a highly consumed food, with FFSL. This finding may promote production of this legume in Chile.

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Ms received 6/2/86; revised 4/20/87; accepted 4/22/87.

This investigation was partially supported by Grant A-1332-8223 from the Departmento de Investigación y Bibliotecas of the University of Chile.

A Research Note

Quality of Moin-moins Prepared from Whole or Dehulled Cowpea (*Vigna unguiculata*) Cultivars

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ABSTRACT

Moin-moins, steamed cowpea (*Vigna unguiculata*) products, made from Texas Cream and Chinese Red peas were evaluated and compared to those from California Blackeye peas (control). Sensory evaluation by 11 West African panelists showed that good quality moin-moins could be prepared from the investigated cowpea cultivars if dehulled. Only Texas Cream pea cultivar produced good quality moin-moin from the whole peas, thus making the strenuous dehulling process unnecessary. Moin-moins made from Texas Cream peas also had higher protein and ash contents. Agtron values were positively correlated ($p < 0.01$) to the sensory color scores.

INTRODUCTION

COWPEAS (*Vigna unguiculata*) are inexpensive sources of protein that are popular in arid and semi-arid regions of the world (Molina et al., 1977; Okaka and Potter, 1979). More than 90% of the world's production of cowpeas occurs in Africa, and in some regions these legumes supply about 80% of the total dietary protein (Dolvo et al., 1976; Aykroyd et al., 1982). An increase in the consumption of cowpeas in the developed nations and other parts of the world could be of dietary advantage (Haytowitz et al., 1981).

Moin-moin is a popular West African steamed cowpea product that has potential for utilization in other parts of the world. Moin-moin is traditionally and preferentially prepared from the dehulled black-eye pea cultivar (Aykroyd et al., 1982), but other cultivars of cow-peas are not commonly utilized. The dehulling of peas is practiced to produce a smooth-textured and light-colored moin-moin with little or no hilium specks. This process is often time and energy consuming (Dolvo et al., 1976, Oyenuga, 1968). Efforts at reducing or eliminating the need for dehulling could be highly beneficial. This study was undertaken to (a) investigate the suitability of utilizing other cowpea cultivars in preparing moin-moin and (b) to compare the qualities of moin-moin made from the whole and dehulled cowpeas.

MATERIALS & METHODS

THREE CULTIVARS of dried matured cowpeas, California Blackeye peas and Texas Cream peas (C.I. Smith Co., Pleasanton, TX) and Chinese Red peas (J&B Seed Co., Brownfield, TX) were procured. The peas were sorted, cleaned and stored in plastic bags at 4.2°C until ready for analyses.

Formulation and preparation of moin-moin

Cowpeas were soaked for 12 hr in distilled water at 25°C to soften the hulls. Soaked peas were hand-rubbed against each other to detach the hulls from the cotyledon. The separated hulls floated to the top by gravity and were drained off. Dehulled peas were then washed, drained and blotted dry with paper (McWatters and Flora, 1980).

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The ingredient composition reported by Adeniji and Potter (1980) was modified to prepare the samples. The composition included 200g whole or dehulled cowpeas as primary ingredients; 150 mL distilled water, 15g tomato paste (Hunt-Wesson Foods, Inc., Fullerton, CA), 8g minced onion (McCormick & Co., Inc., Baltimore, MD), 4g iodized salt and 0.4g red pepper (Food Club, Topco Inc., Skokie, IL) as secondary ingredients. The ingredients were weighed ground and mixed for 2 min. in a Sears Microprocessor controlled Electronic Blender. Mixed samples were poured into aluminum foil containers and steamed in a 15 p.s.i. pressure cooker (Mirro Aluminum Co., Manitowoc, WI) for 45 min.

Chemical analyses

Samples were analyzed in duplicate for moisture, fat, crude protein ($N \times 6.25$) and ash by AOAC (1980) methods. Total carbohydrate was estimated by difference.

Sensory and Agtron analyses

Moin-moin samples were evaluated by 11 semi-trained West African student panelists for color, texture, flavor and overall acceptability on an 8-point verbally anchored rating scale. The training of panelists involved several discussions and evaluations of the quality attributes of moin-moin. After the training, two triangle tests per person were used to select the final panelists according to their discriminating ability.

Samples were taken from the central portion of each container of moin-moin and presented to panelists at room temperature (24°C). Six samples, coded with 3-digit random numbers, were given to each panelist on a circular paper plate to obtain a balanced and unbiased presentation (ASTM, 1968). Four replications were made. Evaluations were performed in comfortable, adequately lighted and individually partitioned booths. Distilled water was provided for oral rinsing between samples. Agtron-M-400-A Reflectance Spectrophotometer was used in evaluating color by the method reported by Shuey and Skarsuane (1973).

Statistical analysis

Data were analyzed by the GLM computer method (SAS, 1981) for analysis of variance, Duncan's New Multiple Range Test and correlation techniques (Steel and Torrie, 1980). Data were tested at the 5% level of significance.

RESULTS & DISCUSSION

Proximate composition

The proximate composition of raw cowpeas and moin-moin samples is presented in Table 1. The raw peas of the three cultivars investigated had similar protein and fat. Texas Cream peas exhibited a higher ash than the other two pea cultivars.

Moin-moin samples prepared from both whole and dehulled Texas Cream peas and whole California Blackeye peas had higher ($p < 0.05$) protein and ash than the dehulled California Blackeye peas (control). Compared to the control, moin-moin from the whole Chinese Red peas had similar protein whereas, the protein value for the dehulled Chinese Red peas was considerably lower. Although fat of all the moin-moin samples was higher than that for the control except that made from the

Table 1—Proximate composition of raw cowpeas and moin-moins[a]

Cowpea/Moin-moin Samples	Moisture (%)	% Dry weight			
		Protein (N x 6.25)	Fat	Ash	Total carbohydrate[b]
Raw Cowpeas					
California Blackeye Peas	11.2[a]	21.5[a]	1.2[a]	3.8[b]	73.5
Texas Cream Peas	10.6[a]	25.2[a]	1.1[a]	4.5[a]	69.2
Chinese Red Peas	10.0[b]	22.4[a]	1.2[a]	3.6[c]	72.8
Moin-Moins					
Whole California Blackeye Peas	70.0[c]	21.5[c]	1.6[a]	7.5[e]	69.4
Dehulled California Blackeye Peas (Control)	71.5[c]	20.6[d]	1.1[b]	8.1[b]	70.2
Whole Texas Cream Peas	72.3[abc]	22.8[b]	1.5[a]	8.3[a]	67.4
Dehulled Texas Cream Peas	74.3[ab]	24.8[a]	1.4[a]	8.4[a]	65.4
Whole Chinese Red Peas	72.0[ab]	20.3[d]	1.4[a]	7.6[d]	70.7
Dehulled Chinese Red Peas	74.9[a]	18.6[e]	0.9[b]	7.8[c]	72.7

[a] Means within the same column under raw cowpeas or moin-moins followed by the same superscript are not significantly different ($p > 0.05$).
[b] Total carbohydrate calculated by difference.

Table 2—Sensory and Agtron Scores for Moin-moin samples prepared from whole and dehulled cowpeas[a]

Moin-moin samples	Sensory scores[g]				Agtron color scores		
	Color	Texture	Flavor	Overall acceptability	Green	Blue	Red
Whole California Blackeye Peas	3.4[c]	3.8[d]	4.8[b]	4.6[b]	39.5[e]	48.3[e]	25.0[d]
Dehulled California Blackeye Peas (Control)	6.7[a]	6.6[a]	6.0[a]	6.7[a]	90.0[a]	95.3[a]	94.0[a]
Whole Texas Cream Peas	6.5[a]	5.4[c]	5.6[ab]	6.1[a]	75.0[c]	65.0[d]	82.5[b]
Dehulled Texas Cream Peas	6.8[a]	6.4[ab]	6.1[a]	6.8[a]	84.0[b]	80.5[b]	91.5[a]
Whole Chinese Red Peas	1.9[d]	2.4[e]	4.0[c]	3.6[c]	23.3[f]	35.0[f]	15.5[e]
Dehulled Chinese Red Peas	4.8[b]	5.6[bc]	5.6[ab]	5.9[a]	64.0[d]	69.5[c]	66.8[c]

[a f] Means within the same column followed by the same superscript are not significantly different ($p > 0.05$).
[g] Sensory scores of 8 = extremely light color, extremely smooth texture, extremely intense flavor or extremely acceptable; whereas, scores of 1 = extremely dark color, extremely coarse texture, extremely weak flavor or extremely unacceptable.

dehulled Chinese Red peas, the fat of peas was generally too low to be considered as important.

Sensory and Agtron values

Panelists gave high light-color scores similar to moin-moin samples made from dehulled California Blackeye peas (control) and both whole and dehulled Texas Cream peas (Table 2). Moin-moins made from whole California Blackeye peas and whole and dehulled Chinese Red peas rated darker in color than the control. Texas Cream pea was the only cultivar investigated that produced a light colored moin-moin from the whole peas. Lighter colors are more desirable in moin-moins (Oyenuga, 1968).

The dehulled peas produced smoother moin-moin samples than the whole peas. Only Texas Cream peas produced a smooth moin-moin product from the whole peas.

Evaluation scores for flavor revealed no differences ($p > 0.05$) for moin-moins made from the control, whole and dehulled Texas Cream peas and dehulled Chinese Red peas. Compared to the control, the whole California Blackeye peas and whole Chinese Red peas produced moin-moins with lower flavor quality.

Moin-moin samples prepared from both whole and dehulled Texas Cream peas and dehulled Chinese Red peas yielded high acceptability scores that were not different ($p > 0.05$) from the control. However, whole California Blackeye peas and whole Texas Cream peas produced moin-moins with lower acceptability.

Agtron color values of moin-moin made from whole peas were lower than those made from dehulled peas for each of the cultivars investigated (Table 2). Like the sensory results, moin-moin samples made from the whole Chinese Red peas had the lowest Agtron scores because of the brown hulls. The green, red and blue Agtron colors had correlation coefficients of 0.96, 0.94, 0.82 ($p < 0.01$), respectively, with sensory color.

CONCLUSION

THE PEAS of the cowpea cultivars investigated were adequate in preparing moin-moin samples of good quality, if dehulled. The Texas Cream pea was the only cultivar that yielded moin-moin of good quality from the whole peas. Preparation of moin-moin from the whole Texas Cream peas rendered the dehulling process obsolete. Further agronomic research is needed to investigate adaptation of the Texas Cream pea cultivar to other regions of the world, especially West Africa.

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Ms received 4/1/87; accepted 4/2/87.

A Research Note

Monitoring Food Product Temperatures during Convective Heating: Implications for Research Procedures

N.F. UNKLESBAY, N.E. BROWN, and M.E. MATTHEWS

ABSTRACT

Turkey rolls were heat processed (105°, 135°, and 165°C) in an electric convection oven with loads of 2, 4, and 6 rolls. Data were analyzed when 50, 66, 83, and 100% of thermocouples (three/roll) reached 77°C. Processing time for 2 rolls, (min/load) and (min/kg), was significantly greater ($P<0.05$) only at 105°C, when 100% versus 50% of thermocouples were monitored. Energy consumed was not different for 2 or 4 rolls. Processing time differed (min/load) but not min/kg, when 4 and 6 rolls were heat processed at 135°C, depending on the percentage monitored. The percentage monitored significantly changed WH/load but not WH/kg, only when a load of 6 rolls was processed. Determining temperatures on the basis of readings from 50 to 100% of thermocouples did not affect energy data.

INTRODUCTION

EFFORTS have been directed toward determining optimal heating conditions in convection ovens (USDA, 1983). Energy use has been correlated with nutrient retention and sensory, microbial, and chemical safety (Klein et al., 1984). Food composition varies and convective heating conditions fluctuate (Hsieh and Matthews, 1986). Thus, the impact of basing operational decisions on various proportions of thermocouple readings within foods requires study.

Some researchers have stated that products were removed at a specific temperature (McNeil and Penfield, 1983); others give a mean temperature and standard deviation (Unklesbay et al., 1983). No reviewed studies included a policy statement about how many thermocouples attained a designated temperature before recording data. When portions of food do not reach the same temperature, simultaneously, implications for food quality are apparent. When energy usage is monitored for a given load size, some products cannot be removed before others. The purpose of this project was to determine the impact of monitoring different numbers of thermocouples, placed into turkey rolls, for three oven temperatures and load sizes.

MATERIALS & METHODS

EXCEPT WHEN SPECIFIED, identical procedures were followed in two laboratories. Turkey rolls were processed according to USDA specifications, by Norbest, Inc. (Salt Lake City, Utah), stored (−18°C) and tempered (4°C) for 48–62 hr. One thermocouple (Type K or C, 28 gauge) was inserted into the geometric center of each roll; two others were located along the longitudinal axis, 2 cm on either side. They were attached to a digital thermometer (Omega No. 2176A) or a potentiometer (Honeywell Braun Electronic). Three oven load sizes (2, 4, or 6 rolls) were used. Each roll was centered in an aluminum pan (Reynolds No.RC1174). A forced-air convection oven (Lang No. ECCO-6) was used. Two pans were placed adjacent to each other on one rack. With two rolls, the rack was 21 cm from the oven bottom; the racks for four rolls, 16 and 31 cm; and the racks for six rolls, 6, 21, and 36 cm. Initial product mass was determined with a digital balance (Mettler No. PS30 or Toledo No. 3210).

Energy (watt hours) was monitored every minute with a digital energy meter (DuPont No. EMT-KWD), from the time the door was closed and one of three oven temperatures (105°, 135°, and 165°C) was set, until all of the thermocouples reached 77°C.

Statistical analysis

Six, 12, and 18, thermocouples were monitored with oven loads of 2, 4, and 6 rolls, respectively. Data were analyzed at four stages—when 50, 66, 83 and 100% of the thermocouples reached 77°C. Time and energy data were analyzed on a mass basis of 1 kg of raw product and on an oven load basis. Analysis of variance procedures were followed with oven temperature and load size as treatments (Snedecor and Cochran, 1980).

RESULTS & DISCUSSION

THE INITIAL mean internal temperature ranged from 7° to 9°C. Initial mean mass of the rolls was 4769 ± 492g. When data based on readings from three to six thermocouples were analyzed for two rolls (Table 1), the only condition which revealed significant ($P<0.05$) differences among the four percentage levels, was the total heat processing time. When six versus three thermocouples were monitored, heat processing time, (min/load) and (min/kg), was significantly ($P<0.05$) greater.

Significant differences ($P<0.05$) were found in heat processing time when four and six rolls were processed at 135°C depending on the percentage of thermocouples monitored (Table 2). Monitoring 100% of the thermocouples gave results (min/load) that were significantly higher ($P<0.05$) than when either 50 or 66% of the thermocouples were monitored. For six rolls, energy usage per load was significantly higher ($P<0.05$) when data from all of the thermocouples were analyzed, versus that from 50 or 66%. When analyzed on an energy per mass basis, these differences did not occur.

The elapsed time for 50% and 100% of the thermocouples reaching 77°C was 10, 25, and 43 min for 2, 4, and 6 rolls, respectively (Table 2). In spite of these differences, when the heating time and energy use were analyzed on a mass basis, all four monitoring schemes were appropriate.

At 105°C the similar time lag was 17 min (Table 1). This time lag was more than twice that at 135°C and 165°C. The mean difference in total energy usage per load at 105°C, based on 100% versus 50% of thermocouples reaching 77°C, was 280 WH or 5% of the total.

Energy consumption for a nonpreheated empty convection oven was 19.62, 24.81 and 29.06 WH/min for oven temperatures of 105°, 135°, and 165°C, respectively. Given the slower rate of energy use at 105°C, the heat processing time differences (Table 1) did not significantly affect energy consumption. Any of the options give in Table 1 would given valid information for energy consumption. However, the highest and lowest product temperatures achieved during any of the trials were 68°C and 94°C. With the exception of six rolls at 135°C

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Table 1—Heat processing conditions for convective heating of two turkey rolls at three oven temperatures[a,b]

Oven temp (°C)	Thermocouples[c] (No.)	(%)	Heat processing time (min/load) Mean	S.E.	(min/kg) Mean	S.E.	Energy usage (WH/load) Mean	S.E.	(WH/kg) Mean	S.E.
105	3	50	245.2^{B}	4.23	24.68^{B}	0.4	5125	400	517	41
	4	66	255.2^{AB}	5.08	25.68^{AB}	0.4	5275	389	532	40
	5	83	258.2^{AB}	5.12	26.00^{AB}	0.4	5342	405	538	42
	6	100	262.5^{A}	5.47	26.42^{A}	0.4	5405	397	545	41
135	3	50	179.7	4.79	17.94	0.4	5834	280	583	29
	4	66	187.3	5.58	18.70	0.5	6036	289	603	30
	5	83	179.7	9.59	17.94	0.9	6066	296	607	30
	6	100	189.1	5.82	18.86	0.5	6077	271	606	28
165	3	50	156.4	3.67	15.68	0.4	6284	217	630	21
	4	66	159.9	3.09	16.02	0.3	6395	270	641	26
	5	83	162.4	1.69	16.29	0.2	6479	307	650	30
	6	100	164.2	1.66	16.46	0.1	6562	336	658	33

[a] N = 6 except for 135°C where N = 5.
[b] Where superscripts (AB) differ for mean values for an oven temperature, values differ significantly ($p<0.05$) from each other.
[c] Three thermocouples were placed in each turkey roll. Percent represents proportion of thermocouples registering an internal temperature of at least 77°C.

Table 2—Heat processing conditions for convective heating of turkey rolls at 135°C with three oven load sizes[a]

Oven load (no. rolls)	Thermocouples[b] (No.)	(%)	Heat processing time (min/load) Mean	S.E.	(min/kg) Mean	S.E.	Energy usage (WH/load) Mean	S.E.	(WH/kg) Mean	S.E.
2[c]	3	50	179.7	4.79	17.94	0.4	5834	280	583	29
	4	66	187.3	5.58	18.70	0.5	6036	289	603	30
	5	83	179.7	9.59	17.94	0.9	6066	296	607	30
	6	100	189.1	5.82	18.86	0.5	6077	271	606	28
4[d]	6	50	190.0^{A}	1.53	9.50	0.1	6945	65	347	3
	8	66	194.0^{A}	3.21	9.70	0.2	7048	67	352	4
	10	83	202.0^{AB}	2.08	10.10	0.1	7279	99	364	5
	12	100	215.0^{B}	6.11	10.75	0.3	7709	120	386	6
6[d]	9	50	190.0^{A}	4.04	6.23	0.2	9329^{A}	129	306	3
	12	66	196.7^{A}	5.78	6.44	0.2	9620^{A}	179	315	5
	15	83	216.3^{AB}	1.33	7.09	0.1	10434^{AB}	142	342	5
	18	100	233.0^{B}	3.61	7.64	0.1	11144^{B}	138	365	3

[a] Where superscripts (AB) differ for mean values for an oven temperature, values differ significantly ($p<0.05$) from each other.
[b] Three thermocouples were placed in each turkey roll. Percent represents proportion of thermocouples registering an internal temperature of at least 77°C.
[c] Number of replicat ons = 6.
[d] Number of replications = 3.

(Table 2), each of these monitoring procedures could be recommended for an energy usage study. However, the high temperature (94°C) would be detrimental to sensory and nutritional qualities. To summarize, studies designed to determine the influence of convective heating on microbial, nutritional, and sensory qualities can only be verified if precise procedures for the placement and monitoring of thermocouples are stated.

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Ms received 12/19/86; revised 4/13/87; accepted 5/29/87.

Contribution from Missouri Agricultural Experiment Station. Journal Series Number 10226.
The turkey rolls were supplied through a contract (DAAK60–84–C–0089) with the Department of the Army, U.S. Army Natick Research, Development, & Engineering Center, Natick, MA. This publication represents the views of the authors and does not purport to reflect the positions of the Department of the Army or the Department of Defense.

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